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Analysis on the substrate specificity and inhibition effect of *Brassica oleracea* glutathione *S*-Transferase

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양배추 유래의 글루타티온 전달효소의 기질 특이성 및 저해 효과 분석

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요 약: 본 연구에서는 식물의 제초제 해독 기구를 알아보기 위해, 양배추 (*Brassica oleracea*)로 부터 글루타티온 전달효소를 정제하고 외래성 이물질에 대한 기질 특이성과 저해 효과를 분석하였다. 양배추로부터 DEAE-Sephacel 컬럼과 GSH-Sepharose 컬럼 크로마토그래피를 이용하여 약 10%의 수율로 글루타티온 전달효소를 정제하였다. 정제된 효소에 대해 분자량을 측정한 결과, SDS-polyacrylamide 겔 전기영동으로 측정한 분자량은 23,000Da을 나타내었으며, 겔 크로마토그래피로 측정한 분자량은 48,000Da을 나타내었다. 따라서 정제된 양배추 글루타티온 전달효소는 소단위체의 분자량이 약 23,000Da의 동종이량체라는 사실을 알 수 있었다. 이 효소의 저해제에 대한 효과를 조사한 결과, *S*-hexyl-GSH와 *S*-(2,4-dinitrophenyl)GSH에 의해 활성이 저해되었다. 양배추 글루타티온 전달효소의 기질특이성은 CDNB와 ETA에서 높은 활성을 보였으며, cumene hydroperoxide에 대한 GSH peroxidase 활성도 나타내었다.

Abstract : To gain further insight into herbicide detoxification of plant, we purified a glutathione *S*-transferase from *Brassica oleracea* (*Bo*GST) and studied its substrate specificity towards several xenobiotic compounds. The *Bo*GST was purified to electrophoretic homogeneity with approximately 10% activity yield by DEAE-Sephacel and GSH-Sepharose column chromatography. The molecular weight of the *Bo*GST 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 *Bo*GST was significantly inhibited by *S*-hexyl-GSH and *S*-(2,4-dinitrophenyl)GSH. The substrate specificity of the *Bo*GST displayed high activities towards CDNB, a general GST substrate and ethacrynic acid. It also exhibited GSH peroxidase activity toward cumene hydroperoxide.

Key words : enzymatic characterization, glutathione *S*-tranferase, *Brassica oleracea,* purification, substrate specificity, inhibition effect.

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1. Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a major family of detoxification enzyme that catalyzes the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic substrate including many herbicides and pesticides.[1,2] Certain GSTs also catalyze peroxidase reactions or isomerization of certain steroids and are involved in hydroxyl peroxidase detoxification or tyrosine metabolism respectively. Other GSTs play an important role in the intracellular transport of numerous nonsubstrate ligands such as auxins and cytokinins or anthocyanins and thus contribute to hormone homeostasis or vacuolar anthocyanin sequestration respectively.

GSTs are distributed in a wide range of organisms from mammal to *E. coli*.[2] Mammalian GSTs can be grouped into at least four distinct classes, alpha, mu, pi and theta according to their structures and catalytic properties.[3] 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.[4-5] Plant GSTs have been concerned in the agricultural chemistry and biochemistry because they are one of the major factors involved in the resistance of a variety of herbicides and insecticides.[6-7] The first GST reported to participate in herbicide metabolism was isolated from maize and characterized in some detail.[8-13] 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.[14] Plant GSTs have also been studied from wheat,[15] tobacco,[16] carnation[17] and broccoli.[18] Despite these efforts, the data on plant GSTs are largely lacking, and little is also known about the biological function, structures and regulations of plant GSTs.

In this study, a GST from the leaves of cabbage (*Brassica oleracea*) was purified, characterized the biochemical properties and compared its properties with those of enzymes from other sources.

2. Materials and Methods

2.1. Materials

The leaves of cabbage (*Brassica oleracea*) used in this study were purchased from Huksuk market (produced in Seosan, Chungcheongnam-do). Reduced glutathione (GSH), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), 1-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 GSH-Sepharose were obtained from Sigma (St. Louis, USA). *S*-(2,4-dinitrophenyl)GSH was synthesized by the method of Schramm *et al.*[19] Benastatin A was gifted by Prof. T. Aoyagi.[20] All other reagents used were of the highest grade commercially available.

2.2. Preparation of enzyme

The leaves of cabbage were homogenized 5 times with 100 mM potassium phosphate buffer (KPB, 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 KPB (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 GSH-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.

2.3. Enzyme activity and kinetic studies

The specific activities of GST were determined by

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measuring the initial rates of the enzyme-catalysed conjugation of GSH with CDNB, DCNB, EPNP and ETA as described by Habig and Jakoby.[21] GSH-dependent peroxidase activity was assayed as described by Mannervik.[22] Protein concentration of the enzyme was determined using a protein assay reagent (Bio-Rad Lab.).

Condition : ① CDNB : 200 mM KPB (pH 6.5) 500 μL, 50 mM GSH 20 μL, 50 mM CDNB 20 μL, enzyme solution 20 μL, H_2O 440 μL, 340 nm, ε= 9.6 mM^{-1} cm^{-1} ② DCNB : 200 mM KPB (pH 7.5) 500 μL, 50 mM GSH 100 μL, 50 mM DCNB 20 μL, enzyme solution 50 μL, H_2O 330 μL, 345 nm, ε=8.5 mM^{-1} cm^{-1} ③ EPNP : 200 mM KPB (pH 6.5) 500 μL, 50 mM GSH 100 μL, 25 mM EPNP 20 μL, enzyme solution 20 μL, H_2O 360 μL, 360 nm, ε=0.5 mM^{-1} cm^{-1} ④ ETA : 200 mM KPB (pH 6.5) 500 μL, 12.5 mM GSH 20 μL, 10 mM ETA 20 μL, enzyme solution 20 μL, H_2O 440 μL, 270 nm, ε=5 mM^{-1} cm^{-1} ⑤ GSH peroxidase activity : 200 mM sodium phosphate buffer (pH 7.0) 500 μL, 50 mM GSH 20 μL, GSH reductase 20 μL, 2.7 mM EDTA 370 μL, enzyme solution 50 μL, 5 mM NADPH 20 μL, 75 mM cumene peroxide 20 μL, 340 nm, ε=6.6 mM^{-1} cm^{-1}

2.4. 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 (I_{50}) was determined from plot of residual activity against inhibitor concentration.

2.5. Heat inactivation assays

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

2.6. 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.[23] Molecular size standards used were blue dextran, yeast alcohol dehydrogenase, bovine serum albumin, trypsin inhibitor and cytochrome c (Sigma).

2.7. Electrophoresis

Denaturing SDS-PAGE was carried out by the method of Laemmli[24] 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.

3. Results

3.1. Purification, homogeneity and molecular weight

The results of the purification of the GST present in *Brassica oleracea* were 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

Table 1. Purification of GST from *Brassica oleracea*

Step	Total Activity (μ mol/min)	Total protein (mg)	Specific Activity (μ mol/min/mg)	Yield (%)	Purification (fold)
Crude extract	8.84	442	0.02	100	1
DEAE-Sephacel	4.27	53.48	0.08	48.3	4
GSH-Sepharose	0.92	0.153	6.01	10.4	301

One unit of enzyme produces 1 μ mol of *S*-(2,4-dinitrophenyl)GSH per min at 30 °C.

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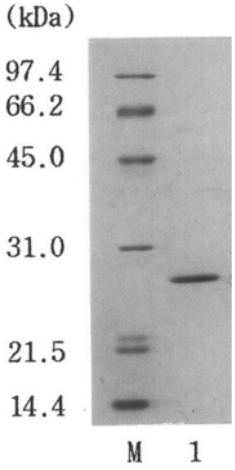

Fig. 1. Electrophoresis of the *Brassica oleracea* GST under denaturing conditions. Denaturing SDS-PAGE was carried out using the method of Laemmli (17) in 12.5% gel. Coomassie blue R-250 was used for staining. Lane M, molecular mass marker (Bio-Rad); lane 1, GSH-Sepharose column chromatography.

Table 2. Substrate specificity of the *Brassica oleracea* GST

Substrates	Specific Activity (μ mol/min/mg)
1-Chloro-2,4-dinitrobenzene	6.01 ± 0.27
1,2-Dichloro-4-nitrobenzene	ND[a]
Ethacrynic acid	7.49 ± 0.15
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	9.85 ± 0.06
Cumene hydroperoxide	1.74 ± 0.04

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

Table 3. Inhibition effect of inhibitors on GSH-CDNB

Inhibitors	I_{50} (M)
S-hexyl-GSH	14.70 ± 0.20
S-methyl-GSH	13.27 ± 0.10
Benastatin A	0.34 ± 0.07
S-(2,4-dinitrophenyl)GSH	2.75 ± 0.11
Ethacrynic acid	0.42 ± 0.06

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

mM NaCl with an increase in specific activity of approximately 4 times. After passage through the GSH-Sepharose column, the specific activity further increased to approximately 301 time, with an overall recovery of around 10.4% of the initial activity.

The purified enzyme gave a single band on electrophoresis in the presence of SDS. Comparison of relative mobility of the enzyme with a standard protein indicated that a molecular weight of the *Brassica oleracea* GST was approximately 23,000 by SDS-PAGE (*Fig.* 1).

3.2. Substrate specificity

Substrate specificity of the *Brassica oleracea* GST toward several compounds is shown in *Table* 2. The enzyme displayed GSH-conjugating activity towards CDNB, EPNP and ETA. On the other hand, there was no detectable activity toward DCNB. The *Brassica oleracea* GST also exhibited GSH peroxidase activity toward cumene hydroperoxide with a specific activity of 1.74 mol min^{-1} per mg of protein.

3.3. Inhibition studies

The inhibition parameters (I_{50}) of various inhibitors, *S*-hexyl-GSH, *S*-methyl-GSH, benastatin A, ETA and *S*-(2,4-dinitrophenyl)GSH for the GSH-CDNB conjugating activity were determined under the standard assay conditions (*Table* 3). The I_{50} value of *S*-hexyl-GSH and *S*-methyl-GSH, a derivative of GSH, for the enzyme was approximately 14 M. The I_{50} of benastatin A, an electrophilic substrate-like compound[20] and *S*-(2,4-dinitrophenyl)GSH, a conjugation product of GSH with CDNB, for the enzyme were 0.34 M and 2.75 M, respectively.

3.4. Thermostability

The thermostability of the enzyme was also investigated by incubation of the enzyme at various

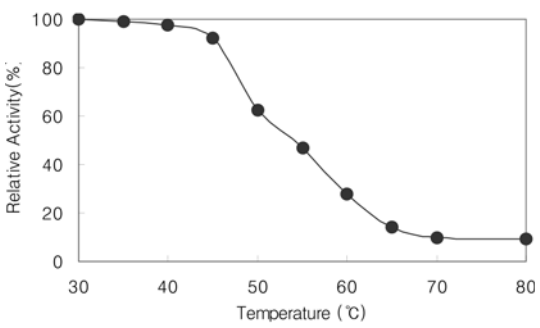

Fig. 2. Thermostability on the *Brassica oleracea* GST. The enzyme was heated at each temperature for 10 mim.

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temperatures for 10 min. The midpoint of the temperature-stability curve was approximately 52 °C for the enzyme (*Fig.* 2). The enzyme was fairly stable to such an 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.

4. Discussion

GST from *Brassica oleracea* 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 *Brassica oleracea* 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 marker, 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 *Brassica oleracea* GST is a homodimer of two equal polypeptides of 23,000. The *Brassica oleracea* 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.[8,25,26] However, it was different from the enzyme of *Tetrahymena thermophila* that was a monomer with a molecular weight of 33,000 to 35,000.[27]

One prominent feature of GST is the presence of a number of isoenzymes within a given species.[1] 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[8] were grouped into phi, zeta, tau and theta.[14] Only one form of GST was also reported from *Xanthomonas campestris*[28] and *E. coli* K-12.[26] The *Brassica oleracea* GST was not significantly active with CDNB, the substrate most often used in the assay of GSTs (*Table* 2). The activity of the enzyme for CDNB was significantly lower than those of the enzymes from mouse, corn and *F. oxysporum*.[8,29,30] On the other hand, it was similar to those of the enzymes from bacteria.[11,26]

The *Brassica oleracea* GST was retained on GSH-affinity column and its apparent K_m value for GSH was calculated at 0.39 mM, which was in general agreement with published K_m^{GSH} values of other GSTs.[31-34] The I_{50} values of the enzyme for *S*-hexyl-GSH and *S*-(2,4-dinitrophenyl)GSH also were within the range reported for other GSTs (*Table* 3). These results suggest that the GSH-binding site of the *Brassica oleracea* 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.[35] In contrast, the K_m value of the *Brassica oleracea* GST for CDNB was 1.56 mM, which was 1 order of magnitude lower than K_m values reported for the enzymes from *Arabidopsis thaliana*[32] and potato.[33] 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.[34] These data indicate a higher affinity of the *Brassica oleracea* GST for electrophilic substrate.

The *Brassica oleracea* GST was not significantly active with DCNB, a marker substrate for the mu-class enzymes (*Table* 2). On the other hand, the enzyme had a significantly high specific activity toward EPNP, a marker substrate for the theta-class of GSTs. Theta-class transferases which were active towards these substrates had been isolated from human liver[36] and broccoli.[18] The purified *Brassica oleracea* GST showed selenium-independent GSH peroxidase activity when cumene hydroperoxide was used as a substrate. The theta-class GSTs purified from *Arabidopsis thaliana* and mouse liver cytosol also exhibited selenium-independent GSH peroxidase activity.[32] On the other hand, *E. coli* B GST showed neither selenium-dependent nor independent GSH peroxidase activity, indicating that the properties of catalytic sites between eukaryote and prokaryote enzymes may be different.[11] The molecular cloning of the GST gene of *Brassica oleracea* is now in progress in order to elucidate the difference in the

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molecular structure between the *Brassica oleracea* GST and enzymes of other sources.

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