A glutathione S-transferase (GST) isozyme from maize (Zea mays Pioneer hybrid 3906) treated with the dichloroacetamide herbicide safener benoxacor (CGA-154281) was purified to homogeneity and partially characterized. The enzyme, assayed with metolachlor as a substrate, was purified approximately 200-fold by ammonium sulfate precipitation, anion-exchange chromatography on Mono Q resins, and affinity chromatography on S-hexylglutathione agarose from total GST activity present in etiolated shoots. The purified protein migrated during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) as a single band with a molecular mass of 27 kD. Using nondenaturing PAGE, we determined that the native protein has a molecular mass of about 57 kD and that the protein exists as a dimer. Two-dimensional electrophoresis revealed only a single protein with an isoelectric point of 5.75 and molecular mass of 27 kD. These results further suggest that the protein exists as a homodimer of two identical 27-kD subunits. The enzyme was most active with substrates possessing a chloroacetamide structure. trans-Cinnamic acid and 1-chloro-2,4-dinitrobenzene were not effective substrates. Apparent $K_m$ values for the enzyme were 10.8 $\mu$M for the chloroacetamide metolachlor and 292 $\mu$M for glutathione. The enzyme was active from pH 6 to 9, with a pH optimum between 7.5 and 8. An apparently blocked amino terminus of the intact protein prevented direct amino acid sequencing. The enzyme was digested with trypsin, and the amino acid sequences of several peptide fragments were obtained. The sequence information for the isolated CST we have designated with a $pI$, isoelectric point; TM buffer, 20 m Tris-HCl (pH 7.8), 5 mM $\beta$-mercaptoethanol.

Members of the GST (EC 2.5.1.18) family of enzymes are best known for their role in the detoxification of various exogenous compounds. These enzymes catalyze the nucleophilic attack of the thiol group of GSH, $\gamma$-glutamylcysteinylglycine, at an electrophilic site of a second substrate. This reaction most frequently results in the covalent linkage of GSH to the second substrate, yielding a GST conjugate, which is generally less toxic than the parent compound. In plants, this conjugation reaction is responsible for the metabolism of several classes of pesticides, including chloroacetamide (Fuerst and Gronwald, 1986; Breaux, 1987; O'Connell et al., 1988), thiocarbamate (Lay and Casida, 1976; Komives et al., 1985), and triazine herbicides (Shimabukuro et al., 1970), to nonphytotoxic forms.

Multiple forms of GST have been identified in a variety of plant species such as chickpea, maize (Zea mays), and sorghum (Dean et al., 1990; Hunaiti and Bassam, 1991; Fuerst and Irzyk, 1992). The isozymes from maize are the best-studied group of plant GSTs and include GST I, GST II, and GST III (Timmerman, 1989). These isozymes have been characterized to varying degrees with respect to molecular mass and subunit composition (Mozer et al., 1983; Wiegand et al., 1986), catalytic activity and substrate specificity (Mozer et al., 1983; Edwards and Owen, 1986; O'Connell et al., 1988), and cDNA or genomic nucleotide sequences (Moore et al., 1986; Shah et al., 1986; Grove et al., 1988).

Herbicide safeners, or antidotes, are compounds used to protect crops such as corn and sorghum from injury by certain thiocarbamate and chloroacetamide herbicides (Hatzios, 1984). In maize and sorghum both GST activity and herbicide metabolism, via GSH conjugation, are stimulated by safener treatment (Lay and Casida, 1976; Fuerst and Gronwald, 1986; Gronwald et al., 1987; Viger et al., 1991).

Benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoazaine; also known as CGA-154281] is a herbicide safener that protects corn from injury by metolachlor [2-chloro-N-(ethyl-6-methylphenyl)-N-(2-methoxy-1-methyl-ethyl) acetamide], a chloroacetamide herbicide commonly used in corn. Treatment of corn with benoxacor causes elevated levels of total GST activity, which subsequently results in the enhanced metabolism of metolachlor to its GSH conjugate (Viger et al., 1991). The total GST activity extracted from benoxacor-treated corn seedlings can be separated by anion-exchange FPLC into several distinct GST activities that differ in both inducibility and substrate specificity (Dean et al., 1991; Fuerst et al., 1993). We have previously described (Fuerst et al., 1993) a GST isozyme that is highly induced by benoxacor treatment and could facilitate the conjugation of metolachlor, but not CDNB (a model GST substrate), with GSH. We designated this isozyme maize GST IV.

In mammalian systems, the induction of GST activity by various compounds has been well characterized (Pickett and

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; FPLC, fast protein liquid chromatography; GST, glutathione S-transferase; GST-C, glutathione S-transferase capable of utilizing 1-chloro-2,4-dinitrobenzene as a substrate; GST-M, glutathione S-transferase capable of utilizing metolachlor as a substrate; PDA, piperaizine diacrylamide; pI, isoelectric point; TM buffer, 20 mM Tris-HCl (pH 7.8), 5 mM $\beta$-mercaptoethanol.
Lu, 1989). In contrast, very little is known about the induction of GST activity in plants, particularly by herbicide safeners (Hatzios, 1984; Clark, 1989). The induction of specific maize GST isozymes by benoxacor treatment provides an excellent system for studying GST induction in plants. Here we report the isolation and partial characterization of benoxacor-inducible GST IV from maize.

**METHODS AND MATERIALS**

**Plant Material**

Corn (Zea mays L. Pioneer hybrid 3906) seeds were planted 2 to 3 cm deep in trays filled with dry vermiculite. The trays were subsequently irrigated with 1 μmol benoxacor (containing 0.01% [v/v] DMSO) until saturated and then incubated at 30°C without light at 100% RH. Seedlings were harvested after 3 d by excising 2- to 3-cm apical sections of shoots. The shoots were immediately frozen in liquid nitrogen and stored at −196°C.

**Enzyme Purification**

The extraction and purification procedures were conducted at 4°C. Frozen shoots (100 g) from benoxacor-treated seedlings were ground to a fine powder and then homogenized for 2 min in 300 mL of 250 mM Tris-HCl (pH 7.8), 0.5 mM DTT, 1 mM EDTA, 0.1 mM PMSF, and 50 mg mL⁻¹ of polyvinylpolypyrrolidone in a Waring blender (high-speed setting). The homogenate was filtered through eight layers of cheesecloth and centrifuged for 15 min at 20,000 g to pellet debris. The supernatant (crude extract) was decanted and adjusted to 40% (NH₄)₂SO₄ saturation by addition of solid (NH₄)₂SO₄. After the solution was stirred for 15 min, it was centrifuged at 20,000 g for 20 min. The supernatant was decanted, adjusted to 70% ammonium sulfate saturation, and centrifuged as previously described.

The pelleted proteins were resuspended in 50 mL of TM buffer and dialyzed overnight against 6 L of the same buffer. The dialysate was clarified by centrifugation at 20,000 g for 10 min and loaded onto a Mono Q-Sepharose FF column (Pharmacia; 2.5 × 15 cm, 25-mL bed volume) equilibrated in TM buffer. The column was washed with 5 column volumes of 20 mM bis-Tris-HCl (pH 6.8), 500 mM NaCl, followed by 5 column volumes of 20 mM Tris-HCl (pH 7.8) at a flow rate of 0.3 mL min⁻¹. Bound protein was eluted with 2.5 mM hexylglutathione, 5 mM GSH in 20 mM Tris-HCl (pH 7.8) at the same flow rate. Fractions containing GST-M activity were pooled and concentrated to approximately one-fifth of the initial volume using a RCF-ConFilt hollow fiber concentrator (Bio-Molecular Dynamics, Beaverton, OR). The affinity-purified protein was subjected to a final purification step on a Mono Q-Superose HR 5/5 column using a 0 to 500 mM NaCl gradient in TM buffer in a total volume of 30 mL at a flow rate of 0.5 mL min⁻¹.

**Evaluation of Molecular Mass, pI, and Homogeneity**

One-dimensional SDS-PAGE of the purified protein was performed by the method of Laemmli (1970) using 12% acrylamide/PDA minigels (Mini Protein II, Bio-Rad) of 0.75 mm thickness. Prior to electrophoresis, the protein and SDS-PAGE marker proteins (Bio-Rad) were boiled in 2× Laemmli sample buffer for 3 min. Protein was visualized by both Coomassie blue R-250 and silver staining (Roubillard et al., 1988). Nondenaturing PAGE was used to determine the molecular mass of the native protein (Hedrick and Smith, 1968). Migration of marker proteins (MW-NDO-500 kit, Sigma) and the purified GST in nondenaturing gels of different acrylamide concentrations were used to calculate the molecular mass of the native protein (Sigma Technical Bulletin MRK-137).

Two-dimensional electrophoresis was conducted according to the method of O’Farrell (1975) using a minigel system (Mini Protein II). First-dimension IEF of the purified protein was conducted with 3000 V h⁻¹ in 1-mm tube gels containing pH 5 to 7 (Bio-Rad) and pH 3 to 10 (Pharmacia) ampholytes blended 4:1, respectively. The pI of the purified protein was determined using marker proteins of known pls (2-D Protein Standards, Bio-Rad). Second-dimension SDS-PAGE was performed as previously described, using a 1-mm-thick, 12% acrylamide/PDA gel. Protein was visualized by silver staining as previously described.

**Enzyme Assays**

The GST-M activity was assayed by measuring the conjugation of GSH and radiolabeled metolachlor at 30°C. The reaction mixture consisted of 25 μmol of Tris-HCl (pH 7.8), 5 μmol of GSH, and 0.05 μmol of [6-¹⁴C]metolachlor (specific
activity 2.0 μCi μmol⁻¹) in a final volume of 500 μL. Enzyme activity (≤0.05 units) was incubated with the reaction mixture for 5 min before initiating the reaction by addition of metolachlor. The reaction was terminated after 30 min by the addition of 50 μL of glacial acetic acid. The reaction mixture was then partitioned against 1 mL of dichloromethane to separate conjugated metolachlor (aqueous phase) from nonconjugated metolachlor (organic phase), and the radiolabeled conjugate in the aqueous phase was quantified by liquid scintillation spectrometry. GST-C activity was assessed spectrophotometrically by measuring change of A₃₄₀ (Habig et al., 1974). The assay mixture consisted of 300 μmol of potassium phosphate (pH 7.3), 3 μmol of CDNB, 15 μmol of GSH, and ≤0.05 unit of enzyme in a final volume of 3 mL. Reactions were initiated by the addition of CDNB, and A₃₄₀ was monitored for 90 s. An extinction coefficient of 9.6 m⁻¹ cm⁻¹ (Habig et al., 1974) was used to quantify conjugation of CDNB and GSH. Both metolachlor and CDNB data were corrected for nonenzymic conjugation.

**Substrate Specificity**

Activity of the enzyme with acetochlor [2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide], alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide], atrazine [6-chloro-N-ethyl-N'-(1-methyl-ethyl)-1,3,5-triazine-2,4-diamine], trans-cinnamic acid, and metolachlor was assayed by quantifying the conjugation of GSH and radiolabeled substrate. The reaction mixture consisted of 300 pmol of Tris-HCl (pH 7.8), 10 pmol of GSH, 0.05 unit of enzyme activity, and 0.05 pmol of either [φ⁻¹⁴C]acetochlor, [φ⁻¹⁴C]alachlor, [Δ⁻¹⁴C]atrazine, [COOH⁻¹⁴C]trans-cinnamic acid, or [φ⁻¹⁴C]metolachlor in a final volume of 500 μL. The reaction mixtures were incubated at 30°C for 5 min before initiating the reaction by addition of substrate. The reaction was allowed to proceed for 60 min and then was terminated by the addition of 50 μL of glacial acetic acid. The reaction product was quantified as previously described for metolachlor. Enzyme activity with CDNB as a substrate was assayed spectrophotometrically as previously described except that the assay mixture consisted of 300 μmol of Tris-HCl (pH 7.8), 0.15 μmol of CDNB, 30 μmol of GSH, and 0.15 unit of enzyme activity in a final volume of 1.5 mL.

**pH Optimum**

Enzyme activity was evaluated from pH 4.5 to 9.0 using three buffer systems: Mes-HCl (pH 4.5–6.5), potassium phosphate (pH 6.0–8.5), and Tris-HCl (pH 7.5–9) at final concentrations of 100 mM. The assays were conducted using [¹⁴C]metolachlor as a substrate as described in "Enzyme Assays," except that the various buffers listed were used instead of Tris-HCl (pH 7.8).

**Kinetic Analysis**

The Kₘ value for metolachlor was determined using approximately 0.05 unit of enzyme activity in a fixed-time assay, with 10 to 100 μM of metolachlor and GSH at a fixed concentration of 10 mM. The Kₘ value for GSH was determined using a fixed-time assay with 0.5 to 10 mM GSH and metolachlor at a fixed concentration of 100 μM. Assays were conducted for 30 min as described in "Enzyme Assays."

**Amino Acid Sequence**

Protein samples were dried by vacuum centrifugation (SpeedVac, Savant, Farmingdale, NY) and redissolved in 200 μL of 50 mM NH₄CO₃, 5 mM CaCl₂. Sequencing-grade trypsin (Sigma) was then added to obtain a protein-to-trypsin ratio of approximately 20:1. The reaction mixtures were capped and incubated for 12 h at 37°C. After digestion, reaction mixtures were reduced to one-fifth of their original volume by vacuum centrifugation and then brought to 600 μL final volume by the addition of 0.01% (v/v) TFA. Peptide fragments generated by the trypsin treatment were resolved by reverse-phase HPLC on a narrow-bore C8 column (SP 300, Applied Biosystems, Foster City, CA) using a linear 0 to 70% acetonitrile gradient containing 0.01% (v/v) TFA at a flow rate of 0.2 mL min⁻¹. Fragment elution was monitored by UV A₂₁₄ and A₂₃₀ nm. Selected fragments were subjected to amino acid sequencing by automated Edman degradation. The sequencing was performed on an Applied Biosystems model 475A liquid-phase protein sequencer by the Laboratory for Biotechnology and Bioanalysis, Washington State University.

**Protein Assay**

Protein concentration was determined by the method of Bradford (1976) or by A₂₈₀. BSA (Pierce Chemical) was used as a protein standard.

**RESULTS AND DISCUSSION**

**Enzyme Purification**

Because GST IV activity is highly induced in benoxacor-treated corn seedlings (Fuerst and Irzyk, 1992), this tissue was used with conventional protein isolation techniques to obtain a homogenous preparation of the enzyme, as determined by silver staining following one- and two-dimensional PAGE. Representative data from purification experiments are presented in Table I. Each purification step yielded increases in the specific activity of the enzyme. Several GST isozymes in corn seedlings are capable of utilizing metolachlor as a substrate (Dean et al., 1991; Fuerst et al., 1993); therefore, enzyme recovery and purification, calculated from the total GST-M activity in crude extracts, is most likely underestimated. Greater than 90% of the total GST-M activity was precipitated between 40 and 70% (NH₄)₂SO₄ saturation.

The presence of multiple GST isozymes in maize displaying GST-M activity necessitated the separation of GST IV from other GSTs. Because we had successfully resolved maize GST activities previously using Mono Q-FPLC (Fuerst et al., 1993), we opted to conduct preparative anion-exchange chromatography as an initial step in GST IV purification. However, the scale-up required for protein purification prompted our use of Mono Q-Sepharose FF instead of Mono Q-FPLC. This chromatography resolved total GST activity into several distinct activities that differed with respect to substrate specific-
ity and elution characteristics. A comparison of GST elution profiles of partially purified protein extracts from benoxacor-treated and control corn seedlings on Mono Q-Sepharose FF are shown in Figure 1. The GST activity that eluted at the highest NaCl concentration could utilize metolachlor, but not CDNB, as a substrate. This characteristic substrate specificity and elution profile were similar to those of the GST IV activity we had previously identified using Mono Q-FPLC (Fuerst et al., 1993). Mono Q-FPLC of the putative GST IV activity obtained by Mono Q-Sepharose FF chromatography provided an elution profile (data not shown) that also supported the assumption that this isozyme was GST IV. Therefore, this GST activity was the focus of our study and was subjected to additional purification steps.

The fractions collected during the initial preparative chromatography that contained maximal GST-M activity were rechromatographed on Mono Q-Sepharose FF to ensure a homogeneous preparation of GST IV activity. Little attempt to recover total GST IV was made during these initial purification steps. Therefore, the relatively low yield of GST IV from these purification steps is a reflection of both the fractionation of total GST-M activity into component GST-M activities and the pooling of only those GST IV fractions that contained the highest levels of GST IV activity (Table I). The enzyme preparations obtained by Mono Q-Sepharose FF chromatography were subjected to semipreparative Mono Q-FPLC to both concentrate the protein and provide additional protein purification. This partially purified GST IV activity was either subjected to additional purification steps or used as the source of enzyme for studies of substrate specificity, pH optimum, and enzyme kinetics.

Affinity chromatography on S-hexylglutathione agarose provided the greatest single-step increase in GST IV purification. This affinity matrix has been used to purify both plant and animal GSTs (Mannervik and Guthenberg, 1981; Williamson and Beverly, 1988). Other affinity matrices, such as bromosulphopthalein-agarose (Mozer et al., 1983) or Cibachrome blue 3GA-agarose (Hunaiti and Bassam, 1991), have also been used to obtain highly purified GST preparations from plants. However, our attempts at using these matrices proved unsatisfactory, because GST IV either did not bind well (bromosulphopthalein) or was nonspecifically bound with unacceptable amounts of other proteins (Cibachrome blue 3GA). S-Hexylglutathione agarose was found to be the most acceptable affinity matrix, but yield was sacrificed for most acceptable affinity matrix, but yield was sacrificed for purity. Under the conditions used for our affinity purification, approximately 30% of loaded activity was recovered. Williamson and Beverly (1988) also reported relatively low yields of enzyme when using this matrix to purify a GST from wheat. The affinity-purified GST IV preparation represented a 10- and 200-fold increase in specific activity of the enzyme, relative to the initial Mono Q-FPLC and crude extract specific activities, respectively. However, the GST IV fractions obtained by affinity purification were found to contain trace amounts of several other proteins when analyzed by SDS-PAGE.

### Table I. Purification of benoxacor-inducible GST IV from etiolated corn shoots treated with 1 μM benoxacor at planting

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity</th>
<th>Total Protein</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units †</td>
<td>mg</td>
<td>units mg⁻¹ of protein</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>Crude extract</td>
<td>145.7</td>
<td>1530</td>
<td>0.095</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>131.3</td>
<td>540</td>
<td>0.24</td>
<td>90</td>
<td>2.6</td>
</tr>
<tr>
<td>Mono Q-Sepharose FF</td>
<td>21.2</td>
<td>17.1</td>
<td>1.2</td>
<td>14.6</td>
<td>13.1</td>
</tr>
<tr>
<td>Mono Q-Superose 1</td>
<td>18.5</td>
<td>10.6</td>
<td>1.7</td>
<td>12.7</td>
<td>18.3</td>
</tr>
<tr>
<td>S-Hexylglutathione</td>
<td>6.2</td>
<td>0.32</td>
<td>19.1</td>
<td>4.2</td>
<td>201.1</td>
</tr>
<tr>
<td>Mono Q-Superose 2</td>
<td>5.2</td>
<td>0.27</td>
<td>19.4</td>
<td>3.6</td>
<td>204.2</td>
</tr>
</tbody>
</table>

† One unit is defined as 1 nmol of metolachlor conjugated min⁻¹. Yield and purification values are based on total GST-M activity in crude extract.

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Benoxacor-Inducible S-Transferase

Figure 2. SDS-PAGE of benoxacor-inducible GST at selected purification steps. Lane 7, Molecular mass standards; lane 1, crude extract; lane 2, protein precipitated by 40 to 70% (NH₄)₂SO₄ saturation; lane 3, Mono Q-Sepharose FF fractions containing GST IV activity; lane 4, Mono Q-Superose 1 fractions containing GST IV activity; lane 5, protein (approximately 2 μg) eluted from the S-heptylglutathione affinity column; lane 6, purified GST IV (approximately 1 μg) from Mono Q-Superose 2 fractions. Protein was visualized by both Coomassie and silver staining. Numbers to the right indicate positions of molecular mass standards in kD.

Figure 3. Nondenaturing PAGE analysis to determine native molecular mass of GST IV. Purified GST IV or protein standards were subjected to nondenaturing PAGE in gels of different acrylamide concentrations. Migration of each protein was plotted against acrylamide concentration. Linear regression was then used to obtain a characteristic slope of \( R_f \) versus acrylamide concentration for each protein. The slopes of the protein standards (○) were subsequently plotted against their respective molecular masses to obtain the calibration curve as shown. The slope calculated for the purified GST (●) was used to estimate native molecular mass based on the calibration curve. Calculated molecular mass of native GST IV is 57.5 ± 3 kD. Protein standards: A, α-lactalbumin, 14.2 kD; B, carbonic anhydrase, 29 kD; C, chicken albumin, 45 kD; D, BSA (monomer), 66 kD; E, BSA (dimer), 132 kD; F, urease (trimer), 272 kD; G, urease (hexamer), 574 kD.

Figure 4. Two-dimensional PAGE electrophoretic pattern of purified benoxacor-inducible GST IV and protein standards. First-dimension IEF was performed by the method of O'Farrell (1975) at 3000 V h⁻¹ in tube gels containing pH 5 to 7 and pH 3 to 10 ampholytes (4:1). Second-dimension SDS-PAGE was performed using a 12% acrylamide/PDA gel. Protein was visualized by silver staining. Purified GST IV is indicated with arrow. Numbers to the left indicate positions of molecular mass standards in kD. Two-dimensional protein standards: A, BSA, pl 5, 66 kD; B, bovine muscle actin, pl 5.5, 43 kD; C, soybean trypsin inhibitor, pl 4.5, 21.5 kD; D, bovine carbonic anhydrase, pl 6.0, 31 kD; E, hen egg

Molecular Mass, Homogeneity, and pI

Purified GST IV was subjected to both nondenaturing PAGE and SDS-PAGE to determine molecular mass and to evaluate purity. The calculated molecular mass of the native protein was 57 ± 3 kD (Fig. 3). This result is similar to those from previous studies of other maize GSTs in which native molecular masses ranging from 45 to 55 kD have been reported (Guddewar and Dautermann, 1979; Mozer et al., 1983; Edwards and Owen, 1986; O'Connell et al., 1988). Analysis of the purified protein preparations by SDS-PAGE and silver staining revealed the presence of a single protein band with a molecular mass of 27 kD (Fig. 2, lane 6). Because most plant and animal GSTs are known to exist as hetero- or homodimers (Mannervik and Danielson, 1988; Clark, 1989), the determination of a single protein by SDS-PAGE suggested that GST IV is a dimer composed of two 27-kD subunits. Mozer et al. (1983), using SDS-PAGE, characterized maize GST I as a homodimer of 29-kD subunits and GST II as a heterodimer comprised of 27- and 29-kD subunits. Similarly, maize GST III has been described as a homodimer consisting of 26-kD subunits (Moore et al., 1986; O'Connell et al., 1988). Additional analysis of purified GST IV by two-dimensional PAGE (Fig. 4) indicated that a single protein was present in the purified protein preparation. This result suggests...
that functional GST IV is a homodimer of identical 27-kD subunits.

Determination of the pl for GST IV was accomplished by comparing the migration of the purified GST with the migration of protein standards of known pl across the IEF gel (Fig. 4). Using this technique we determined a pl of 5.75 for the protein under the denaturing conditions used for IEF.

**pH Optimum**

The effect of pH on GST IV activity was evaluated using metolachlor as a substrate. GST-M activity was greatest between pH 7 and 8, indicating a broad pH optimum (Fig. 5). Various pH optimum values have been reported for other maize GST activities. Frear and Swanson (1970) reported a relatively discrete pH optimum between pH 6.6 and 6.8 for GST activity in a semicrude protein extract from maize assayed with atrazine as a substrate. In contrast, Guddewar and Dautermann (1979), also using atrazine as a substrate, reported a relatively broad pH optimum between 8 and 8.5 for a purified maize GST.

A marked effect of pH on nonenzymic rates of conjugation was also found. As pH was increased past 8.0, using either Tris or phosphate buffers, increased nonenzymic conjugation was also observed. This effect has previously been reported for both chloroacetamide (Leavitt and Penner, 1979) and triazine (Frear and Swanson, 1970) compounds. Consequently, we routinely conducted our standard assays with metolachlor at pH 7.8 to minimize excessive nonenzymic conjugation.

**Substrate Specificity**

Enzyme activity was evaluated with compounds known to be substrates of various maize GSTs. These included atrazine, CDNB, trans-cinnamic acid, and the chloroacetamide analogs metolachlor, acetochlor, and alachlor. The assays were conducted with specific enzyme and substrate concentrations to determine substrate specificity under equivalent conditions. Maximal activity of the enzyme was observed with the chloroacetamide substrates (Table II). As expected, because of their structural similarities, rates of enzymic conjugation were similar among these three substrates. In contrast, CDNB and trans-cinnamic acid were not detectably conjugated. Atrazine could also function as a substrate but was conjugated at approximately one-tenth of the rate determined for the chloroacetamide compounds.

Although the GST-mediated conjugation of the tested substrates with GSH is known to be mechanistically similar, i.e. the nucleophilic attack of the thiol group of GSH at an electrophilic site of the second substrate, the observed differences in GST IV substrate specificity may be attributed to the position of the electrophilic sites within the various substrates. Conjugation of GSH with the chloroacetamides, atrazine, and CDNB occurs by the substitution of the electrophilic chloro group of these substrates with GSH (Frear and Swanson, 1970; Leavitt and Penner, 1979; Mannervik and Danielson, 1988). These addition sites represent chlorosubstituted alkyl groups for the chloroacetamides, a chlorosubstituted aryl group for CDNB, and a chlorosubstituted heterocyclic amine for atrazine. The conjugation of GSH and trans-cinnamic acid occurs by the addition of GSH to the olefinic double bond of cinnamic acid that is adjacent to an electrophilic carbonyl group (Diesperger and Sandermann, 1979). This represents an \( \alpha,\beta \) double bond immediately adjacent to an aryl group. These structural differences, and the differences in enzyme activity observed with the different substrates, suggest that maize GST IV is most active with substrates having electrophilic alkyl groups. GSTs specific for various structural characteristics have been well documented in mammalian systems (Mannervik and Danielson, 1979), and their structural specificity has been utilized for their classification (Boyland and Chasseaud, 1969). However, the fact that atrazine can act as a substrate for GST IV suggests limited usefulness of this type of classification for plant GSTs.

**Kinetic Analysis**

The activity of the enzyme as a function of various metolachlor and GSH concentrations is shown in Figure 6. At a fixed GSH concentration of 10 mm, the enzyme exhibited Michaelis-Menten kinetics in response to changes in metolachlor concentration. Enzyme activity was linearly depend-

Table II. GST IV activity with various GST substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Activity (nmol min(^{-1}) mg(^{-1}) of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetochlor</td>
<td>2.8 ± 0.4(\text{a})</td>
</tr>
<tr>
<td>Alachlor</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CDNB</td>
<td>n.d.</td>
</tr>
<tr>
<td>trans-Cinnamic acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>2.1 ± 0.1</td>
</tr>
</tbody>
</table>

\(\text{a}\) Average value ± se.  
\(\text{b}\) n.d., Not detectable.
Figure 6. Kinetic analysis of GST IV activity. Enzyme activity as a function of metolachlor concentration was assayed in the presence of 10 mM GSH. Enzyme activity as a function of GSH concentration was assayed in the presence of 0.1 mM metolachlor. Metolachlor (A) and GSH (B) data are presented as double-reciprocals of plots. Plotted values represent averaged results from two experiments. Calculated $K_m$ values are 10.8 $\mu$m for metolachlor and 292 $\mu$m for GSH.

Table III. Partial amino acid sequences of plant GSTs and maize GST IV

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize GST I</td>
<td>7-29</td>
<td>Y G A V M S W L T R C A T L E E A G S D Y E</td>
</tr>
<tr>
<td>Maize GST III</td>
<td>7-29</td>
<td>G M P L S P N V V V N K L F</td>
</tr>
<tr>
<td>Maize GST IV (fragment 1)</td>
<td></td>
<td>*** V ***</td>
</tr>
<tr>
<td>Wheat</td>
<td>8-30</td>
<td>G H P M L T N V A * V L L F * * V * A E Y *</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>7-25</td>
<td>G A V M S W N V * * C * * * * * *</td>
</tr>
<tr>
<td>Bladder campion</td>
<td>7-29</td>
<td>G N P R S T A T O * V L Y * Y * K H L E F *</td>
</tr>
<tr>
<td>Maize GST I</td>
<td>54-70</td>
<td>V P A L Q D G D L Y L F E S R A</td>
</tr>
<tr>
<td>Maize GST III</td>
<td>54-70</td>
<td>* * * * V * * * E * * * *</td>
</tr>
<tr>
<td>Maize GST IV (fragment 2)</td>
<td></td>
<td>*** V * E * * * T * * * *</td>
</tr>
<tr>
<td>Wheat</td>
<td>54-70</td>
<td>M * G F Q * * * V * * * *</td>
</tr>
<tr>
<td>Bladder campion</td>
<td>53-69</td>
<td>* * * * E * * * V * * * *</td>
</tr>
<tr>
<td>Maize GST IV (fragment 3)</td>
<td></td>
<td>V A Q F M P V G A G A P K</td>
</tr>
</tbody>
</table>

Amino Acid Sequence

Initial attempts to sequence the purified protein were unsuccessful because of apparent N-terminal blocking. Trypsin digestion of the purified protein generated fragments that yielded sequence information. The alignment of the GST IV sequence data to currently available plant GST sequences is shown in Table III (Grove et al., 1988; Dudler et al., 1991; Sharad et al., 1991; Kutchan and Hochberger, 1992). Fragment 1 had strong homology to maize GST I. Fragment 2 had strong homology to both maize GST I and GST III. Limited homology with constitutively expressed GSTs of wheat, sugarcane, and bladder campion most likely reflects the presence of amino acids that are generally conserved among plant GSTs. The sequence obtained for fragment 3 was not homologous to maize GST I or GST III and indicates that GST IV is a unique maize GST isozyme.

Summary

GST IV represents a previously unidentified GST in maize. This isozyme shares many characteristics with other maize GSTs, including molecular mass, subunit size, and conserved regions of amino acids. However, GST IV differs significantly from known maize GSTs with respect to substrate specificity and nonconserved regions of amino acids. The $K_m$ value depends on the amount of protein added as well as on incubation time (data not shown). The apparent $K_m$ value for metolachlor determined by Lineweaver-Burk double-reciprocal plots was 10.8 ± 0.4 $\mu$m (Fig. 6A). This value is 1 to 2 orders of magnitude lower than $K_m$ values reported for maize GST I (Mozer et al., 1983; Edwards and Owen, 1986) or maize GST III (O'Connell et al., 1988) with metolachlor, or the analog alachlor, as substrates. Although other GST isozymes are induced in response to benoxacor treatment, this low $K_m$ value indicates a high affinity of GST IV for metolachlor and supports the idea that the induction of this particular isozyme is partially responsible for the ability of benoxacor to protect maize from metolachlor injury. The $K_m$ for GSH was calculated at 292 ± 47 $\mu$m (Fig. 6B), which is in general agreement with published GSH $K_m$ values for plant GSTs and may reflect conservation of the GSH-binding site (Clark, 1989).
determined for metolachlor, 10.8 μm, is much lower than previously reported \( K_a \) values determined for other maize GSTs using herbiocidal chloroacetamide substrates. Both the inducibility of GST IV by the herbicide safener benoxacor and the high affinity of GST IV for metolachlor suggest that this particular isozyme has an important role in the benoxacor-mediated protection of maize from metolachlor injury.

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**LITERATURE CITED**


