Glutathione S-Transferase Activity in Nontreated and CGA-154281-Treated Maize Shoots*

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Fast protein liquid chromatography (anion exchange) was used to separate glutathione S-transferase isozymes in nontreated etiolated maize shoots and those treated with the herbicide safener CGA-154281 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine. Nontreated shoots contained isozymes active with the following substrates: *trans*-cinnamic acid (1 isozyme), atrazine (3 isozymes), 1-chloro-2,4-dinitrobenzene (1 isozyme), metolachlor (2 isozymes) and the sulfoxide derivative of S-ethyl dipropylcarbamothioate (2 isozymes). Pretreatment of shoots with the safener CGA-154281 (1 μM) had no effect on the activity of the isozymes selective for *trans*-cinnamic acid and atrazine but increased the activity of the constitutively-expressed isozymes that exhibit activity with 1-chloro-2,4-dinitrobenzene, metolachlor and the sulfoxide derivative of S-ethyl dipropylcarbamothioate. The safener pretreatment also caused the appearance of one new isozyme active with 1-chloro-2,4-dinitrobenzene and one new isozyme active with metolachlor. The results illustrate the complexity of glutathione S-transferase activity in etiolated maize shoots, and the selective enhancement of glutathione S-transferase isozymes by the safener CGA-154281.

Introduction

GST enzymes are dimeric, multifunctional proteins that conjugate a wide variety of electrophilic, hydrophobic compounds to GSH [1, 2]. The enzymes are found in mammals [1, 2], insects [3], and plants [4–21] and are thought to play a major role in the detoxification of xenobiotics. In most organisms studied, GSTs have been found to exist in multiple forms [1, 3, 21]. In the cytosol of rat liver, more than ten GST isozymes have been detected [2]. These isozymes are due to the expression of multiple genes and the differential hybridization of subunits to form hetero- and homodimers [1, 2].

It is well established that GSTs play an important role in the detoxification of certain herbicides in plants [4, 7–14, 16, 18, 21]. Maize is tolerant to atrazine primarily because it contains GST (atrazine) activity that catalyzes the conjugation of the herbicide to GSH [18, 20, 21]. GSTs also play a role in the tolerance of maize to alachlor and EPTC [10, 15, 17], and the tolerance of sorghum to metolachlor [4, 13].

Herbicide safeners (also referred to as herbicide antidotes) are chemical compounds used to protect certain grasses, in particular maize and sorghum, from injury by thiocarbamate (*e.g.* EPTC) and chloroacetanilide (*e.g.* metolachlor, alachlor) herbicides [12, 22]. Evidence suggests that safeners act by enhancing GST activity and thereby accelerating herbicide metabolism in the protected plant [4, 13, 22]. Pretreatment with the dichloroacetamide safener, dichlormid, caused a two-fold increase in GST (EPTC-sulfoxide) activity in crude extracts from maize roots [10, 15]. Dichlormid, as well as other safeners, have been shown to

Abbreviations: GST, glutathione S-transferase activity; GSH, reduced glutathione; GST (atrazine), GST activity measured with atrazine as substrate; EPTC, S-ethyl dipropylcarbamothioate; EPTC-sulfoxide, the sulfoxide derivative of EPTC; GST (EPTC-sulfoxide), GST activity measured with EPTC-sulfoxide as substrate; GST (alachlor), GST activity measured with alachlor as substrate; GST (metolachlor), GST activity measured with metolachlor as substrate; CGA-154281, 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine; PVPP, polyvinylpolypyrrolidone; CDNB, 1-chloro-2,4-dinitrobenzene; FPLC, fast protein liquid chromatography; GST (CDNB), GST activity measured with CDNB as substrate; GST (*trans*-cinnamic acid), GST activity measured with *trans*-cinnamic acid as substrate.

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increase GST (alachlor) activity in maize [16, 21] and GST (metolachlor) activity in sorghum [4, 13]. In both maize and sorghum, this increase in GST activity is due to the induction of GST isozymes by the safeners.

CGA-154281 (Fig. 1) is a new dichloroacetamide herbicide safener that protects maize from injury by the chloroacetanilide herbicide metolachlor [23]. The safener accelerates the detoxification of metolachlor in maize shoots by increasing GST (metolachlor) activity [23].

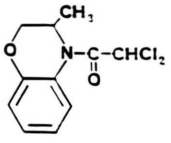

Fig. 1. Structure of CGA-154281.

The objectives of this study were to: 1) isolate GST isozymes from etiolated maize shoots using FPLC-anion exchange chromatography; 2) characterize the specificity of the isozymes using several substrates (CDNB, metolachlor, atrazine, EPTC-sulfoxide, *trans*-cinnamic acid); and 3) determine the effect of the herbicide safener CGA-154281 on the activity of GST isozymes in etiolated maize shoots.

Materials and Methods

Chemicals

[U-^{14}C]metolachlor (2-chloro-N-(ethyl-6-methyl-phenyl)-N-(2-methoxy-1-methylethyl) acetamide), specific activity, 21.5 μCi/μmol, CGA-154281 (4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine) were provided by CIBA-GEIGY Corp. (Greensboro, NC). N-(1-^{14}C-propyl) EPTC (S-ethyl dipropylcarbamothioate) with a specific activity of 35 μCi/μmol was provided by ICI Americas Inc. (Mountain View, CA). [^{14}C]-EPTC-sulfoxide was prepared and purified as described by Casida *et al.* [24]. [3-^{14}C]-*trans*-cinnamic acid was purchased from Research Products International Corp. (Mount Prospect, IL). GSH was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Plant material

Maize (*Zea mays* L. 'Pioneer 3906') seeds were planted in plastic trays containing vermiculite and watered with either deionized water or a 1 μM aqueous solution of CGA-154281 (500 ml solution per 750 ml dry vermiculite). The trays were covered with aluminum foil and the seeds were germinated and grown for 3 days in the dark at 30 °C and 70% RH. Two-cm apical sections of the etiolated shoots were excised and stored in liquid N_2 until extracted.

FPLC-anion exchange chromatography

Twenty-five frozen (−196 °C) 2.0-cm apical sections were ground to a fine powder using a mortar and pestle. PVPP (1.25 g) and 25 ml of extraction buffer (0.2 M Tris-HCl, pH 7.8) were added to the powder and the slurry was again briefly ground. All other procedures for FPLC-anion exchange chromatography were as described by Dean *et al.* [4]. The elution profiles presented represent typical results of experiments repeated two or more times.

GST activity

GST (CDNB) activity and GST (metolachlor) activity were measured as described by Gronwald *et al.* [13]. GST (EPTC-sulfoxide) activity was measured by adding 15 μl of 6.8 mM [^{14}C]EPTC-sulfoxide (specific activity, 0.75 μCi/μmol) in ethanol to 60 μl of 0.1 M potassium phosphate buffer (pH 6.8), 75 μl of the enzyme fraction, and 7.5 μl of 182 mM GSH. GST (*trans*-cinnamic acid) activity was measured by adding 50 μl of 1.2 mM [^{14}C]*trans*-cinnamic acid (specific activity, 1.84 μCi/μmol) in water to 50 μl of 0.2 M Tris-HCl (pH 7.5), 70 μl of the enzyme fraction and 5 μl of 175 mM GSH. GST (atrazine) activity was measured by adding 20 μl of 0.2 mM [^{14}C]atrazine (specific activity, 4.5 μCi/μmol) in water to 80 μl of 100 mM MES; pH 6.4, 80 μl of the enzyme fraction, and 20 μl of 100 mM GSH. BSA was included in the GST (atrazine) assay medium at a final concentration of 3% (w/v). All assay solutions [excluding the GST (CDNB) assays] were incubated for 1 to 2 h at 30 °C. Assays were terminated by the addition of trichloroacetic acid or dichloromethane (2 X volume). Assay solutions were extracted with dichloromethane and the radioactivity remaining in the aqueous phase was quantified

by liquid scintillation spectrometry. The water soluble product of the GST (EPTC-sulfoxide) assay was identified as the glutathione conjugate of EPTC-sulfoxide on the basis of co-chromatography with an authentic standard on silica gel TLC plates (LK 60 F Whatman) using the solvent systems described by Lay and Casida [15]. The water soluble product of the GST (*trans*-cinnamic acid) assay was identified as the glutathione conjugate of *trans*-cinnamic acid based on co-chromatography with an authentic standard using HPLC [C-18, reverse-phase, acetonitrile gradient (20–80%) containing 0.05% H_3PO_4] and TLC [silica gel plates (LK60 F Whatman) developed with 1-butanol:acetic acid:water (2:1:1, v/v/v)]. Previous studies with maize had identified the water soluble conjugate formed during the GST (atrazine) and GST (metolachlor) assays as the glutathione conjugate of the herbicide [7, 11, 25].

Results

The FPLC (anion exchange) elution profiles of GST activity from nontreated and CGA-154281-treated etiolated maize shoots are shown in Fig. 2. Because the same fractions had activity with more than one substrate, the activity peaks were numbered separately for ease of identification and discussion. The GST activity from nontreated maize shoots contained one peak of GST (*trans*-cinnamic acid) activity (peak 1) and one peak of GST (CDNB) activity (peak 3) (Fig. 2A). At least three peaks of GST (atrazine) activity (peaks 6, 7 and 11) were observed as well as two peaks of GST (EPTC-sulfoxide) (peaks 4 and 9) and GST (metolachlor) (peaks 5 and 10) activity.

The elution profile for GST activity from CGA-154281-treated maize shoots revealed that safener treatment has little or no effect on GST (*trans*-cinnamic acid) activity (peak 1) or the three peaks of GST (atrazine) activity (peaks 6, 7 and 11) (Fig. 2B). The GST (CDNB) peak (peak 3) in the CGA-154281-treated tissue was increased 1.7-fold over the same peak in the nontreated tissue, and at least one new GST (CDNB) peak (peak 8) was induced by the safener treatment. The GST (EPTC-sulfoxide) and GST (metolachlor) activities of peaks 4 and 5 were increased approximately 5- and 7-fold, respectively, in response to safener treatment. The GST (EPTC-sulfoxide) and the GST

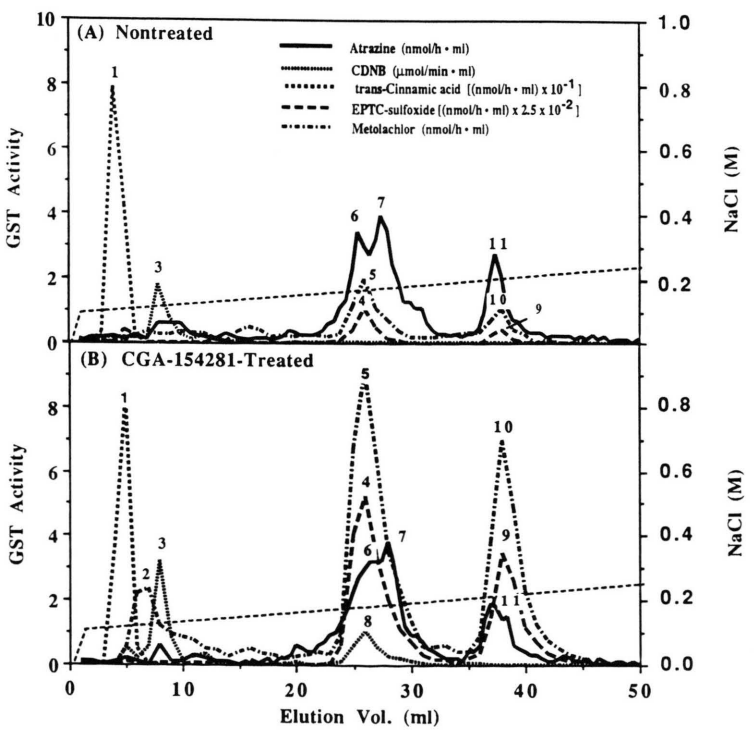

Fig. 2. FPLC-anion exchange (Mono-Q) chromatograph of GST activities, using various substrates, from nontreated (A) or CGA-154281-treated (B) etiolated, 3 day old maize shoots. The GST (*trans*-cinnamic acid) and the GST (EPTC-sulfoxide) activities measured in the fractions were 10- and 40-fold higher, respectively, than the values plotted. See Materials and Methods for experimental details.

(metolachlor) activities of peaks 9 and 10 were increased approximately 7- and 10-fold, respectively, in response to safener treatment. At least one new peak of GST (metolachlor) activity (peak 2) was seen in the safener-treated maize shoots. In all replications of the elution profiles, the GST (EPTC-sulfoxide) and GST (metolachlor) activities of peaks 4 and 5 always coincided, and these activities were enhanced in response to the safener. Likewise, the GST (EPTC-sulfoxide) and GST (metolachlor) activities of peaks 9 and 10 always coincided and both were increased in response to the safener treatment. Although two peaks active with atrazine (peaks 6 and 11) co-eluted with peaks active with EPTC-sulfoxide (peaks 4 and 9) and metolachlor (peaks 5 and 10), it appeared that the GST (atrazine) peaks were distinct. In contrast to those peaks active with EPTC-sulfoxide or metolachlor, the peaks active with atrazine were not enhanced by safener treatment.

The activities of the various GST peaks exhibited a wide range. As expected, the highest activities were found for the substrate CDNB. The activity of peak 1 for *trans*-cinnamic acid was quite high as well. Of the herbicide substrates, the GST (EPTC-sulfoxide) peaks had the highest activity. This probably reflects the high concentration of EPTC-sulfoxide (0.65 mM) used in the assays compared to atrazine (20 μM) and metolachlor (5 μM).

Discussion

On the basis of activity with five different substrates (three herbicides, two non-herbicides), it is apparent that both nontreated and safener-treated etiolated maize shoots contain several different GST isozymes (Fig. 2A and B). On the basis of reactivity with five substrates, at least nine peaks of GST activity were detected in nontreated shoots and at least eleven in safener-treated shoots.

The results of this study clearly show that treatment with CGA-154281 causes a selective enhancement of GSTs in etiolated maize shoots. While the safener increased GST (CDNB) activity of peak 3 and induced a small peak (peak 8) of GST (CDNB) activity, the primary effect of safener treatment was to enhance the activity of those peaks exhibiting activity with metolachlor (peaks 5 and 10) and EPTC-sulfoxide (peaks 4 and 9). A previous report indicated that CGA-154281 protected maize against metolachlor injury by increasing GST (metolachlor) activity [23]. This study shows that this is due to the ability of the safener to induce a new isozyme that exhibits activity with metolachlor (peak 2) and enhance the activity of two other constitutive GSTs (peaks 5 and 10) that are active toward metolachlor as a substrate.

GST isozymes that conjugate GSH to the olefinic double bond of *trans*-cinnamic acid have been reported in pea (*Pisum sativum* L.) seedling leaves [5, 6, 26], potato (*Solanum tuberosa* L.) tuber slices [5], and in cultured cells of alfalfa (*Medicago sativa* L.) [6], soybean (*Glycine max* L.) [5, 26], parsley (*Petroselinum hortense*) [5, 26] and Black Mexican Sweet maize [26]. The GST (*trans*-cinnamic acid) activity from these species was typically found in the microsomal fraction, however, in pea seedlings and cultured soybean cells, activity was also found in the cytosol [5]. In this study, the GST (*trans*-cinnamic acid) activity of etiolated maize shoots was associated with both the soluble (Fig. 2) and microsomal fraction (data not shown). Our results contrast with those of Edwards and Owen [8] who reported that GST (*trans*-cinnamic acid) occurred only in the microsomal fraction from Black Mexican Sweet maize cell cultures. The soluble GST (*trans*-cinnamic acid) activity was not affected by the herbicide safener CGA-154281 (Fig. 2) and was completely separate from the GST (CDNB) activity and the GSTs active with herbicides.

In atrazine-tolerant plants, the conjugation of atrazine to GSH is catalyzed by GST [11, 18]. GST enzymes that conjugate atrazine to GSH have been partially-purified and characterized from maize [8, 11, 20, 21]. Depending on the report, maize contains two [20] or three [8] GST (atrazine) isozymes. Our results indicate that etiolated maize shoots contain three GST (atrazine) isozymes (peaks 6, 7 and 11) which appear to be distinct from other GST activities, including those for the herbicide substrates EPTC-sulfoxide and metolachlor. Edwards and Owen [7] also concluded that different GST isozymes(s) were responsible for detoxifying atrazine and metolachlor. Their conclusion was in part based on the finding that GST(atrazine) and GST(metolachlor) activities were present in maize leaves but only GST(metolachlor) activity was present in maize cell cultures [7]. They suggested that GST (atrazine) isozyme(s) were lost upon primary dedifferentiation in maize cells. However, in a later study [8], these authors

separated GST isozymes from maize leaf extracts using chromatofocusing and observed three GST peaks that exhibited reactivity with both metolachlor and atrazine. Although GST activity for both substrates appeared to co-elute, it was not clear whether there were one or two isozymes under each peak. Our results indicate that the GST isozymes reactive toward metolachlor are distinct from those having activity with atrazine.

There are two GST(metolachlor) isozymes in nontreated maize shoots (Fig. 2A, peaks 5 and 10). Safener treatment caused an increase in GST (metolachlor) activity under these peaks and induced a third isozyme which had activity toward metolachlor (Fig. 2B, peak 2). These results agree with other reports indicating that there are three isozymes (GST I, GST II, GST III) in maize that have activity with chloroacetanilides [16, 21]. However, it is difficult to make a direct comparison between peaks 3, 5, and 10, and the three isozymes previously reported [16, 21]. An elution profile (anion exchange, DEAE-Sepharose) has been published for GST I and GST II [16], and reference to GST III was made in subsequent papers [17, 27, 28] but an elution profile for all three isozymes in maize has not been published.

The detoxification of EPTC proceeds through two steps [10, 12, 15]. The herbicide is first converted to a sulfoxide derivative, presumably catalyzed by a cytochrome P-450 monooxygenase or a peroxidase. The sulfoxide, which is the active form of the herbicide, is then conjugated with GSH. Though EPTC-sulfoxide conjugation to GSH is known to occur in plants, there is a lack of consensus as to whether the reaction is predominantly non-enzymatic or is mediated by a GST [10, 12]. GST (EPTC-sulfoxide) activity has been measured in crude extracts from maize roots [10, 15], but has not been purified in this species. Based on the data in Fig. 2, it appears that there are two isozymes (peaks 4 and 9) in etiolated maize shoots that are active with EPTC-sulfoxide. Both isozymes are induced by CGA-154281 and both co-elute with the GST (metolachlor) activity in peaks 5 and 10.

In maize, safeners are used primarily to confer protection to chloroacetanilide and thiocarbamate herbicides [22]. The dichloroacetamide safener, dichlormid, protects maize against injury from both herbicide classes [22] and it has been hypothesized that this is because dichlormid enhances GST (EPTC-sulfoxide) and GST(metolachlor) activity [10, 12, 15]. This report shows that the dichloroacetamide safener CGA-154281, which protects maize from injury by metolachlor [23], enhances the activity of two GST (metolachlor) peaks (peaks 5 and 10). The fact that this safener also enhances the activity of two GST (EPTC-sulfoxide) peaks suggests that it, like dichlormid, may confer protection against injury by thiocarbamate herbicides. Because peaks 4 and 5 and peaks 9 and 10 co-elute and are comparably induced by the safener, it is possible that they may represent two isozymes that exhibit cross-reactivity with both herbicide substrates. However, further work is required to determine whether this is the case.

Many of the GST activities reported in this study have been previously detected in crude extracts isolated from maize and other plants [21]. In a few studies, GSTs have been separated by chromatographic techniques (anion exchange, chromatofocusing) but only two substrates were assayed; CDNB and chloroacetanilide herbicide (metolachlor or alachlor) [4, 13, 16, 17] or metolachlor and atrazine [7–9]. This is the first report to show a composite elution profile for GSTs that exhibit activity toward three herbicide substrates (atrazine, EPTC-sulfoxide, metolachlor), CDNB, and the natural substrate *trans*-cinnamic acid. The results clearly show the complexity of the GST isozyme profile in etiolated maize shoots.

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