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Nitrate reductase inactivating factor from rice cells in suspension culture

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With a crude extract of cultured rice cells, there was no direct relationship between the activity of NADH nitrate reductase measured and the amount of cell extract used, when the amount was large. It appeared that some factor in the cell extract inactivated nitrate reductase. The inactivating factor could be separated from nitrate reductase by $(NH_4)_2SO_4$ precipitation. The factor seemed to be protein: 1) it was precipitable with $(NH_4)_2SO_4$, heat labile, and pronase treatment caused loss of activity; 2) cycloheximide reduced the formation of the inactivating factor. The activity of this factor fluctuated during the growth period. The existence of this inactivating factor was further investigated in various other cultured cells.

Nitrate reductase has been shown to have a relatively rapid turnover in vivo; a half-life of approximately 4 hr has been measured in corn leaves (*15*) and in tobacco cells in suspension culture (*20*). It is also well established that leaves from a variety of higher plants lose nitrate reductase activity in darkness and regain it in light (*3, 9, 13, 16*). In cultured cells of tobacco (*2*) and rice (*8*), the enzyme activity fluctuates greatly during cell growth.

Rapid fluctuations in nitrate reductase activity can be brought about by changes in relative rate of synthesis or activation and breakdown or inactivation. Thus increased activity can be due to decreased breakdown or to increased activation or synthesis. Conversely, decreased activity can be due to increased breakdown or inactivation, or decreased synthesis.

Recently, Travis (*16*) demonstrated that the disappearance of nitrate reductase in barley leaves during darkness was inhibited by cycloheximide. This indicated that protein synthesis was required for loss of nitrate reductase activity, and suggested the existence in barley leaves of a system which inactivated nitrate reductase. Wallace found and purified a nitrate reductase inactivating enzyme, apparently specific for the NADH nitrate reductase enzyme complex, in the mature root extract of maize seedlings (*17, 18*). Kadam (*5*) found a protein-like inhibitor of nitrate reductase in rice roots.

The present paper reports the existence and properties of a nitrate reductase inactivating factor in cultured rice cells, and the changes in activity of the inactivating factor with cell growth.

Materials and methods

Culture methods

Rice cells were grown as previously described (*7*, *8*). Stock cultures were initiated from the callus, and fresh cells in the stock culture (700 to 800 mg) were quantitatively inoculated into 50 ml of R-2 medium in 200 ml Erlenmeyer flasks with a volute cup made of nichrome wire. The flasks were set on a gyratory shaker agitated at 120 rpm under fluorescent light of 200 to 300 lux at 26°C.

Preparation of cell-free extracts

The fresh cells were harvested and washed on Miracloth, then homogenized with a mortar and pestle, with 0.1 M potassium phosphate buffer (pH 7.5) containing 6 mM cysteine as extraction medium. The ratio of fresh weight to volume of extraction medium (w/v) was 1 : 3 for the cultured rice cells. The homogenate was centrifuged at 10,000 $\times g$ for 15 min. The supernatant was used as the crude extract for assay. The $(NH_4)_2SO_4$ fractionation procedure used by Wallace (*17*) was followed to separate the inactivating factor. Precipitates at 0–40% saturation (nitrate reductase fraction) and 40–70% (inactivating factor fraction) were obtained. The precipitates were dissolved in a small volume of 0.1 M phosphate buffer (pH 7.5). Then this was dialyzed for 4 hr (4°C) against a large volume of 10 mM phosphate buffer (pH 7.5, four times). The volume of the dialyzed solution was adjusted with the same buffer to one-fourth of the original extract volume after dialysis.

Assay of nitrate reductase

Nitrate reductase was assayed with NADH as an electron donor (*8*). The reaction mixture contained 1.0 ml of 0.1 M potassium phosphate (pH 7.5), 0.2 ml of 0.1 M potassium nitrate and 0.5 ml of 1 mM NADH. The addition of 0.2 ml of enzyme solution containing about 1 mg of protein initiated the reaction. After incubation for 20 min at 28°C, the reaction was stopped by adding 0.2 ml of 1 M zinc acetate and 2.0 ml of 99% ethanol. After centrifugation, 2.0 ml of the supernatant was added to 1.0 ml of 1% sulfanilamide in 1 N HCl and 1.0 ml of 0.01% N-1-naphthylethylenediamine dihydrochloride. After 15 min, nitrite formation was estimated from the absorbance at 550 nm (*8*).

Investigation of the nitrate reductase inactivating factor

The activity of the nitrate reductase inactivating factor was assayed as follows. The 40 to 70% $(NH_4)_2SO_4$ precipitate from the cell extract was dissolved in 0.1 M phosphate buffer as described above. The inactivating factor fraction, which contained 1 to 3 mg of protein, was first mixed with an equal volume of nitrate reductase fraction containing about 2 mg of protein freshly prepared from 2nd day cells from another culture. After incubation at 28°C for 1 hr, the mixture was employed as the enzyme solution in the nitrate reductase assay system. As a control, a part of the inactivating factor solution was placed for 10 min in a boiling water bath and assayed in the same way. The inactivating activity was known from the difference in the nitrate reductase activities estimated with the unboiled and boiled solutions.

Assay of other enzymes

Standard assay methods were used to measure the activity of other enzymes, including $FMNH_2$ nitrate reductase (*19*), NADH cytochrome *c* reductase (*19*), nitrite reductase (*4*) and glutamate dehydrogenase (*12*). Soluble protein in cell extracts and enzyme solutions was determined by the method of Lowry et al. (*6*).

Results and discussion

Changes in nitrate reductase activity during the growth of cultured rice cells

Fig. 1 illustrates the results of three representative cultures grown at different times. These showed similar tendencies. Growth could be divided into three stages on the basis of fresh weight increment (Fig. 1-A). Fresh weight hardly increased in the early stage (till the 2nd day), then increased vigorously in the middle stage (days 3 to 10) and ceased thereafter.

Changes in nitrate reductase activity in crude and fractionated extracts were examined during growth, and the results are shown in Fig. 1-B (with the crude extracts) and Fig. 1-C (with both crude and fractionated extracts). The enzyme activity changed throughout growth in the same manner in both the crude and the

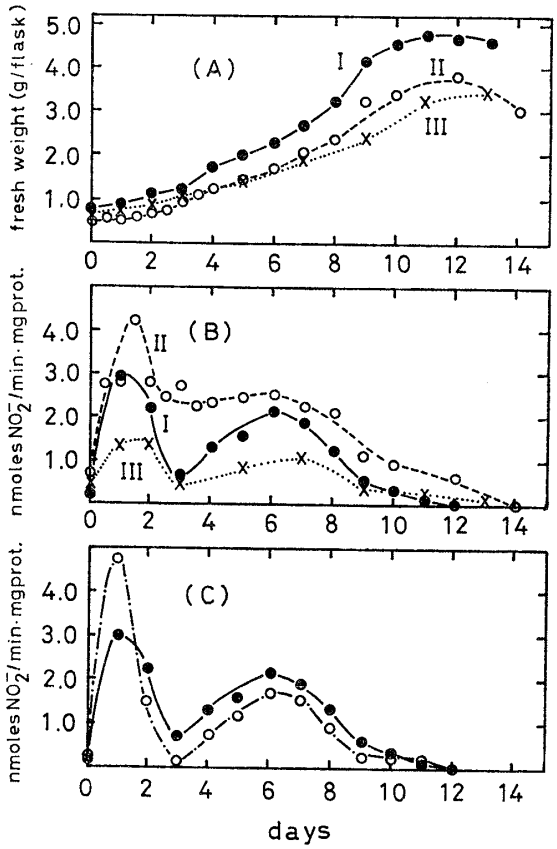

Fig. 1. *Growth pattern and the changes in nitrate reductase activity of cultured rice cells.* (A) Growth of rice cells and (B) nitrate reductase activity in the crude extracts during the growth of three different cultures (I, II and III). (C) Changes in nitrate reductase activity of culture I, assayed with crude extracts (-●-) and with fractionated extracts by 0 to 40% $(NH_4)_2SO_4$ saturation (-•○•-).

fractionated extracts. It is interesting that there are two peaks in activity during the growth period (Fig. 1-B, C). The first one appeared immediately after inoculation as a sharp increase and a quick decrease of activity, and the second one appeared as a gentle slope. These results suggest the possibility that two different types of nitrate reductase may exist throughout the growth period.

Fig. 1-C showed that the nitrate reductase activity of fractionated nitrate reductase extracts were always slightly lower than those of the crude extracts except for the 1st day. These results seem incomprehensible because the enzyme activity should be higher after the elimination of the inactivating factor fraction. A possible explanation for this, now under investigation, is that the loss of nitrate reductase activity might occur in different ways during fractionation in distinctive growth period. It is well-known that nitrate reductase in higher plants is quite unstable, and difficult to purify (*1*, *5*, *15*).

Existence and separation of the nitrate reductase inactivating factor

Fig. 2 shows the relation between nitrate reductase activity estimated and the amount of cell extract in the assays. The extracts were prepared from rice cells homogenized with 3 times the volume (w/v) of 0.1 M phosphate buffer (pH 7.5) to cell fresh weight at distinctive growth stages.

With small amounts of cell extracts, there was a direct relationship between the amount of extract assayed and the nitrate reductase activity measured, but with large amounts of extract, there was no direct relationship between them. This tendency became stronger with the cells of progressive days of culture. It appeared that some factor in the cell extract inactivated nitrate reductase.

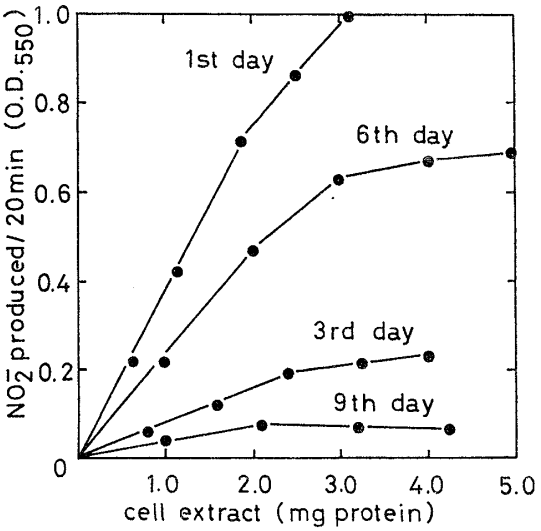

Fig. 2.

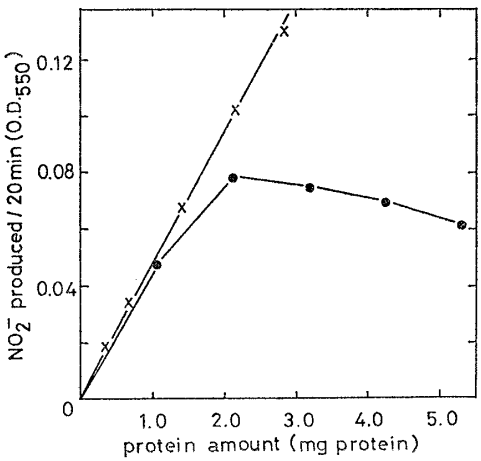

Fig. 3.

Fig. 2. *Relationship between the activity of nitrate reductase and the amount of crude extract.* On each sampling day shown in the figure, the cells were harvested and their crude extracts used for assay of nitrate reductase.

Fig. 3. *Nitrate reductase activity before and after fractionation with* $(NH_4)_2SO_4$. The crude extract (10,000×*g* supernatant) before fractionation (-●-), and the fraction precipitated by 0 to 40% saturation with $(NH_4)_2SO_4$ (-×-) of 9th day cells were examined.

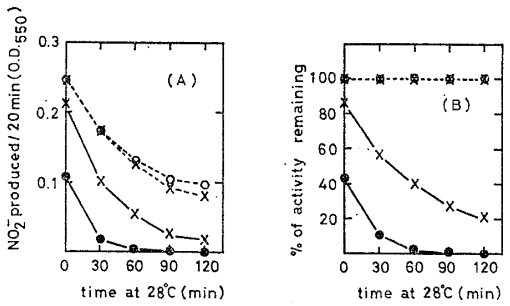

Fig. 4. *Effect of preincubation of nitrate reductase with the inactivating factor on the activities of nitrate reductase.* (A) Nitrate reductase activity remaining after incubation with the inactivating factor or the control. (B) The percentage expression of the nitrate reductase remaining after incubation with the factor or the control. A nitrate reductase fraction containing 1.70 mg protein isolated from 2nd day cells was mixed with the inactivating factor fraction obtained from 9th day cells (-●-), 1.80 mg protein) or 13th day cells (-×-, 1.30 mg protein). After preincubation at 28°C for the times indicated, the mixture was used as the enzyme solution in the nitrate reductase assay system. Inactivating factor fractions treated for 10 min in a boiling water bath (--○--, 9th day; --×--, 13th day) were used as controls.

In order to examine whether a factor such as Wallace found in maize roots (*17*) exists in cultured rice cells, an extract of 9th day cells was fractionated with $(NH_4)_2SO_4$, and nitrate reductase activity was measured. The results are shown in Fig. 3. The nitrate reductase was largely precipitated by 0–40% saturation. With this fraction, a linear relationship was obtained between the protein amounts and the nitrate reductase activity even with large amounts of protein, indicating that nitrate reductase could be separated from the inactivating factor by fractionating with $(NH_4)_2SO_4$.

When more $(NH_4)_2SO_4$ was added to the extract, the factor responsible for the inactivation of nitrate reductase was found in the precipitation by 40 to 70% saturation. The effects of preincubation of the inactivating factor with nitrate reductase on nitrate reductase activity was examined. The results are shown in Fig. 4. The loss of nitrate reductase activity was markedly intensified by this preincubation. The inhibition of nitrate reductase after 60 min preincubation was calculated to be 95% and 60% in 9th day and 13th day cells, respectively. The extent of inactivation was different at different growth stages.

When a crude extract from 9th day cells, heated for 10 min in a boiling water bath, or the concentrated dialyzable fraction, was added to the nitrate reductase assay system, the nitrate reductase activity was not inhibited at all.

Characterization of the nitrate reductase inactivating factor

In order to confirm that the inhibition of nitrate reductase observed was not simply due to consumption of NADH by certain systems in the cell extract, potassium nitrate was removed from the reductase assay system. Then the amount of NADH consumed was determined by measuring the decrease in extinction at 340 nm. Some consumption was detected with the crude extract, but hardly any with either the nitrate reductase fraction or the inactivating factor fraction, indicating that nitrate reductase inactivation by the inactivating factor fraction was not due to any

Table 1 *Effect of pronase treatment on nitrate reductase inactivating factor*

Preincubation time (min)	Pronase	Activity of inactivating factor (% loss of NR activity/time unit)
0	None	14
	Added	8
30	None	30
	Added	7

Pronase (Kaken Chemicals, 0.023 unit) was mixed with the inactivating factor (40–70% saturation fraction) prepared from the 9th day cells in a test tube at 25°C for 2 hr. Then the incubated solution was added to the nitrate reductase assay system.

Table 2 *Effect of cycloheximide on the activities of nitrate reductase (NR) and nitrate reductase inactivating factor (NRI)*

Days after inoculation	Cycloheximide[a]	Growth (g fresh weight)	NR activity[b] (nmoles NO_2^-/min, mg protein)	NRI activity[c] (% loss of NR activity/hr, mg protein)
1	None	0.84	1.29	47
2	None	1.19	2.75	41
	Added	0.96	2.04	33
3	None	1.82	2.07	74
	Added	1.11	1.57	60
4	None	2.31	2.27	63
	Added	1.28	1.42	36

[a] Cycloheximide at a concentration of 1.0 ppm was added on the 1st day of culture.
[b] Nitrate reductase activity was assayed with crude enzyme solution on each day.
[c] The standard nitrate reductase for the assay of the inactivating factor was prepared from 2nd day cells.

Table 3 *Specificity of the inactivating factor for various enzymes*

Enzymes assayed	Fraction[a] used as enzyme solution	Initial specific activity (per mg protein)	Influence of inactivating factor (% loss of NR activity/hr)
Expt. I			
NADH nitrate reductase	I	2.29 nmoles NO_2^- produced/min	50
$FMNH_2$ nitrate reductase	I	0.59 nmoles NO_2^- produced/min	49
Nitrite reductase	II	6.60 nmoles NO_2^- produced/min	None
Glutamate dehydrogenase	II	0.64 unit	None
Expt. II			
NADH nitrate reductase	I	1.05 nmoles NO_2^- produced/min	27
NADH cytochrome *c* reductase	I	0.97 unit	29

[a] The fractions used as enzyme solutions were: I, 0 to 40% $(NH_4)_2SO_4$ precipitate and II, 40 to 70% $(NH_4)_2SO_4$ precipitate of 2nd day cell extracts. Individual enzyme solutions prepared from 2nd day cells were preincubated with the inactivating factor prepared from 10th day cells for 60 min.

specific NADH consumption system. Nitrite reductase in the inactivating factor fraction did not have any influence on the assay of nitrate reductase.

The observation that the inactivating factor could be precipitated with $(NH_4)_2SO_4$ and was heat labile, indicated that it might be of protein nature. The inactivating factor was treated with pronase (0.023 unit) dissolved in 0.5 ml of 0.1 M phosphate buffer (pH 7.5). This was added to the inactivating factor fraction (1.5 ml) obtained from 9th day cells. The mixture was incubated at 25°C for 2 hr and tested for its inactivation activity. The activity after incubation was reduced to one-fourth of that without pronase treatment (Table 1). In addition, the effects of cycloheximide on the formation of the inactivating factor and nitrate reductase in growing cells were investigated. Cycloheximide at a concentration of 1.0 ppm was added to the culture one day after inoculation, when the formation of nitrate reductase was most intensive (Fig. 1). The results are shown in Table 2. The activities of both the inactivating factor and nitrate reductase were reduced by the addition of cycloheximide. These results also suggest that the inactivating factor might be a protein.

In the nitrate reductase of higher plants, $FMNH_2$ can be used as an alternative electron donor for NADH (*10*, *11*, *15*, *17*). Wray and Filner (*19*) have shown that nitrate in the medium induces NADH cytochrome *c* reductase in barley leaves. They proposed that the barley leaf nitrate reductase was an enzyme complex with at least two components, and that the over-all reaction of the enzyme complex was the reduction of nitrate to nitrite with NADH as an electron donor.

The specificity of the nitrate reductase inactivating factor from rice cells was investigated, and the results are shown in Table 3. The inactivating factor prepared from 10th day cells inactivated not only NADH nitrate reductase but also $FMNH_2$ nitrate reductase and NADH cytochrome *c* reductase, but not nitrite reductase and glutamate dehydrogenase prepared from 2nd day cells. These results showed that inactivation by this factor is not the same as that by proteases such as chymotrypsin and trypsin which have broad specificity for various proteins.

Table 4 shows the changes in the activity of the inactivating factor during the growth of rice cells. Its activity was detectable throughout all growth stages. The

Table 4 *Changes in the activity of the nitrate reductase inactivating factor during the growth of the cultured rice cells*

Days in culture	% loss of NR activity/hr (A)	Amount of protein in inactivating factor fraction (mg) (B)	NRI activity $\left(\frac{\%\text{ loss of NR activity/hr}}{\text{mg protein}}\right)$ (A)/(B)
2	53	2.70	20
4	92	2.40	38
6	33	2.80	12
8	25	3.10	8
9	98	1.80	54
11	71	2.14	33
13	58	1.30	45
15	54	0.80	67

Nitrate reductase was prepared from 2nd day cells.

Table 5 *The existence of nitrate reductase inactivating factor in various cultured cells*

Cultured cells	Growth (g fresh weight)	NR activity (nmoles NO_2^-/min, mg protein)	NRI activity (% loss of NR activity/hr, mg protein)
1) the 4th day after inoculation			
rice	1.37	0.45	24
wheat	2.30	1.71	21
soybean	7.94	0.59	42
peanut	2.52	0.08	83
tobacco	3.21	1.38	28
Ruta	4.82	1.41	25
2) the 10th day after inoculation			
rice	3.05	0.53	25
wheat	5.95	0.20	39
soybean	24.60	0.00	231
peanut	12.40	0.00	161
tobacco	6.99	1.74	13
Ruta	18.20	0.04	8

The inactivating factor (40 to 70% saturation fraction) of these cells on each day was preincubated for 60 min with the nitrate reductase (0 to 40% saturation fraction) prepared from 2nd day rice cells.

amount fluctuated, being low during the middle of the culture period and high earlier and later in the period. It reached a maximum in 4th day and 9th day cells, when the nitrate reductase activity was relatively low.

The existence of the nitrate reductase inactivating factor was further investigated in various cultured cells: wheat, soybean, peanut, tobacco and *Ruta graveolens*. The inactivating factor fractions (40–70% saturation) prepared from 4th day and 10th day cells were preincubated with the nitrate reductase fraction (0–40% saturation) prepared from 2nd day rice cells. Then the activity of the inactivating factor was measured in the same manner as above. The activity of nitrate reductase in crude extracts from various cells were also assayed.

The results are shown in Table 5. The existence of the factor was confirmed in all these cultured cells. Its activity was very high in peanut and soybean cells, while the nitrate reductase activity was very low or undetectable in these cells. It seemed that inactivation by the factor occured strongly in crude extracts. In the case of rice cells, possible inactivation of nitrate reductase in crude extracts during the assay period of 20 min was calculated to be 10% at the most, from a consideration of both the time and the amount of protein.

Through the experiments described here, the existence of a factor which inactivates the nitrate reductase complex was ascertained in cultured rice cells, and the factor seemed to be protein. Moreover, the activity of this factor was observed to fluctuate throughout the growth period; it reached maxima on the 4th day and the 9th day when the nitrate reductase activity was low. The existence of the inactivating factor was also confirmed in various other cultured cells. The factor may be concerned with one of the systems which regulate nitrate reductase activity in intact plants. This is now under investigation.

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