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Properties of a Maize Glutathione S-Transferase That Conjugates Coumaric Acid and Other Phenylpropanoids[1]

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A glutathione S-transferase (GST) enzyme from corn (*Zea mays* L. Pioneer hybrid 3906) that is active with *p*-coumaric acid and other unsaturated phenylpropanoids was purified approximately 97-fold and characterized. The native enzyme appeared to be a monomer with a molecular mass of approximately 30 kD and an apparent isoelectric point at pH 5.2. The enzyme had a pH optimum between 7.5 and 8.0 and apparent K_m values of 4.4 and 1.9 mM for reduced glutathione (GSH) and *p*-coumaric acid, respectively. In addition to *p*-coumaric acid, the enzyme was also active with *o*-coumaric acid, *m*-coumaric acid, *trans*-cinnamic acid, ferulic acid, and coniferyl alcohol. In addition to GSH, the enzyme could also utilize cysteine as a sulfhydryl source. The enzyme activity measured when GSH and *trans*-cinnamic acid were used as substrates was enhanced 2.6- and 5.2-fold by the addition of 50 μM *p*-coumaric acid and 7-hydroxycoumarin, respectively. ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopic analysis of the conjugate revealed that the enzyme catalyzed the addition of GSH to the olefinic double bond of *p*-coumaric acid. Based on the high activity and the substrate specificity of this enzyme, it is possible that this enzyme may be involved in the in vivo conjugation of a number of unsaturated phenylpropanoids.

GSTs (EC 2.5.1.18) are a family of enzymes that conjugate GSH to a wide variety of compounds that are lipophilic and possess an electrophilic center. Typically, these enzymes are dimeric proteins that exist in several isoenzymatic forms. It is generally believed that GSTs play a role in the intracellular detoxification of xenobiotics and toxic compounds produced endogenously (Mannervik, 1985; Clark, 1989). In plants, GST enzymes have received a great deal of attention because of their role in herbicide metabolism, and the GST enzymes from corn (*Zea mays* L.) have been characterized to the greatest extent (Timmerman, 1989). Corn shoots contain a number of GSTs that are active with chloroacetanilide herbicides (alachlor and metolachlor), and many of these GSTs are induced by chemical compounds known as herbicide safeners (Mozer et al., 1983; Edwards and Owen, 1986, 1988; O'Connell et al., 1988; Dean et al., 1991; Fuerst et al., 1993; Irzyk and Fuerst, 1993). Herbicide safeners are chemical compounds that are known to protect corn and sorghum from injury by selected herbicides (Hatzios, 1989). Corn tissue also contains enzymes that are active against the herbicides atrazine (Frear and Swanson, 1970; Guddewar and Dauterman, 1979; Timmerman, 1989; Dean et al., 1991) and EPTC sulfoxide (Lay and Casida, 1976; Dean et al., 1991).

In addition to the herbicide substrates, corn tissue has also been shown to contain GST enzymes that are active with CA (Edwards and Owen, 1987; Dean et al., 1991; Dean and Machota, 1993). This is significant, since CA is a compound produced endogenously by the plant and may represent a natural substrate of the enzyme. In corn, the enzyme that is responsible for the conjugation of CA is distinct from the enzymes that are active with the various herbicides and the general substrate CDNB (Edwards and Owen, 1987; Dean et al., 1991). Recently, it was shown that corn shoots contain several GST isozymes that are active with CA; however, some of these enzymes are only detected in the presence of 4-CA and 7-HC, which appear to serve as activators of the enzymes (Dean and Machota, 1993). Although the corn GST enzymes responsible for herbicide metabolism have been purified and characterized to varying degrees, very little research has focused on the corn GST enzymes that may be involved in the conjugation of natural substrates such as CA.

In addition to corn, several other plant species have also been shown to contain a GST enzyme that will presumably catalyze the addition of GSH to the olefinic double bond of CA, and depending on the species, the enzyme activity could be found in either the soluble fraction, the microsomal fraction, or both (Diesperger and Sandermann, 1978, 1979; Edwards and Dixon, 1991). Diesperger and Sandermann (1979) partially characterized the enzyme from pea

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Abbreviations: CA, *trans*-cinnamic acid; 4-CA, 4-hydroxycinnamic acid (*p*-coumaric acid); CDNB, 1-chloro-2,4-dinitrobenzene; δ_H and δ_C, proton chemical shift and carbon-13 chemical shift, respectively; EPTC, S-ethyl dipropylcarbamothioate; EPTC sulfoxide, the sulfoxide derivative of EPTC; GST, GSH S-transferase; GST followed by a compound in parentheses, the compound in parentheses indicates the substrate used to measure the enzyme activity; 7-HC, 7-hydroxycoumarin; HNE, 4-hydroxynonenal; PAL, Phe ammonia-lyase; TPBO, *trans*-4-phenyl-3-buten-2-one.

seedlings and reported that this species contained both a soluble and microsomal GST (CA) enzyme. In addition, it was reported that, when intact log-phase soybean suspension cultures were incubated with [^{14}C]CA in the absence of GSH, a 6% conversion of CA to the GSH conjugate was observed (Diesperger and Sandermann, 1979). These results indicate that in some plant species the GST (CA) enzyme may function in vivo to produce GSH conjugates of CA. Although detailed studies involving alternative substrates were not conducted, it was indicated that 4-CA would also serve as a substrate for the pea microsomal GST (Diesperger and Sandermann, 1979). More recently, Edwards and Dixon (1991) partially characterized a GST (CA) enzyme from suspension-cultured cells of French bean (*Phaseolus vulgaris*) and other legume species. The cytosolic enzyme was inhibited by GST-specific inhibitors (tridiphane and sulfobromophthalein), and the enzyme from legumes cross-reacted with an antiserum raised against a corn GST that is involved in herbicide detoxification (Edwards and Dixon, 1991). Edwards and Dixon (1991) also showed that the enzyme from suspension-cultured cells of bean was induced following exposure of the cells to a fungal elicitor; therefore, this enzyme may be involved in plant defense responses. Although feeding experiments with exogenous [^{14}C]CA were unable to identify a GSH conjugate of CA in either elicited or nonelicited bean suspension cultures (Edwards et al., 1990), it is possible that the endogenous substrate of this enzyme may be other unsaturated compounds that are generated during stress responses. Recently, it was shown that the corn GST enzyme active with CA is also active with 4-CA and a number of other phenylpropanoids; however, this activity has not yet been characterized (Dean and Orlofsky, 1993).

To gain some insight into the possible significance of a plant GST enzyme active with phenylpropanoids, the purpose of this research was to characterize the corn shoot GST enzyme that was responsible for the in vitro conjugation of 4-CA and CA with regard to enzyme molecular mass, subunit composition, pH optimum, substrate specificity, enzyme activation, kinetics, and identity of the product.

MATERIALS AND METHODS

Plant Material and Chemicals

Corn (*Zea mays* L. Pioneer hybrid 3906) seeds were grown in vermiculite in the dark for 3 d as described by Dean et al. (1991). Shoots were excised and stored at −70°C before enzyme extraction. [U-^{14}C]4-CA was enzymatically synthesized from L-[U-^{14}C]Tyr (New England Nuclear) by PAL isolated from *Rhodotorula glutinis* (Sigma). The reaction medium consisted of 1 mM L-[U-^{14}C]Tyr (specific activity 35.7 μCi/μmol), 0.1 M Tris-HCl (pH 8.5), and 0.05 unit of PAL. The reaction was allowed to proceed at 30°C for at least 8 h and was terminated by the addition of 0.1 volume of 32.5% (w/v) TCA. The reaction medium was extracted twice with 2 volumes of ether to separate the [^{14}C]4-CA from the L-[^{14}C]Tyr. The ether phase was collected and evaporated under N_2, and the residual [^{14}C]4-CA was dissolved in methanol. Examination of the product by TLC on silica gel plates (LK6, Whatman) developed in *n*-butanol:acetic acid:water (2:1:1, v/v/v) revealed that the reaction and extraction procedure resulted in pure [^{14}C]4-CA. Greater than 80% of the L-[^{14}C]Tyr was recovered as [^{14}C]4-CA.

[^{14}C]CA was either purchased from Research Products International Corp. (Mt Prospect, IL) ([3-^{14}C]CA, specific activity 45–60 mCi/mmol) or synthesized from L-[U-^{14}C]Phe (New England Nuclear) by PAL as described above, except that the reaction product was extracted with methylene chloride. Coumarin, 7-HC, coniferyl alcohol, sinapic acid, and TPBO were purchased from Aldrich. The 4-HNE was generously provided by Dr. H. Esterbauer (Institut für Biochemie, Universität Graz, Graz, Austria). All other chemicals were purchased from Sigma.

Enzyme Assays

GST activity, using CDNB as a substrate, was determined in the presence of 3 mM GSH and 3 mM CDNB as described by Gronwald et al. (1987). GST activity with TPBO was measured as described by Habig et al. (1974). GST activity with HNE was measured using the method described by Ålin et al. (1985). GST (CA) activity was determined in the presence of 3.0 mM GSH and 2.0 mM CA as described by Dean et. al. (1991). GST (4-CA) activity was determined with either a radiochemical assay or a colorimetric assay that utilized 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent) to measure the amount of nonconjugated GSH that remained in the assay medium (Ellman, 1959). The medium for the radiochemical assay consisted of 3 mM [U-^{14}C]4-CA (specific activity 0.125–1.0 μCi/μmol), 3 mM GSH, 0.2 M Tris-HCl (pH 8.0), and extract (approximately 0.38 μg of protein) in a final volume of 180 μL. The reaction medium was incubated at 30°C for 45 min and the assay was terminated by the addition of 20 μL of 32.5% (w/v) TCA. Nonconjugated 4-CA was removed from the aqueous phase by extracting twice with 2 volumes of ether. Radioactivity remaining in the aqueous phase was determined by liquid scintillation spectrometry. To determine the sulfhydryl specificity of the enzyme, GSH was replaced in the radiochemical assay medium by either Cys, CoA, DTT, or 2-mercaptoethanol.

The colorimetric assay used was a modification of the assay described by Boyland and Chasseaud (1967) and Wadleigh and Yu (1987). The colorimetric assay medium consisted of 3 mM 4-CA, 3 mM GSH, 0.2 mM Tris-HCl (pH 8.0), and extract (approximately 2 μg of protein) in a final volume of 1 mL. Following incubation at 30°C for 45 min, 50 μL of the reaction medium was added to 3 mL of 5,5′-dithio-bis(2-nitrobenzoic acid) (0.8 mM in 0.1 M K-PO_4, pH 7.4) and A_{412} was measured. The 4-CA in the colorimetric assay was replaced by other compounds to test the substrate specificity of the enzyme. Loss of GSH during the colorimetric assay did not occur when the enzyme was incubated with GSH in the absence of a GSH acceptor. Nonenzymatic conjugation determined with boiled (20 min) enzyme blanks was not detected with the colorimetric assay and was less than 10% of the enzymatic rate when determined with the radiochemical assay. A correction was made in the conjugation rates observed in the enzymatic

radiochemical assay to account for the nonenzymatic rates of conjugation.

The procedure described by Dean and Machota (1993) was used to determine whether either the GST (4-CA) or GST (CA) activity of the enzyme was activated by either 50 μM 4-CA or 7-HC.

GST Purification

All procedures were performed at 0 to 4°C. The following buffers were used: buffer A, 0.2 M Tris-HCl (pH 7.8), 1 mM EDTA, 10% (w/v) polyvinylpolypyrrolidone; buffer B, 20 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 M $(NH_4)_2SO_4$; buffer C, 20 mM Tris-HCl (pH 7.8), 1 mM EDTA; buffer D, 20 mM Tris-HCl (pH 7.8); buffer E, 20 mM Tris-HCl (pH 7.8), 1 M NaCl.

Frozen shoots (70 g) were homogenized with buffer A (212 mL) in a blender at high speed for 1 min. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000*g* for 20 min. The supernatant was decanted and adjusted to 80% ammonium sulfate with solid ammonium sulfate. After the solution was stirred for 30 min, it was centrifuged at 20,000*g* for 20 min. The resulting pellet was resuspended in 40 mL of buffer B, stirred for 30 min, and centrifuged at 20,000*g* for 20 min to remove insoluble materials. The resulting supernatant was loaded at 7.5 mL/min onto a phenyl-Sepharose CL-4B column (2.5 × 12 cm; Pharmacia) equilibrated with buffer B. The column was washed for 30 min with buffer B, and GST activity was eluted from the column with a 600-mL reverse ammonium sulfate gradient from 100% buffer B to 100% buffer C. The flow rate was 7.5 mL/min and the fraction size was 15 mL. The fractions containing the greatest amount of GST (4-CA) activity were pooled (30 mL) and desalted on Sephadex G-25 columns (PD-10, Pharmacia) equilibrated with buffer D. The eluant was then loaded onto a Q-Sepharose FF column (1.5 × 10 cm; Pharmacia) equilibrated with buffer D. The column was washed for 30 min with buffer D, and the GST (4-CA) activity was eluted with a 250-mL gradient from 100% buffer D to 100% buffer E. The flow rate was 3 mL/min and the fraction size was 3 mL. The fractions containing the greatest amount of GST (4-CA) activity were pooled (12 mL) and concentrated to 2 mL in a Centriprep-10 concentrator (Amicon, Inc., Beverly, MA). The concentrate was then loaded onto a Sephacryl S-200 gel filtration column (1.5 × 45 cm; Pharmacia) equilibrated with buffer D. The GST (4-CA) activity was eluted with buffer D at a flow rate of 0.3 mL/min. The fraction size was 0.75 mL. The gel filtration column was calibrated using BSA (66 kD) carbonic anhydrase from bovine erythrocytes (29 kD), and Cyt *c* from horse heart (12.4 kD). The fractions containing the greatest amount of GST (4-CA) activity from the gel filtration column were pooled (3.75 mL) and frozen at −70°C until used in enzyme assays.

SDS-PAGE

Samples were prepared for SDS-PAGE as described by Laemmli (1970). A Mini-Protean II system (Bio-Rad) was used with a 0.75-mm, 12% slab gel run at 200 V. Following the electrophoresis, the proteins were visualized by staining with Coomassie brilliant blue. Molecular mass markers included phosphorylase *b* (97.4 kD), BSA (66.2 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD) (Bio-Rad).

pH Optimum

The effect of pH on the purified GST (4-CA) activity was examined for pH values from 6.5 to 10.0. The enzyme was purified as described in "GST Purification." The following buffers at a concentration of 0.2 M were used to achieve the desired pH values: Mes (pH 6.5–7.0), K-PO_4 (pH 7.5–8.0), Tris (pH 8.5–9.0), and Gly (pH 9.5–10.0). The GST activity was determined with the radiochemical assay using [^{14}C]4-CA as a substrate as described in "Enzyme Assays."

Kinetics

The apparent K_m value of the purified enzyme for 4-CA was determined using various concentrations of [^{14}C]4-CA in the radiochemical assay as described in "Enzyme Assays" in the presence of 10 mM GSH. The apparent K_m value for GSH was determined using various concentrations of GSH in the radiochemical assay in the presence of 3 mM [^{14}C]4-CA. Apparent K_m values were calculated from double-reciprocal plots.

TLC

Reaction products formed during in vitro radiochemical GST assays were analyzed on silica gel TLC plates (LK6, Whatman) developed in *n*-butanol:acetic acid:water (2:1:1, v/v/v). Radioactivity on TLC plates was visualized with a Bioscan (Washington, DC) imaging scanner. To detect unlabeled conjugates, the plates were sprayed with a solution of 0.2% (w/v) ninhydrin in butanol to detect the amino group of GSH or a solution of sodium cobaltinitrite in acetic acid to visualize phenolics and conjugates of phenolics (Bhatia et al., 1971). Since the Tris-HCl buffer used in the GST assays was detected with the spray reagent described by Bhatia et al. (1971) and interfered with the detection of the phenylpropanoid conjugates, a buffer system consisting of 50 mM Hepes was used in assay mixtures that were to be analyzed with the spray reagents on TLC plates.

NMR Spectroscopy

The water-soluble products formed during the in vitro radiochemical GST (4-CA) assay were concentrated under nitrogen at 70°C and loaded onto several silica gel Sep-Pak cartridges (375 nmol product/cartridge; Millipore) equilibrated with isopropanol. Each cartridge was washed with 5 mL of isopropanol followed by 10 mL of 80% methanol. The 80% methanol fractions (approximately 90% of the product) were pooled and evaporated to dryness under N_2 at 70°C. The dried product was then dissolved in a minimal amount of water and strip loaded onto a silica gel TLC plate. TLC was performed and the product located as described in "TLC." The product was eluted from the silica gel with methanol:water (2:1) and evaporated to dryness under N_2 at 70°C. ^{1}H-NMR and ^{13}C-NMR spectra were

obtained with a Varian (Sunnyvale, CA) XL-300 NMR spectrometer operating at 300 and 75.4 MHz, respectively. GSH and the GSH conjugate of 4-CA were run in $[^2H]H_2O$ using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. 4-CA was run in a mixture of $[^2H]CHCl_3$/$[^2H]CH_3OH$ using tetramethylsilane as an internal standard.

Protein Assays

The protein concentration was determined by the method of Bradford (1976) using BSA as a protein standard. The protein concentration of column effluents was measured spectrophotometrically at 280 nm.

RESULTS

GST Purification, Molecular Mass, and pI

Partial purification of the enzyme was achieved by a combination of ammonium sulfate precipitation, hydrophobic-interaction, anion-exchange, and gel filtration chromatography (Fig. 1). The gel filtration column was calibrated with protein molecular mass standards and the results were used to determine that the native molecular mass of the GST (4-CA) protein was approximately 32 kD (Fig. 1C, inset). The purification procedure resulted in a 97-fold purification of the enzyme with a greater than 5% yield (Table I). Further purification of the enzyme using an HPLC mono-Q anion-exchange column according to the method of Dean et al. (1990) and a chromatofocusing column (Pharmacia) according to the method recommended by the manufacturer did not result in any additional increase in the specific activity. However, the chromatofocusing column was used to determine that the pI of the GST enzyme was at pH 5.2 (data not shown). Other researchers have reported the successful isolation of corn GSTs using affinity chromatography (Mozer et al., 1983; Irzyk and Fuerst, 1993); however, we were unable to obtain satisfactory results for the GST (4-CA) enzyme using GSH-agarose, Cys-agarose, *S*-hexylglutathione-agarose, bromosulfophthalein-agarose, or a variety of dye ligand columns.

SDS-PAGE analysis of the purified fraction revealed the presence of a major band that corresponded to a protein with a molecular mass of 30 kD (Fig. 2).

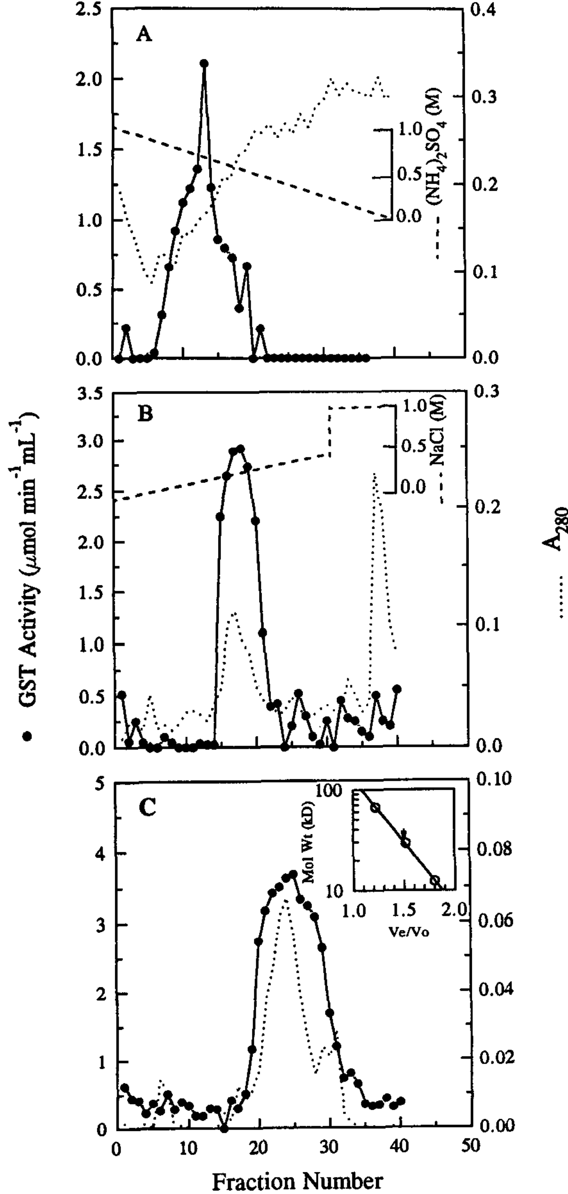

Figure 1. Chromatographic purification of a corn GST active with 4-CA. Chromatography of GST activity on phenyl-Sepharose hydrophobic-interaction (A), Q-Sepharose FF anion-exchange (B), and Sephacryl S-200 gel filtration (C) columns. The inset in C shows the calibration curve for the gel filtration column. The standards (O) used to generate the curve were: BSA, 66 kD; carbonic anhydrase, 29 kD; and Cyt *c*, 12.4 kD. The arrow indicates the location of the peak of the GST activity. The GST activity was determined with 4-CA as a substrate using the colorimetric assay as described in "Materials and Methods."

pH Optimum, Kinetics, and Factors Affecting the GST Assay

The effect of pH on the GST activity was determined for a pH range of 6.5 to 10. The pH optimum of the GST (4-CA) enzyme was determined to be between 7.5 and 8.0 (data not shown).

The apparent K_m values for GSH and 4-CA were determined from double-reciprocal plots to be 4.4 and 1.9 mM, respectively (Fig. 3).

The activity increased as the protein concentration in the assay medium increased from 0.19 to 9.5 μg/mL assay medium, with no increase in activity above 14.3 μg/mL (data not shown). The enzyme activity increased in a linear fashion as the assay time was increased up to 45 min but began to level off between 60 and 120 min (data not shown). Preliminary investigations revealed that 97% of the GST activity detected with 4-CA as a substrate was detected in the soluble fraction of maize extracts.

Substrate Specificity

In addition to 4-CA, the enzyme was also active with *o*-coumaric acid, *m*-coumaric acid, ferulic acid, and co-

Table I. *Purification of the GST (4-CA) enzyme from etiolated corn shoots*

The GST (4-CA) enzyme was extracted from 70 g of etiolated corn shoots and the activity was measured with the radiochemical assay as described in "Materials and Methods."

Fraction	Total		Specific Activity	Purification	Yield
	Activity	Protein			
	μmol min⁻¹	*mg*	*μmol min⁻¹ mg⁻¹*	*-fold*	%
Crude extract[a]	44.7	232.2	0.19	1	100
Ammonium sulfate	15.6	105.2	0.15	0.79	34.9
Phenyl-Sepharose	7.2	2.0	3.6	18.9	16.1
Q-Sepharose FF	3.3	0.37	8.9	46.8	7.4
Sephacryl S-200	2.4	0.13	18.5	97.4	5.4

[a] The enzyme activity in the crude extract was measured after desalting on Sephadex G-25 columns.

niferyl alcohol. Measurable activity was not observed when caffeic acid, sinapic acid, coumarin, or 7-HC were examined as substrates with the colorimetric assay (Table II). Other compounds that could not serve as substrates for the enzyme included TPBO (activity measured with the method described by Habig et al., 1974) and HNE (activity measured with the method described by Ålin et al., 1985).

In addition to GSH, the enzyme could also utilize Cys as a sulfhydryl source when the acceptor molecule was 4-CA (Table III). The activity was very low to undetectable when either CoA, 2-mercaptoethanol, or DTT was used as the sulfhydryl source (Table III).

Enzyme Activation

In addition to the substrates listed above, the purified GST enzyme was also active with CA (Table IV). The enzyme activity with CA was approximately 650-fold less than the activity observed with 4-CA (Table IV). The activity of the enzyme with [^{14}C]CA could be enhanced 2.6-fold by the addition of 50 μM unlabeled 4-CA or 5.2-fold by the addition of 50 μM 7-HC (Table IV). When [^{14}C]4-CA was

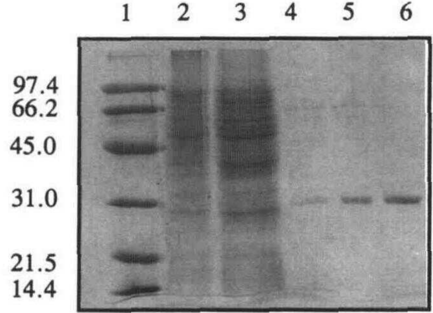

Figure 2. SDS-PAGE analysis of proteins from each stage of the GST purification procedure. Fractions were subjected to SDS-PAGE on 12% gels and visualized by Coomassie blue staining. Lane 1, Protein standards; lane 2, crude extract; lane 3, proteins precipitated between 25 and 80% ammonium sulfate saturation; lane 4, pooled fractions from the phenyl-Sepharose column; lane 5, pooled fractions from the Q-Sepharose FF column; lane 6, pooled fractions from the Sephacryl S-200 column. Approximately 15 μg of protein were loaded in lanes 2 to 4, and approximately 5 μg were loaded in lanes 5 and 6. Numerical values to the left are the molecular masses (in kD) of the protein standards.

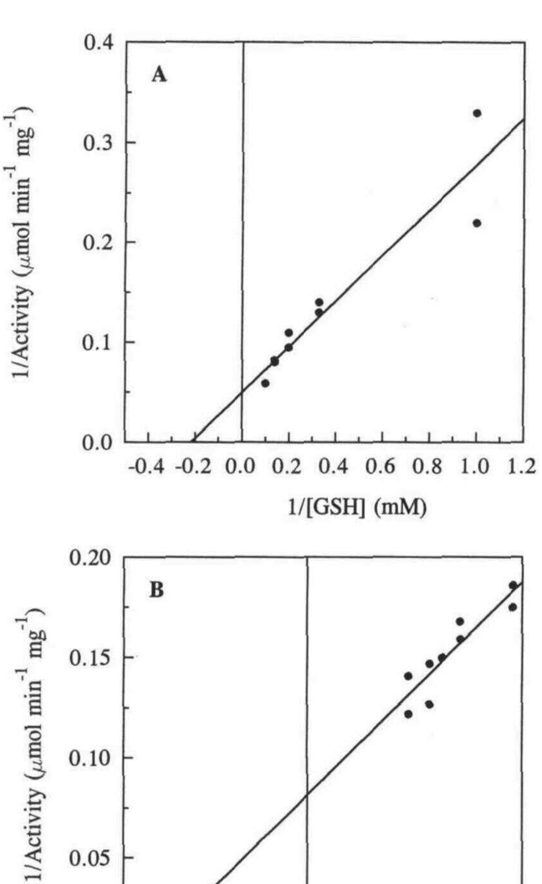

Figure 3. Kinetics analysis of GST (4-CA) activity. Double-reciprocal plots of GST (4-CA) activity versus either GSH concentration (A) or [^{14}C]4-CA concentration (B) were generated in the presence of 3 mM [^{14}C]4-CA and 10 mM GSH, respectively. The GST (4-CA) activities were determined with the radiochemical assay as described in "Materials and Methods." Data points represent the means of three replications, and points from two separate experiments are plotted on each graph. The apparent K_m values were calculated as 4.4 mM for GSH and 1.9 mM for 4-CA.

Table II. *GSH acceptor specificity of the GST (4-CA) enzyme*

The enzyme was purified as described in "Materials and Methods." The GST activities (with the exception of TPBO and HNE) were determined in the presence of 3 mM GSH and 3 mM acceptor. Activity was determined colorimetrically by using DTNB to measure the amount of nonconjugated GSH remaining in the assay medium. The enzyme activity with TPBO was determined by measuring the change in A_{290} according to the method of Habig et al. (1974), and the activity with HNE was determined by measuring the change in A_{224} according to the method of Ålin et al. (1985).

Substrate	Structure	Specific Activity[a]	Percentage of Activity with 4-CA
		μmol min⁻¹ mg⁻¹ protein	
p-Coumaric Acid (4-CA)		56.3 ± 4.8	100
m-Coumaric Acid		32.5 ± 4.6	58
o-Coumaric Acid		56.6 ± 2.1	101
Caffeic Acid		nd[b]	0
Ferulic acid		19.6 ± 1.3	35
Sinapic acid		nd[b]	0
Coniferyl alcohol		16.7 ± 1.2	30
Coumarin		nd[b]	0
7-HC		nd[b]	0
TPBO		nd[b]	0
HNE		nd[b]	0

[a] Values are means of three replicates ± SD. [b] Not detected.

Table III. *Sulfhydryl specificity of the GST (4-CA) enzyme*

The enzyme was purified and the GST (4-CA) activities were determined with the radiochemical assay as described in "Materials and Methods."

Sulfhydryl	Specific Activity[a]	Percentage of Activity with GSH
	µmol min⁻¹ mg⁻¹ protein	
GSH	19.2 ± 0.6	100
Cys	3.9 ± 0.1	20
CoA	0.76 ± 0.02	4
2-Mercaptoethanol	0.05 ± 0.03	0.3
DTT	nd[b]	0

[a] Values are means of three replicates ± SD. [b] Not detected.

used as a substrate, the addition of 7-HC did not result in any significant increase in enzyme activity (Table IV).

TLC Analysis of Conjugates

TLC analysis revealed that two main water-soluble reaction products of [^{14}C]4-CA were formed during the in vitro GST assay conducted at pH 8.0 in the presence of GSH (Fig. 4A). The major peak (approximately 78% of the radioactivity in the aqueous phase) had an R_F of 0.33 and the minor peak (approximately 22% of the radioactivity in the aqueous phase) had an R_F of 0.46 with the solvent system used in this study. The distribution of radioactivity between these two peaks was dependent on the pH of the assay medium. At a pH of 9.5, approximately 50% of the radioactivity in the aqueous phase was found in the R_F 0.33 product and 50% was found in the R_F 0.46 product (data not shown). Only the product with an R_F of 0.33 was formed nonenzymatically when GSH and [^{14}C]4-CA were incubated together for several hours in the presence of 100 mM NaOH (data not shown). A single water-soluble product of [^{14}C]4-CA was formed during the in vitro assay when Cys was used as the sulfhydryl source (Fig. 4B). The Cys conjugate of [^{14}C]4-CA had an R_F of 0.38. Nonconjugated [^{14}C]4-CA had an R_F value of 0.83 (Fig. 4C). The GSH conjugates of *o*-coumaric acid, *m*-coumaric acid, ferulic acid, and coniferyl alcohol formed during the colorimetric assay were also examined on TLC plates. In all cases, when compared to the controls, the complete assay mixtures contained only one additional spot detected with the spray reagent of Bhatia et al. (1971). The R_F values of the conjugates of *o*-coumaric acid, *m*-coumaric acid, and coniferyl alcohol were all 0.34, and the R_F value of the ferulic acid conjugate was 0.30. A single radioactive product with an R_F value of 0.48 was formed when the purified enzyme was incubated with GSH and [^{14}C]CA (data not shown), and the identity of the product did not change when activity was measured in the presence of 4-CA and 7-HC, which serve as activators of the enzyme (Dean and Machota, 1993).

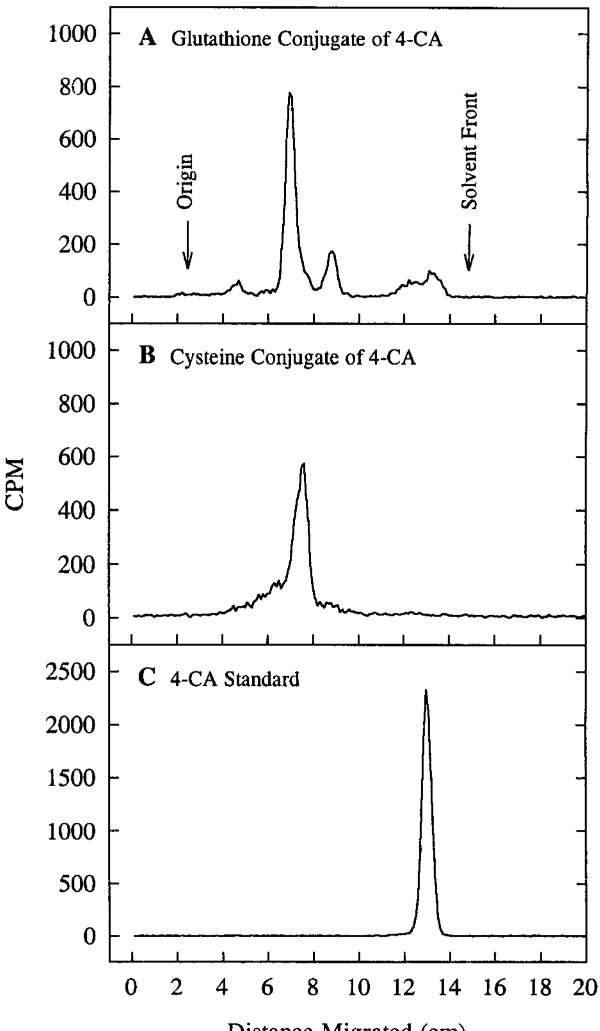

Figure 4. TLC analysis of the GSH and Cys conjugates of [^{14}C]4-CA formed by the GST (4-CA) enzyme. An aliquot of the aqueous phase (following ether extraction) from the radiochemical GST (4-CA) assay medium was spotted onto silica gel TLC plates, and the plates were developed in *n*-butanol:acetic acid:water (2:1:1, v/v/v). The GST assays were conducted in the presence of either GSH (A) or Cys (B). For comparison, a standard of [^{14}C]4-CA was subjected to the same TLC analysis (C). Radioactivity on the plates was detected with a Bioscan imaging scanner.

Table IV. *Effect of 4-CA and 7-HC on the GST (CA) and GST (4-CA) activity of the corn GST enzyme*

The enzyme was purified and the GST activity in the presence or absence of 4-CA and 7-HC was measured with the [^{14}C]CA or [^{14}C]4-CA radiochemical assays described in "Materials and Methods."

Substrate	Activator (50 µM)	Specific Activity	Activation
		nmol min⁻¹ mg⁻¹	*-fold*[a]
CA	None	25.8 ± 3.7	1
CA	4-CA	67.0 ± 3.1	2.6
CA	7-HC	134.5 ± 4.7	5.2
		µmol min⁻¹ mg⁻¹	
4-CA	None	16.9 ± 1.2	1
4-CA	7-HC	17.9 ± 0.5	1.1

[a] Increase in activity compared to the activity in the absence of the activator.

Characterization of the 4-CA Conjugate

The enzyme-catalyzed conjugate of [^{14}C]4-CA that had an R_F value of 0.33 (Fig. 4A) was analyzed by ^{1}H-NMR and ^{13}C-NMR spectroscopy. We were unable to obtain sufficient quantities of the minor product (R_F value of 0.46; Fig. 4A) for NMR analysis. In the ^{1}H-NMR spectrum, the conjugate displayed four proton signals in the aromatic region corresponding to two protons at both the *ortho* and *meta* positions (δ_H 7.10 and 6.70, respectively). These chemical shift values were not significantly different from the values (doublets at δ_H 7.41 and 6.84, respectively) obtained with 4-CA, indicating that substitution did not take place in the aromatic region of the molecule. In contrast, the characteristic *trans*-olefinic protons of 4-CA occurring at δ_H 6.25 (1H, d, J = 13.3 Hz) and 7.63 (1H, d, J = 13.3 Hz) were no longer observed in the conjugate.

In the ^{13}C-NMR spectrum, the signals in the aromatic region of 4-CA (δ_C 130.1 and 115.9 for the *ortho* and *meta* positions, respectively) were essentially unaffected (δ_C 131.3 and 113.0 for the *ortho* and *meta* positions, respectively) by the conjugation of GSH to the molecule. However, the spectrum for the 4-CA conjugate did not contain signals that corresponded to the signals observed in the spectrum for the C-2 and C-3 positions of nonconjugated 4-CA, which resonate at δ_C 114.6 (d) and 145.8 (d), respectively. The spectrum of the conjugate contained new signals at δ_C 46.1 (t) and 33.9 (d), which were attributed to the C-2 and C-3 positions, respectively, of the conjugate. Figure 5 shows the proposed structure of the conjugate based on the results obtained through NMR spectroscopy.

DISCUSSION

Several studies have reported the partial purification of a GST (CA) enzyme from various plant tissues, but in most cases only a single chromatographic step was utilized. Edwards and Dixon (1991) reported a 12-fold purification of the GST (CA) enzyme from bean using ammonium sulfate precipitation and hydrophobic-interaction chromatography. Diesperger and Sandermann (1979) reported a partial purification of the soluble GST (CA) from pea using gel filtration, and the corn GST (CA) enzyme was separated from the corn GST enzymes that were active with herbicide substrates and CDNB using anion-exchange FPLC (Dean et al., 1991). In addition, it was shown that anion-exchange chromatography could be used to separate the GST (CA) enzyme(s) that can be detected in the absence of an activator from those GST (CA) enzymes that can only be detected in the presence of an activator (Dean and Machota, 1993). In most of these reports, the extent of the purification was not shown, nor was it discussed. The purification reported in this study is believed to represent the most extensive purification of any GST enzyme that is known to be active with phenylpropanoids.

The native molecular mass of the GST (4-CA) enzyme was determined through gel filtration to be approximately 32 kD (Fig. 1C, inset). Since the SDS-PAGE analysis of the purified fraction contains a major band corresponding to a protein with a molecular mass of 30 kD, it appears that the corn GST (4-CA) enzyme exists as a monomer with a molecular mass of approximately 30 kD. From gel filtration studies, Diesperger and Sandermann (1979) reported that the soluble GST (CA) enzyme from pea had a molecular mass of 37 kD and that the enzyme had a tendency to aggregate into dimeric and tetrameric forms. In all of the gel filtration experiments performed in our study, we never observed aggregation of the GST (4-CA) enzyme. Typically, GST enzymes are found to exist as dimers. In corn, GST I, which was described as a constitutive enzyme active with both CDNB and the herbicide alachlor, has been characterized as a homodimer consisting of 29-kD subunits (Mozer et al., 1983). GST II is a heterodimer (Mozer et al., 1983; Jepson et al., 1994) composed of 27- and 29-kD subunits. GST II was originally detected only in extracts from safener-treated maize and is an enzyme active with both CDNB and alachlor (Mozer et al., 1983). GST III is a maize enzyme that is active with CDNB, alachlor, and metolachlor, and it has been reported to be a homodimer consisting of 26-kD subunits (O'Connell et al., 1988). Recently, another GST designated, GST IV, has been purified from benoxacor-treated maize (Irzyk and Fuerst, 1993). Characterization of this enzyme has indicated that it is a homodimer consisting of identical 27-kD subunits. GST IV is an enzyme reported to be active with acetochlor, alachlor, and metolachlor but not CA or CDNB. Compared to the other known maize GST enzymes, the GST (4-CA) enzyme has the unique property of existing as a monomer rather than a dimer. It remains to be seen whether the subunits of the other maize GST enzymes share any homology with this monomeric GST enzyme.

The corn GST (4-CA) enzyme has a pH optimum between 7.5 and 8.0 (data not shown), which is very similar to the pH optimum reported for the soluble pea GST enzyme that is active with CA (Diesperger and Sandermann, 1979). An increase in the nonenzymatic rate of GSH conjugation as the pH of the assay medium increased has been reported for the herbicide substrates alachlor, metolachlor, atrazine, and EPTC sulfoxide (Frear and Swanson, 1970; Lay and Casida, 1976; Leavitt and Penner, 1979; Irzyk and Fuerst, 1993), as well as for many of the general substrates (Habig et al., 1974; Habig and Jakoby, 1981). In addition, Diesperger and Sandermann (1979) reported an increase in the nonenzymatic rate of the conjugation of GSH to CA. However, we did not observe a measurable increase in the nonenzymatic rate of GSH and 4-CA conjugation as the pH

γ Glu
Cys — S
Gly
1 COOH
2
3
OH

Figure 5. Proposed structure of the GSH conjugate of 4-CA (major peak in Fig. 4A).

values increased from 6.5 to 10 in our assay medium, and at a pH value of 8, the nonenzymatic rate was less than 10% of the enzymatic rate.

The apparent K_m value for 4-CA was determined to be 1.9 mM. Since the concentration of 4-CA in plant cells may only be as high as 0.25 mM (Loake et al., 1991), the GST (4-CA) enzyme appears to have a low affinity for this substrate. However, this enzyme may still be active in vivo, since it has a high specific activity with 4-CA and may represent as much as 1% of the total protein (calculated from data in Table I) found in etiolated corn shoots. The apparent K_m value for CA was determined to be 50 μM (data not shown). This value is lower than the K_m value for CA reported for the GST (CA) enzymes from other species (Diesperger and Sandermann, 1979; Edwards and Dixon, 1991). Although the affinity of the corn enzyme for CA is greater than the affinity of the enzyme for 4-CA, the specific activity of the enzyme is substantially greater with 4-CA (Table IV).

In the presence of GSH, the enzyme was found to have activity with 4-CA, *o*-coumaric acid, *m*-coumaric acid, CA, ferulic acid, and coniferyl alcohol (Tables II and IV). In all of these cases, activity measured with the enzyme assay corresponded to the appearance of a water-soluble product that could be detected on TLC plates. Unfortunately, the colorimetric assay is not sensitive enough to detect activity with all possible substrates. For example, activity with CA can easily be measured with a radiochemical assay (Table IV) but is not observed with the colorimetric assay. Therefore, it is possible that some of the other compounds listed in Table II may also serve as substrates for the enzyme even though activity was not detected with the colorimetric assay.

In addition to the indication that the pea microsomal enzyme may be active with 4-CA, virtually nothing is known regarding the substrate specificity of other plant GST enzymes that are active with CA. It has been shown that a GST enzyme(s) extracted from rat liver is active with a variety of α,β-unsaturated compounds, including several that are naturally occurring (Boyland and Chasseaud, 1967), and a GST enzyme(s) from fall armyworm [*Spodoptera frugiperda* (J.E. Smith)] larvae has been shown to be active with a variety of α,β-unsaturated plant allelochemicals (Wadleigh and Yu, 1987). Therefore, it seems probable that screening of additional plant secondary compounds may reveal the existence of several other substrates for the corn GST (4-CA) enzyme.

Previous work has shown that the corn GST (CA) enzyme does not have activity with CDNB, metolachlor, atrazine, or EPTC sulfoxide (Dean et al., 1991), and in the present investigation, it was determined that the enzyme did not have activity with TPBO or HNE. TPBO is a general substrate typically used to measure the activity of GST enzymes with α,β-unsaturated carbonyl compounds (Habig et al., 1974; Habig and Jakoby, 1981; Mannervik, 1985), and HNE is a major product of lipid peroxidation in rat liver microsomes (Esterbauer, 1982). The failure of all of these compounds to serve as substrates for the enzyme may indicate that this GST is specific for naturally occurring secondary compounds and not for products of lipid peroxidation or synthetic compounds.

In addition to GSH, the GST (4-CA) enzyme has also been shown to have substantial activity with Cys as a sulfhydryl source (Table III). Typically, GST enzymes are very specific in their requirement for GSH (Clark, 1989), and the corn GST (4-CA) enzyme appears to be unique in its ability to use Cys as a substrate. It is interesting that two enzymes described as *S*-cysteinyl-hydroxychlorpropham transferase enzymes from oat (*Avena sativa* L.) have also been reported to have activity with both GSH and Cys (Rusness and Still, 1977b). Since both 4-CA and ferulic acid were found to be effective inhibitors of the *S*-cysteinyl-chlorpropham transferase, it was suggested that these compounds may also serve as substrates for the enzyme (Rusness and Still, 1977a). Still and Rusness (1977) concluded that an aryl-thioether bond was formed between Cys and 4-hydroxychlorpropham. In our study, NMR spectroscopy of the major enzymatic reaction product was used to determine that GSH was added to the olefinic double bond of 4-CA and not the aromatic ring. This is the same type of conjugate formed by the soluble and microsomal enzymes from pea (Diesperger and Sandermann, 1979) and the microsomal enzyme from parsley cells (Diesperger and Sandermann, 1978) when CA is used as the substrate. Therefore, the GST (4-CA) enzyme from corn appears to have a catalytic activity that is distinctively different from the activity of the *S*-cysteinyl-chlorpropham transferase from oat.

When compared to other maize GST enzymes, the enzyme described in this investigation has some unusual properties: The enzyme has been found to exist as a monomer rather than a dimer; the enzyme will utilize Cys as well as GSH as a sulfhydryl source; and when activity is measured with CA as a substrate, the enzyme can be activated in vitro by 4-CA and 7-HC. This enzyme may be involved in the in vivo conjugation of a number of phenylpropanoids or other α,β-unsaturated phenolics that are produced as defense compounds in response to UV radiation, wounding, infection, low-temperature stress, or other environmental factors. It is also possible that the enzyme could detoxify allelochemicals produced by other plants. In fact, appreciable amounts of unsaturated phenylpropanoids have been found in soil and are known to inhibit the germination and growth of some plants (Rice, 1987). Since most phenolics show some degree of phytotoxicity, they are rarely found free in the plant (Harborne, 1979). Conjugation of these phenolics to GSH may reduce their toxicity and increase their water solubility such that they can be safely sequestered in the vacuole. The elevated levels of phenolics produced during times of stress may regulate the activity of the enzyme, since it has been shown that this enzyme and other GST (CA) enzymes (Dean and Machota, 1993) can be activated by 4-CA and 7-HC.

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