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SHORT COMMUNICATION

An f-Type Thioredoxin from *Arabidopsis thaliana* Leaves

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Thioredoxin, a small redox protein with an active site disulfide/dithiol, is ubiquitous in bacteria, plants, and animals and functions as a reducing agent and modulator of enzyme activity. A thioredoxin has been purified to electrophoretic homogeneity from the leaves of *Arabidopsis thaliana* using procedures such as DE-52 ion exchange chromatography, Sephadex G-50 gel filtration, Q-Sepharose ion exchange chromatography, and DEAE-Sephadex A-25 chromatography. The purified thioredoxin was determined to be a single band on SDS-PAGE, and its molecular weight was estimated to be 21 KDa, which was much larger than those of most other known thioredoxins. It was proved to be an f-type thioredoxin, since it could activate fructose-1,6-bisphosphatase, but it could not activate $NADP^+$-malate dehydrogenase. As a protein disulfide reductase, it could reduce the disulfide bonds contained in insulin. As a substrate, it showed a Km value of 20.2 μM on *Escherichia coli* thioredoxin reductase, and it had an optimal pH of 8.0. The molecular weight of the purified f-type thioredoxin is not consistent with those of the five divergent h-type thioredoxins already identified by cDNA cloning. The purified f-type thioredoxin is the first example isolated from *A. thaliana*.

Keywords: *Arabidopsis thaliana*, purification, thioredoxin

Introduction

Thioredoxins are small ubiquitous proteins with an exposed active site of a redox disulfide in the structure Cys-Gly-Pro-Cys, and have been identified from diverse organisms such as *Escherichia coli* and photosynthetic prokaryotes (Gleason and Holmgren, 1988), higher plants (Florencio *et al.*, 1988; Agostino and Hatch, 1993; Brugidou *et al.*, 1993; Hodges *et al.*, 1994), and mammals (Wollman *et al.*, 1988; Tonissen *et al.*, 1989). Thioredoxin has been implicated in a variety of physiological processes. It acts as a hydrogen donor for various reductive enzymes, such as protein disulfide oxidoreductases, photosynthetic regulatory factors, a subunit of T7 DNA polymerase, essential components of the assembly of filamentous bacteriophages, and possibly protein disulfide isomerase (Holmgren, 1985; Holmgren, 1989). In addition, it has been found to play an important role in defense against oxidative stress, either by reducing protein disulfide bonds produced by various oxidants or by scavenging reactive oxygen species (Spector *et al.*, 1988; Fernando *et al.*, 1992; Natsuyama *et al.*, 1992; Mitsui *et al.*, 1992). Thioredoxin also modulates the DNA binding of transcription factors, including the cytoplasmic factors NF-κB and AP-1 (Abate *et al.*, 1990; Schreck *et al.*, 1991; Schenck *et al.*, 1994). The adult T cell leukemia derived factor (ADF), which is produced by many human T cell lymphotropic virus-I-transformed T cells and EBV-transformed B cells, has been identified to be thioredoxin (Taniguchi *et al.*, 1996).

Reduced thioredoxins were demonstrated to activate many enzymes, including four enzymes of the reductive pentose phosphate cycle (Calvin cycle), namely: fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, NADH-glyceraldehyde-3-phosphate dehydrogenase, and phosphoribulokinase (Holmgren, 1985). They have also been found to activate $NADP^+$-malate dehydrogenase, phenylalanine ammonia-lyase, ATPase activity associated with the chloroplast coupling factor, and ribulose bisphosphate carboxylase (Holmgren, 1985). The best-characterized plant thioredoxin system was from spinach chloroplasts (Schürmann *et al.*, 1981) carrying two types of thioredoxin: the f-type, which could activate fructose-1,6-bisphosphatase exclusively, and the m-type, which could activate $NADP^+$-malate dehydrogenase (but also fructose-1,6-bisphosphatase with different kinetics). Since then, thioredoxin f has been designated as the fraction

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most effective in activating fructose-1,6-bisphosphatase and thioredoxin m has been designated as the fraction most effective in activating $NADP^+$-malate dehydrogenase (Whittaker and Gleason, 1984). Usually, nonchloroplast plant thioredoxins were designated h, as the system was originally described only in heterotrophic higher plant tissues such as wheat seeds (Johnson *et al.*, 1987), soybeans (Berstermann *et al.*, 1983), and dark-grown carrot calli (Johnson *et al.*, 1987).

In *Arabidopsis thaliana*, thioredoxins of multiple functions have never been studied on a protein level. However, five divergent clones encoding cytoplasmic thioredoxin h were isolated from *A. thaliana* cDNA libraries (Rivera-Madrid *et al.*, 1995). Each one has an open reading frame of 110-138 amino acids. Three of them contain a variant active site of Trp-Cys-Pro-Pro-Cys in place of the canonical Trp-Cys-Gly-Pro-Cys sequence described for thioredoxins in prokaryotes and eukaryotes. In this paper, a thioredoxin was purified and characterized from the leaves of *A. thaliana*. It was shown to be a f-type thioredoxin, which activated fructose-1,6-bisphosphatase, and has an atypical molecular mass.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA), insulin (bovine pancreas), 5,5'-dithio-2-nitrobenzoic acid (DTNB), NADPH, $NADP^+$, dithiothreitol, fructose-1,6-bisphosphate, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, oxaloacetic acid, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, ammonium persulfate, and SDS were purchased from Sigma Chemical Company (St. Louis, USA). Sephadex G-50, Q-Sepharose, and DEAE-Sephadex A-25 were also obtained from Sigma Chemical Company (St. Louis, USA). DE-52 was a product of Whatman International Ltd. (Maidstone, UK). The protein markers for SDS-PAGE were obtained from Bio-Rad Laboratories. (Hercules, USA). Vermiculite, perlite, and peat moss were obtained from the gardening shop in Chuncheon, Korea. *E. coli* thioredoxin reductase was a kind gift from Prof. J.A. Fuchs, Department of Biochemistry, The University of Minnesota, St. Paul, Minnesota, USA. All other chemicals and reagents used were of the highest grade commercially available.

Plant Material

Seeds of *A. thaliana* ecotype Columbia were kindly provided by Prof. Hong-Gil Nam, Pohang University of Science and Technology, Pohang, Korea. The seeds were cultivated in soil, a 1 : 1 : 1 mixture of vermiculite, perlite, and peat moss. Cultivation conditions were 26°C and 60% humidity in a growth chamber.

Enzyme Assay

Thioredoxin. Thioredoxin catalyzes NADPH-dependent reduction of the disulfide bond in DTNB (Luthman and Holmgren, 1982). The assay mixture contained 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 μg/mL BSA, 0.5 mM DTNB, and 0.24 mM NADPH in a volume of 500 μL. The enzyme source was added into the sample cuvette, whereas buffer A [50 mM Tris-HCl-1 mM EDTA, pH 8.0] was added into the reference cuvette. The reaction was initiated by adding thioredoxin reductase. An increase in absorbance at 412 nm was directly monitored using spectrophotometer. Thioredoxin activity was expressed as (A_{412}/min).

Fructose-1,6-bisphosphatase (FBPase). FBPase was first activated during incubation with thioredoxin, and the resulting FBPase activity was measured after dilution into the reaction mixture by a coupled spectrophotometric test following $NADP^+$ reduction (Le Marechal *et al.*, 1992). The activation medium contained 100 mM Tris-HCl, pH 7.9, 5 mM dithiothreitol, a source of FBPase and thioredoxin. After incubation at 20°C for 5 min, a 10 μl aliquot was used to determine the activity of FBPase in 1 mL of a reaction medium containing 100 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 1.5 mM $MgSO_4$, 2.5 mM fructose-1,6-bisphosphate, 1.5 U glucose-6-phosphate dehydrogenase, 6 U phosphoglucose isomerase, and 1 mM $NADP^+$. The activity was followed spectrophotometrically by monitoring the increase in absorbance at 340 nm.

$NADP^+$-malate dehydrogenase (MDH). The activation medium contained 100 mM Tris-HCl (pH 7.9), 5 mM dithiothreitol, a source of MDH, and thioredoxin. After incubation at 20°C, a 5 μL aliquot was used to determine the activity of MDH at 30°C in medium containing 100 mM Tris-HCl (pH 7.9), 0.2 mM NADPH, and 0.7 mM oxaloacetic acid. The amount of NADPH oxidized was measured by monitoring the decrease in absorbance at 340 nm (Le Marechal *et al.*, 1992).

Purification

All purification steps were performed at 4°C un-

less otherwise stated. Protein content was determined according to the method of Lowry *et al.* (1951). Dilute protein solutions were concentrated by centrifugation with Amicon Centriprep. Dialysis was done with Spectrum membrane tubing (MWCO: 6,000-8,000). The *Arabidopsis* leaves (30 g) were disrupted by using a glass bead beater, resuspended in 470 mL of buffer B [20 mM Tris-HCl (pH 8.0)-2 mM EDTA], and filtrated through four layers of cheesecloth. Crude extract (Fr1) was obtained after centrifugation. Fr1 was loaded on a DE-52 column (2.5×20 cm) equilibrated with buffer B. 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 B. Active fractions were pooled and concentrated (Fr2). Fr2 was then loaded onto a Sephadex G-50 column (2.8×100 cm) equilibrated with buffer B. Active fractions of this were pooled and concentrated (Fr3). Fr3 was loaded onto a Q-Sepharose column (1.5×10 cm) equilibrated with buffer B. Active fractions of this were pooled and concentrated (Fr4). Fr4 was loaded onto a DEAE-Sephadex A-25 column (1.5×10 cm) equilibrated with buffer B. Elution was carried with a linear gradient of 0 to 0.5 M NaCl in buffer B. Fractions containing thioredoxin activity were pooled and concentrated (Fr5).

Reduction of Insulin

Thioredoxin-catalyzed reduction of insulin by dithiothreitol was monitored as a turbidity increase at 650 nm (Holmgren, 1979). This method was used to demonstrate the ability of thioredoxin to function as a protein disulfide reductase.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis on vertical polyacrylamide slab gels (16×18 cm) or pre-casting 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

Thioredoxins were purified from several plant species and identified to have multiple biological functions. Until recently, no information has been obtained on a thioredoxin from *A. thaliana* on a protein level. However, at least five divergent h-type thioredoxins were found to exist in *A. thaliana*, which were estimated by cDNA cloning.

Purification of Thioredoxin

The protein thioredoxin has been purified to a homogeneous state from *A. thaliana* leaves. Since thioredoxin activity was shown to be highest in 2-week-grown leaves in a preliminary test, 2-week-grown leaves were harvested for the purification. The crude extract (Fr1) was sequentially subjected to DE-52 chromatography, Sephadex G-50 gel filtration, Q-Sepharose chromatography, and DEAE-Sephadex A-25 chromatography. On the profile of DEAE-Sephadex A-25 chromatography, the unique activity peak appeared to coincide with a protein peak (data not shown). Starting with 30 g of *Arabidopsis* leaves, 4.5 mg of thioredoxin was obtained with an overall yield of 0.015%. The purified thioredoxin could be reduced by *E. coli* thioredoxin reductase. The purity of the isolated enzyme (Fr5) was monitored by performing a pre-cast SDS-polyacrylamide gel electrophoresis (4-20% Tris-glycine gel), in which Fr5 showed a single stained band (Fig. 1). This indicates that a thioredoxin was successfully purified from the leaves of *A. thaliana*. To verify the identity of thioredoxin purified from *A. thaliana* leaves, enzyme activities were measured under various reaction con-

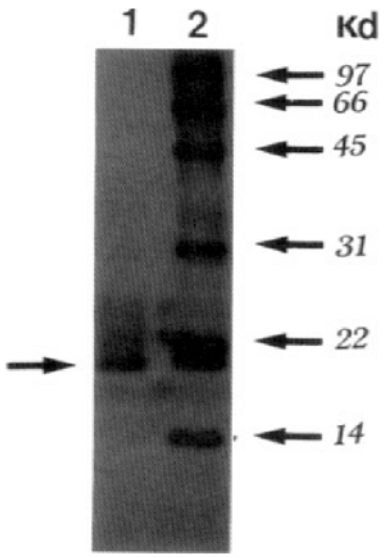

Fig. 1. The electrophoretic pattern of the purified thioredoxin from the leaves of *A. thaliana*. The purified sample was analyzed on a 15% SDS-PAGE gel and stained with silver nitrate. Lane 1, the purified thioredoxin; Lane 2, molecular marker.

Table 1. Activity of the purified Arabidopsis thioredoxin under various conditions.

Conditions	Thioredoxin Activity*	Relative Activity (%)
Complete	0.0224	100
-BSA	0.0257	115
-DTNB	0	0
-NADPH	0	0

Thioredoxin activity was assayed according to the DTNB reduction in the presence of thioredoxin reductase and NADPH as described in 'MATERIAL AND METHODS'. Its activity was expressed as ΔA_{412}/min.

ditions in which individual components were deleted from the complete assay mixture. As shown in Table 1, the result indicated that the purified thioredoxin absolutely required NADPH and thioredoxin reductase (in this case, *E. coli* thioredoxin reductase) to reduce the disulfide bond contained in DTNB.

Molecular Weight

The molecular weight of *A. thaliana* thioredoxin purified in this study was estimated to be about 21 KDa on SDS-PAGE. Most thioredoxins had been generally shown to be heat-stable proteins of approximately 12 KDa. It indicates that the purified thioredoxin has large molecular mass. Based on the estimation from cDNA clones, *Arabidopsis* h-type thioredoxins also contain typical molecular mass (Rivera-Madrid *et al.*, 1995). The functional significance of this atypical thioredoxin remains to be elucidated.

Evidence for f-Type Thioredoxin

Plant thioredoxins were originally classified into two groups such as f-type and m-type thioredoxins (Schürmann *et al.*, 1981). F-type thioredoxin activates frutose-1,6-bisphosphatase whereas m-type thioredoxin activates $NADP^+$-malate dehydrogenase. In later years, h-type thioredoxin was identified as non-chloroplast plant thioredoxins (Johnson *et al.*, 1987). To decide whether the purified thioredoxin was f-type or m-type, activation of fructose-1,6-bisphosphatase and $NADP^+$-malate dehydrogenase was examined. Fructose-1,6-bisphosphatase and $NADP^+$-malate dehydrogenase were partially purified from a crude extract from *A. thaliana* leaves according to ion-exchange chromatography and gel filtration (data not shown). As shown in Fig. 2, the purified *A. thaliana* thioredoxin was able to activate fructose-1,6-bis-

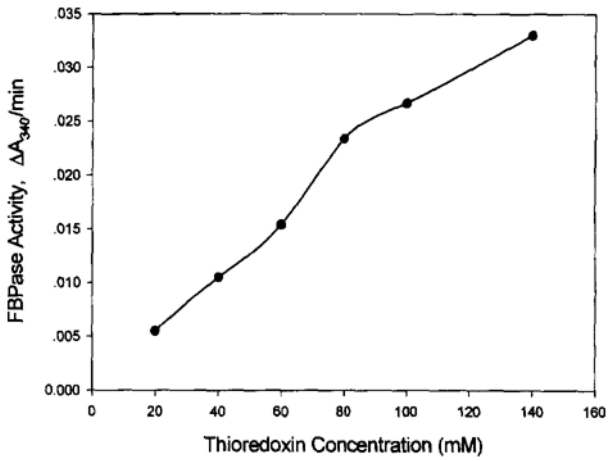

Fig. 2. Activation of *Arabidopsis* fructose-1,6-bisphosphatae by the purified thioredoxin from the leaves of *A. thaliana*. Activation experiments were carried out as described in 'MATERIALS and METHODS'. The activity of fructose-1,6-bisphosphatase was expressed as ΔA_{340}/min.

phosphatase. Conversely, it could not activate $NADP^+$-malate dehydrogenase. These results suggest that the *Arabidopsis* thioredoxin is classified as an f-type.

Reduction of Insulin Disulfides

Thioredoxin from *E. coli* was shown to catalyze the reduction of insulin disulfides by dithiothreitol (Holmgren, 1979). A quantitative assay was developed which measures the rate of insulin reduction spectrophotometrically at 650nm, as turbidity forms from the precipitation of the free insulin B chain. To estimate the ability of the f-type *Arabidopsis* thioredoxin to function as a protein disulfide reductase, its insulin precipitation activity was determined in the presence of dithiothreitol. The results shown in Fig. 3 indicate that the f-type *Arabidopsis* thioredoxin is able to reduce insulin. Turbidity formation increased in a thioredoxin concentration-dependent manner, indicating that the f-type thioredoxin acted as a protein disulfide reductase.

Kinetic Parameter and Optimal pH

The f-type *Arabidopsis* thioredoxin is a substrate for *E. coli* thioredoxin reductase, since its purification was based on its interaction with *E. coli* thioredoxin reductase. Kinetic measurements of the reaction of the f-type *Arabidopsis* thioredoxin with *E. coli* thioredoxin reductase were carried out by using a Model UV-2401 Shimadzu spectrophotometer at

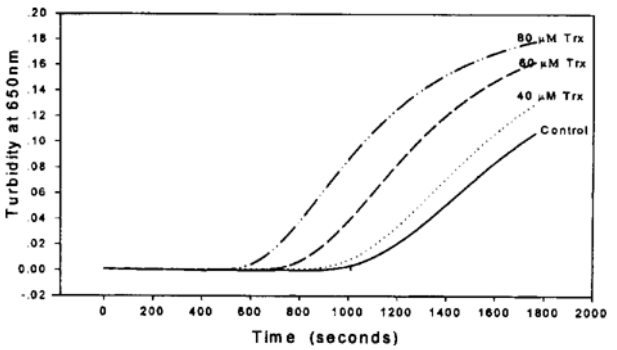

Fig. 3. Reduction of insulin disulfides by the f-type thioredoxin from the leaves of *A. thaliana*. The experiment was carried out as described in 'MATERIALS and METHODS'. Absorbance at 650 nm was measured as an indicator of turbidity produced from the reduction of insulin.

25°C (Fig. 4). The Km value obtained from the Lineweaver-Burk plot was estimated to be 20.2 μM. The Km value of *E. coli* thioredoxin on *E. coli* thioredoxin reductase was determined to be 0.67 μM (Sa *et al.*, 1996). The optimal pH of the f-type *Arabidopsis* thioredoxin was determined to be 8.0 (data not shown).

In this article, we purified and characterized an f-type thioredoxin from the leaves of *A. thaliana*. It could react with *E. coli* thioredoxin reductase. However, its physiological role could not yet be defined. It might play an important role in the regulation of the Calvin Cycle in photosynthesis. An interesting question is whether the f-type thioredoxin

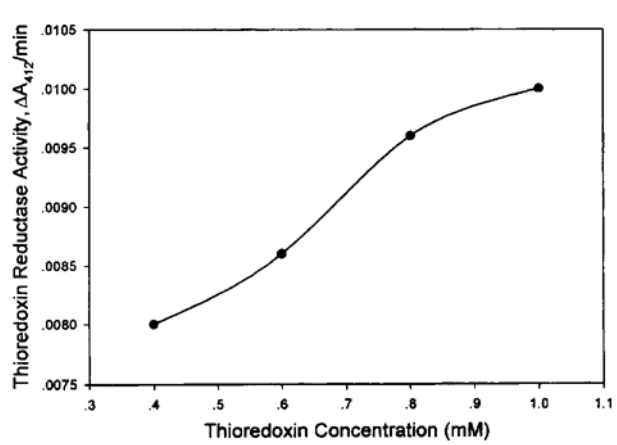

Fig. 4. Reduction of various concentrations of the f-type *Arabidopsis* thioredoxin with *E. coli* thioredoxin reductase. From this curve, the Km value was calculated according to the Lineweaver-Burk plot. The activity of thioredoxin reductase (TR) was expressed as ΔA_{412}/min.

acts as a cofactor of ribonucleotide reductase in *A. thaliana*. The purified thioredoxin is the first example in *A. thaliana* on a protein level, although there are five known h-type thioredoxins on the gene level. Considering the larger molecular mass of the f-type *Arabidopsis* thioredoxin, its interaction with *E. coli* thioredoxin attracts an interest. The subcellular location of the purified f-type thioredoxin remains unclear. Thioredoxin activity gradually decreases as *Arabidopsis* leaves age (Sa *et al.*, 1998). Further investigation would explain the physiological role of the f-type *Arabidopsis* thioredoxin in cellular actions, especially photosynthesis.

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