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Spinach Nitrate Reductase

PURIFICATION, MOLECULAR WEIGHT, AND SUBUNIT COMPOSITION

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ABSTRACT

Nitrate reductase was purified about 3,000-fold from spinach leaves by chromatography on butyl Toyopearl 650-M, hydroxyapatite-brushite, and blue Sepharose CL-6B columns. The purified enzyme yielded a single protein band upon polyacrylamide gel electrophoresis under nondenaturing conditions. This band also gave a positive stain for reduced methylviologen-nitrate reductase activity. The specific NADH-nitrate reductase activities of the purified preparations varied from 80 to 130 units per milligram protein. Sucrose density gradient centrifugation and gel filtration experiments gave a sedimentation coefficient of 10.5 S and a Stokes radius of 6.3 nanometers, respectively. From these values, a molecular weight of 270,000 ± 40,000 was estimated for the native reductase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the denatured enzyme yielded a subunit band having a molecular weight of 114,000 together with a very faint band possessing a somewhat smaller molecular weight. It is concluded that spinach nitrate reductase is composed of two identical subunits possessing a molecular weight of 110,000 to 120,000.

Assimilatory nitrate reductase (NADH-nitrate oxidoreductase, EC 1.6.6.1) catalyzes the first step of nitrate assimilation. Elucidation of its molecular, catalytic, and regulatory properties is, therefore, crucial for our understanding of the assimilation process (1). Thanks to the development of improved methodologies such as blue Sepharose affinity chromatography (27), nitrate reductase has recently been purified from various higher plants (3, 4, 12, 15, 16) as well as from a number of fungi and algae (7, 11, 24–26). However, the properties so far reported for the enzyme purified from higher plants are rather variable especially with respect to mol wt and quaternary structure. For example, nitrate reductase from barley leaves (12) and that from squash cotyledons (19) have been shown to have mol wt of 221,000 and 230,000, respectively, and to be composed of two subunits, whereas the enzyme purified from spinach leaves has been reported to have a mol wt of 197,000 and consist of three subunits possessing mol wt of 45,000, 85,000, and 120,000 (16). It seems, therefore, necessary to refine further the purification procedure for higher plant nitrate reductase.

This paper reports the purification of nitrate reductase from spinach leaves by a method in which butyl Toyopearl 650-M, an amphiphilic gel, was used in addition to blue Sepharose CL-6B as chromatography adsorbents. This method has permitted us to isolate an apparently homogeneous enzyme preparation having a specific activity higher than those reported previously for spinach nitrate reductase. Evidence is also presented that the spinach enzyme is composed of two identical subunits having a mol wt of 110,000 to 120,000.

MATERIALS AND METHODS

Materials. Fresh leaves of spinach (*Spinacia oleracea*) were obtained from the field plot of Faculty of Horticulture, Chiba University. The following chemicals were purchased from sources indicated in parentheses: NADH (Boehringer Mannheim); methylviologen (British Drug House); specially purified guanidine hydrochloride (Nakarai Chemical Co., Kyoto); blue dextran 2000 and blue Sepharose CL-6B (Pharmacia); Toyopearl HW 55 and butyl Toyopearl 650-M (Toyo Soda Co., Tokyo); and polyclar AT (Gokyo Sangyo Co., Tokyo). All other chemicals used were of highest quality available commercially. The sources of marker proteins used were as follows: horse heart Cyt *c*, aldolase, bovine liver catalase, and ferritin from Boehringer Mannheim; ovalbumin and BSA from Mann; chymotrypsinogen from Serva; yeast alcohol dehydrogenase and *Escherichia coli* β-galactosidase from Sigma; and thyroglobulin from Pharmacia.

Enzyme Assays. The reaction mixture for NADH-nitrate reductase assay contained 25 mM K-phosphate (pH 7.5), 5 mM KNO_3, 100 μM NADH, and enzyme in a final volume of 1 ml. The reaction was started by adding NADH, run for 15 min at 30°C, and stopped by adding 1 ml of 1.5 N HCl containing 1% sulfanilamide followed by 1 ml of 0.02% *N*-1-naphthylethylendiamine dihydrochloride. The red color developed was measured at 540 nm and the amount of nitrite formed was determined from a standard curve. A control experiment in which the enzyme was omitted was also performed. One unit of NADH-nitrate reductase produced 1 μmol nitrite/min. Alcohol dehydrogenase (18) and catalase (5) were assayed by the published methods.

Protein Determination. Protein was determined by the method of Bradford (2) using BSA as a standard.

Polyacrylamide Gel Electrophoresis. Polyacrylamide disc gel electrophoresis under nondenaturing conditions was carried out as described by Davis (6) using 7.5% and 5% cross-linked gels and 50 mM Tris-glycine buffer (pH 8.3). The current applied was 2 mamp per tube. Protein bands were visualized by staining with Coomassie blue R-250. Nitrate reductase in the electrophoresis gel was detected by staining for reduced methylviologen-nitrate reductase activity by the method of Solomonson *et al.* (24). To estimate the subunit mol wt of the purified enzyme, SDS-PAGE was performed by using Laemmli's buffer system (13). The sample was pretreated with 6 M guanidine hydrochloride (8) and the denatured protein was alkylated with monoiodoacetic acid (8). The alkylated protein was dialyzed against 8 M urea and then against 0.1% SDS, followed by further treatments as described

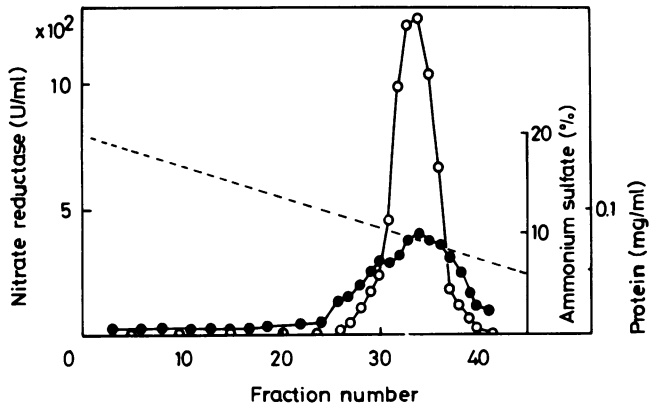

FIG. 1. A typical elution profile in butyl Toyopearl 650-M chromatography of partially purified nitrate reductase. The 2nd butyl Toyopearl eluate fraction containing 20% $(NH_4)_2SO_4$ (see text) was applied to a butyl Toyopearl column (1.8 × 30 cm) equilibrated with 250 mM buffer containing 20% $(NH_4)_2SO_4$. After washing the column with the same buffer, nitrate reductase was eluted with a liner $(NH_4)_2SO_4$ gradient from 20 to 0% saturation in 250 mM buffer. Fractions equal about 3 ml. Flow rate was adjusted to about 0.6 ml/min. (○), NADH-nitrate reductase activity was expressed as μmol NO_2^- produced/min·ml. (●), Protein concentration was expressed as μg/ml.

by Weber and Osborn (28) with bromophenol blue as tracking dye. Electrophoresis was carried out on a 10% cross-linked slab gel (10 cm long, 1 mm thick) by applying a current of 15 mamp for 15 min and then 20 mamp until the dye front moved closely to the bottom of the slab. Protein bands were stained with Coomassie blue R-250. The mol wt of protein bands were estimated from their mobilities relative to those of four marker proteins, *i.e. E. coli* β-galactosidase (subunit M_r[1], 116,000), BSA (M_r, 68,000), ovalbumin (M_r, 43,000), and chymotrypsinogen (M_r, 25,000).

Determination of Sedimentation Coefficient. The sedimentation coefficient of the purified reductase was determined by sucrose density gradient centrifugation as described by Martin and Ames (14) with bovine liver catalase (11.3 S) and yeast alcohol dehydrogenase (7.4 S) as reference standards. A solution of the sample or a standard protein was layered over 4.8 ml of a linear sucrose gradient from 5 to 15% (w/v) in 25 mM K-phosphate (pH 7.5) containing 1 mM 2-ME, 1 mM EDTA and 50 μM FAD. The gradient was centrifuged at 40,000*g* at 4°C for 10 h in a Hitachi RPS 50-2 rotor. After centrifugation, 8-drop fractions were collected from the bottom of the centrifuge tube and assayed for NADH-nitrate reductase, catalase, or alcohol dehydrogenase activity.

Determination of Stokes Radius. The Stokes radius of the purified enzyme was determined by a modification of the method of Siegel and Monty (23). A Toyopearl HW-55 column (1.8 × 90 cm) was equilibrated with 25 μM K-phosphate (pH 7.5) containing 1 mM 2-ME, 1 mM EDTA, and 50 μM FAD. Approximately 1 mg of each standard protein, dissolved in 0.5 ml of the equilibration buffer, was applied to the column. Elution was conducted with the equilibration buffer at a flow rate of 15 ml/h, and 2-ml fractions were collected. Protein elution was monitored by measuring the absorption at 280 nm. The Stokes radii of the standard proteins used are 8.5 nm for thyroglobulin, 6.1 nm for ferritin, 4.6 nm for yeast alcohol dehydrogenase, 3.5 nm for BSA, and 2.75 nm for ovalbumin (11, 23). Blue dextran 2000 was used to determine void volume of the column. The radius of purified nitrate reductase was then determined on this calibrated column.

RESULTS

Purification of Nitrate Reductase. All steps were conducted at 0 to 4°C. Spinach leaves (1500 g) were homogenized for 2 min in a Waring Blendor with 2000 ml of 250 mM K-phosphate (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride, 5% isopropyl alcohol, 1 mM 2-ME, 1 mM EDTA, 5 mM KNO_3, 20 μM FAD, and 126 g of polyclar AT, and the homogenate was squeezed through a broad cloth with the aid of a hand press. The fluid thus obtained was centrifuged at 10,000*g* for 15 min and the supernatant ('crude extract') was saved. Solid $(NH_4)_2SO_4$ was slowly added to the crude extract to 20% saturation with constant stirring. During this process the pH of the solution was kept to 7.5 by occasional addition of 1 N NH_4OH. The solution was then mixed with 400 ml of butyl Toyopearl 650-M, which had been equilibrated with 250 mM K-phosphate (pH 7.5) containing 20% saturated $(NH_4)_2SO_4$, 1 mM 2-ME, 1 mM EDTA, and 20 μM FAD ('250 mM buffer'). After stirring the mixture for 30 min, the gel was recovered by centrifugation and washed several times with 250 mM buffer until virtually no protein could be washed out of the gel. The washed gel was poured into a column (3.5 × 46 cm) and nitrate reductase was eluted from the column with 250 mM buffer containing no $(NH_4)_2SO_4$. Peak fractions having high nitrate reductase activities were pooled ('1st butyl Toyopearl eluate').

The phosphate concentration of the 1st butyl Toyopearl eluate

[1] Abbreviations: M_r, mol wt; 2-ME, 2-mercaptoethanol; FAD, flavin adenine dinucleotide.

Table I. *Purification of Nitrate Reductase from Spinach Leaves*

The purification procedure from 1500 g of spinach leaves is described in the text. These values are means of two experiments.

Step	Volume	Total Protein	Total Activity	Specific Activity	Recovery	Purification
	ml	*mg*	*units*	*units/mg protein*	*%*	*-fold*
Crude extract	2320	10,240	359	0.035	100	1.0
1st butyl Toyopearl eluate	110	776	244	0.315	68	9.0
Hydroxylapatite-brushite eluate	80	157	198	1.26	55	36
Blue Sepharose eluate	77	10	156	15.6	43	446
2nd butyl Toyopearl eluate	47	2.4	112	47.2	31	1350
3rd butyl Toyopearl eluate	7	0.7	68	103	19	2950

fraction was reduced to 75 mM by dilution with water containing 1 mM 2-ME, 1 mM EDTA, and 20 μM FAD. The diluted solution was mixed with 110 ml of a hydroxyapatite-brushite mixture (1:3, v/v) and the mixture was stirred for 10 min. The gel was recovered by decantation and washed several times with 75 mM K-phosphate (pH 7.5) containing 1 mM 2-ME, 1 mM EDTA, and 20 μM FAD ('75 mM buffer') until practically no protein could be washed out. The washed gel was poured into a column (5.5 × 20 cm) and nitrate reductase was eluted from the column with 250 mM buffer containing no $(NH_4)_2SO_4$. Peak fractions having high reductase activities were combined ('hydroxyapatite-brushite eluate').

The hydroxyapatite-brushite eluate fraction was directly applied to a blue Sepharose CL-6B column (2.5 × 24 cm) equilibrated with 75 mM buffer. Upon washing the column with 75 mM buffer, a large amount of protein having no nitrate reductase activity was eluted. The reductase still tightly adsorbed was then eluted with 300 mM K-phosphate (pH 7.5) containing 1 mM 2-ME, 1 mM EDTA, 50 μM FAD, 300 mM KNO_3, and 20% glycerol and active fractions were pooled ('blue Sepharose eluate').

Solid $(NH_4)_2SO_4$) was added to the blue Sepharose eluate fraction to 20% saturation and the mixture was applied to a butyl Toyopearl column (1.8 × 30 cm) which had been equilibrated with 250 mM buffer (containing 20% saturated ammonium sulfate). After washing the column with the same buffer, nitrate reductase was eluted with 500 ml of a linear ammonium sulfate gradient from 20 to 0% saturation in 250 mM buffer. A typical elution profile obtained in this step is shown in Figure 1. The combined active fractions ('2nd butyl Toyopearl eluate') was again made 20% saturated with respect to $(NH_4)_2SO_4$ and subjected to a third butyl Toyopearl column chromatography essentially as described above. The nitrate reductase-containing fractions were combined, concentrated by ultrafiltration through an Amicon model 12, Diaflo ultrafilter ('3rd butyl Toyopearl eluate'), and used as the purified preparation.

As summarized in Table I, the purification method described above usually led to a 3000-fold increase in specific NADH-nitrate reductase activity from the crude extract, and the overall yield was approximately 20%. The entire purification procedure could be completed within 4 to 5 d. No protease activity could be detected in the final preparation when checked by the method of Wittenbach (29) using azocasein as substrate.

Purity and Specific Activity. As shown in Figure 2A, the purified preparation gave a single protein band when subjected to polyacrylamide disc gel electrophoresis under nondenaturing conditions. The use of 7.5% and 5% cross-linked gels both gave essentially similar results. This protein band also showed a positive stain for reduced methylviologen-nitrate reductase activity (Fig. 2B). The specific NADH-nitrate reductase activity of the purified preparations varied from 80 to 130 units/mg protein; an average value of 87 was obtained for four preparations.

Native and Subunit Mol Wt. When the purified preparation was subjected to gel filtration through a calibrated Toyopearl HW-55 column, nitrate reductase activity was eluted at a position corresponding to a Stokes radius (r_s) of 6.3 nm (Fig. 3). Centrifugation of the preparation in a sucrose concentration gradient from 5 to 15% yielded a sedimentation coefficient ($s_{20,w}$) of 10.5 S, relative to the two reference standards used, *i.e.* bovine liver catalase (11.3 S) and yeast alcohol dehydrogenase (7.4 S) (data not shown). According to Siegel and Monty (23), the mol wt of a protein (M_r) is related to r_s and $s_{20,w}$ by the following equation:

$$M_r = 6\pi r_s \eta N s_{20,w}/(1 - v\rho)$$

where N is Avogadro number, η is the viscosity of the medium, v is the partial specific volume of the protein, and ρ is the density of the medium. The ρ value was determined to be 1.00115 g/cm^3, and the η and v values were assumed to be 0.01002 g s^{-1} cm^{-1} and 0.725 cm^3/g, respectively, as had been assumed by De la Rosa *et al.* (7) for *Ankistrodesmus braunii* nitrate reductase. From these values and the r_s and $s_{20,w}$ values determined above,

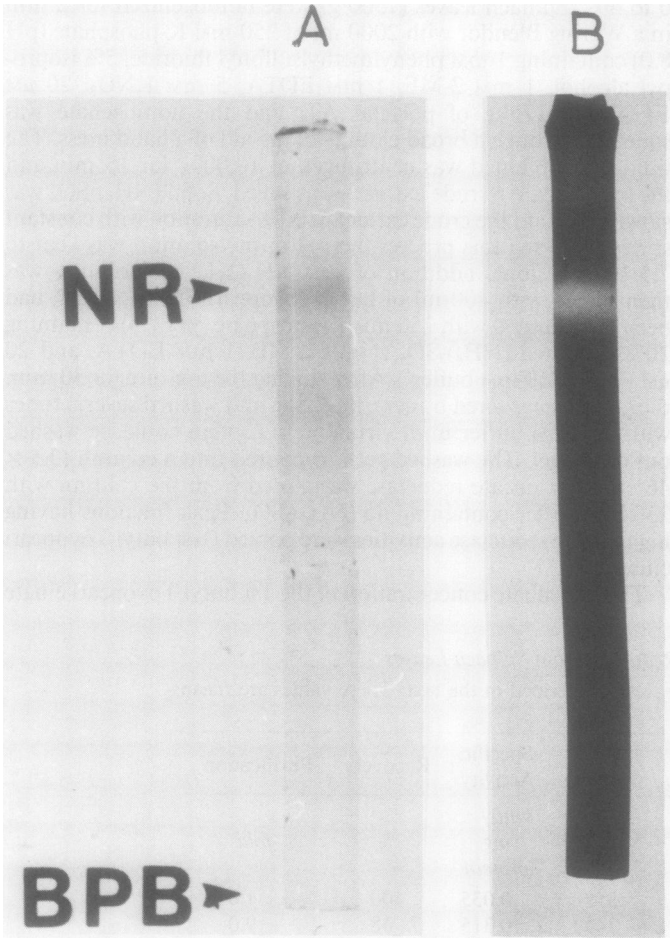

FIG. 2. Polyacrylamide disc gel electrophoresis of purified nitrate reductase. Electrophoresis of purified nitrate reductase was carried out on 7.5% cross-linked polyacrylamide disc gels in 50 mM Tris-glycine buffer (pH 8.3) as described in "Materials and Methods." A, 10 μg of protein was loaded and the gel was stained for protein; B, 80 nunits of reduced methyl viologen-nitrate reductase activity was loaded and the gel was stained for the activity.

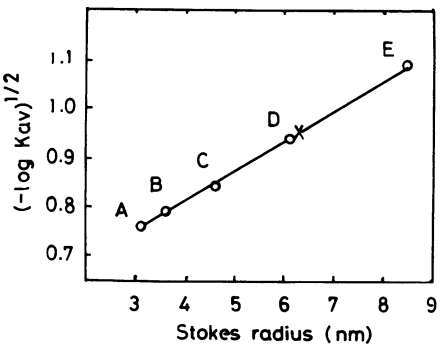

FIG. 3. Determination of Stokes radius of purified nitrate reductase. The determination was performed by gel filtration on a Toyopearl HW-55 column as described in "Materials and Methods." Ovalbumin (A), BSA (B), yeast alcohol dehydrogenase (C), ferritin (D), and thyroglobulin (E) were used as standards of known Stokes radii. X, position where nitrate reductase activity was eluted.

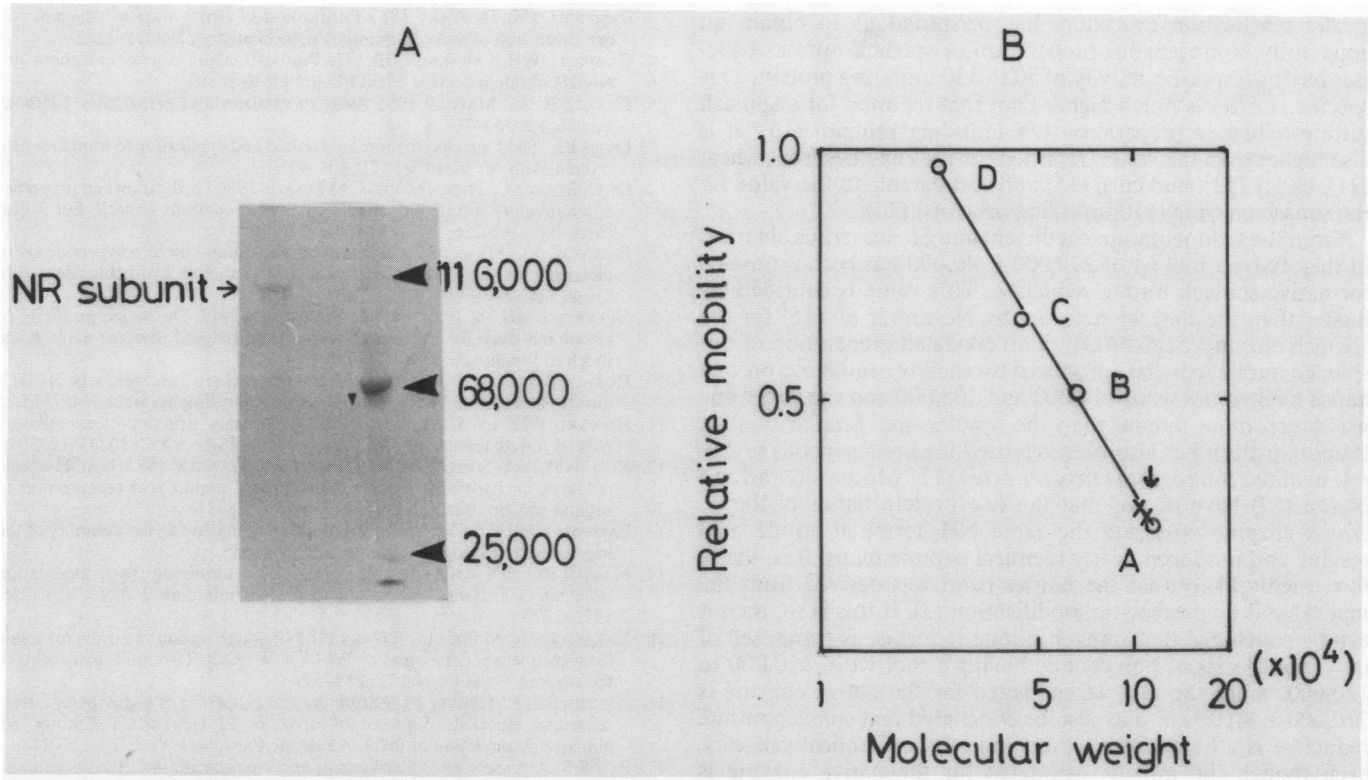

FIG. 4. A, SDS-PAGE analysis of the purified nitrate reductase (NR) subunit. Mol wt standards used were: β-galactosidase (116,000); BSA (68,000); and chymotrypsinogen (25,000). B, Estimation of subunit mol wt of purified NR by SDS-PAGE. Experimental conditions are described in "Materials and Methods." Mol wt standards used were: (A), β-galactosidase; (B), BSA; (C), ovalbumin (43,000); and (D), chymotrypsinogen. NR gave two bands having relative mobilities indicated by X; of the two bands, the one shown by an arrow was much more intensely stained than the other.

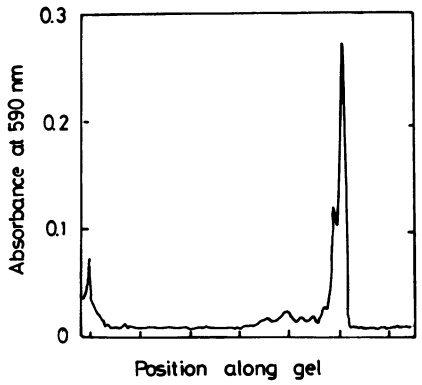

FIG. 5. Densitometric scan of SDS-polyacrylamide gel after electrophoresis. Purified nitrate reductase was electrophoresed in subunit state (Fig. 4A). The destined gel was scanned at 590 nm at slit width of 0.1 mm, scanning speed of 4.5 cm/min, and chart speed of 10 cm/min. The peak corresponds to the nitrate reductase subunit band in the gel.

a mol wt of 270,000 was calculated for native spinach nitrate reductase. However, since it is likely that determinations of sedimentation coefficient and Stokes radius by the methods employed involve 5 to 10% errors, it can only be stated that the mol wt of the spinach enzyme is 270,000 ± 40,000.

To estimate the subunit mol wt, the purified enzyme was denatured and alkylated with monoiodoacetic acid. When this sample was subjected to SDS-PAGE, two protein bands were detected (Fig. 4A) and their apparent mol wt were estimated to be 114,000 and 105,000 from their mobilities relative to four marker proteins (Fig. 4B). The M_r = 114,000 band was much more intense than the M_r = 105,000 band; densitometry of the stained bands indicated that the former was about 40 times more abundant than the latter (Fig. 5). It is likely that the minor band was a proteolytically modified product of the intact subunit. In any case, it does not seem unreasonable to assume that the subunit of spinach nitrate reductase has a mol wt of 110,000 to 120,000.

This subunit mol wt, coupled with the mol wt of 270,000 ± 40,000 estimated for the native enzyme, suggested that the enzyme is composed of two identical subunits, even though the subunit mol wt estimated (110,000–120,000) is only 41 to 44% of the native mol wt calculated above (270,000).

DISCUSSION

The purification method developed in this study is characterized by the use of butyl Toyopearl 650-M, an amphiphilic gel, as a unique chromatography adsorbent in addition to blue Sepharose CL-6B. Butyl Toyopearl was initially manufactured as an adsorbent for membrane proteins, Sakihama *et al.* (22) have recently reported that several soluble proteins such as Cyt *c* can be effectively adsorbed to this gel in the presence, but not in the absence, of 0.7 M $(NH_4)_2SO_4$. We have found that spinach nitrate reductase can also be adsorbed to this gel in the presence of 20% saturated $(NH_4)_2SO_4$ and this adsorption is not affected by the presence of 300 mM KNO_3. Therefore, butyl Toyopearl seems to be a convenient tool for purification of higher plant nitrate reductase. Another advantage of the use of this gel is that the enzyme remains in solution during adsorption and elution, a fact enabling one to avoid adverse effects such as irreversible inactivation that can be caused by precipitation. In the present procedure, phenylmethylsulfonyl fluoride was included in the extraction buffer to prevent proteolytic attack on the reductase during the purification.

The purification procedure has permitted us to obtain an apparently homogeneous preparation of spinach nitrate reductase having a specific activity of 80 to 130 units/mg protein. This specific activity is much higher than that reported for a spinach nitrate reductase preparation (24 units/mg protein) (16). It is also higher than the values reported for the enzymes from wheat (21), barley (12), and corn (15) and comparable to the value for the squash enzyme (110 units/mg proteins) (20).

From the sedimentation coefficient and Stokes radius obtained in this study, a mol wt of 270,000 ± 40,000 has been estimated for native spinach nitrate reductase. This value is considerably higher than the mol wt reported by Notton *et al.* (16) for the spinach enzyme. SDS-PAGE of an alkylated preparation of our spinach nitrate reductase produced two nearly contiguous protein bands having mol wt of 114,000 and 105,000 and the larger one was much more intense than the smaller one. Similar doublet staining pattern has also been reported for homogeneous preparation of the *Neurospora crassa* enzyme (17). Moreover, Pan and Nason (17) have shown that the two protein bands of the *N. crassa* enzyme possessed the same NH_2-terminal amino acid residue and produced nearly identical peptide maps. It is, therefore, highly likely that the fainter band was derived from the major band by proteolytic modification (3). If this is so, then it can be concluded that spinach nitrate reductase is composed of only one species of polypeptide having a mol wt of 110,000 to 120,000. Since the mol wt estimated for the native enzyme is 270,000 ± 40,000, it may also be concluded that spinach nitrate reductase is a homodimer consisting of two identical subunits, even though the mol wt estimated for the native enzyme is somewhat larger than expectation. In contrast to our finding, Notton *et al.* (16) have reported that SDS-PAGE of purified spinach nitrate reductase gave three polypeptide bands having mol wt of 45,000, 85,000, and 120,000. It is likely that their preparation was partially modified by proteolysis (3).

The conclusion drawn above that spinach nitrate reductase is composed of two identical subunits having a mol wt of 110,000 to 120,000 is compatible with the results reported for the enzymes from barley (12), squash (19), *N. crassa* (10), and *Rhodotorula glutinis* (9). These enzymes have also been shown to be homodimers consisting of two identical subunits having mol wt ranging from 100,000 to 145,000. It is, therefore, tempting to assume that most assimilatory nitrate reductases possess similar mol wt and subunit composition in their undegraded state. However, it should be noted that the enzymes purified from *Aspergillus nidulans* (26), *Chlorella vulgaris* (11), and *A. braunii* (7) have been reported to consist of 4, 4, and 8 subunits, respectively.

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