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Regulation of Corn Leaf Nitrate Reductase[1]

I. IMMUNOCHEMICAL METHODS FOR ANALYSIS OF THE ENZYME'S PROTEIN COMPONENT

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ABSTRACT

NADH:nitrate reductase was extracted from corn leaves (*Zea mays* L. W64A × W182E) and purified on blue Sepharose. After the nitrate reductase was further purified by polyacrylamide gel electrophoresis, it was used to immunize mice and a rabbit. Western blots of crude leaf extracts were used to demonstrate monospecificity of the mouse ascitic fluids and the rabbit antiserum. The electrophoretic properties of purified corn and squash NADH:nitrate reductases in both native and denatured states were shown to be similar using western blotting with mouse ascitic fluid. The corn leaf enzyme has a 115,000 polypeptide subunit like that of squash. Western blots could detect 3 to 10 nanograms of nitrate reductase protein. But the detection of proteolytic degradation products using western blotting was inconsistent and remains to be established. An enzyme-linked immunosorbent assay (ELISA) was developed for quantifying nitrate reductase protein in the crude extracts of corn leaves. Using a standard curve based on nitrate reductase activity, the ELISA for corn nitrate reductase could detect 0.5 to 10 nanograms of nitrate reductase protein and was adequately sensitive for quantitative analysis of nitrate reductase in crude extracts of leaves even when activity levels were very low. When the ELISA was used to compare the nitrate reductase protein content of corn roots and leaves, these tissues were estimated to contain 0.24 to 0.5 and 4 to 5 micrograms nitrate reductase protein/gram root and leaf, respectively.

Higher plant nitrate reductase exists in several forms, but NADH:NR[2] is most common (3, 6). The biochemical properties of squash NADH:NR have now been described in detail (3, 5, 16). By combining our studies on squash with those on spinach and barley (11, 12), it can be concluded that native NADH:NR of both mono- and dicots is a dimeric enzyme composed of two identical subunits and each subunit contains one 110 to 115,000 polypeptide, one FAD, one heme-Fe (Cyt *b*) and one Mo-containing cofactor (Mo-pterin) (3, 16). Despite these recent advances in knowledge of NR biochemistry, the regulation of NR remains poorly understood. Two characteristics of NR contribute to the difficulty encountered when trying to study the enzyme's regulation. First, the complex structure of the enzyme and its well-known *in vitro* lability make it difficult to draw meaningful conclusions concerning enzyme synthesis and turnover just from activity measurements (6). Second, NR is a highly efficient catalyst with a specific activity for squash and spinach homogeneous NADH:NR found to be 100 μmol/min·mg protein (3, 12, 14, 16). Thus, a leaf need contain only a very small amount of NR in order to provide adequate capacity for reducing the available nitrate. Squash cotyledons and spinach leaves, even when highly enhanced in NR activity, appear to contain only a few μg of NR/g tissue (3, 12, 16). Biochemical methods are inadequate for evaluating these low levels of enzyme protein and the most effective approach would appear to be via immunochemistry.

Immunochemistry has been widely applied in the study of NR (6). Using immunoinactivation of NR activity as a comparative assay, higher plant NR were shown to have antigenic determinants in common which established these enzymes as homologous family having been derived from the same ancestral form (6, 19). Immunoprecipitation assays have been used to characterize and to quantify NR forms present in crude and purified fractions (6, 8, 20). In general, these assays lack the sensitivity needed to quantify small changes in NR in crude extracts and have only been useful in evaluating changes taking place over a period of many h or even d (20). These limitations can now be overcome by application of more sensitive immunochemical methods to the analysis of NR protein in crude extracts of plant tissues.

Western blotting is a recently developed immunochemical method with high sensitivity and great utility (9, 21). In western blotting, one takes advantage of the resolving power of nondenaturing and denaturing (SDS) PAGE and combines it with antibody-specific detection of antigens by transferring the resolved proteins from the PAGE gel to a nitrocellulose membrane (21). This method has been used for the study of NR polypeptides in NR-deficient mutants of soybean (18); for qualitative analysis of the synthesis and degradation of NR protein in barley leaves and roots (20); and to demonstrate the cross-reactivity of soybean NR with antibodies against *Chlorella* NR (23). Western blotting has permitted the estimation of the mol wt of the NR polypeptide in crude extracts (20, 23). Thus, western blotting appears, adequately sensitive to detect NR and, possibly, to evaluate the degree of intactness of the enzyme's polypeptide in order to detect proteolytic degradation of NR. Although western blotting has been used for quantitative analysis in some cases (9), its usefulness as a quantitative assay for NR protein has not been established.

A better immunochemical method for quantitative assays is the ELISA (6). The ELISA was originally developed for quantitative analysis of animal proteins in sera and other crude protein

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[2] Abbreviations: anti-NR, antibodies specific for nitrate reductase; ELISA, enzyme-linked immunosorbent assay; FAD, flavin adenine dinucleotide; IgG, immunoglobulin G; M_r, relative molecular mass; NR, nitrate reductase.

mixtures (7). Over the past several years, this laboratory has developed an ELISA for quantifying NR in crude extracts of squash using rabbit antibodies raised against squash cotyledon NADH:NR (3, 4, 6). This assay is designed after systems developed by Engvall and most directly from the ELISA described for serum ferritin (1, 7). The ELISA for NR is a solid-phase, double-sandwich type and noncompetitive, which eliminates the need for a large stock of pure antigen (4, 6). This method yields a low background and is not interfered with by peroxidase activity present in plant crude extracts (4, 6, and WH Campbell, unpublished data). The ELISA for NR can detect 1 ng or less of NR protein in squash crude extracts by using a standard curve prepared with homogeneous squash cotyledon NR of known protein content (3, 4, 6).

MATERIALS AND METHODS

Plant Material. Corn seeds (*Zea mays* L. W64A × W182E) from the Wisconsin Seed Foundation, Madison, WI, were imbibed with deionized H_2O for 24 h (120 g of dry seeds yields 100 g of leaf tissue at 5 d) and planted in vermiculite in a growth chamber (14 h day at 25°C). Light was provided by cool white fluorescent and incandescent bulbs with a total irradiance of 27 W/m^2 when measured from 380 to 750 nm with an ISCO SR spectroradiometer. A Hoagland solution (20 mM K-phosphate, 2 mM calcium nitrate, 1 mM $[NH_4]_2SO_4$, 1 mM $MgCl_2$, 0.2 mM Fe-EDTA, and micronutrients, supplemented with 35 mM NH_4NO_3) was given each of 2 d prior to harvest. Leaves were harvested when 5 d old and 4 to 6 h after the light period began.

Extraction, Purification, and Assay of NR. Extraction and purification procedures were carried out at 4°C. Blue Sepharose was synthesized and maintained as previously described (5, 6, 16). Corn leaves were blended at high speed in 100 g batches with 4 volumes of 0.1 M K-phosphate, pH 8.0, 7.5 mM cysteine, 1 mM EDTA, 0.01 mM FAD, 0.025% (w/v) casein, and 10 g polyvinylpolypyrrolidone. The resulting slurry was filtered and squeezed through 4 layers of cheese cloth and 1 layer of Miracloth and centrifuged. The crude extract was poured over glass wool, then added to an appropriate amount of blue Sepharose (1 g gel/4 g tissue), which had been equilibrated with 0.1 M K-phosphate, pH 7.5, 1 mM EDTA, and 0.01 mM FAD (buffer A). After stirring for 1 h at 4°C, the gel was collected by vacuum filtration and washed 4 times batchwise with 4 volumes of 0.1 M K-phosphate, pH 8.0, 3.5 mM cysteine, 1 mM EDTA, and 0.01 mM FAD. This was followed by two washes with buffer A. The blue Sepharose was resuspended in 100 ml of buffer A and packed into a 2.5 cm diameter column. The NR was eluted with 0.1 mM NADH in buffer A. Fractions with NADH-NR activity greater than 0.3 unit/ml were pooled. The NR was precipitated with 45% (w/v) $(NH_4)_2SO_4$ and stored at −20°C. The NADH:NR activity was assayed as previously described (5). One unit of NR activity was 1 μmol nitrite formed/min.

Gel Electrophoresis. Native- or nondenaturing-PAGE was done as previously described (16). Both tube gels (5 mm inner diameter × 80 mm) and slab gels (8 × 8 cm, 0.5 mm thick) were used. To stain for protein, the gel was fixed in TCA and stained with Serva blue R-250 (16). For detecting methyl viologen:NR activity, the gel was immersed in 21 mM K-phosphate (pH 7.5), 0.7 mM EDTA, 8.3 mM K-nitrate, 0.5 mM methyl viologen, and 2.8 mM sodium dithionite. Against a blue background, an achromic band quickly formed at the location of NR. The NR band was preserved by transferring the gel to 7.5% (w/v) triphenyltetrazolium chloride.

Denaturing- or SDS-PAGE was done as previously described (16). Partially purified NR and standards (Sigma mol wt 200-kit) were denatured with SDS as described (16). For crude extracts, leaves were extracted in 0.25 M Tris adjusted to pH 7.8 with 0.1 M phosphoric acid, containing 7.5 mM cysteine, 1 mM EDTA, and 0.01 mM FAD. To each ml of crude extract, 0.02 g SDS, 0.01 ml 2-mercaptoethanol, and 0.01 ml glycerol were added, and then denaturation was achieved by boiling in a water bath for 5 min. After electrophoresis, the gels were removed from the casing and prepared for western blotting (see below) or stained for protein as previously described (16).

Antibodies Specific for NR. The immunization of mice followed an established protocol (22). Partially purified NR was purified to homogeneity by native PAGE as previously described for squash NADH:NR (19). Twenty units of corn NR, which had been purified by the blue Sepharose procedure, were electrophoresed on native gels. The gel portion containing NR was located with the methyl viologen stain and excised by a conservative cut in order to avoid contaminating proteins. Just prior to injection, the excised gel was emulsified in a syringe with 9 volumes of complete Freund's adjuvant. The emulsion was injected intraperitoneally into an 8 week-old female mouse (strain A/J, Jackson Laboratories, Bar Harbor, ME). Each mouse was given 0.2 ml of the emulsion (4 units NR/mouse/injection). Injections were made on d 0, 14, 21, 28, and 35. Ascitic fluid was collected from the peritoneal cavity with a 19 gauge needle (22). Collections were made before the injection on d 21 and every 7 to 10 d thereafter until cessation of fluid production. Three to 10 ml of ascitic fluid/tap·mouse were collected. The fluid was centrifuged at 15,000g for 20 min at 4°C and stored at −20°C.

For the production of rabbit antiserum, 40 units of blue-Sepharose-purified NR was electrophoresed, excised, and emulsified as described above for mice. A previously unimmunized male New Zealand White rabbit (1–1.5 kg) was given 9 subcutaneous injections of 0.4 ml each on d 0 and 21. Complete Freund's adjuvant was used in the first injection, incomplete adjuvant was used on d 21. High titer for immunoinactivation of NR was obtained on d 35 and the rabbit was exsanguinated. The blood, to which 1% heparin had been added, was centrifuged and 50 ml of antiserum was obtained. The antiserum was stored at −20°C.

Purification of IgG and Preparation of Conjugate. The IgG fractions from mouse ascitic fluid and rabbit serum were prepared by affinity chromatography (10). Protein A Sepharose was equilibrated with 40 mM Na-phosphate (pH 8.0), 0.15 M NaCl, and 1 mM EDTA (PBS). Five ml of ascitic fluid or 10 ml of serum was applied to the column. After the column had been washed with 40 ml of PBS, IgG was eluted with 4 ml of 150 mM NaCl containing 0.58% (v/v) acetic acid (pH 2.7). Conjugation of IgG to horseradish peroxidase (Sigma Type VI, RZ = 3.1) was performed according to Wilson and Nakane (24). Peroxidase (4 mg) was resuspended in 1 ml deionized H_2O. Freshly prepared sodium meta/periodate (0.2 ml of a 10 mM solution) was added and stirred for 20 min. This solution was dialyzed against 1 mM sodium acetate buffer, pH 4.4, overnight at 4°C. Conjugation was begun by the addition of 0.02 ml of 0.2 M sodium carbonate, pH 9.5, to the peroxidase-aldehyde solution, followed by 4 mg IgG, adjusted to pH 9.5. The reaction mixture was stirred for 2 h at room temperature. The mixture was then reduced with 0.1 ml sodium borohydride (4 mg/ml) by standing for 2 h without stirring at 4°C. The conjugate was separated from any unreacted material on a 0.9 × 30 cm column of Sepharose 6B equilibrated in PBS (1). Fractions of 1.5 ml were collected and A at 403 and 280 nm were measured for the fractions. Fractions with a ratio of A_{403}:A_{280} between 0.3 and 0.6 were pooled, BSA was added to give a final concentration of 10 mg/ml, and 0.2 ml aliquots were stored at −20°C.

Western Blotting of NR. Methods used were modified from several sources (2, 9, 21). Samples (crude extracts or partially purified-NR) were electrophoresed as decribed above. The polyacrylamide gels were soaked for 0.5 h in chilled transfer buffer

consisting of 15.6 mM Tris, 120 mM glycine (pH 8.3), and 20% (v/v) methanol. Gels were placed in sandwich fashion in a gel holder as follows: 3 mm thick fiber pad, 2 layers No. 1 Whatman filter paper, gels, 1 sheet of 0.45 μm nitrocellulose membrane (presoaked in deionized H_2O), 2 layers of filter paper, and another fiber pad. The gel holder was secured and placed in a trans-blot electrophoretic transfer cell (American Bionuclear, Emeryville, Ca) such that the nitrocellulose was anodal to the gel. Transfer occurred at 80 V with cooling coil at 4°C for 3.5 to 4 h or at 10 V overnight. For blot development a series of incubations were undertaken. Buffers used depended on the source of anti-NR. For mouse ascitic fluid, 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl was used, with gelatin as the blocking protein. For rabbit antiserum, the buffer was 10 mM K-phosphate, pH 7.2, 0.15 M NaCl, with BSA as the blocking protein. Incubations were as follows:

a. The nitrocellulose with bound proteins was soaked in buffer for 10 min.

b. Unbound sites were blocked by incubation with 3% gelatin for 45 min or BSA overnight.

c. The nitrocellulose sheet was washed with water for 1 min, then transferred to 60 ml of antibody buffer containing 0.5 ml anti-NR and 1% BSA or gelatin. For mouse ascitic fluid overnight incubation was optimal, while for rabbit serum 5 h incubation was sufficient.

d. Unbound antibody was removed by washing twice for 10 min with buffer containing 0.05% Tween-20.

e. Goat anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugate diluted 1/3000 or goat anti-rabbit IgG-glucose oxidase conjugate diluted 1/200 was bound to the mouse or rabbit antibody in 1% gelatin or BSA buffer during a 3 h incubation.

f. The nitrocellulose sheet was washed twice for 10 min with buffer containing 0.05% Tween-20.

g. For color development of the horseradish peroxidase conjugate, a solution containing 60 mg 4-chloro-1-naphthol dissolved in 20 ml methanol and mixed with 0.015% H_2O_2 (w/v) in 100 ml buffer was incubated with the nitrocellulose sheet for 20 to 30 min, after which the reaction was terminated by washing the sheet with deionized H_2O. Alternatively for color development of the glucose oxidase conjugate, the nitrocellulose was incubated overnight in 0.17% glucose (w/v) and 5 mM nitro blue tetrazolium in 10 mM K-phosphate, pH 7.2, and 150 mM NaCl.

h. The nitrocellulose sheets were air dryed and preserved between polyester sheets.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA was developed from an ELISA for the analysis of squash NR (3, 4, 6, and WH Campbell, unpublished data). The ELISA was done as follows:

a. The wells of a 96-well NUNC 1 microtiter plate (Vangard, Neptune, NJ) were coated with 0.125 ml/well of 0.01 mg/ml rabbit IgG in PBS and incubated for 3 h at room temperature then stored overnight at 4°C.

b. Unbound IgG was removed by inverting the plate and washing wells with PBS twice.

c. Unbound sites were blocked with 0.25 ml/well of 0.5% (w/v) BSA in PBS (PBS) and left at room temperature 1 to 3 h.

d. The wells were washed twice with PBS and samples of 0.1 ml, appropriately diluted with PBA/PBS (1:1 mixture), were incubated in the wells for 2.5 h at 40°C.

e. Samples were removed and the wells were washed 3 times with PBS.

f. Diluted rabbit anti-NR IgG-horseradish peroxidase conjugate (0.125 ml/well) was incubated in each well for 2 h.

g. Conjugate was removed and wells were washed 3 times with PBS.

h. Color development was initiated by adding 0.1 ml/well of a solution containing 55 mg 2,2′-azino bis(3-ethyl-benzthiazoline sulfonic acid) diammonium salt (ABTS) in 100 ml of 0.1 M citric acid, pH 4.0, with 0.05% H_2O_2. After 12 min at room temperature, the reaction was terminated by adding 0.05 ml/well of 1 M citric acid, pH 2.0.

i. *A* at 405 nm was determined with a Bio Tek EIA plate reader (model EL 307) with an Apple II plus microcomputer for data acquisition and a BASIC program for data analysis.

Calculation of NR Protein. Since homogeneous corn leaf NADH:NR was not available for use as a standard for calibration of either the ELISA or the western blots, these immunochemical methods were standardized based on the NR activity of the crude extracts and purified enzyme preparations. However, in order to make a clear presentation of results, it is useful to express the results as amounts of NR protein. This was done by assuming that the specific activity of homogeneous corn leaf NADH:NR is 100 units/mg enzyme protein; thus 1 unit of NR activity is equivalent to 10 μg NR protein. Since both squash and spinach NADH:NR have a specific activity of 100 units/mg enzyme protein (12, 16), it is reasonable to expect corn leaf NADH:NR to approach this limit when purified to homogeneity.

Protein Determination. A dye-binding protein assay adapted for microtiter plates was used with a Bio Tek EIA plate reader and an Apple II plus microcomputer for data acquisition running a BASIC program for data analysis (15).

Sources of Chemicals. Biochemicals were obtained from Sigma Chemical Corp. Protein A-Sepharose and gels for molecular sieving were from Pharmacia Fine Chemicals. The supplies for PAGE, Tween-20 protein assay kit, and goat anti-rabbit and -mouse IgG horseradish peroxidase conjugates were from Bio-Rad Laboratories. Goat anti-rabbit IgG-glucose oxidase conjugate was from Cappel Worthington of Cooper Biomedical Inc., Malvern, PA. Complete and incomplete Freund's adjuvants were from Difco Laboratories. Deionized H_2O was used in the preparation of all solutions.

RESULTS AND DISCUSSION

Purification of NR. The purification of corn leaf NR to homogeneity has not been described, despite this goal having been achieved for NR from several other higher plants (6, 12, 16). Our objective in this study was to refine blue Sepharose affinity chromatography of corn leaf NADH:NR, which we had introduced several years ago (6), such that large quantities of tissue could be handled and high yield of high specific activity enzyme could be obtained. The NR was purified 800-fold to a specific activity of 20 units/mg protein with yields approaching 60%.

Production and Characterization of Antibodies for NR. For the immunization of animals, the blue-Sepharose-purified NR was purified to homogeneity in order to obtain monospecific preparations of antibodies. This was achieved by electrophoresing partially purified NR by native-PAGE and excising the gel portion containing NR as revealed by the methyl viologen:NR activity stain (19). Since polyacrylamide has no apparent adverse affect on the animals and may enhance antigenicity, gel containing NR can be emulsified with adjuvant and injected into the animals without the need for separating NR from gel. The essential criterion of this approach is that no other protein contaminant of the blue Sepharose preparations co-electrophorese with NR in native-PAGE. This had been demonstrated for squash NR in extensive PAGE studies of partially purified forms of this enzyme using gels of different acrylamide concentration (3, 6). When this type of analysis was done on corn leaf NR, we found that NR was not co-electrophoresing with another protein (data not shown). Corn leaf NADH:NR was estimated to have $M_r = 230,000 \pm 20,000$ by using four proteins of known mol wt and squash NADH:NR as standards (data not shown).

Mice were immunized with NR as an alternative to rabbits, which require large amounts of enzyme for immunization. After

succeeding in immunizing mice with corn NR, mice were also immunized with soybean NAD(P)H:NR which was available in only very small quantities (18). Thus the mouse system appears to be very useful for obtaining anti-NR against NR forms available in only small quantities. Strain A/J mice were selected since this strain has been reported to yield large volumes of ascitic fluid when immunized intraperitoneally (22). We obtained about 15 ml of high titer ascitic fluid per mouse (Table I). When the ascitic fluid was analyzed by the Ouchterlony double diffusion method, only a single precipitin band was obtained *versus* partially purified corn lean NR (data not shown). Thus, by this criterion monospecific antibodies had been obtained, but this is not a highly sensitive method and does not work with crude extract of plant tissues because the amount of NR is too low to yield immunoprecipitates. As will be shown below, western blotting and ELISA methods are better tests for establishing the specificity of antibodies.

A rabbit was immunized in order to obtain a large quantity of high titer antiserum for developing an ELISA for corn NR. Several rabbits had been previously immunized with squash NR and the same procedure was followed for corn NR (4, 5, and WH Campbell, unpublished data). After the subcutaneous injections of NR, the animal's serum was found to have a strong ability to immunoinactivate NR and subsequently, 50 ml of high titer antiserum was obtained. The specificity of this rabbit anti-NR preparation was determined as described below.

Western Blotting for Corn Leaf NR. The specificity of the mouse and rabbit anti-NR preparations was investigated using native- and SDS-PAGE western blots of blue-Sepharose-purified corn leaf and homogeneous squash cotyledon NR. These forms of NR were immuoprecipitated and immunoinactivated by anti-NR prepared against squash NR, thus they share antigenic determinants (19). Furthermore, squash NADH:NR has been characterized in detail and the M_r of the native enzyme and its subunit are known (16). Thus, squash NR could be used as an unambiguous comparative standard, which was needed since homogeneous corn NR was not available. Corn and squash NR were found to yield the same electrophoretic pattern when western blots were prepared from native- and SDS-PAGE gels (Fig. 1). The complex banding patterns observed in native-PAGE western blots of corn and squash NR (Fig. 1, A and B) result from the natural tendency of NR to exist as both dimer and tetramer, which can be separated by native-PAGE (3, 6, 16), and appear similar to native-PAGE gels stained for protein and NR activity which were previously published for squash NR (14). Since these polymeric forms of NR are non-covalent, SDS-PAGE of NR yields a single protein staining band when proteolysis has not occurred (3, 16), which appeared to be the case for the results presented in Figure 1C and 1D for denatured corn and squash NR. For squash NADH:NR, the M_r of the enzyme's subunit has been determined to be 115,000 (3, 6, 16) and the results of Figure 1,C and D, establish a similar M_r for the corn leaf NADH:NR subunit.

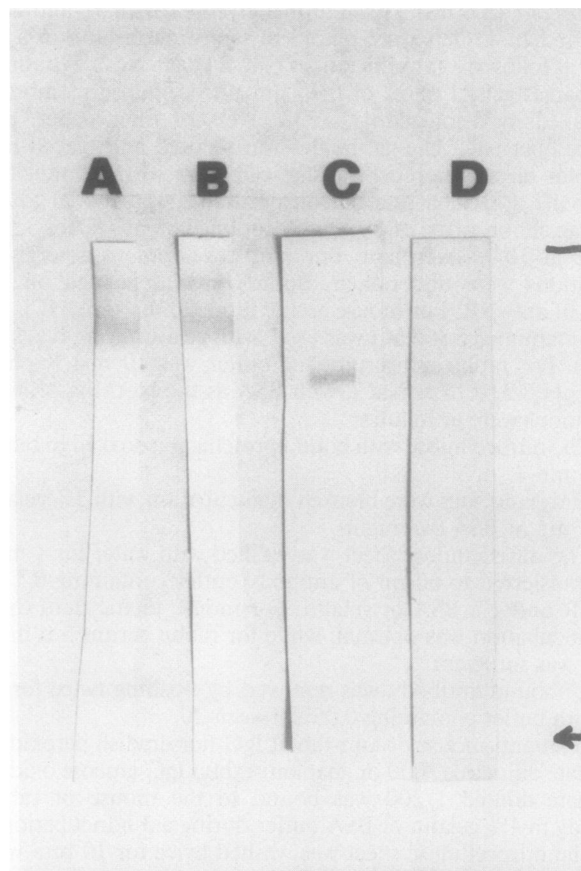

FIG. 1. Western blots of blue-Sepharose-purified corn leaf NADH:NR and homogeneous squash cotyledon NADH:NR. Native-PAGE western blots of 1.5 units of corn NR (A) and 1.0 unit of squash NR (B). SDS-PAGE western blots of 0.001 unit of corn NR (C) and 0.01 unit of squash NR (D). Mouse ascitic fluid anti-NR (corn) and goat anti-mouse IgG conjugated to peroxidase were used to develop the blots. Origin is marked by a dash and the front by an arrow.

Table I. *Collection of Mouse Ascitic Fluid: Volume and Titer*

Ascitic fluid titer was determined by diluting in PBS and incubating 0.22 ml with 20 μl purified NR (final activity = 0.1 unit/ml) for 10 min at 25°C.

Time of Collection	Volume[a]	Dilution for 50% Inhibition
d	*ml*	
0	11	1/200
4	8	1/300
6	12.5	1/1000
11	8	1/1200
14	9	1/1000
18	3	1/1100
21	1.2	1/500

[a] Pooled from 2 mice.

When crude extracts were electrophoresed on native-PAGE and western blotted, endogenous peroxidase activity of the corn leaf extracts yielded stained bands in addition to the NR bands, which was confirmed in controls where anti-NR was omitted from the blot incubations (Fig. 2, A and B). This was overcome by switching the second antibody-conjugate from peroxidase to glucose oxidase (Fig. 2, C and D). Thus, monospecificity of the antibody preparations was established by the single staining band found on native-PAGE western blots using crude extracts of corn leaf. Since the anti-NR preparations used in this study were prepared against native NR, the native-PAGE western blot of crude extract is a key method for determining specificity of these anti-NR. Only those mouse ascitic fluids and rabbit antiserum, which yielded a single band in native-PAGE western blots of crude extract, were used in this study. It is recommended that other investigators using immunochemical methods for study of plant proteins adopt a similar criterion for monospecificity of antibodies to be used.

Although we did not use western blots for quantitative determination of NR content of leaves, it was useful to assess the detection limit of this method. The limit for detecting NR in native-PAGE western blots of crude extracts was found to be 0.0003 unit of NR activity or about 3 ng of NR protein. This

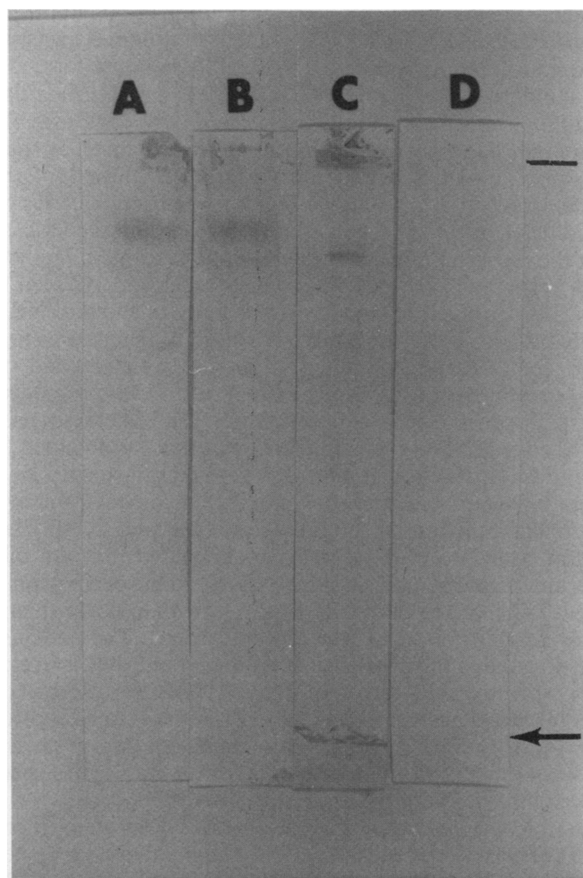

FIG. 2. Comparison of conjugates for native-PAGE western blots of crude extracts of corn leaves. Identical gels were prepared using 0.006 unit of crude corn leaf NADH:NR. Blots A and D served as controls and were incubated with 1% (w/v) BSA in PBS instead of anti-NR. Blots B and C were incubated with rabbit anti-NR serum. Blots A and B were developed with goat anti-rabbit IgG conjugated to peroxidase and stained by peroxidase assay. Blots C and D were developed with goat anti-rabbit IgG conjugated to glucose oxidase and stained by glucose oxidase assay. Origin is marked by a dash and front by an arrow.

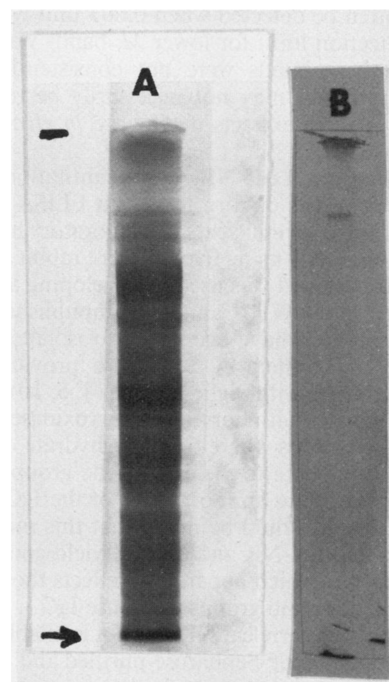

FIG. 3. Denatured crude extract of corn leaf on SDS-PAGE gel stained for protein (A) and SDS-PAGE western blot of an identical gel (B). The gel contained 50 μg of crude protein. Blot was developed with mouse anti-NR and goat anti-mouse IgG conjugated to peroxidase. Origin is marked by a dash and the front by an arrow.

high sensitivity is adequate to determine presence of NR protein in extracts which yield NR activities near the detection limit of the *in vitro* assay (6). In addition, it approaches the detection limit others have found for western blotting (9, 21).

In order to do SDS-PAGE western blotting on crude extracts of corn leaves, the extraction buffer had to be changed to eliminate potassium, which precipitates SDS, and this was accomplished by switching to the Tris-phosphate buffer. When mouse anti-NR was used to develop SDS-PAGE western blots of corn leaf crude extracts, only a single stained band was found (Fig. 3B). This band, which is identical in mobility to the band found in SDS-PAGE western blots of blue-Sepharose-purified corn and homogeneous squash NR, is the intact 115,000 polypeptide of the subunit of native NR. A similar conclusion was drawn for SDS-PAGE western blots of barley leaf and soybean cotyledon NR, although the M_r were designated 110,000 and 98,000, respectively (20, 23). When a SDS-PAGE gel of crude extract, which had been stained for protein, was compared to the blot of a similar gel, it could be concluded that the large mass of protein separating in the region of the NR band did not prevent detection of NR on the blot (Fig. 3). However, other investigators have noted that a biased transfer of proteins from the gel to the nitrocellulose takes place with greater efficiency of transfer for lower mol wt proteins (9, 21). We tested this by comparing SDS-PAGE gels stained for protein before transfer to identical ones stained after transfer. When we analyzed both crude extracts of corn leaf and mol wt standards, transfer was found to be biased in favor of low mol wt proteins (data not shown).

In some cases the 115,000 NR band on SDS-PAGE western blots could clearly be resolved into a doublet, which was especially true when rabbit anti-NR was used to develop SDS-PAGE western blots. Moreover, rabbit anti-NR revealed bands staining at lower M_r in blots of some crude extract samples. In general, lower M_r bands were only observed when large amounts of NR activity had been electrophoresed, but in some samples these low M_r bands were as intense as the high M_r bands. These additional bands may have resulted from proteolytic degradation of NR, which occurred either *in vivo* or *in vitro*. In SDS-PAGE gels of highly purified NR from squash, barley, spinach, and other higher plants, a doublet of bands at 100,000 to 120,000 have often been reported, and characteristic breakdown products of about 70,000 and 40,000 have also been found (6). The *in vivo* existence of degraded forms of the NR polypeptide has yet to be established. They were not found in the SDS-PAGE western blots of crude extracts of barley and soybean (20, 23). However, the ability to detect the degradation products of NR in crude extract SDS-PAGE western blots will depend on the sensitivity of the anti-NR preparations for sequential determinants of the NR polypeptide, which presumably are fewer in number in the degradation products.

Although the biased transfer of proteins in SDS-PAGE western blots limits the utility of this method for quantitation of NR protein, it is still useful to consider the detection limit. For mouse anti-NR, 0.0007 unit of NR activity was the detection limit for the intact NR polypeptide, which would correspond to 7 ng of NR protein. In general, more NR was required for clear detection of a doublet band with mouse anti-NR such that 0.002 to 0.003 unit was the limit of detection of the high M_r doublet (20–30 ng). For rabbit anti-NR, 0.001 to 0.002 unit of NR activity was required to detect the high mol wt band (10–20 ng), while a

doublet could often be detected when 0.003 unit was on the gel (30 ng). The detection limit for lower M_r bands was difficult to estimate, since these bands were not consistently found. In addition, their intensity may not necessarily be related to the activity of NR in these extracts if they are *in vivo* degradation products.

An ELISA for Corn Leaf NR. For quantitation of the NR protein in crude extracts of corn leaves an ELISA method was used. Over a period of several years this laboratory has developed an ELISA for squash NR using five different rabbit anti-NR sera and the experience gained was used in developing an ELISA for corn NR (3, 4, 6, and WH Campbell, unpublished data). Although several methods have been used to isolate IgG, affinity chromatography on protein A Sepharose provides the most consistent preparations with highest yields (4, 6, 10). For preparing anti-NR conjugates with horseradish peroxidase, the method of Nakane and coworkers where the carbohydrate of peroxidase is oxidized with periodate to yield aldehyde groups, which can then be coupled with free amino groups of the IgG, was found to be best (24). But it should be noted that this method is only effective with rabbit anti-NR and did not yield good conjugates with mouse anti-NR, which apparently reflects the low number or inaccessability of amino groups in mouse IgG.

Since homogeneous corn leaf NR was not available, the ELISA was standardized with blue-Sepharose-purified and crude extract NR (Fig. 4). Although the two standard curves were not exactly the same, the least square fitted lines were nearly parallel with slopes of 900 and 800 A_{405}/unit NR activity for purified and crude, respectively. Corn leaf crude extract NR was also analyzed with a large number of dilutions over a broader range of activities and a similar linear standard curve was found (Fig. 5). The decreased ELISA response to NR in crude extracts *versus* purified enzyme might be explained by matrix effects such as interference of crude extract components with the binding of NR to the anti-NR IgG coated to the plate. When squash crude extracts were compared to homogeneous NR, the ELISA response formed the same standard curve (3 and WH Campbell, unpublished data). This difference between squash and corn ELISA responses probably reflects the higher NR concentrations obtained in squash cotyledon extracts, which permits a greater dilution of these crude extracts, and the absence of additives from squash crude extracts, such as cysteine, which may interfer with the ELISA.

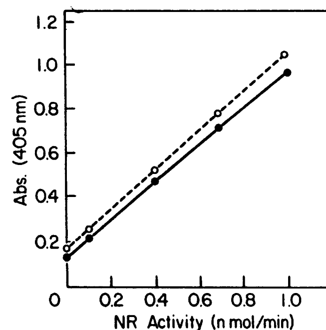

FIG. 4. ELISA standard curves for blue-Sepharose-purified NADH:NR and crude extract. Both the purified (O----O) and crude (●——●) NR were diluted to 0.1, 0.4, 0.7, and 1.0 munit/well using 0.005 g BSA/ml PBS. The anti-NR-peroxidase conjugate was diluted 1/500.

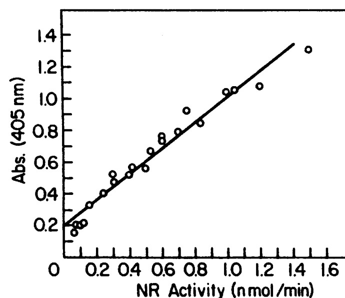

FIG. 5. ELISA standard curve for corn leaf crude extract. The crude extract NR was diluted from 0.06 to 1.5 munits/well using 0.005 g BSA/ml PBS. The anti-NR-peroxidase conjugate was diluted 1/500.

But the most important consideration is that the difference in ELISA response between corn crude extract and purified NR cannot be attributed to a lack of specificity of the anti-NR for the NR protein. If the rabbit anti-NR used in Figure 4 were not specific for NR and contained antibodies which would bind other components of the crude extract, the ELISA response to crude extract would have been greater than the response to purified enzyme. This type of ELISA response was obtained with one anti-NR, which had been prepared against squash NR thought to be pure but later shown to contain a contaminant (4). By comparing the results of our studies of the squash NR ELISA with the corn NR ELISA, we have concluded that the rabbit antiserum prepared against corn NR is monospecific for this antigen and that the corn NR ELISA can be used to quantify the NR protein found in corn crude extracts. The conclusion concerning the monospecificity of the rabbit anti-NR is in agreement with the results found in native-PAGE western blots described above. Thus, an ELISA can also be used to establish the specificity of an antibody preparation and probably offers sensitivity equal to the western blot in detecting antibodies specific for proteins other than the antigen of interest.

In terms of quantifying the amounts of NR protein in crude extracts of corn leaves, we faced a difficulty in that homogeneous corn leaf NADH:NR was not available for analysis in the ELISA. While it was adequate to use squash homogeneous NADH:NR for the qualitative comparisons made in western blotting, the cross reactivity is too low to be useful for quantitative comparisons (data not shown). Thus, the ELISA for corn NR can only be related to the amount of NR activity in extracts by standard curves such as shown in Figures 4 and 5. But for clarity in presentation of results, it is highly convenient to convert the activity based standard curve to an NR protein basis as described in the Materials and Methods. For the standard curves shown in Figures 4 and 5, 0.001 unit of NR activity would be equivalent to 10 ng of NR protein. The lower limit for detection of corn leaf NR by this ELISA is about 0.00005 unit or 0.5 ng of NR protein (Fig. 5). This sensitivity corresponds very closely to the limit found in western blots, but it is clear that the ELISA is more sensitive by nearly a factor of 10, which proved very useful in the physiological studies of corn leaf NR (17). Since the assay requires 0.1 ml of sample, the sensitivity range was from 0.0005 to 0.015 unit/ml (Fig. 5). This sensitivity range is adequate for the analysis of crude extracts containing NR activities as low as 0.001 unit/ml.

ELISA Analyses of Corn Root NR. The NR activity of corn roots is low as compared to the leaf on a fresh weight basis: 0.017 to 0.05 and 0.25 to 0.33 unit/g tissue for roots and leaves, respectively; but on a protein basis the two tissues are about equal: 0.009 to 0.02 and 0.011 to 0.025 unit/mg protein for roots and leaves, respectively (13). These data indicate that NR activity is present to the same degree relative to total proteins in both tissues, but roots contain about 14% of the protein found in leaves on a fresh weight basis. However, the activity in crude extracts of roots can be doubled by extracting in the presence of casein, which appears to stabilize NR activity in roots and leaves of corn (6). We compared the NR activities and ELISA determination of NR protein in extracts made in the presence and absence of 0.5% (w/v) casein in Tris-phosphate buffer containing

all the additives described in the Materials and Methods (Table II). These data were collected at a higher anti-NR-peroxidase conjugate concentration than the normal leaf assay (1/300 *versus* 1/500). This change made little difference in the slope of the ELISA response for leaf and root extracts (without casein), which averaged 550 and 540 A_{405}/unit, respectively, over conjugate dilutions of 1/300, 1/400, and 1/500. The casein containing extracts were increased by 70% in ELISA response at 1/300 as compared to 1/500 conjugate dilution with an averaged slope of 380 and 210 A_{405}/unit for leaves and roots, respectively. These results indicate that casein interferes with the ELISA assay of both leaf and root NR, but that this can be overcome to some degree by increasing the concentration of the anti-NR-peroxidase conjugate.

When the leaf extract with no casein is used as a standard, the leaves were estimated to contain 4 to 5 μg NR protein/g while roots contained 0.24 μg NR protein/g (Table II). The two root extracts had the same amount of NR protein/g root, but the casein containing extract had twice as much activity and its NR specific activity was twice as high (Table II). The results in Table II for NR protein content of the roots are based on assuming that leaf and root NR have identical antigenic determinants. Alternatively, root and leaf NR might have shared only some antigenic determinants leading to an underestimation of the NR protein content of the roots. The comparasion of ELISA slopes for leaf and root extracts containing casein indicates that root NR has about 50% of the antigenicity of leaf NR. Thus, the roots seem more likely to contain about 0.5 μg of NR protein. Moreover, the roots extracted without casein may contain inactive NR protein which retains its antigenic properties. However, it should be noted that the NR content of corn roots may be complicated by the presence of more than one form of NR (13). Spinach root NR was found to be about 20% as antigenic as spinach leaf NR when anti-NR against spinach leaf enzyme was used in immunoinactivation assays (8). Barley root NR could be recognized by barley leaf anti-NR in SDS-PAGE western blots (20). Thus, it appears that immunochemical assays, especially the ELISA, can be useful in analyzing root NR and have revealed that corn root has about 0.5 μg NR protein/g while leaves contain about 5 μg/g.

Table II. *Nitrate Reductase Activity and Protein from Corn Leaves and Roots*

Nitrate reductase protein (NRP) was calculated from the ELISA by assuming a specific activity for corn leaf NR = 100 units/mg protein.

	ELISA Slope	NR Activity	NR Protein	Specific Activity
	A_{405}/unit	*unit/g*	*μg/g*	*units/mg NRP*
I. LEAF				
No casein	540	0.47	4.7	100
With casein	470	0.42	3.6	120
II. ROOT				
No casein	570	0.022	0.24 [0.5][a]	90 [44]
With casein	260	0.048	0.24 [0.5]	200 [96]

[a] Data in brackets were calculated by assuming 50% cross-reactivity between root NR and leaf anti-NR.

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