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Purification and Characterization of Thioredoxin f from Pea Leaves

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Abstract: Thioredoxin f from pea leaves was purified to homogeneity and characterized. The purification steps involved ammonium sulfate fractionation, heat treatment, Sephadex G-75 and G-50 gel filtration, and hydroxyapatite and DEAE ion exchange chromatography. The monomeric molecular weight of purified pea thioredoxin f determined by SDS polyacrylamide gel electrophoresis was 12,000. The purified protein was active in the presence of reducing agents, such as dithiothreitol, at an alkaline pH (7.8~8.5). It was stable against heat such that more than 40% of its maximum activity remained after treatment at 90℃ for 10 min. Pea thioredoxin f was able to reduce insulin and was specific only to pea chloroplast fructose-1,6-bisphosphatase.

Key words: Fructose-1,6-bisphosphatase, Pea thioredoxin f.

Thioredoxins are heat stable, low molecular weight (12,000) proteins that play important function in the regulation of prokaryotic and eukaryotic organisms. Thioredoxins have an active site composed of Cys-Gly-Pro-Cys, and exist either in a reduced form with a dithiol, or in an oxidized form when the half cysteine residues form an intracellular disulfide bridge (Holmgren, 1985; Brandes *et al.*, 1993). Reduced thioredoxins reduce specific disulfide groups on biosynthetic target enzymes, such as ribonucleotide reductase and methionine sulfoxide reductase (Holmgren, 1985).

In plant chloroplasts there are at least two thioredoxins which regulate light dependent carbon metabolism by modulating the redox status of target enzymes. Thioredoxin m regulates malate dehydrogenase and thioredoxin f activates fructose-1,6-bisphosphatase in the stroma of chloroplasts (Wolosiuk *et al.*, 1977). Light mediated activation of these enzymes occurs via a ferredoxin/thioredoxin system *in vivo* which converts oxidized forms into reduced forms of the enzymes (Buchanan, 1980). Plant thioredoxins have been purified mainly from spinach leaves (Buc *et al.*, 1984; Marcus *et al.*, 1991). Recent work on spinach thioredoxin f suggests that Cys-46 acts as a nucleophile which attacks the disulfide bridge of target enzyme (Brandes *et al.*, 1993).

In this study, we purified and characterized thioredoxin f from pea leaves, as a preliminary work to elucidate light dependent regulation mechanism of photosynthetic enzymes in higher plants.

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Materials and Methods

Materials

Pea seeds (*Pisum sativum* L. Giant) were purchased from Jungang Seed Co. (Seoul, Korea) and grown in a greenhouse for 4 weeks. Harvested pea leaves were used immediately or stored at −70℃. Sephadex G-50 and G-75 were purchased from Pharmacia Fine Chemicals, and DE-52 (DEAE-Cellulose) was obtained from Whatman Biosystems. Phosphoglucoseisomerase (from yeast) was supplied by Boehringer Mannheim and sodium dodecyl sulfate was obtained from Bethesda Research Laboratories. NADP, reference proteins, and other chemicals were purchased from Sigma Chemical Co.

Purification of chloroplastic and cytosolic fructose-1,6-bisphosphatase

Chloroplastic and cytosolic fructose-1,6-bisphosphatases (FBPase) were purified from pea leaves as described previously (Bhoo and Hahn, 1989; Cho and Hahn, 1991; Lee *et al.*, 1994). In summary, one kilogram of pea leaves was homogenized and the clarified homogenate was fractionated with 40~60% ammonium sulfate. The preparation obtained from ammonium sulfate fractionation was dialyzed against a 0.02 M sodium phosphate buffer, pH 7.5, containing 0.4 mM EDTA and 50 mM NaCl. The dialyzed sample was clarified and applied to a DEAE Bio-gel A column and eluted with the same buffer containing a gradient of NaCl. The active fractions with chloroplastic FBPase activity were further purified by Sephadex G-200 chromatog-

raphy. For cytosolic FBPase purification, the active fractions from DEAE Bio-gel A chromatography were subjected to hydroxylapatite chromatography. Fractions containing cytosolic FBPase activity were further purified by Sephadex G-200 chromatography. The final enzyme preparations for both chloroplastic and cytosolic FBPase appeared homogeneous based on SDS-polyacrylamide gel electrophoresis (Cho and Hahn, 1991; Lee *et al.*, 1994).

Purification of thioredoxin f

Thioredoxin f was purified from frozen pea leaves by a minor modification of the method of Schürmann *et al.* (1981). Unless otherwise mentioned, all preparative procedures were carried out at 4℃. Benzoylated dialysis membrane (1,200 Da cutoff, Sigma) was used for dialysis.

Preparation of crude extracts: One kg of pea leaves was homogenized with one liter of 0.1 M sodium phosphate buffer, pH 7.5, supplemented with 2 mM EDTA and 0.4 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was filtered through four layers of gauze and the filtrate was centrifuged (7000 g, 30 min). The supernatant was fractionated with 30~70% ammonium sulfate. The precipitate collected by centrifugation was dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 7.5, containing 50 mM NaCl.

Heat treatment: The active fractions obtained from ammonium sulfate fractionation were heat-treated at 65℃ for 8 min, then immediately cooled in ice. Insoluble proteins were removed and the supernatant was precipitated with ammonium sulfate (80%). The precipitate was dissolved in a minimal amount of 0.1 M sodium-phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.5 mM EDTA.

Gel filtration on Sephadex G-75: The preparation obtained from heat treatment was applied to a Sephadex G-75 column (4×40 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 0.15 M NaCl. The column was eluted with the same buffer and the active fractions were pooled. The fractions containing thioredoxins were dialysed with 0.02 M sodium phosphate buffer, pH 7.0, containing 0.4 mM EDTA for 4 h. A typical elution profile of Sephadex G-75 chromatography was shown in Fig 1.

Hydroxyapatite chromatography: The dialysed sample was clarified by centrifugation and the supernatant was applied to a hydroxyapatite column (1×10 cm) equilibrated with a 0.02 M sodium phosphate buffer, pH 7.0, containing 0.4 mM EDTA. After washing the column with the same buffer, proteins were eluted with a 0.02~0.30 M sodium phosphate gradient buffer, pH 7.0, containing 0.4 mM EDTA. An Elution profile of hydroxyapatite chromatography was shown in Fig. 2.

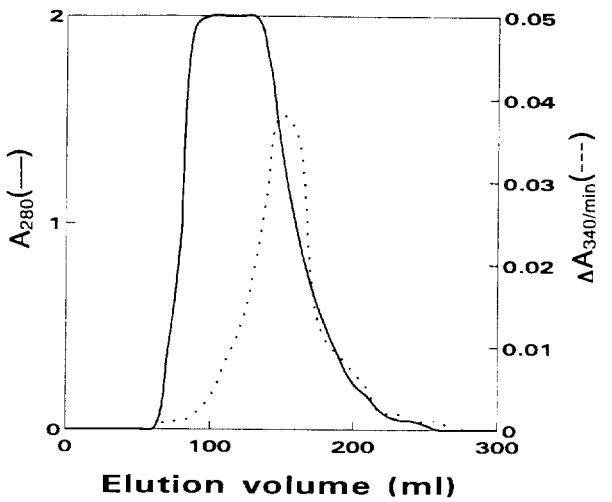

Fig. 1. Elution profile of thioredoxin f from Sephadex G-75 column chromatography. After heat treatment, fractions were loaded onto a Sephadex G-75 column equilibrated with 0.1 M sodium phosphate buffer, pH 7.5, containing 0.5 mM EDTA and 0.15 M NaCl. Fractions were collected and the thioredoxin f activity was determined.

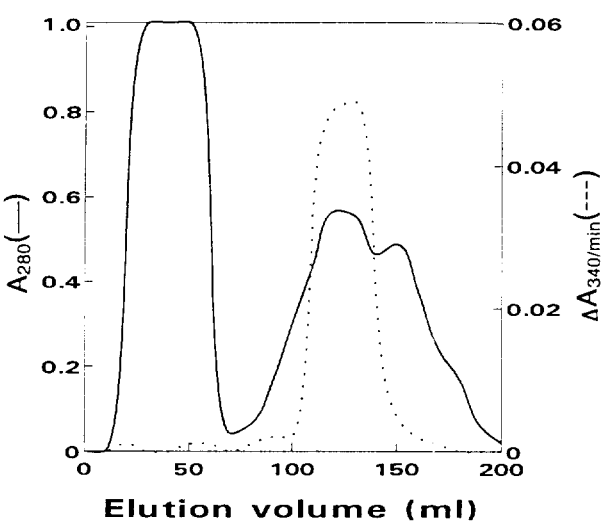

Fig. 2. Elution profile of thioredoxin f from hydroxyapatite chromatography. Active fractions from Sephadex G-75 column chromatography were dialysed, then applied to a hydroxyapatite column equilibrated with 0.02 M sodium phosphate buffer, pH 7.0. Proteins were eluted with a 0.02~0.30 M sodium phosphate gradient buffer, pH 7.0.

Gel filtration on Sephadex G-50: The active fractions from hydroxyapatite chromatography were precipitated using 80 % ammonium sulfate. After centrifugation, the precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, supplemented with 0.1 M NaCl, 0.4 mM EDTA, then applied to a Sephadex G-50 column (4×40 cm). The column was eluted with the same

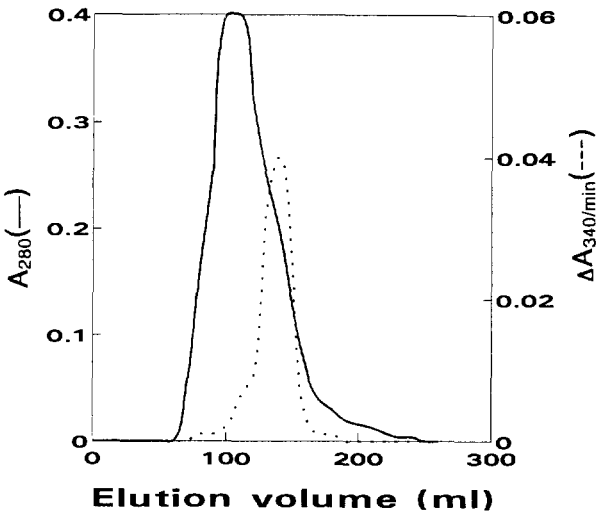

Fig. 3. Elution profile of thioredoxin f from Sephadex G-50 column chromatography. Fractions from hydroxyapatite chromatography were concentrated with 80% ammonium sulfate. Precipitates were dissolved in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, then applied to a Sephadex G-50 column equilibrated with the same buffer. Fractions containing thioredoxin f activity were pooled.

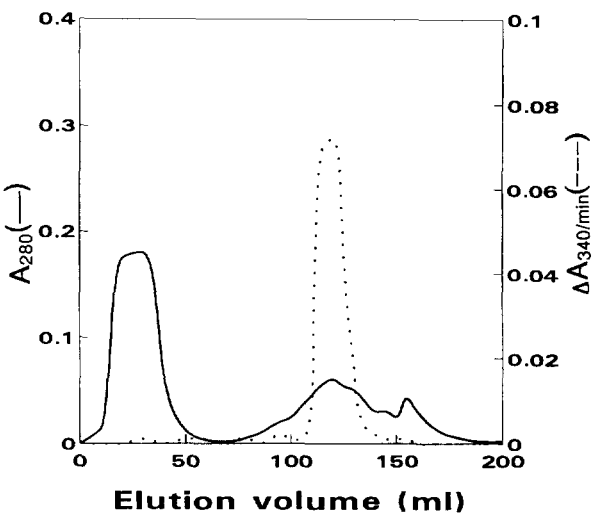

Fig. 4. Elution profile of thioredoxin f from DE-52 column chromatography. Active fractions obtained from Sephadex G-50 chromatography were dialysed in 0.02 M Tris-HCl, pH 7.8, containing 0.02 M NaCl, then applied to a DE-52 column equilibrated with the same buffer. The column was washed with the same buffer, then eluted with 200 ml of a linear gradient from 0.02 M to 0.4 M NaCl. Fractions were collected and thioredoxin f activity was determined.

buffer and the active fractions were pooled. An elution profile of Sephadex G-50 chromatography was shown in Fig. 3.

DE-52 chromatography: The active fractions from Sephadex G-50 chromatography were dialysed against 0.02 M Tris-HCl, pH 7.8, containing 0.02 M NaCl for 4 h. The dialysed sample was centrifuged and the clarified supernatant was applied to a DE-52 column (1× 10 cm) equilibrated with 0.02 M Tris-HCl, pH 7.8, containing 0.02 M NaCl. After washing the column with the same buffer, proteins were eluted with 200 ml of the same buffer containing a linear gradient of 0.02~ 0.4 M NaCl (Fig. 4).

Table 1. Purification of thioredoxin f from pea leaves. Enzyme activity was expressed as the change in absorbance at 340 nm per min. Specific activity equals to enzyme activity per mg protein

Fractions	Total proteins (mg)	Total activity ($\Delta A_{340/min}$)	Specific activity ($\Delta A_{340/min/mg}$) $\times 10^{-2}$
Crude extract	4450	1.80	0.04
Ammonium sulfate	1850	1.40	0.08
Heat treatment	230	1.15	0.50
Sephadex G-75	75	0.92	1.22
Hydroxyapatite	25	0.74	2.96
Sephadex G-50	5	0.60	12.00
DE-52	1.2	0.40	33.00

Measurement of enzyme activity

Chloroplastic FBPase activity was determined according to the method of Zimmerman *et al.* (1976). Reaction mixtures consisted of 100 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.3 mM NADP, 0.6 u glucose-6-phosphate dehydrogenase, 1.2 u phosphoglucoseisomerase, and an enzyme preparation containing FBPase. The reaction was started by addition of 0.6 mM fructose-1,6-bisphosphate. Cytosolic FBPase was similarly assayed, except the buffer was Tris-HCl buffer, pH 7.0. Enzyme activities were expressed as the change in absorbance at 340 nm per min. Thioredoxin f activity was estimated as follows: Purified FBPase was preincubated with a thioredoxin f preparation and 10 mM dithiothreitol in the same reaction buffer. After 10 min, the reaction mixtures were incubated for 15 min at 30℃. Thioredoxin f activity was determined by an increase in FBPase activity.

Alternatively, thioredoxin activity was assayed spectrophotometrically (OD_{400}) by measuring insulin reduction which caused turbidity formation due to precipitation of free insulin B chains (Holmgren, 1979; Cho and Hahn, 1991).

Analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method

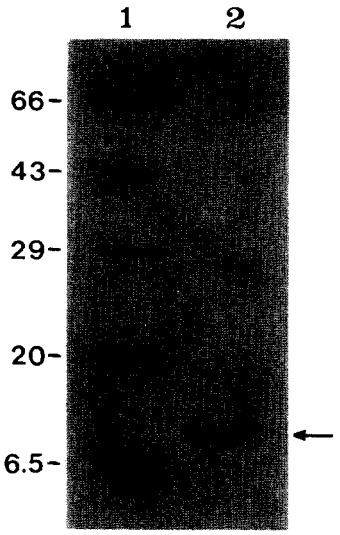

Fig. 5. SDS-polyacrylamide gel electrophoresis of thioredoxin f from pea leaves. Lane 1 : reference proteins; Lane 2 : purified thioredoxin f from pea leaves. Reference proteins were bovine serum albumin (Mr, 66,000), ovalbumin (Mr, 43,000), carbonic anhydrase (Mr, 29,000), trypsin inhibitor (Mr, 20,100) and aprotinin (Mr, 6,500).

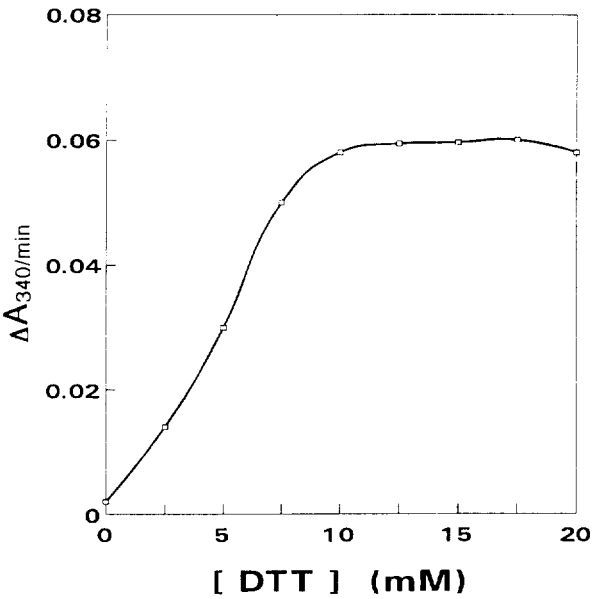

Fig. 6. Effect of dithiothreitol on the activity of pea thioredoxin f. Different amounts of dithiothreitol were added to assay mixtures. Assay mixtures contained 5 μM fructose-1,6-bisphosphatase, 20 μM thioredoxin f, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.3 mM NADP, 1.2 u phosphoglucoseisomerase, 0.6 u glucose-6-phosphate dehydrogenase, and 0.6 mM fructose-1,6-bisphosphate.

of Laemmli (1970). The slab gels consisted of 18% acrylamide running gel and 5% acrylamide stacking gel. Proteins were stained by Coomassie brilliant blue G. The molecular weight of thioredoxin f was estimated by SDS-PAGE using size marker proteins (bovine serum albumin, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 29 kD; trypsin inhibitor, 20 kD; aprotinin, 6.5 kD). The protein concentration was determined either by measuring the absorbance at 280 nm, or by the dye binding assay of Bradford (1976).

Results and Discussion

Thioredoxins are key regulator proteins for various light dependent photosynthetic enzymes in plant chloroplasts. Thioredoxin f was purified from pea leaves. Table 1 summarizes the purification steps of pea thioredoxin f. The final preparation was purified approximately 800 fold. The most effective step for the purification of pea thioredoxin f was DE-52 ion exchange chromatography, which separates thioredoxin f from other thioredoxins, including thioredoxin m (data not shown). Fig. 5 shows the SDS-PAGE pattern of purified thioredoxin f, indicating that the final preparation after DE-52 chromatography was homogeneous with a monomeric molecular weight of 12,000 which corresponds to spinach thioredoxin f (Schürmann *et al.*, 1981; Crawford, 1986). This preparation was used for all procedures for the characterization of pea thioredoxin f.

Chloroplastic FBPase is activated by reduced thioredoxin f (Schürmann *et al.*, 1981). Thus, FBPase activation by thioredoxin f in the presence of different concentrations of the reducing agent dithiothreitol was tested at pH 8.0. Fig. 6 shows the effect of dithiothreitol on thioredoxin activity. The activity of thioredoxin f gradually increased with an increase in dithiothreitol concentration. Thioredoxin was almost fully activated by more than 10 mM dithiothreitol, which is similar to chloroplastic FBPase without thioredoxin f (Cho and Hahn, 1991). It is nothworthy that cytosolic FBPase had no effect on purified thioredoxin f in the presence and absence of dithiothreitol (data not shown). Chloroplastic FBPase is distinct from the cytosolic enzyme in several ways. The most striking difference is that chloroplastic FBPase is activated by light (Buchanan, 1980). It is also activated by reducing agents, an alkaline pH, and Mg^{2+}, which mimicks conditions in the stroma of chloroplasts upon light illumination. Thus, the effect of pH on the activation of chloroplastic FBPase by thioredoxin f was examined in the presence of dithiothreitol. Fig. 7 shows that the activation of chloroplastic FBPase by thioredoxin f was pH dependent. The enzyme was activated only at an alkaline pH with the optimum pH range from 7.8~8.5. These are lower values than for chloroplastic FBPase in which the optimum pH is approximately 9.0 (Cho and Hahn, 1991). Pea thioredoxin f did not activate chloroplastic FBPase at pH values lower than 7.0. Cytosolic FBPase is only active at a neutral pH (Kelly *et al.*, 1982; Ladror *et*

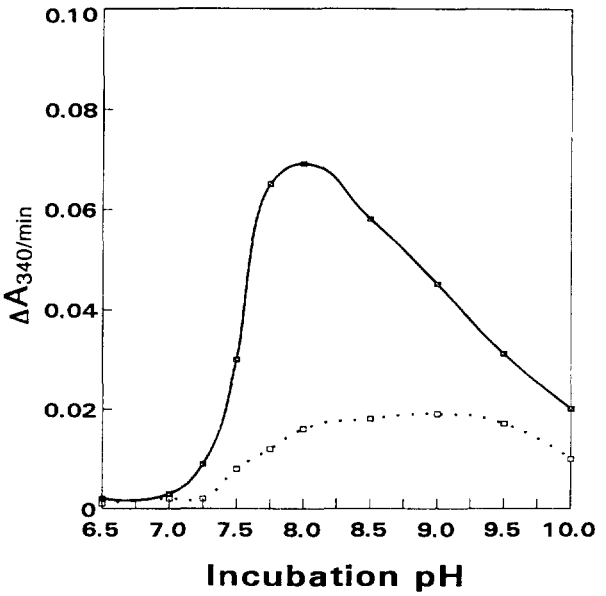

Fig. 7. Effect of pH on the activity of pea thioredoxin f. Thioredoxin f activity was assayed by fructose-1,6-bisphosphatase activation at different pH values in the presence of 10 mM dithiothreitol. 0.1 M Tris-HCl buffers, and 0.1 M sodium phosphate buffers were used for pH ranges from 6.0 to 7.0 and 7.0 to 9.0, respectively. Fructose-1,6-bisphosphatase activity without thioredoxin f (----); with thioredoxin f (20 μM) (——).

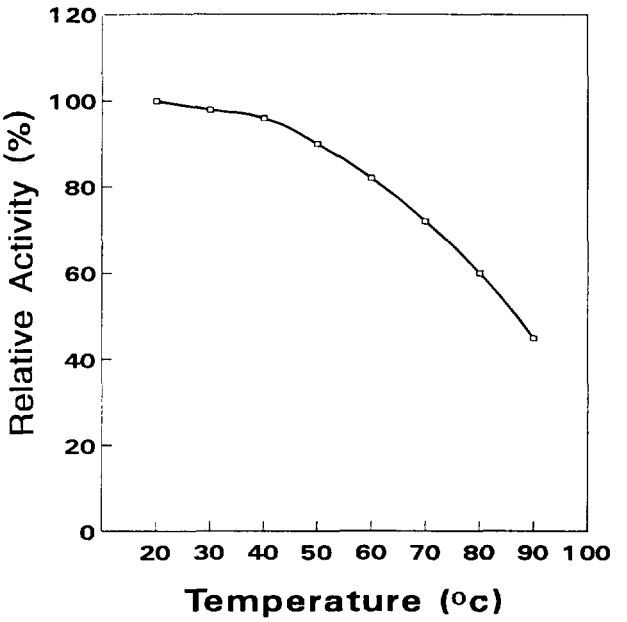

Fig. 8. Heat stability of pea thioredoxin f. Purified thioredoxin f was incubated in a water bath with a temperature range from 30℃ to 80℃ for 10 min. After rapid cooling in an ice bath, the heat treated sample was added to an assay mixture to measure thioredoxin f activity.

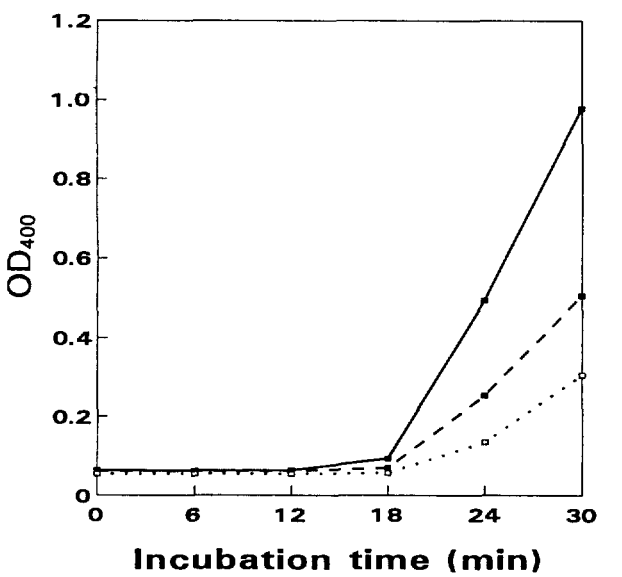

Fig. 9. Insulin reduction by pea thioredoxin f. Insulin reduction was assayed by turbidity formation at 25℃. Assay mixtures contained 10 mM dithiothreitol, 0.05 mM insulin, and thioredoxin f. Turbidity formation was measured spectrophotometrically at 400 nm. Insulin reduction without thioredoxin (-----) ; with 5 μM thioredoxin f (-----); 10 μM of thioredoxin f (——).

al., 1990; Lee *et al.*, 1994).

Bacterial and mammalian thioredoxins are resistant to heat (Laurent *et al.*, 1964; Larson and Larsson, 1972; Cho and Hahn, 1991). In this regard the thermal stability of purified pea thioredoxin f was examined. Results indicated that thioredoxin f was very stable against heat. Even after treatment at 90℃ the protein was still active with more than 40% of the maximum activity (Fig. 8). However, pea thioredoxin f was relatively less stable than bacterial thioredoxins. For example, *E. coli* thioredoxin was stable against heat with more than 95% of its activity remaining after treatment at 95℃ for 10 min (Cho and Hahn, 1990). This exceptional heat stability of *E. coli* thioredoxin is due to the fact that more than 75% of the protein consists of well defined secondary structures (Holmgren *et al.*, 1975; Eklund *et al.*, 1984; Katti *et al.*, 1990). The molecule contains a 5-stranded β-sheet surrounded by 3 α-helices. Present data for pea thioredoxin f suggest that plant thioredoxins resemble procaryotic thioredoxins, but are somewhat different in secondary structures and tertiary folds.

Thioredoxins can serve as hydrogen donors for *E. coli* ribonucleotide reductase, and can reduce insulin disulfides (Holmgren, 1985). *E. coli* thioredoxin accelerates insulin reduction approximately 20 fold at pH 7.0 in the presence of dithiothreitol. Therefore, the activities of bacterial and mammalian thioredoxins can be determined spectrophotometrically by turbidity formation due to the precipitation of the free insulin B chains (Holmgren, 1979; Cho and Hahn, 1990). In the present study pea thioredoxin f was tested to determine its capability for insulin reduction in the presence of

dithiothreitol. Results shown in Fig. 9 indicate that purified pea thioredoxin f is able to reduce insulin. Turbidity formation was increased in a thioredixin concentration dependent manner (Fig. 9). However, in contrast to *E. coli* thioredoxin, reduction of insulin by thioredoxin f required a time lag of approximately 20 min, indicating that the structure of thioredoxin f is different from other bacterial and mammalian thioredoxins. Recent data suggest that the Cys-46 of spinach thioredoxin f is the nucleophile required to attack the disulfide of the substrate protein (Brandes *et al.*, 1993).

In conclusion, we purified and characterized thioredoxin f from pea leaves. Purified thioredoxin f was homogeneous with a molecular weight of 12,000 as determined by SDS polyacrylamide gel electrophoresis. The protein was active in the presence of dithiothreitol at alkaline pH values. It was also heat-stable and specific only to chloroplastic FBPase. Purified pea thioredoxin f was able to reduce insulin, as are other bacterial and mammalian thioredoxins.

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