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A Nitrate Reductase Inactivating Enzyme from the Maize Root[1]

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ABSTRACT

The nitrate reductase in the mature root extract of 3-day maize (*Zea mays*) seedlings was relatively labile *in vitro*. Insoluble polyvinylpyrrolidone used in the extraction medium produced only a slight increase in the stability of the enzyme. Mixing the mature root extract with that of the root tip promoted the inactivation of nitrate reductase in the latter. The inactivating factor in the mature root was separated from nitrate reductase by $(NH_4)_2SO_4$ precipitation. Nitrate reductase was found in the 40% $(NH_4)_2SO_4$ precipitate, while the inactivating factor was largely precipitated by 40 to 55% $(NH_4)_2SO_4$. The latter fraction of the mature root inactivated the nitrate reductase isolated from the root tip, mature root, and scutellum. The inactivating factor, which has a Q_{10} 15 to 25 C of 2.2, was heat labile, and hence has been designated as a nitrate reductase inactivating enzyme. The reduced flavin mononucleotide nitrate reductase was also inactivated, while an NADH cytochrome *c* reductase in nitrate-grown seedlings was inactivated but at a slower rate. The inactivating enzyme had no influence on the activity of nitrite reductase, glutamate dehydrogenase, xanthine oxidase, and isocitrate lyase. The activity of the nitrate reductase inactivating enzyme was not influenced by nitrate and was also found in the mature root of minus nitrate-grown seedlings.

Nitrate reductase has been shown in several higher plants to have a relatively rapid turnover *in vivo*. A half-life for its decay of approximately 4 hr has been measured in excised corn leaves (14) and in a tobacco cell suspension (19). Most investigators have also noted the lability of the enzyme *in vitro* but have considered this to be the result of inhibitory substances in crude extracts. The inactivation of the enzyme could, however, be due to the action of the protease responsible for the *in vivo* decay of the enzyme.

In the primary root of the 3-day maize seedling, it has been shown by Oaks *et al.* (9) that the nitrate reductase in the mature root has a more rapid rate of decay ($t_{0.5}$ 2 hr) than that in the younger root tip cells ($t_{0.5}$ 3 hr). It was also shown that the mature root enzyme was much more labile *in vitro*. The use of cysteine in the extraction medium, while effective in stabilizing the root tip nitrate reductase, had only a partial effect on the mature root enzyme (17). Thus, in the older root cells, there is either a higher content of inhibitory substances, which cause a gradual inactivation of nitrate reductase *in vitro*, or some proteolytic degradation of the enzyme. An investigation of these two possibilities is described in this paper.

MATERIALS AND METHODS

Zea mays seedlings of the hybrid variety DSC1 (Wf9 × 38-11) were grown for 3 days on 5 mM nitrate, as described previously (9). The seeds were supplied by the DeKalb Shand Seed Company, Tamworth, 2340, Australia. The root tip (0–2 cm of the primary root), mature root (remainder of the primary root), and the scutellum were used as experimental material. The samples were extracted with a mortar and pestle and 50 mM phosphate, EDTA 0.5 mM, and cysteine 5 mM (root samples only) as extraction medium (pH 7.5). The ratio of fresh weight to volume of extraction medium (w/v) was 1:4 for the root tip, 1:3 for the mature root, and 1:10 for the scutellum. The resultant extracts (27,000*g* supernatant) had an equivalent protein content. The $(NH_4)_2SO_4$ fractionation procedure used by Schrader *et al.* (14) was followed, and the precipitates were dissolved in one-fifth of the original extract volume of 25 mM phosphate, pH 7.5.

Nitrate reductase was assayed with both NADH (9, 17) and $FMNH_2$[2] (18) as electron donor. A component of the crude extract which was precipitated by 55 to 75% $(NH_4)_2SO_4$ interfered in the assay of $FMNH_2$ nitrate reductase by causing a disappearance of the product nitrite. The dithionite used to reduce FMN was also involved. Since the extract factor was heat-stable, the utilization of nitrite is apparently nonenzymic. Interference in the $FMNH_2$ nitrate reductase assay was avoided by isolating the nitrate reductase [0–40% $(NH_4)_2SO_4$] and inactivating enzyme [40–55% $(NH_4)_2SO_4$]. An alternative assay procedure was also employed for $FMNH_2$ nitrate reductase in which NADP and an NADP reductase from barley leaves were used to generate reduced FMN (14).

In the assay procedure for nitrite reductase, benzyl viologen, used as an electron donor, is also reduced by dithionite (3). With the maize root and scutella extracts, it was again found that a nonenzymic loss of nitrite, due to the presence of dithionite, caused serious interference in the assay. Nitrite reductase was precipitated between 40 and 75% $(NH_4)_2SO_4$, hence overlapping the fraction shown above to mediate the chemical dissipation of nitrite. Instead of dithionite, an *Azotobacter* transhydrogenase system (8) was included in the nitrite reductase assay to reduce benzyl viologen.

Standard assay methods were used for the other enzymes investigated, glutamate dehydrogenase (17), isocritrate lyase (2), NADH cytochrome *c* reductase (18), and xanthine oxidase (1). For the spectrophotometric assays, a Unicam SP800 re-

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[2] Abbrevations: $FMNH_2$: reduced flavin mononucleotide; Polyclar AT: insoluble polyvinyl pyrrolidone.

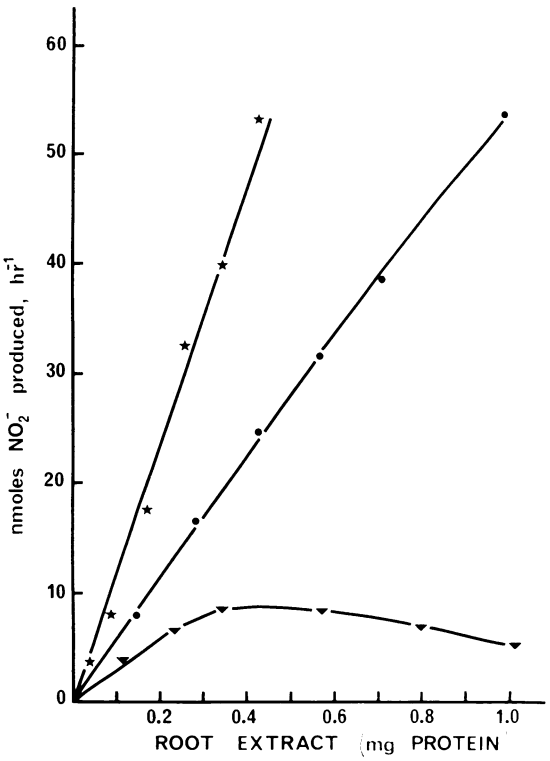

FIG. 1. Influence of the amount of root extract assayed on the activity of nitrate reductase. The crude extracts (27,000*g* supernatants) of the root tip (●, 0–2 cm of the primary root) and mature root (▼, remainder of primary root) of 3-day maize seedlings were compared. Data are also presented for a mature root fraction precipitated with 20 to 40% saturation with $(NH_4)_2SO_4$ (★).

cording spectrophotometer with scale expansion accessory was used. Polyclar AT was obtained from Croda Federal Chemicals, Richmond 3121, Australia, and prepared as described by Loomis and Battaile (7).

RESULTS AND DISCUSSION

Evidence for the *in vitro* Inactivation of Nitrate Reductase. The loss of nitrate reductase activity in the mature root extract of 3-day maize seedlings was temperature- and time-dependent. At 0 C, 80% of the activity was lost in 24 hr, while at 25 C the $t_{0.5}$ was 0.5 hr. The root tip enzyme was stable at 0 C and the $t_{0.5}$ at 25 C was 5 hr. With the root tip extract, there was a direct relationship between the amount of extract assayed and the activity of nitrate reductase measured (Fig. 1). At low levels of extract protein, a similar pattern was obtained for the mature root. However, with higher levels of mature root extract, there was a decreased activity of nitrate reductase. It appeared that some factor in the mature root extract, when present at high concentrations, inactivated nitrate reductase. When the mature root extract was treated with $(NH_4)_2SO_4$, the nitrate reductase was largely precipitated at 40% saturation. With this fraction of the mature root, there was a linear relationship between enzyme level and nitrate reductase activity (Fig. 1). The isolated enzyme was also more stable, the $t_{0.5}$ at 25 C was 2 hr. The inactivating factor, which was separated from nitrate reductase by this $(NH_4)_2SO_4$ fractionation step, could either be a protein molecule not precipitated by 40% saturation with $(NH_4)_2SO_4$ or a nonprotein molecule.

Phenolic substances have been shown to be inhibitory to nitrate reductase in some plant extracts (5, 16). In these cases, it was found that the Polyclar AT could be used in the extraction medium to adsorb such compounds and alleviate interference caused by them. With the radish cotyledon Stulen *et al.* (16) also found a poor relationship between extract level and nitrate reductase activity, as described above for the mature maize root, but with the employment of Polyclar AT a direct relationship between enzyme concentration and activity was obtained. In the present investigation, Polyclar AT (50% of tissue fresh weight) used in the extraction medium for the mature root resulted in only a 13% reduction in the *in vitro* loss of nitrate reductase. Further, it did not improve the relationship between the amount of mature root extract assayed and nitrate reductase activity measured. Thus phenolic or related inhibitory substances are not entirely responsible for the *in vitro* inactivation of the nitrate reductase from the mature root.

When the mature root extract was incubated with the root-tip extract, an increased rate of inactivation of the nitrate reductase in the latter was observed (Fig. 2). (The nitrate reductase in the mature root extract was first eliminated by preincubation at 25 C for 2 hr.) Thus the inactivating factor from the mature root was also capable of inactivating the root tip nitrate reductase.

Isolation and Preliminary Characterization of the Nitrate Reductase Inactivating Enzyme. When the mature root extract was further fractionated with $(NH_4)_2SO_4$, the factor responsibile for the inactivation of nitrate reductase was precipitated by 70% saturation with the salt. Most of the activity was recovered in the 40 to 55% $(NH_4)_2SO_4$ fraction. When the 40 to 70% fraction of the mature root was incubated at 25 C with the root tip nitrate reductase [0 to 40% $(NH_4)_2SO_4$ fraction], a marked inactivation of the latter was observed (Fig. 3). The inactivation of nitrate reductase appeared to follow a first order reaction with a Q_{10} 15 to 25 C of 2.2.

The activity of the mature root factor was destroyed after 5 min in a boiling water bath or after treatment at pH 1.3 for 30 min (0 C). Since the inactivating factor can be precipitated with $(NH_4)_2SO_4$ and is heat labile, it appears to be a protein and will subsequently be referred to as a nitrate reductase inactivating enzyme. Its further purification is in progress.

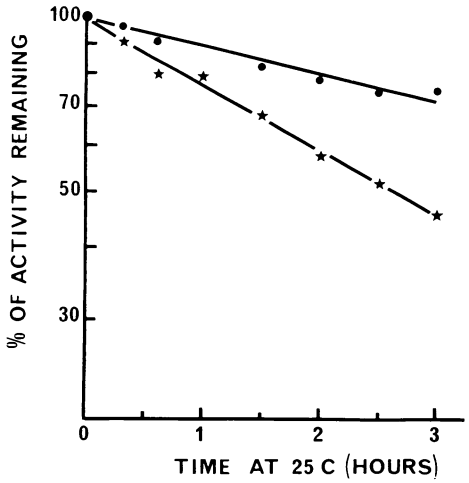

FIG. 2. Effect of the mature root extract on the *in vitro* stability of the root tip nitrate reductase. The loss of nitrate reductase in the root tip extract (●) and in a mixture (1:1) of the root tip and mature root extracts (★) is shown. The nitrate reductase in the mature root extract was inactivated by preincubation for 2 hr at 25 C. The protein levels (mg) were root tip 0.47 and mature root 0.51, and the initial nitrate reductase activities (nmoles NO_2^- produced/hr) were root tip 28 and mixture of root tip and mature root 25.

From the data presented in Figure 3, the activity of the inactivating enzyme was calculated to be 26 units of nitrate reductase inactivated per hr·mg protein (one unit of nitrate reductase was taken as the amount of the enzyme that will produce 1 nmole NO_2^-/hr). The mature root inactivating enzyme was also active on the nitrate reductase from the mature root and scutellum. No such inactivating factor was identified in the root tip and scutella extracts. The inactivating enzyme was also found in the mature root of seedlings grown in the absence of nitrate. Nitrate (30 mM), when added in the assay of the inactivating enzyme [$(NH_4)_2SO_4$ fractions], did not protect nitrate reductase against inactivation. In spinach (13) it has been shown that flavin adenine dinucleotide protects nitrate reductase against heat inactivation. Flavin adenine dinucleotide (0.1 mM), however, did not protect nitrate reductase against the inactivating enzyme.

Specificity of Nitrate Reductase Inactivating Enzyme. In the higher plant, $FMNH_2$ can be used as an alternative electron donor to NADH for nitrate reductase (10, 12, 14). It has been shown in the barley leaf by Wray and Filner (18) that nitrate also induces an NADH cytochrome *c* reductase. On a sucrose density gradient, the inducible NADH cytochrome *c* reductase was shown to co-sediment with NADH nitrate reductase and $FMNH_2$ nitrate reductase activity. Wray and Filner proposed that the barley leaf nitrate reductase was an enzyme complex with at least two components, an NADH cytochrome *c* reductase and an $FMNH_2$ nitrate reductase, and that the over-all reaction of the enzyme complex was the reduction of nitrate to nitrite with NADH as an electron donor. A similar model for the nitrate reductase enzyme in the higher plant had earlier been proposed by Schrader *et al.* (14). It has also been demonstrated in *Neurospora* (15) that the NADPH nitrate reductase in this case and a nitrate-inducible NADPH cytochrome *c* reductase were co-sedimented in a sucrose density gradient.

The mature root protein fraction, which was shown above to inactivate NADH nitrate reductase, also inactivated the $FMNH_2$ nitrate reductase and NADH cytochrome *c* reductase from nitrate-grown seedlings. When the $FMNH_2$ nitrate reductase from the scutellum [40% $(NH_4)_2SO_4$ precipitate] was incubated at 25 C with the mature root inactivating enzyme [40 to 55% $(NH_4)_2SO_4$ precipitate], approximately 50% of the $FMNH_2$ nitrate reductase activity was lost in 2 hr (Fig. 4). With an NADP reductase system instead of dithionite to reduce FMN (see "Materials and Methods"), the effect of the inactivating enzyme on $FMNH_2$ nitrate reductase was confirmed (Table I).

The NADH cytochrome *c* reductase from nitrate-grown seedlings was also inactivated by the mature root inactivating enzyme but at a slower rate (Fig. 5). At 25 C there was a similar loss of NADH nitrate reductase and NADH cytochrome *c* reductase from the 40% $(NH_4)_2SO_4$ fraction of the scutellum ($t_{0.5}$, 12 hr). In the presence of the inactivating enzyme, the $t_{0.5}$ for nitrate reductase was 2 hr and that for NADH cytochrome *c* reductase, 7 hr. However, the inactivation of NADH cytochrome *c* reductase from seedlings grown in the absence of nitrate was not increased by the mature root inactivating enzyme.

In contrast to the situation in the barley leaf, where growth on 5 mM nitrate gave a 3- to 4-fold increase in the level of NADH cytochrome *c* reductase, as reported previously (18), only a slight increase (<50%) was found in the level of the enzyme in the root tip and scutellum of 3-day maize seedlings grown on the same level of nitrate. A higher total level of NADH cytochrome *c* reductase was found in the root and scutellum than in the leaf (Wallace, unpublished data), and thus the nitrate-inducible NADH cytochrome *c* reductase may

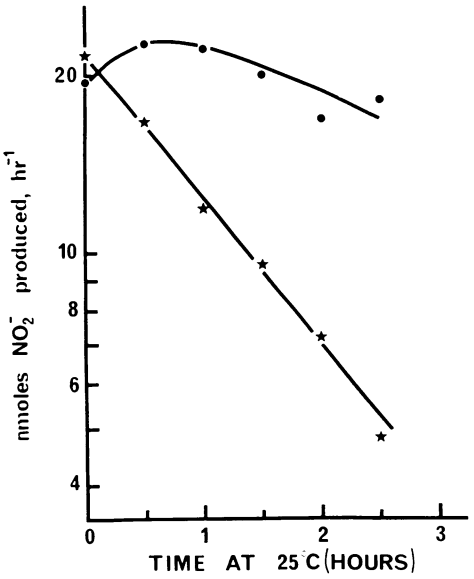

FIG. 3. The identification of a fraction of the mature root extract which can inactivate NADH nitrate reductase. Nitrate reductase activity was measured during incubation of the root tip enzyme [20–40% $(NH_4)_2SO_4$ precipitate, ●] and root tip enzyme mixed with mature-root inactivating factor [40–70% $(NH_4)_2SO_4$ precipitate, ★]. The root tip sample used contained 0.15 mg of protein, and the mature root fraction, 0.29 mg.

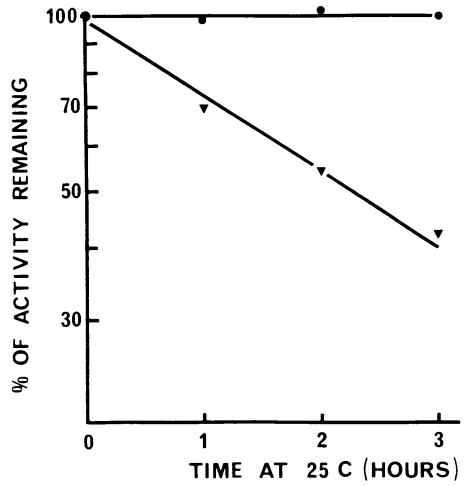

FIG. 4. Effect of the inactivating enzyme on $FMNH_2$ nitrate reductase. $FMNH_2$ nitrate reductase activity was measured during incubation of the scutella enzyme [●, 0–40% $(NH_4)_2SO_4$ precipitate] and scutella enzyme incubated with the mature root inactivating enzyme [▼, 40–55% $(NH_4)_2SO_4$ precipitate]. The scutella sample used contained 0.19 mg of protein, and the mature root fraction, 0.07 mg, and the initial nitrate reductase activity in the scutella sample alone and in the mixture with the mature root fraction was 21 nmoles NO_2^- produced/hr.

represent only a smaller fraction of the total enzyme. Since an effect of the inactivating enzyme on NADH cytochrome *c* reductase was demonstrated only in nitrate-grown seedlings, it appears to be acting only on the inducible fraction, which is believed to be a component of the nitrate reductase enzyme complex.

In heat-inactivation studies and tests with *p*-chloromercuribenzoate, previous workers (14, 18) have been able to demonstrate the inactivation of NADH cytochrome *c* reductase and NADH nitrate reductase under conditions where

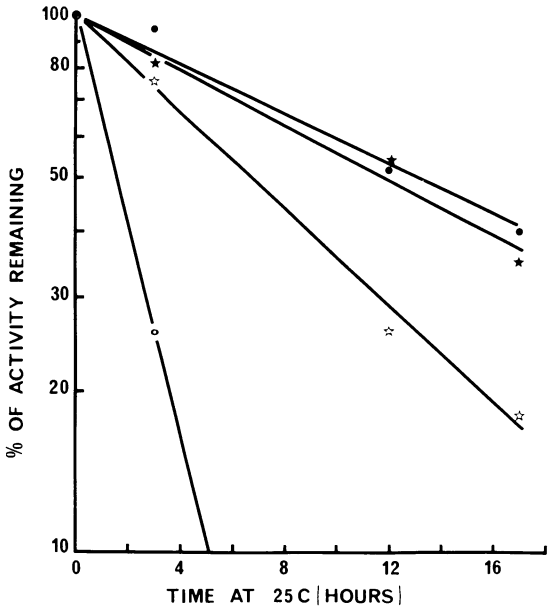

FIG. 5. A comparison of the influence of the inactivating enzyme on NADH nitrate reductase and NADH cytochrome *c* reductase. A scutella sample [0 to 40% $(NH_4)_2SO_4$ precipitate, 0.20 mg of protein] was used for the enzymes and a mature root sample [40–75% $(NH_4)_2SO_4$ precipitate, 0.19 mg of protein] for the inactivating enzyme. NADH nitrate reductase activity is shown for the scutella sample incubated alone (●) and mixed with the inactivating enzyme (○). Similarly, for the NADH cytochrome *c* reductase, the symbols are scutella enzyme alone (★) and mixed with inactivating enzyme (☆). The initial enzyme activities were: nitrate reductase (nmoles NO_2 produced/hr) in scutella sample, 51, and with inactivating enzyme, 55, and cytochrome *c* reductase (nmoles Cyt *c* reduced/min) in scutella sample, 33, and with inactivating enzyme, 32.

the $FMNH_2$ nitrate reductase was relatively unaffected. The inactivating enzyme, however, is active on the total nitrate reductase enzyme complex.

The inactivating enzyme was not found to have an effect on a number of other enzymes (Table I). These were incubated at 25C for 3 hr and the loss of activity of each in the absence of the inactivating enzyme was <10%. The activity of nitrite reductase and of glutamate dehydrogenase was not influenced by the inactivating enzyme. These two enzymes were recovered in the 40 to 75% $(NH_4)_2SO_4$ fraction. The test on glutamate dehydrogenase was conducted on the mature root, since this region of the primary root of the maize seedling has a relatively high content of the enzyme (17). The inactivating enzyme from the mature root did not have any influence on the *in vitro* activity of isocitrate lyase from the scutellum, an enzyme which is induced during the mobilization of the seed's fat reserves (6) and which in some seedlings is subsequently inactivated. The assay of this enzyme was conducted on the crude extract, since the isocitrate lyase in the 40% $(NH_4)_2SO_4$ precipitate was relatively labile. Xanthine oxidase, a flavoprotein with Fe and Mo components, reported to be similar to $FMNH_2$ nitrate reductase in fungi (4), was also not affected.

CONCLUSION

The inactivating enzyme found in the mature root of 3-day maize seedlings is apparently specific for the NADH nitrate reductase enzyme complex. Since it was not detected in the root tip and scutellum, which have the highest level of nitrate reductase and do exhibit *in vivo* decay of the enzyme, the inactivating enzyme demonstrated does not appear to be the protease involved in the normal turnover of nitrate reductase. A similar nitrate reductase inactivating enzyme has been found in the scutellum of older seedlings (Wallace, unpublished data). Its presence in older cells may indicate a role in preventing accumulation of nitrate reductase under conditions where active assimilation of nitrate is not required but where nitrate does induce nitrate reductase (9). The synthesis of the inactivating enzyme, however, in being uninfluenced by nitrate must be controlled by some other metabolic or hormonal factor.

Since this study was commenced, Pan and Marsh (11) have reported a protein-like macromolecule in the maize root which inactivated nitrate reductase in leaf extracts.

Table I. *Specificity of the Inactivating Enzyme*

The fractions reported below were: I, 27,000*g* supernatant; II, 40% $(NH_4)_2SO_4$ precipitate of I, and III, 40 to 75% $(NH_4)_2SO_4$ precipitate of I. In the assay of the inactivating enzyme, conditions were used which gave an approximately 50% inactivation of NADH nitrate reductase in 2 hr, and other enzymes were tested at levels which were rate-limiting in their own assays.

Enzyme	Source	Fraction	Initial Specific Activity	Influence of Inactivating Enzyme
			mg protein	*% loss of activity/2 hr*
NADH nitrate reductase	Root tip	II	210 nmoles NO_2^- produced hr	62
	Scutellum	II	257 nmoles NO_2^- produced hr	49
NADH cytochrome *c* reductase	Scutellum	II	166 nmoles cytochrome *c* reduced min	11
$FMNH_2$ nitrate reductase				
(a) Dithionite reduced FMN	Scutellum	II	109 nmoles NO_2^- produced hr	54
(b) NADPH diaphorase system	Scutellum	II	136 nmoles NO_2^- produced hr	44
Nitrite reduced (BV)	Scutellum	III	62 nmoles NO_2^- produced hr	None
Isocitrate lyase	Scutellum	I	3.2 μmoles glyoxylate produced hr	None
Glutamate dehydrogenase	Mature root	III	207 nmoles NADH oxidized min	None
Xanthine oxidase	Sigma grade I from buttermilk		0.56 μmole uric acid formed min	None

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