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A Second Thioltransferase from Chinese Cabbage: Purification and Characterization

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Thioltransferase, also known as glutaredoxin, was previously purified and characterized from Chinese cabbage (*Brassica campestris* ssp. *napus* var. *pekinensis*). However, in the process of gel filtration on Sephadex G-75, there were two activity peaks. In this study, a second thioltransferase (TTase CC-2) in the minor peak of the Sephadex G-75 elution profile was further purified using affinity chromatography on an S-hexylglutathione-agarose column by eluting with buffer solution containing 2.5 mM S-hexylglutathione. It showed a single band on SDS-PAGE indicating that TTase CC-2 is electrophoretically homogeneous. The molecular weight of TTase CC-2 was estimated to be about 22,000 daltons, and its isoelectric point was determined to be 6.73. Its size appears to be atypical and much larger than that of the first thioltransferase (TTase CC-1) from Chinese cabbage, and it can utilize 2-hydroxyethyl disulfide, S-sulfocysteine, and insulin as substrates. S-sulfocysteine was found to be a superior substrate for TTase CC-2. TTase CC-2 also displayed the reducing activity for non-disulfides such as dehydroascorbic acid. Its optimum pH was 8.5, which was consistent with that of TTase CC-1. TTase CC-2 activity was greatly activated by L-cysteine and reduced glutathione, and was found to be less heat-stable compared with TTase CC-1. Molecular and physiological differences between TTase CC-1 and TTase CC-2 remain to be elucidated. Chinese cabbage is the first plant which is known to contain two kinds of thioltransferases.

Keyword: Chinese Cabbage (*Brassica campestris* ssp. *napus* var. *pekinensis*), Glutaredoxin, Purification, Thioltransferase.

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Introduction

Thioltransferase (TTase), also called glutaredoxin (Grx), is a small and heat-stable protein that acts as a multifunctional glutathione-dependent disulfide oxidoreductase. TTase reductively cleaves a variety of disulfides including protein disulfides and low-molecular-mass disulfides in the presence of reduced glutathione (GSH) (Holmgren, 1976; Axelsson *et al.*, 1978; Hatekeyama *et al.*, 1984). TTase controls the ratio of cellular thiol and disulfide and regulates various other activities. In fact, activities of pyruvate kinase (Axelsson and Mannervik, 1983), papain (Bushweller *et al.*, 1992), iodothyronine 5′-deionase (Goswami and Rosenberg, 1985), ornithine decarboxylase (Flamgni *et al.*, 1989), and glyceraldehyde-3-phosphate dehydrogenase (Lind *et al.*, 1998) were reported to be affected by TTase. TTase (or Grx), together with glutathione and glutathione reductase, was shown to couple the oxidation of NADPH to the reduction of ribonucleotide (Holmgren, 1979), sulfate (Tsang, 1981), and methionine sulfoxide (Fuchs, 1977). TTase was also identified to be able to reduce non-disulfide substrates such as dehydroascorbate (Wells *et al.*, 1990) and alloxan (Washburn and Wells, 1997). Recently, OxyR transcription factor, which is activated through the formation of a disulfide bond, has been found to be deactivated by TTase (Zheng *et al.*, 1998). TTase was detected within HIV-1, and was implicated in the regulation and/or maintenance of protease activity in HIV-1 infected cells (Davis *et al.*, 1997).

TTases from mammalian cells, plant cells, and microbial cells are small proteins with the active site sequence of –Cys-Pro-Phe(Tyr)-Cys- (Höög *et al.*, 1983). TTase was isolated and characterized in a number of prokaryotic and eukaryotic species including *E. coli* (Holmgren, 1979), yeast (Gan *et al.*, 1990; Kim *et al.*, 1998), vaccinia virus (Ahn and Moss, 1992), bovine (Luthman and Holmgren, 1982), rabbit (Hopper *et al.*, 1989), pig (Gan and Wells,

1987a), rat (Axelsson *et al.*, 1978), human (Padilla *et al.*, 1995), spinach (Morell *et al.*, 1995), rice (Sha *et al.*, 1997a), kale (Sa *et al.*, 1998), *Arabidopsis thaliana* seed (Cho *et al.*, 1998a), and *Cryptococcus neoformans* (Sa *et al.*, 1997). TTase cDNAs were isolated and their nucleotide sequences were determined from pig (Yang *et al.*, 1989), human (Padilla *et al.*, 1995), and rice (Sha *et al.*, 1997b). The yeast *Saccharomyces cerevisiae* has been reported to contain two TTase genes, which are required for protection against reactive oxygen species (Luikenhuis *et al.*, 1998). In *v-jun*-transformed chicken embryo fibroblasts, the expression of the TTase mRNA could be induced, indicating that TTase is a direct target of v-Jun (Goller *et al.*, 1998). Glutathione S-transferase purified to electrophoretic homogeneity from bovine lens was found to display TTase activity, catalyzing the transthiolation reaction between GSH and 2-hydroxyethyl disulfide (Dal Monte *et al.*, 1998).

Recently, we purified and characterized a 12 kDa TTase (now designated TTase CC-1) from Chinese cabbage (Cho *et al.*, 1998b). It could utilize 2-hydroxyethyl disulfide, S-sulfocysteine, α-chymotrypsin, insulin, and trypsin as substrates in the presence of GSH, and also contained dehydroascorbate reductase activity. In this article, we report that a second TTase (designated TTase CC-2) has been identified, isolated, and characterized from Chinese cabbage (*Brassica campestris* ssp. *napus* var. *pekinensis*). The molecular size of TTase CC-2 is much larger than that of TTase CC-1.

Materials and Methods

Plant material Fresh Chinese cabbage (*Brassica campestris* ssp. *napus* var. *pekinensis*) was purchased in October from a local market at Chuncheon, Korea, and was immediately used for experiments. It was thoroughly washed prior to the purification process.

Chemicals Bovine serum albumin (BSA), reduced glutathione (GSH), glutathione reductase (yeast), Tris, Coomassie Brilliant Blue R-250, Sephadex G-75, N,N'-methylenebisacrylamide, S-hexylglutathione, S-hexylglutathione-agarose, acrylamide, NADPH , SDS, EDTA, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Co. (St. Louis, USA). Dehydroascorbic acid and 2-hydroxyethyl disulfide (HED) were from Aldrich Chemical Co. (Milwaukee, USA). DEAE-cellulose was purchased from Whatman International Ltd. (Maidstone, England). Molecular weight standards for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, USA). All other chemicals and reagents were of the highest grade commercially available.

Enzyme assay Since thioltransferase contained transhydrogenase activity, its activity was measured spectrophotometrically at 340 nm by the use of glutathione reductase as a coupling enzyme (Höög *et al.*, 1986). The reaction was performed in a total volume of 0.4 ml in two cuvettes, each of which contained 100 μg/ml of BSA, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris·HCl, 2 mM EDTA, pH 8.8. To each cuvette was added 40 μl of 15 mM 2-hydroxyethyl disulfide dissolved in 0.02 M Tris·HCl, pH 8.8. The absorbance at 340 nm was recorded for several minutes to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between GSH and 2-hydroxyethyl disulfide. Enzyme was added to the sample cuvette and distilled water to the control cuvette. The change in absorbance resulting from the oxidation of NADPH was then recorded with time and its activity was expressed as ΔA_{340}/min. The K_m value of the enzyme was determined by the method of Lineweaver and Burk in the standard assay system containing various concentrations of 2-hydroxyethyl disulfide.

To test whether the purified thioltransferase contains dehydroascorbate reductase activity, dehydroascorbic acid was used as a substrate. Dehydroascorbate reductase activity was measured by means of the spectrophotometric assay described by Stahl *et al.* (1983), which is based on the change in absorbance at 265.5 nm as dehydroascorbic acid is reduced to ascorbic acid. The reaction mixture consisted of 0.1 M Tris·HCl, pH 8.8, 2 mM EDTA, 1 mM glutathione, 0.8 mM dehydroascorbic acid, and TTase CC-2 (purified from Chinese cabbage) in a total volume of 0.5 ml. The reaction was initiated by adding dehydroascorbic acid and was linear for up to 2 min at 20°C.

Determination of isoelectric point Isoelectric focusing was performed in vertical slab gels using ampholines in the pH range of 3–10. The isoelectric point was determined from a calibration curve using isoelectric point markers. The calibration proteins included phycocyanin (pI, 4.45, 4.65, 4.75), β-lactoglobulin B (pI, 5.10), bovine carbonic anhydrase (pI, 6.00), human carbonic anhydrase (pI, 6.50), equine myoglobin (pI, 7.0), human hemoglobin A (pI, 7.10), human hemoglobin C (pI, 7.50), lentil lectin (pI, 7.8, 8.0, 8.2), and cytochrome C (pI, 9.6).

Protein determination Protein concentration was determined according to the procedure of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard. The protein content in fractions obtained during the chromatographic process was determined by measuring absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis SDS-polyacrylamide gel electrophoresis with 10–20% polyacrylamide gradient precasting gel was run according to the procedure described by Laemmli (1970). The gels were stained with silver nitrate or Coomassie Brilliant Blue R-250.

Purification of thioltransferase CC-2 All purification procedures were carried out at 4°C by the following steps.

Step 1. Preparation of crude extract: Fresh Chinese cabbage (500 g) was ground up and disrupted using a glass bead-beater in a total volume of 600 ml buffer A solution (0.02 M Tris·HCl, 2 mM EDTA, pH 8.8). After centrifugation for 90 min at 4500 rpm, the supernatant (Fraction I) was obtained.

Step 2. DEAE-cellulose chromatography: Fraction I was applied to a column (2.5 × 17 cm) of DEAE-cellulose pre-equilibrated with buffer A. Proteins were eluted with a linear

gradient of 0–0.5 M NaCl in buffer A (total volume, 800 ml) using a flow rate of 1.44 ml/min. The fractions containing thioltransferase activity (133 ml) were pooled and concentrated to 10 ml by centrifugation with Amicon Centriprep 3000 (Fraction II).

Step 3. Sephadex G-75 gel filtration: Fraction II was applied to a Sephadex G-75 column (2.5 × 70 cm) and then eluted with buffer A. The flow rate was 0.27 ml/min. The fractions (96 ml) containing thioltransferase activity were pooled (Fraction III).

Step 4. Affinity chromatography on S-hexylglutathione-agarose: Fraction III was applied to an S-hexylglutathione-agarose column (1 × 5 cm). The column was stepwise eluted with 15 ml buffer A, each containing 2.5 mM, 5 mM, and 10 mM S-hexylglutathione. The fractions containing thioltransferase activity were pooled (Fraction IV).

Results and Discussion

Purification of TTase CC-2 from Chinese cabbage Previously, a TTase was purified to electrophoretic homogeneity from Chinese cabbage (Cho *et al.*, 1998b). It was purified by the combination of ion-exchange and gel filtration column chromatography. However, two peaks possessing TTase activity appeared in the elution profile of the gel filtration on Sephadex G-75. The major peak was additionally subjected to further chromatographic steps to isolate the purified thioltransferase (TTase CC-1). In this study, the minor peak was pooled and subjected to an affinity chromatographic column containing S-hexylglutathione-agarose to purify a second thioltransferase (TTase CC-2). Its transhydrogenase activity was eluted with buffer A solution containing 2.5 mM S-hexylglutathione. Its purity was examined by SDS-polyacrylamide gel electrophoresis (Fig. 1) and it showed a single protein band, indicating that Fraction IV was in a homogeneous state. This confirmed that TTase CC-2 was successfully purified from Chinese cabbage and was clearly different from TTase CC-1.

To verify the identity of the purified TTase CC-2, the individual component was deleted in a complete assay (Table 1). It indicated that the transhydrogenase activity of TTase CC-2 absolutely required HED, GSH, and glutathione reductase for its full action. These requirements are consistent with those of most thioltransferases for transhydrogenase activity. It was also shown that the HED reduction activity was linearly proportional to the concentration of TTase CC-2 (data not shown).

Molecular weight and isoelectric point The molecular weight of TTase CC-2 from Chinese cabbage was estimated to be about 22,000 daltons on SDS-PAGE (Fig. 1). Its size appeared to be much larger than that of TTase CC-1 (12,000 daltons). TTases had been generally found to be heat-stable proteins of approximately 12 kDa containing a GSH-dependent redox-active disulfide bond

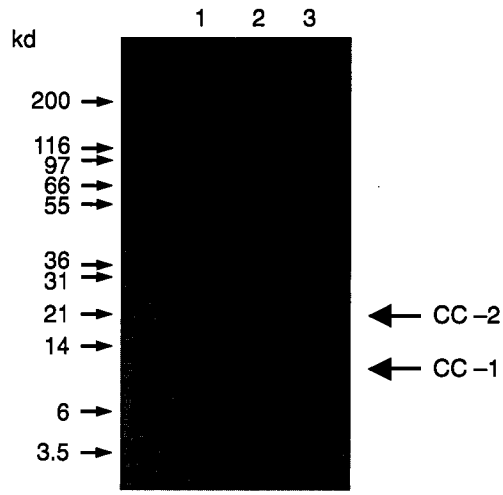

Fig. 1. Electrophoretic patterns of TTase CC-1 and TTase CC-2 on SDS-polyacrylamide gel. Electrophoresis was performed in 10–20% SDS-PAGE. Proteins were stained with silver nitrate. Lane 1, protein markers; lane 2, TTase CC-1; lane 3, TTase CC-2.

Table 1. Activity of TTase CC-2 purified from Chinese cabbage under various deleted conditions. The purified thioltransferase activity was measured in the standard assay and expressed as relative activity.

Conditions	Thioltransferase activity (ΔA_{340}/min)	Relative activity (%)
Complete	0.020	100
– bovine serum albumin	0.014	70
– 2-hydroxyethyl disulfide	0	–
– reduced glutathione	0	–
– glutathione reductase	0	–

(Wells *et al.*, 1993). Glutaredoxin 1 (Grx1), originally purified from *Escherichia coli*, is a small and acidic protein containing a single disulfide bond which can be reduced to the dithiol by GSH, GR, and NADPH (Holmgren, 1979). Two additional glutaredoxins, Grx2 and Grx3, were purified and characterized from *E. coli* (Åslund *et al.*, 1994). The molecular weight of Grx2, 27,000, is atypical for glutaredoxins, whereas Grx3 has a molecular weight of 10,000. The molecular size of TTase CC-2 is comparable to that of Grx2 from *E. coli*, and appears to be atypically large among the thioltransferases isolated so far.

Isoelectric focusing gel electrophoresis was performed (data not shown). The isoelectric point of TTase CC-2 was determined to be about 6.73, indicating that TTase CC-2 is relatively neutral. This appears to be different from most thioltransferases which possess an acidic character.

Substrate specificity and kinetic properties Substrate specificity of TTase CC-2 was examined under saturating concentrations of substrates (Table 2). It could utilize HED, S-sulfocysteine, and insulin as substrates, but not L-cystine. Previously, TTase CC-1 was shown to contain broad specificity for protein disulfides (Cho *et al.*, 1998b). S-sulfocysteine was found to be a superior substrate for TTase CC-2. Transhydrogenase activity of TTase CC-2 was examined under various concentrations of HED (Fig. 2). Kinetic parameters were obtained by a Lineweaver-Burk plot. TTase CC-2 was estimated to have K_m and V_{max} values of 1.24 mM and 0.07 ΔA_{340}/min, respectively. The K_m value of TTase CC-1 is 0.05 mM on HED (Cho *et al.*, 1998b). These values indicate that TTase CC-2 contains much less affinity for HED than TTase CC-1.

Non-disulfide reducing activity Mammalian TTases have significant dehydroascorbate reductase activity (Wells *et al.*, 1990). The purified TTase CC-2 of Chinese cabbage could utilize dehydroascorbic acid as a substrate, whereas it could not utilize alloxan. Non-disulfide reducing activity of TTase CC-2 was examined under various concentrations of dehydroascorbic acid (Fig. 3). From the Lineweaver-Burk plot, it was found to have K_m and V_{max} values of 0.51 mM and 0.15 ΔA_{340}/min, respectively. The K_m value of TTase CC-2 is comparable to that of TTase CC-1 (0.79 mM), indicating that TTase CC-2 has relatively higher affinity for dehydroascorbic acid. Also, TTase CC-2 appears to be very specific for non-disulfides.

pH optimum The activity of TTase CC-2 from Chinese cabbage as a function of pH was determined using the standard assay mixture. The maximal activity of the TTase CC-2 was observed at about pH 8.5 (Fig. 4), which was consistent with that of TTase CC-1. Most of known thioltransferases have basic pH optima and the optimal pH

Table 2. Transhydrogenase activity of TTase CC-2 under saturating concentrations of several substrates.

Substrate	Thioltransferase activity (ΔA_{340}/min)	Relative activity (%)
2-Hyderoxyethyl disulfide	0.0140	100
S-Sulfocysteine	0.0025	175
Insulin	0.0025	18
L-Cystine	0	–

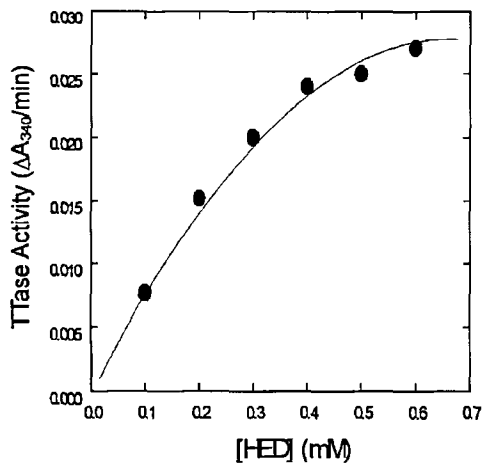

Fig. 2. Concentration effect of 2-hydroxyethyl disulfide on the transhydrogenase activity of TTase CC-2 from Chinese cabbage.

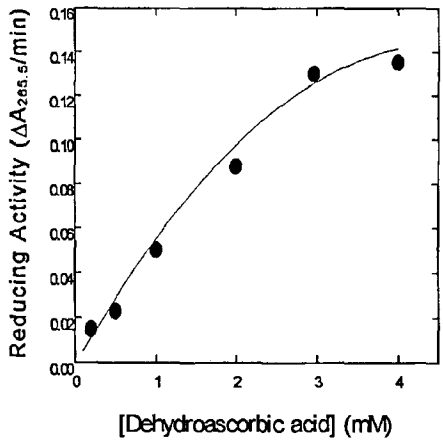

Fig. 3. Dehydroascorbate reductase activity of TTase CC-2 purified from Chinese cabbage. Dehydroascorbate reductase activity was assayed as described in Materials and Methods.

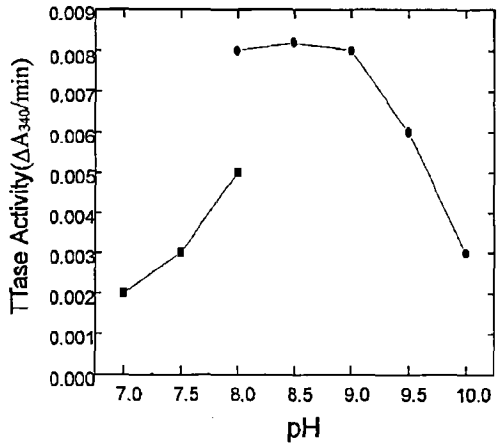

Fig. 4. pH-Dependence of the TTase CC-2-catalyzed transhydrogenation reaction. The initial velocities were monitored in the standard assay system with buffer consisting of 0.1 M sodium phosphate (pH 7.0–8.0) (—■—) and 0.1 M Tris·HCl (pH 8.0–10.0) (—●—).

values of pig, bovine, and rat TTase are about pH 8.5, 8.5, and 9.0, respectively (Hatekeyama *et al.*, 1984; Gan and Wells, 1986; 1987b).

Activation by thiol compounds Since various thiol compounds were found to activate several thioltransferases, the effect of a few thiol compounds was tested on the TTase CC-2 from Chinese cabbage (Table 3). A 3.5-fold increase in enzymatic activation was observed in the presence of 0.5 mM 2-cysteine. TTase CC-2 was also greatly activated by reduced glutathione (about 2-fold increase). The activity of TTase CC-1 was about 1.6-fold by reduced glutathione (0.5 mM) (Cho *et al.*, 1998b). However, L-cysteine had no effect on the activity of TTase CC-1 (Cho *et al.*, 1998b). TTase CC-1 and CC-2 appear to have different activation patterns by monothiol compounds.

Heat stability TTase is generally believed to be heat-stable. In this regard, the thermal stability of the purified TTase CC-2 was examined. The purified enzyme was incubated in a water bath for 30 min with a temperature range from 20–80°C. After rapid cooling in an ice bath, the heat-treated sample was added to a standard assay mixture. As shown in Fig. 5, when incubated at 40°C through 70°C the enzyme activity was reduced to 60–70% of that at 20°C incubation. At temperatures higher than 70°C, the activity rapidly decreased. This indicates that TTase CC-2 is less stable at high temperatures compared with TTase CC-1, which was previously shown to be very heat-stable (up to 95°C) (Cho *et al.*, 1998b). The two TTases of Chinese cabbage have different heat stabilities.

In the present article, we described the purification and some properties of a second thioltransferase (TTase CC-2) from Chinese cabbage. This is the first example of a plant which contains two kinds of thioltransferases. However, the physiological differences of the two thioltransferases remain uncertain in Chinese cabbage. The relatively different specificities of the two thioltransferases may explain the presence of multiple thioltransferases. In *E. coli*, there are three kinds of glutaredoxins (thioltransferases). One of them may act as a cofactor of ribonucleotide reductase. Also, the yeast *Saccharomyces cerevisiae* was found to contain two kinds of thioltransferases, the physiological meaning of which is not yet known. Since the molecular size of TTase CC-2 is relatively large, it may contain other unknown functions. Further investigation should be done to elucidate the physiological role of TTase CC-2 in Chinese cabbage and the molecular differences between TTase CC-1 and TTase CC-2.

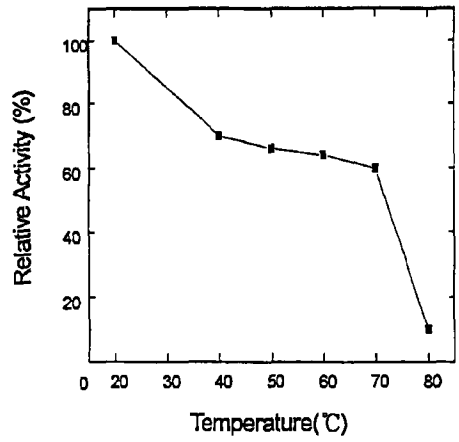

Fig. 5. Heat stability of TTase CC-2 purified from Chinese cabbage. The thioltransferase was incubated in a water bath for 30 min with a temperature range from 20–80°C. After rapid cooling in an icebox, the heat-treated sample was added to an assay mixture to measure thioltransferase activity.

Table 3. Effects on Chinese cabbage TTase CC-2 activity by thiol compounds.

Thiol compounds	Relative activity (%)
None	100
Reduced glutathione	208
L-Cysteine	347
β-Mercaptoethanol	144
Dithiothreitol	108

The purified TTase CC-2 was pre-incubated at 30°C for 30 min with 0.5 mM thiol compounds. The enzymatic activity of the incubation mixtures was measured in the standard assay and expressed as relative activity.

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