Separation of the complete thioredoxin pattern of soybean leaves (*Glycine max*) by high-performance anion-exchange chromatography on Mono Q

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ABSTRACT

Chromatographic studies are essential for assessing the physiological roles of the multiple thioredoxins (TRs) found in plants. Green leaves of the soybean (*Glycine max*) were shown to contain six different thioredoxins, which could be separated by anion-exchange chromatography. Three different thioredoxin activities were obtained by DEAE-cellulose chromatography (TRI, TRII and TRIII), which were further resolved by high-performance anion-exchange chromatography on a Mono Q column into TRIa, TRIb, TRIIa, TRIIb and TRIIc; TRIII could not be subdivided into additional thioredoxin species. In isolated chloroplasts of soybean leaves only TRIa and TRIb have been detected.

INTRODUCTION

Thioredoxins are ubiquitous, heat- and acid-stable proteins composed of about 110 amino acids. Their active centre contains a tetrapeptide, –Cys–Gly–Pro–Cys–, which enables the proteins to transfer reducing equivalents through reversible cysteine–cystine changes in a wide variety of biochemical reactions [1]. In plants, thioredoxins participate as protein disulphide reductase in he light–dark regulation of carbon dioxide fixation. For example, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase and ribulose 5-phosphate kinase are activated in the light via reductive cleavage of regulatory disulphide bridges by reduced thioredoxin [2]. On the other hand, it has been demonstrated recently that thioredoxins can be inactivated by sulphitolysis of their own disulphide bond [3]. Based on this observation, a biochemical hypothesis of sulphur dioxide toxicity in plants has been developed [4]. All these properties make the analysis of plant thioredoxins a continuing and challenging task.

Since the discovery of multiple thioredoxins in spinach [5–7], it has been assumed that only the chloroplasts of a plant cell contain thioredoxins, in particular as the cytosolic thioredoxins originally described in spinach [6] were later recognized as artefacts [8]. Thus, all the many thioredoxins fractionated from total leaf extracts of other plants (*e.g.*, six proteins in maize [8] have usually been associated with chloroplasts. However, Langlotz *et al.* [9] described clearly non-chloroplastic thioredoxins in green algae (*Scenedesmus obliquus*), and Bodenstein-Lang *et al.* [10] also found thioredoxins in mammalian and plant mitochondria.

A knowledge of the complete thioredoxin pattern of an organism and of the intracellular distribution of these proteins is obviously required in order to define the specifities and numerous physiological functions of thioredoxins in plant cells. Here we describe a systematic analysis of the thioredoxins present in soybean leaves by high-performance anion-exchange chromatography [fast protein liquid chromatography (FPLC)].

EXPERIMENTAL

All chemicals and reagents were of the highest available purity and were obtained from Merck,

Biomol or Serva. Standard chromatographic steps were carried out at 5–10°C, using a Uvicord SII (Pharmacia–LKB) UV detector operating at 280 nm. The FPLC system used (Pharmacia–LKB) was equipped with a UV 1 optical unit and was operated at ambient temperature. Soybean (Maple Arrow variety) plants were grown in a greenhouse under standard conditions.

Thioredoxin assay

The assay is based on thioredoxin-dependent activation of NADP–malate dehydrogenase (EC 1.1.1.82) of spinach. The assay conditions and enzyme preparation have been described in detail previously [9,11]. A Uvikon 930 spectrophotometer (Kontron) was used in the enzyme assays.

Preparation of thioredoxins from soybean leaves

Unless noted otherwise, all steps were carried out at 4°C. About 100 g of leaves from 8–12 week-old plants were extracted with 250 ml of extraction buffer (100 mM Tris–HCl–1 mM EDTA, pH 7.5) in a Waring blender and the homogenate was filtered through three layers of Miracloth. Heat precipitation (70°C, 3 min) denatured or inactivated endogenous enzymes which could react with thioredoxin. Precipitated proteins were removed by centrifugation (20 min at 38 700 g). Ammonium sulphate (90% saturation) was then added to the supernatant to precipitate the thioredoxins, which were collected by centrifugation (20 min at 38 700 g). The pellet was redissolved in 20 mM ammonium acetate buffer–2 mM EDTA (pH 8.6) and dialysed against 2 × 3 l of the same buffer.

DEAE-Cellulose chromatography. A DEAE-cellulose column (Whatman DE-52; 8 × 2.8 cm I.D.) equilibrated with 20 mM ammonium acetate buffer–2 mM EDTA (pH 8.6) separated three different thioredoxin activities, TRI, TRII and TRIII. TRI was found in the void volume, whereas TRII and TRIII were resolved in a linear salt gradient of 0–300 mM NaCl in 400 ml of column buffer (flow-rate, 50 ml/h). Each thioredoxin fraction was further purified separately by the following chromatographic steps.

CM-Cellulose chromatography. A CM-cellulose column (Whatman CM-52; 5 × 2.8 cm I.D.) was equilibrated with 10 mM sodium acetate buffer (pH 4.6). Bound thioredoxins were eluted in a linear salt gradient of 50–250 mM NaCl in 400 ml of column buffer (flow-rate, 30 ml/h). TRI, TRII and TRIII all eluted at 200 mM NaCl in independent runs. Active fractions were collected, neutralized with 1 M NaOH and concentrated by ultrafiltration on an Amicon YM 5 membrane.

Gel filtration. The individual thioredoxin pools (TRI, TRII and TRIII) were chromatographed on a Sephadex G-50 column (140 × 1.5 cm I.D.) equilibrated with 50 mM Tris–HCl buffer–100 mM NaCl (pH 7.5) at a flow-rate of 10 ml/h. Active fractions were collected and dialysed against 10 l of 20 mM Tris–HCl buffer (pH 8.3) to prepare the different thioredoxins for the final chromatographic step.

Mono Q chromatography. FPLC on a Mono Q HR 5/5 column equilibrated with 20 mM Tris–HCl buffer (pH 8.3) was performed in a standard gradient of 0–200 mM NaCl in 20 ml of column buffer at a flow-rate of 0.5 ml/min and room temperature. Fractions of 0.5 ml were collected.

Thioredoxins in extracts of soybean chloroplasts

Isolation of chloroplasts from soybean leaves was done by analogy with the method of Mourioux and Douce [12]. The pelleted chloroplasts were suspended in Mono Q column buffer, disrupted by freezing at −70°C. The lysate was heat treated (3 min, 70°C) and centrifuged for 15 min at 38 700 g. The supernatant was dialysed against 5 l of 20 mM Tris–HCl buffer (pH 8.3) and analysed by FPLC on a Mono Q column as described above.

RESULTS AND DISCUSSION

Thioredoxins of soybean leaves

Plants possess a complex thioredoxin system with up to six different isoproteins; in contrast, non-photosynthetic organisms contain only one or two thioredoxins [1,2]. All these proteins exhibit a molecular weight of about 12 000 and isoelectric points between 4 and 6. Although the heat and acid stability of the thioredoxins can be exploited in their purification, steps which also remove target enzymes, thus faciliating thioredoxin assays, further purification and characterization of the individual thioredoxins are difficult owing to their close similarity. Chromatography of the heat-stable soybean leaf proteins on DEAE-cellulose resulted in the separation of three different thioredoxin acitivities, which

were numbered by their sequence of elution (Fig. 1). (We advocate that the functional thioredoxin classification f or m [6–8,13] is not used as long as the cellular functions of proteins are unknown). TRI remained unbound and was found in the void volume, whereas TRII and TRIII eluted at 70 and 200 mM NaCl, respectively, in a linear salt gradient. A DEAE-cellulose column has also been used successfully in analysing thioredoxins from spinach leaves [6], barley leaves [13], wheat flour [14] and soybean seeds [15]. It can be regarded as the most general initial separation step for thioredoxin fractionation, but not sufficient, however, to resolve all isothioredoxins reliably.

Before taking advantage of the high resolving power of FPLC on Mono Q, each individual thioredoxin separated by DEAE-cellulose chromatography had to be further purified. This could be achieved by conventional CM-cellulose chromatography followed by a Sephadex G-50 gel filtration as described under Experimental. No differences between the chromatographic behaviours of TRI,

TRII and TRIII were observed. In separate runs all thioredoxins eluted at 200 mM NaCl in a linear salt gradient from CM-cellulose and the gel filtration indicated a molecular weight of about 12 000 for all of them (not shown).

The number and pattern of thioredoxins in soybean leaves could be extended and completed by FPLC on Mono Q (Figs. 2 and 3). The elution characteristics are summarized in Table I. In these experiments, the TRI and TRII fractions were subdivided into two (TRIa and b) and three (TRIIa, b and c) protein species, respectively. TRIII eluted as a single peak without evidence of additional TRIII species (Fig. 3). The quality of separation decreased substantially at other pH values (e.g., pH 7.5 or 9.0) and it turned out that less than 10 mg of total protein had to be applied to achieve complete separation of the individual thioredoxin species. Virtually the same chromatographic profiles were obtained when the preparation of leaf thioredoxins was carried out in the presence of protease inhibitors [0.5 mM phenylmethylsulphonyl fluoride (PMSF), 2
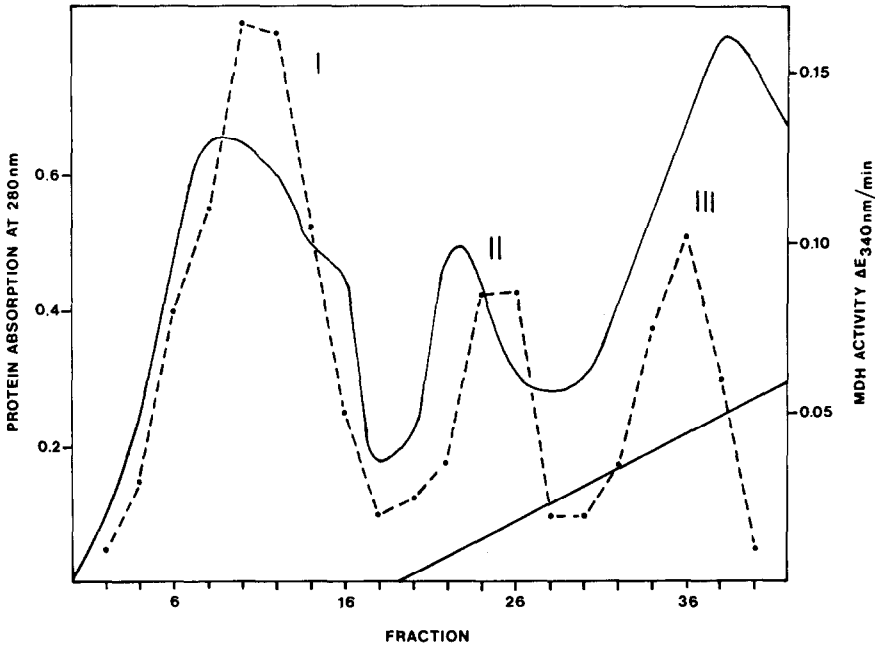

Fig. 1. DEAE-cellulose chromatography of thioredoxins in a heat-stable soybean leaf extract. A linear salt gradient (straight line) of 0–300 mM NaCl was applied within 400 ml of column buffer (20 mM ammonium acetate–2 mM EDTA, pH 8.6). Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.
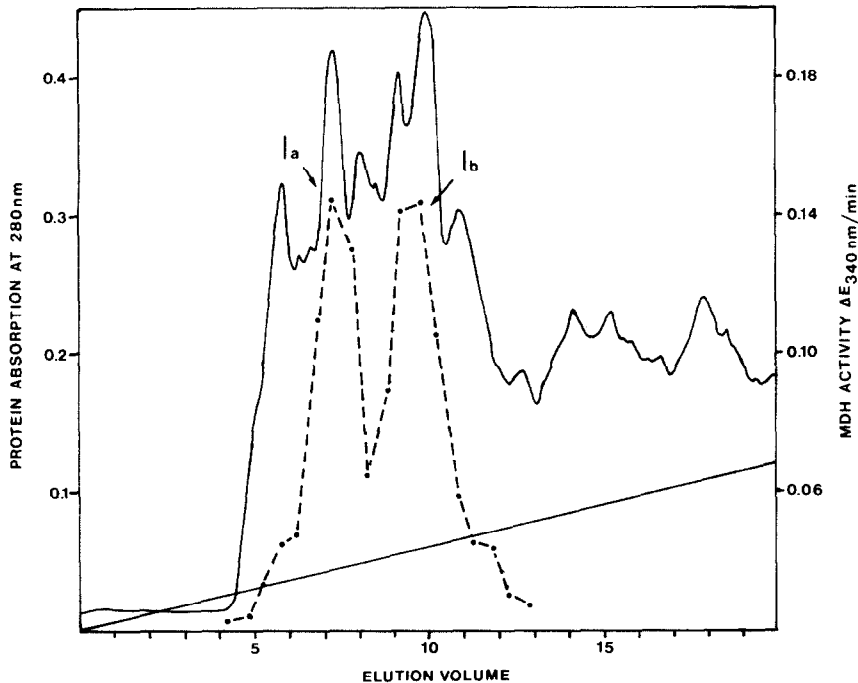
Fig. 2. Subdivision of TRI by Mono Q FPLC. A linear gradient (straight line) of 0–200 mM NaCl in 20 ml of 20 mM Tris–HCl column buffer (pH 8.3) was used for the separation. Solid line, protein absorption at 280 nm. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent increase of NADP–malate dehydrogenase (MDH) activity.

mM aminocaproic acid and 2 mM benzamidine–HCl], minimizing the risk that any of the above thioredoxins arise by artefactual, proteolytic degradation. Subsequently, each soybean leaf thioredoxin (TRIa and b, TRIIa, b and c and TRIII) was further analysed by Mono S FPLC. No indication of any additional thioredoxin species was observed in these cation-exchange analyses, in which every thioredoxin was chromatographed separately under identical conditions [50 mM sodium acetate buffer (pH 4.0); data not shown].

The present results indicate a total of six thioredoxins in green soybean leaves. A variety of other chromatographic techniques have been applied to the problem of resolving these six isoproteins, including chromatography on Blue Sepharose, hydroxyapatite and Fractogel TSK DEAE-650 S. None of these materials reached the quality of protein fractionation given by FPLC, nor could any further protein fraction be resolved (unpublished data). It therefore appears that FPLC is currently the superior, and probably the only, method for

analysing thioredoxin patterns in plants. Other workers have also described six different thioredoxins in maize by the combined use of Mono Q and Mono S FPLC [8]. However, we recommend that thioredoxin patterns, previously described in higher plants without using FPLC techniques, should certainly be reinvestigated for their completeness.

Thioredoxins in isolated soybean cell organelles

Thioredoxins in plants have mostly been assigned to chloroplasts [5–7]. Non-chloroplastic thioredoxins have not been considered, although a likely cytosolic function of thioredoxin, ribonucleotide reduction, has been demonstrated in soybean cells [16]. A knowledge of the complete thioredoxin pattern in soybean leaves should now allow the individual cellular species to be classified. Two thioredoxins have been observed in isolated soybean chloroplasts (Fig. 4). The chromatographic behaviours of these two chloroplastic thioredoxin were identical with the behaviour of TRIa and TRIb in the standard Mono Q chromatography, described
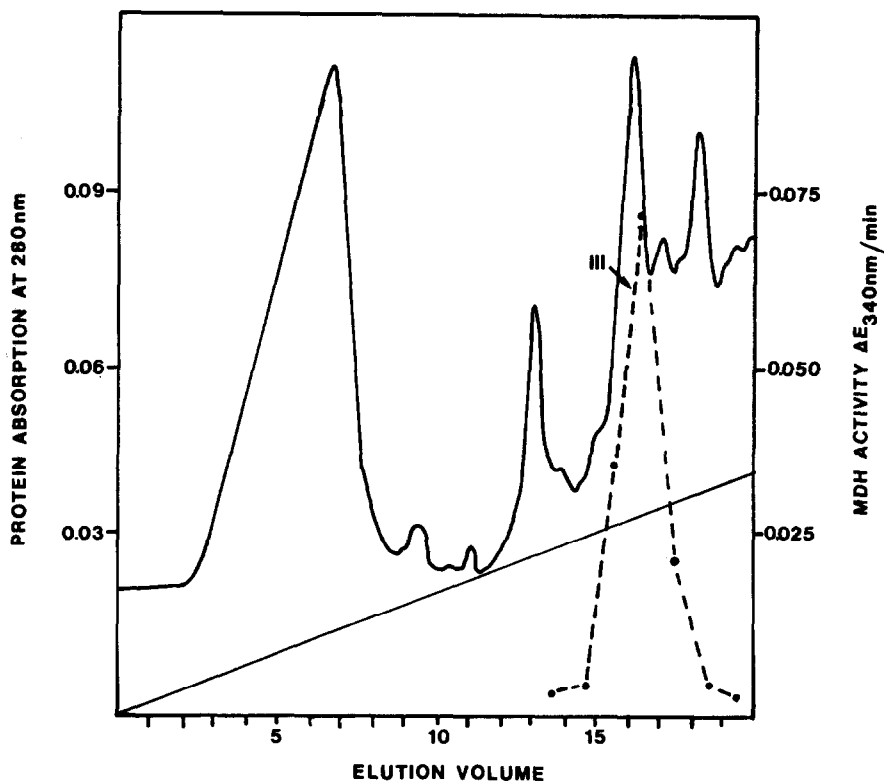
Fig. 3. Analysis of TRIII by Mono Q FPLC after preceding purification by DEAE-cellose and Sephadex G-50 gel filtration chromatography. A linear gradient (straight line) of 0–200 mM NaCl in 20 ml of Tris–HCl column buffer (pH 8.3) was applied. Solid line, protein absorption at 280 nm. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent increase of NADP-malate dehydrogenase (MDH) activity.

above. This allows the specification of TRIa and TRIb as the chloroplastic species in a soybean leaf cell. Spinach chloropasts also possess two thiore-

TABLE I

ANION-EXCHANGE CHROMATOGRAPHY OF SOYBEAN LEAF THIOREDOXINS ON DEAE-CELLULOSE AND BY FPLC ON MONO Q

DEAE-cellulose		Mono Q	
TR fraction	NaCl molarity for elution	TR fraction	NaCl molarity for elution
TRI	(Unbound)	TRIa	75 mM
		TRIb	95 mM
TRII	70 mM	TRIIa	85 mM
		TRIIb	115–120 mM
		TRIIc	135–140 mM
TRIII	200 mM	TRIII	170 mM

doxins [6,17,18]; chloroplasts of other plants have not been analysed for thioredoxin so far.

A preliminary analysis of mitochondria from soybean leaves indicates that TRIIb and TRIIc are located in these cell organelles [19]; one mitochondrial thioredoxin had been observed in potato tuber mitochondria [10]. The intracellular localization of TRIIc and TRIII remains to be established. We expect that at least one of these thioredoxins is of cytosolic origin, to function as the essential hydrogen donor for soybean ribonucleotide reduction [16] as it does in green algae [20].

Interestingly, soybean leaves and seeds contain the same number of thioredoxins, which are very similar but not identical in their chromatographic behaviour. Changes in the thioredoxin pattern should indeed be expected during morphogenesis from resting and germinating seeds to light-regulated green plants. Further, thioredoxin profiles
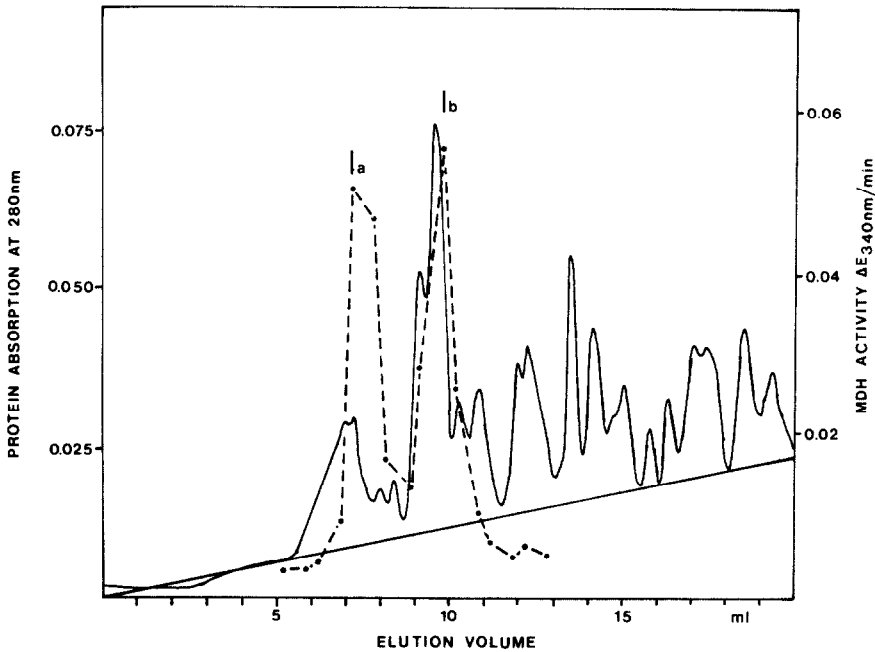
Fig. 4. Mono Q chromatography of a heat-treated protein extract from soybean chloroplasts. Only proteins separated within the standard linear salt gradient (straight line) are illustrated. Thioredoxin activity (dashed line) was observed by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.
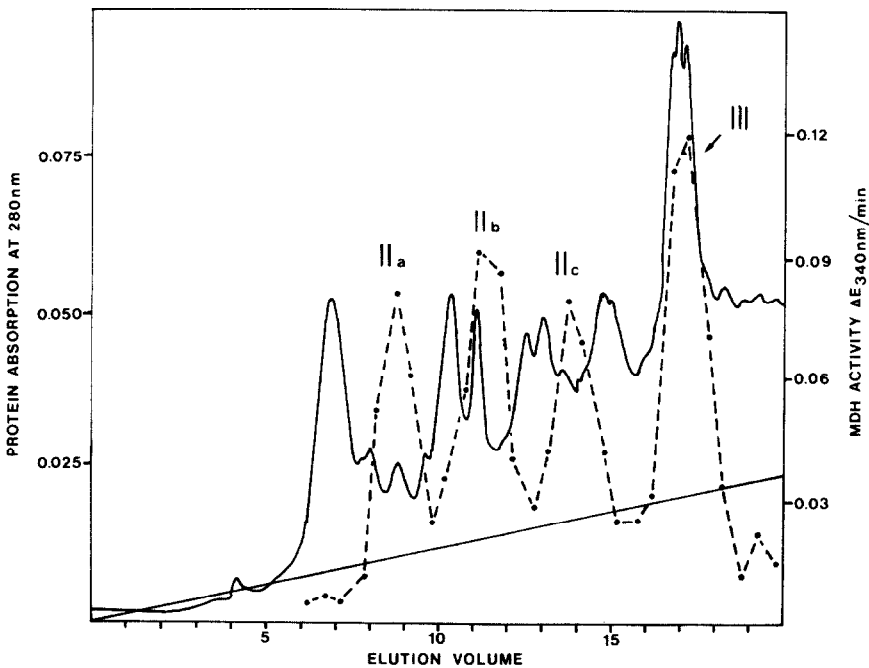
Fig. 5. Separation of a TRII–TRIII mixture by Mono Q FPLC. The TRII–TRIII mixture was eluted together from DEAE-cellulose at a 300 mM NaCl concentration and further enriched by CM-cellulose and gel filtration chromatography. A linear salt gradient (straight line) of 0–200 mM NaCl in 20 ml of 20 mM Tris–HCl column buffer (pH 8.3) was used to resolve the mixture. Thioredoxin activity (dashed line) was measured by the thioredoxin-dependent activation of NADP–malate dehydrogenase (MDH). Solid line, protein absorption at 280 nm.

may be subject to environmental influences, *e.g.* sulphur dioxide cause sulphitolysis and inhibition [3,4]. Studies of the thioredoxin profiles at different stages of development or under different environmental conditions demand a facile and reproducible analytical scheme. To this end, DEAE-cellulose chromatography of soybean thioredoxins was further simplified by first isolating TRI, which is present in the void volume, and then TRII and TRIII together by a one-step elution with 300 m*M* NaCl. The TRII–TRIII pool has been similarly prepared for Mono Q FPLC by a CM-cellulose and gel filtration step as already described for the individual TRI, TRII and TRIII fractions (see Experimental). TRIIa, TRIIb, TRIIc and TRIII could all be separated by one standard Mono Q FPLC (Fig. 5). The two chloroplastic thioredoxins have to be analysed separately in this scheme because TRIb and TRIIa cannot be resolved reproducibly enough by one standard Mono Q run (see Table I). Optimum conditions of Mono Q chromatography are currently being investigated to achieve the analysis of all soybean thioredoxins in one run with a minimum of preceding purification steps.

ACKNOWLEDGEMENTS

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