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A Method for the Separation and Partial Purification of the Three Forms of Nitrate Reductase Present in Wild-Type Soybean Leaves[1]

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ABSTRACT

A rapid and simple purification method was used to separate and purify nitrate reductases (NR) from Williams soybean leaves. Blue Sepharose columns were sequentially eluted with 50 millimolar NADPH and 50 millimolar NADH, thus separating NAD(P)H:NR from NADH:NRs. Subsequent purification of the collected peaks on a fast protein liquid chromatography-Mono Q column enabled separation of two NADH:NRs. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the subunit relative molecular mass for all three NR forms (constitutive NAD(P)H:NR [pH 6.5], EC 1.6.6.2; constitutive NADH:NR [pH 6.5], EC not assigned; and inducible NADH:NR [pH 7.5], EC 1.6.6.1) was approximately 107 to 109 kilodaltons. All three NRs showed similar spectra with absorption maxima at 413 and 273 nanometers in the oxidized state, and with the characteristics of a cytochrome *b* type heme upon reduction with NADH (absorption maxima at 556, 527, and 424 nanometers). The technique developed provides an improved separation of the three NR forms from soybean leaves. The similarity of the NRs with regard to their cytochrome b_{556} type heme content and in relative molecular mass indicated that other differences must exist to account for the different kinetic and physical properties previously reported.

Nitrate reductases from higher plants have strikingly similar properties (6). It is not surprising, therefore, that polyclonal antibodies raised against barley or squash NR[4] showed cross-reactivity with NR from various plants (16, 17). Some differences in structure must exist, however, since monoclonal antibodies showed differential binding to NR from different plants (11). The structure of plant NR has been extensively studied in *Chlorella vulgaris* (18), and squash (*Cucurbita maxima*) (13). Nitrate reductase appears to be a dimer with a subunit relative molecular mass of 100 to 115 kD, and with one Cyt *b* and one molybdenum cofactor per subunit (13).

Nitrate reductase from soybean has been partially purified by several laboratories (4, 7, 12, 14, 20, 21). A NAD(P)H:NR and a NADH:NR have been partially purified from soybean leaves and cotyledons (4, 7, 12, 15). Studies with a NR mutant of soybean (nr_1) indicated that the NADH:NR isolated from nitrate-grown wild-type (cv Williams) soybean leaves was a mixture of two NR enzyme forms (20). One was the substrate inducible NADH (pH 7.5) NR (EC 1.6.6.1, formerly designated iNR) and the second was a constitutive NR which preferentially used NADH as an electron donor and had a pH optimum of 6.5 (EC number not assigned, formerly designated c_2NR). Separation of these two enzymes on Blue Sepharose has not been previously accomplished from nitrate-grown wild-type soybean plants. Use of the nr_1 mutant line, which lacks both the NAD(P)H:NR, EC 1.6.6.2 (formerly designated c_1NR) and the constitutive NADH:NR (pH 6.5) enzyme form, was necessary to demonstrate that the Blue Sepharose NADH eluted peak from wild-type plants was a mixture of NR forms (20). The presence of constitutive NADH (pH 6.5) and inducible NADH (pH 7.5) NR forms was also verified by comparing two additional soybean mutants which lacked the NADH (pH 6.5) form present in Williams soybean (19). Additional constitutive NR mutants, in a different soybean background (cv Bragg), have been recently reported (5), but they have not been biochemically characterized. Although the mutants have been invaluable in providing evidence for multiple NR forms in soybean, the separation of these forms from the wild-type parent is desirable to allow relative quantification and characterization from a single genotype.

The objective of this study was to develop a procedure to successfully separate the NR enzyme forms from wild-type (cv Williams) soybean leaves which contain all three forms. This procedure entails a rapid process involving initial separation on Blue Sepharose followed by subsequent purification using FPLC with a Mono Q column.

MATERIALS AND METHODS

Chemicals. Cibacron Blue 3-FGA[5] was from Polyscience, Warrington, PA. NADPH and FAD were from United States Biochemicals, Cleveland, OH. Acrylamide, Tris, mercaptoethanol, and glycine were from Bio-Rad. Other biochemicals were from Sigma. All other chemicals were of analytical grade and purchased locally. Blue Sepharose 4B-CL was prepared using the method of Bohme *et al.* (1).

Plant Growth and Leaf Extraction. Soybean plants (*Glycine max* [L.] Merr. cv Williams) were grown in a greenhouse as previously described (20). Three hundred g of unifoliolate leaves from 12- to 15-d-old plants were frozen with liquid N_2, ground

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[4] Abbreviations: NR, nitrate reductase; FPLC, fast protein liquid chromatography; U, unit (= 1 μmol substrate converted per min at 30°C).

[5] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the vendor or product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other vendors or products that may also be suitable.

with a mortar and pestle, and imbibed in 1.5 L of ice-cold extraction medium (50 mM Tris-HCl, 1 mM EDTA, 10 mM L-cysteine, 10% [v/v] glycerin, 0.5% [w/v] casein, and 1% [w/v] PVP [insoluble polyvinylpolypyrrolidone], adjusted to pH 8.0 with phosphoric acid). The tissue was disrupted using a Polytron homogenizer, filtered through Miracloth fortified with four layers of cheesecloth, and centrifuged for 15 min at 18,600*g*.

Blue Sepharose Chromatography. The supernatant, following the centrifugation step noted above, was mixed thoroughly with 70 g of suction dried Blue Sepharose, and the pH of the slurry was adjusted to 7.0 with phosphoric acid. After rigorous stirring for 1 h, Blue Sepharose was collected on a Buchner funnel, and washed several times with 200 ml washing buffer (50 mM K-phosphate pH 7.4, 1 mM EDTA, 1 mM DTT, 7 μM FAD, 10% (v/v) glycerin, 0.01 mM Leupeptin, 2.0 mM *p*-amino-benzamidine, 1.0 mM phenylmethylsulfonylfluoride, and 0.065 mM Na-*p*-tosyl-L-lysine chloromethyl ketone. The washed gel was packed into a column (3.5 cm diameter), and eluted with 200 ml of 50 mM NADPH in washing buffer, followed by 200 ml of 50 mM NADH in washing buffer. Active fractions of the two elutions were pooled separately, precipitated with ammonium sulfate (300 g/L), and stored at −18°C. Precipitates were collected when needed by centrifugation (18,600*g* for 15 min), resuspended in 40 ml equilibration buffer (which was the same as the washing buffer except that 50 mM Tris-HCl [pH 7.5] was used in place of the phosphate buffer), and concentrated to 3 to 5 ml in a stirred cell using a PM 30 membrane (Amicon Corp.).

Ion Exchange Chromatography. A FPLC Mono Q-column (Pharmacia Inc.), attached to a Waters solvent delivery system (Waters Assoc.), was used. The column was equilibrated with equilibration buffer, and kept at 0°C. Three ml samples (from NADPH and NADH elutions from Blue Sepharose) were loaded, and the enzymes were eluted with a linear gradient of 0 to 400 mM NaCl and 4 mM EDTA in washing buffer (pH 7.0). The flow rate was 0.75 ml/min, and the elution volume was 24 ml. Active fractions were collected and concentrated on Centricon-30 concentrators (Amicon) and stored at −18°C.

SDS-PAGE. SDS-PAGE was done according to Laemmli (8). Sets of low and high mol wt SDS-PAGE standards were obtained from Bio-Rad. The gels were stained with Comassie brilliant blue R-250.

Enzyme Assays and Protein Determination. NR activities were determined as described earlier (20). Cyt *c* reductase activity was measured by increase in absorbance at 550 nm (22). Protein was measured by the Bradford method (2).

RESULTS

Separation of constitutive NADH(P)H:NR (pH 6.5) from constitutive NADH:NR (pH 6.5) plus inducible NADH:NR (pH 7.5) activity was achieved by sequential elution from Blue Sepharose with NADPH and NADH in the first step of purification (Table I). The Blue Sepharose yielded partially purified NR enzymes with specific activities of 1.45 (NAD[P]H:NR, pH 6.5), 3.26 (NADH:NR, pH 6.5), and 3.44 U mg^{-1} protein (NADH:NR, pH 7.5) (Table I). Recoveries from Blue Sepharose ranged from 17 to 28%, and the purification was 132- to 202-fold of the initial activities (Table I). Calculation of recoveries and fold-purification, however, are distorted by the fact that initial activities represent the sum of NR activity in a mixture of the isozymes, thus overestimating the initial activity of each isozyme.

FPLC Mono Q ion exchange column chromatography resulted in a nearly homogenous preparation of the NAD(P)H, pH 6.5 NR form with a specific activity of 12.8 U mg^{-1} protein, corresponding to a 1164-fold purification and a 15% recovery (Table I). The elution profile of NADH:NRs from the Mono Q column was complex (Fig. 1). Fractions 13 and 14 contained a NR which

Table I. *Purification of Nitrate Reductases from Soybean Leaves*

Step	Total Activity[a]	Specific Activity	Purification	Recovery
	units[b]	*units mg⁻¹ protein*	*-fold*	%
	constitutive NAD(P)H:NR (pH 6.5)			
Crude extract	26.6	0.011	1	100
Blue Sepharose	7.2	1.46	132	27
Mono Q	3.9	12.8	1164	15
	constitutive NADH:NR (pH 6.5)			
Crude extract	56.0	0.023	1	100
Blue Sepharose	9.7	3.26	142	17
Mono Q	2.4	18.8	817	4
	inducible NADH:NR (pH 7.5)			
Crude extract	36.4	0.017	1	100
Blue Sepharose	10.2	3.44	202	28
Mono Q	2.5	41.8	2459	7

[a] NAD(P)H:NR activity was measured at pH 6.5 with 100 mM KNO_3; NADH:NRs were measured at pH 6.5 or pH 7.5, as indicated, both in the presence of 10 mM KNO_3. [b] Unit = 1 μmol substrate converted min^{-1} at 30°C.

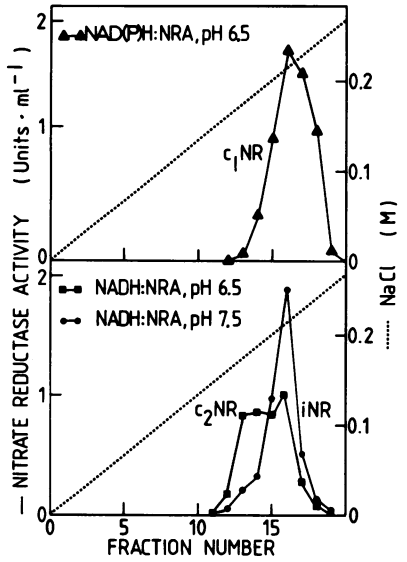

FIG. 1. Elution profile of soybean NRs from Mono Q columns. Separate Mono Q columns were loaded with fractions previously resolved on Blue Sepharose by sequential elution with NADPH and NADH. For details see "Materials and Methods."

was three to four times higher in activity at pH 6.5 than at pH 7.5. This is consistent with the properties of NADH, pH 6.5 NR, and thus we collected these two fractions. Nitrate reductase activity in fraction 15 was about the same when measured at low and high pH, indicating that this tube contained a mixture of both NADH NRs. Therefore, this fraction was not used. Fractions 16 and 17 were more active when assayed at pH 7.5 than at pH 6.5 and were collected as inducible NADH, pH 7.5 NR. Specific activities were 18.8 U mg^{-1} protein for constitutive NADH, pH 6.5 NR, and 41.8 U mg^{-1} protein for inducible NADH, pH 7.5 NR (Table I). Absorbance at 280 nm and 413 nm of the eluates could not be used to distinguish between constitutive and inducible NADH NRs, since the FAD present in the buffers bound to the column and co-eluted with the NRs as one broad peak (results not shown).

All three NRs had one predominant band on SDS-PAGE (Fig. 2). The NADH, pH 7.5 NR, however, appeared to migrate a

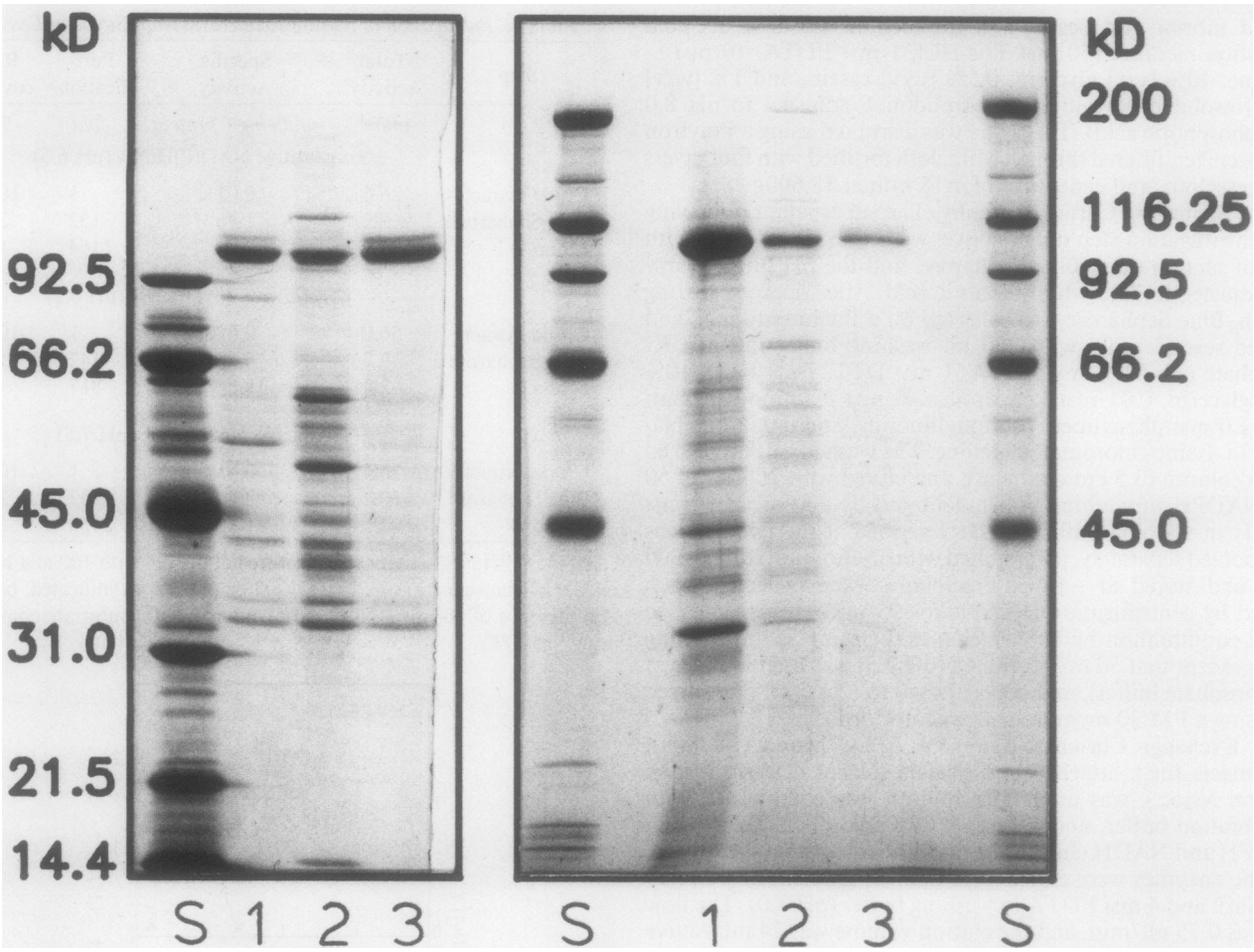

FIG. 2. SDS-PAGE of purified soybean NRs. S, Standards; lane 1, constitutive NAD(P)H:NR (pH 6.5); lane 2, constitutive NADH:NR (pH 6.5); lane 3, inducible NADH:NR (pH 7.5); all purified through FPLC Mono Q columns. Left panel shows low molecular mass standards and 10 μg protein lane^{-1} for NR forms (lanes 1, 2, and 3); right panel shows high molecular mass standards with 15, 10, and 7 μg protein lane^{-1} for NR forms (lanes 1, 2, and 3, respectively).

little slower, implicating a slightly larger subunit size. Although Figure 2 (right panel) indicates that constitutive NAD(P)H NR has a smaller subunit size than constitutive NADH NR, this effect is mainly due to overloading. Repeated runs on SDS-PAGE confirmed that constitutive NRs had approximately the same subunit size, while the inducible subunit was slightly larger. Estimation of relative molecular mass gave values ranging between 107 (constitutive NRs) and 109 kD (inducible NR). An impurity of about 34 kD and several fine bands were present in all preparations, especially in the constitutive NRs (Fig. 2). Specific activities varied considerably, inducible NR showed highest specific activity, and NAD(P)H NR the lowest (Table I).

Cyt *b* type heme was shown by UV/visible spectra to be a common component of the three NR isozymes from soybean (Fig. 3). The initial oxidized forms had peaks at 273 and 413 nm (Fig. 3). Addition of NADH reduced the enzymes, revealing peaks at 424, 527, and 556 nm, characteristic of *b*-type Cyt. Reoxidation of the enzymes with $NaNO_3$ generated the original spectrum in visible light (Fig. 3). The ratio of A_{273}/A_{413} was 1.9 for constitutive NAD(P)H NR, 3.5 for constitutive NADH NR, and 1.8 for inducible NADH NR (Fig. 3).

DISCUSSION

An improved separation and purification of soybean NR isozymes enabled us to further characterize the three NR isozymes which had been partially purified earlier (20). Blue Sepharose column chromatography (14) proved to be an excellent method for separation of NAD(P)H:NR from NADH:NRs. This rapid step (approximately 4 h) achieved a purification ranging from 132- to 202-fold. Separation on a FPLC Mono Q anion exchange column required only 30 min run time, and yielded a degree of purification ranging from 800- to 2500-fold. Moreover, a separation of NADH pH 6.5 and pH 7.5 NR forms was partially achieved. Previously published procedures failed to separate all three isozymes from soybean (4, 7, 14, 20), and only through use of the nr_1 mutant and two subsequent mutants was tentative identification of the three NR forms possible (10, 19, 20). The importance of a rapid purification procedure has previously been demonstrated in barley (3). It is likely that our method may prove successful for the purification of NR enzymes from other plants with a less labile enzyme.

SDS-PAGE indicated that all three NRs had similar subunit sizes (107–109 kD). It cannot be ruled out, however, that the minor 34 kD band is the subunit of the constitutive NRs. Additional studies such as Western Blot and further chromatographic techniques may clarify this (9). Soybean NRs, as most NRs from higher plants, are probably composed of two identical subunits, since sucrose density gradients and native PAGE gels indicated a molecular mass that was higher than SDS-PAGE (20). Also, inducible NADH (pH 7.5) NR from soybean has

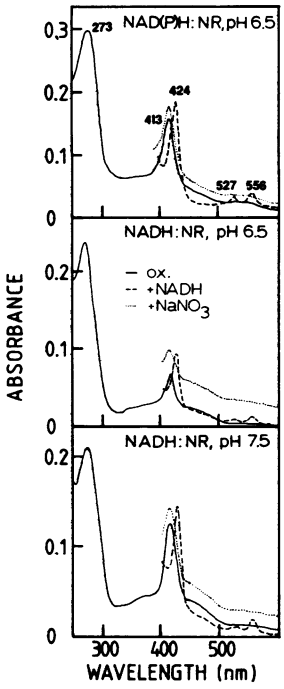

FIG. 3. Absorption spectra of purified soybean NRs. Enzymes from two purifications were combined in semi microcuvettes in a total volume of 0.5 ml. The initial spectra were zeroed against buffer and run against air as reference. The enzymes were reduced by addition of solid NADH, and reoxidized by addition of solid KNO_3.

similar R_F values to corn NR on native PAGE (P Robin, WH Campbell, personal communication). Thus, all three soybean NRs have similarities to NRs from other higher plants (13). A further indication for this hypothesis comes from the presence of Cyt b_{556} type heme in all three preparations (Fig. 3). Although subunit size of the soybean NRs were almost identical (Fig. 2), migration on native PAGE and sucrose density gradients was markedly different (14, 20). Inhibitor studies also point to differences at the nitrate reducing site of the native enzyme (10). We conclude that differences affecting charge and hydrophobicity of the apoprotein, rather than subunit size, are responsible for the different kinetic and physical properties among the three soybean NRs.

The relationship between the NR form induced in soybean cotyledons of norflurazon-treated, nitrate-grown soybean (21), to the three NR forms currently identified in leaves is unclear. The subunit relative molecular mass of norflurazon-treated, nitrate-grown soybean was 98 kD (21), which is less than any of the three NR forms we've studied. The cotyledonary NR (21) induced in the presence of norflurazon required nitrate for induction and, as such, resembles our inducible NADH NR form; however, our inducible NR form has a relative molecular mass of approximately 109 kD. The relationship between cotyledonary NRs and leaf NRs remains to be resolved.

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