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Characterization of a Novel Glutathione S-Transferase from *Pseudomonas* sp. DJ77

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Abstract: A novel glutathione S-transferase from *Pseudomonas* sp. DJ77 was expressed in *E. coli* and purified by glutathione-affinity chromatography. The enzyme was composed of two identical subunits. The molecular size of the enzyme was 42 kDa by sephadex G-150 gel permeation chromatography and Mr of each subunit was 23 kDa by sodium dodecylsulfate-polyacrylamide gel electrophoresis. pI value of the enzyme was approximately 5.8 by isoelectric focusing. This enzyme showed the highest activity toward 1-chloro-2,4-dinitrobenzene as the electrophilic substrate. The relative activities toward *p*-nitrobenzyl chloride and 1,2-dichloro-4-nitrobenzene were 3.8% and 1.3% of the activity toward 1-chloro-2,4-dinitrobenzene, respectively. K_m and V_{max} values for 1-chloro-2,4-dinitrobenzene calculated by Lineweaver-Burk plot were 0.76 mM and 14.81 μmol/min/mg, respectively, and those for glutathione were 6.23 mM and 64.93 μmol/min/mg, respectively. The enzyme showed highest glutathione S-transferase activity at pH 8.0 and was stable between pH 6.0 and 9.0. The enzyme retained its activity up to 35℃ for 90 min but was unstable above 45℃.
Key words: glutathione S-transferase, kinetic property, *Pseudomonas*, purification.

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of enzymes that catalyze the conjugation of glutathione (GSH) with various compounds carrying an electrophilic center (Chasseaud, 1979; Jakoby and Habig, 1980). Mammalian cytosolic GSTs are known to be involved in detoxification of xenobiotics. It is also known that rat liver GSTs serve as ligandins for compounds such as bilirubin, 3,6-dibromosulfophthalein and indocyanine green (Litwack *et al.*, 1971). Mammalian GST isozymes are classified into at least five groups, alpha, mu, pi, theta and microsomal, according to their primary structures, immunological cross-reactivities and catalytic characteristics (Mannervik *et al.*, 1985; Meyer *et al.*, 1991).

Extensive studies were performed on the mammalian GSTs from humans, mice and rats. Recently, GSTs were identified or purified from bacteria (Di Ilio *et al.*, 1988; Piccolomini *et al.*, 1989; Di Ilio *et al.*, 1989; Iizuka *et al.*, 1989; Di Ilio *et al.*, 1991; Orser *et al.*, 1993; Hofer *et al.*, 1994). Amino acid sequence analysis revealed that the amino acid sequences of bacterial GSTs are greatly different from those of mammallian GSTs showing homologies below 20%, but bacterial GSTs show 45~55% sequence homologies (Wilce and Parker, 1994). The functions of bacterial GSTs are unclear, but some bacterial GSTs are considered to be involved in degradation of biphenyls or polycyclic aromatic hydrocarbons (Orser *et al.*, 1993; Hofer *et al.*, 1994).

Recently, a novel putative GST gene *phnC* was cloned from *Pseudomonas* sp. DJ77, which can degrade polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and biphenyls or aromatic compounds with a single benzene ring such as toluene or benzoate (Kim *et al.*, 1992; Shin, 1992). The genes involved in the degradation of phenanthrene were cloned into a gene cluster pHENX7 which contains *phnCDEFG*. *phnC* was subcloned from pHENX7 into pSP13. The putative amino acid sequence of *phnC* showed a high homology to those of bacterial GSTs and the cell extract of *E. coli* carrying cloned *phnC* gene exhibited GST enzymatic activity toward GSH and CDNB as substrates. In this study, *phnC* gene was expressed in *E. coli* and the product of *phnC* was purified with glutathione-affinity chromatography. Its biochemical and catalytic properties were examined and compared with the characteristics of other known GSTs.

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Materials and Methods

Bacterial species

E. coli XL1-BLUE which contains the pSP13 plasmid was used for the expression of the *phnC* gene. pSP13 plasmid is pBLUESCRIPT SK(+) which contains the *phnC* gene in its multicloning site (Shin, 1992).

Chemicals

Glutathione, epoxy-activated Sepharose 6B, ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, *p*-nitrophenethyl bromide, *p*-nitrobenzyl chloride and bromosulfophthalein were purchased from the Sigma Co., Ltd (St. Louis, USA). 1-Chloro-2,4,-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) were from EGA-Chemie (Germany). *trans*-4-Phenyl-3-buten-2-one was obtained from the Aldrich Chemical Co. Inc. (Milwakee, USA). Isopropyl thiogalactopyranoside (IPTG) and marker proteins for SDS-PAGE and gel permeation chromatography were obtained from Boehringer Mannheim (Mannheim, Germany). Sephadex G-150 and Blue dextran 2000 were purchased from the Pharmacia Co., Ltd. (Uppsala, Sweden).

Preparation of cell extract

E. coli possessing pSP13 plasmid was cultured in Luria-Bertani media which contained 50 μg/ml of ampicillin and 10 μg/ml of tetracycline at 37℃. IPTG was added to the culture in a concentration of 40 μg/ml 5 h after the start of culture. After 6 h of further culture, the cells were harvested by centrifugation and washed several times with 20 mM potassium phosphate buffer (pH 7.0). The cells were disrupted by sonication for 40 min (30 sec of bursts followed by 30 sec of resting) at 50 W with a US-50 (nihonseiki) sonicator. Sonicated cell suspension was then centrifuged at 12000 rpm for 30 min and the supernatant was taken. The cell extract was stored at −70℃ until use.

Purification of GST

A GSH-Sepharose affinity column was prepared by the method as described by Simons and Vander Jagt (1977). The crude cell extract was applied to the GSH-affinity column (1×4 cm) which was pre-equilibrated with buffer A (20 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA). After loading the crude cell extract, the column was washed with buffer A until unbound proteins were completely eluted. The enzyme was eluted with Buffer B (50 mM Tris-Cl, pH 9.6, 15 mM GSH, 1 mM EDTA). The eluted fractions which exhibited GST activities were pooled and dialyzed against 20 mM potassium phosphate buffer (pH 7.0) for 10 h (5 h for each batch). The dialyzed enzyme solution was stored at −70℃ until use.

Assay for GST activity, kinetic properties and substrate specificity

The enzyme activity was assayed with GSH and 1-chloro-2,4-dinitrobenzene (CDNB). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 1 mM GSH, 1 mM CDNB and enzyme. The reaction was monitored at 25℃ by the increase in absorbance at 340 nm, which is the result of synthesis of S-(2,4-dinitrophenyl)glutathione from GSH and CDNB. The kinetic values of GST for GSH and CDNB were determined by the procedure as follows. The concentration of GSH (0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mM) or CDNB (0.25, 0.3, 0.4, 0.5, 0.65, 0.75, 0.85, 1.0, 1.5 and 2.0 mM) was varied while the concentration of the other substrate was fixed at 1.0 mM. K_m and V_{max} were calculated from the Lineweaver-Burk plot of the results.

The enzymatic activities were assayed toward CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, ethacrynic acid, *trans*-4-phenyl-3-buten-2-one, *p*-nitrophenethyl bromide, *p*-nitrobenzyl chloride, and bromosulfophthalein as electrophilic substrates by the method of Habig *et al.* (1974). CDNB and other electrophilic substrates were dissolved in ethanol and added to the reaction mixture because they are insoluble in water.

Determination of molecular size

Protein concentrations were determined by Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard. SDS-PAGE was performed by the method of Laemmli (1970). The gel was stained with Coomassie brilliant blue. Marker proteins used for the determination of subunit molecular size were α-lactalbumin (14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), albumin from egg (45,000) and albumin from bovine (66,000).

The molecular size of the enzyme was estimated by gel permeation of a Sephadex G-150 column (2.6×60 cm). The enzyme was eluted with 10 mM sodium phosphate buffer (pH 7.0) and the elutates were collected at a flow rate of 26 ml/h by observing the profile at 280 nm with a single path monitor UV-1 (Pharmacia Co. Ltd.). Marker proteins for the determination of molecular size were aldolase (158,000), bovine serum albumin (68,000), chymotrypsinogen (25,000) and cytochrome C (12,500). The void volume of the column was determined by using Blue dextran 2000.

N-terminal residues determination and isoelectric focusing

The N-terminal amino acid sequence of the purified enzyme was determined automatically with a Beckman PI-200 automatic amino acid sequencer.

Isoelectric focusing was performed by the method described previously (Giulian *et al.*, 1984). The polyacrylamide gel containing ampholyte (pH 3.5~10) was pre-run at 150 V for 30 min prior to the loading of samples. The enzyme sample was loaded into the gel and was electrophoresized at 200 V for 2.5 h. After the electrophoresis, the gel was cut into slices and each slice was suspended in 1 ml of 10 mM KCl. The pI of the enzyme was determined by measuring the pH of the KCl solution of the gel slice containing the enzyme.

Effect of temperature and pH on the activity of the enzyme

The enzyme activity was monitored at 25, 35, 45, 50, 60 and 70℃. The inactivation of the enzyme by temperature was observed at 25, 35, 40, 45, 50℃. The pH-dependence of the enzyme activity and stability was determined by measuring the activity of the enzyme in 0.1 M sodium acetate buffer (pH 5.0~6.0), 0.1 M sodium phosphate buffer (pH 6.0~8.0) and 0.1 M Tris-Cl buffer (pH 8.0~9.0).

Results

Molecular size and pI of GST

The purification steps are shown in Table 1. The single step purification with GSH-affinity chromatography yielded a single protein and a 43 fold purification of the GST. 9.5 mg of purified protein was obtained from a two-liter culture. The purified protein showed GST activity toward GSH and CDNB as substrates. The specific activity was 10.37 U/mg, in which 1 U of GST activity is defined as 1 μmol of conjugate formation per 1 min. The Mr of PhnC subunit estimated by SDS-PAGE was 23 kDa (Fig. 1) and the Mr determined by Sephadex G-150 gel permeation chromatography was 42 kDa. These results suggested that PhnC was a homo-dimeric enzyme. Mr of PhnC was similar to those of bacterial and mammalian cytosolic GSTs (40~50 kDa). The pI value of the enzyme determined by isoelectric focusing was approximately 5.8.

Table 1. Summary of purification of the glutathione S-transferase

Step	Protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
1 Crude extract	510	122.4	0.24	100	1
2 GSH-affinity	9.36	97.1	10.37	79.3	43.2

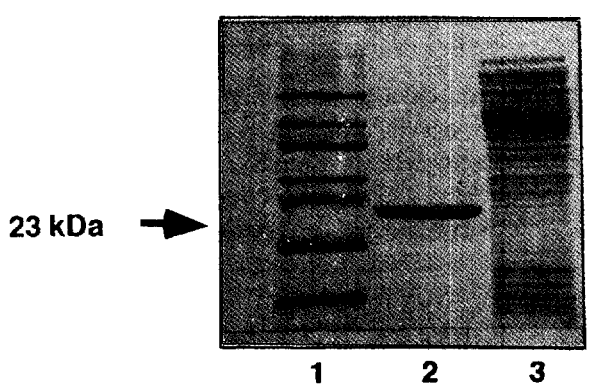


Fig. 1. SDS-Polyacrylamide gel electrophoresis. 12% T, 2.5% C; lane 1, size marker; lane 2, purified enzyme solution; lane 3, crude cell extract.

Table 2. Specific activity of GST toward different substrates. Enzyme assays were done in 0.1 M sodium phosphate buffer of corresponding pH. GSH-concentration was 5 mM except towart CDNB (1 mM), ethacrynic acid and *trans*-4-phenethyl-3-buten-2-one (0.25 mM)

Substrate	Concentration (mM)	pH	λ_{max} (nm)	Extinction coefficient ($mM^{-1} \cdot cm^{-1}$)	Specific activity (μmol/min per mg)
1-Chloro-2,4-dinitrobenzene	1.0	6.5	340	9.6	10.596(100)[a]
1,2-Dichloro-4-nitrobenzene	1.0	7.5	345	8.5	0.133(1.25)
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	5.0	6.5	360	0.5	0.063(0.60)
Ethacrynic acid	0.2	6.5	270	5.0	ND[b]
trans-4-Phenyl-3-buten-2-one	0.05	6.5	290	−24.0	ND
p-Nitrophenethyl bromide	0.1	7.5	310	1.2	ND
p-Nitrobenzyl chloride	1.0	6.5	310	1.9	0.404(3.81)
Bromosulfophthalein	0.03	7.5	33C	4.5	0.008(0.07)

[a]Numbers in parentheses indicate relative activity compared with CDNB.
[b]ND : Not detected.

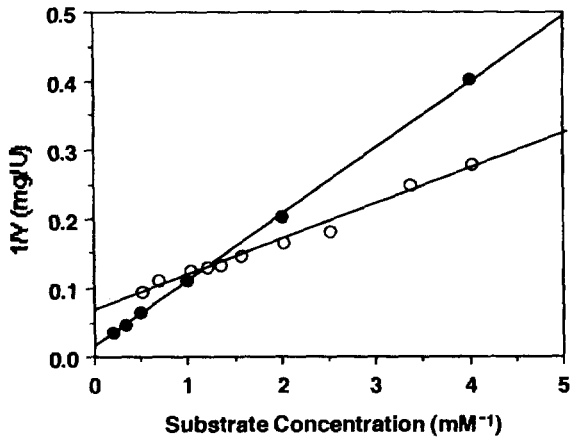


Fig. 2. Lineweaver-Burk plot of kinetic results of PhnC. (○) GSH concentration fixed (1 mM) and CDNB concentration varied (0.25, 0.3, 0.4, 0.5, 0.65, 0.75, 0.85, 1.0, 1.5 and 2.0 mM). (●) CDNB concentration fixed (1 mM), GSH concentration varied (0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 mM).

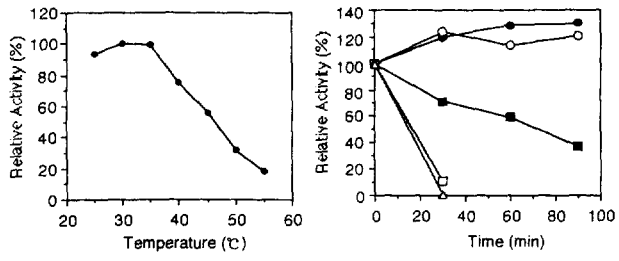
Nine N-terminal residues were determined with a Beckman PI-200 automatic amino acid sequencer. It was Met-Lys-Leu-Phe-Ile-Ser-Pro-Gly-Ala-Cys-Ser which was identical to the amino acid sequence deduced from the *phnC* nucleotide sequence (Shin, 1992).

Substrate specificity and kinetic properties

Enzymatic activity was determined toward GSH and various electrophilic compounds as substrates (Table 2). The purified GST showed maximal activity toward CDNB with specific activity 10.6 μmol/min/mg. K_m and V_{max} values for CDNB calculated from Lineweaver-Burk plot were 0.76 mM and 14.81 μmol/min/mg, respectively, and those for GSH were 6.23 mM and 64.93 μmol/min/mg, respectively (Fig. 2). The enzyme showed a little GST activity toward 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(*p*-nitrophenoxy) propane, *p*-nitrobenzyl chloride and bromosulfophthalein, and no enzymatic activity was observed toward ethacrynic acid, *trans*-4-phenyl-3-buten-2-one and *p*-nitrophenethyl bromide.

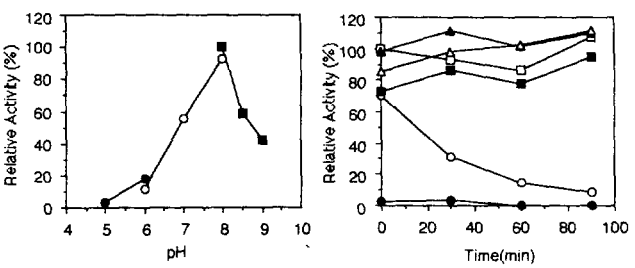
Effects of temperature and pH on the enzyme activity

Enzymatic activity and stability was measured at various temperature (Fig. 3). Enzymatic activity was highest at 30℃ and decreased above 40℃ (Fig. 3A). The enzyme was stable below 35℃ but was quickly inactivated above 45℃ (Fig. 3B). The effect of pH on the activity and the stability of the enzyme was observed at various pH buffers (Fig. 4). The highest activity was shown at pH 8.0 (Fig. 4A) and PhnC was stable for 90 min between pH 6.0 and pH 9.0, but was unstable below pH 5.0 (Fig. 4B).



(A) (B)

Fig. 3. The effect of temperature on the activity and stability of PhnC. (A) The reactions were proceeded at 25, 35, 45, 50, 60, 70℃. The enzymatic activity at 25℃ was taken as 100%. (B) The enzymatic activities were measured at 25℃ after incubation of PhnC at 25℃ (●), 35℃ (○), 40℃ (■), 45℃ (□), 50℃ (△) for 0, 30, 60, 90 min. The activity with incubation time 0 min at each temperature was taken as 100%.



(A) (B)

Fig. 4. The effect of pH on the activity and the stability of PhnC. (A) The enzyme reactions were proceeded in various buffers of different pH. The buffers used were 0.1 M sodium acetate buffer (●, pH 5.0, 6.0), sodium phosphate buffer (○, pH 6.0, 7.0, 8.0), Tris-Cl buffer (■, pH 8.0, 8.5, 9.0). The activity in 0.1 M Tris-Cl buffer, pH 8.0, was taken as 100%. (B) The enzymatic activities were measured in 0.1 M sodium phosphate buffer after incubation in buffers of various pH for 0, 30, 60, 90 min. The buffers used were 0.1 M sodium acetate buffer, pH 4 (●), pH 5 (○), sodium phosphate buffer, pH 6 (■), pH 7 (□), Tris-Cl buffer, pH 8 (▲), pH 9 (△). The activity at pH 7.0 at time 0 was taken as 100%.

Discussion

A novel GST (PhnC) was purified from *E. coli* carrying a cloned *phnC* gene which was derived from *Pseudomonas* sp. DJ77 through a GSH-affinity Sepharose column. A single band of protein was identified by SDS-PAGE. The molecular size of monomer was 23 kDa, which matched well with the calculated Mr from the nucleotide sequence of *phnC*. However, the Mr determined by the Sephadex G-150 gel permeation chromatography was 42 kDa. These results indicated that PhnC was a homo-dimeric enzyme, which is common to other known bacterial GSTs (Di Ilio *et al.*, 1988; Piccolomini *et al.*, 1989; Iizuka *et al.*, 1989; Di Ilio *et al.*, 1991). Also, the dimeric formation of the enzyme was similar to mammalian cytosolic GSTs

which are homo- or hetero-dimeric enzymes (Wilce and Parker, 1994). Mr of PhnC was similar to those of bacterial and mammalian cytosolic enzymes ranging between 40 and 50 kDa. The pI value of PhnC was 5.8, which is well matched with the pI value (5.5) calculated from the amino acids sequence deduced from the nucleotide sequence. Nine N-terminal amino acid residues were exactly matched with those deduced from the nucleotide sequence. This result confirmed that the *phnC* gene was expressed in *E. coli* and its product showed GST enzymatic activity.

The substrate specificity toward various electrophilic chemicals showed that PhnC had the highest activity toward CDNB and little or no activities toward other chemicals. The specific activity of PhnC toward CDNB was 10.6 μmol/min/mg protein. This value was much greater than those of bacterial GSTs from *E. coli* B (Iizuka *et al.*, 1989), *Serratia marscens* (Di Ilio *et al.*; 1991) and *Proteus mirabilis* (Di Ilio et al., 1988). PhnC was similar in the substrate specificity to other bacterial GSTs, but different from mammalian cytosolic GSTs, which have broader spectra of substrate specificities (Jakoby and Habig, 1980). This enzyme had no tyrosine residue in the N-terminal region that is thought to be crucial to enzyme catalysis in mammalian cytosolic GSTs (Kong *et al.*, 1992; Reinemer *et al.*, 1992; Atkins *et al.*, 1993). It was reported that the tyrosine residue in the N-terminal region of the GST from *E. coli* is not crucial for catalysis (Nishida *et al.*, 1994). These results may represent the difference in the active site structures or the catalytic mechanism between bacterial and mammalian cytosolic GSTs. Little is known about the catalytic mechanism and three-dimensional structure of bacterial GSTs. Further research on the crystallization and the function of PhnC will give us the information of relationship between the structure and the function of bacterial GSTs.

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