Enzyme-Modified Phytotoxic Structure of Thiadiazolidine Compounds

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A simple model thiadiazolidine, 5-(4-methoxycarbonylmethylthio-phenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one, was synthesized and its structural modification investigated using glutathione S-transferase (GST) and an esterase preparation isolated from *Echinochloa utilis*. The objective is a better understanding of the metabolic activation of peroxidizing thiadiazolidine compounds. The model thiadiazolidine with an ester group (thiadiazolidine ester) was isomerized by GST to a more phytotoxic triazolidine structure (triazolidine ester). Both the thiadiazolidine and the more active triazolidine ester were hydrolyzed by *Echinochloa* esterase to less active free acid compounds, 5-(4-carboxymethylthiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (thiadiazolidine acid) and 4-(4-carboxymethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (triazolidine acid), respectively. The thiadiazolidine acid, however, was only slightly converted into the triazolidine acid in the presence of GST. It is concluded that the thiadiazolidine ester was isomerized in *Echinochloa* to give the triazolidine acid through the triazolidine ester. Since the triazolidine ester exhibited the highest phytotoxic peroxidizing activity GST is considered as an activating enzyme for phytotoxicity and esterase as a detoxifying enzyme to reduce phytotoxic activity. Accordingly, phytotoxic thiadiazolidine-ester type herbicides may be produced by an interplay of isomerizing GST and esterase activity contributing to herbicide selectivity among plant species.

Introduction

Peroxidizing compounds exert their phytotoxic activities by destruction of cell membranes and ethane formation. *p*-Nitrodiphenyl ethers, cyclic imides and 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones (thiadiazolidin-ones) are known as inhibitors of protoporphyrinogen oxidase (protox) (Matringe *et al.*, 1989; Nicolaus *et al.*, 1993 ; Sato *et al.*, 1994 a). Due to protox inhibition protoporphyrin IX (proto IX) may accumulate in the cell. Then, the protox IX is sensitized by light leading to formation of reactive radicals which degrade cellular constituents. *N*-Aryl-3,4,5,6-tetrahydroisophthalimides (isoimides) and 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones are converted into corresponding *N*-aryl-3,4,5,6-tetrahydrophthalimides (imides) and 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (triazolidin-ones), respectively, by an enzymatic reaction in the plants (Hoshi *et al.*, 1993 a; Sato *et al.*, 1994 b; Sato *et al.*, 1995). It has been reported that thiadiazolidin-ones are converted into corresponding triazolidin-one-thiones in the presence of GST and SH compounds. For example, 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one is isomerized to 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione in the presence of equine GST and several SH substrates (Iida *et al.*, 1995). When both triazolidin-one-thiones and thiadiazolidin-ones are assayed with protox isolated from etiolated maize, the former exhibits about a 100-fold stronger inhibition than the latter (Sato *et al.*, 1994 b).

Hoshi *et al.* (1993 b) have reported that both 5-(4-chloro-2-fluoro-5-methoxycarbonyl-methyl-thiophenylimino)-3,4-tetramethylene-1,3,4-

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thiadiazolidin-2-one and 4-(4-chloro-2-fluoro-5-methoxycarbonyl-methylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione are enzymatically converted into 4-(4-chloro-2-fluoro-5-carboxymethyl-thiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione when present in a culture of *Echinochloa utilis* and *Scenedesmus acutus*, or when added to a spinach homogenate. Recently, Shimizu *et al.* (1994 and 1995) have reported that 5-(4-chloro-2-fluoro-5-methoxycarbonylmethyl-thiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one is changed into its isomeric triazolidine, 4-(4-chloro-2-fluoro-5-methoxycarbonylmethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione, in the presence of a crude GST preparation from velvetleaf (*Abutilon theophrasti*).

Information described above may indicate that the conversion of thiadiazolidin-ones into triazolidin-one-thiones is caused by GST and the esterified thiadiazolidin-one is modified into triazolidin-one-thione having a free carboxylic acid moiety by means of both GST and an esterase in plants. To clarify whether GST is active on the ester or the free acid, a simple thiadiazolidine, 5-(4-methoxycarbonylmethylthiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (**I**) and its three possible metabolites (**II, III** and **IV**; see structures in Fig. 1) were prepared. The structural modification of the thiadiazolidin ester (**I**) was investigated using a GST preparation and an enriched esterase preparation both isolated from *E. utilis*.

Materials and Methods

Synthesis of compounds

Thiadiazolidines (**I** and **II**) and triazolidines (**III** and **IV**, see Fig. 1) were prepared according to Wakabayashi *et al.* (1976), Yamaguchi *et al.* (1987) and Sato *et al.* (1994 a).

1) 5-(4-Methoxycarbonylmethylthiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (**I**)

1-(4-Methoxycarbonylmethylthiophenylthiocarbamoyl)-hexahydropyrida-zine (3.3 g, 10 mmol) and pyridine (2.4 g, 30 mmol) were dissolved in CH_2Cl_2 (30 ml), and trichloromethyl chloroformate (1 g, 5 mmol) in CH_2Cl_2 (5 ml) was added dropwise to the above solution at 0°C. The mixture was stirred for 12 hr at room temperature, followed by decantation into ice water. The CH_2Cl_2 layer was separated from the aqueous phase, dried over Na_2SO_4 and the solvent evaporated *in vacuo*. The residue was chromatographed over silica gel (100 g, $CHCl_3$ as mobile phase) to give 4.0 g of a yellow oil.

IR υ_{max} (NaCl) cm^{-1}: 2900, 1720, 1680, 1620, NMR $\delta_H(CDCl_3)$: 1.83 (2H, m), 1.91 (2H, m), 3.61 (2H, s), 3.71 (3H, s), 3.74 (4H, m), 6.87 (2H, d), 7.39 (2H, d).

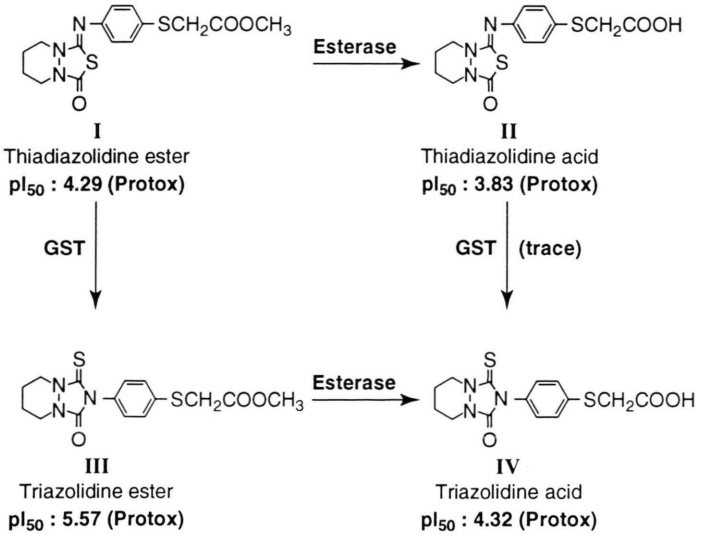


Fig. 1. Proposed metabolic pathway of thiadiazolidine ester (**I**).

2) 5-(4-Carboxymethylthiophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (**II**)

5-(4-Methoxycarbonylmethylthiophenylimino)-3,4-tetramethylene-1,3,4-triazolidin-2-one (60 mg, 0.17 mmol) was stirred in a 4 N HCl solution (4 ml) for 12 hr at room temperature. The reaction mixture was extracted with ethyl acetate. Then the organic layer was dried over Na_2SO_4 and the solvent evaporated *in vacuo*. The residue was chromatographed over silica gel (45 g, CH_2Cl_2 as mobile phase) to give 20 mg of yellow crystals, m.p. 101–102°C. IR ν_{max} (KBr) cm^{-1}: 3200, 2960, 1610

NMR $\delta_H(CDCl_3)$: 1.81 (2H, m), 1.91 (2H, m), 3.65 (2H, s), 3.74 (4H, m), 6.90 (2H, m), 7.41 (2H, m).

3) 4-(4-Methoxycarbonylmethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (**III**)

A mixture of 1-(4-methoxycarbonylmethylthiophenylcarbamoyl)-2-methoxycarbonyl-hexahydropyridazine (2 g, 5.2 mmol) and sodium acetate (0.1 g) in xylene (40 ml) was refluxed for 5 hr. After evaporation, the residue was washed with water and recrystallized from ethyl acetate to give 1.3 g colorless crystals, m.p. 124–125°C.

IR ν_{max} (KBr) cm^{-1}: 2930, 1730, 1500 NMR $\delta_H(CDCl_3)$: 1.99 (4H, m), 3.71 (2H, s), 3.72 (2H, t), 3.75 (3H, s), 4.04 (2H, t), 7.41 (2H, m), 7.49 (2H, m).

4) 4-(4-Carboxymethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (**IV**)

4-(4-Methoxycarbonylmethylthiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (0.6 g, 1.7 mmol) in 10% KOH/CH_3OH solution (15 ml) was stirred for 2 hr at 60°C. After methanol was evaporated in *vacuo*, the residue was acidified with 1N HCl. The collected precipitate was washed with water and then recrystallized from ethyl acetate to give 0.38 g of colorless crystals, m.p. 156–157°C.

IRν_{max} (KBr) cm^{-1}: 3470, 2900, 1710 NMR$\delta_H(CDCl_3)$: 1.98 (4H, m), 3.72 (2H, m), 3.73 (2H, s), 4.04 (2H, m), 7.42 (2H, m), 7.52 (2H, m).

Extraction and enrichment of enzymes from Echinochloa utilis

E. utilis seedlings were germinated in a glass chamber for 7 days at 27°C under a light-dark regime of daylight for 12 hr (light intensity of 11,000 lux) and darkness for 12 hr. Protein amount was determined according to the Lowry method using bovine serum albumin as standard.

Isomerase: Shoots of *E. utilis* were frozen under liquid nitrogen and ground to a powder. The powder was suspended into 200 ml of tris(hydroxymethyl)aminomethane (Tris-HCl) (0.2 M, pH 7.8) containing EDTA (1 mM) and 7.5% polyvinylpolypyrrolidone (w/v). The suspension was centrifuged at 10,000 x *g* for 10 min, and the upper phase was subjected to ammonium sulfate fractionation. A 0–70% $(NH_4)_2SO_4$ precipitate was pooled and redissolved into potassium phosphate buffer (0.01 M, pH 7.3). After dialysis the solution was applied to a DEAE-Sepharose column (1.9 x 260 mm; Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with potassium phosphate buffer (0.1 M, pH 7.3). A crude isomerase fraction was obtained by stepwise elution using potassium phosphate buffer with increasing concentration from 0.01 M to 0.25 M. Fractions of crude *Echinochloa* GST (7 ml; approx. 1–2 mg protein/ml) containing the highest isomerization activity were pooled. To remove esterase activity from the crude *Echinochloa* GST, the fraction was applied to an affinity column. Bromosulfophthalein-glutathione was attached to a Sepharose matrix by conjugating it with cyanogen bromide-activated Sepharose as indicated by the manufacturer. The affinity column (1.3 x 50 mm) was equilibrated with potassium phosphate buffer (0.05 M, pH 7.3). After the column was washed with 25 ml of the buffer solution, the bound GST activity was eluted with a buffer (0.05 M, pH 7.3) containing reduced glutathione (5 mM).

Esterase: All steps in enzyme extraction and enrichment were carried out at 0–4°C. 115 g of harvested E. utilis shoots were cut into small pieces and homogenized with 400 ml potassium phosphate buffer (0.1 M, pH 7.0) using a glass kitchen mixer twelve times for 10 sec with low speed. The homogenate was centrifuged at 17,000 x *g* for 10 min. A 30–70% $(NH_4)_2SO_4$ precipitate was prepared from the resulting supernatant and redis-

solved in Tris-HCl buffer (0.02 M, pH 8.0) containing 10% glycerol (v/v) and 1% EDTA. The 30–60% $(NH_4)_2SO_4$ fraction was layered on top of DEAE-Sepharose column (1.7 x 260 mm) (Pharmacia) which had been equilibrated with Tris-HCl buffer (0.02 M, pH 8.0) containing 10% glycerol (v/v) and 1% EDTA at room temperature. The esterase fraction was eluted with Tris-HCl buffer (0.02 M, pH 8.0) containing NaCl (0.1 M), 10% glycerol (v/v) and 1% EDTA and detected by UV absorbance at 280 nm. Fractions (14.5 ml ; approx. 1–2 mg protein/ml) containing the highest esterase activity were pooled.

Standard assay of enzyme activities

Isomerization activity: Activity was determined by the isomerization rate of 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4- thiadiazolidin-2-one to 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (Sato *et al.*, 1994 a). 1 mg of lyophilized of enzyme preparation, glutathione (GSH); (0.1 mM) and substrate at 0.1 mM were dissolved in 0.5 ml of potassium phosphate buffer (0.05 M, pH 6.8). This solution was incubated at 30°C for 18 hr. The converted product was analyzed by a Shimadzu LC-6A HPLC system equipped with ODS-1251-SK column (4.6 x 250 mm, Senshu Scientific Co., Tokyo, Japan). The mobile phase was acetonitrile/H_2O (3:2) at a flow rate of 1 ml/min. The amount of compounds was determined by comparison of the integrated area of the eluted peaks with a standard curve. GST activity was measured spectrophotometrically at 340 nm by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The 1-ml assay solution was prepared with 100 µl of enzyme solution and 900 µl of potassium phosphate buffer (0.2 M, pH 6.5), containing GSH (1 mM) and CDNB (1 mM).

Esterase activity: It was determined by hydrolysis of 4-(4-methoxycarbonylmethyl-thiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (**III**) to 4-(4-carboxymethyl-thiophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (**IV**). The reaction mixture contained 1 mg of lyophilized enzyme preparation and 0.1 mM of substrate in 0.5 ml of potassium phosphate buffer (0.05 M, pH 6.8). After incubation for 18 hr at 30°C, the reaction solution was analyzed by the same HPLC system as used for the determination of isomerase activity. Carboxylesterase activity was measured spectrophotometrically at 405 nm by using *p*-nitrophenyl acetate (PNPA) as a substrate. The 2-ml assay solution contained 1.8 ml of PNPA (1 mM), 100 µl of Tris-HCl buffer (1 M, pH 8.0) and 100 µl of enzyme fraction. This solution was incubated at 30°C for 3 min.

Conversion experiments using compounds **I** *to* **IV**

0.5 ml of potassium phosphate buffer (0.05 M, pH 6.8) including 1 mg of lyophilized enzyme preparation, GSH (0.1 mM) and 0.1 mM of substrate, was incubated at 30°C for 18 hr. The reaction mixture was analyzed by the same HPLC system as used for the determination of isomerase activity.

Hydrolysis experiments using compounds **I** *to* **IV**

1 mg of lyophilized enzyme preparation and 0.1 mM of substrate were dissolved in 0.5 ml of potassium phosphate buffer (0.05 M, pH 6.8). This solution was incubated at 30°C for 18 hr. The reaction solution was analyzed by the same HPLC system as applied for the determination of isomerase activity.

Determination of protoporphyrinogen-IX oxidase inhibition

Preparation of protoporphyrinogen-IX and determination of protox from maize were carried out according to Nicolaus *et al.* (1993). The protox inhibition activity of the compounds is expressed as pI_{50} (Protox), the negative logarithm of the pI_{50} value of protox inhibition.

Determination of ethane formation

According to Ogino *et al.* (1993), an adequate amount of each test compound in ethanol was added to 70 ml of a 24-hr old S. *acutus* cell culture with a density of 2 µl packed cell volume/ml cell suspension in a 100 ml reaction flask containing $NaHCO_3$(5 mM). After incubating under continuous illumination (fluorescent lamps, approx. 16,000 lux) at 22°C for 20 hr, the amount of ethane evolved was determined by a Shimadzu GC-6A gas chromatography system equipped with a flame-ionization detector. The I_{50} (Eth), the molar concentration giving half of the hypothetical maxi-

mum of light-induced ethane formation produced by S. *acutus* in a 20-hr incubation period, was determined through double-reciprocal plots (Böger and Wakabayashi, 1995). These values have also been called "activity values" (Lambert *et al.*, 1983; Ogino *et al.*, 1993).

Chemicals for synthesis and fine chemicals

Starting chemicals for synthesis of compounds (**I** to **IV**) were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Fine chemicals, such as equine GST, porcine esterase, GSH, PNPA, CDNB, bromosulfophthalein-glutathione and protoporphyrin IX were supplied by Sigma Chemical Co., St Louis , MO, USA. Cyanogen bromide-activated Sepharose and DEAE-Sepharose were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Results and Discussion

GST and esterase from E. utilis

The results of a typical extraction and purification procedure are shown in Table IA and IB. For *Echinochloa* GST and esterase, an overall purification of about 32-fold and a 3-fold enrichment was obtained, respectively. Along the purification of *Echinochloa* GST, GST activity coincided with isomerase activity which converted thiadiazolidine type compounds into triazolidines (see GST specific activities of GST and isomerase in Table IA). Esterase activity was removed from the *Echinochloa* GST fraction.When preparing *Echinochloa* esterase, carboxylesterase activity coincided with esterase activity which hydrolyzed the ester of compound (**III**) to free carboxylic acid of compound (**IV;** see specific activities in Table IB). Although the *Echinochloa* esterase preparation still contained GST activity, isomerization activity was not detected in the absence of GSH (Table IB).

Table I. Isolation and purification of enzymes.

A. Purification of GST froom *Echinochloa utilis*

Fraction	Isomerase		Esterase	Purification (x-fold) Assay system		
	GST specific activity (units[a]/mg protein)	Isomerase specific activity (units[b]/mg protein)	Carboxylesterase specific activity (units[c]/mg protein)	GST	Isomerase	Carboxyl-esterase
Crude supernatant	3420	0.7	3900	1	1	1
$(NH_4)_2SO_4$ precipitate	3780	0.7	4320	1.1	1	1.1
DEAE-Sepharose	24960	4.3	6660	7.3	6.1	1.7
Affinity column	110460	18	0	32.3	25.7	0

[a] One unit is defined as one nmol conjugate of 1-chloro-2,4-dinitrobenzene with reduced glutathione per hour.
[b] One unit is defined as one nmol conversion of 5-(4-bromophenylimino)-3-4-tetramethylene-1,3,4-thiadiazolidin-2-one into 4-(4-bromophenyl)-1-2-tetramethylene-1,2,4-thiadiazolidin-3-one-5-thione per hour.
[c] One unit is defined as one nmol hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol per hour.

B. Enrichment of esterase from *Echinochloa utilis*

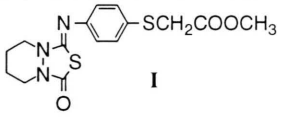
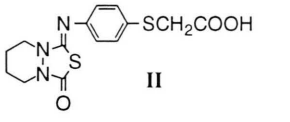
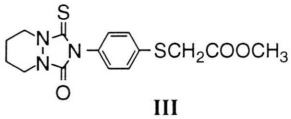
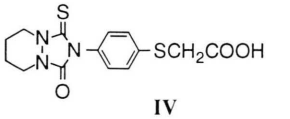
Fraction	Isomerase		Esterase	Purification (x-fold) Assay system		
	GST specific activity (units[a]/mg protein)	Carboxylesterase specific activity (units[c]/mg protein)	Esterase specific activity (units[d]/mg protein)	GST	Carboxyl-esterase	Esterase
Crude supernatant	816	2520	2.6	1	1	1
$(NH_4)_2SO_4$ precipitate	856	2400	4.7	1	1	1.8
DEAE-Sepharose	840	7500	8.6	1	3	3.3

[d] One unit is defined as one nmol hydrolysis of triazolidine ester (**III**) to triazolidine acid (**IV**) per hour.

Thiadiazolidines (**I** *and* **II**) *and triazolidines* (**III** *and* **IV**) *peroxidizing compounds*

Our model compounds, thiadiazolidine ester (**I**) and its three possible metabolites (**II**, **III** and **IV**) were assayed by inhibition of protox isolated from maize etioplasts and by light-induced ethane formation by S. *acutus*. The results are shown in Table II. Although both phytotoxic activities of compounds (**I, II, III** and **IV**) were rather weak compared to the reference thiadiazolidine, 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one, and the reference triazolidine, 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (Sato *et al.*, 1994a; see footnote of Table II), all compounds (**I**) to (**IV**) exhibited protox inhibition and ethane formation, the esters being more active than the free acids.

Table II. Peroxidizing activity of thiadiazolidines and triazolidines.

Compounds	pI_{50} (Protox)	pI_{50} (Ethane)
 **I**	4.29	4.39
 **II**	3.83	4.03
 **III**	5.57	5.29
 **IV**	4.32	4.51

Note: More active reference compounds are 5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (pI_{50}:5.30 (Protox) and 6.43 (Ethane)) and 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione (pI_{50}:7.89 (Protox) and 6.90 (Ethane)).

Conversion of thiadiazolidines (**I** *and* **II**) *into triazolidines* (**III** *and* **IV**) *in the presence of GSTs*

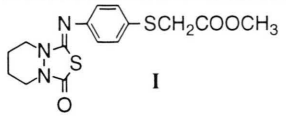
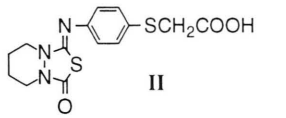
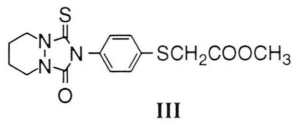
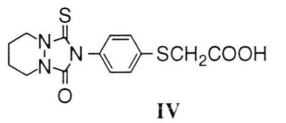
The conversion of the thiadiazolidine ester (**I**) into its isomeric triazolidine ester (**III**), was investigated in the presence of equine GST and isolated *Echinochloa* GST. Results of these conversion experiments are summarized in Table III. In the presence of equine GST and GSH, thiadiazolidine ester (**I**) (line no. 3) was isomerized to triazolidine ester (**III**) (98.2% of starting ester (**I**)), while thiadiazolidine acid (**II**) (line no. 8) was only slightly converted into triazolidine acid (**IV**) (2% of starting acid (**II**)). In these experiments, no isomerization reaction of thiadiazolidines (**I** and **II**) to triazolidines (**III** and **IV**) was observed in the absence of GSH (line nos. 2 and 7). No structural modifications of triazolidine ester (**III**) (line no. 13) and triazolidine acid (**IV**) (line no. 18) occurred in the presence of equine GST and GSH. In the presence of isolated *Echinochloa* GST and GSH, thiadiazolidine ester (**I**) (line no. 4) was converted into the triazolidine ester (**III**) (42.0% of starting ester (**I**)) while thiadiazolidine acid (**II**) (line no. 9) was only very slightly converted into the triazolidine acid (**IV**). Apparently the isolated *Echinochloa* GST had esterase activity, because neither the thiadiazolidine ester (**I**) nor triazolidine ester (**III**) were hydrolyzed in the presence of this enzyme. (line nos. 4 and 14). Additionally, both the heat-denatured equine GST (data not documented) and the isolated *Echinochloa* GST did not catalyze any structural modification of thiadiazolidines (**I** and **II**) and triazolidines (**III** and **IV**) (line nos. 5, 10, 15 and 20).

GST is considered to be the enzyme catalyzing isomerization of thiadiazolidine ester (**I**) to triazolidine ester (**III**). Since the thiadiazolidine acid (**II**) having a carboxyl group at the *N*-phenyl moiety was not easily isomerized to the triazolidine acid (**IV**), this isomerization reaction is dependent on a thiadiazolidine ester as substrate.

Hydrolysis of thiadiazolidine ester (**I**) *and triazolidine ester* (**III**) *to respective thiadiazolidine acid* (**II**) *and triazolidine acid* (**IV**) *in the presence of esterases*

The hydrolysis of thiadiazolidine ester (**I**) and triazolidine ester (**III**) to thiadiazolidine acid (**II**) and triazolidine acid (**IV**) respectively was investi-

Table III. Conversion of thiadiazolidines in the presence of purified glutathione S-transferases (GST).

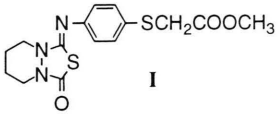
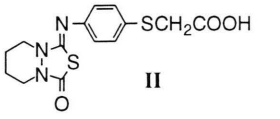
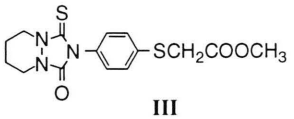
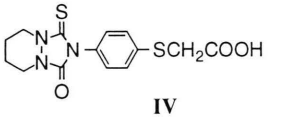
Compounds	Line no.	Additions	Thiadiazolidine SCH_2COOCH_3 (I)	Thiadiazolidine SCH_2COOH (II)	Triazolidine SCH_2COOCH_3 (III)	Triazolidine SCH_2COOH (IV)
			Molar percent			
 I	1	(−) Enzyme equine GST	99.0	n.d.	n.d.	n.d.
	2	(+) GST	98.6	n.d.	n.d.	n.d.
	3	(+) GST (+) GSH *Echinochloa* GST	1.7	n.d.	98.2	n.d.
	4	(+) GST (+) GSH	57.4	n.d.	42.0	n.d.
	5	Heat-denatured	98.9	n.d.	n.d.	n.d.
 II	6	(−) Enzyme equine GST	n.d.	99.3	n.d.	n.d.
	7	(+) GST	n.d.	99.0	n.d.	n.d.
	8	(+) GST (+) GSH *Echinochloa* GST	n.d.	96.0	n.d.	2.0
	9	(+) GST (+) GSH	n.d.	99.3	n.d.	0.5
	10	Heat-denatured	n.d.	99.7	n.d.	n.d.
 III	11	(−) Enzyme equine GST	n.d.	n.d.	99.6	n.d.
	12	(+) GST	n.d.	n.d.	99.0	n.d.
	13	(+) GST (+) GSH *Echinochloa* GST	n.d.	n.d.	98.3	n.d.
	14	(+) GST (+) GSH	n.d.	n.d.	99.1	n.d.
	15	Heat-denatured	n.d.	n.d.	99.7	n.d.
 IV	16	(−) Enzyme equine GST	n.d.	n.d.	n.d.	99.1
	17	(+) GST	n.d.	n.d.	n.d.	99.3
	18	(+) GST (+) GSH *Echinochloa* GST	n.d.	n.d.	n.d.	99.6
	19	(+) GST (+) GSH	n.d.	n.d.	n.d.	99.2
	20	Heat-denatured	n.d.	n.d.	n.d.	99.6

n.d.: not detected

gated in the presence of *Echinochloa* and porcine esterase, respectively. Results of these conversion experiments are summarized in Table IV. With *Echinochloa* esterase present, thiadiazolidine ester (**I**) (see line no. 4 in Table IV) was hydrolyzed to thiadiazolidine acid (**II**) (57.4% of starting ester (**I**)). About 35% of triazolidine ester (**III**) (line no. 14) was hydrolyzed to give triazolidine acid (**IV**) under the same condition. Although our impure esterase preparation still contained GST activity (see Table I, 1st col.), no structural modification of both thiadiazolidine acid (**II**) and triazolidine acid (**IV**) was found (line nos. 4, 9 and 19) since no GSH was included in the assay. Our data show that the esterase itself does not exhibit isomerization activity. In the presence of porcine esterase, thiadiazolidine ester (**I**) and triazolidine ester (**III**) were completely hydrolyzed to their free carboxylic acids (see line nos. 2 and 12). In the hydrolysis experiments with compounds (**I** and **III**), paraoxon (*O*, *O*-diethyl *O*-*p*-nitrophenyl phosphate, 10^{-6} M), an esterase inhibitor (Carino and Montgomery, 1968), reduced the conversion ratio of thiadiazolidine ester (**I**) and triazolidine ester (**III**) into hydrolyzed products (**II** and **(IV)** (line nos. 3, 5, 13 and 15). Additionally, the heat-denatured *Echinochloa* esterase preparation could not produce a structural modification of thiadiazolidines (**I**) and (**II**) and triazolidines (**III**) and (**IV**) line nos. 6, 10, 16 and 20).

In the experiment using enriched *Echinochloa* esterase, paraoxon inhibited the hydrolysis not completely since the 10^{-6}M concentration may not be sufficient for inhibition due to less sensitivity

Table IV. Hydrolysis of thiadiazolidines and triazolidines in the presence of esterases.

Compounds	Line no.	Additions	Thiadiazolidine SCH_2COOCH_3 (I)	Thiadiazolidine SCH_2COOH (II)	Triazolidine SCH_2COOCH_3 (III)	Triazolidine SCH_2COOH (IV)
			Molar percent			
 I	1	(−) Enzyme	99.3	n.d.	n.d.	n.d.
		Porcine esterase				
	2	(+) esterase	n.d.	99.6	n.d.	n.d.
	3	(+) esterase (+) Paraoxon*	91.3	8.5	n.d.	n.d.
		Echinochloa esterase				
	4	(+) esterase	42.6	57.4	n.d.	n.d.
	5	(+) esterase (+) Paraoxon*	68.6	31.2	n.d.	n.d.
	6	Heat-denatured	98.3	n.d.	n.d.	n.d.
 II	7	(−) Enzyme	n.d.	99.5	n.d.	n.d.
		Porcine esterase				
	8	(+) esterase	n.d.	99.1	n.d.	n.d.
		Echinochloa sterase				
	9	(+) esterase	n.d.	98.7	n.d.	n.d.
	10	Heat-denatured	n.d.	99.3	n.d.	n.d.
 III	11	(−) Enzyme	n.d.	n.d.	99.6	n.d.
		Porcine esterase				
	12	(+) esterase	n.d.	n.d.	n.d.	99.3
	13	(+) esterase (+) Paraoxon*	n.d.	n.d.	95.6	3.6
		Echinochloa esterase				
	14	(+) esterase	n.d.	n.d.	64.5	35.5
	15	(+) esterase (+)Paraoxon*	n.d.	n.d.	80.3	19.7
	16	Heat-denatured	n.d	n.d	99.5	n.d.
 IV	17	(−) Enzyme	n.d.	n.d.	n.d.	99.6
		Porcine esterase				
	18	(+) esterase	n.d.	n.d.	n.d.	99.2
		Echinochloa esterase				
	19	(+) esterase	n.d.	n.d.	n.d.	99.3
	20	Heat-denatured	n.d.	n.d.	n.d.	99.8

n.d.: not detected. * Paraoxon (10^{-6} M).

to paraoxon. The *Echinochloa* esterase preparation is considered as being instrumental to catalyze hydrolysis of the ester type of compounds (**I**) and (**III**) to compounds (**II**) and (**IV**), respectively.

E. utilis is susceptible to both thiadiazolidine herbicides and their isomeric triazolidine herbicides (Sato *et al.*, 1994 a; Iida *et al.*, 1995; Shimizu *et al.*, 1995). We have already reported that thiadiazolidines are converted into triazolidines having strong phytotoxic activity by isomerase GST (Iida *et al.*, 1995; Sato *et al.*, 1995). The thiadiazolidine ester (**I**), our model compound in this paper, was isomerized to triazolidine ester (**III**) by GST exhibiting higher phytotoxic activity. The thiadiazolidine ester (**I**) and its activated triazolidine ester (**III**) were hydrolyzed by *Echinochloa* esterase to less active compounds, thiadiazolidine acid (**II**) and triazolidine acid (**IV**), respectively. The free thiadiazolidine acid (**II**), however, was only slightly converted into triazolidine acid (**IV**). These findings indicate that the thiadiazolidine ester (**I**) is modified to give the triazolidine acid (**IV**) through the triazolidine ester (**III**) as intermediate in *Echinochloa*. Since the triazolidine ester (**III**) exhibits the highest phytotoxic activity, the isomerizing GST can be considered to be an activating enzyme for phytotoxicity and the esterase as a detoxifying enzyme to reduce phytotoxic activity of the triazolidine ester (**III**).

Phytotoxic activity of thiadiazolidine-ester type of herbicides like fluthiacet-methyl, 5-(4-chloro-2-fluoro-5-methoxycarbonylmethyl-thiophenyl-imino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one (Shimizu *et al.*, 1995), may interact with the

isomerizing GST and esterase in the plants, contributing to herbicide selectivity among plant species (Iida *et al.*, 1995; Shimizu *et al.*, 1995).

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