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Stabilization of Nitrate Reductase in Maize Roots by Chymostatin[1]

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ABSTRACT

Nitrate reductase (NR) in maize (*Zea mays* cv W64A × W182E) roots has been stabilized *in vitro* by the addition of chymostatin to extraction buffer. Contrary to previous observations, levels of NR were higher in the mature root than in root tip sections when chymostatin was included in the extraction buffer. Two forms of NR were identified, an NADH monospecific NR found mainly in the 1cm root tip and an NAD(P)H bispecific NR found predominantly in mature regions of the root. During the first 10 days of seedling growth, NR activity in the root ranged from 50 to 80% of the activities found in the leaf (a maximum of 2.4 micromoles NO_2^- produced per hour per gram fresh weight was measured at 4 days).

Nitrate reductase (NR[3]:EC 1.6.6.1) is the first enzyme involved in the assimilation of nitrate in plants. It catalyzes the conversion of nitrate to nitrite. In maize (*Zea mays* cv W64A × W182E) roots a protease, referred to as CIP (corn inactivating protein), has been found to selectively inactivate NR (17, 19, 21, 22). CIP is the main protease in the mature portion of the root (20). It appears to be inactive in the root tip and leaf tissues. NR, on the other hand, has been found to be reasonably stable and to have higher recoverable activities in root tips and leaf tissues relative to mature root sections (1, 7, 8, 17, 20). CIP is stable over a greater pH and temperature range than NR. Thus, the low extractable NR activity and the apparent short half-life of NR in mature regions of the maize root (8) could be an artifiact of CIP activity.

A variety of methods have been used to try to stabilize maize root NR *in vitro* (18, 19). In particular, the addition of PMSF and/or casein (3%) to the extraction buffer has been found to confer some stability to the enzyme. We have identified a protease inhibitor, chymostatin, which effectively stabilizes NR in maize roots. Chymostatin is a low mol wt compound which has been isolated from culture filtrates of *Streptomyces* species (14–16). It has been found to inhibit many serine proteases, including chymotrypsin, and a few cysteine proteases. By using chymostatin in our extraction buffer we have been able to characterize some of the properties of NR in maize roots.

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[3] Abbreviations: NR, nitrate reductase; CIP, corn inactivating protein; NRA, nitrate reductase activity.

MATERIALS AND METHODS

Growth Conditions of Plants

Agar Grown Plants

Maize kernels (*Zea mays* cv W64A × W182E) were germinated in petri plates (15 cm diameter) containing 1% (w/v) agar made up in 1/10 Hoagland solution modified to contain 10 mM KNO_3. The plates were incubated in the dark for 48 h at 28 °C. The 3 cm long roots were well dried with paper towels and cut into two sections, a 1 cm root tip section and a mature section consisting of the remaining 2 cm next to the kernel. These sections were frozen in liquid N_2, ground to a fine powder with a mortar and pestle and kept at −70 °C for up to 3 d.

Hydroponically Grown Plants

Plants were germinated in the same manner as described above with the exception that KNO_3 was omitted from the germination media. The seedlings were then transferred to aerated hydroponic boxes (10 × 20 × 8cm) which contained 1 L of 1/10 Hoagland solution. Plants were grown with either continuous light or with a 16 h light/8 h dark cycle at 28 °C. A supplement of 10 mM KNO_3 was added where required. At harvest the roots were cut into three consecutive sections, a 1 cm tip, a section consisting of the adjacent 2 cm of the root and a third section that began 3 cm from the tip and included the rest of the root up to the kernel.

Nitrate Reductase Activity Assay

Tissue samples were extracted at 4 °C with the high pH Tris-HCl buffer developed by Kuo *et al.* (6). The extraction buffer included Tris-HCl (25 mM, pH 8.5), EDTA (1 mM), flavin adenine dinucleotide (20 μM), BSA (1% w/v), DTT (1 mM), and cysteine (10 mM). Leupeptin (10 μM) was added to buffer used to extract leaf tissue and chymostatin (10 μM dissolved in DMSO) was usually used to extract root tissue. When comparisons were made of different concentrations of chymostatin, DMSO alone was added to the extraction buffer of the control roots. One gram of frozen powder was ground with 4 mL of extraction buffer in a mortar. The extracts were centrifuged at 10,000*g* for 20 min at 4 °C, filtered through Miracloth, and kept on ice until measured. The assay mixture

Table I. *Stabilization of Root NR with Chymostatin*

Plants were grown with 10 mM KNO_3, harvested, and assayed as described in "Materials and Methods." The initial activities are presented in the first column under each root section heading.

Treatment	Nitrate Reductase Activity		
	1 cm	2–3 cm	Upper portion
	μmol NO_2^- produced·h^{-1}·g^{-1} fresh wt		
Agar grown plants			
NADH	1.26	0.17	
NADPH	0.26	0.03	
NADH and NADPH	1.29	0.09	
Hydroponically grown plants			
NADH	2.42 (22)[a]	2.08 (28)	2.13 (15)
NADPH	0.90 (15)	2.08 (30)	2.55 (23)
NADH and NADPH	2.04 (3)	2.31 (30)	2.59 (23)
Hydroponically grown plants + 10 μM chymostatin			
NADH	1.96 (4)	3.08 (5)	4.26 (0)
NADPH	1.17 (46)	2.62 (7)	4.25 (16)
NADH and NADPH	1.72 (1)	3.44 (1)	4.58 (10)

[a] The values in parentheses represent the percent loss of activity after 2 h on ice.

consisted of 0.2 mL Hepes buffer (0.65 M, pH 7.0), 0.2 mL KNO_3 (0.1M), and 0.2 mL extract. When NADPH activity was to be measured, 0.1 mL oxaloacetic acid (2.64 mg/mL H_2O) was also added to the reaction mixture. This addition enabled an NADH:malate dehydrogenase in the root extract to remove any NADH formed from NADPH by endogenous phosphatases. Water was added to bring the volume of the mixture up to 1.4 mL. The reaction was started with the addition of 0.1 mL NADH (3.6 mg/mL) and/or 0.1 mL NADPH (4.2 mg/mL) both of which were made up in 0.04 M KPO_4, pH 7.0. The mixture was incubated at 28 °C for 20 min. The reaction was stopped with the addition of 0.1 mL alcohol dehydrogenase (0.5 mg/mL 0.1 M KPO_4, pH 7.0) and 0.1 mL 2% (v/v) acetaldehyde. In our hands, alcohol dehydrogenase (Boehringer Mannheim) oxidized both NADH and NADPH. After 2 min, 1 mL of 1% (w/v) sulfanilamide in 1 N HCl and 1 mL of 0.01% (w/v) *N*-1-naphthylene-diamine-dihydrochloride in water were added to produce a color reaction with the NO_2^- produced by the assay. After 30 min the samples were measured spectrophotometrically at 540 nm. Each experiment was repeated at least three times and the assays performed within each experiment were done in triplicate. One unit of NR is defined here as the amount of NR which catalyzes the formation of 1 μmol of NO_2^- per hour. One unit of CIP is equivalent to the amount of CIP required to inactivate 1 unit of NR per hour.

Corn Inactivating Protein Inhibition Assay

Assays were set up with 0.2 mL water, 25 μL pure NADH:NR (0.5 unit) obtained from a blue Sepharose column (10), 10 μL CIP (1.5 units) prepared as described by Shannon and Wallace (12) and 1 μL of chymostatin at the required dilution (10 nmol to 0.01 pmol). Control tubes contained water, NR and either 10 μL CIP and 1 μL DMSO or just 1 μL DMSO. The tubes were mixed and incubated at 28 °C for 15 min. At this time the other components required for an NR assay were added and the tubes incubated for a further 10 min. Color development reagents were added and the results were measured spectrophotometrically. This experiment was repeated with similar results obtained.

Preparation of Antiserum

Crude antiserum that had been made against purified maize leaf NADH:NR (10) was used. After the blue Sepharose affinity step (10) this NR was further purified by passage over a DEAE-cellulose column followed by native gel electrophoresis before injection into the rabbits. The antiserum was precipitated by 45% saturation with ammonium sulfate at 4 °C for 30 min. It was then centrifuged at 10,000*g* for 15 min and the pellet was washed twice with 1.75 M ammonium sulfate. The pellet was resuspended in a volume of 10 mM $KP0_4$ (pH 8.0) equivalent to the initial volume of the crude antiserum, and then dialyzed overnight against the same buffer.

RESULTS

Stabilization of Root Nitrate Reductase Activity

Maize was grown under two different conditions. The first was on agar plates containing 10 mM KNO_3 for 48 h in the dark. At this age the roots were approximately 3 cm long. When the roots were cut into sections and assayed for NR activity, it was found that the majority of the extractable activity was in the root tip (Table I). This activity appeared to be predominantly from an NADH monospecific form of NR. A small amount of NAD(P)H bispecific activity was also present.

Alternatively, the kernels were germinated on agar plates in the dark in the absence of KNO_3. After 48 h seedlings were transferred to an aerated hydroponic system containing 1/10 Hoagland solution and 10 mM KNO_3 for an additional 24 h in the light. When NR activity (NRA) was measured in these

roots it was found that the NADH:NRA was similar in all sections of the root and that it was approximately double the activity found in the root tips of the agar grown plants. Higher levels of NADPH:NRA were found in the mature regions of the root relative to the root tip region. Because the NADH and NADPH:NRAs were not additive, the NADPH:NRA in the mature root sections is likely to be from a bispecific form of the enzyme.

A number of different protease inhibitors were tested in our system. One of these, chymostatin, greatly improved the stability of the NR obtained from all regions of the root (Table I). With this inhibitor NR activity was found to be higher in mature root sections than in root tips. When the extracts with chymostatin were left on ice for 2 h essentially all of the activity in the mature root sections was recovered. Without chymostatin, approximately 25% of the activity was lost over that period of time. Chymostatin appears to be less effective in stabilizing the root tip enzyme(s).

The optimal concentration of chymostatin required to stabilize root NRA was determined for the 1 cm tip and 2 to 3 cm mature root section of hydroponically grown plants. The NR activity of the 1cm tip assayed without chymostatin was set at 100.0 (Table II). All other activities are relative to this value. The addition of DMSO alone to the extraction buffer had little effect on the NRA. Additions of chymostatin had little effect on the NRA recovered from root tip sections. In the mature regions of the root, however, a large enhancement of NRA was seen. The highest activity was recovered when 10 μM chymostatin was included in the extraction buffer. Consequently this concentration was used routinely in preparations of root NR.

Inhibition of CIP by Chymostatin

Purified preparations of maize leaf NR (10) and maize root CIP (12) were used to examine the effect of chymostatin on CIP activity. A series of dilutions of chymostatin in DMSO from 10 nmol to 0.01 pmol were tested. From this experiment it was found that 10 pmol of chymostatin were required to inactivate 1.5 units of CIP (Table III). Wallace (18) required 7.5 mmol of PMSF to inhibit 1.5 units of CIP.

Table II. *Concentration of Chymostatin Required to Stabilize NADH:NRA in Maize Roots*

After germination maize seedlings were grown in a hydroponic system for 24 h with 10 mM KNO_3. The roots were harvested and assayed as described in "Materials and Methods."

Treatment	Relative NADH:NRA	
	1 cm	2–3 cm
Regular buffer	100[a]	94
+ DMSO alone	95	99
+ 1.0 mM chymostatin	110	150
+ 0.1 mM chymostatin	100	161
+ 0.01 mM chymostatin	107	242
+ 0.005 mM chymostatin	127	183

[a] The relative value of 100.0 is equivalent to 1.05 μmol NO_2^- produced h^{-1} g^{-1} fresh wt.

Table III. *Effect of Chymostatin on CIP Activity*

Purified preparations of NADH:NR from maize leaves and CIP from corn roots were incubated with or without chymostatin and then assayed for the remaining NRA. DMSO was present in all treatments.

Amount of Chymostatin	Nitrate Reductase Activity		
	NR	NR + CIP	NR + CIP + chymostatin
pmol	*μmol NO_2^- produced·h^{-1}*		
10	0.53	0.16	0.51
1	0.55	0.19	0.18

Inhibition of NR Activity by Antiserum

Antiserum made against maize leaf NADH:NR or preimmune serum was added in a series of dilutions to crude extracts from maize shoots and roots. The results are shown in Figure 1. The shoot extract was used undiluted and at a dilution of 1 part crude extract to 3 parts extraction buffer. Root activities were measured with either NADH or NADPH.

Figure 1 shows that both root and shoot activities are inhibited in a similar manner by the antiserum. This suggests that all of the enzymes, including the NAD(P)H form, have common epitopes. The preimmune serum had no effect on the NRA of either roots or shoots.

Root and Shoot NR Activity in Seedlings

Maize kernels were germinated on agar plates and transferred to a hydroponic system (day 2). They were then placed in a growth chamber with a 16 h light/8 h dark regime at 28 °C. At 24 h before harvest, KNO_3 was added to the medium to give a final concentration of 10 mM. This was done over a period of 10 consecutive days. The whole root and whole shoot above the coleoptile of the induced plants were harvested 7 h into the light period. Shoot NRA was shown to be higher than that of the roots, and to peak at 5 days of age (Fig. 2). In the root NADH:NRA peaked at 4 d. NADPH:NRA peaked just prior to that. The NRA in the roots reached up to 80% of the shoot activity at 4 d of age on a micromoles per gram fresh weight basis. In other experiments with plants grown in continuous 10 mM KNO_3 instead of a 24 h pulse, similar results were obtained (data not shown).

DISCUSSION

The presence of CIP has made the study of NR in maize roots difficult. Early studies with maize roots showed that NRA was highest in the root tip sections. The mature root (all tissue excluding the 1 cm tip) was found to contain very little NRA. In experiments designed to look at the turnover of NR, the enzyme in mature sections had a much higher rate of turnover than the tip enzyme (1, 8, 17, 19). Our results with the agar grown roots confirm these earlier findings. The use of a hydroponic system greatly increased NRA throughout the root, particularly in mature root sections. This may have been the result of both an increase in NO_3^- availability to the plant in a hydroponic system as compared to an agar plate, and the increased age of the plants. Stability was still a

problem though in the mature sections of these roots. The addition of chymostatin not only further increased the recovered levels of NRA in the mature root, but also stabilized the activity over a 2 h period. Although the effect of CIP on NR *in vivo* is not known, the specific cleavage of NR by CIP *in vitro* is well documented (2, 13, 17, 18, 19, 21, 22). Thus its inhibition by chymostatin probably explains the increased stability of NR in root extracts.

Two forms of NR in maize root crude extracts were first identified by Redinbaugh and Campbell (11). We also appear to have found the same forms: a stable NADH form and a more labile NAD(P)H form. The NADH form of NR was predominant in the root tip whereas the mature portion contained mostly the bispecific form. Though corn leaves do not contain a bispecific form of the enzyme, the NR isolated from this tissue appears to have epitopes that are similar to both forms of the root enzyme as determined by antiserum inhibition tests. Unlike our results, NAD(P)H forms of NR have been found to be immunologically different from their NADH monospecific counterparts in other species. For example, mutant barley leaves which lack the usual NADH:NR but contain an NAD(P)H form of NR in its place are not inhibited to the same extent by antibodies made to the wild-type NR (3, 5).

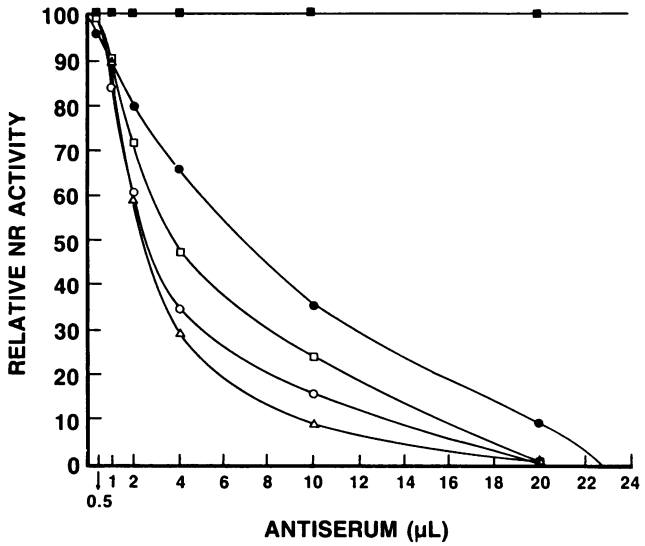

Figure 1. Inhibition of maize leaf and root NRA by antiserum made against purified maize leaf NR. Crude extracts were prepared from 6-day-old shoots or whole roots which had been grown hydroponically with 10 mM KNO_3. To 1 mL of crude extract 1 mL of diluted antiserum was added. The antiserum was brought up to 1 mL with 10 mM KPO_4, pH 8.0. The extract/antiserum mixture was left for 30min on ice and then assayed for NRA. Shoot extracts were used both undiluted (●) and at one-quarter strength (○) and measured for NADH:NRA. Root extracts were measured for their activity with either NADH (△) or NADPH (□). Both roots and shoots were checked for their inhibition by preimmune serum. Data for the root NADH:NRA values is shown (■). Values for shoot NRA and root NADPH:NRA were also insensitive to preimmune serum (data not shown). The NRA measurements of each extract after 30 min on ice without any antiserum was set to 100.0 and all other activities set relative to it.

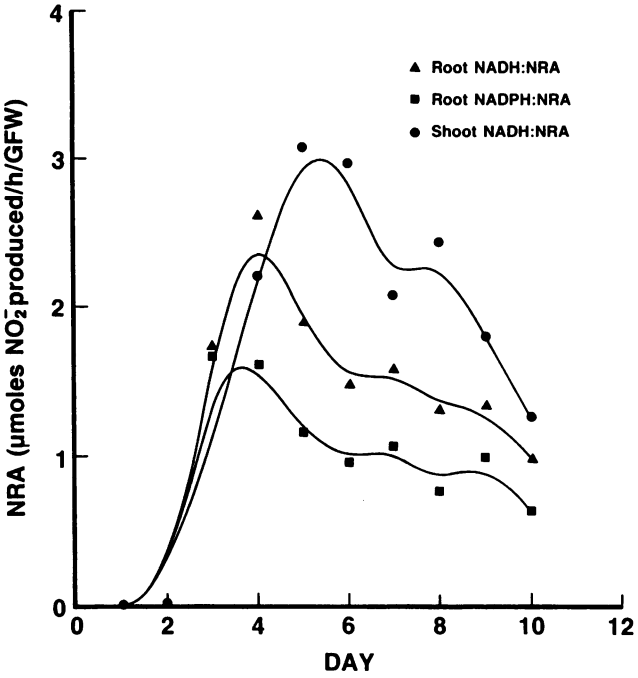

Figure 2. NR activity in maize seedlings. Plants were germinated on agar plates for 2 d and then transferred to a hydroponic system for a further 8 d. Twenty-four hours before harvest, KNO_3 was added to the growth media to give a final concentration of 10 mM. Whole roots or shoots were extracted as described in "Materials and Methods" and assayed for NRA.

The high proportion of root to shoot NRA found in seedlings suggests the importance of maize roots in nitrate reduction. Pate suggested that under steady state conditions approximately one-third of the nitrogen shipped to the shoots from maize roots was reduced (9). The results of Gojon *et al.* (4) using $[^{15}N]NO_3^-$ agreed with this figure. However, during induction they found that the roots were carrying out 70% of the whole plant nitrate reduction. Earlier results with maize roots (8, 19) indicated that there were very low levels of NR in maize roots making interpretation of these *in vivo* results difficult. The present experiments show, however, that roots do have the capacity to carry out substantial levels of nitrate reduction.

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