Identification and localization of the first glutaredoxin in leaves of a higher plant

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Abstract Glutaredoxin(thioltransferase) has been identified and purified to homogeneity from spinach leaves. Its cytosolic localization was demonstrated by chromatographic and immunological analysis of extracts from isolated spinach chloroplasts and mitochondria, respectively. Spinach glutaredoxin shows a significant crossreactivity with antibodies raised against *E. coli* glutaredoxin and possesses a specific thioltransferase activity comparable to that of the *E. coli* protein. Minor thioltransferase activities (less than 10% of total leaf activity) have been observed in spinach chloroplasts which are probably due to the presence of trypsin inhibitor and thioredoxins (TRf and TRm).

Key words: Spinach leave; Glutaredoxin; Thioredoxin; Localization

1. Introduction

Glutaredoxin was discovered in *Escherichia coli* as an alternative reducing substrate of ribonucleotide reductase, key enzyme of deoxyribonucleotide biosynthesis [1]. Originally, thioredoxin had been regarded as the exclusive substrate in this process [2]. Both proteins possess two redox-active cysteine residues in their active sites formed by the sequences Cys-Gly-Pro-Cys in thioredoxin and Cys-Pro-Tyr(Phe)-Cys in glutaredoxin [3]. Whereas the presence of thioredoxin and glutaredoxin in virus, bacterial, and mammalian cells was already shown many years ago, the presence of glutaredoxin besides thioredoxin in plant cells was not elucidated prior to recent findings of Minakuchi et al. They identified the first plant glutaredoxin in rice, which was detected in seeds and aleurone layers, but not in leaves, by cDNA cloning and Northern blot analysis [4]. Here we describe the direct purification of glutaredoxin from spinach leaves and its intracellular localization.

2. Materials and methods

All chemicals and reagents were of highest purity available. Glutathione was obtained from Merck, glutathione reductase from Sigma and β-hydroxyethyl disulfide (HED) from Aldrich. Spinach was obtained from local markets. Spinach thioredoxins TRm and TRf were generous gifts of Dr. Peter Schürmann, University of Neuchatel, Switzerland. A sample of antibodies raised against *E. coli* glutaredoxin by Dr. Arne Holmgren, Karolinska Institute Stockholm, Sweden, is gratefully acknowledged. Glutaredoxin and thioredoxin from *E. coli* were prepared by published procedures [14,15].

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2.1. Glutaredoxin(thioltransferase) assay

The GSH-disulfide oxidoreductase activity of glutaredoxin which catalyses the reduction of β-hydroxyethyl disulfide (HED) by GSH was applied to identify the corresponding protein in spinach leaves [5]. The assay was coupled with glutathione reductase and the consumption of NADPH was monitored at 340 nm. One unit was defined as the oxidation of 1 μmol NADPH per minute.

$$GSH + XSSX \rightarrow GSSX + XSH$$

(XSSX = β-hydroxyethyl disulfide)

$$GSH + GSSX \xrightarrow{\text{glutaredoxin}} GSSG + XSH$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{glutathion reductase}} 2\ GSH + NADP^+$$

2.2. Dehydroascorbate reductase assay

Dehydroascorbate reductase activity was measured by a recently described assay [6]. The formation of ascorbate was followed at 265 nm.

2.3. Thioredoxin assays

Thioredoxins have been detected by their stimulatory effects on two chloroplast enzymes, NADP-malate dehydrogenase and fructose-1,6-bisphosphatase from spinach leaves. Purification of these two enzymes and the assay conditions have been described elsewhere [7,8].

2.4. Purification of glutaredoxin from spinach leaves

Unless otherwise noted, all operations were performed at 4°C. In a Waring Blender 200 g of spinach leaves were extracted in 100 ml buffer of 20 mM Tris/Cl, buffer pH 8.7, containing 2 mM EDTA. The homogenate was passed through three layers of Miracloth and centrifuged at 15,000 rpm for 10 min. The green-yellow supernatant was directly applied onto a DEAE-cellulose ion exchange column (2.5 × 8.0 cm; flow rate 40 ml/h; fraction size 10 ml) which was equilibrated with 20 mM Tris/Cl, 2 mM EDTA, pH 8.7. Glutaredoxin was eluted in a linear salt gradient of 0–0.5 M NaCl in 2 × 200 ml buffer. Fractions with thioltransferase activity were pooled and concentrated to a final volume of 5 ml by Aquacide II (Calbiochem). Gel filtration was performed on a Superdex 75 HR 16/60 column (Pharmacia) equlibrated with 50 mM Tris/Cl buffer, pH 8.3, containing 150 mM NaCl and 1 mM EDTA. Fractions of 5 ml were collected at a flow rate of 1.5 ml/min. Fractions with thioltransferase activity were dialyzed against 20 mM Tris/Cl buffer, pH 8.3, and applied to a MonoQ HR5/5 ion exchange column (Pharmacia). A FPLC-system was used to elute glutaredoxin in a linear gradient from 0.2–0.35 M NaCl in 30 ml buffer. Fractions of 1 ml were collected at a flow rate of 1 ml/min. Homogenous glutaredoxin was obtained by rechromatography on the MonoQ column under identical conditions. Purity of the preparation was analysed by 12% SDS-polyacrylamide gel electrophoresis in a tricine buffer system [9].

2.5. Analysis of glutaredoxin in spinach chloroplasts

Isolation of chloroplasts from spinach leaves followed the method described by Mourioux and Douce [10]. The chloroplasts were extracted in hypotonic buffer (20 mM Tris/Cl, 2 mM EDTA, pH 8.7). Organelle debris was removed by centrifugation at 15,000 rpm for 15 min. The DEAE-cellulose ion exchange chromatography of the chloroplast extract was performed under identical conditions as for the leaf extract described above.

2.6. Immunological assays

Enzyme linked immunosorbent assays (ELISA) have been performed

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with antibodies raised against *E. coli* glutaredoxin, and following essentially the published procedure [11]. Anti-rabbit IgG peroxidase conjugate and ABTS (2,2'-azino-di(3-ethylbenzothiazolinosulfonic acid)) from Sigma were used to determine the crossreactivity of the first antibody. Product formation was determined photometrically at 410 nm after 20 min.

3. Results

The thioltransferase assay specific for glutaredoxin using GSH and hydroxyethyldisulfide as substrates, has been applied to identify glutaredoxin in spinach leaves. Although no thioltransferase activity could be detected in crude leaf extracts, DEAE-cellulose anion exchange chromatography was performed and revealed three fractions of the salt gradient containing peaks of thioltransferase activity which are numbered acccording to their elution sequence (Fig. 1a). Peak III represents about 90% of the total activity. The very high absorbance at 280 nm is due to the presence of coloured components in the fractions. The chemically determined protein content is much lower (see Table 1).

The protein extract of isolated spinach chloroplasts was analysed by a separate DEAE-cellulose anion exchange chromatography under identical conditions (Fig. 2). The elution profile demonstrates that the minor thioltransferase activities (peak I and peak II) found in the leaf extract are of chloroplastic origin. Because of the similarity of the active sites of glutaredoxin and thioredoxin the elution positions of the two chloroplast thioredoxins TRm and TRf have been determined in activation assays with NADP malate dehydrogenase and alkaline

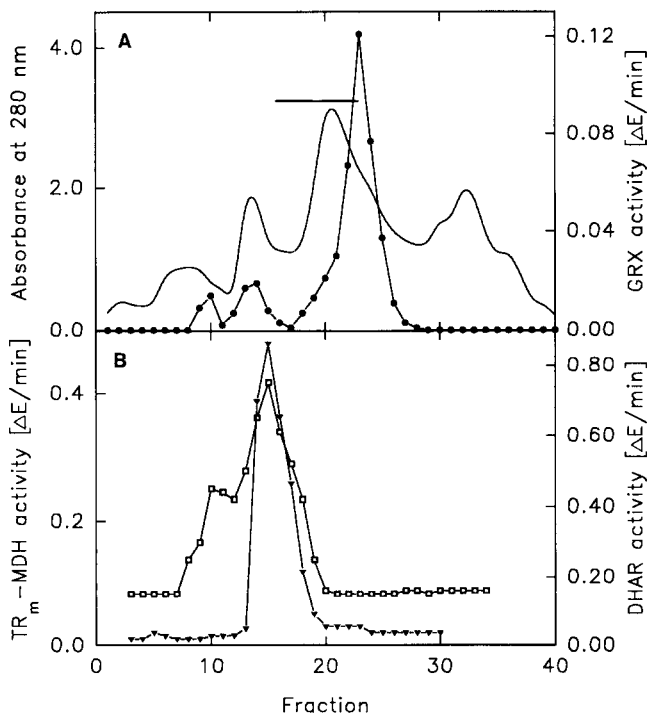

Fig. 1. Fractionation of spinach leaf proteins by DEAE cellulose chromatography. The elution profile of the salt gradient from 0–0.5 M NaCl is presented. (a) Solid line: Protein absorption at 280 nm. Glutaredoxin (GRX) activity (●) was measured with the thioltransferase assay. (b) TR_m (▼) was determined by the NADP malate dehydrogenase (MDH) activation assay, dehydroascorbate reductase (DHAR) activity (□) was followed by the formation of ascorbate.

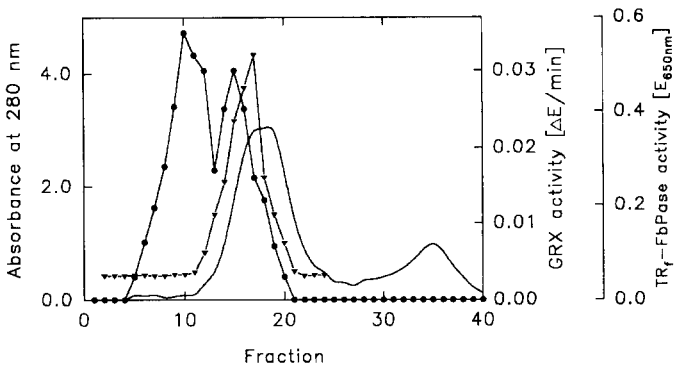

Fig. 2. Fractionation of a protein extract from isolated spinach chloroplasts by DEAE cellulose chromatography. Solid line: Protein absorption at 280 nm, thioltransferase activity (●) of glutaredoxin (GRX), TR_f was measured by the fructose-1,6-bisphosphatase (FbPase) activation assay (▼).

fructose-1,6-bisphosphatase. We observed that the thioltransferase activity of peak II coincides with the activities of TRm and TRf whereas peak I and peak III are free of thioredoxin activity (Figs. 1b and 2). Wells et al. described that *E. coli* glutaredoxin possesses dehydroascorbate reductase activity; a feature which was not observed with the cloned glutaredoxin from rice seeds. Because of these findings dehydroascorbate reductase activity in our preparations was measured. Fig. 1b shows that dehydroascorbate reductase coelutes with peak I and II but that peak III does not contain this enzymatic activity.

The fractions of peak III were concentrated to a final volume of 5 ml and subjected to size exclusion chromatography. Thioltransferase activity eluted in a single peak with a molecular mass of about 25 kDa. Finally, the enzyme was purified to homogeneity by two sequential MonoQ ion exchange chromatography steps under identical conditions. Glutaredoxin eluted as a symmetrical peak at 100 mM NaCl of the salt gradient, and no additional thioltransferase isoforms were observed along this procedure. The purification scheme shows that this procedure yields 20 μg of homogenous thioltransferase with a specific activity of 10 U/mg from 200 g spinach leaves (Table 1).

SDS-polyacrylamide gel electrophoresis (Fig. 3) revealed a molecular mass of 12 kDa for spinach thioltransferase. This figure, in comparison to the result obtained by size exclusion chromatography, indicates that the thioltransferase comprises two identical subunits. Under reducing conditions (1 mM dithiothreitol) the thioltransferase eluted from the Superdex 75 column with a molecular mass of about 12 kDa. Thus, the enzyme appears to form disulfide-linked dimers in the absence of reducing conditions (data not shown).

In Table 2 the specific thioltransferase activities of different thioredoxins and of *E. coli* glutaredoxin have been compared with the purified thioltransferase from spinach leaves. Obviously, *E.coli* glutaredoxin and spinach thioltransferase (peak III) possess comparable specific activities, whereas the thioltransferase activities of thioredoxins are seveal orders of magnitudes lower. Nevertheless, TRm and TRf do show very low but measurable thioltransferase activities, which may explain the thioltransferase activity of peak II.

Antibodies raised against *E. coli* glutaredoxin have permitted

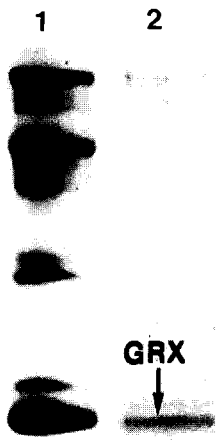

Fig. 3. SDS Polyacrylamide gel electrophoresis of spinach glutaredoxin. Lane 1: Marker proteins from top to bottom: bovine serum albumin, 65 kDa; ovalbumin, 45 kDa; chymotrypsinogen, 25 kDa; cytochrome *c*, 12.5 kDa. Lane 2: Spinach glutaredoxin.

to investigate the immunological relations of all thioltransferase activities observed in spinach leaves by ELISA assays (Table 3). In comparison with the native glutaredoxin of *E. coli* the thioltransferase of peak III shows a remarkable crossreactivity indicating common structural features. Peak I was not recognized by the antibodies but with peak II a significant signal was obtained. The crossreactivity of peak II explains the positive signal observed with the chloroplast extract. Surprisingly, in contrast to TRm and *E. coli* thioredoxin, TRf of spinach chloroplast appears immunolgically related to *E. coli* glutaredoxin. Thus the low thioltransferase activity of peak II may be due to TRm and TRf whereas the positive immunological signal of the chloroplast extract is due to only TRf. No crossreactivity of the antiserum with a protein extract of isolated mitochondria of spinach leaves has been observed, indicating that the peak III thioltransferase resides in the cytosol of spinach leaf cells.

Discussion

Glutaredoxin has long been known as a redox active protein in viruses and phages, bacteria, and animal cells [3]. The presence of glutaredoxin in plants was only recently demonstrated by Minakuchi et al. who found glutaredoxin in rice seeds but not in leaves [4]. In this study several lines of evidences have been obtained to proof that plant leaves contain glutaredoxin.

The glutaredoxin specific thioltransferase activity was used to search for glutaredoxin in spinach leaf extracts. Surprisingly, in the homogenate of spinach leaves glutaredoxin activity could not be detected. It was of advantage to avoid ammonium sulfate precipitation steps before performing a DEAE-cellulose anion exchange chromatography. With this chromatography three different thioltransferase activities: peak I, II and III (Fig. 1a) were observed. Peaks I and II were also recovered in a protein extract obtained from isolated spinach chloroplasts which indicates their chloroplastic origin (Fig. 2). In comparison to peak III, peak I and II comprise together only 10% of the total thioltransferase activity in the spinach leaf.

The major transhydrogenase activity (peak III) was enriched by size exclusion chromatography and homogeneous enzyme was obtained by high performance ion exchange chromatography. SDS-polyacrylamide electrophoresis (Fig. 3) indicated a molecular mass of 12 kDa which is within the typical range of the known glutaredoxins from other organisms [3]. The measured specific activity of this spinach thioltransferase is comparable to the specific activity of *E.coli* glutaredoxin (Table 2). Wells et al. found that *E. coli* glutaredoxin possesses a significant dehydroascorbate reductase activity [12]. Such an activity could not be observed with the isolated spinach protein, in accordance with the fact that rice seed glutaredoxin failed to show this activity, too. It may be concluded that plant glutaredoxins are not acting as dehydroascorbate reductase. Spinach leaf glutaredoxin and *E. coli* glutaredoxin have in common that their thioltransferase activity is heat stable (data not shown) and that they do not activate NADP-malate dehydrogenase and fructose-1,6-bisphosphatase (Figs. 1 and 2). The relationship of the spinach thioltransferase to *E. coli* glutaredoxin is demonstrated by its crossreactivity with antibodies raised against *E. coli* glutaredoxin (Table 3). Thus, the thioltransferase activity of peak III is definetely due to spinach glutaredoxin which is localized in the cytosol because it was not observed in extracts of isolated chloroplasts (Fig. 2) and isolated spinach leaf mitochondria (Table 3).

Peak II contained the two thioredoxins TRm and TRf of spinach chloroplasts (Figs. 1 and 2). Isolated TRm and TRf showed minor thioltransferase activities (Table 2) and TRf was recognized by the antibodies raised against *E. coli* glutaredoxin (Table 3), which indicates that the thioltransferase activity in peak II is probably due to these two thioredoxins. In a recent publication we have shown that soybean chloroplast dehydroascorbate reductase is identical with trypsin inhibitor [6].

Table 1
Purificaton of glutaredoxin from spinach leaves

	Total protein (mg)	Specific activity (μmol·min^{-1}·mg^{-1})	Purification
Spinach leaf homogenate	50	n.d.	
DEAE cellulose eluate	12	0.13	1
Chromatograpy on Superdex 75	3.4	0.33	2.5
Chromatography on Mono Q	0.1	8.9	6.8
Rechromatography on Mono Q	0.02	10	77

n.d., not detectable.

Table 2
Comparison of the specific thioltransferase activities of different redox proteins

	Specific activity (μmol·min^{-1}·mg^{-1})
E. coli glutaredoxin	8.9
E. coli thioredoxin	0.12
Spinach glutaredoxin	10.0
Spinach thioredoxin m	0.0038
Spinach thioredoxin f	0.0045
Soybean trypsin inhibitor	0.0013

Table 3
Immunological crossreactivity of different proteins and protein extracts with antibodies raised against *E. coli* glutaredoxin

	Protein content per Elisa assay (μg)	Absorbance at 410 nm
Control with bovine serum albumin	500	0.096
E. coli glutaredoxin	10	0.835
Spinach leaf extract	600	0.557
Spinach chloroplast extract	700	0.397
Spinach mitochondria extract	680	0.102
DEAE cellulose eluate peak I	90	0.110
DEAE cellulose eluate peak II	89	0.447
DEAE cellulose eluate peak III	90	0.634
Spinach thioredoxin m	10	0.123
Spinach thioredoxin f	10	0.563
E. coli thioredoxin	10	0.108
Soybean trypsin inhibitor	50	0.112

For experimental details see section 2.

Isolated trypsin inhibitor of soybean possesses a low but measurable thioltransferase activity (Table 2) which may indicate that the thioltransferase activity in peak I as to be the trypsin inhibitor of spinach. Further experiments are necesssary to substantiate this interpretation. In summary, it has been shown for the first time that plant leaves contain glutaredoxin, which is localized in the cytosol. About the physiological functions of glutaredoxin can only be speculated for the moment but it seems to be very probable that it is involved in the cytosolic ribonucleotide reduction of plant cells [13].

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