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Short Communication

Immunochemical Characterization of Nitrate Reductase Forms from Wild-Type (cv Williams) and nr_1 Mutant Soybean[1]

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ABSTRACT

Soybean (*Glycine max* L. Merr.) leaves contain two forms of nitrate reductase (NR)—NAD(P)H:NR and NADH:NR. Wild-type (cv Williams), nr_1 mutant and an unrelated cultivar (Prize) were grown with either no N source or with nitrate. Crude extracts were assayed for NR activities and the enzyme forms were purified on blue Sepharose. Analyses were done by polyacrylamide gel electrophoresis and 'Western blotting' using antibodies specific for NR. NAD(P)H:NR was identified as the constitutive NR present in wild-type and Prize, but was absent from the mutant. All three soybean lines contained nitrate-inducible NADH:NR with highest activity at pH 7.5. The results showed that NAD(P)H:NR and constitutive NR were one in the same and confirmed the presence of NADH:NR with pH 7.5 optimum.

Evans and Nason (4) originally isolated and purified NR[3] from the primary leaves of soybean. They found soybean NR to use NADPH and NADH equally well and to have a pH optimum of 6. This enzyme has been designated NAD(P)H:NR (EC 1.6.6.2). Beevers *et al.* (2) reinvestigated higher plant NR and found that most plants contained NADH:NR (EC 1.6.6.1) with a pH optimum of 7.5. The pyridine nucleotide specificity of soybean NR was found to be influenced by extraction in the presence of cysteine (NADH-dependent activity was increased with cysteine), but the pH optimum was 6.25 with either electron donor. Most interestingly, the K_m for nitrate was decreased when soybean leaves were extracted in cysteine containing buffer (2). Jolly *et al.* (5) were able to separate and purify two forms of NR from the leaf extracts of soybean. One form was an NAD(P)H:NR with a pH optimum of 6.5 and high K_m for nitrate and nearly identical to the soybean NR isolated by Evans and Nason (4). The other was an NADH:NR with a low K_m for nitrate but a pH optimum of 6.5 and was not found unless cysteine or another thiol-reducing agent was present during extraction (5). Separation and purification of these two forms of soybean NR using blue-Dextran Sepharose provided a quick and easy way to obtain purified preparations of these enzymes (3).

Nelson *et al.* (6, 7) have generated and characterized a mutant of soybean which has decreased NR activity in young leaves. Based on growth of soybeans with either urea or nitrate, a physiological distinction has been identified between NR activities of soybean. When urea grown plants were analyzed, NR activity was found in the wild-type (cv Williams) but not in the mutant (nr_1) and this NR was called constitutive since it did not require nitrate for expression (6). Leaves from nitrate-grown nr_1 mutant plants had NR activity and it was called inducible since nitrate was required for expression. Most interestingly, the NR activity induced by nitrate in nr_1 has a pH optimum of 7.5 and a low K_m for nitrate (6, 7). Since nr_1 has normal xanthine dehydrogenase activity, inducible NR and, therefore, normal molybdenum-pterin cofactor, the mutation can not be an alteration of this cofactor (7). However, constitutive NR may contain an altered molybdenum-pterin cofactor since it has been found to be resistant to inhibition by tungstate at concentrations adequate to inhibit the inducible NR activity (1). The loss of constitutive NR activity in mutant nr_1 might be due to mutation of the enzyme's structural gene(s), one of its regulatory genes, or a gene for an unknown cofactor requirement.

An alternative means for characterizing proteins can be achieved via immunochemical methods when monospecific antibodies are available. Previously, monospecific antibodies raised against squash NADH:NR were used to inhibit the NR activity of soybean NAD(P)H: and NADH:NR as well as several other higher plant NADH:NR, which demonstrated these NR forms shared antigenic determinants and were derived from the same ancestor (10). Monospecific antibodies against soybean NAD(P)H:NR have been raised in mice and used to characterize soybean NR forms (9). This communication presents biochemical and immunochemical characterization of the NR forms present in wild-type and nr_1 mutant Williams soybean.

MATERIALS AND METHODS

Plant Material. Seeds of soybean (*Glycine max* L. Merr.) of wild-type (cv Williams), nr_1 mutant (previously LNR-2, see Ref. 6) and Prize (cv from W. A. Burpee Seed Co., Warminister, PA) were germinated in vermiculite in water and grown in growth chambers (6, 9). Each day the plants were subirrigated with nutrient solution: some plants were fed a normal nitrate-containing nutrient mixture and induced with 50 mM KNO_3 on the last 3 d of growth, while another group of plants were fed a nutrient

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[3] Abbreviations: NR, nitrate reductase.

mixture lacking nitrate or other N source but the concentration of ions was adjusted (6, 9). Unifoliolate leaves were harvested on the 8th d after sowing, which is chronologically younger than previously described material but this difference is due to growth chamber conditions (6).

Extraction, Purification, and Assays of Enzymes. Leaves were blended in 0.1 M K-phosphate (pH 7.4), 10 mM cysteine, 1 mM EDTA, 10 μM flavin adenine dinucleotide with 0.5% (w/v) casein (5 ml/g leaf). After centrifugation, crude extract was mixed for 15 min with blue Sepharose in a ratio of 1 g/3 g leaf. After NR was bound, the gel was washed seven times (5 ml/g gel each wash) with extraction buffer containing 1 mM DTT instead of cysteine and omitting casein. Washed gel was poured into a column and first eluted with 50 μM NADPH in wash buffer (3 ml/g gel) and then with 50 μM NADH in wash buffer (3 ml/g gel). Fractions with NR activity were pooled and precipitated by adding 273 mg $(NH_4)_2SO_4$/ml. NAD(P)H:NR was assayed with 0.2 mM NADPH and 100 mM KNO_3 in pH 6.5 K-phosphate, while NADH:NR was assayed with 0.2 mM NADH and 10 mM KNO_3 in pH 7.5 K-phosphate as previously described (3, 5). Protein was assayed with the Bio-Rad dye-binding method.

Gel Electrophoresis. PAGE was done with 7.5% acrylamide/ 2.7% cross-linker and stacking gels as previously described (8). After electrophoresis, gels were stained for protein and dehydrogenase activity or cut into 2-mm slices and assayed for NR activity (8, 9). One gel was sliced and one-half of each slice was assayed for NAD(P)H:NR and other half for NADH:NR. These assays were done by incubating slices in standard assay mixtures, to which 10 μM flavin adenine dinucleotide had been added, for 60 to 120 min and determining nitrite content after removing the gel slice.

Immunochemical Methods. Monospecific antibodies against NAD(P)H:NR were raised by immunizing mice with enzyme isolated in PAGE gel slices after purification on blue Sepharose (9). Monospecific antibodies against corn leaf NADH:NR were provided by J. Remmler. For 'Western blotting', crude extracts, which had been prepared without casein, and purified NR were electrophoresed by PAGE and, subsequently, electrophoretically transferred to nitrocellulose; then the nitrocellulose was incubated with specific antibody for either soybean NAD(P)H:NR or corn NADH:NR followed by incubation with an appropriate second antibody/peroxidase conjugate (Bio-Rad Laboratories, Richmond, CA) and stained for peroxidase activity (11, 12).

RESULTS

Crude extracts from leaves of wild-type, nr_1 mutant and Prize soybean grown with and without nitrate were assayed for NR activity with NADPH at pH 6.5 and NADH at pH 7.5 (Table I). The extracts from the wild-type grown with or without nitrate and the extract from nitrate-grown nr_1 mutant were purified using blue Sepharose affinity chromatography (Fig. 1). For wild-

Table I. *Nitrate Reductase Activities in Crude Extracts of Soybean Leaves*

Source	N Nutrition	Electron Donor (Assay pH)	
		NADPH (6.5)	NADH (7.5)
		nmol NO_2^- min^{-1} g^{-1} leaf	
Wild-type	None	92	73
Wild-type	Nitrate	340	305
nr_1	None	2	3
nr_1	Nitrate	2	82
Prize	None	58	45
Prize	Nitrate	315	315

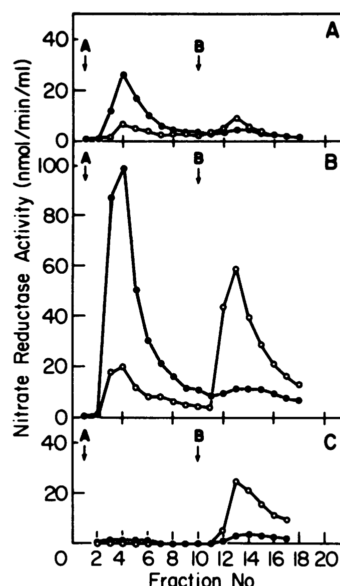

FIG. 1. Profiles for elution of soybean nitrate reductases from blue Sepharose. After the gel was loaded with enzyme, washed, and packed into a column, it was first eluted with 0.05 mM NADPH (arrow A) and then with 0.05 mM NADH (arrow B). NAD(P)H:NR and NADH:NR activities were assayed as described in "Materials and Methods" and are designated as NADPH/pH 6.5 (●——●) and NADH/pH 7.5 (○——○). A, Wild-type grown without N source. B, Wild-type grown with nitrate. C, nr_1 mutant grown with nitrate.

type plants grown without nitrate, NADPH eluted 30% of the original NR activity, while NADH eluted only 3% (Fig. 1A). The NADPH-eluted fraction had properties similar to those reported for NAD(P)H:NR; for example, it was most active with NADPH at pH 6.5. For wild-type plants grown with nitrate (Fig. 1B), NADPH eluted 20% of the original NADPH-NR activity, while NADH eluted only 0.4% of this activity. When the NADH-NR activity is considered, 6% was eluted by NADPH while 18% was eluted by NADH (Fig. 1B). This second enzyme form which is eluted by NADH from the blue Sepharose column is highly specific for NADH as electron donor and is most active at pH 7.5. Qualitatively similar results were obtained when Prize NR activities were purified on blue Sepharose (9).

For the nr_1 mutant grown on nitrate, the elution of the blue Sepharose column with NADPH yielded no NR activity (Fig. 1C). When the column was eluted with NADH, 30% of the original NADH-NR activity and about 30% of the original NADPH-NR activity was recovered. However, it should be noted that the nr_1 mutant was very low in NADPH-NR activity (Table I). The NR eluted by NADH was highly specific for NADH and most active at pH 7.5, which indicates this enzyme form is an NADH:NR like the one found in the NADH elution of nitrate-grown, wild-type leaf extracts (Fig. 1B). No attempt was made to purify the NR activities from the extracts of nr_1 mutant plants grown without nitrate because these activities were too low.

For nitrate-grown, nr_1 mutant leaf extracts, the NADH-eluted NR was concentrated by $(NH_4)_2SO_4$ precipitation, electrophoresed by PAGE and the resulting gels sliced into 2-mm sections. Assays for NADPH- and NADH-NR activity showed only one peak on these gels, which had an R_F of 0.2 relative to dye front. This R_F was the same as has been found for NADH:NR isolated from the Prize cultivar and, in fact, is about the same relative mobility found for NADH:NR from corn, spinach, and squash when electrophoresed in this system (8 and unpublished data). NAD(P)H:NR isolated from Prize has an $R_F = 0.3$ (9). Thus,

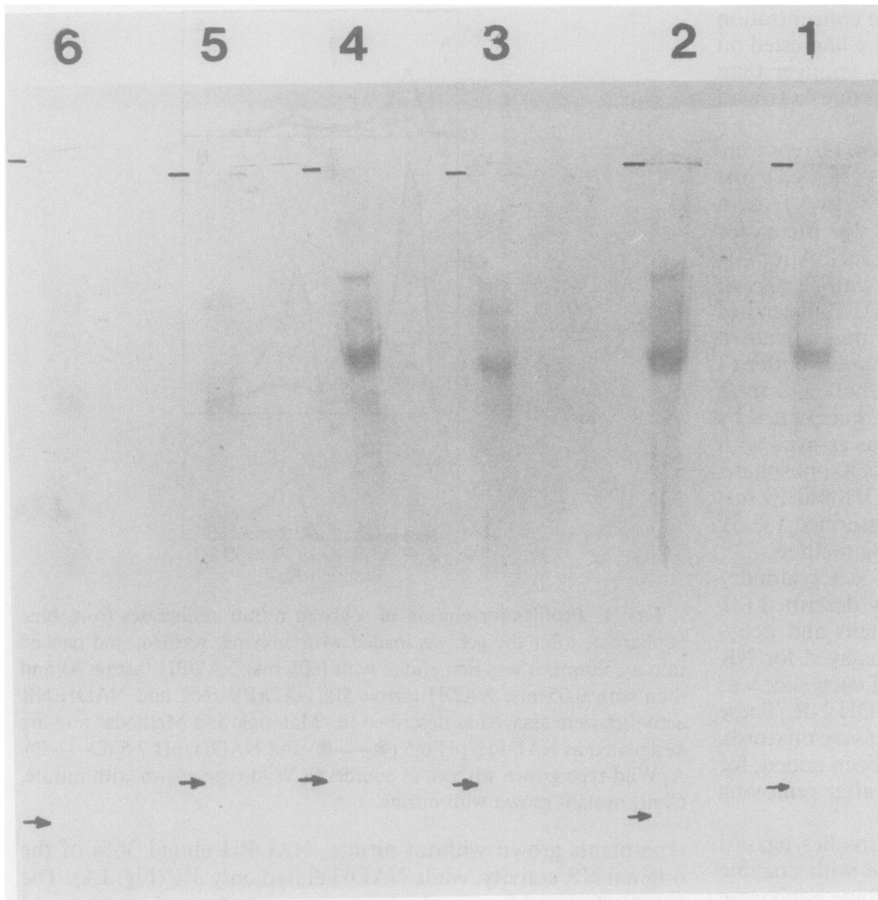

FIG. 2. 'Western blots' for crude extracts of soybean leaves. Sample volume was 0.1 ml and NR activity in nmol min^{-1} ml^{-1} is given in brackets. Lane 1, Prize grown without N [9]. Lane 2, Prize grown with nitrate [17]. Lane 3, Wild-type grown without N [4]. Lane 4, Wild-type grown with nitrate [13]. Lane 5, nr_1 mutant grown without N [0.5]. Lane 6, nr_1 mutant grown with nitrate [2.7]. For Prize and wild-type NAD(P)H:NR activity is given, while for nr_1 mutant the NADH:NR activity is given. The dash (—) near top of each lane indicates the origin of the gel, while the arrow near the bottom indicates the dye-front.

PAGE appears to be a useful system for separation of the two soybean NR forms.

Advantage of this ability of PAGE was taken in applying the immunochemical method known as Western blotting. In Figure 2, a Western blot comparing the crude extracts of Prize, Williams, and nr_1 is presented. For plants grown without nitrate, extracts of Prize (lane 1) and wild-type (lane 3) have only one band, which ran with an $R_F = 0.3$ and has been correlated with NAD(P)H:NR activity in gel slices of Prize (9). For plants grown with nitrate, extracts of Prize (lane 2) and wild-type (lane 4) have two bands: one with an $R_F = 0.2$ and one with an $R_F = 0.3$. The lower mobility band corresponds to the position of NADH:NR in these gels as was described above for gel slice experiments. Unfortunately, the nr_1 mutant plant extracts (lanes 5 and 6) yielded no clearly discernible bands with antibody against either soybean or corn NR.

Western blotting was also done with the purified NR fractions obtained from the blue Sepharose columns (Fig. 3). For wild-type plants grown without nitrate, the NADPH- and NADH-eluted fractions (lane 1 and 2) have only one band and it has an $R_F = 0.3$. For nitrate-grown wild-type plants, the NADPH- and NADH-eluted fractions (lanes 3 and 4) have two bands: one with an $R_F = 0.2$ and the other with an $R_F = 0.3$. The nitrate-grown nr_1 mutant extract eluted with NADH was only faintly stained (lane 5), but the ammonium sulfate concentrated sample from this fraction gave better staining (lanes 6 and 7). In lane 6, the strongly stained band has an $R_F = 0.2$ and also stains with the antibody against corn NADH:NR, which indicates this fraction contains mainly NADH:NR. However, in lane 7 where this fraction is applied at 5 times higher concentration, a band at higher mobility is clearly present. Although this second band has a mobility similar to that found for NAD(P)H:NR, very little activity was found in this region of the gel when assayed for NR. The NADPH elution of the column for nitrate-grown nr_1 mutant, which had no NR activity, was precipitated and assayed by Western blot; however, no stained bands could be detected (data not shown).

DISCUSSION

This paper is an attempt to reconcile the biochemical data on soybean NR activities with the physiological and genetic data. Briefly, the biochemical data show that soybean leaf extracts contain two forms of NR: an NAD(P)H:NR and an NADH:NR (3, 5). Both these NR forms had a pH optimum of 6.5 (3–5). In the physiologic/genetic experiments, generation of the nr_1 mutant confirmed that a constitutive NR was present in urea-grown plants of the wild-type but was absent from the mutant (6, 7). Furthermore, the mutant had nitrate-inducible NR activity, while both constitutive and inducible NR activities were found in nitrate-grown wild-type plants (6, 7). The nitrate-induced NR activity of nr_1 mutant leaves was active with NADH and had an optimum of 7.5 (7). Thus, it appeared that the mutant contained an NR form similar to the one found in the leaves of most plants, but one not previously found in the leaf extracts of soybeans.

Using physiological conditions different from previous studies (*i.e.* comparison of plants grown without N source to nitrate-grown plants), it was found that the nr_1 mutant had NR activity only when nitrate was present, while wild-type (cv Williams) and Prize soybeans had NR activity in the absence of N and increased levels when grown with nitrate (Table I). For purifying the NR activities in these extracts, an improved affinity chromatography method, utilizing blue Sepharose, was applied where both NADPH- and NADH-eluted fractions were obtained, which provided higher specific activity NR forms (9). For wild-type, the NADPH-eluted fraction was an NAD(P)H:NR which was

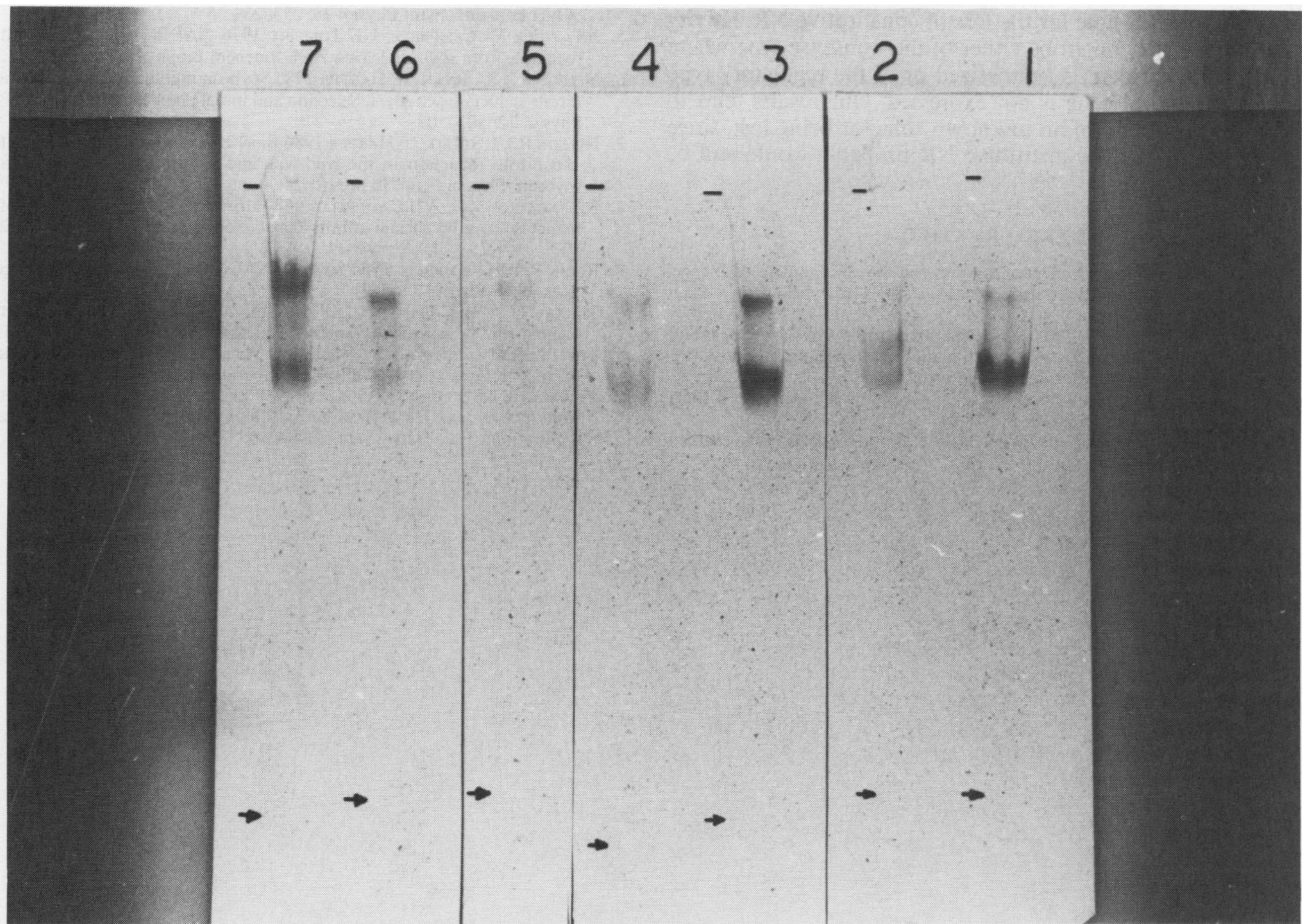

FIG. 3. Western blot for purified soybean nitrate reductases. Sample volume varied from 0.01 to 0.15 ml and NR activity applied is given, in brackets, as nmol min^{-1}. Lane 1, NADPH-eluted fraction from wild-type grown without N [300]. Lane 2, NADH-eluted fraction from wild-type grown without N [200]. Lane 3, NADPH-eluted fraction from wild-type grown with nitrate [1800]. Lane 4, NADH-eluted fraction from wild-type grown with nitrate [400]. Lane 5, NADH-eluted fraction from nr_1 mutant grown with nitrate [300]. Lane 6, Same as lane 5 except [600]. Lane 7, Same as lane 5 except [3000]. The same designations are used for origin and dye-front as in Figure 2.

most active with NADPH at pH 6.5, but this form was absent from the nr_1 mutant (Fig. 1). Thus, it is now clear that the constitutive NR present in wild-type and Prize is NAD(P)H:NR. For nitrate-grown plants of wild-type and nr_1 mutant, the NADH-eluted fraction was an NADH:NR which was most active with NADH at pH 7.5 (Fig. 1). The improved purification scheme permitted demonstration that the NADH:NR (7.5) type was present in not only the nr_1 mutant but also in the wild-type and an unrelated cultivar (Prize). However, it remains to be determined if there are two NADH:NR forms in soybean leaves (*i.e.* one with a pH optimum of 6.5 and one with pH 7.5 optimum).

These biochemical experiments were supported by immunochemical data obtained with antibodies against soybean NAD(P)H:NR and corn NADH:NR. Western blots showed that the wild-type and Prize soybean, which were grown without N, had only a single band of cross-reactive material and it electrophoresed with the same mobility as NAD(P)H:NR. When grown on nitrate, a second band of cross-reactive material was found and it had a mobility similar to NADH:NR. These results are consistent with a constitutive NAD(P)H:NR being present under all conditions of N nutrition and with an inducible NADH:NR being present only when nitrate is the N source. Thus, leaves of nitrate-grown soybean plants contain both enzyme forms, but the relative proportion will depend on age of the seedling and other factors (3–7).

In the immunochemical analyses of the nr_1 mutant, no cross-reactive material could be detected in crude extracts, which indicated that the pool of NR protein being made was small in concert with the low level of NR activity in these plants. Cross-reactive material was found in the purified NADH:NR from the mutant and it had an electrophoretic mobility consistent with the other NADH:NR from soybean. This purified fraction also contained another band of cross-reactive material, which was in a lesser amount than the NADH:NR and had an electrophoretic mobility similar to NAD(P)H:NR. Since the fraction eluted from blue Sepharose with NADPH contained no cross-reactive material, it is possible that any residual cross-reactive material derived from the mutant NAD(P)H:NR gene is eluting from the column with NADH. But the higher mobility material in lane 7 of Figure 3 could be the same band as stains at $R_F = 0.3$ in lanes 2 and 4, which are also NADH-eluted fractions. The origin of this material is unknown and requires further study since it could be breakdown products of NADH:NR or an artifact of the method.

For the nr_1 mutant soybean, it can be concluded that it contains an NADH:NR, which is similar in its biochemical and immunochemical properties to the same enzyme form in the wild-type and Prize soybean. The mutant contains no detectable NAD(P)H:NR activity and contains no cross-reacting material which purifies as if it were the NAD(P)H:NR of this tissue. Thus,

the mutation responsible for the loss of constitutive NR activity (*i.e.* NAD(P)H:NR) might be either of the nonsense type where no recognizable protein is synthesized or of the regulatory type where the structural gene is not expressed. Our results tend to rule out the possibility of an unknown cofactor being lost, since the apo-protein for the constitutive NR probably would still be made in that case.

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