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Characterization of Thioltransferase from Kale

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Thioltransferase, also known as glutaredoxin, is an enzyme that catalyzes the reduction of a variety of disulfides, including protein disulfides, in the presence of reduced glutathione. Thioltransferase was purified from kale through ammonium sulfate fractionation, DE-52 ion-exchange chromatography, Sephadex G-75 gel filtration, and Q-Sepharose ion-exchange chromatography. Its molecular size was estimated to be about 31,000 daltons on SDS-PAGE. The purified enzyme has an optimum pH of about 8.0 with 2-hydroxyethyl disulfide as a substrate. The enzyme also utilizes L-sulfocysteine, L-cystine, bovine serum albumin, and insulin as substrates in the presence of GSH. The enzyme has K_m values of 0.24–0.67 mM for these substrates. The enzyme was partly inactivated after heating at 80°C or higher temperature for 30 min. The enzyme was stimulated by various thiol compounds such as reduced glutathione, dithiothreitol, L-cysteine, and β-mercaptoethanol. This is a second example of a plant thioltransferase which was purified and characterized.

Keywords: *Brassica oleracea* L. var. *acephala*, Kale, Thioltransferase.

Introduction

Thioltransferase (TT), also known as glutaredoxin (GRX) or transhydrogenase, is a monomeric enzyme that catalyzes the reduction of low molecular weight disulfides and some protein disulfides in the presence of reduced glutathione (Racker, 1955; Axelsson *et al.*, 1978). It is a small protein containing the active site -Cys-Pro-Tyr(Phe)-Cys- which is conserved among various organisms such as *E. coli*, vaccinia virus, yeast, and mammalian cells (Höög *et al.*, 1983; Gan, Z.-R. *et al.*, 1990; Ahn and Moss, 1992; Minakuchi *et al.*, 1994). It was originally identified as an alternative hydrogen donor for the reduction of ribonucleotide reductase in an *E. coli* mutant deficient in thioredoxin. Since then, it has been postulated to regulate various cytosolic enzymes, such as pyruvate kinase, papain, and ornithine decarboxylase (Axelsson and Mannervik, 1983; Hatakeyama *et al.*, 1986; Flamigni *et al.*, 1989), via interaction with the redox status of glutathione. In addition, thioltransferase was shown to be involved in some kinds of important reactions: glutathione-dependent deiodination of thyroxine to triiodothyronine (Goswami and Rosenberg, 1985), reduction of dehydroascorbate (Wells *et al.*, 1990), regeneration of oxidatively damaged proteins (Mieyal *et al.*, 1991; Terada, 1992), and reduction of methionine sulfoxide (Fuchs, 1977; Fuchs and Carlson, 1981).

The genes encoding thioltransferase (glutaredoxin) were cloned and their nucleotide sequences were determined in pig liver (Yang and Wells, 1989), *E. coli* (Sandberg *et al.*, 1991), human brain (Fernando *et al.*, 1994), and rice (Minakuchi *et al.*, 1994). High level expression of human thioltransferase gene was accomplished in *E. coli* (Chrestenson *et al.*, 1995). However, little is known about the regulation of thioltransferase gene expression. The first plant thioltransferase has been identified and purified to homogeneity from spinach leaves (Morell *et al.*, 1995). Spinach thioltransferase showed a significant cross-reactivity with antibodies raised against *E. coli* glutaredoxin (thioltransferase). However, thioltransferase has never been purified and characterized from other plant sources.

In this article, we report the purification and properties of a thioltransferase from kale, a second plant thioltransferase isolated.

Materials and Methods

Plant material Fresh kale (*Brassica oleracea* L. var. *acephala*) was purchased from a commercial market at Chuncheon, Korea. It was washed thoroughly prior to the purification process.

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Chemicals Bovine serum albumin (BSA), insulin (bovine pancreas), S-sulfocysteine, L-cystine, L-cysteine, glutathione (GSH), glutathione reductase (yeast), NADPH, dithiothreitol, Tris, Sephadex G-75, and Q-Sepharose were products of Sigma Chemical Co (St. Louis, USA). Dehydroascorbic acid and 2-hydroxyethyl disulfide (HED) were from Aldrich Chemical Co. (Milwaukee, USA). DE-52 was a product of Whatman. Acrylamide, N,N-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED), ammonium persulfate, SDS, silver nitrate, and Coomassie Brilliant Blue R-250 were also obtained from Sigma Chemical Co. (St. Louis, USA). SDS-PAGE molecular weight standards were obtained from Bio-Rad (Richmond, USA). All other chemicals and reagents used were of the highest grade commercially available.

Enzyme assay Thioltransferase (glutaredoxin) catalyzes the reduction of certain disulfides by glutathione and thus has GSH-disulfide-transhydrogenase (Racker, 1955; Nagai and Black, 1968) or oxidoreductase activity (EC 1.8.4.4 and 1.8.4.1). When the reaction is coupled to NADPH and glutathione reductase, the overall reaction may be characterized as an NADPH-dependent disulfide reduction. In the present study, 2-hydroxyethyl disulfide (HED) was used as a substrate (Holmgren, 1985). Two quartz semimicro cuvettes with 1 cm light path contained 500 μl of mixture at room temperature. To both cuvettes were added 50 μl of 15 mM HED, 100 μg/ml bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris-HCl (pH 8.0)-2 mM EDTA. The absorbance of the NADPH was recorded at 340 nm for 2 min to ensure that both cuvettes were balanced with respect to the nonenzymatic spontaneous reaction between GSH and HED. Enzyme was added to the sample cuvette and an equal volume of buffer was added to the reference cuvette. The decrease in absorbance was then recorded for a few minutes. The result was calculated as ΔA_{340}/min. To examine substrate specificity, other disulfide substrates such as L-sulfocysteine, L-cystein, bovine serum albumin and insulin were used instead of HED. To test whether the purified thioltransferase contains dehydroascorbate reductase activity or not, dehydroascorbic acid was used as a substrate.

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

Preparation of crude extract Fresh kale (1 kg) was ground up, and disrupted by using a glass bead beater in a total volume of 250 ml buffer A (0.05 M Tris-HCl, pH 8.7, 1 mM EDTA). After centrifugation, the supernatant (fraction 1) was obtained for next purification step.

Purification of thioltransferase All purification procedures were carried out at 4°C. Solid ammonium sulfate (60% saturation) was added to fraction 1. The mixture was stirred for 2 h. The supernatant was obtained after centrifugation for 30 min at 10,000 *g*. Additional amount of solid ammonium sulfate was added to the supernatant to make 80% saturation. The precipitate was collected by centrifugation. It was dissolved in buffer A by stirring, and the solution (fraction 2) was dialyzed against 5 l of buffer A.

Fraction 2 was applied to a column of DEAE-cellulose pre-equilibrated with buffer A. The column was eluted with a linear gradient of 0–0.5 NaCl in buffer A. Active factions were pooled and concentrated by centrifugation with Amicon Centriprep (fraction 3).

Fraction 3 was applied and purified with a gel filtration chromatography of Sephadex G-75 column (2.5 × 4.8 cm) preequilibrated with buffer A. The fractions containing thioltransferase activity were pooled (fraction 4).

Fraction 4 was further purified with a Q-Sepharose column (2.5 × 7 cm) pre-equilibrated with buffer A. The column was eluted with a linear gradient of 0–0.5 M NaCl in buffer A. The fractions containing thioltransferase activity were pooled, dialyzed, and concentrated (fraction 5).

SDS-polyacrylamide gel electrophoresis SDS-polyacrylamide gel electrophoresis (16 × 18 cm) with a 12% running gel was performed according to the procedure described by Laemmli (1970). The gels were stained with silver staining or Coomassie Brilliant Blue R-250.

Results and Discussion

Thioltransferases (glutaredoxins) were purified and characterized from various organisms including microorganisms and mammals. However, thioltransferase purified from spinach leaves (Morell *et al.*, 1995), a unique plant thioltransferase, was recently identified at the protein level. This paper describes the purification and some properties of thioltransferase from kale, a second example of plant thioltransferases.

Purification of thioltransferase from kale A thioltransferase was purified to homogeneity from kale according to the procedure described in the section on Materials and Methods. Its purity was examined on SDS-polyacrylamide gel elecrophoresis (Fig. 1), and it showed a major band of molecular mass 31,000 daltons. Its molecular size is comparable to that of glutaredoxin 2 (27,000 daltons) from *Escherichia coli* (Åslund *et al.*, 1994). It is larger than the first known plant thioltransferase (12,000 daltons) from spinach leaves (Morell *et al.*, 1995).

Requirements for the action of the purified thioltransferase To verify the identity of the purified thioltransferase from kale, individual components in complete assay were deleted (Table 1). It indicates that the kale thioltransferase absolutely requires glutathione and glutathione reductase for its full activity. In the absence of 2-hydroxyethyl disfulfide as a substrate, a little activity was observed possibly due to the presence of albumin as a plausible substrate. These results verify that the purified kale thioltransferase is a genuine one. Then, the kale thioltransferase was characterized in various aspects.

Effect of enzyme concentration The effect of enzyme concentration was measured in the complete assay

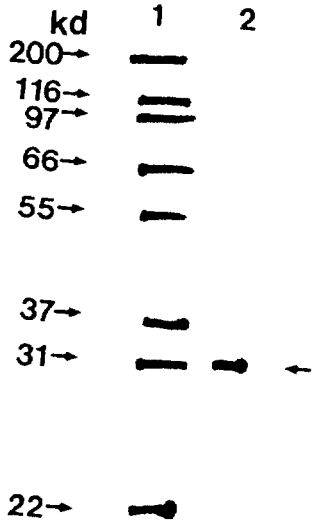

Fig. 1. SDS-polyacrylamide gel electrophoresis of thioltransferase purified from kale. The proteins were analyzed on 12% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue R-250. Lane 1, protein markers; lane 2, kale thioltransferase (Fraction 5).

Table 1. Activity of the purified kale thioltransferase under various deleted conditions. The purified thioltransferase was measured in the standard assay system and also expressed as relative activity.

Conditions	Thioltransferase activity (ΔA_{340}/min)	Relative activity (%)
Complete	0.096	100
– BSA	0.080	83.3
– Yeast glutathione reductase	0.005	5.2
– GSH	0	0
– HED	0.008	8.3

medium. The oxidation of NADPH in the assay was proportional to the amount of enzyme added in the range between 1 to 5 μg (data not shown). Therefore, the amount of enzyme used in the following characterizations of the kale thioltransferase was restricted to this range.

Kinetic properties Activity of the kale thioltransferase was tested using different concentrations of a substrate, 2-hydroxyethyl disulfide. The data were plotted according to Lineweaver and Burk (1/V versus 1/S) (1934), and the Michaelis–Menten constant, K_m, was calculated from the intercept and the slope of the line (Fig. 2). The K_m for 2-hydroxyethyl disulfide was estimated to be 3.3×10^{-4} M, and its V_{max} was measured to be 0.125 (ΔA_{340}/min) at the used enzyme concentration. This K_m value is very similar to those obtained with *E. coli* and calf thymus thioltransferases, 0.26 and 0.38 mM, respectively (Luthman and Holmgren, 1982).

Substrate specificity The kale thioltransferase catalyzed the reaction between glutathione and various disulfides. 2-Hydroxyethyl disulfide, S-sulfocysteine, L-cystine, bovine serum albumin, and insulin seemed to be equally good substrates for the enzyme, since they gave similar K_m values, 0.24–0.67 mM (Table 2). They also gave similar catalytic efficiencies, although that for 2-hydroxyethyl disulfide was higher than those for other substrates.

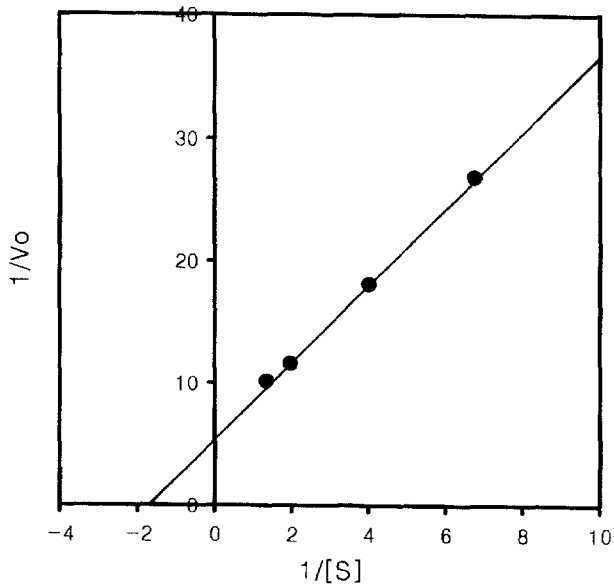

Fig. 2. The Lineweaver–Burk plot showing the HED reduction activity of kale thioltransferase as a function of substrate concentration. HED reduction activity was assayed as described in the section on Materials and Methods.

Table 2. Values of Michaelis constant of thioltransferase for various disulfides. The reaction mixture, containing 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH and 0.1 M Tris-Cl, 2 mM EDTA buffer, pH 8.0, and various amounts of disulfides in a total volume of 500 μl, and the absorbance at 340 nm was followed. Kinetic parameters, K_m and V_{max}, were obtained by plotting Lineweaber–Burk curves.

Substrates	K_m (mM)	V_{max} (ΔA_{340}/min)	Catalytic efficiency (V_{max}/K_m)
HED	0.33	0.125	0.379
S-Sulfocysteine	0.67	0.091	0.136
L-Cystine	0.48	0.071	0.148
BSA	0.26	0.055	0.212
Insulin	0.24	0.045	0.188

Effect of thiol compounds Since thioltransferase is involved in thiol/disulfide exchange, effects of various thiol compounds such as dithiothreitol, L-cysteine, β-mercaptoethaol, and reduced glutathione were examined on the kale thioltransferase (Table 3). They activated the

kale thioltransferase by about two- to fivefold when preincubated prior to the assay. Previously, thiol compounds were found to activate some animal thioltransferases. In our work, they could activate thioltrasferase purified from *Cryptococcus neoformans*. However, the activation mechanism remains unclear.

Table 3. Activation of the kale thioltransferase by thiol compounds. The purified thioltransferase was preincubated at 30°C for 30 min containing 0.5 mM thiol compounds. The enzymatic activity of the incubation mixtures was measured in the standard assay and expressed as relative activity.

Thiol compounds	Relative activity (%)
None	100
Reduced glutathione	232
Dithiothreitol	176
L-Cysteine	493
Mercaptoethanol	176

pH optimum The maximal activity of the kale thioltransferase was observed at about pH 8.0 (Fig. 3), when 2-hydroxyethyl disulfide was used as a substrate. This optimum pH is similar to that for bovine liver thioltransferase (Hatekeyama *et al.*, 1984)

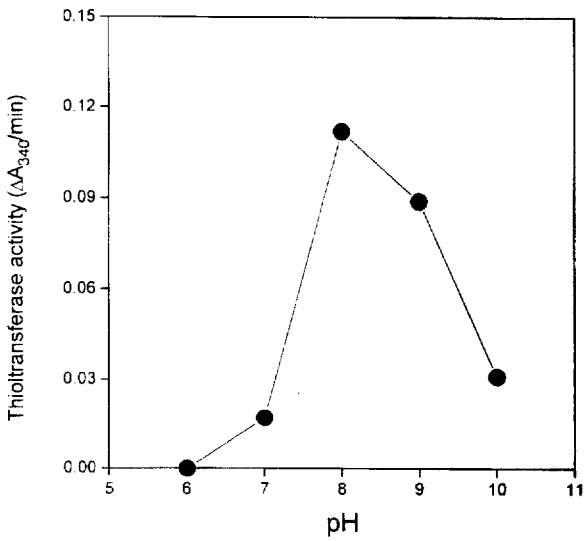

Fig. 3. Effect of pH on HED reduction activity of the kale thioltransferase. It was assayed at different pH values in the presence of 0.1 M sodium phosphate (pH 6–7) or 0.1 M Tris-HCl (pH 8–10).

Heat stability Thioltransferase is generally considered as a heat-stable protein. To examine the heat stability of the kale thioltransferase, the purified enzyme was incubated for 30 min at different temperatures prior to the assay. At temperatures up to 60°C, almost complete activity was observed (Fig. 4). However, the enzyme, incubated at 80°C and 90°C for 30 min, showed about 40% of the initial activity. These indicate the relatively thermostable nature of the enzyme.

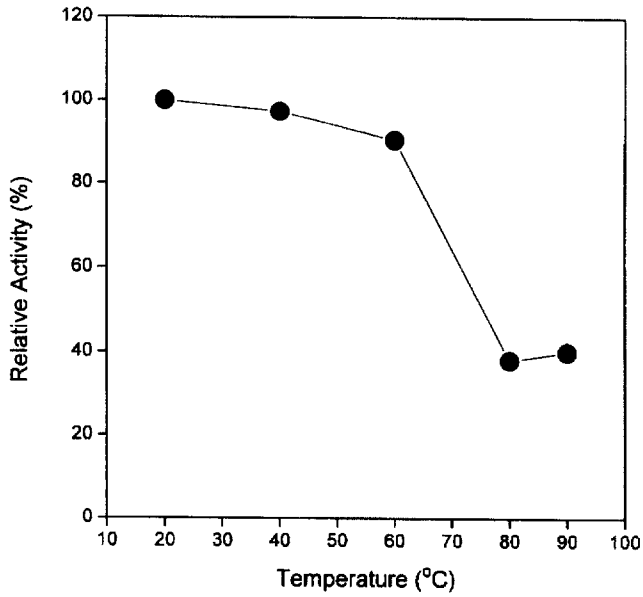

Fig. 4. Heat stability of the kale thioltransferase. The kale thioltransferase was incubated in a water bath with a temperature range from 20°C to 90°C for 30 min. After rapid cooling in an ice box, the heat-treated sample was added to an assay mixture to measure thioltransferase activity.

Lack of dehydroascorbate reductase activity Some thioltransferases were found to contain dehydroascorbate reductase activity. Then, to test whether the kale thioltrasferase has this activity or not, dehydroascorbate reductase assay (Trömper *et al.*, 1994) following the formation of ascorbate at 265 nm was performed using the kale thioltransferase. However, no dehydroascorbate reductase activity was observed with the kale thioltrasferase, indicating the kale thioltransferase lacks the activity.

In this article, we described the purification and some properties of thioltransferase from kale. It was found to be relatively large compared most other thioltransferase identified so far. Its active site structure and reaction mechanism will be investigated in the future. The precise physiological role of plant thioltransferase remains to be elucidated. It may regulate the enzyme activatities involved in the CO_2 fixation of photosynthesis via thiol-disulfide exchange, as thioredoxin does. It could also be involved with sulfhydryl homeostasis and growth.

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