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-Sephadex 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-transferase, 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, bilirubin 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 lettuce (Lactuca sativa L.) used in this study was purchased from Huksuk market. Glutathione (GSH), 1-
chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (ETA), 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP). DEAE-Sepharose 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 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 × 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-Sepharocolumn (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 EDTA and 1 mM 2-mercaptoethanol (buffer A). The dialyzed solution was applied to a DEAE-Sephalocolumn (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 4°C or on ice. The enzymes were stored at −70°C 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-nitrophenoxyl) propane and ETA (ethacrynic acid) as described by Habig and Jakoby (Habig et al., 1981). GST-dependent peroxidase activity was assayed as described by Mannervik (Mannervik, 1985). Kinetic studies with GSH and CDNB were performed at 30°C as described by Ivanetich and Goold (Ivanetich et al., 1989). The enzyme was preincubated for 2 min at 30°C 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 30°C. The concentration of inhibitor giving 50% inhibition (I50) 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 30°C.

**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 SDSPAGE 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. GST-conjugating activity toward CDNB in the crude extract was very low. Following chromatography on DEAE-Sephalothe 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

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

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

One unit of enzyme produces 1 µmol of S-(2,4-dinitrophenyl)glutathione per min at 30°C.
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 (I₅₀) 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 I₅₀ value of S-hexylGSH and S-methylGSH, a derivative of GSH, for the enzyme was approximately 15 µM. The I₅₀ 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 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.
Purification of Glutathione S-transferase from Lactuca sativa

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 *Km* value for GSH was calculated at 0.42 mM, which was in general agreement with published *Km* values of other GSTs (Aceto et al., 1993; Bartling et al., 1993; Hahn et al., 1994; Kong et al., 1997). The *I50* 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 (Reinemeyer et al., 1996). In contrast, the *Km* 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 *I50* value for benastatin A, an electrophilic substrate-like compound (Table 3) also was significantly lower than the *I50* 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-nitrophenoxypy)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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