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Partial Characterization of Glutathione S-Transferase Isozymes Induced by the Herbicide Safener Benoxacor in Maize[1]

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The effects of the dichloroacetamide safener benoxacor on maize (*Zea mays* L. var Pioneer 3906) growth and glutathione *S*-transferase (GST) activity were evaluated, and GST isozymes induced by benoxacor were partially separated, characterized, and identified. Protection from metolachlor injury was closely correlated with GST activity, which was assayed with metolachlor as a substrate, as benoxacor concentration increased from 0.01 to 1 μM. GST activity continued to increase at higher benoxacor concentrations (10 and 100 μM), but no further protection was observed. Total GST activity with metolachlor as a substrate increased 2.6- to 3.8-fold in response to 1 μM benoxacor treatment. Total GST activity from maize treated with or without 1 μM benoxacor was resolved by fast protein liquid chromatography anion-exchange chromatography into four major activities, designated activity peaks A, B, C, and D in their order of elution. These GST activity peaks were enhanced to varying degrees by benoxacor. Activity peak B showed the least induction, whereas activity peak A was absent constitutively and thus highly induced by benoxacor. In contrast to earlier reports, there appear to be not one, but at least two, major constitutive isozymes (activity peaks A and D) having activity with metolachlor as substrate; there were at least three such isozymes in benoxacor-treated maize (activity peaks A, C, and D). The elution volumes of activity peaks A, B, C, and D were compared with those of partially purified maize GST I and GST II; also, the reactivity of polypeptides in these activity peaks with antisera to GST I or GST I/III (mixture) was evaluated. Evidence from these experiments indicated that activity peak B contained GST I, and activity peak C contained GST II and GST III. Activity peaks A and D contained unique GSTs that may play a major role in metolachlor metabolism and in the safening activity of benoxacor in maize. Isozymes present in activity peaks A and D were not detected in earlier reports because of the very low activity with the artificial substrate 1-chloro-2,4-dinitrobenzene. Immunoblotting experiments also indicated the presence of numerous unidentified GST subunits, including multiple subunits in chromatography fractions containing single peaks of GST activity; this is indicative of the likely complexity and diversity of the maize GST enzyme family.

GST enzymes catalyze the conjugation of GSH to electrophilic, lipophilic molecules. The GSH conjugates formed are hydrophilic and frequently have reduced toxicity and reactivity. GST enzymes are found in a variety of eukaryotic organisms and typically are comprised of two polypeptide subunits with molecular masses of approximately 25 kD each. GST enzymes may be homodimeric or heterodimeric, and various combinations of subunits have been observed (Jakoby and Keen, 1977; Shimabukuro, 1985; Mannervik and Danielson, 1988; Clark, 1989; Lamoureux and Rusness, 1989). GST enzymes in plants are responsible for the species selectivity of many herbicides (Shimabukuro, 1985; Lamoureux and Rusness, 1989).

A diversity of xenobiotic chemicals induce GST in mammals, insects, and plants (Boyer, 1989; Clark, 1989; Dean et al., 1990). Similarly, several herbicide safeners, chemicals that protect crops from herbicide injury, stimulate the conjugation of herbicides with GSH by inducing GST activity in maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* [L.] Moench). This increased GST activity appears to be the basis for protection from EPTC (Lay et al., 1975), metolachlor[2] (Fuerst and Gronwald, 1986; Gronwald et al., 1987; Dean et al., 1990; Viger et al., 1991b), and alachlor (Mozer et al., 1983). Benoxacor, the herbicide safener for maize studied in this report, stimulated maize GST-M activity and decreased levels of unmetabolized metolachlor in seedlings (Kreuz et al., 1989; Viger et al., 1991b).

Several papers concerning the purification and characterization of maize GST isozymes I, II, and III have been published in the past decade. Unfortunately, they provide incomplete and sometimes contradictory information about these isozymes. Mozer and colleagues (Mozer et al., 1983; Jaworski et al., 1984) separated and purified two maize GST isozymes, designated GST I and II, by anion-exchange and BSP chromatography. GST I was reported to be a homodimer of 29-kD subunits having activity with both CDNB and

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[2] Herbicides and safeners used: metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide; benoxacor (also known as CGA-154281), 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine; flurazole, phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate; EPTC, *S*-ethyldipropyl carbamothioate.

Abbreviations: BSP, bromosulfophthalein-glutathione-agarose affinity column; CDNB, 1-chloro-2,4-dinitrobenzene; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; GST-C, glutathione *S*-transferase activity assayed with CDNB as substrate; GST-M, glutathione *S*-transferase activity assayed with metolachlor as substrate; TB, 20 mM Tris-HCl (pH 7.8), 5 mM β-mercaptoethanol.

alachlor as substrates. GST II was reported to be a heterodimer, composed of 27- and 29-kD subunits, and active with both substrates. The 29-kD subunit of GST II was apparently the same as the 29-kD GST I subunit because the N-terminal sequences of both subunits were identical. GST I was constitutively expressed, and its activity was increased by treatment with the safeners flurazole and dichlormid. In contrast, GST II was only detected in extracts from safener-treated maize (Mozer et al., 1983).

We are not aware of any other published research concerning GST II. Subsequently, however, Moore et al. (1986) reported, without supporting data or explanation, that GST II was a dimer with two 27-kD subunits. We have received verbal confirmation from a member of this research group that GST II is a homodimer (K.M. O'Connell, personal communication).

A third GST isozyme, GST III, was isolated and characterized by O'Connell et al. (1988). GST III was reported to be a homodimer with 26-kD subunits having activity with alachlor, metolachlor, and CDNB as substrates. GST III accounted for more than 80% of the total maize GST activity when alachlor or metolachlor were used as substrates. GST I and II were retained on the BSP column, and GST III was not, which is how separation was achieved (K.M. O'Connell, personal communication). GST III was detected using alachlor and CDNB as substrates. Mozer et al. (1983) did not detect GST III because only GST-C assays were conducted on chromatography fractions, and the GST-C activity of GST III is 20 to 30 times less than that of GST I or II, and GST III was lost during BSP chromatography (K.M. O'Connell, personal communication).

Anion-exchange FPLC was subsequently used to separate several GST isozymes; five different substrates were used to assay GST in each fraction (Dean et al., 1991). Benoxacor treatment induced GST activities to varying degrees, and some activities were not induced at all. No attempt was made to identify GST I, II, and III.

The goal of this research effort is to identify and definitively characterize the major GST isozymes of maize that contribute significantly to herbicide selectivity and safening. The research reported here establishes a foundation for this effort: safener-induced GST isozymes were partially purified and characterized, and preliminary identification of these isozymes was obtained. The specific objectives of this study were (a) to evaluate the effect of benoxacor concentration on maize growth and GST activity; (b) to evaluate the effect of benoxacor on the GST isozyme profile of maize using the FPLC procedure of Dean et al. (1990); (c) to partially characterize the isozymes with respect to their activity with CDNB and metolachlor as substrates, and with respect to their reactivity with antisera to GST I and GST I/III (mixture); and (d) to identify GST I, II, and III among the GST activity peaks separated by FPLC.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L. var Pioneer 3906) seed was planted 2 to 3 cm deep into trays filled with dry vermiculite to a depth of 12 cm. The trays were subsequently watered to saturation with or without 1 μM aqueous benoxacor. For flurazole treatments, maize seed was treated as described by Mozer et al. (1983). Flurazole-coated seeds were planted as previously described. Trays were incubated without light at 30°C with 100% RH. Etiolated seedlings were harvested after 3 d by excising 2 to 3 cm of apical sections of shoots. The shoots were immediately frozen in liquid nitrogen and stored at −196°C.

Crude Extract

All steps were conducted at ≤4°C. Five grams of frozen tissue were ground to powder in a chilled mortar and homogenized in 5 volumes of a solution containing 250 mM Tris-HCl (pH 7.8), 1 mM Na_2EDTA, 0.1 mM PMSF, 0.5 mM 2-mercaptoethanol, and 50 mg mL^{-1} PVPP. The homogenate was filtered through eight layers of cheesecloth and then centrifuged at 20,000*g* for 15 min to pellet debris. The supernatant was designated as the "crude extract."

GST-M Assay

This assay was similar to that of Viger et al. (1991b). The 500-μL reaction mixture contained 100 μL of the enzyme extract or chromatography fraction, 100 mM Tris-HCl (pH 7.8), 10 mM GSH, and 100 μM [^{14}C]metolachlor (specific activity 2 μCi μmol^{-1}). The reaction was initiated by adding [^{14}C]metolachlor. The reaction mixture was incubated for 30 min at 30°C and was stopped by adding 50 μL of glacial acetic acid. The reaction mixture was partitioned once against 2 volumes of methylene chloride to remove unmetabolized metolachlor. A 200-μL aliquot of the aqueous phase was removed, and the quantity of [^{14}C]metolachlor-GSH conjugate formed was determined by liquid scintillation counting. Reported GST-M activity values were corrected for nonenzymic conjugation.

GST-C Assay

The 3.0-mL reaction mixture contained 100 μL of the enzyme extract or chromatography fraction, 65 mM potassium phosphate (pH 7.4), 1 mM GSH, and 1 mM CDNB. The mixture was incubated at ambient temperature (21°C), and the reaction was started by the addition of CDNB. The rate of change in A_{340} was determined immediately. Reported GST-C activity values were corrected for nonenzymic conjugation.

Protein was quantified according to the method of Bradford (1976) using BSA as a protein standard. All experiments were repeated, and representative results are shown.

Effect of Benoxacor Concentration in Growth Medium on Growth Responses and GST-M Activity

Growth analysis was conducted to determine the concentration of benoxacor required to protect maize from metolachlor injury. Thirty maize seeds were planted 5 cm deep in dry vermiculite in square pots 12 cm on each side and 12 cm deep. Pots were watered to saturation with aqueous treatment solutions containing 100 μM metolachlor plus 0, 0.01, 0.1, 1,

10, or 100 μM benoxacor. The concentration of metolachlor applied was chosen based on preliminary experiments that showed that it caused severe growth inhibition. There was also a control treatment solution with no metolachlor and no benoxacor. Treatments containing both chemicals were applied as a single solution. Pots were covered with aluminum foil and placed in a growth chamber at 30°C with a PPFD of 165 $\mu E\ m^{-2}\ s^{-1}$ (14 h d^{-1}). The foil was removed after 2 d. Shoot height above the vermiculite surface was measured as an indicator of herbicide injury 11 d after planting, at approximately the two-leaf stage.

Plant material was grown similarly for GST-M analysis, but shoots were harvested 3 d after planting. A crude extract was prepared, and GST-M activity was assayed.

Effect of Metolachlor and CDNB Concentration in Assay Medium on GST Activity

Previous experiments with sorghum (Gronwald et al., 1987) indicated that substrate concentrations affect the relative GST activity assayed in control versus safener-treated seedling tissue. We conducted a similar experiment to determine whether the same is true using benoxacor treatment in maize. Maize seedlings were grown with or without 1 μM benoxacor and a crude extract was prepared. The metolachlor concentration in the assay medium ranged from 0.1 to 100 μM, and the CDNB concentration ranged from 10 to 1000 μM.

FPLC Chromatography of Total GST Activity

All steps were conducted at ≤4°C. The crude extract was adjusted to 0.3% (w/v) streptomycin sulfate to precipitate nucleic acids and centrifuged at 40,000*g* for 20 min. The supernatant was subjected to ammonium sulfate fractionation, and proteins precipitating between 30 and 70% ammonium sulfate saturation were pelleted by centrifugation at 40,000*g* for 30 min. The proteins were resuspended in 7.5 mL of TB buffer and desalted on prepacked Sephadex G-25 columns (PD-10, Pharmacia). The protein solution was subsequently filtered through 0.22-μm membrane filters (Millipore) and loaded (about 15 mg of total protein) onto a Mono-Q anion-exchange column (Pharmacia HR 5/5; Pharmacia LKB Biotechnology, Inc.) equilibrated in TB buffer. After loading, the column was washed with 5 column volumes of TB buffer. FPLC was conducted using the procedure developed by Dean et al. (1990). Protein was eluted at a flow rate of 0.5 mL min^{-1} using a stepped gradient of NaCl dissolved in TB buffer: 0 to 50 mM NaCl in 1 mL, 50 to 180 mM NaCl in 34 mL, and 180 to 500 mM NaCl in 16 mL. Increasing NaCl concentration beyond 500 mM did not elute any additional GST activity (data not shown). Protein elution was monitored by A_{280}. Fractions (1 mL) were collected and assayed for GST-M and GST-C activity. FPLC was conducted using Beckman models 100A and 110A HPLC pumps and a Rainin "Dynamax" titanium analytical mixer with <1.2 mL of mixing volume. GST isozyme retention times and separations with this system differed slightly from those of Dean et al. (1991).

DEAE-Sepharose Chromatography of Total GST Activity

All steps were conducted at ≤4°C. Partially purified GST I and II were obtained using the protein extraction and DEAE-Sepharose chromatography procedure developed by Mozer et al. (1983). Protein elution was monitored by A_{280}. Fractions (10 mL) were collected and assayed for GST-C activity.

FPLC Chromatography of GST I and II

All steps were conducted at ≤4°C. Fractions containing GST I and II collected during DEAE-Sepharose chromatography were dialyzed overnight against 6 L of TB buffer. The dialysate was loaded onto the FPLC Mono-Q column as previously described. The column was washed with 1 volume of TB buffer, and protein was eluted as previously described. Fractions (1 mL) were collected and assayed for GST-C activity.

Electrophoresis and Immunoblotting

Fractions containing GST I and II separated by DEAE-Sepharose chromatography of flurazole-treated maize and four major GST activity peaks separated by FPLC chromatography of benoxacor-treated maize were analyzed by electrophoresis and immunoblotting. One-dimensional SDS-PAGE of protein samples was performed according to the method of Laemmli (1974) on 15% acrylamide minigels (Bio-Rad Protean II) at 200 V. Two-dimensional electrophoresis was conducted according to the method of O'Farrell (1975) using a miniformat system (Bio-Rad). First dimension IEF was performed for 3000 V h^{-1} using tube gels containing pH 5 to 7 (Bio-Rad) and pH 3 to 10 (Pharmacia) ampholytes blended 4:1, respectively. Second dimension SDS-PAGE was performed as previously described using 12% acrylamide gels.

Following electrophoresis, gels were briefly equilibrated in transfer buffer and electrophoretically transferred to 0.45-μm pore-size nitrocellulose membranes (Bio-Rad) using a semidry transfer apparatus (Bio-Rad SD blotter) operating at 10 V. GST cross-reactive proteins were probed with rabbit anti-GST I or anti-GST I/III (mixture) antisera (provided by C.P.D. Tu) containing 2% (w/v) nonfat dry milk. Goat anti-rabbit antiserum conjugated to alkaline phosphatase (Sigma) was used as the secondary antibody. Localization of the antisera complexes was accomplished using nitroblue tetrazolium and 5-bromo-4-chloroindoxyl phosphate as alkaline phosphatase substrates (Blake et al., 1984).

RESULTS AND DISCUSSION

Effect of Benoxacor Concentration in Growth Medium on Growth Responses and GST-M Activity

In the absence of benoxacor, 100 μM metolachlor reduced shoot height to less than 20% of the untreated control (Table I). The severe growth inhibition observed is not representative of levels of injury observed in the field, which would be very low (Viger et al., 1991a). When benoxacor concentration was increased from 0.01 to 1 μM, shoot height increased to approximately 86% of the control (Table I). This demonstrates

the high level of protection from metolachlor injury afforded by relatively low concentrations of benoxacor. Shoot height did not increase further at 10 or 100 μM benoxacor (Table I). In most subsequent studies, 1 μM benoxacor was used because this was the lowest concentration that provided the maximum level of protection. Lower concentrations might not have been sufficiently active, and higher concentrations might have caused irrelevant secondary physiological effects.

Increased GST-M activity was correlated with increased shoot height as benoxacor concentration in the growth medium increased from 0.01 to 1 μM (Table I). However, it is surprising that GST-M activity continued to increase at 10 and 100 μM benoxacor, because these concentrations did not provide protection any greater than did 1 μM benoxacor (Table I). If we assume that safening activity is due primarily to induction of GST activity, as indicated in studies of the mode of action of benoxacor (Kreuz et al., 1989; Viger et al., 1991b) and other safeners (Gronwald et al., 1987; Gronwald, 1989; Fuerst and Lamoureux, 1992), there is a need to explain why further increases in GST-M titer did not lead to greater protection. Two explanations are suggested: (a) The inhibition of growth that was seen even at very high benoxacor concentrations was the result of inhibition of growth by metolachlor during germination or emergence, before maximal induction of GST-M by benoxacor; alternatively, (b) at such high metolachlor concentrations, further increases in GST-M titer did not completely prevent metolachlor from reaching shoot meristematic regions. Thus, some extracellular movement of metolachlor might occur no matter how rapidly intracellular detoxification occurs. Metolachlor reaching the meristematic regions would be expected to lead to inhibition of growth (Fuerst, 1987).

Effect of Metolachlor and CDNB Concentration in Assay Medium on GST Activity

The treated/control ratio for GST is greater when metolachlor is used as a substrate than when CDNB (a standard synthetic GST substrate) is used (Table II); this preferential enhancement of GST-M activity by safeners was also observed in sorghum (Gronwald et al., 1987). There was no clear trend in the effect of CDNB concentration on the treated/control ratio (Table II). However, as the concentration of metolachlor in the assay medium increased, the treated/control ratio of GST activity tended to increase (Table II). Because GST isozymes with relatively high affinity for metolachlor would be saturated at relatively low metolachlor concentrations, the increases in GST activity measured at high metolachlor concentrations were probably due to low-affinity forms of GST. Thus, the increase in the treated/control ratio at higher concentrations of metolachlor can be interpreted as indicating that isozymes having a relatively low affinity for metolachlor were induced slightly more by safener treatment than isozymes with a high affinity. In contrast, in oxabetrinil-treated sorghum, the safener-treated/control ratio decreased from 21.8 to 4.6 as metolachlor concentration was increased from 0.5 to 500 μM (Gronwald et al., 1987). Thus, high-affinity isozymes may be induced more than low-affinity isozymes in sorghum, the opposite of maize.

Table II. *Effect of metolachlor and CDNB concentration in the assay medium on estimated GST activity in maize grown with or without 1 μM benoxacor in the growth medium*

Substrate	Substrate	Enzyme Activity		Ratio Safened/Control
		Safened[a]	Control	
	μM	*pmol min⁻¹ mg⁻¹ of protein*		
Metolachlor	0.1	1.2 ± 0.1[b]	0.5 ± 0.01	2.6
	1	11.4 ± 1.1	3.6 ± 0.4	3.2
	10	71.3 ± 4.8	21.5 ± 0.3	3.3
	100	172.4 ± 11.7	45.0 ± 10.7	3.8
		μmol min⁻¹ mg⁻¹ of protein		
CDNB	10	1.1 ± 0.4	0.7 ± 0.0	1.6
	100	7.4 ± 0.0	4.1 ± 0.1	1.8
	1000	34.8 ± 0.0	21.3 ± 0.4	1.6

[a] Safened, Treated with 1 μM benoxacor in growth medium.
[b] SE.

FPLC Chromatography of Total GST Activity

Four major GST activities were separated by Mono-Q FPLC and are designated and referred to as peaks A, B, C, and D, in order of elution during chromatography (Fig. 1). Peak A was not detected constitutively, but in benoxacor-treated

Table I. *Effect of benoxacor concentration in the growth medium on height of 11-d-old maize seedlings and GST-M activity in 3-d-old maize seedlings*

Results are the average of two experiments ± SE. The shoot height of the untreated control was 11.1 cm.

Metolachlor Concentration	Benoxacor Concentration	Shoot Height	GST-M Activity	
μM	μM	*% of control*	*pmol min⁻¹ mg⁻¹ of protein*	*Ratio treated/control*
0	0	100 ± 0.9	32 ± 6	1
100	0	12.0 ± 1.4	53 ± 1	1.7
100	0.01	16.2 ± 2.6	51 ± 3	1.6
100	0.1	57.9 ± 6.6	82 ± 1	2.6
100	1	85.7 ± 9.7	107 ± 0.2	3.4
100	10	79.9 ± 5.1	125 ± 1	3.9
100	100	83.3 ± 4.9	150 ± 6	4.8

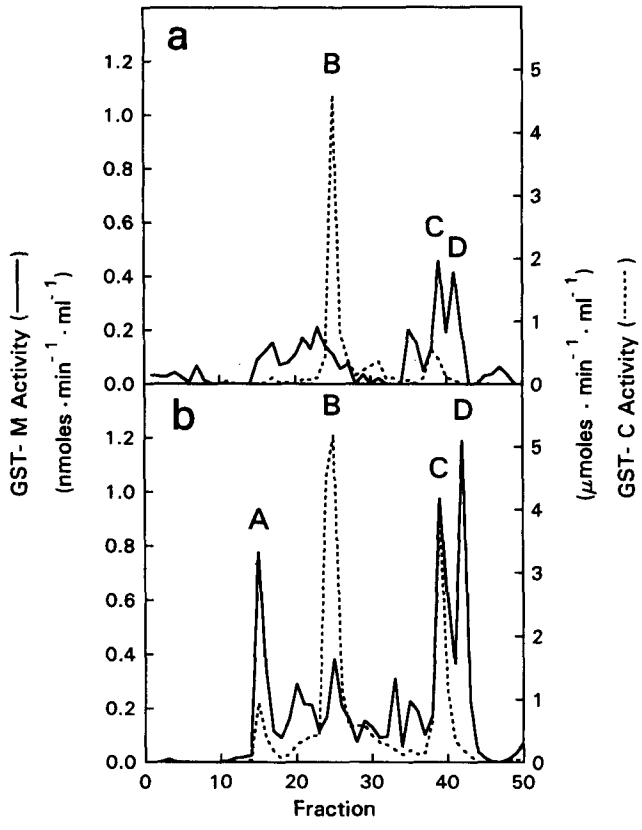

Figure 1. FPLC elution profile for total GST activity in control (a) and 1 μM benoxacor-treated (b) maize. Fractions were assayed using metolachlor (——) or CDNB (- - -) as substrate.

maize, peak A had substantial GST-M activity but low GST-C activity. Peak B was constitutively present at high levels and had the greatest GST-C activity of all activity peaks. It also had low GST-M activity and was induced by benoxacor treatment to a lesser extent than the other major GST activity peaks. Peak C was constitutively present, was induced by benoxacor, and had activity with both substrates. However, GST-C activity appears to have been induced to a greater extent than GST-M activity in peak C. This observation, which suggests that two differentially induced isozymes may be present, was consistent among several experiments and also was documented by Dean et al. (1991). Peak D was constitutively present and was induced by benoxacor and had relatively high levels of GST-M activity and no detectable GST-C activity.

These observations confirm that several GST isozymes are present in maize, that these isozymes differ in substrate specificity, and that activity of specific GST isozymes are induced to varying degrees by the safener benoxacor. The presence of two major GST-C activity peaks and three major GST-M activity peaks in safener-treated maize corresponds well with previous reports concerning maize GST isozymes (Mozer et al., 1983; Dean et al., 1991). Further chromatography and immunoblotting studies (see below) were conducted in an effort to identify GST I, II, and III among the GST activity peaks in the FPLC chromatography profile.

DEAE-Sepharose Chromatography of Total GST Activity

The procedure of Mozer et al. (1983) was used to partially purify GST I and II. Untreated maize had a single peak of GST-C activity, which corresponds to GST I (Fig. 2; Mozer et al., 1983). In contrast, flurazole-treated maize had two peaks of GST-C activity. The first activity peak corresponds to GST I, and the second activity peak corresponds to GST II, as designated by Mozer et al. (1983). Like flurazole, benoxacor treatment also induced a second peak of GST-C activity, corresponding to GST-II (Fig. 2).

FPLC Chromatography of GST I and II

Fractions from DEAE-Sepharose chromatography containing GST I and II were individually chromatographed by Mono-Q FPLC. GST I from both control and safener-treated maize eluted as a single activity peak (Fig. 3a). GST I and peak B both had very high levels of GST-C activity and had similar FPLC elution volumes; thus, we conclude that peak B contains GST-I. GST II from flurazole- and benoxacor-treated maize eluted in fractions close to those of peaks C and D (Figs. 1 and 3b). We conclude that peak C contains GST II, based on the presence of GST-C activity in peak C and GST II and the lack of GST-C activity in peak D. Thus, the two major peaks of GST-C activity resolved by FPLC, peaks B and C (Fig. 1), correspond well with GST-I and II, respectively, as resolved by DEAE-Sepharose chromatography both in our work (Fig. 2) and in the work of Mozer et al. (1983).

Because GST III has not been identified in any published chromatography profiles, it is difficult to positively identify which activity peak in Figure 1 contains GST III. GST III was reported to be the major maize isozyme with GST-M activity (O'Connell et al., 1988), which allows us to exclude the possibility that peak B is GST III because it had very low GST-M activity. Because GST I, II, and III all have GST-C activity (Mozer et al., 1983; Moore et al., 1986; O'Connell et al., 1988), and because the isozyme present in peak D had

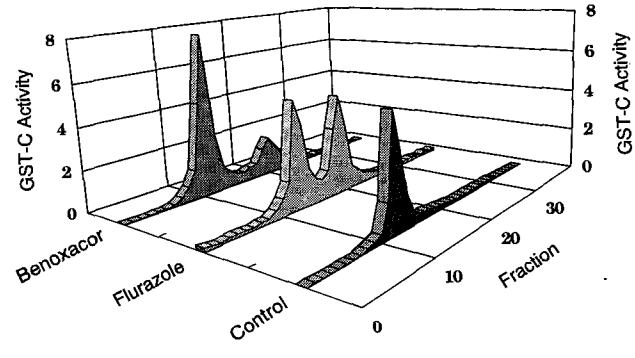

Figure 2. DEAE-Sepharose elution profile for total GST activity in control, flurazole-treated, and benoxacor-treated maize following the isolation and chromatography procedure of Mozer et al. (1983). The GST-C activity peak from the control corresponds to GST I; the activity peaks in safener-treated maize correspond to GST I and GST II in the order of elution. GST-C activity units are μmol min^{-1} mL^{-1}.

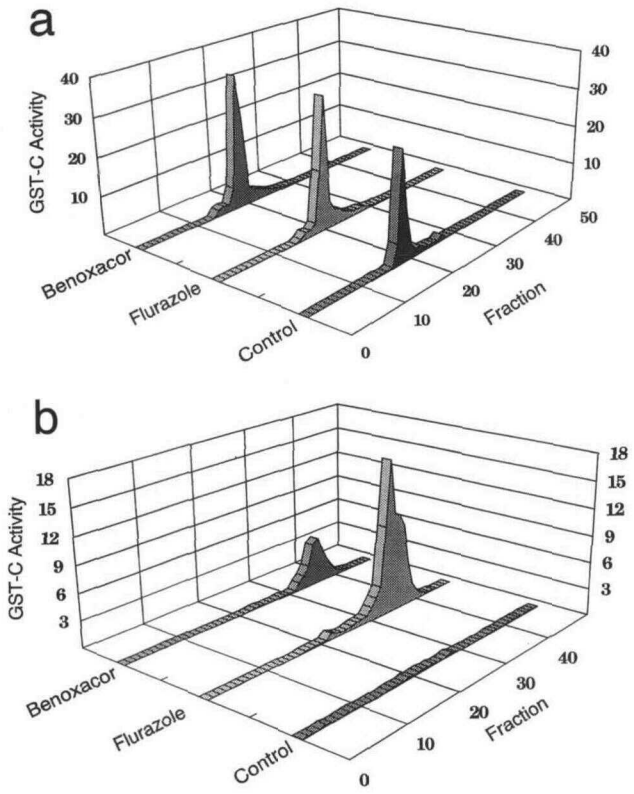

Figure 3. FPLC elution profile of GST I (a) and GST II (b) from maize. Partially purified GST I and II were obtained by DEAE-Sepharose chromatography of total maize GST (Fig. 2). GST-C activity units are μmol min^{-1} mL^{-1}.

no detectable GST-C activity in the FPLC profile (Fig. 1) or in purified form (Irzyk and Fuerst, 1993), we can exclude the possibility that peak D is GST III. (The GST isozyme present in peak D has been designated "GST IV"; unique partial amino acid sequences of GST IV demonstrate unambiguously that it is not GST III [Irzyk and Fuerst, 1993].) Based on evidence presented thus far, it is possible that peak A or C contain GST III. Because peak A is not constitutively present and GST III was isolated from untreated maize (O'Connell et al., 1988), it is unlikely that peak A contains GST III. This evidence, in addition to immunological evidence presented below, excludes the possibility that peak A contains GST III.

Electrophoresis and Immunoblotting

Experiments were conducted with antisera to GST I and GST I/III (mixture) in further attempts to identify GST I, II, and III in our FPLC chromatography profile (Fig. 1). Fractions from peaks A, B, C, and D were subjected to electrophoresis, blotted, and probed with the antisera. GST-I antiserum reacted with polypeptides of about 28 kD from fractions 24 and 39, corresponding to peaks B and C (Fig. 4a), which we previously concluded contained GST I and II, respectively. Like GST I antiserum, GST I/III antiserum also showed a positive reaction with polypeptides of about 28 kD from peaks B and C, and, in addition, it showed a positive reaction

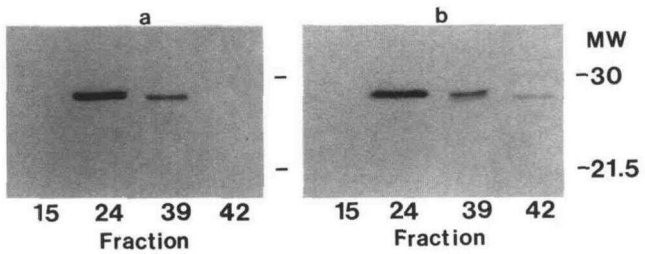

Figure 4. Immunoblots of GSTs from benoxacor-treated maize seedlings. Total GST activity was separated by anion-exchange FPLC (Fig. 1). Fractions containing GST activity were subjected to one-dimensional SDS-PAGE and electrophoretically transferred to nitrocellulose. Blots were probed with GST I antiserum (a) or GST I/III antiserum (b). Fractions 15, 24, 39, and 42 correspond to GST activity peaks A, B, C, and D, respectively (Fig. 1). Numbers to the right indicate molecular mass in kD.

with a second polypeptide from peak C (band is barely visible) and with a polypeptide from FPLC fraction 42, corresponding to peak D (Fig. 4b). With higher protein loadings, both GST I and GST I/III antisera reacted strongly with the polypeptide from fraction 42 (data not shown). The reaction of these antisera only with polypeptides of about 28 kD confirms the presence of GST subunit polypeptides in these fractions. However, the reactivity of these antisera with several polypeptides also indicates that these antisera are not highly specific for GST I or the GST I/III mixture.

These antisera did have some specificity among GST subunits because neither antiserum reacted with polypeptides from FPLC fraction 15, corresponding to peak A (Fig. 4). This is significant because it allows us to conclude that peak A does not contain GST I or III subunits. Based on substrate specificity, we previously concluded that either peak A or C contains GST III. Thus, by process of elimination, we conclude that peak C probably contains GST III.

When total GST from maize was analyzed by SDS-PAGE and immunoblotting, at least nine polypeptides in the 27- to 29-kD range reacted with GST I antiserum (Fig. 5). This is indicative of the likely complexity of the maize GST enzyme family and verifies the presence of numerous GST subunit types, which would be expected based on the multiple GST

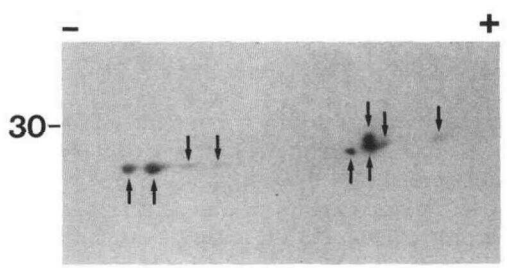

Figure 5. Immunoblot of total GSTs from maize seedlings. Total protein was extracted from 3-d-old maize seedlings and subjected to two-dimensional SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose. Blots were probed with antiserum raised against GST I. The 30-kD marker is indicated on the left.

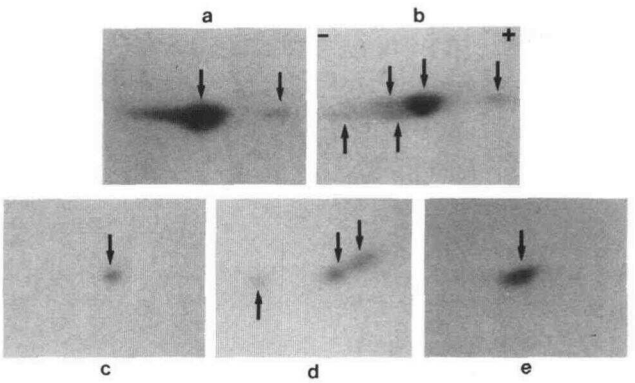

Figure 6. Immunoblot of chromatography fractions containing GST activity. Partially purified GST I (a) and GST II (b) from DEAE-Sepharose fractions of flurazole-treated maize and FPLC fractions 25 (c), 39 (d), and 42 (e), corresponding to GST activity peaks B, C, and D, respectively, from benoxacor-treated maize. Polypeptides were subjected to two-dimensional SDS-PAGE and electrophoretically transferred to nitrocellulose. Blots were subsequently probed with antiserum to GST I.

activity peaks observed in FPLC chromatography of total maize extracts (Fig. 3; Dean et al., 1991).

Chromatography fractions from GST I, GST II, and peaks B, C, and D were also analyzed by two-dimensional SDS-PAGE and immunoblotting with GST I antiserum (Fig. 6). (Activity peak A was not evaluated because no reactive polypeptides are present in this fraction [Fig. 4].) This study revealed additional reactive polypeptides of about 28 kD that were not separated by one-dimensional SDS-PAGE (Fig. 4). GST I and II fractions from DEAE-Sepharose contained, respectively, two and perhaps five reactive polypeptides each (Fig. 6, a and b). Thus, "GST I" and "GST II" separated by DEAE-Sepharose probably contain multiple GST isozymes. Only one reactive polypeptide was observed in fractions from peaks B and D (Fig. 6, c and e), but three reactive polypeptides were observed in the fraction from activity peak C (Fig. 6d). The latter observation is significant because it is consistent with the contention that peak C could contain both GST II and III.

The cross-reactivity of the GST I and GST I/III antisera with other polypeptides is probably due to sequence homologies among various GST isozymes. GST I and III share 45% amino acid sequence homology (Grove et al., 1988). Thus, a portion of the antibody population in these polyclonal antisera probably recognizes epitopes common to subunits of both GST I and III and probably other GSTs as well.

Several lines of evidence support the contention that peak C contains both GST II and GST III. Mono-Q FPLC of partially purified GST II indicated that peak C contains GST II (Fig. 3b), whereas substrate specificity and immunoblotting data indicated that peak C contains GST III. The possible presence of at least two isozymes in peak C was further supported by the differential induction of GST-C and GST-M activities in peak C (Fig. 1) as well as the presence of three polypeptides from peak C that reacted with GST I antiserum (Fig. 6d) and two polypeptides from peak C that reacted with GST I/III antiserum (Fig. 4b).

Benoxacor-treated maize contains at least three major GST isozymes (peaks A, C, and D) having activity with metolachlor as a substrate (Fig. 1b). At least two of these safener-induced GST isozymes (peaks A and D) were not detected in the work of Mozer et al. (1983) because enzyme purification was based on GST-C assays; isozymes present in peaks A and D (Fig. 1) would not have been detected because of their very low GST-C activity; apparently GST III also was not detected because of its low GST-C activity. We have also observed that peak D (GST IV) is lost when protamine sulfate is used to precipitate nucleic acids before chromatographic separations (data not shown). Perhaps this is why it was not reported in the studies of GST III (O'Connell et al., 1988). Consequently, the estimate that GST III contains more than 80% of the total constitutive GST-M activity in maize (O'Connell et al., 1988) may be in error because of loss of GST IV during sample preparation. Thus, two major isozymes (GST III and IV), rather than just one, may be responsible for most of the constitutive GST-M activity in maize.

In conclusion, we have partially separated and characterized several GST isozymes of maize. Results indicate that GST activity peak B from FPLC of total maize GST (Fig. 1) contains GST I, and peak C probably contains both GST II and III. Activity peaks A and D do not contain GST I, II, or III and, thus, contain unique GSTs. Activity peaks A, C, and D contain isozymes that appear to play a major role in maize metabolism of metolachlor and in the safening activity of benoxacor. Immunoblotting experiments indicated that fractions containing GST I and GST II from DEAE-Sepharose and activity peak C from FPLC contain multiple GST subunits. Although the significance and multiplicity of these enzymes in maize is clearly indicated, only three of these, GST I, III, and IV, have been purified, and even these have not yet been characterized in detail. The work presented here will facilitate further research to identify and more fully characterize these and other members of the maize GST isozyme family.

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