Isomerization of Peroxidizing Thiadiazolidine Herbicides Is Catalyzed by Glutathione S-Transferase

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Thiadiazolidine-converting activity (isomerase), detected in a 45–75% ammonium sulfate precipitate from corn seedlings extracts, was purified by chromatography on hydroxyapatite and by anion exchange on Mono Q Sepharose. Two fractions 1 and 2 with isomerase activity were separated on Mono Q by combination of a stepwise elution and continuous salt gradient; fraction 2 eluting at higher salt concentrations was found the most active. Total activity could be enhanced by treatment of seedlings with naphthalic anhydride. Both fractions containing isomerase activity were further purified by glutathione-(GSH) agarose affinity chromatography and characterized by their specificity for different thiadiazolidines. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel filtration revealed that the isomerase of fraction 2 consists either of a homodimer or a heterodimer of two proteins with apparent molecular weights of 28 and 31 kDa, respectively. The protein pattern as well as the strict dependence of activity on thiol groups (GSH or dithiothreitol) suggested a glutathione S-transferase (GST) catalyzing the thiadiazolidine conversion. Further evidence was obtained by measuring reactions specific for GSTs in both purified fractions, namely the conjugating activity for 1-chloro-2,4-dinitrobenzene (CDNB), atrazine and metazachlor. While no atrazine turnover was found, metazachlor and CDNB conjugation occurred rapidly. Both fractions differed in their activities to several GST substrates with fraction 2 being more effective in metazachlor but less active in CDNB conjugation. Inhibitors specific for GST-catalyzed reactions also inhibited thiadiazolidine conversion confirming that isomerizing activity is attributed to a GST form. We conclude that GST isoforms with different affinities towards thiadiazolidines have been isolated. CDNB activity, molecular weight, the protein pattern on SDS-PAGE as well as the amino acid sequence of one of its polypeptides suggest that fraction 1, less active in thiadiazolidine isomerization, is identical to GST I. The second peptide of this fraction was resistant to Edman degradation probably due to N-terminal blockage. The properties of the high isomerase activity found in fraction 2 are in agreement with characteristics of a GST previously termed as isoform II.

Chemical names and abbreviations: Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; CDNB, 1-chloro-2,4-dinitro-benzene; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent; DTT, dithiothreitol; GSH, reduced glutathione; GST, glutathione **S**-transferase; indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetic acid; metazachlor, 2-chloro-*N*-(2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-ylmethyl)acetamide; NA, naphthalic anhydride, (naphthalene-1,8-dicarboxylic acid anhydride); NEM, *N*-ethylmaleimide; proto, protoporphyrin IX; protox, protoporphyrinogen-IX oxidase; SB, sulfobromophthalein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; thiadiazolidines, 5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones; thiadiazolidine no.**1**, 5-(4-bromophenylimino)-3,4,-tetramethylene-1,3,4-thiadiazolidin-2-one; thiadiazoldidine no. **4**, 5-[4-(4-chlorobenzyloxy)phenylimino]-3,4-tetramethylene-1,3,4-thiadiazolidine-2-thione; triazolidines, 4-aryl-1,2 tetramethylene-1,2,4-triazolidin-3-one-5-thiones; triazolidine-3,5-dithiones, 4-aryl-1,2-tetramethylene-1,2,4-triazolidine-3,5-dithiones; (see Table III for formof all thiadiazolidines and triazolidines); tridiphane, 2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane; Tris, tris(hydroxymethyl)-aminomethane.

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Introduction

The primary target of peroxidizers has been identified as protoporphyrinogen-IX oxidase, an enzyme instrumental in the tetrapyrrole biosynthetic pathway (Matringe *et al.*, 1989; Duke *et al.*, 1991; Nicolaus *et al.*, 1993; Wittkowski and Halling, 1989). Protox inhibitors bind competitively *vs.* protoporphyrinogen and inhibition constants in the nanomolar range have been reported (Camadro *et al.*, 1991; Nicolaus *et al.*, 1995). Inhibition of protox results in accumulation of protoporphyrin IX (Matringe and Scalla, 1988; Lydon and Duke, 1988; Sandmann and Böger, 1988; Wittkowski and Halling, 1988). Although growth is affected by interruption and deregulation of porphyrin biosynthesis, the typical strong phytotoxic effects of peroxidizers are observed in the light (Kunert and Böger, 1981; Wakabayashi *et al.*, 1988). The accumulated proto IX is photoactivated with subsequent induction of toxic oxygen species (for details see Böger and Sandmann, 1990; Böger and Wakabayashi, 1995). A radical chain reaction causes membrane lipid degradation, loss of pigments, and destruction of cellular organization. Typical long-term effects are chlorosis and desiccation of plant tissues.

Differences in proto IX accumulation were found to correlate with the herbicidal injury obtained (Nandihalli *et al.*, 1992; Sherman *et al.*, 1991; Watanabe *et al.*, 1993). Tolerance against peroxidizers requires an antioxidative radical-scavenging system or a metabolic breakdown of the herbicide (Böger and Sandmann, 1993; Cole, 1994). Indeed, soybean and peanuts are tolerant against diphenyl ethers due to detoxification catalyzed by glutathione *S*-transferase (GST), an enzyme causing resistance against different xenobiotica in many cereal crops (Eastin, 1971; Frear *et al.*, 1983). In a recent study we presented data on the bioconversion of a new class of peroxidizing compounds, the 5-arylimino-3,4-tetramethylene 1,3,4-thiadiazolidin-2-ones (= thiadiazolidines), into their isomeric 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (= triazolidines) (Iida *et al.*, 1994; 1995; Sato *et al.*, 1994). Surprisingly, the short-term phytotoxicity differed markedly between thiadiazolidines and triazolidines. Triazolidines have been found to be stronger inhibitors of protox, therefore causing higher accumulation of proto IX than their thiadiazolidine couples. Similar long-term phytotoxic effects of isomers were shown to depend on the interconversion of thiadiazolidines into triazolidines representing a mechanism of herbicide activation. Formerly, a hydrolyzation step was suggested (Iida *et al.*, 1994; Sato *et al.*, 1994) but further studies indicated that a GST could be involved in the bioactivation of thiadiazolidines (Iida *et al.*, 1995; Shimizu *et al.*, 1994). Cellular activation of peroxidizers may be of importance for herbicidal selectivity since GSTs have only been described as detoxifying enzymes while a function in herbicide activation is not yet known. Thiocarbamates, a class of herbicides metabolized by GST, are structurally related to thiadiazolidines. Conceivably, GST could recognize thiadiazolidines as a substrate and activate them in plants.

GST isoforms have been described and partially characterized by their specificity for different substrates, their purification characteristics, and by response to safeners (Dean *et al.*, 1991; Fuerst *et al.*; Timmermann, 1989). GST I, III, and IV isoforms are present constitutively, with GST III and GST IV exhibiting high activities for chloroacetamides. Treatment of plants with safeners increases the activity of GST isoforms present in non-treated plants and induces additional isoforms, one of them designated GST II (Mozer *et al.*, 1983). All isoforms exist as dimers of polypeptides with a molecular weight range of 20 to 30 kDa for the monomers. An additional class of GSTs, identified by their specificity to atrazine, differ in apparent molecular weight and complete purification has not yet been reported (Guddewar and Dauterman, 1979; Timmermann, 1989).

The objective of the present study was to purify the enzyme responsible for bioactivation of thiadiazolidines, to characterize it and to confirm its identity as a GST. We call this enzyme *thiadiazolidine isomerase* and present evidence that this is the GST II isoform.

Materials and Methods

Preparation of the crude extract

Corn seedlings (*Zea mays* cv. Anjou) were grown at 30 °C for 6 days in the dark on vermiculite watered every second day. For safener treatment during germination the watering solution contained 0.1 mM naphthalic anhydride. 100 g of

shoots (without seeds) were frozen in liquid nitrogen, ground to a fine powder and homogenized in 0.05 M potassium phosphate buffer, pH 6.8, containing 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.2% (w/v) polyvinylpolypyrrolidone and 0.1 mM phenylmethylsulfonyl fluoride using a Waring blendor for 5 sec with low speed.

The homogenate was filtered through two layers of gauze and centrifuged for 15 min at 10,000 x *g*. The supernatant was kept on ice and subjected to fractionation by ammonium sulfate of an initial concentration of 45%, followed by a salting out step of 45–75% ammonium sulfate saturation. Both protein fractions were pelleted at 20,000 x *g* for 15 min, resuspended in buffer (A) (0.01 M potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol), and then dialyzed against buffer (A) overnight at 4°C. The 45–75% precipitate contained about 80% of total thiadiazolidine-converting activity and was used for further purification.

Purification of isomerase activity

The dialyzed protein fraction was loaded on a hydroxyapatite SC column (Serva, prepared suspension) equilibrated with buffer (A). The thiadiazolidine-converting activity (isomerase) coeluted with the unbound fraction. After a 4-hr dialysis against buffer (B) (0.02 M 1,3-Bis-Tris propane buffer, pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol) the protein fraction was separated by FPLC on a Mono Q Sepharose anion-exchange column HR 10/10 (= Mono Q; Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden) by a combination of stepwise and discontinuous sodium chloride gradients (in buffer B) as indicated in Fig.1. 2-ml fractions collected at a flow rate of 0.5 ml/min. Fractions at salt concentrations of 0.1 M and 0.2 to 0.3 M NaCl containing isomerase activity were pooled, desalted on a PD 10 column (Pharmacia) into 0.05 M potassium phosphate, pH 6.8, containing of 1 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 10% (v/v) glycerol (buffer C), and subjected separately to glutathione (GSH)-affinity chromatography (GSH-Sepharose 4B, Pharmacia). The affinity-chromatography matrix (1 ml) was equilibrated in buffer (C) and the protein bound by incubation for 30 min at room temperature. Elution from a column was performed with 0.05 M Tris/HCl, pH 7.8, containing 1 mM EDTA, 5 mM GSH, 10% (v/v) glycerol. Fractions of 500 µl were collected and assayed for thiadiazolidine conversion.

To identify the major peptide responsible for isomerase activity protein fractions obtained by GSH-Sepharose 4B chromatography were subjected to sulfobromophthalein glutathione-affinity chromatography after change to buffer (C). Binding of more than 90% of the isomerase activity was obtained while about 50% of the protein was not retained. Elution was carried out as for GSH-affinity chromatography (see above). Active samples were used for the determination of thiadiazolidine specificity, atrazine and metazachlor conjugation as well as for CDNB turnover.

SDS-polyacrylamide gel electrophoresis; molecular weight calculation

Fractions obtained by affinity-chromatography from both safener-treated and control plants were analyzed for purity, molecular weight and qualitative amounts of different peptides by SDS-PAGE according to Laemmli (1970). The separating gel consisted of 15% acrylamide and was run for 6 h at 15 mA.

After GSH-affinity chromatography fraction no. 2 was loaded directly onto a Superose 12 column (Pharmacia, FPLC), equilibrated with 0.05 M potassium phosphate, pH 6.8, 1 mM DTT, 1 mM EDTA and 1 mM $MgCl_2$ to determine its native apparent molecular weight. The column was calibrated with cytochrome C (12 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase from yeast (150 kDa) and blue dextran (2,000 kDa). A flow rate of 0.5 ml/min was used and fractions of 1 ml were collected.

Assays

Thiadiazolidine conversion was determined by separation of the produced triazolidine from the thiadiazolidine by HPLC. The conversion reaction was carried out at 30°C for 30 min (unless otherwise stated) in a final volume of 200 µl. The standard assay contained 1 mM GSH, 0.1 mM thiadiazolidine no. **1** (for structure and number see Table III), buffered in 0.05 M potassium phosphate, pH

6.8. The reaction was stopped with two volumes of ethyl acetate followed by a 1-min centrifugation for complete phase separation. The upper triazolidine- and thiadiazolidine-containing phase was collected and the lower aqueous phase reextracted. Pooled extracts were dried under nitrogen, dissolved in acetonitrile (50 µl) and subjected to HPLC analysis using a C_{18} reversed-phase column (Nucleosil, 5µm; Macherey & Nagel, Düren, Germany). Acetonitrile/H_2O (3:2, v/v) was used as the mobile phase at a flow rate of 1 ml/min. The amount of triazolidine was determined by comparing the integrated areas of the eluted peaks with a calibration standard. Some non-enzymatic conversion was observed at basic pH but it was low at the pH used in the assay. (A total of 0.09 nmol triazolidine was formed per h starting with 0.1 mM thiadiazolidine).

CDNB turnover was measured spectrophotometrically by increase of absorbance at 340 nm with bovine liver GST (Sigma, Munich) as a positive control (Drotar *et al.*, 1985). The 1-ml assay mixture contained 100 µmol potassium phosphate, pH 7.0, 2 µmol CDNB, 2 µmol GSH and 50 µl of enzyme solution. Most of the CDNB-conversion activity was found in the protein fractions eluted from Mono Q at 0.1 M NaCl. Protein fractions were diluted 5-fold with potassium phosphate before assaying for CDNB activity. Inhibition of CDNB and thiadiazolidine turnover was determined by addition of inhibitor stock solutions in acetonitrile or water at start of the assay.

Atrazine conjugation was carried out according to Guddewar and Dauterman (1979) with slight modification. The assay mixture contained in a final volume of 200 µl 20 µmol potassium phosphate, pH 7.0, 1 µmol GSH, 0.1 µmol atrazine [^{14}C]atrazine diluted 1000-fold with unlabelled atrazine) and the protein sample obtained by affinity chromatography. The reaction mixture was incubated at 35 °C for 2 h and stopped by addition of 500 µl $CHCl_3$. The solution was extracted a second time with chloroform and the water phase (containing the GSH-conjugated atrazine) counted for radioactivity after mixing with Ultima Gold scintillation cocktail (Packard Instruments); conjugation of metazachlor was measured similarly. The 200-µl assay contained 20 µmol potassium phosphate, pH 7.0, 1 µmol GSH, 50 nmol metazachlor and protein as indicated and was incubated at 35°C for 2 h. Non-conjugated metazachlor was extracted twice with 500 µl hexane. Both phases were collected and counted for radioactivity. The values obtained in the presence of protein were corrected for the non-enzymatic conjugation determined without protein for both atrazine and metazachlor conjugation.

Protein concentration was measured after Bradford (1976) using bovine serum albumin as standard.

Peptide sequencing

Protein samples obtained from sulfobromophthalein GSH-agarose were desalted by PD 10 into ammonium bicarbonate buffer (5 mM, pH 7.0) and concentrated in a speedvac. The peptides were separated by SDS-PAGE on a 15% acrylamide gel and blotted to a PVDF membrane for 1 h according to LeGendre and Matsudaira (1989). Peptides were visualized by Coomassie staining, predominant bands cut off and subjected to automated Edman degradation (477A Protein sequencer, Applied Biosystems).

Chemicals and statistics

All chemicals used were of highest purity available; for HPLC analysis acetonitrile with an UV cut below 180 nm (J. T. Baker, Deventer, The Netherlands) was used. Sulfobromophthalein, sulfobromophthalein-GSH agarose and indomethacin were obtained from Sigma Chemicals, Munich, Germany. [Phenyl-U-^{14}C] metazachlor (11.6 mCi/mmol = 429 MBq/ mmol) was a gift from BASF AG, Limburgerhof, Germany. [^{14}C]-Atrazine (16 mCi/mmol = 592 MBq/mmol) was from Amersham Buchler, Braunschweig, Germany. Unless indicated otherwise the data documented in the tables represent mean values from three independent experiments, the figures show a typical experiment. The maximum standard deviation is given in the table legends.

Results and Discussion

Purification data

The purification scheme for both untreated (control) and NA(safener)-treated corn seedlings

Table I. Purification of thiadiazolidine-converting activity from naphthalic anhydride-treated (+NA) and non-treated corn seedlings (control).

Fraction	Total activity [units]		Specific activity [units/mg protein]		Purification x-fold	
	Control	(+) NA	Control	(+) NA	Control	(+) NA
(a) Ammonium sulfate precipitate	7780	19740	6	11	1	1
(b) Hydroxyapatite	9000	15840	71	18	12	6
(c) Mono Q						
0.1 M NaCl (1)	460	840	28	23	5	2
0.2 M NaCl (2)	1290	5600	37	56	6	5
(d) GSH-affinity (1)	158	322	350	428	58	38
GSH-affinity (2)	276	500	13200	25250	2200	2360
(e) SB-affinity (2)	182	303	27600	60600	4600	5660

A unit is defined as 1 nmol of 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thione formed per hour (= product of compound no. **1**, see Table III). The table represents a typical experiment. Purification factors refer to the ammonium sulfate precipitate. The number in parenthesis denote the fraction referring to the activity peaks obtained after separation on Mono Q of Fig. 1. At start the standard assay contained 1 mM glutathione, 0.1 mM thiadiazolidine.

is shown in Table I. Isomerase activity *in vitro* could first be assayed after concentration of a homogenate by a two-step ammonium sulfate precipitation. Using thiadiazolidine no. **1** a specific activity of 5–10 nmol of the triazolidine produced per mg protein x h was found in the second salt precipitate (45–75%) prepared from nontreated plants (Table I). Both the total and specific activity was enhanced in NA-treated corn. We also assayed other fractions for thiadiazolidine conversion, such as thylakoids and membrane debris obtained by differential centrifugation but could not detect any substantial conversion rate (data not shown). Accordingly, the 45–75% ammonium sulfate precipitate was purified further representing 80% of total activity. The crude extract was chromatographed on a hydroxyapatite column giving about a 15-fold enrichment at maximum for untreated plants. Isomerase activity was not retained by the material and a slight increase in total activity was found which may reflect the loss of inhibiting factors by the first purification step as discussed by Guddewar and Dauterman (1979). Two further steps in sequence allowed purification and identification of the GST isoform mainly responsible for thiadiazolidine conversion. Mono Q anionic exchange chromatography revealed that at least two activities were present in extracts from corn seedlings which contribute to a different extent to thiadiazolidine isomerization. One activity peak eluted at 0.1 M NaCl, the second peak in a concentration range of 0.25–0.3 M NaCl (Table I, Fig. 1). These peaks are called fraction 1 (low salt) and 2 (high salt), referring to 0.1 M and 0.25 M NaCl, respec-

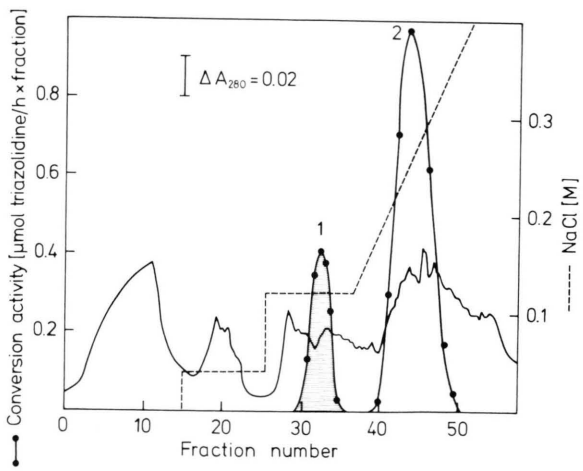


Fig. 1. FPLC elution profile of Mono Q chromatography of thiadiazolidine-converting activity (●–●) from a hydroxyapatite-purified fraction of corn seedlings treated with 0.1 mM naphthalic anhydride. 40 mg of protein were loaded and eluted by a stepwise and continuous NaCl gradient (– – –). Straight line = 280-nm absorbance. Fraction volume 2 ml; enzyme activity was determined as described in Materials and Methods.

tively. Fraction 1 contained about 50–65% of the activity of fraction 2.

Activity of fraction 2 increased **vs.** activity of fraction 1 by treatment of the seedlings with NA (Fig. 1 shows an elution profile obtained by safener treatment). Generally, isomerase activity in the low-salt fraction increased 2-fold only while activity 2 was enhanced up to 5- fold. Because of the split of the activity (see line c of Table I) a decrease in the enrichment factor was found by anion-exchange chromatography but this step separated isoforms with thiadiazolidine-converting activity as demonstrated by SDS-PAGE (see below).

Previous experiments have shown that isomerase activity was dependent on thiol containing agents such as DTT, thioglycollate or GSH (Iida *et al.*, 1995; Shimizu *et al.*, 1994). These results suggested a thiol as a cofactor for the reaction. Therefore, GSH-affinity chromatography should be advantageous for further purification. Both fractions of activity were applied individually to GSH Sepharose and quantitative binding was obtained. Although this purification step was accompanied by a high loss of activity it resulted in the best single-step purification, represented by both the enrichment factor as well as the protein pattern analyzed by SDS-PAGE (Fig. 2, lane 2 and 3, where only three or four bands were present). The specific activities of fraction 1 and 2 clearly demonstrate that the main thiadiazolidine-converting activity is attributed to the peptides found in fraction 2. Separation by SDS-PAGE revealed that fraction 1 (lane 2) differed markedly from fraction 2 (lane 3) with respect to protein content and apparent molecular weight of the respective peptides although comparable activities had been loaded. Four dominant peptides of which two were present in a similar quantity were detected in fraction 2, with apparent molecular weights from 25–31 kDa. Similar molecular weights were obtained for the peptides of fraction 1 but one peptide of about 30 kDa was dominant. A purification of nearly 95% is estimated for the latter fraction. Calculation of the *native* molecular weight of isomerase activity from fraction 2 by gel filtration resulted in a molecular weight of 60 ± 3 kDa (data not shown). Therefore, the isomerase exists either as a homodimer or as a heterodimer of two peptides of similar size which is consistent with the protein

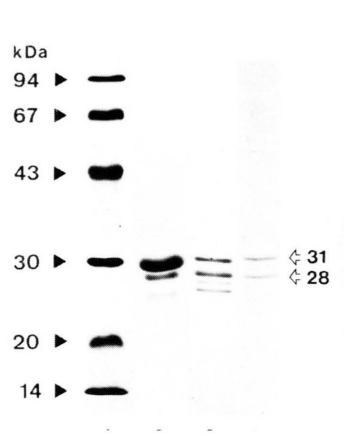


Fig. 2. SDS-PAGE of various fractions from corn seedlings obtained after purification as described in Materials and Methods. Lane 1, marker protein; lane 2, fraction 1 after GSH-affinity chromatography; lane 3, fraction 2 after GSH-affinity chromatography; lane 4, fraction 2 additionally purified by sulfobromophthalein-GSH affinity chromatography.

pattern known for all the GSTs identified yet (Miller *et al.*, 1994; Moore *et al.*, 1986; Mozer *et al.*, 1983; Timmerman, 1989).

Our main interest was to find those peptides which contribute to the main activity found in fraction 2 and to further characterize both fractions. Purification of fraction 2 was continued with a second GSH-affinity chromatography, SB-GSH agarose, described as a matrix for separation of different GST isoforms (32). GST I and II, but not the isoforms III and IV, have been reported to be retained by SB-GSH agarose (Fuerst *et al.*, 1993; Mozer *et al.*, 1983; O'Connell *et al.*, 1988). An additional 2-fold purifica-tion was obtained by binding the isomerase activity to the material indicating that GST I or II catalyze isomerization of thiadiazolidines. The proteins are shown in Fig. 2, lane 4, for a fraction obtained from safener-treated corn.

Influence of sulfhydryl reagents

For more information about the isomerization reaction we examined the influence of sulfhydryl reagents on the thiadiazolidine-converting activity in purified fractions from corn seedlings (Table II). In absence of a thiol almost no formation of a corresponding triazolidine (in our case 4-(4-bromophenyl)-1,2-tetramethylene-1,2,4-triazolidine-3-

Table II. Influence of sulfhydryl reagents on thiadiazolidine converting activity of purified fractions from corn seedlings.

Additions	Specific activity [nmol triazolidine formed from thiadiazolidine no.**1**/mg protein x h]*)	
	Fraction 1	Fraction 2
Control, (−) SH reagent	28	822
(+) Glutathione		
0.1 mM	232	10720
0.5 mM	352	13440
1.0 mM	369	13200
5.0 mM	376	10800
(+) DTT		
1 mM	196	2100
5 mM	358	9800
(+) 1 mM NEM	42	1230
(+) 10 mM NEM	15	820
(+) 10 mM DTNB	17	760
(+) 5 mM GSH, 1 mM DTT	258	4600

Fraction 1, 2 refer to affinity chromatography fractions eluted with 0.1 M and 0.2–0.3 M NaCl from Mono Q (see Table I), respectively. Fractions were incubated for 30 min with the compounds indicated before starting the assay by addition of 0.1 mM thiadiazolidine no. **1**. A heat-inactivated aliquot was included into each assay. For determination of inhibition by NEM and DTNB, 0.1 mM GSH was added after the preincubation to start the assay. Reaction time was 2 h at 30 °C. Maximum standard deviation was less than ± 15%.
*) See Table III for structure.

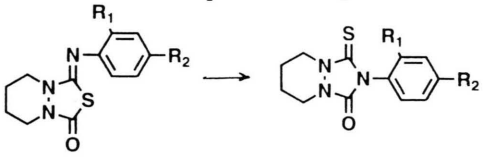
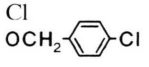
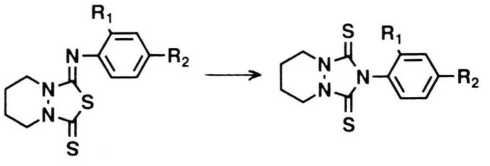
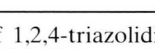
one-5-thione) was detected. This is in agreement with data reported earlier about the isomerization characteristics in crude extracts (Iida *et al.*, 1995; Shimizu *et al.*, 1994). The little activity obtained for both fractions is most likely due to remaining DTT in the protein extract after buffer exchange to a DTT- free buffer. Isomerase activity was dramatically enhanced by addition of GSH with an optimum concentration range of 0.5–1 mM. DTT could substitute GSH by a certain extent but a higher concentration was necessary. Both NEM and DTNB strongly inhibited isomerase activity. These chemicals indicate either the involvement of the protein sulfhydryl groups in the isomerization process or the necessity for thiols as a cofactor. Therefore DTT was included in all buffers used for purification and isomerase activity assayed routinely in the presence of 1 mM GSH.

Thiadiazolidine conversion specificity

Thiadiazolidine conversion *in vivo* was dependent on the 5-arylimino moiety catalyzed by *Echinochloa utilis* seedlings (Sato *et al.*, 1994). While some of the compounds, e. g. no. **1** used in this study and the closely related chlorophenyl analogue (compound no. **2** of Table III), were isomerized rapidly, others had twice this half-life time. Thiadiazoline no. **4** and the thiadiazolidine-2-thiones were not isomerized (Iida *et al.*, 1995). Provided one enzyme or an enzyme family are involved in the isomerization, the reaction characteristics obtained *in vivo* should be reflected by the purified enzyme. So, we checked whether a structure-activity relationship observed for *Echinochloa* or spinach homogenate is also found with the fractions purified by affinity-chromatography from corn (Table III A). Although the specific activities of fraction 1 and 2 differed the pattern of the conversion rates was similar in both fractions and are in complete agreement with the *in vivo* data we reported previously (Iida *et al.*, 1995; Sato *et al.*, 1994). Triazolidine-3,5-dithiones as isomerization products of thiadiazolidines-2-thiones were barely detected; some other metabolites were present in low amounts but have not been identified yet. No alteration in specificity was observed whether extracts from control or safener-treated plants were used (data not shown). Obviously, thiadiazolidine specificity is common for several isoforms of the isomerase present in intact seedlings or plant homogenates.

It has been shown previously that equine and bovine GST can convert thiadiazolidines into triazolidines (Iida *et al.*, 1995). Provided thiadiazolidine isomerization is catalyzed by a GST in plants, the GST from bovine may exhibit a similar specificity for thiadiazolidines as the purified isomerase from corn. Therefore bovine liver GST was assayed whether its specificity was similar to the plant enzyme focussing on the *N*-aryl residue of the compounds (Table III). The three thiadiazolidines, nos. **1**, **2** and **3**, were converted with high activity by fraction 2 while fraction 1 exhibited very low activity. Fraction 2 from safener-treated plants was 5 to 30-fold more active than the bovine enzyme. Neither no. **4** nor the thiadiazolidine-2-thiones (nos. **5–8**) were converted substantially by all three enzyme samples assayed.

Table III. Comparison of thiadiazolidine-conversion specificity of two purified fractions from corn seedlings with bovine glutathione S-transferase.

Compound			Specific activity [unit / mg protein]		
			Fraction 1	*Fraction 2*	*Bovine GST*
A. Thiadiazolidin-2-ones					
No.	R_1	R_2			
1	H	Br	306	62930	2121
2	H	Cl	306	77980	1889
3	CH_3	Cl	194	26970	2721
4	H		3	3564	308
B. Thiadiazolidine-2-thiones					
No	R_1	R_2			
5	H	Br	3	4774	43
6	H	Cl	3	2332	155
7	CH_3	Cl	2	n. d.	23
8	H		1	n. d.	178

A unit is defined as 1 nmol of 1,2,4-triazolidin-3-one-5-thione, the product of group (A) compounds or 1,2,4-triazolidine-3,5-dithione, the product of group (B) compounds, formed per hour. The table represents a typical experiment. Corn seedlings were treated with 0.1 mM NA during growth and prepared as described. Fractions 1, 2 refer to the activity obtained after elution from Mono Q at 0.1 M and 0.2 M NaCl, respectively, followed by GSH-affinity chromatography. Reaction time was 2 h at 30°C; n. d., not detectable. Numbers 1–8 refer to the thiadiazolidines used at start of the conversion expts.

Protein pattern, dependence of isomerase activity on GSH, as well as the activity profile for different thiadiazolidines in comparison to bovine GST strongly support earlier findings that the isomerase activity is attributed to a GST (Shimizu *et al.*, 1994). GSTs belong to an enzyme family that catalyze the nucleophilic attack of GSH to electrophilic substrates (Cole, 1994; Timmerman, 1989). They exist as polypeptide dimers, each polypeptide of a molecular weight of 20 to 30 kDa (Jepson *et al.*, 1994), which agrees with the protein pattern obtained for both fractions of thiadiazolidine isomerase activity. Because a variety of isoenzymes exists differing in their substrate specificity, a broad range of substrates are recognized by plants determined by the set of GSTs present in the cell (Cole, 1994; Dean *et al.*, 1991). Isoenzymes can be differentiated by their acceptance of substrates, their purification characteristics, apparent molecular weight and the amino-acid sequence of their subunits (Dean *et al.*, 1991; Fuerst *et al.*, 1993; Timmerman, 1989). It has been pointed out that several isoenzymes were only detected when other substrates than the experimental model substrate CDNB are used (Dean *et al.*, 1991; Fuerst *et al.*, 1993). So, thiadiazolidine isomerization could be either a new side reaction of a known GST isoform or representing the activity of an isoform not yet detected due to lack of activity for common GST substrates. To prove this assumption we in-

Table IV. Specificity of purified thiadiazolidine-converting activity from corn for GST substrates.

Substrate	Specific activity Fraction 1	Fraction 2
Thiadiazolidine no.**1**	327 nmol/mg protein x h	13227 nmol/mg protein x h
CDNB	4894 μmol/mg protein x h	2642 μmol/mg protein x h
[^{14}C]Metazachlor	2.1 nmol/mg protein x min	90 nmol/mg protein x min
[^{14}C]Atrazine	not detectable	not detectable

Metazachlor conjugation was determined by hexane extraction of the assay mixture after a 2-h reaction time in presence of fractions purified by GSH-affinity chromatography (lines **d** of Table I) and measurement of radioactivity in the hexane (metazachlor) and water (GSH-metazachlor) phase, respectively. For determination of atrazine conjugation the assay mixture was extracted twice with chloroform and the water phase counted for radioactivity. Protein content was 10 μg and 2 μg for fraction 1 and 2, respectively, with the exception of the CDNB assay, in which 1 μg of fraction 1 was used. For reaction volume see "Assays" in Materials and Methods. Standard deviation was 28% for metazachlor conjugation and less than 15% for CDNB and thiadiazolidine conversion. Thiadiazolidine no.**1**, 0.1 mM (see Table III for structure); metazachlor, 0.25 mM; atrazine, 0.5 mM; CDNB, 2 mM.

vestigated whether both purified fractions can utilize typical GST substrates and moreover, whether GST inhibitors also influence thiadiazolidine conversion. Substrates for different isoforms in plants are triazines, chloroacetamides, CDNB as the most common model substrate, and *trans*-cinnamic acid.

Specificity for GST substrates

Atrazine, metazachlor and CDNB were chosen to assay GST activity in isomerase fractions (Table IV). Both fractions contained GST activity but their activity for CDNB, metazachlor and thiadiazolidine no. **1** differed markedly. A high rate of CDNB conjugation was measured in fraction 1 while the specific activity for metazachlor was considerably higher in fraction 2. CDNB is known to be a substrate conjugated rapidly by GST I (Dean *et al.*, 1991; Fuerst *et al.*, 1993; Mozer *et al.*, 1983). Fraction 2 showed less CDNB conversion in comparison to fraction 1 (depending on the preparation we found 50–80% of the turnover measured for fraction 1) which can be interpreted either by a single isoform highly effective in chloroacetamide and thiadiazolidine conversion with little specificity for CDNB or by different isoenzymes within one fraction contributing independently to the turnover of the substrates examined. Specific CDNB-conversion activity of fraction 2 remained constant with further purification (on an SB-affinity column, see Table I). Atrazine conjugation was not detected indicating the absence of the so-called atrazine-dependent GSTs in both fractions (see below and refs. Guddewar and Dauterman, 1979; Timmerman, 1989).

GST *inhibitors and isomerase activity*

Inhibition experiments gave conclusive proof that GST activity present in our purified fractions matches with isomerase activity and that identical enzymes exhibit both isomerase and GST activity (Table V, A). Various GST inhibitors were assayed and as a control the effect of inhibitors on CDNB turnover was determined (Table V, B). It has been reported that GST isoenzymes are differently inhibited by various inhibitors thereby facilitating the identification of isoforms (Lamoureux and Rusness, 1986). In our experiments all inhibitors used affected isomerization with sulfobromophthalein being the most effective on both thiadiazolidine and CDNB turnover. Inhibition by SB confirms the purification characteristics of fraction 2, since binding of activity on SB/GSH-affinity chromatography was obtained. Tridiphane showed weak inhibition of thiadiazolidine conversion even at high concentrations, which is consistent with the absence of atrazine-conjugating activity in fraction 2. Tridiphane, or more specific, a GSH conjugate of tridiphane, was found a strong inhibitor for at-

Table V. Influence of inhibitors of glutathione-S transferase on thiadiazolidine isomerization and CDNB conjugation in purified fractions from corn seedlings.

Conversion of thiadiazolidine no.**1***)	
Addition	Specific activity [μmol triazolidine formed from thiadiazolidine no.**1** per mg protein and hour]
Thiadiazolidine, 0.1 mM	73
(+) Tridiphane, 0.1 mM**)	51
(+) Hexylglutathione,	
0.5 mM	55
2.0 mM	42
(+) Protoporphyrin IX, 0.2 mM	57
(+) Indomethacin, 0.05 mM	48
(+) Sulfobromophthalein, 0.05 mM	26

B. Conversion of CDNB	
Addition	Specific activity [μmol CDNB/mg protein x h]
CDNB, 2 mM	4200
(+) Tridiphane, 0.1 mM	3240
(+) Hexylglutathione, 0.5 mM	2910
(+) Indomethacin, 0.05 mM	1370
(+) Sulfobromophthalein, 0.05 mM	546

*) See Table III for structure.
) Inhibition by tridiphane was less (10%) if the assay was carried out without GSH but in presence of 5 mM DTT. Because NA-treated seedlings were used CDNB conjugation was higher than the data given in Table IV. The maximum standard deviation for Part A and B was 31 and 23%, respectively. Reaction time for conversion of thiadiazolidine no.1** and CDNB was 1 h and 5 min at 30°C, respectively. Fraction 2 purified by SB-affinity chromatography was assayed for thiadiazolidine and CDNB conversion; the assay included a protein content of 0.5 and 2 μg, respectively.

razine conjugation and CDNB reaction by GSTs (Lamoureux and Rusness, 1986). Most likely the small inhibition found under our standard assay conditions is caused by a tridiphane-GSH complex formed by GST activity during the reaction time. We did not distinguish between tridiphane and tridiphane-GSH but inhibition of isomerization by tridiphane was even less when the assay was performed in the absence of GSH (in this case the thiol group was provided by DTT since it does not conjugate with tridiphane). Comparable results were obtained for inhibition studies with fraction 1 (data not shown). It is concluded that atrazine-dependent GSTs are absent both in fraction 1 and 2. Because CDNB turnover in fraction 2 was relatively slow per volume the protein content in the assay for CDNB turnover was increased compared to the thiadiazolidine conversion experiments. This should facilitate tridiphane-GSH formation in a shorter time but nevertheless CDNB turnover was poorly affected by tridiphane.

Conclusions

GST I from corn has been described to be present constitutively with some increase after treatment of seedlings with safeners. It elutes at lower salt concentrations than other isoforms (II, III, IV) from anionic exchange columns and exist as a homodimer of 2 x 29 kDa (Fuerst *et al.*, 1993; Jepson *et al.*, 1994; Moore *et al.*, 1986; Mozer *et al.*, 1983; Timmerman, 1989). We conclude that fraction 1 contains GST I (Fig. 2, lane 2) which contributes to thiadiazolidine isomerization to a minor extent.

To confirm that fraction 1 is GST I, we reproduced the purification procedure of Mozer *et al.*, 1983 and Fuerst *et al.*, 1993 and compared CDNB conjugation and thiadiazolidine conversion (data not shown). Fraction 1 corresponded to GST I while fraction 2 was found to elute comparable to GST C or D according to Fuerst *et al.* (1993). Activity peaks C and D have been reported to be resolved incompletely, therefore overlapping activity profiles were obtained for CDNB and metolachlor representing contamination with different isoforms. Immunoblotting with antisera raised against GST I and III revealed the presence of multiple isoenzymes in those fractions previously designated GST I and II. From the utilization of different substrates as well as from the response to safener treatment it was concluded that peak C and D refers to GST II and III, and peak D refers to GST IV. GST III and IV are constitutively present with an activity increase by safener treatment (Fuerst *et al.*, 1993; Miller *et al.*, 1994). GST II was only detectable by CDNB conjugation after safener treatment (Mozer *et al.*, 1983). GST III and IV are the isoforms which contribute mainly to chloroacetamide tolerance in corn because of their high activity in alachlor and metolachlor conjugation. Although isomerase activity in fraction 2 is constitutively present, is enhanced by NA treatment, and coincided with metazachlor activity, GST III and IV are unlikely to be the instrumental isoforms. Firstly, the apparent molecular weights of the two main peptides in fraction 2 are different from those reported for GST III and IV which have a reported molecular weight of 25 and 27 kDa, respectively (Miller *et al.*, 1994; Moore *et al.*, 1986; O'Connell *et al.*, 1988). Secondly, isomerase activity was bound to SB-agarose, which is inconsistent with the purification characteristics, namely that GST III and IV are *not* retained on this matrix. Although different chromatography conditions may influence binding the inhibition of thiadiazolidine conversion by SB demonstrates a binding of the inhibitor to the enzyme (Table V) which corresponds with retention of the isomerase by SB-coupled agarose. Since fraction 2 eluted under similar conditions as a peak C containing both GST II and III according to Fuerst *et al.* (1993) we may assume that thiadiazolidine conversion is catalyzed by GST II. GST II was shown to utilize the chloroacetamide alachlor and was identified as a heterodimer of 27 and 29 kDa polypeptides (Fuerst *et al.*, 1993; Jepson *et al.*, 1994; Moore *et al.*, 1986; O'Connell *et al.*, 1988). This would be conclusive with our observations with the exception that our apparent molecular weights were slightly larger and thiadiazolidine isomerase was constitutively present. It is possible that a low GST II activity is constitutively present in corn but is detectable only by its (high) isomerase activity. When assayed by CDNB conjugation GST II activity can be determined in preparations from safener-treated seedlings only.

15 N-terminal amino acids from the 31-kDa peptide were sequenced and gave complete homology to GST I as reported (Met-Ala-Pro-Met-Lys-Leu-Tryp-Gly-Ala-Val-Met-Ser-Trp-Asn-Val; comp. ref. Shah *et al.*, 1986) exactly as has been shown for the 29-kDa peptide of GST II (Jepson *et al.*, 1994; Moore *et al.*, 1986; Timmerman, 1989). The second peptide of GST II purified from plants has been reported to be resistant to Edman degradation and we also failed to sequence our 28-kDa peptide because of N-terminal blockage. Jepson *et al.* (1994) recently reported the nucleotide sequence of a cDNA fragment encoding for the 27-kDa subunit of GST II. It has been discussed that different isoforms are formed by new association of subunits resulting in new substrate specificity. The GST-II subunits form the homodimers of GST I and IV, respectively (Jepson *et al.*, 1994; Miller *et al.*, 1994). Firstly, thiadiazolidine isomerase could consist of a subunit homologue of GST I and of the second peptide of the GST-II fraction (Jepson *et al.*, 1994; Mozer *et al.*, 1983). It is, secondly, conceivable that the safener induces new peptides which combine with peptides present constitutively thereby increasing thiadiazolidine conversion. Thirdly, at the moment it can not be excluded that *only* the 28-kDa peptide catalyzes thiadiazolidine isomerization.

It could be shown that thiadiazolidine isomerization is catalyzed by different isoenzymes of GSTs in corn. The reaction differs from typical GST reactions so far that apparently GSH is not conjugated to the herbicide as intermediate but acts as a cofactor of the isomerization (see Iida *et al.*, 1995; Sato *et al.*, 1994). Our findings indicate that one isoenzyme is mainly responsible for thiadiazolidine conversion having characteristics as those described for GST II. Although NA treat-

ment increased isomerase activity, a constitutive conversion activity is present in corn seedlings. However, in untreated plants GST II or the respective polypeptides present in this fraction can only be detected by using high-affinity substrates like our thiadiazolidines.

It would be of interest whether weeds and crop differ in their ability to activate peroxidizing compounds, depending on the presence of the respective GST isoform. This may allow the development of thiadiazolidines which are converted more rapidly in weeds than in the crop possibly in combination with a safener enhancing bioactivation of the pro-herbicide in weeds only. Future studies should include the examination of GST response to different isoimide type compounds on the molecular level as well as the design of thiadiazolidine-triazolidine couples being markedly different in their inhibitory activity on protox.

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