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Short communication

An L-Type Thioltransferase from *Arabidopsis thaliana* Leaves

Tae-Soo Kim, Young-Wook Cho, Joon-Chul Kim, Chang-Duck Jin, Tae-Jin Han[1], Soo-Sun Park[2], and Chang-Jin Lim*

Division of Life Sciences, Kangwon National University, Chuncheon 200-701, Korea
[1] Department of Biology, Hallym University, Chuncheon 200-702, Korea
[2] The National Academy of Sciences, Seoul 137-044, Korea

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Thioltransferase, also called glutaredoxin, is a general GSH-disulfide reductase of importance for redox regulation. Previously, the protein thioltransferase, now called S-type thioltransferase, was purified and characterized from *Arabidopsis thaliana* seed. In the present study, a second thioltransferase, called L-type thioltransferase, was purified to homogeneity from *Arabidopsis thaliana* leaves. The purification procedures included DEAE-cellulose ion-exchange chromatography, Sephadex G-50 gel filtration, and glutathione-agarose affinity chromatography. The purified enzyme was confirmed to show a unique band on SDS-PAGE and its molecular weight was estimated to be 26.6 kDa, which appeared to be atypical compared with those of most other thioltransferases. It could utilize 2-hydroxyethyl disulfide, S-sulfocysteine, and insulin as substrates, and also contained dehydroascorbate reductase activity. Its optimum pH was 8.5 and its activity was greatly activated by L-cysteine. When it was kept for 30 min, it appeared to be very stable up to 70°C. It was activated by $MgCl_2$ and, on the contrary, inhibited by $ZnCl_2$, $MnCl_2$, and $AlCl_3$.

Keywords: *Arabidopsis thaliana*, Thioltransferase, Glutaredoxin, Dehydroascorbate reductase activity.

Introduction

Thioltransferase (TTase), also called glutaredoxin (Grx), reductively cleaves a variety of disulfides including protein disulfides and low-molecular mass disulfides in the presence of reduced glutathione (GSH) (Hatekeyama *et al.*, 1984). TTase either controls the ratio of cellular thiol and disulfide or regulates various enzyme activities. In fact, activities of papain (Bushweller *et al.*, 1992), OxyR transcription factor (Zheng *et al.*, 1998), and glyceraldehyde-3-phosphate dehydrogenase (Lind *et al.*, 1998) were affected by TTase. TTase (or Grx) was originally shown to couple the oxidation of NADPH to the reduction of ribonucleotides (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). TTase was detected within HIV-1, and shown to implicate in the regulation and/or maintenance of protease activity in HIV-1 infected cells (Davis *et al.*, 1997). The 23kDa TTase of *Schizosaccharomyces pombe* was recently shown to contain glutathione S-transferase activity (Kim *et al.*, 1999).

TTases from mammalian cells, plant cells, and microbial cells are small and heat-stable proteins with the active site sequence of -Cys-Pro-Phe(Tyr)-Cys-. Since plant TTase was identified from spinach (Morell *et al.*, 1995), it was purified and characterized from various plant organisms such as rice (Sha *et al.*, 1997), kale (Sa *et al.*, 1998a), Chinese cabbage (Cho *et al.*, 1998a; 1999), and *Arabidopsis thaliana* seed (Cho *et al.*, 1998b). The S-type TTase of *Arabidopsis thaliana* seed is a heat-stable protein with a molecular mass of 22 kDa and a pI of 4.8. It was found to contain broad specificity for substrates ranging from low-molecular disulfides (S-sulfocysteine and cystine) to protein disulfides (trypsin and insulin). It also utilizes non-disulfides such as dehydroascorbic acid and alloxan. In this article, we purified and characterized a second 26.6 kDa TTase, called L-type, from *Arabidopsis thaliana* leaves. Some properties of the purified protein are reported below.

* To whom correspondence should be addressed.
Tel: 82-361-250-8514; Fax: 82-361-242-0459
E-mail: cjlim@cc.kangwon.ac.kr

Materials and Methods

Chemicals Bovine serum albumin (BSA), insulin (bovine pancreas), S-sulfocysteine, L-cystine, glutathione (reduced), glutathione reductase (yeast), S-hexylglutathione, NADPH, dithiothreitol, Tris, L-cysteine, β-mercaptoethanol, iodoacetamide, Sephadex G-50, and glutathione-agarose were purchased from Sigma Chemical Co. (St. Louis, USA). Dehydroascorbic acid and 2-hydroethyl disulfide (HED) were from Aldrich Chemical Co. (Milwaukee, USA). DEAE-cellulose was a product of Whatman International Ltd. (Maidstone, England). Molecular weight standards for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad Laboratories (Richmond, USA). All other chemicals and reagents were of the highest grade commercially available.

Plant material *Arabidopsis thaliana* ecotype Columbia was cultivated in soil, a 1:1:1 mixture of vermiculite, perlite, and peat moss. The cultivation conditions consisted of a temperature of 26°C and 60% moisture in a growth chamber.

Thioltransferase assay Since thioltransferase contained transhydrogenase activity, its activity was measured spectrophotometrically at 340 nm by the use of glutathione reductase (GR) as a coupling enzyme (Holmgren, 1979; Sa *et al.*, 1998b; Park *et al.*, 1999). In a total volume of 0.5 ml, two cuvettes, each containing 100 μg/ml of bovine serum albumin, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris·HCl − 2 mM EDTA, pH 8.0 were monitored for several minutes 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 water to the control cuvette. The change in the absorbance was then recorded with time and the activity was expressed as ΔA_{340}/min.

Purification All purification procedures were carried out at 4°C unless otherwise stated. Dilute protein solutions were concentrated by centrifugation with Amicon centriprep. Dialysis was done with Spectrum membrane tubing (MWCO: 6000–8000). The *Arabidopsis* leaves grown in 3 weeks were disrupted by using a glass bead beater and resuspended in 20 mM Tris·HCl − 2 mM EDTA, pH 8.0 (buffer A), and filtrated through four layers of cheesecloth. Crude extract (Fr1) was obtained after centrifugation. Fr1 was loaded on a DEAE-cellulose column (2.5 × 20 cm) equilibrated with buffer A. The column was washed with the same buffer until the protein content of the effluent returned to a baseline level. Elution was carried out with a linear gradient of 0 to 0.5 M NaCl in buffer A. Active fractions were pooled and concentrated (Fr2). Fr2 was then loaded onto a Sephadex G-50 column (2.8 × 100 cm) equilibrated with buffer A. Active fractions were collected and concentrated (Fr3). Fr3 was loaded onto a glutathione-agarose affinity column (1 × 4 cm) equilibrated with buffer A. Elution was performed with a step gradient of 5, 10, and 20 mM S-hexylglutathione in buffer A. The 10 mM fraction containing thioltransferase activity was concentrated (Fr4).

Determination of protein concentration Protein contents in Fr1–4 were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. During purification, absorbance at 280 nm was measured to determine protein contents in chromatographic fractions.

SDS-polyacrylamide gel electrophoresis Electrophoresis on vertical polyacrylamide slab gels (16 × 18 cm) or pre-cast gels were performed in the presence of SDS by the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R-250 or silver nitrate.

Results and Discussion

Purification of an L-type thioltransferase from *Arabidopsis thaliana* leaves Previously, an S-type TTase was purified to electrophoretic homogeneity from the seeds of *Arabidopsis thaliana* (Cho *et al.*, 1998b). Its molecular weight was estimated to be 22 kDa, and it showed the reducing activity on various substrates such as HED, S-sulfocysteine, cystine, trypsin, and insulin. In this study, an L-type thioltransferase was purified according to procedures that included ion-exchange chromatography, gel filtration, and affinity chromatography. Fr3, collected and concentrated from a Sephadex G-50 column, was subjected to an affinity chromatographic column containing glutathione-agarose. The transhydrogenase activity of an L-type thioltransferase was eluted with buffer A containing 10 mM S-hexylglutathione. Its purity was examined by SDS-polyacrylamide gel electrophoresis (Fig. 1A) and it showed a single protein band, indicating that Fr4 was in a homogeneous state. This confirmed that an L-type TTase was successfully purified from *Arabidopsis thaliana* leaves. The purified L-type thioltransferase was clearly shown to migrate differently from the purified S-type thioltransferase on native gel (Fig. 1B). This indicates that the TTase from *Arabidopsis thaliana* leaves is different to the TTase from *Arabidopsis thaliana* seeds. The gene encoding an L-type TTase would be expressed only in the leaves.

To verify the identity of the purified L-type TTase, the individual component was deleted in a complete assay (data not shown). It indicated that the transhydrogenase activity of L-type TTase 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 L-type TTase (data not shown).

Molecular weight The molecular weight of L-type TTase from *Arabidopsis thaliana* leaves was estimated to be about 26.6 kDa on SDS-PAGE (Fig. 1A). This size appeared to be larger than that of S-type TTase (22 kDa). TTases had been generally found to be heat-stable proteins of approximately 12 kDa containing a GSH-dependent redox-active disulfide bond (Wells *et al.*, 1993). The molecular weight of *E. coli* Grx2, 27 kDa, is atypical for

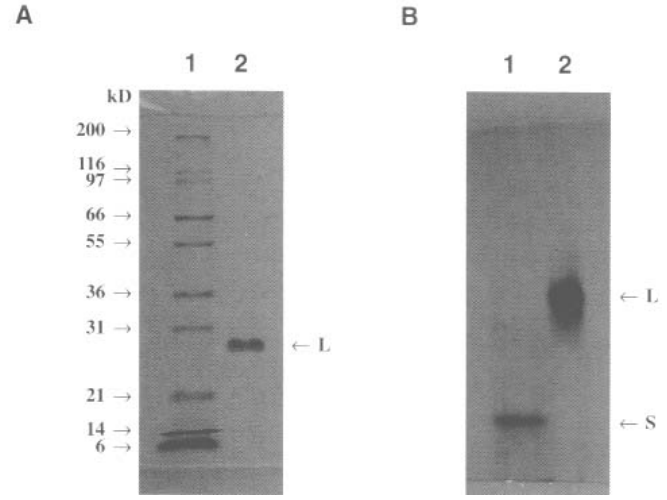

Fig. 1. A. Electrophoretic pattern of the purified L-type thioltransferase from *Arabidopsis thaliana* leaves. The purified samples were analyzed on 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, SDS-PAGE protein standards; Lane 2, the L-type TTase of *Arabidopsis thaliana* leaves. **B.** Native electrophoresis patterns on the 8% polyacrylamide gel of the two purified thioltransferases from *Arabidopsis thaliana*. Lane 1, the S-type TTase from seed; lane 2, the L-type TTase from leaves.

TTases, whereas *E. coli* Grx3 has a molecular weight of 10 kDa. The molecular weight of L-type TTase is comparable to that of Grx2 from *E. coli*, and appears to be atypically large among the thioltransferase isolated so far. Previously, the molecular weight of TTase CC-2 from Chinese cabbage was also reported to be about 22 kDa (Cho *et al.*, 1999).

Substrate specificity and kinetic properties The substrate specificity of the L-type TTase was examined under various concentrations of substrates (Table 1). It could utilize HED, S-sulfocysteine, and insulin. The S-type TTase was shown to contain specificity for trypsin and insulin (Cho *et al.*, 1998b). The K_m values of the L-type TTase on HED, S-sulfocysteine, and insulin were estimated to be 22.2 mM, 2.0 mM, and 0.4 mM, respectively. However, the catalytic efficiencies of the L-type TTase were found to be highest on S-sulfocysteine.

Mammalian TTases have significant dehydroascorbate reductase activity (Wells *et al.*, 1990). The purified L-type TTase of *Arabidopsis thaliana* utilized dehydroascorbic acid as a substrate (Table 1) with a K_m value of 1.6 mM indicating that it has a relatively low affinity for this substrate.

pH optimum The activity of the L-type TTase from *Arabidopsis thaliana* as a function of pH was determined using the standard assay mixture. The maximal activity of the L-type TTase was observed at about pH 8 (data not shown). The optimal pH of the S-type TTase was estimated to be pH 8.5 (Cho *et al.*, 1998b). Most of known TTases have basic pH optima and the optimal pH values of pig, bovine, and rat TTases are about pH 8.5, 8.5, and 9.0, respectively (Cho *et al.*, 1999).

Effects of thiol compounds Since various thiol compounds were found to activate several TTases, the effect of a few thiol compounds was examined on the L-type TTase from *Arabidopsis thaliana* (Table 2). An about 7-fold increase on the enzymatic activity was observed in the presence of 0.5 mM L-cysteine. However, the L-type TTase was greatly inactivated by β-mercaptoethanol and dithiothreitol. In particular, no activity of the L-type TTase was observed in the presence of dithiothreitol. Therefore, the activation of the L-type TTase depends on the kind of monothiol compounds.

Heat stability TTase is generally believed to be heat-stable. In this regard, the thermal stability of the purified L-type TTase was examined. The purified enzyme was incubated in a waterbath for 30 min with a temperature

Table 1. Kinetic parameters of the L-type thioltransferase purified from *Arabidopsis thaliana* leaves.

Substrates	K_m (mM)	V_{max} (μmoles/min)	Catalytic efficiency (V_{max}/K_m)
Disulfide substrates			
2-Hydroxyethyl disulfide	22.2	0.20	9.00
S-Sulfocysteine	2.0	81.11	40.56
Insulin	0.4	0.04	0.10
Non-disulfide substrate			
Dehydroascorbic acid	1.6	0.04	0.03

The reaction mixture contained 100 μg/ml BSA, 1 mM GSH, 6 μg/ml yeast glutathione reductase, 0.4 mM NADPH, and 0.1 M Tris·HCl-2 mM EDTA buffer (pH 8.0), and various amounts of disulfides in a total volume of 0.5 ml, and change in the absorbance at 340 nm was measured. The K_m and V_{max} values were obtained from Lineweaver-Burk plots.

range of 50–90°C. After rapid cooling in an ice box, the heat-treated sample was added to a standard assay mixture. As shown in Fig. 2, at temperatures higher than 70°C, the activity rapidly decreased. This indicates that the L-type TTase is relatively unstable compared with other known thioltransferases.

Inactivation by iodoacetamide To test the effect of iodoacetamide on the activity of the L-type TTase, it was incubated with various concentrations of iodoacetamide (Fig. 3). At 1 mM or higher concentrations of iodoacetamide, the activity of the L-type TTase was drastically decreased. It reconfirms that the sulfhydryl residue of L-cysteine exists in the active center of TTases.

Table 2. Effects of thiol compounds and metal ions on the L-type thioltransferase activity purified from *Arabidopsis thaliana* leaves.

Thiol compounds	Relative activity (%)
None	100
Reduced glutathione	120
L-Cysteine	694
β-Mercaptoethanol	23
Dithiothreitol	0

The purified L-type thioltransferase was pre-incubated at 30°C for 30 min with 0.5 mM thiol compounds. The enzymatic activities of the incubation mixtures were measured using the standard assay and expressed as relative activities.

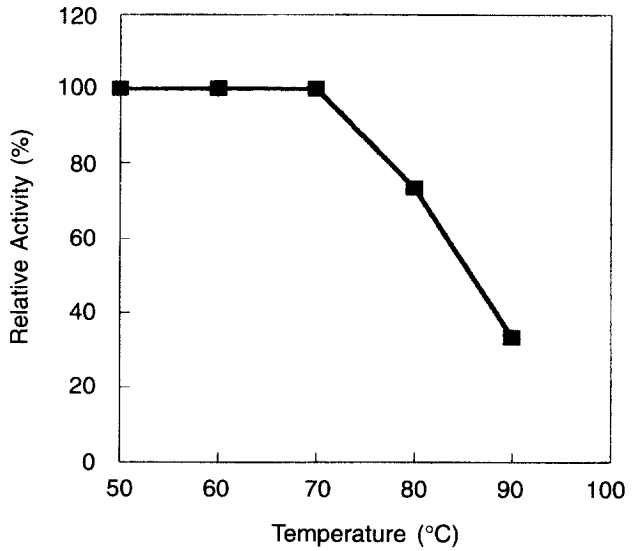

Fig. 2. Heat stability of the L-type thioltransferase purified from *Arabidopsis thaliana* leaves. The thioltransferase was incubated in a waterbath with a temperature range of 50–90°C for 30 min. After rapid cooling in an ice box, the heat-treated samples were added to the standard assay system.

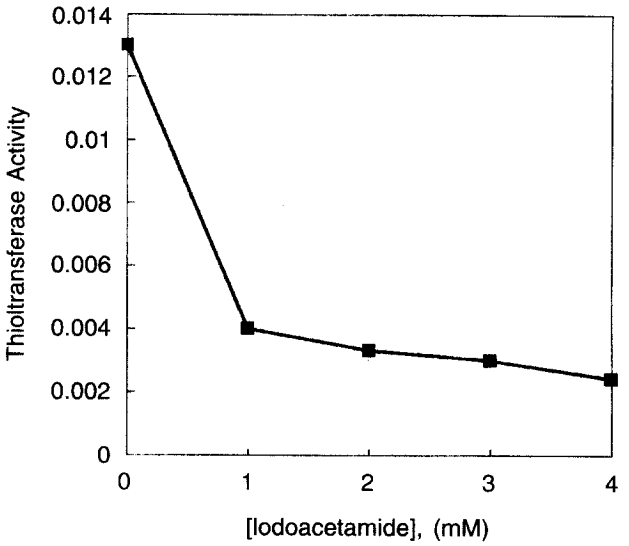

Fig. 3. Inactivation of the purified L-type thioltransferase by iodoacetamide. After incubation with various concentrations of iodoacetamide, the initial velocities of thioltransferase activity were monitored in the standard assay system. Thioltransferase activity is represented as ΔA_{340}/min.

Conclusions

In the present article, we described the purification and some properties of the L-type TTase from *Arabidopsis thaliana*, which is a second TTase identified from this plant species. The S-type TTase exists in the seeds of *Arabidopsis thaliana*, whereas the L-type TTase is found in its leaves. This is the first example of finding different TTases in different locations of the same plant. The yeast *Saccharomyces cerevisiae* has also been found to contain two kinds of TTases, the physiological meaning of which is not yet known. Further investigation should be done on *Arabidopsis thaliana* to elucidate the physiological role of the L-type TTase in its leaves and the molecular difference between its S-type and L-type TTases.

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