PURIFICATION AND PROPERTIES OF NITRITE REDUCTASE FROM SPINACH LEAVES

J. CÁRDENAS, J.L. BAREA, J. RIVAS and C.G. MORENO
Departamento de Bioquímica, Facultad de Ciencias y CSIC, Universidad de Sevilla, Spain

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1. Introduction

In algae, as in green tissues of higher plants [1–3], nitrite is directly reduced to ammonia by the action of a single specific protein, nitrite reductase. The enzyme from different sources has been partially purified and characterized, and its intracellular localization has been determined. Of particular relevance has been the finding that it is a chloroplast enzyme and the identification of ferredoxin as its electron carrier; methyl viologen can also act as an efficient artificial substitute for ferredoxin. Physiologically, a variety of electron donor systems are effective as the source of reducing power for the six electrons reaction catalyzed by ferredoxin-nitrite reductase: H_2-hydrogenase, NADPH-ferredoxin NADP reductase, and illuminated grana. Nitrite can thus act as a terminal electron acceptor in the noncyclic photosynthetic electron transport chain, and its reduction can be stoichiometrically coupled with oxygen evolution and phosphorylation [1–3]. Recent reports from this laboratory [4–6] have shown that iron–but not molybdenum–is a component of nitrite reductase and that the activity level of the enzyme in the cells specifically increases in response to the iron content of the nutrient solution.

This paper describes a method for the purification to homogeneity of spinach nitrite reductase, as well as some of its most relevant physicochemical properties, i.e. molecular weight, absorption spectrum and metal content.

2. Materials and methods

A crude homogenate of spinach leaves was prepared as described by San Pietro and Lang [7]. Precipitation with acetone, extraction of the protein precipitate, dialysis of the extract and removal of ferredoxin by adsorption on DEAE-cellulose were carried out according to Ramírez et al. [3, 8]. Adsorption of the enzyme on a DEAE-cellulose bed and its elution were also carried out according to the same authors. The eluate was treated with $(NH_4)_2SO_4$ between 45 and 70% saturation. The precipitate obtained after centrifugation was dissolved in 2 ml of 50 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl and passed through a Sephadex G-100 column (1.5 × 80 cm) previously equilibrated with the Tris-NaCl solution. 2 ml fractions were collected, and the most active ones were pooled, concentrated to 1 ml using a DEAE-cellulose column, and finally purified in a preparative electrophoresis procedure, using a 7.5% polyacrylamide gel column of 10 cm height at pH 9.5; 2 ml fractions were collected. The three most active fractions were pooled and used for testing homogeneity, recording the absorption spectrum, and analysing the metals content. Both electrophoretic purification and test homogeneity were carried out with Shandon electrophoresis equipment. After electrophoresis, gels were stained with 1% Amido Black 10 B in 7% acetic acid and destained by acetic acid at 7%. The nitrite reductase activity was directly assayed on the gel cylinders by the procedure of Cárdenas [9].

Molecular weight was estimated by gel filtration according to Andrews [10]. Plot of results was obtained by representing elution volume versus mole-

Table 1
Purification of nitrite reductase from spinach.

Fraction	Vol (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (mU/mg)
I. Homogenate	1320	20,400	680	100	33
II. Acetone precipitate, dialysed and freed of ferredoxin.	174	2,550	587	86	230
III. Eluate from a DEAE-cellulose bed.	23	357	420	62	1,210
IV. Double precipitation with $(NH_4)_2SO_4$ and filtration on Sephadex G-100.	70	35	250	37	7,140
V. Preparative electrophoresis on polyacrylamide gel.	6	1.3	44	6.5	33,850

cular weight of known molecular weight protein (γ-globulin, M.W. = 160,000; serum albumin, M.W. = 67,000; ovalbumin, M.W. = 45,000 and cytochrome *c*, M.W. = 12,400). Separation was carried out on a column of Sephadex G-200 (2.5 × 40 cm). The proteins were eluted with 50 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl. The average molecular weight, as well as the sedimentation coefficient, was also calculated by the method of Martin and Ames [11]. Nitrite reductase and several other proteins of known molecular weight (γ-glubulin, M.W. = 160,000; serum albumin, M.W. = 67,000; chymotrypsinogen, M.W. = 25,000) were sedimented on a linear sucrose gradient from 5 to 20%.

The absorption spectrum was measured with a Beckman DK-2A recording spectrometer. Colorimetric iron assay was made with *o*-phenanthroline according to Ballentine and Burford [12]. Atomic absorption analysis was carried out in a Unicam SP 90, ser. 2, recording spectrophotometer. In the spectrographic determinations a Hilger E 498 spectrograph was used. Calculations were made after Albi and Vioque [13].

Nitrite reductase was assayed with methyl viologen chemically reduced by $Na_2S_2O_4$ according to Ramírez et al. [3, 8]. Protein was determined by the method of Lowry et al. [14]. One enzyme unit is defined here as one micromole of nitrite disappeared per minute. All chemicals were of analytical grade.

3. Results and discussion

Nitrite reductase was purified about 1,000-fold as summarized in table 1. Fig. 1 shows the homogeneity of the purified enzyme, as revealed by disc polyacrylamide electrophoresis. The single protein band observed was coincident with nitrite reductase activity assayed directly on the cylinder gel.

The absorption spectrum of pure nitrite reductase (fig. 2) presents two peaks at about 380 and 570 nm, and a shoulder at 292 nm. Thus, there is no indication of flavin peaks, a finding consistent with those reported by Losada et al. [15], Ramírez et al. [8] and Shin and Oda [16] (cf. [17]) for the spinach enzyme and suggesting that this protein may differ from the soybean enzyme described by Nason et al. [18] and Roussos and Nason [19]. It has a striking similarity to that reported by Asada et al. [21] for the sulfite reductase from spinach leaves, another MVH-dependent iron–protein which also lacks flavin.

Figs. 3 and 4 depict the results obtained in estimating the molecular weight of nitrite reductase by gel filtration on Sephadex G-200 and by sucrose density gradient sedimentation, respectively. In both methods the calculated molecular weight was about 63,000 daltons. These results agree well with those reported by Hewitt et al. [22] for the enzymes from spinach and marrow, and by Zumft et al. [23] for that

Fig. 1. Photograph of stained polyacrylamide gel after electrophoresis of spinach nitrite reductase. The direction of electrophoresis was from top (−) to bottom (+).

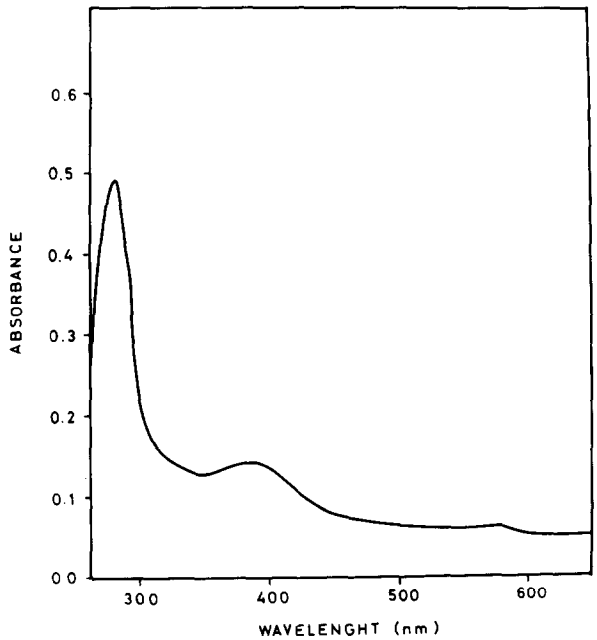

Fig. 2. Absorption spectrum of homogeneous spinach nitrite reductase.

of Chlorella. The sedimentation coefficient ($s_{20,w}$) was calculated to be 4.9 S.

The iron content of several different preparations of homogeneous reductase from spinach, as measured by atomic absorption spectrophotometry and by spectrographic and colorimetric methods, was 2 atoms of iron per molecule of enzyme (table 2). Neither Mn nor Cu could be detected in these preparations by atomic absorption and spectrographic methods. The presence of iron is consistent with our recent demonstration [4–6] of the role of this metal in the activity of nitrite reductase and with the inhibitory effects of KCN and *o*-phenanthroline [1–3, 9]. Preliminary experiments suggest that this enzyme is not a hemoprotein. The failure to detect Mn and Cu is also consistent with our studies in Chlorella using ^{64}Cu and ^{52}Mn [9]. However the results described here are in contrast with the reported requirements of Mn and Cu for the activity of this enzyme obtained from other sources [18–20] but consistent with reports [8, 9, 24] that the activity of nitrate reductase from spinach, marrow and Chlorella is not affected by Mn or Cu.

The properties of spinach nitrite reductase are very similar to those found by us [25] in the squash enzyme and by Zumft [26] in the Chlorella one.

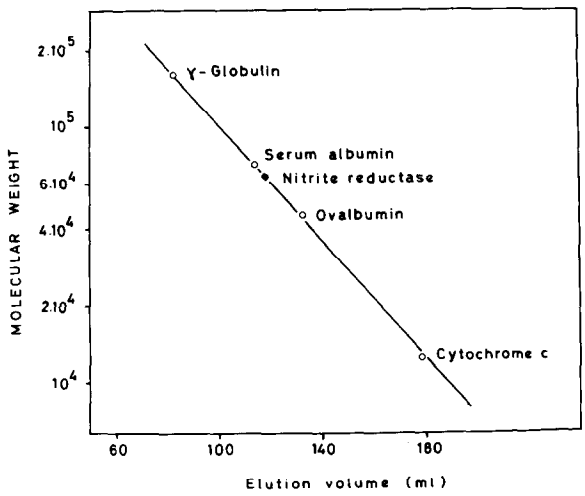

Fig. 3. Estimation of molecular weight of spinach nitrite reductase by filtration on a Sephadex G-200 column.

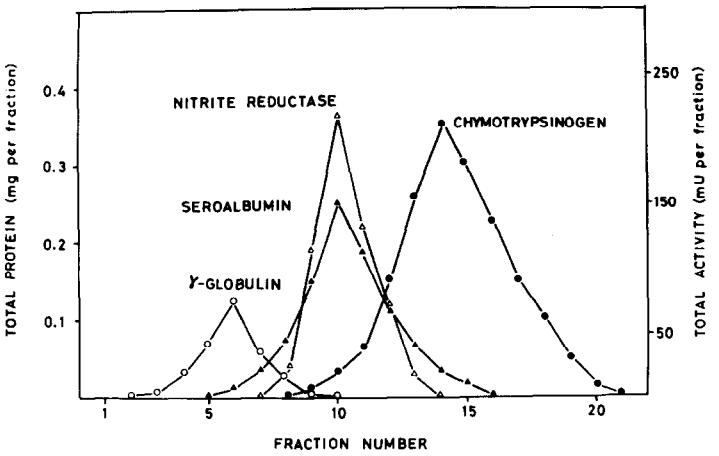

Fig. 4. Sedimentation pattern of spinach nitrite reductase by sucrose density gradient centrifugation. Fraction 1 corresponds to the gradient bottom.

Table 2
Iron content of nitrite reductase.

Exp.	Protein (mg)	Iron (μg)	Iron/protein (atoms/molecule)
I	0.33	0.55	1.8
II	0.49	1.00	2.3
III	0.46	0.80	2.3
IV	0.86	1.50	1.7
V	0.54	0.96	2.0
VI	0.61	1.12	2.0

The analyses were made by atomic absorption method using different preparations of nitrite reductase purified to homogeneity. Calculations are referred to 63,000 as molecular weight of the enzyme.

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