IDENTIFICATION OF A PROTEIN FACTOR INVOLVED IN DITHIOTHREITOL ACTIVATION OF NADP MALATE DEHYDROGENASE FROM FRENCH BEAN LEAVES

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1. Introduction

NADP malate dehydrogenase catalyses the reduction of oxaloacetate to malate in leaves of C_3 and C_4 plants; its activity is restricted to the chloroplast [1,2]. However, in the extracts the enzyme can only be detected after incubation in the presence of reducing compounds such as dithiothreitol or lipoic acid [2,3].

In the present paper, we show that an activating factor, easily separable from the enzyme is also necessary for activation of NADP malate dehydrogenase from French bean leaves.

A protocol for partial purification of the factor is described. Filtration through Sephadex G-50 shows it to have a low molecular weight. Its precipitation by ammonium sulphate, its retention on DEAE cellulose and its destruction by pronase strongly suggest it to be a protein.

2. Methods

2.1. *Plant material*

The methods for cultivation and stabilization of the French bean (*Phaseolus vulgaris L.* var. *Contender*) have been described elsewhere [4].

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2.2. *Extraction and purification of the enzyme*

Extraction was carried out on lyophilized leaves with 100 mM phosphate buffer, pH 7, containing 10 mM sodium thioglycolate and 1 g of polyclar AT per g of plant material, using the protocol previously described [5].

The proteins were purified by precipitating with ammonium sulphate (80% saturation) and then filtering through Sephadex G-25 or G-50 gels equilibrated with 100 mM phosphate buffer pH 7.0. Protein assay was carried out by Lowry's method [6].

2.3. *Extraction and purification of the protein factor*

100 g of fresh plant material were homogenized in 300 ml of 100 mM phosphate buffer, pH 7, containing 10 mM mercaptoethanol and 1.5 g of polyclar AT using an 'Ultra Turrax' homogeneizer. After deaeration by bubbling with nitrogen, the extract was freed from gross cellular debris by filtering through gauze then centrifuged at 48 000 × g for 20 min. The supernatant was then transferred to a water bath at 100°C for 2 min. The coagulated proteins were removed by centrifuging; those remaining in the extract were precipitated by adding $(NH_4)_2SO_4$ to give 80% saturation and allowing sedimentation to take place for 2 h. The residue obtained after centrifuging at 5000 × g for 10 min was taken up in 25 ml 100 mM phosphate buffer, pH 7.5. Filtration through Sephadex G 50 gel equilibrated with the above buffer gave 70 ml of

effluent which was added to the top of a DEAE cellulose column (1 X 10 cm) prepared in 10 mM phosphate buffer, pH 7.5. Elution was carried out at 50 ml/h using a linear NaCl gradient of 0–0.4 M. The effluent was collected in 2.5 ml fractions and the activity of each determined.

2.4. *Determination of NADP malate dehydrogenase activity*

In extracts, the NADP malate dehydrogenase has to be activated by a dithiol compound; we used 10 mM dithiothreitol in the presence of bovine serum albumin (1 mg/ml). For the determination of activities, the reaction medium contained in a volume of 3 ml of 50 mM phosphate buffer, pH 8.0, 5 μmol oxaloacetate; 0.6 μmol NADPH and 1.5 μmol ethylenediamine tetra-acetate. Optical density measurements at 340 nm were made at 30°C using a Beckman Acta C II double beam spectrophotometer.

2.5. *Detection of the activating factor*

The activating factor in the purified extracts was detected by adding an aliquot of an enzymatic preparation obtained by filtration through Sephadex G-50 gel equilibrated with 100 mM phosphate buffer, pH 7.0, 10 mM dithiothreitol and determining the enzymatic activity as previously described.

3. Results and discussion

In the French bean leaf extracts purified by precipitation with $(NH_4)_2SO_4$ and filtration through Sephadex G-25, no NADP malate dehydrogenase activity could be detected. The latter manifested itself after addition of dithiothreitol, increased and became steady after 150 min contact at 22°C (fig.1). In the effluent from Sephadex G-50 equilibrated with 100 mM phosphate buffer, pH 7, on the other hand, the reducing agent was without effect. This result might be attributed to irreversible denaturing of the biocatalyst during filtration through this type of gel or to the elimination of a low molecular weight substance indispensable for the activation.

To decide between these two hypotheses, we did the experiment described in fig.2. A 1 ml sample concentrated by $(NH_4)_2SO_4$ precipitation was gel

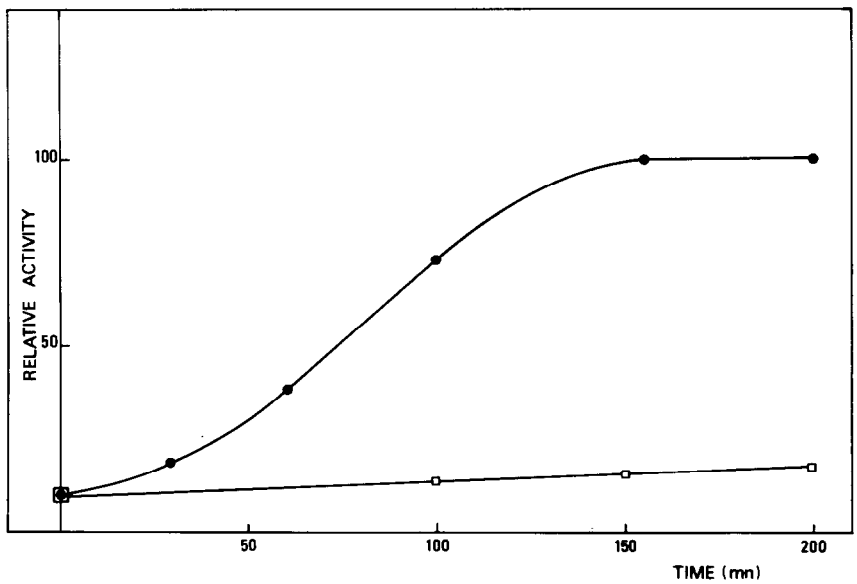

Fig.1. Time course of the activation of inactive NADP malate dehydrogenase filtered through Sephadex G-25 (•—•) or G-50 (□—□) gel. The gels were equilibrated with 100 mM phosphate buffer, pH 7.0. Activation was carried out in the same buffer at 22°C in the presence of 10 mM dithiothreitol, and above serum albumin (1mg/ml). 1 ml of this medium contained 3.75 mg protein. The determination of enzymic activity is described under 'Methods'.

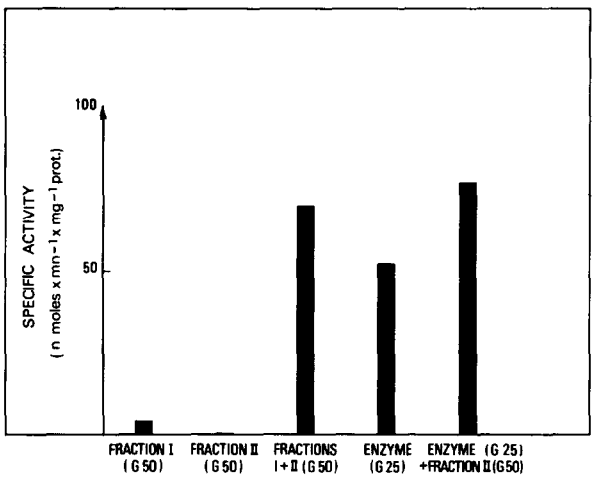

Fig.2. Comparison of specific activities of extracts obtained by filtration through Sephadex G-50 and G-25 gels. The Sephadex G-50 gel used in this experiment had a void volume (V_0) of 26 ml and an internal volume (V_i) of 42 ml. During filtration of a 1 ml sample concentrated by $(NH_4)_2SO_4$ precipitation, two fractions of the effluent were collected after the void volume; fraction I between 0 and 4.4 ml and fraction II between 8.4 and 21 ml. Activation was carried out at 22°C in 100 mM phosphate buffer pH 7.0, in the presence of bovine serum albumin (1 mg/ml) and 10 mM dithiothreitol. To 200 μl of fraction I were added 1800 μl of fraction II or the same quantity of buffer in the case of the reference samples. The enzymatic activity was determined at its maximum value.

filtrated on Sephadex G-50. Two fractions of the effluent were collected after the void volume; fraction I between 0 and 4.4 ml and fraction II between 8.4 and 21 ml. Fraction II, which possessed no enzymatic activity of its own, permitted activation by dithiothreitol of fraction I. Figure 2 also shows that in the presence of the same fraction II, an enzymatic extract obtained from filtration on Sephadex G 25 attained a markedly higher activity level. Thus, NADP malate dehydrogenase from French bean leaves requires for its activation not only dithio-

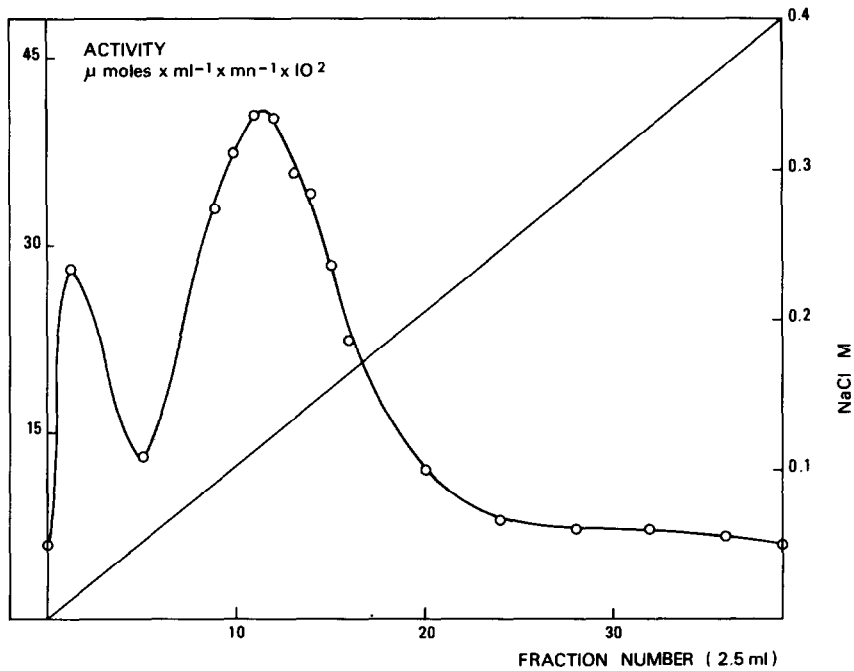

Fig.3. Elution diagram for the activating factor from a DEAE–cellulose column. The linear ionoc strength gradient was obtained by progressively mixing 50 ml of 10 mM phosphate buffer with 50 ml of the same buffer 0.4 M in NaCl. The factor was detected by adding 1 ml of Sephadex G-50 filtered enzymatic extract and 10 mM dithiothreitol, to 1 ml of each fraction and determining the enzyme activity as described previously.

Table 1
Influence of pronase on the activating factor

	Nature of test	Activity expressed as % of maximum activity
I	Enzymatic extract + dithiothreitol + buffer	17
II	Enzymatic extract + dithiothreitol + activating factor	100
III	Enzymatic extract + dithiothreitol + activating factor treated with pronase	9
IV	Enzymatic extract + dithiothreitol + activating factor heated for 1 min at 100°C	53

The inactive enzyme was obtained by filtering through Sephadex G-50 gel. To 500 μl (1.77 mg protein) of this extract were added 100 μl of dithiothreitol, 100 mM and I, 400 μl buffer; II, 400 μl activating factor solution (80 μg protein); III, 400 μl activating factor solution treated with pronase (1 mg/ml) for 20 min at 37°C. The protease was denatured by heating for one minute at 100°C and removed by centrifuging; IV, 400 μl activating factor solution heated for one minute at 100°C.

threitol but also one or several substances whose molecular weight lie between 5000 and 10 000; the latter being held back during filtration through Sephadex G-50 but not during filtration through Sephadex G-25. In addition, under our experimental conditions, their concentration in the extract purified on Sephadex G-25 was insufficient to obtain maximum enzyme activity.

Chromatography on DEAE–celluose of the fraction prepared as indicated in the Methods section gave the elution diagram shown in fig.3. Activation by dithiothreitol of the enzyme prepared using Sephadex G-50 was obtained for two zones whose maxima were at 0.01 M and 0.12 M NaCl. Only the most strongly adsorbed and highly efficacious fractions of the molecular species were examined. To confirm that the latter was a protein, we treated it with pronase. Because of the long incubation period necessary for activation of the biocatalyst and the risk of it being destroyed by the protease, the latter was denatured by heating for one minute at 100°C before adding it to the inactive enzyme. Examination of table 1 shows that this treatment resulted in complete loss of activating power. This is not due to destruction of the factor by the thermal treatment since, when heated alone to this temperature, it conserved more than half its initial efficiency.

These results reveal the existence of at least one factor which is indispensable for the activation of NADP malate dehydrogenase; they strongly suggest that the factor is a protein, for it can be precipitated by $(NH_4)_2SO_4$, retained by DEAE–cellulose and, above all, rendered inactive by incubation with pronase. The participation of protein factors in chloroplastic biocatalyst activation mechanisms has already been reported for sedoheptulose 1,7-diphosphatase [7] and ribulose diphosphate phosphatase [8] in C_3 type plants and for pyruvate-P_i-dikinase in C_4 type plants [9]. Nevertheless, the disparity in the molecular weights reported suggests that the action of these factors is relatively specific. We propose to check this point for the NADP malate dehydrogenase protein factor in the French bean. At the moment we are continuing this work with an examination of its properties, but also of those of the second compound separated by chromatography on DEAE–cellulose.

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