FERREDOXIN-SEPHAROSE AFFINITY CHROMATOGRAPHY FOR THE PURIFICATION OF ASSIMILATORY NITRITE REDUCTASE

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1. Introduction

Assimilatory nitrate reduction in higher plants and algae proceeds in two steps: the first stage is catalyzed by nitrate reductase (NAD(P)H-nitrate oxidoreductase, EC 1.6.6.2.) and yields nitrate, and the subsequent step is catalyzed by nitrite reductase (ferredoxin-nitrite oxidoreductase, EC 1.6.6.4) to yield ammonia with the use of six electrons [1,2]. Recently nitrate reductase has been obtained in a highly purified state by the use of affinity chromatography in which FAD [3], Blue dextran [4], NADH [5], and *p*-nitroaniline, respectively [6] were used as specific or general ligands coupled to Sepharose gels. To date no attempts have been made to purify nitrite reductase (NiR) by affinity chromatography, although NiR has been prepared in a highly purified form by conventional purification procedures such as ion exchange chromatography, gel filtration and preparative gel electrophoresis [7], the yields of the enzyme were, however, rather low and the procedures were time-consuming. In this communication, we describe a simple and efficient method for the purification of NiR from a higher plant by application of ferredoxin-Sepharose affinity chromatography and some properties of the purified preparation.

2. Materials and methods

1 kg of leaves and green stalks of green spinach (*Spinacea oleracea* L.) were homogenized in a Waring blendor with 2 litres of water with addition of 0.6 g of Tris to maintain the pH of the homogenate between 7.0 and 7.5. The homogenate was centrifuged in a basket centrifuge with a cotton cloth filter to remove unbroken materials. To 1 litre of the green extract, 600 g wet DEAE-cellulose were added and stirred gently for 30 min and then the ion exchanger, now green, was collected on a Büchner funnel. The enzyme was eluted from the ion exchanger with 0.05 M Tris-HCl buffer containing 0.8 M NaCl and 10 mM 2-mercaptoethanol (ME). The eluates were mixed with glycerol to give a 10% final concentration. Ammonium sulfate (243 g/litre) was added to the glycerol solution and the precipitate removed by centrifugation. The supernatant was then brought to 75% saturation with ammonium sulfate (516 g/litre). The precipitate was collected by centrifugation and dialyzed against 0.03 M Tris-HCl buffer, pH 7.5, containing 10% glycerol, and 10 mM ME (the standard buffer). The enzyme solution was applied to a DEAE-cellulose column (9.6 × 37 cm) equilibrated with standard buffer. The column was eluted with a linear NaCl concentration gradient formed with 5.5 litres of the standard buffer and 5.5 litres of the same buffer supplemented with 0.4 M NaCl. The active fractions were pooled and concentrated with a membrane filter (G-10T, Bioengineering Co., Tokyo). The concentrated solution was again fractionated with ammonium sulfate in the same manner as described for the previous step. The precipitate was dialyzed against the standard buffer. The dialyzed solution was then chromatographed on a DEAE-Sephadex A-50 column (5 × 47 cm), equilibrated with the standard buffer. The column was eluted with a linearly increasing concentration of potassium phosphate formed with

3 litres of the standard buffer and 3 litres of the same buffer containing 0.35 M potassium phosphate, pH 7.5. The enzyme fractions were pooled and concentrated with a membrane filter. 7 ml of the enzyme solution was desalted on a Sephadex G-25 column (1.8 X 30 cm), equilibrated with 5 mM potassium phosphate buffer, pH 7.5. The desalted solution (16 ml) was applied to a ferredoxin-Sepharose 4B column (1.6 X 20 cm), equilibrated with the desalting buffer. The enzyme was eluted by application of 0.4 M NaCl in the same buffer. Ferredoxin-Sepharose 4B was prepared as follows. Sepharose 4B was activated by CNBr according to the method of March et al. [8]. The CNBr-activated gel (25 ml) was suspended in 45 ml of 0.1 M sodium bicarbonate, pH 8.0, containing 42 mg of spinach ferredoxin and kept strirring for 20 h. After filtration and washing, the gel was resuspended in the bicarbonate buffer supplemented 0.1 M glycine and stirred gently for 20 h to saturate the remaining free coupling ligands. 1.2 mg of ferredoxin were fixed per ml of the Sepharose under these conditions. Spinach ferredoxin was prepared by a combination of the methods of Mayhew [9] and Buchanan and Arnon [10] and finally purified by gel filtration on Sephadex G-75. The ferredoxin obtained had an absorbance ratio at 422 nm/275 nm above 0.5. All operations were conducted at 0–5°C.

NiR activity was assayed by the dithionite-methyl viologen method [11,12]. A unit of enzyme is defined as the amount required to reduce 1 μmol nitrite per min. Protein was determined by the method of Lowry et al. [13] with bovine serum albumin as the standard. Disc electrophoresis and SDS-acrylamide gel electrophoresis were performed according to Davis [14] and Method 1 (Standard Method) of Weber et al. [15], respectively.

3. Results and discussion

The availability in quantity of a homogeneous enzyme preparation is prerequisite to structural studies and the elucidation of reaction mechanism. Work on assimilatory NiR has been much handicapped by the lack of suitable methods for obtaining purified enzyme in quantity. The purification procedure described here seems to be instrumental for this purpose. The ferredoxin-Sepharose gel was easily prepared and could be used repeatedly provided the column is washed with 0.1 M potassium phosphate buffer, pH 7.5, after each run. It was important to minimize the time taken for desalting and affinity chromatography since the enzyme is unstable under conditions where it could be adsorbed on the gel. To minimize inactivation of the enzyme, phosphate buffer, glycerol and ME were placed beforehand into the tubes where the active fractions would be collected to give final concentrations of 0.05 M buffer, 15% glycerol and 10 mM ME. NiR has been shown to be completely stable in the presence of glycerol and ME [11,12].

Table 1 summarizes the purification procedure for NiR from 22 kg of green spinach tissues. The elution profile of the enzyme from affinity chromato-

Table 1
Purification of nitrite reductase from spinach green tissues

Purification step	Protein (g)	Activity (units)	Specific activity	Yield (%)	Purification (factor)
Homogenate	381.000	25 600	0.067	100	1
1st Ammonium sulfate fractionation	123.000	24 300	0.197	95	2.9
DEAE-cellulsoe	22.800	22 300	0.979	87	15
2nd Ammonium sulfate fractionation	10.900	17 900	1.640	70	25
DEAE-Sephadex A-50	3.600	17 500	4.870	68	73
Ferredoxin-Sepharose	0.086	6040	70.000	24	1045

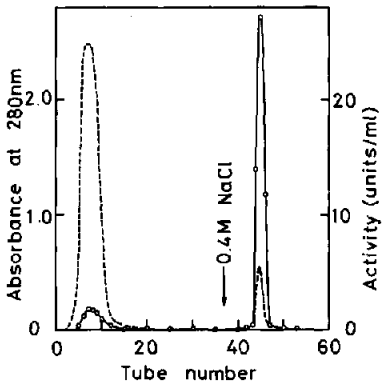

Fig.1. Elution profile for NiR from a ferredoxin-Sepharose column (1.6 × 20 cm). 5.0 ml fractions were taken into tubes containing 2.5 ml potassium phosphate buffer, pH 7.5, glycerol and ME to give 0.05 M buffer, 15% glycerol and 10 mM ME at a final concentration, respectively. Absorbance at 280 nm (- - -), enzymic activity (o - o - o).

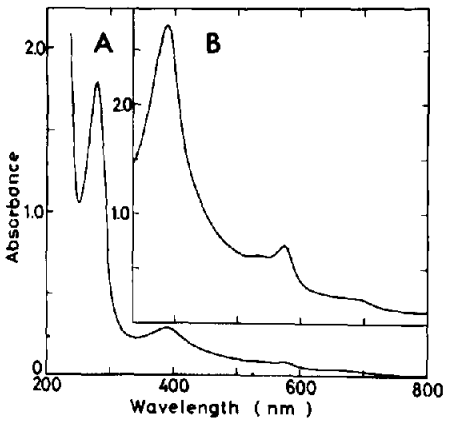

Fig.2. Absorption spectrum of spinach NiR. (A) 1.6 mg protein/ml, (B) 16 mg protein/ml, both in 0.05 M phosphate buffer, pH 7.5, measured in 1-cm light path with a Shimazu MPS 5000 spectrophotometer.

graphy is given in fig.1. The enzyme was purified 1000-fold with a yield of 24%. The specific activity of the final preparation was 70 units/mg protein which is comparable with the highest values for NiR purified by conventional procedures from *Cucurbita pepo* [16]. The latter enzyme was reported to be homogeneous on polyacrylamide gel electrophoresis [16]. Our purified enzyme was almost homogeneous but two other faint protein bands were detected on gel electrophoresis. Further purification is under way. The mol. wt. of the enzyme was estimated to be 62 000 by SDS-gel electrophoresis. Its absorption spectrum is shown in fig.2; there are absorption maxima at 280, 389, 535, 578 and near 695 nm. The spectrum is similar to those reported for the enzyme from *Chlorella* [7], barley roots [12], *Cucurbita pepo* [16] and spinach leaves [17].

It has been well established that the flavoprotein and iron–sulfur protein electron transport components of the enzyme system form a complex under certain conditions. Affinity chromatography has been developed for these flavoproteins taking advantage of these characteristics [18,19]. The nature of binding of the enzyme to ferredoxin-Sepharose is not clear, but the affinity between NiR and ferredoxin, described for the first time here, is understandable because of a pivotal role of ferredoxin as reducing agent in the photosynthetic apparatus [20] from which electrons are derived for nitrite reduction [2]. These findings provide additonal interests in enzymological and physiological aspects of NiR.

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