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Short Communication

Purification of Squash NADH:Nitrate Reductase by Zinc Chelate Affinity Chromatography[1]

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ABSTRACT

NADH:nitrate reductase (EC 1.6.6.1) was isolated and purified from the green cotyledons of 5-day-old squash seedlings (*Cucurbita maxima* L.). The 10-hour purification procedure consisted of two steps: direct application of crude enzyme to blue Sepharose and specific elution with NADH followed by direct application of this effluent to a Zn^{2+} column with elution by decreasing the pH of the phosphate buffer from 7.0 to 6.2. The high specific activity (100 micromoles per minute per milligram protein) and high recovery (15–25%) of electrophoretically homogeneous nitrate reductase show that the enzyme was not damaged by exposure to the bound zinc. With this procedure, homogeneous nitrate reductase can be obtained in yields of 0.5 milligram per kilogram cotyledons.

The rate-limiting step in the assimilation of nitrate by higher plants is the reduction of nitrate to nitrite (3). In most plants, this two electron transfer is catalyzed by NADH:NR[2] (EC 1.6.6.1) (2). In addition to the physiological reaction, NR has several partial activities, including the reduction of nitrate with reduced viologen dyes and the reduction of artifical acceptors (Cyt *c*, ferricyanide, etc.) with NADH (3). NR is considered to be a large, complex protein, containing FAD, Cyt *b*, and Mo as prosthetic groups (2).

Affinity chromatography on blue-dye agaroses has proven to be a very effective means of purifying NR (1, 3, 6, 15). By combining affinity chromatography with conventional purification methods, algal and fungal NR have been obtained with specific activities of 90 to 225 units/mg protein (2, 3, 10). When higher plant NR has been purified using blue-dye affinity chromatography, specific activities of 2 to 10 units/mg protein were obtained for NR from corn and squash (1), wheat (11), and barley (4), with yields of 30 to 50%. The combination of blue-dye affinity chromatography with conventional methods resulted in a specific activity of 25 units/mg protein for spinach NR, but the yield was 7% (6). While the lower specific activity found for higher plant NR could mean this enzyme is of lower inherent activity than algal and fungal forms, it could also mean that higher plant NR is less pure. Gel electrophoresis has shown that squash NR purified using blue Sepharose is probably only 10% pure (12).

In 1975, Porath and coworkers (9) developed a new purification technique, which they have termed metal-chelate affinity chromatography. The affinity media consists of what is essentially a half EDTA molecule attached to Sepharose and will bind metal ions such as Zn^{2+} and Cu^{2+}. Proteins with high affinity for metal ions will bind to the metal-chelate column, and may be subsequently eluted by chelators or by changing the ionic strength or pH (9). Squash and other higher plant NR are inhibited by heavy metals and require chelators to protect the enzyme during purification (3, 6, 12, 14). As one demonstration of the affinity of squash NR for heavy metals, it was shown that the enzyme could be bound to and eluted from a Zn^{2+} column (14). We have found that a more specific elution of NR from the Zn^{2+} column may be achieved by lowered pH.

MATERIALS AND METHODS

Plant Material. Squash (*Cucurbita maxima* L. cv Buttercup) were grown in vermiculite as previously described (1). NR was induced by irrigating the plants with a Hoagland solution supplemented with 50 mM nitrate each of 2 d prior to harvest. The cotyledons were harvested 5 d after sowing.

Treatment of Buffer and Salts. Prior to their use for the preparation of NR, deionized H_2O, 1 M K-phosphate (pH 7.5), 4 M NaCl, and saturated ammonium sulfate were individually passed over 10-ml columns of 8-hydroxyquinoline-controlled pore glass (Pierce Chemical Co.). The treated solutions were stored in plastic containers which had been soaked in HCl and rinsed with treated deionized H_2O.

Gel Synthesis. Blue Sepharose was made as described (1). Biscarboxymethylamino-Sepharose 4B was synthesized by coupling iminodiacetic acid to oxirane-activated Sepharose (14). The Zn^{2+} columns were generated by passing 10 ml of 7 mM $Zn(SO_4)$ over a 2-ml gel column.

Enzyme Assays. NADH:NR, methylviologen:NR, and Cyt *c* reductase were assayed as previously described (12–14), except that cysteine was replaced with 50 μM EDTA.

PURIFICATION OF NITRATE REDUCTASE

All procedures were carried out at 4°C. The cotyledons (700–900 g) were blended in 200-g batches with 1 volume of extraction buffer containing 100 mM K-phosphate (pH 7.5), 1 mM EDTA, and 70 to 90 g of insoluble PVP. The slurry was passed through eight layers of cheesecloth, then centrifuged at 9000*g* for 20 min. The resulting crude extract was filtered through glass wool to remove lipid and mixed with sufficient blue-Sepharose (equilibrated with extraction buffer) to bind 80 to 90% of the crude extract NADH:NR activity. After stirring for 45 min, the blue-Sepharose was collected by vacuum filtration and washed batchwise successively with 3 × 300 ml extraction buffer containing 10

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[2] Abbreviations: NR, nitrate reductase; FAD, flavin adenine dinucleotide.

μM FAD, then 4 × 300 ml buffer A (100 mM K-phosphate [pH 7.5], 10 μM FAD). The blue-Sepharose was then packed into a 2.5-cm diameter column. NR was eluted with 100 μM NADH in buffer A. Fractions containing >0.3 unit/ml NR activity were loaded directly onto a 2 ml Zn^{2+} column (equilibrated with buffer A). After loading, the Zn^{2+} column was washed with 75 mM K-phosphate (pH 7.0) containing 1 M NaCl and 10 μM FAD. NR was eluted with the same buffer (pH 6.2). Fractions containing more than 4 to 5 units/ml NADH:NR activity were pooled. For storage, the purified NR was precipitated with an equal volume of saturated ammonium sulfate and kept at −20°C.

RESULTS AND DISCUSSION

The ideal tissue for large scale preparation of higher plant NADH:NR should be easy to grow in large quantities and be high yielding of a stable enzyme. The 5-d-old green cotyledon of squash may best meet these qualifications, since it is easy to grow and yields a crude extract relatively rich in a NR with a half-life of 17 h at 0°C. In contrast, the instability of NR from barley (4, 16), corn (1), and wheat (11) has interfered with purification efforts, while, on the other hand, the stable NR of spinach is less desirable because growth of large quantities of young seedlings is not easy (3, 6). Immunochemical assays have indicated that squash and spinach NR have only small differences in structural determinants, while corn is less similar but still closely related (13). Inasmuch as these NR are also closely related in biochemical properties, squash NADH:NR may be a form typical of those found in most higher plants (1–3, 12, 13).

The results of a two-step procedure for the purification of squash cotyledon NADH:NR are given in Table I. The initial purification step is similar to the batchwise procedure developed earlier (1), with a few modifications. A change necessary to permit use of the Zn^{2+} column was to omit EDTA from the final washes of the NR bound to blue-Sepharose. Because the absence of chelators leaves NR vulnerable to heavy metal inhibition (14), buffer and salts were passed over a 8-hydroxyquinoline-glass bead column. This treatment appeared to free the solutions of heavy metals, but this assumption has not been proven. Also, the amount of blue-Sepharose used is carefully adjusted so that not all of the NR in the crude extract binds, increasing competition for dye sites by NR and other enzymes. Finally, the blue-Sepharose has been washed more thoroughly than previously; a total of about 20 volumes of buffer in seven batches are used. These changes may account for the increased specific activity reported here for the blue-Sepharose purified NR (Table I) *versus* previous reports (1, 3).

As soon as the NR is eluted from blue-Sepharose and assayed, it is loaded onto the Zn^{2+} column, minimizing the time NR is standing. Since the Zn^{2+} column has high capacity for NR (about 100 units/ml gel) and gives a high yield, the resulting NR solutions contain about 100 μg protein/ml. The appearance of a reddish-orange band during the loading of the blue-Sepharose purified enzyme onto the gel illustrates the concentration of NR on these columns. The NR is not eluted by the wash with 1 M NaCl in buffer A, but is eluted when the pH of the elution buffer is adjusted to 6.2.

For seven different preparations of NR, the specific activity averaged 100 ± 20 units/mg protein, which is in the range reported for homogeneous preparations of algal and fungal NR (2). A 40

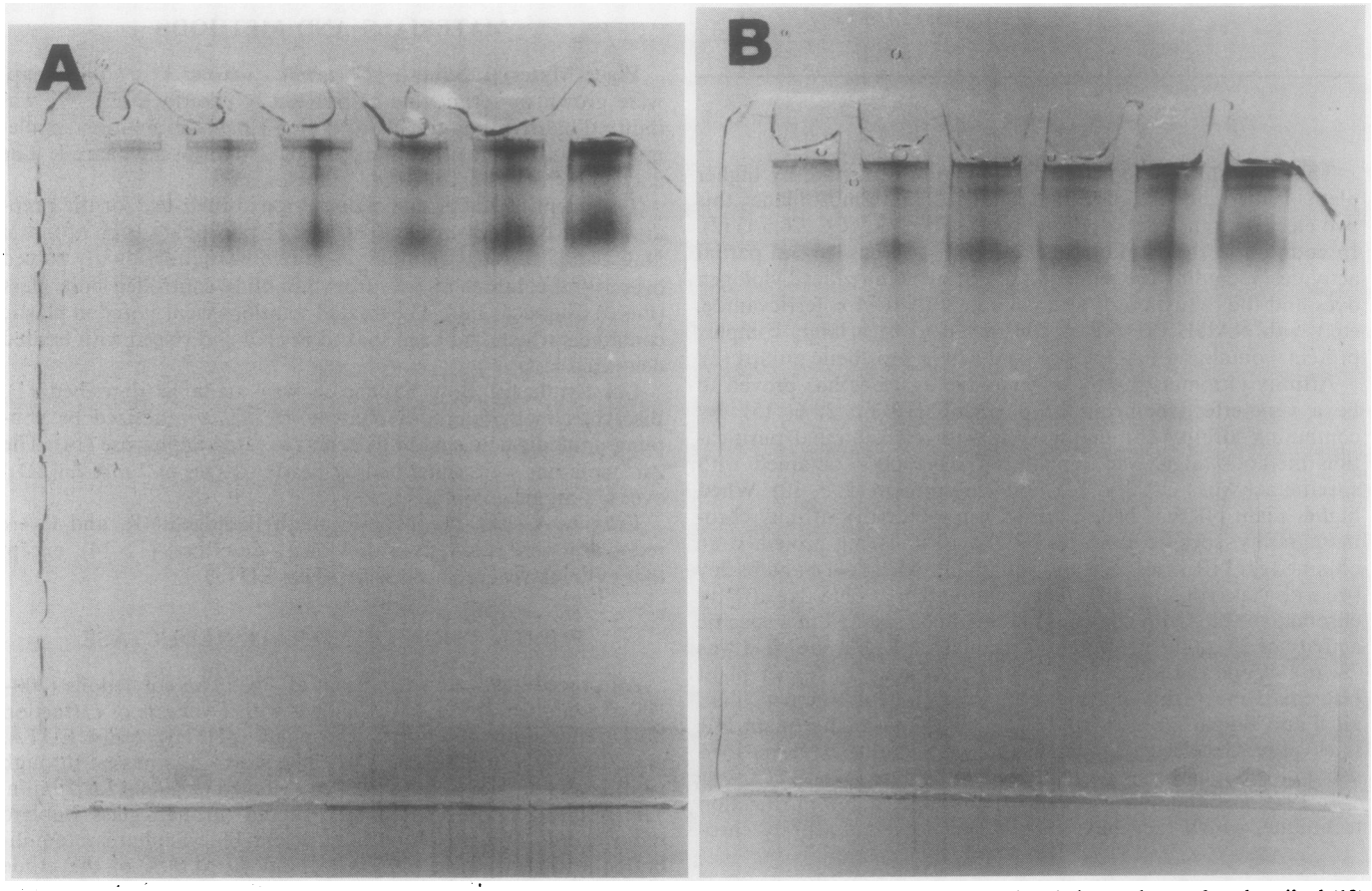

FIG. 1. Polyacrylamide gel electrophoresis of NADH:NR. Identical 7% gels with stacking gels were prepared and electrophoresed as described (12). The gels were stained for protein (A) with Serva blue R and for NADH dehydrogenase activity (B) with NADH and nitroblue tetrazolium (5, 12). NR (specific activity = 97 units/mg protein) was applied as measured amounts of protein of, from left to right, 1.5, 3, 4.5, 6, 7.5, and 15 μg.

Table I. *Purification of NADH:NR from Squash*

One unit of activity is 1 μmol nitrite formed/min at 30°C. Protein was estimated with a dye-binding assay (Bio-Rad Laboratories) using BSA as standard (Sigma fraction V).

Step	Total Activity	Specific Activity	Purification	Recovery
	units	*units/mg protein*	*-fold*	*%*
Crude extract	344	0.03	1	100
Blue Sepharose	186	19	575	54
Zn^{2+} column	86	110	3,300	25

to 50% recovery from each of the two steps means that an overall recovery of 15 to 25% is achieved, resulting in a yield of about 0.5 mg of NR/kg cotyledons. Gel electrophoresis was used to test purity of these high specific activity preparations (Fig. 1). The only protein staining bands found on these gels were also found to stain for NR activities, as shown for the dehydrogenase activity stain. Both bands showing dehydrogenase activity also stained for methylviologen:NR activity (gel not shown). The amount of NR applied to these gels was varied from 1.5 to 15 μg to illustrate the high degree of homogeneity (Fig. 1). The two-band NR pattern observed on these gels has been observed with highly purified barley and spinach NADH:NR (4, 6), and *Neurospora crassa* NADPH:NR (8). Recent analyses for mol wt of these NR bands on gels differing in amounts of acrylamide monomer have shown that the major band is 240 kD, while the minor band is 460 kD (5). Thus, the minor band may be a dimeric form of the major NR species and may reflect a tendency of highly purified NR to polymerize. The electrophoretic properties of these preparations of NR are under further investigation.

In addition to the electrophoretic characterization, consideration should be given to the stability and catalytic properties of these preparations of NR. For the most purified NR, Cyt *c* reductase activity (measured as 2 electron equivalents transferred/min) was 1.4 times greater than the NADH:NR, while methylviologen reductase activity was 2.1 times greater than NADH:NR. While no standard has been established for the ratios of partial activities to complete activity of NR, these ratios are similar to those reported for homogeneous algal and fungal NR (1, 2, 8, 10). If NR which had been frozen as the ammonium sulfate precipitate is reconstituted in solutions of 50 to 100 μg protein/ml, the NADH:NR activity has a half-life of 23 h at 0°C. While the enzyme appears to be slightly more stable in the presence of EDTA, the presence of a chelator is not a necessity, which indicates that Zn^{2+} is not a significant contaminant and did not leach from the column into the enzyme preparation. Similar results were found for rat liver nucleoside diphosphatase, another enzyme purified by metal-chelate affinity chromatogaphy (7).

In conclusion, we have developed a simple two-step procedure which allows for the isolation of approximately 500 μg of electrophoretically homogeneous NR from about 800 g squash cotyledons in less than 10 h. The specific activity of these preparations is about 100 units/mg protein, greater than any previously reported for higher plant NR. This procedure should allow us to perform the physical and structural studies of higher plant NR needed for a more complete understanding of nitrate reduction in higher plants.

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