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NITRATE REDUCTASE INHIBITOR OF RICE PLANTS

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Summary

Two fractions of nitrate reductase inhibitor activities were found in extracts of primary and regenerated roots of nitrate-grown rice seedlings. The inhibitor was proteinaceous in nature and specific to nitrate reductase. The main site of action of the inhibitor was the NADH:cytochrome *c* reductase component of nitrate reductase. NADH was able to protect the NADH:nitrate reductase against the inhibitor.

Introduction

The presence of a nitrate reductase inactivating factor(s) has been reported in roots of corn seedlings [1—3], roots of rice seedlings [4] and suspension cultures of rice [5] and Paul's Scarlet Rose [6]. The inactivating factor in corn roots was a protein with a molecular weight of 44 000 [2]. Kadam et al. [4] suggested that in the rice plant the inactivating factor might play an important role in regulating nitrate assimilation in the plant. In this work the nitrate reductase inactivating enzyme in rice roots was isolated and its properties and mode of action were studied.

Materials and Methods

Plant materials. Rice plants (*Oryza sativa* L. var IR8) were grown in culture solution [7] in the greenhouse. Six days after the start of germination the primary roots were harvested and the seedlings left to grow in the same culture. After another six days the leaves and regenerated roots were harvested.

Leaf and root extracts. Leaves and roots were washed in cold distilled water and then macerated separately with chilled mortar and pestle in chilled grinding

media containing 0.1 M potassium phosphate (pH 7.5), 5 μM FAD and 1 mM cysteine hydrochloride. Each homogenate was filtered through 3 layers of cheese-cloth and centrifuged at 20 000 $\times g$ for 20 min. The 20 000 $\times g$ supernatant of the leaf extract was passed over a Sephadex G-25 column at 4°C. The eluate containing nitrate reductase activity was designated as fraction I. The nitrate reductase inhibiting enzyme of roots was obtained in the 40—60% saturation ammonium sulphate fraction of the root extract and was used for inhibitor studies.

Nitrate reductase and inhibitor assays. NADH:nitrate reductase and NADH:cytochrome *c* reductase activities were assayed by the methods of Wray and Filner [8]. Reduced benzyl viologen:nitrate reductase and $FMNH_2$:nitrate reductase activities were assayed by the methods of Garrett and Nason [9] and Paneque [10], respectively. Intact tissue nitrate reductase was assayed by the method of Jaworski [11].

Inhibitor activity was assayed by incubating a sample of the fraction I (adjusted to contain 100 ± 10 nmol NO_2^- produced/h per ml NADH:nitrate reductase activity) with the inhibitor preparation at 24°C for 10 min. The inhibitor samples were adjusted to contain nitrate reductase inhibitor causing 0—80% inhibition of nitrate reductase in the linear reaction range. Inhibitor activity was measured by comparing nitrate reductase activity before and after 10 min incubation and expressed as inhibitor units. One inhibitor unit is equivalent to 1% inhibition of nitrate reductase activity under the assay conditions.

Protein measurement. Protein was measured by the method of Lowry et al. [12].

Results and Discussion

Cell-free nitrate reductase activity of roots of rice seedlings was very low. After 12 h of induction cell-free NADH: nitrate reductase activity of secondary roots was 10 times lower than intact tissue root nitrate reductase activity (Fig. 1) and 40 times lower than cell-free nitrate reductase activity of leaves of the same rice seedlings. Fig. 1 also shows that the induction of cell-free NADH:nitrate reductase activity of roots reached a plateau after 4 h. In the case of aseptically cultured whole seedlings the induction curve of NADH:nitrate reductase was linear for about 18 h (Shen, T.-C., unpublished data).

A nitrate reductase inhibitor was precipitated by $(NH_4)_2SO_4$ fractionation of nitrate-grown rice root extract. The main fraction of nitrate reductase inhibitor activity precipitated at 40—60% $(NH_4)_2SO_4$ saturation (Table I). Higher inhibitor activity was recorded in nitrate-grown regenerated roots (317 inhibitor units/mg protein) than in nitrate-grown primary roots (119 inhibitor units/mg protein).

Sephadex G-200 chromatography of the 40—60% ammonium sulphate fraction of the root extract indicated the presence of two inhibitors in the regenerated roots of nitrate-grown seedlings (Fig. 2). Primary and regenerated roots of minus-nitrate-grown seedlings and primary roots of nitrate-grown seedlings did not show the complete development of the second inhibitor. This

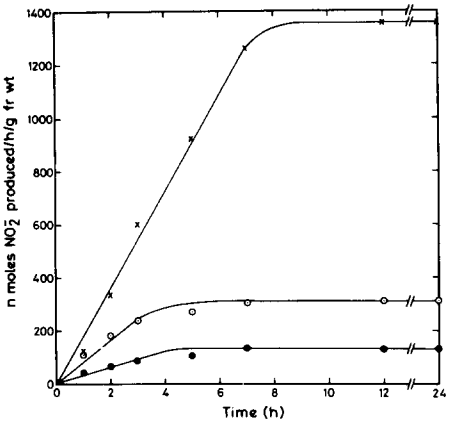

Fig. 1. The induction of NADH:, reduced benzyl viologen:, and intact-tissue:nitrate reductase activities in rice roots. Primary rice roots of the 5-day-old seedlings grown in a minus-nitrate culture medium were used for induction. At time zero they were transferred to the nitrate culture medium and the roots were aerated. Roots were harvested at various times for NADH (⊛), reduced benzyl viologen (⊙), and intact-tissue (X) nitrate reductase assays.

main inhibitor activity appears to be nitrate induced.

The inhibitor was completely destroyed after 5 min incubation at 100°C but could be stored at −5°C for 3 weeks with little loss of activity. Fig. 3 shows that the inhibitor retained 50% of its original activity after 10 min incubation at 64°C. Addition of bovineserum albumin did not protect NADH:nitrate reductase against the inhibitor. The inhibitor had no effect on the activities of α-amylase (rice grain) and lactate dehydrogenase (rabbit muscle). Wallace [3] demonstrated the degradation of casein by the maize nitrate reductase inactivating enzyme and suggested that the inactivation of nitrate reductase might be due to its proteolytic degradation. In the case of rice roots, though the inhibitor does not seem to be a general proteolytic enzyme, the possibility cannot be ruled out that it may act as a proteinase on nitrate reductase specifically.

It was found that the NADH:cytochrome *c* reductase and $FMNH_2$:nitrate

TABLE I

AMMONIUM SULPHATE FRACTIONATION OF AN EXTRACT OF NITRATE-GROWN REGENERATED ROOTS

The crude root extract was fractionated by $(NH_4)_2SO_4$ precipitation at various percentages of saturation. Each fraction was then assayed for nitrate reductase and inhibitor activities.

Saturation (%) $(NH_4)_2SO_4$	Nitrate reductase activity (nmol NO_2^- produced/h)	Inhibitor activity (units)
0— 25	60	0
25— 30	0	0
30— 35	0	17
35— 40	0	208
40— 60	0	911
60— 70	0	333
70— 80	0	50
80—100	0	0

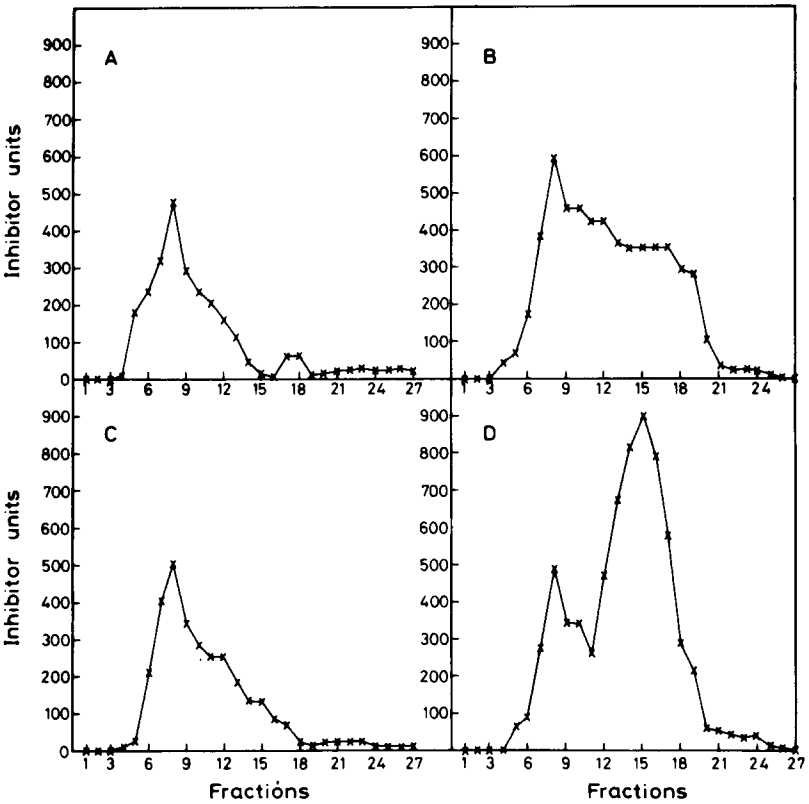

Fig. 2. Sephadex G-200 chromatography of the nitrate reductase inhibitor of rice roots. The 40—60% saturation $(NH_4)_2SO_4$ precipitates of roots were processed for Sephadex G-200 chromatography. (A) Primary roots of minus-nitrate-grown seedlings. (B) Primary roots of nitrate-grown seedlings. (C) Regenerated roots of minus-nitrate-grown seedlings. (D) Regenerated roots of nitrate-grown seedlings.

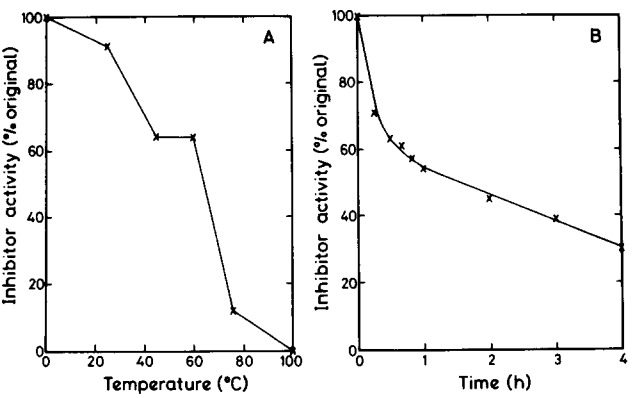

Fig. 3. Effect of temperature on nitrate reductase inhibitor activity. (A) The inhibitor was incubated for 10 min at each of the various temperatures before its activity was determined. (B) Change in activity of the inhibitor incubated at 40° C.

reductase activities were inhibited to the same extent as NADH:nitrate reductase by the inhibitor. However, reduced benzyl viologen:nitrate reductase activity was only very slightly affected. Wallace [13] reported that the nitrate reductase inhibitor from maize root inactivated the NADH:cytochrome *c* reductase activity at a greater rate than the $FADH_2$:nitrate reductase component. However, the same inhibitor preparation inactivated only the NADH: cytochrome *c* reductase of *Neurospora*. The above evidences suggest that the cytochrome *c* reductase component of nitrate reductase is generally inactivated by the inhibitor. However, the actual effect of the inhibitor on the $FADH_2$, $FMNH_2$ and reduced benzyl viologen:nitrate reductase activities may depend on the site of action of these hydrogen donors on the molecule which could differ in nitrate reductase of different origins.

During the course of our work we have found that when NADH:nitrate reductase activity of crude root extract was assayed under normal conditions there was a linear rate of nitrate reduction in the first 20 min of assay followed by a rapid inactivation of the enzyme. When a mixture of leaf NADH:nitrate reductase and the inhibitor was incubated with NADH and nitrate under the assay conditions, the same phenomenon was observed. Fig. 4B shows that when the NADH:nitrate reductase of leaf extract was pretreated with the inhibitor the former was inactivated by a zero order reaction of progressive increase in inhibition. NADH only partially protected the nitrate reductase from inactivation. Fig. 4A further confirmed the previous observation that during the first 20 min of the assay period there were linear rates of nitrate reduction followed by a rapid loss of the nitrate reductase activity. It did not show a progressive inhibition of the enzyme during assay. The kinetics of these changes do not appear to suggest that nitrate protected nitrate reductase during the assay period. However, an unknown mode of action of the inhibitor may be involved.

The activity of the maize root inhibitor was dependent on both the concentration of the inhibitor and the amount of nitrate reductase [2,3]. Our results

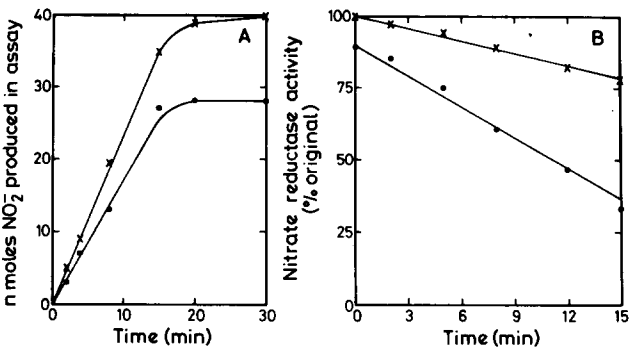

Fig. 4. Effect of NADH on inhibitor activity. (A) Leaf fraction I was incubated with root nitrate reductase inhibitor at 24° C for 10 min in the presence (X) or absence (•) of 0.1 mM NADH. After that the NADH:nitrate reductase activity in the mixtures was assayed at 30° C. Nitrite production during the 30 min of nitrate reductase assay is shown in the figure. (B) Leaf fraction I was incubated with the root inhibitor at 24° C in the presence (X) or absence (•) of 0.1 mM NADH. At various time intervals, aliquots were removed from the mixture for NADH:nitrate reductase assay. Enzyme assay was for 15 min. In both (A) and (B) the concentrations of NADH in the nitrate reductase assay mixture were adjusted to be equal for both ±NADH treatments.

showed however that the activity of rice root inhibitor was dependent on the concentration of the inhibitor but independent of the amount of nitrate reductase (Fig. 5). It appears that the mode of action of the rice inhibitor was such that a fixed amount or concentration of inhibitor brought about the same percentage of inactivation of nitrate reductase regardless of the activity of the latter. This suggested a competitive inhibition. However, it was found that the inhibitor did not act by competing with nitrate reductase for NADH or nitrate (Fig. 6).

Nitrate reductase of leaf fraction I precipitated mostly at 35—40% $(NH_4)_2SO_4$ saturation (Table II). After mixing a sample of leaf fraction I with a sample of root inhibitor preparation the total nitrate reductase activity from fraction I was reduced to 49% of the original activity in 10 min. When nitrate reductase in the mixture was precipitated with $(NH_4)_2SO_4$ the enzyme appeared mostly in the 30—35% saturation fraction, probably due to the presence of inhibitor in the 35—60% saturation fractions (Table II). At this stage the total nitrate reductase activity recovered was only 16% of the original activity. This low percentage of recovery could be due to the high activity of the inhibitor in its concentrated form after $(NH_4)_2SO_4$ precipitation. Attempts to separate mixed leaf nitrate reductase and root inhibitor by Sephadex G-200 chromatography were unsuccessful. Nitrate reductase eluted at the same fraction as the first peak of inhibitor activity. This suggests that the molecular weight of the inhibitor could be much higher than the molecular weight of the nitrate reductase inactivating enzyme of maize which was reported to be 44 000 [3].

Due to the lack of success in separating nitrate reductase from the inhibitor, it is difficult to evaluate the nitrate reductase activity in rice roots, or whether the inactivation of nitrate reductase by the inhibitor is a reversible process. Addition of ferricyanide to inhibitor-treated preparations of nitrate reductase did not reactivate the nitrate reductase.

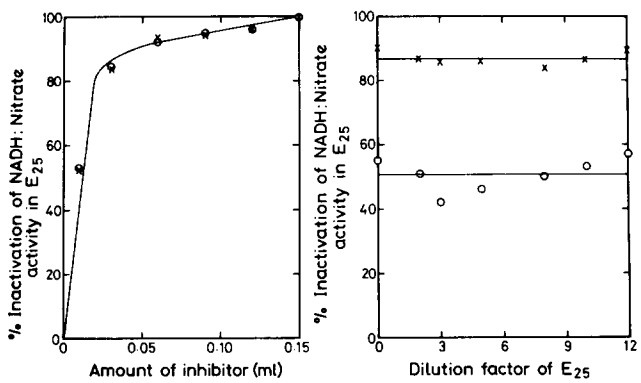

Fig. 5. Inactivation of leaf nitrate reductase by root extract. (A) Two levels of leaf fraction I, 120 nmol NO_2^- produced/h per ml (X), and 60 nmol NO_2^- produced/h per ml (○) were incubated with various amounts of root inhibitor extract at 24°C for 10 min followed by NADH:nitrate reductase assay. The original inhibitor extract contained 1.8 mg protein/ml. As control, fraction I was pretreated with phosphate buffer alone. (B) Various nitrate reductase preparations ranging in activity from 1200 nmol NO_2^- produced/h per ml (0 times dilution) to 100 nmol NO_2^- produced/h per ml (12 times dilution) were incubated with 0.1 ml (X) and 0.025 ml (○) root inhibitor preparation, followed by NADH:nitrate reductase assay. As control, various dilutions of fraction I were pretreated with phosphate buffer alone.

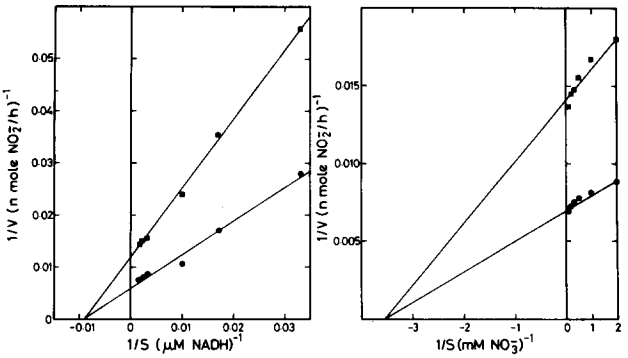

Fig. 6. Double reciprocal plots of nitrate reductase activity versus NADH and KNO_3 concentrations. Leaf fraction I was assayed for NADH:nitrate reductase with various amounts of NADH (A) and KNO_3 (B), in the presence (■) or absence (●) of inhibitor. In each case nitrate produced was determined after 5 min incubation.

As mentioned earlier reduced benzyl viologen:nitrate reductase activity was only inactivated by about 12% by the inhibitor. It appears that the actual nitrate reductase activity in rice roots could be of the same order of magnitude as the reduced benzyl viologen:nitrate reductase activity shown in Fig. 1. The in vivo nitrate reductase activity (Fig. 1) would then be about 3 times higher than the estimated nitrate reductase activity of about 450 nmol NO_2^- produced/h per g fresh wt. It was reported by Ferrari and Varner [14] that with the addition of alcohol the measured in vivo nitrate reductase activity was about 1.5 times higher than the measured in vitro NADH:nitrate reductase activity of the tissue. However, this still shows that the nitrate reductase activity of rice roots is very low. There is also no indication whether the low level of nitrate reductase

TABLE II

AMMONIUM SULPHATE FRACTIONATION OF RICE LEAF NITRATE REDUCTASE PREPARATION BEFORE AND AFTER TREATMENT WITH ROOT NITRATE REDUCTASE INHIBITOR

A preparation of leaf fraction I was divided into two portions. One was fractionated by $(NH_4)_2SO_4$ precipitation. The other was mixed with a Sephadex G-25 fraction containing nitrate reductase inhibitor from nitrate-grown roots. After 10 min at 24°C the mixture was fractionated by $(NH_4)_2SO_4$ precipitation and the NADH:nitrate reductase and inhibitor activities in each fraction were determined.

$(NH_4)_2SO_4$ Saturation (%)	Nitrate reductase activity of untreated fraction I (nmol NO_2^- produced/h)	Nitrate reductase and nitrate reductase inhibitor activities from mixture of fraction I and root extract	
		Nitrate reductase activity (nmol NO_2^- produced/h)	Inhibitor activity (units)
0— 25	133	33	0
25— 30	120	47	198
30— 35	600	150	230
35— 40	4200	80	390
40— 60	1520	0	1200
60— 70	13	0	370
70— 80	0	0	66
80—100	0	0	0

was due to a low rate of synthesis or to irreversible inactivation of the enzyme by the inhibitor.

References

1 Pan, Y.T. and Marsh, H.V., Jr. (1972) Plant Physiol. 49, S274
2 Wallace, W. (1973) Plant Physiol. 52, 197—201
3 Wallace, W. (1974) Biochim. Biophys. Acta 341, 265—276
4 Kadam, S.S., Gandhi, A.P., Sawhney, S.K. and Naik, M.S. (1974) Biochim. Biophys. Acta 350, 162—170
5 Yamaya, T. and Ohira, K. (1976) Plant Cell Physiol. 17, 633—641
6 James, D.M., Abbott, A.J., Hewitt, E.J. and Clements, E. (1976) Rep. Long Ashton Res. Stn. for 1976, 61—62
7 Shen, T.C. (1969) Plant Physiol. 44, 1650—1655
8 Wray, J.L. and Filner, P. (1970) Biochem. J. 119, 715—725
9 Garrett, R.H. and Nason, A. (1969) J. Biol. Chem. 244, 2870—2882
10 Paneque, A., Del Campo, F.F., Ramirez, J.M. and Losada, M. (1965) Biochim. Biophys. Acta 109, 79—85
11 Jaworski, E.G. (1971) Biochem. Biophys. Res. Commun. 43, 1274—1279
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Wallace, W. (1975) Biochim. Biophys. Acta 377, 239—250
14 Ferrari, T.E. and Varner, J.E. (1970) Proc. Natl. Acad. Sci. U.S.A. 65, 729—736