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# EVIDENCE FOR THE EXISTENCE OF SEVERAL ENZYME-SPECIFIC THIOREDOXINS IN PLANTS

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## 1. Introduction

Thioredoxin, a small molecular weight protein, functioning as hydrogen carrier in DNA synthesis has recently been identified to be an indispensable factor in the light activation of certain regulatory enzymes of plant chloroplasts [1]. In the presence of thioredoxin and ferredoxin-thioredoxin reductase, photochemically-reduced ferredoxin is able to activate enzymes like NADP malate dehydrogenase [2] and fructose 1,6-bisphosphatase (FBPase) [3], both enzymes restricted to the chloroplasts [3,4]. In vitro reduced ferredoxin and ferredoxin-thioredoxin reductase can be replaced by the non-physiological sulfhydryl reagent dithiothreitol (DTT), thus eliminating the need for light, but not the need for thioredoxin [3,5,6]. During our efforts to isolate and purify thioredoxin from different plant species (spinach, sorghum and French beans) and plant materials (leaves and roots) we have realized that there are several forms of thioredoxin which are specific for the activation of either NADP malate dehydrogenase or FBPase. Similar findings have recently been reported by [7].

We describe here a simple method for the separation of different forms of thioredoxin which is applicable to various plant materials. In addition our results indicate that the different forms of thioredoxin are enzyme specific. However a given form of thioredoxin isolated from one plant species can activate the respective enzyme isolated from another plant species and vice versa.

## 2. Methods

### 2.1. *Plant material*

French beans (*Phaseolus vulgaris* L. var. Contender) and Sorghum (*Sorghum dochna* F. var. INRA 450) were cultivated as in [8] and the harvested material stored lyophilized; spinach (*Spinacea oleracea*) was purchased on the local market and used fresh.

### 2.2. *Isolation of thioredoxins*

Lyophilized material, 3 g (Bean and Sorghum) or 30 g fresh material (Spinach) were blended in 100 ml 100 mM $KP_i$ buffer (pH 7.0) containing 0.1% 2-mercaptoethanol in a Waring blender. The homogenate was filtered through Kleenex tissues and centrifuged for 20 min at 30 000 × *g*. In leaf thioredoxin preparations the supernatant was heated in a boiling water bath until heavy precipitation occurs (to ~85°C) and then immediately cooled in ice. This thermal treatment is carried out in order to eliminate NADP malate dehydrogenase and FBPase from the protein solution. The precipitate is removed by centrifugation for 5 min at 30 000 × *g*. To the clean supernatant $(NH_4)_2SO_4$ is added and proteins precipitating between 40% and 80% saturation are collected by centrifugation for 5 min at 30 000 × *g*. The precipitated proteins are redissolved in a minimal amount of blending buffer and applied to a Sephadex G-50 column ($V_0$ = 35 ml) equilibrated in 10 mM Tris–HCl (pH 7.5). The column is eluted by gravity flow and fractions of 2 ml are collected. Those fractions containing thioredoxins were pooled and passed over

a DEAE-cellulose (Cellex-Biorad) column 1 × 10 cm equilibrated in 10 mM Tris–HCl (pH 7.5). The thioredoxins are eluted from the DEAE cellulose with a 0–300 mM NaCl linear gradient in the equilibration buffer.

### 2.3. *Isolation of enzymes*

FBPase was purified from spinach leaves as in [3]. NADP malate dehydrogenase was isolated from spinach or sorghum leaves by the following method: 100 g leaf material was blended filtered and centrifuged as for the thioredoxin isolation except that the thermal treatment was omitted. The clarified homogenate was fractionated by $(NH_4)_2SO_4$. Proteins precipitating between 40 and 80% $(NH_4)_2SO_4$ were collected by centrifugation, dissolved in a minimal volume of blending buffer and then stored frozen. For use a small aliquot of the concentrated protein solution was separated from the thioredoxin on a Sephadex G-50 column ($V_0$ = 35 ml) equilibrated in 200 mM $KP_i$ buffer (pH 7.0). The first protein fractions emerging from the column were free of thioredoxin and used as enzyme solution.

### 2.4. *Determination of enzyme activities*

The enzymes were activated by preincubating them for the indicated times with the column fractions containing the thioredoxins reduced directly with dithiothreitol. The activity of the NADP malate dehydrogenase was then determined spectrophotometrically as in [5]. The FBPase activity was followed by measuring colorimetrically the $P_i$ released [3].

## 3. Results and discussion

When isolating thioredoxin from bean leaves, following the protocol outlined above, we realized that after the gel filtration of the protein solution through Sephadex G-50 the two test enzyme systems, NADP malate dehydrogenase and FBPase are activated by different clearly separated protein fractions. Whereas the FBPase is activated by fractions corresponding to proteins of ~20 000 mol. wt, the NADP malate dehydrogenase is activated by fractions corresponding to proteins of ~12 000 mol. wt (fig.1A). Identical separation patterns have been found consistently whether using preparations

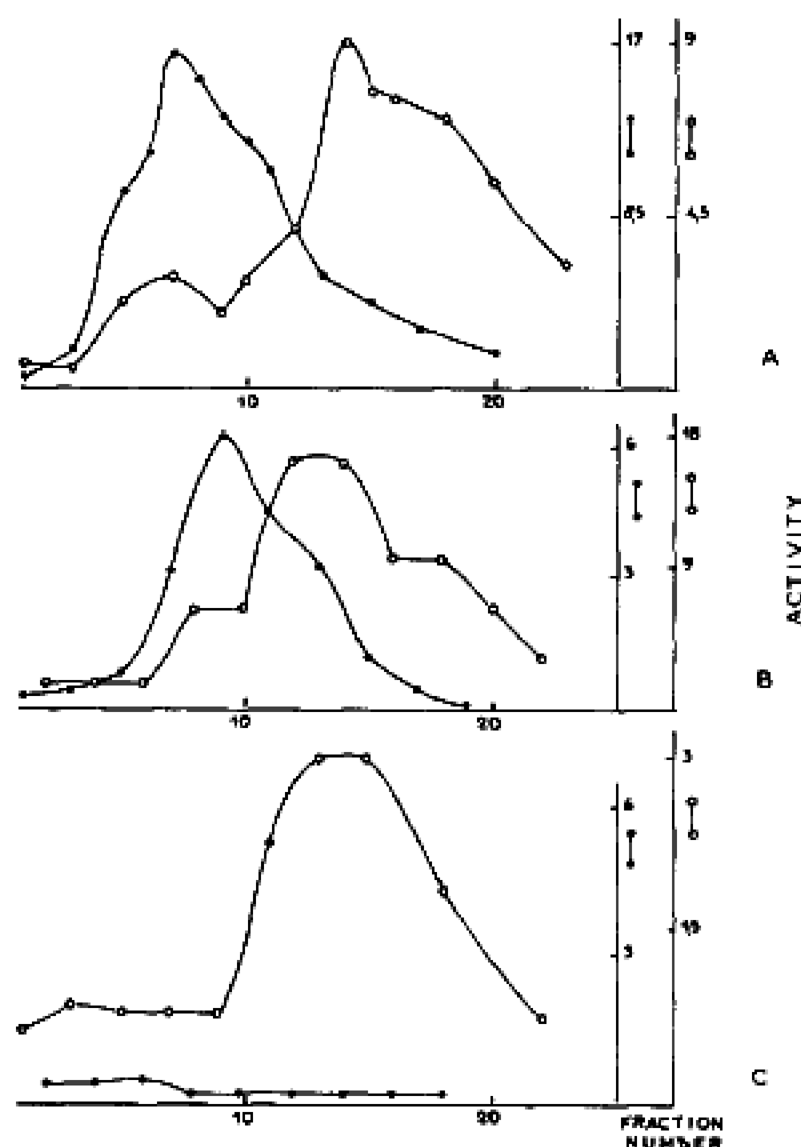


Fig.1. Sephadex G-50 gel filtration: elution profiles for thioredoxin activity from bean leaves (A), sorghum leaves (B) and bean roots (C), tested with NADP malate dehydrogenase (○——○) and FBPase (●——●). All incubations and reactions were done at 30°C and the activities are expressed as nmol NADPH oxydized/min for NADP malate dehydrogenase and as nmol $P_i$ released/min for the FBPase.

(A) NADP malate dehydrogenase: 200 µl column fraction were incubated with 150 µg partially purified enzyme and 10 mM DTT in a final volume of 310 µl for 90 min. After that time 100 µl were tested as in [5]. FBPase: 200 µl column fraction were incubated with 3.7 µg enzyme in 450 µl containing (in µmol): Tris–HCl (pH 7.9), 50; EDTA-Na, 0.05; $MgSO_4$, 0.5, and DTT, 2.5. After 15 min the reaction was started by adding 50 µl 60 mM fructose 1,6-bisphosphatase and stopped after 15 min by the addition of 2 ml of a mixture of reagents used for $P_i$ analysis.

(B) NADP malate dehydrogenase: 100 µl column fraction were incubated with 75 µg enzyme for 140 min in a final volume of 160 µl and 50 µl used to test the enzyme activity. The other conditions were as for A. FBPase: 250 µl column fraction were used, the other conditions were as for A.

(C) NADP malate dehydrogenase: 100 µl column fraction were incubated with 150 µg enzyme for 95 min in a total of 210 µl and 100 µl tested for activity. The other conditions were as for A. FBPase: conditions as for A.

from bean leaves, sorghum leaves (fig.1B) or spinach leaves (data not shown). The higher molecular weight thioredoxin coming off the column first and activating mainly the FBPase, corresponds to thioredoxin *f*, the smaller molecular weight thioredoxin, activating the NADP malate dehydrogenase corresponds to thioredoxin *m*, this nomenclature being in line with that in [7] where similar observations were made independently.

It has been reported that spinach roots also contain thioredoxin that is able to activate FBPase [9] using DTT to reduce thioredoxin. In an attempt to compare the root thioredoxin with the thioredoxins found in photosynthesizing green tissue we isolated thioredoxin from bean and spinach roots. After the gel filtration on Sephadex G-50 the root material yields only one thioredoxin activity peak using the NADP malate dehydrogenase (fig.1C). When the fractions were tested with FBPase there was no thioredoxin activity peak detected under the experimental conditions used a result which is in contrast to the reported activation of FBPase with root thioredoxin [9]. The activity peak with NADP malate dehydrogenase corresponds to proteins of ~12 000 mol. wt. On a protein basis the activation by this thioredoxin is 5–10-times weaker than with leaf thioredoxin at the same level of purification. This smaller specific activity may be the reason for the FBPase not to be activated by this protein under our experimental conditions, NADP malate dehydrogenase being a more sensitive test system. The thioredoxin isolated from the roots resembles somehow the thioredoxin *c* reported [7] which is a non-chloroplast thioredoxin and only partly activates FBPase and NADP malate dehydrogenase.

From the results presented so far it can be concluded that leaves contain at least two types of thioredoxins that distinguish themselves by their molecular weight and by their specificity towards enzymes. Roots contain only one, a low molecular weight form of thioredoxin, which under our conditions is able to partly activate NADP malate dehydrogenase only.

A further separation of the different forms of thioredoxins has been attempted by ion-exchange chromatography. The Sephadex G-50 fractions containing thioredoxin *f* and thioredoxin *m* were either combined or separately fractionated on a DEAE-cellulose column. The activity profile obtained for the bean thioredoxins (fig.2A) is representative for the results after ion-exchange chromatography. We find consistently one thioredoxin *f* peak when testing the column fractions with FBPase. This peak elutes at the

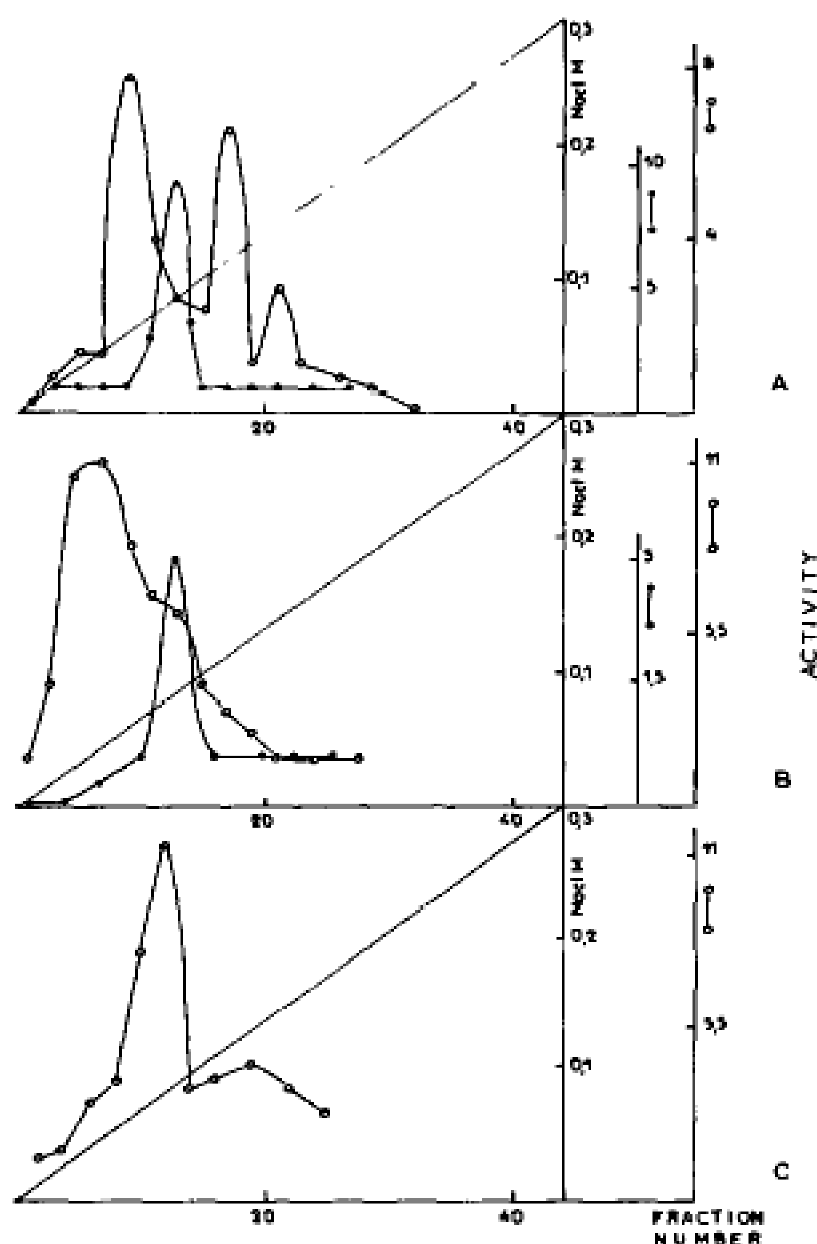


Fig.2. DEAE-cellulose column chromatography: elution profiles for thioredoxin activity from bean leaves (A), sorghum leaves (B) and bean roots (C) tested as indicated in the legend to fig.1.

(A) NADP malate dehydrogenase: conditions as for fig.1A. FBPase: 250 µl column fraction were used, the other conditions were as for fig.1A.

(B) NADP malate dehydrogenase: 100 µl column fraction were used in a final volume of 210 µl and the enzyme incubated for 150 min. The other conditions were as for fig.1A. FBPase: conditions as for fig.1B.

(C) NADP malate dehydrogenase: except for an incubation time of 150 min conditions were as for fig.1A.

same molarity of NaCl whether high molecular weight thioredoxin (thioredoxin *f*) was applied alone to the column or together with low molecular weight thioredoxin (thioredoxin *m*).

The low molecular weight form of thioredoxin, thioredoxin *m*, separated by ion-exchange chromatography always yields multiple peaks. This is the case with bean leaves (fig.2A), spinach leaves [4] or sorghum leaves (fig.2B). As can be seen in fig.2A there are two about equal activity peaks when assaying the fractions with NADP malate dehydrogenase for the presence of thioredoxin *m*. It can be clearly seen that the two thioredoxin *m* peaks as well as the thioredoxin *f* peak elute from the column at distinctly different molarities, representing therefore three proteins with differing charges. A comparison of the elution profiles of the three different plant materials used indicates, that there are always three main activity peaks, although not very well resolved in the experiment shown for sorghum (fig.2B). Better resolution was achieved in other experiments as in [10]. Furthermore for each plant material the molarity needed for the elution of a particular thioredoxin varies.

Root-thioredoxin, chromatographed on DEAE-cellulose does yield a single peak when tested for with the NADP malate dehydrogenase (fig.2C). It elutes at a molarity different from the ones of the leaf thioredoxins, which lets us assume that it is a protein different from the ones in the leaves and having another function than activating photosynthetic enzymes.

Figure 2A demonstrates further very nicely that the thioredoxins are enzyme specific when added in small amounts. However they are, as far as the studied plants are concerned, not plant specific, since throughout this investigation we have used spinach FBPase and interchangeable spinach or sorghum NADP malate dehydrogenase to test the column fractions with no difference in the results.

In conclusion we can confirm the report [7] of the existence of several enzyme specific forms of thioredoxin. In addition to the results in [7] we are able to distinguish two low molecular weight thioredoxins, both activating the NADP malate dehydrogenase equally well. The different forms of thioredoxin are found in all three plants tested whether $C_3$-type (bean, spinach) or $C_4$-type plant (sorghum). In roots there is only a low molecular weight form of thioredoxin. The specific leaf thioredoxins activate enzymes from different plants interchangeably.

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**References**

[1] Buchanan, B. B., Wolosiuk, R. A. and Schurmann, P. (1978) Trends Biochem. Sci. in press.
[2] Wolosiuk, R. A., Buchanan, B. B. and Crawford, N. A. (1977) FEBS Lett. 81, 253–258.
[3] Schurmann, P. and Wolosiuk, R. A. (1978) Biochim. Biophys. Acta 522, 130–138.
[4] Jacquot, J-P., Vidal, J. and Gadal, P. (1977) Planta 137, 89–90.
[5] Jacquot, J-P., Vidal, J. and Gadal, P. (1976) FEBS Lett. 71, 223–227.
[6] Kagawa, T. and Hatch, M. D. (1977) Arch. Biochem. Biophys. 184, 290–297.
[7] Buchanan, B. B., Wolosiuk, R. A., Crawford, N. A. and Yee, B. C. (1978) Plant Physiol. 61, 38 S.
[8] Vidal, J., Cavalié, G. and Gadal, P. (1976) Plant Sci. Lett. 7, 265–270.
[9] Buchanan, B. B. and Wolosiuk, R. A. (1976) Nature 264, 669–670.
[10] Vidal, J., Jacquot, J-P., Membre, M. and Gadal, P. (1978) Plant Sci. Lett. 11, 305–310.