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Purification and Biochemical Properties of Glutathione S-Transferase from *Lactuca sativa*

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A glutathione S-transferase (GST) from *Lactuca sativa* was purified to electrophoretic homogeneity approximately 403-fold with a 9.6% activity yield by DEAE-Sephacel and glutathione (GSH)-Sepharose column chromatography. The molecular weight of the enzyme was determined to be approximately 23,000 by SDS-polyacrylamide gel electrophoresis and 48,000 by gel chromatography, indicating a homodimeric structure. The activity of the enzyme was significantly inhibited by S-hexylGSH and S-(2,4-dinitrophenyl) glutathione. The enzyme displayed activity towards 1-chloro-2,4-dinitrobenzene, a general GST substrate and high activities towards ethacrynic acid. It also exhibited glutathione peroxidase activity toward cumene hydroperoxide.

Keywords: Enzymatic characterization, Glutathione, S-tranferase, Homodimer, *Lactuca sativa*, Lettuce, Purification, Substrate specificity

Introduction

Glutathione S-transferase (GST, EC 2.5.1.18) is a family of multifunctional proteins, catalyzing the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds including alkyl- and aryl halides, epoxides, esters and alkenes (Mannervik *et al.*, 1988). Certain GSTs can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity. Others catalyze the isomerization of certain steroids and play an important role in the intracellular transport of numerous hydrophobic

nonsubstrate ligands such as bile acids, billirubin and a number of drugs.

GSTs are distributed in a wide range of organisms from mammal to *E. coli* (Fahey *et al.*, 1991). Mammalian GSTs can be grouped into at least four distinct classes, alpha, mu, pi and theta according to their structures and catalytic properties (Mannervik *et al.*, 1992). The enzymes have been extensively purified from mammals such as human, mouse, cattle and rat, and their structure, function and physiological significance have been studied in detail (Beckett *et al.*, 1993; Beckett *et al.*, 1994; Armstrong, 1997). Plant GST has been concerned in the agricultural chemistry and biochemistry because it is one of the major factors involved in the resistance of a variety of herbicides and insecticides (Lamoureux *et al.*, 1980; Leurs *et al.*, 1989). The first GST reported to participate in herbicide metabolism was isolated from maize and characterized in some detail (Mozer *et al.*, 1983; Moore *et al.*, 1986; Grove *et al.*, 1988; Izuka *et al.*, 1989; Irzyk *et al.*, 1993, 1995). GSTs play roles in plants, having been implicated in herbicide resistance, being inducible by pathogens and/or dehydration, showing direct binding of auxins and catalyzing the formation of anthocyanins (Sheehan *et al.*, 2001). Plant GSTs have also been studied from wheat (Riechers *et al.*, 1997), tobacco (Droog *et al.*, 1995), carnation (Itzhaki *et al.*, 1993) and broccoli (Lopez *et al.*, 1994). Despite these efforts, the data on plant GSTs are largely lacking, and little is known about the biological function, structures and regulations of plant GSTs. In this study, a GST from the leaves of lettuce (*Lactuca sativa*) was purified, characterized the enzymatic properties and compared its properties with those of enzymes from other sources. This study is the first report on glutathione S-transferase in lettuce (*Lactuca sativa*), and it will be of great value in designing new herbicides having a high selectivity.

Materials and Methods

Materials The leaves of lattuce (*Lactuca sativa* L.) used in this study was purchased from Huksuk market. Glutathione (GSH), 1-

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chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ETA), 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), DEAE-Sephacel and glutathione-Sepharose were obtained from Sigma (St. Louis, USA). *S*-(2,4-dinitrophenyl) glutathione was synthesized by the method of Schramm *et al*. Benastatin A was gifted by Prof. T. Aoyagi (Aoyagi *et al*., 1992). All other reagents used were of the highest grade commercially available.

Preparation of enzyme The leaves of lettuce were homogenized 5 times with 100 mM potassium phosphate buffer (pH 7.0) containing 5 mM polyethylene glycol in a waring blender for 1 min. The mixture was centrifuged at $20,000 \times g$ for 30 min, yielding the crude extracts. This solution was dialyzed 3 times with changes every 8 hrs against 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The dialyzed solution was applied to a DEAE-Sephacel column (25×250 mm) equilibrated with buffer A. After washing the column with buffer A, bound proteins were eluted with a linear gradient of 0-500 mM NaCl in buffer A at 0.4 ml/min. The active fractions were pooled, dialyzed against buffer A and loaded onto a 15-ml column of glutathione-Sepharose equilibrated with buffer A. The column was exhaustively washed with the same buffer. The enzyme was eluted with 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH and dialyzed against buffer A. This dialyzed purified enzyme was used for next experiment. Unless otherwise indicated, all purification procedures were performed either at 4ºC or on ice. The enzymes were stored at −70ºC until use.

Enzyme activity and kinetic studies The specific activities were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with CDNB (1-chloro-2,4-nitrobenzene), DCNB (1,2-dichloro-4-nitrobenzene), EPNP (1,2-epoxy-3-(*p*-nitrophenoxy) propane and ETA (ethacrynic acid) as described by Habig and Jakoby (Habig *et al*., 1981). GSH-dependent peroxidase activity was assayed as described by Mannervik (Mannervik, 1985). Kinetic studies with GSH and CDNB were performed at 30ºC as described by Ivanetich and Goold (Ivanetich *et al*., 1989). The enzyme was preincubated for 2 min at 30ºC with a desired concentration of CDNB. Nonenzymatic reaction rates were subtracted from the reaction rate in the presence of enzyme. The concentrations of enzyme used for initial rate studies was varied in the range of 1-50 µg/ml. The concentrations of GSH and CDNB were varied in the range of 0.075-1 mM and 0.1-1 mM, respectively. Kinetic parameters were obtained from hyperbolic saturation curves by least squares fit of the initial velocity data to the equation of rapid equilibrium random sequential Bi Bi mechanism. Protein concentration of the enzyme was determined

using a protein assay reagent (Bio-Rad Lab., Richmond, USA).

Inhibition studies The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1 mM GSH and the inhibitor for 2 min and initiating the reaction by addition of 1 mM CDNB at 30ºC. The concentration of inhibitor giving 50% inhibition (I_{50}) was determined from plot of residual activity against inhibitor concentration.

Heat inactivation assays The enzyme was incubated at each temperature for 10 min at a protein concentration of 50 µg/ml in 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM DTT and 10 mM EDTA, to prevent the oxidative inactivation. The remaining activity was assayed in 100 mM potassium phosphate buffer (pH 6.5) with 1 mM GSH and 1 mM CDNB at 30ºC.

Molecular size determination The molecular weight was also determined using fast protein liquid chromatography (FPLC) on a Superdex 200 HR column (Pharmacia Biotech, Sweden) according to the method described previously (Hong *et al*., 1999). Molecular size standards used were blue dextran, yeast alcohol dehydrogenase, bovine serum albumin, trypsin inhibitor and cytochrome c (Sigma).

Electrophoresis Denaturing SDS-PAGE was carried out by the method of Laemmli (1970) in 12.5% gels. The molecular-mass makers were SDS molecular weight standard markers (Bio-Rad) that contains phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Coomassie Blue R-250 was used for staining (Kim *et al*., 2002).

Results

Purification, homogeneity and molecular weight The results of the purification of the GST present in *Lactuca sativa* was summarized in Table 1. GSH-conjugating activity toward CDNB in the crude extract was very low. Following chromatography on DEAE-Sephacel of the crude extract, a single peak of GST activity was eluted between 100 and 150 mM NaCl with an increase in specific activity of approximately 7 times. After passage through the GSH-Sepharose column, the specific activity further increased to approximately 403 times, with an overall recovery of around 9.6% of the initial activity.

The purified enzyme gave a single band on electrophoresis in the presence of SDS. Comparison of relative mobility of the

Table 1. Purification of glutathione *S*-transferase from *Lactuca sativa*

Step	Total activity (µmol/min)	Total protein (mg)	Specific activity (µmol/min per mg)	Yield (%)	Purification (fold)
Crude extract	8.41	621	0.01	100	1
DEAE-Sephacel	5.43	77.57	0.07	65	7
GSH-Sepharose	0.81	0.201	4.03	9.6	403

One unit of enzyme produces 1 µmol of *S*-(2,4-dinitrophenyl)glutathione per min at 30ºC.
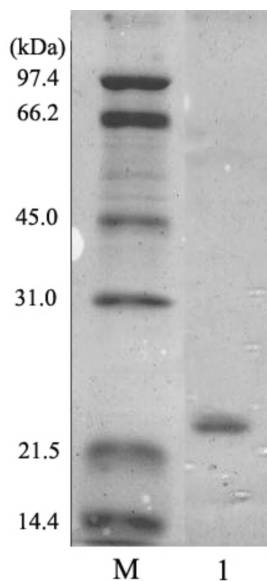
Fig. 1. Electrophoresis of the *Lactuca sativa* GST under denaturing conditions. Denaturing SDS-PAGE was carried out using the method of Laemmli (1970) in 12.5% gel. Coomassie blue R-250 was used for staining. Lane M, molecular mass marker (Bio-Rad); lane 1, GSH-Sepharose column chromatography.

enzyme with a standard protein indicated that a molecular weight of the *Lactuca sativa* GST was approximately 23,000 by SDS-PAGE (Fig. 1).

Substrate specificity Substrate specificity of the *Lactuca sativa* GST toward several compounds was shown in Table 2. The enzyme displayed GSH-conjugating activity towards 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-epoxy-3-(*p*-nitrophenoxy) propane(EPNP) and ethacrynic acid (ETA). On the other hand, there was no detectable activity toward 1,2-dichloro-4-nitrobenzene. The *Lactuca sativa* GST also exhibited glutathione peroxidase activity toward cumene hydroperoxide with a specific activity of 0.32 µmol min^{-1} per mg of protein.

Inhibition studies The inhibition parameters (I_{50}) of various inhibitors, *S*-hexylGSH, *S*-methylGSH, benastatin A, ethacrynic acid and *S*-(2,4-dinitrophenyl)glutathione for the GSH-CDNB conjugating activity were determined under the standard assay conditions (Table 3). The I_{50} value of *S*-hexylGSH and *S*-methylGSH, a derivative of GSH, for the enzyme was approximately 15 µM. The I_{50} of benastatin A, an electrophilic substrate-like compound (Aoyagi *et al.*, 1992) and *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with CDNB, for the enzyme were 0.41 µM and 3.4 µM, respectively.

Thermostability The thermostability of the enzyme was also investigated by incubation of the enzyme at various temperatures for 10 min. The midpoint of the temperature-stability curve was approximately 53°C for the enzyme (Fig. 2). The enzyme was stable to such incubation at temperatures

Table 2. Substrate specificity of the *Lactuca sativa* glutathione *S*-transferase

Substrates	Specific Activity (µmol/min per mg)
1-Chloro-2,4-dinitrobenzene	4.03 ± 0.23
1,2-Dichloro-4-nitrobenzene	ND[a]
Ethacrynic acid	6.21 ± 0.10
1,2-Epoxy-3-(p-nitrophenoxy)propane	8.78 ± 0.03
Cumene hydroperoxide	1.32 ± 0.01

Values are Means ± S.D., generally based on n≥5.
ND[a], No detected activity.

Table 3. Inhibition effect of *S*-hexyl-GSH, *S*-methyl-GSH, benastatin A, *S*-(2,4-dinitrophenyl)glutathione and ethacrynic acid on GSH-CDNB conjugation of the *Lactuca sativa* glutathione *S*-transferase

Inhibitors	I_{50} (µM)
S-hexyl-GSH	15.20 ± 0.10
S-methyl-GSH	12.30 ± 0.07
Benastatin A	0.41 ± 0.10
S-(2,4-dinitrophenyl)glutathione	3.40 ± 0.03
Ethacrynic acid	0.28 ± 0.04

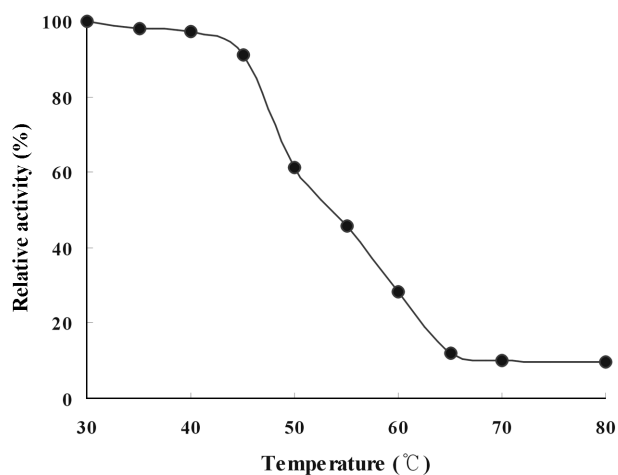Values are Means ± S.D., generally based on n≥5.

Fig. 2. Thermostability on the *Lactuca Sativa* GST. The enzyme was heated at each temperature for 10 mim.

up to 45°C. Above 50°C, its activities declined rapidly as the temperature increased, but the enzyme was not completely inactivated even at 80°C.

Discussion

Glutathione *S*-transferase from *Lactuca sativa* was purified to apparent homogeneity with the use of standard techniques, i.e.

anion exchange chromatography and affinity chromatography. The molecular weight of the purified *Lactuca sativa* GST was estimated to be 23,000 by SDS-polyacrylamide gel electrophoresis. On the other hand, runs of the purified enzyme in size-exclusion chromatography together with molecular weight markers indicated that the activity eluted at a point corresponding to a protein of approximately 48,000. It is then concluded that the active form of the *Lactuca sativa* GST is a homodimer of two equal polypeptides of 23,000. The *Lactuca sativa* GST seemed to be similar to those of mammalian, plant and microorganism enzymes, all of which are dimers with a molecular weight of 40,000 to 60,000 (Mozer *et al.*, 1983; Kong *et al.*, 1991; Nishida *et al.*, 1994). However, it was different from the enzyme of *Tetrahymena thermophila* that was a monomer with a molecular weight of 33,000 to 35,000 (Overbaugh *et al.*, 1988).

One prominent feature of GST is the presence of a number of isoenzymes within a given species (Mannervik *et al.*, 1988). According to the sequence comparisons, substrate specificities, sensitivities to inhibitors, *N*-terminal amino acid sequence and exon-intron compositions , the majority of GST purified from plant (Mozer *et al.*, 1983) were grouped into phi, zeta, tau and theta (Sheehan *et al.*, 2001). Only one form of GST was also reported from *Xanthomonas campestris* (Di Ilio *et al.*, 1993) and *E. coli* K-12 (Nishida *et al.*, 1994). The *Lactuca sativa* GST was not significantly active with CDNB, the substrate most often used in the assay of GSTs (Table 2). The activity of the enzyme toward CDNB was significantly lower than the enzymes from mouse, corn and *F. oxysporum* (Lee *et al.*, 1981; Mozer *et al.*, 1983; Ando *et al.*, 1988). On the other hand, it was similar to those of the enzymes from bacteria (Izuka *et al.*, 1989; Nishida *et al.*, 1994).

The *Lactuca sativa* GST was retained on GSH-affinity column and its apparent K_m value for GSH was calculated at 0.42 mM, which was in general agreement with published K_m^{GSH} values of other GSTs (Aceto *et al.*, 1993; Bartling *et al.*, 1993; Hahn *et al.*, 1994; Kong *et al.*, 1997). The I_{50} values of the enzyme for *S*-hexylGSH and *S*-(2,4-dinitrophenyl) glutathione also were within the range reported for other GSTs (Table 3). These results suggest that the GSH-binding site of the *Lactuca sativa* GST may be similar to those of other GSTs. Three-dimensional structure of GST from plant (*Arabidopsis thaliana*) showed an overall similarity to other GSTs particularly in the GSH-binding domain (Reinemer *et al.*, 1996). In contrast, the K_m value of the *Lactuca sativa* GST for CDNB was 1.42 mM, which was 1 order of magnitude lower than Km values reported for the enzymes from Arabidopsis thaliana (Bartling *et al.*, 1993) and potato (Hahn *et al.*, 1994). Its I_{50} value for benastatin A, an electrophilic substrate-like compound (Table 3) also was significantly lower than the I_{50} value reported for human pi-class GST (Kong *et al.*, 1997). These data indicate a higher affinity of the *Lactuca sativa* GST for electrophilic substrate.

The *Lactuca sativa* GST also was not significantly active with 1,2-dichloro-4-nitrobenzene, a marker substrate for the mu-class enzymes (Table 2). However, the enzyme had a significantly high specific activities towards 1,2-epoxy-3-(*p*-nitrophenoxy)propane, a marker substrate for the theta-class of GSTs. Theta-class transferase which were active towards these substrates had been isolated from human liver (Meyer *et al.*, 1991) and broccoli (Lopez *et al.*, 1994). The purified *Lactuca sativa* GST showed selenium-independent glutathione peroxidase activity when cumene hydroperoxide was used as a substrate. The theta-class GSTs purified from Arabidopsis thaliana and mouse liver exhibited selenium-independent glutathione peroxidase activity (Bartling *et al.*, 1993; Hiratsuka *et al.*, 1995). On the other hand, *E. coli* B GST showed neither selenium-dependent nor independent glutathione peroxidase activity, indicating that the properties of catalytic sites between eukaryote and prokaryote enzymes may be different (Izuka *et al.*, 1989). The molecular cloning of the GST gene of *Lactuca sativa* is now in progress in order to elucidate the difference in the molecular structure between the *Lactuca sativa* GST and enzymes of other sources.

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