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The occurence of a nitrate reductase inactivating factor in extracts of *Spirodela polyrrhiza*

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Abstract

NADH-nitrate reductase (NR) had a low activity immediately after extraction from 14 day-old cultures of *Spirodela polyrrhiza* with the basic extraction solution (50 mM K-phosphate buffer, pH 7.5, 5 mM cysteine and 3 mM EDTA) whereas nitrite reductase (NiR) activity in the same extract was very high. Increasing the EDTA or cysteine concentration or replacing cysteine by dithiothreitol had no effect on the level of NR activity. The addition of 3% bovine serum albumine (BSA) and 0.4 mM phenylmethyl sulfonyfluoride (PMSF) or 3% BSA and 1.5% polyvinylpolypyrrolidone (PVPP) to the extraction solution greatly increased the NR activity. The enzyme activity increased 40 times in the presence of 1.5% PVPP with 3% BSA and 0.4 mM PMSF. The highest NR activity (over 100-fold) was found in the presence of 3% caseine. The probable nature of the factor inactivating nitrate reductase in *S. polyrrhiza* extracts is discussed.

Key words: NR inactivator, nitrate reductase, Spirodela polyrrhiza

INTRODUCTION

In vitro determinations of NADH-nitrate reductase activity (EC 1.6.6.1) in tissue extracts from higher plants is often difficult due to either the instability of the enzyme in tissue homogenates or the increased activity of NR inactivating proteins (Yamaya et al. 1980). It has been shown recently (Schrader et al. 1974, Wallace 1975, Yamaya and Ohira 1976, Jolly and Tolbert 1978, Robin 1979, Lewis et al. 1982) that the addition of casein or BSA to the extraction solution increases and stabilizes the NR activity, protecting the enzyme from specific inhibitors. Similarly, *in vitro* determinations of NR from water plants from the *Lemnaceae* family

is connected with the necessity of using certain protective substances in the extraction solution. Ferguson (1969) showed a high NR activity in *S. oligorrhiza* in the presence of 1% PVPP. Similar results for *S. oligorrhiza* were attained by Vijayaraghavan et al. (1982) by adding 3% BSA to the extraction solution. Lakshimi and Maheshwari (1979) used 1.5% PVPP and 100 mM cysteine when extracting NR from *Lemna paucicostata*. In our experiments, both PVPP and BSA as well as increasing the cysteine concentration were not very effective in preventing the loss of NR activity during the extraction of the enzyme. Therefore, studies were done on determining the NR activity in *S. polyrrhiza* extracts in the presence of BSA, casein, PVPP, PMSF and their combinations, as these are substances which may be capable of protecting NR from inactivating factors. At the same time, the *in vitro* activity of nitrite reductase (EC 1.6.6.4), extracted with the same solution as NR, was studied although it is known that NiR is a rather stable enzyme in tissue homogenates.

MATERIAL AND METHODS

Sterile cultures of *Spirodela polyrrhiza* (L.) Schleiden were grown in a photothermostat room using a medium containing nitrate as the sole source of nitrogen. The cultures were in 300 cm^3 Erlenmayer flasks containign 100 cm^3 of the medium, given 16 h light (10.3 $W \times m^{-2}$, obtained from white fluorescent tubes) at 25°C and 8 h darkness at 21°C. The composition of the medium, in mM, was as follows: KNO_3 — 3; $Ca(NO_3)_2 \cdot 4H_2O$ — 2; $MgSO_4 \cdot 7H_2O$ — 1; NaH_2PO_4 — 1; $NaNO_3$ — 1; Fe-citrate — 0.0045 and microelements in amounts as given in a previous paper (Tatkowska and Buczek 1983). The level of sucrose was kept at 0.1% and the pH was adjusted to 6.6. Media were buffered against a drop in pH during plant growth by the addition of sterilized solid $CaCO_3$ (50 mg per flask).

The extractions of the enzyme were performed from 14 day-old sterile *S. polyrrhiza* cultures (exponential phase). The plants were washed, blotted dry and 1 g of fresh weight was homogenised in 4 cm^3 of extraction solution with a cold mortar and pestle. The basic enzyme extraction medium contained 50 mM K-phosphate buffer (pH 7.5), 5.0 mM cysteine and 3mM EDTA. The following substances were added to the basic extraction medium at the concentrations given in the Tables: polyvinylpolypyrrolidone (insoluble), bovine serum albumin, casein and phenylmethyl sulfonylfluoride. The homogentae was strained through cheesecloth and centrifuged at 20 000 $\times$ g (15 min), the resulting supernatant was used for the assay of NR and NiR activities. All of the operations described above were performed in a temperature range of 0-+4°C.

The nitrate reductase activity was assayed in triplicate for 30 min at 27°C in a final volume of 2.0 cm^3 solution containing: 1 cm^3 100 mM K-phosphate (pH 7.5), 0.2 cm^3 0.1 mM KNO_3, 0.2-0.6 cm^3 of enzyme extract, 0.2 cm^3 2 mM NADH (freshly prepared in 25 mM K-phosphate, pH 7.5) and H_2O was added just before use to a final volume of 2.0 cm^3. After incubation, the reaction was stopped by addition of 1 cm^3 0.1 M Zn-acetate and left standing 15 min in a refrigerator. The incubation mixture was then centrifuged at 10 000 × g for 10 min and nitrate determined in the supernatant. Nitrate was determined by adding to the sample 1 cm^3 of 1% (w/v) sulphanilamide in 1 N HCl and 1 cm^3 0.01% (w/v) of N-(1-naphtyl)--ethylenediamine dihydrochloride and distilled water to a final volume of 5 cm^3. After standing 30 min, the nitrite was determined by measuring the absorbance at 540 min.

The nitrite reductase activity was assayed in triplicate for 30 min at 27°C in a final volume of 3 cm^3. The reaction mixture contained: 1 cm^3 100 mM K-phosphate buffer (pH 6.8), 0.5 cm^3 1 mM $NaNO_2$, 0.3 cm^3 H_2O, 0.2 cm^3 enzyme extract and 0.5 cm^3 methyl viologen (6 mg MV per 10 cm^3 K-phosphate, pH 6.8) and sodium dithionite (72 mg $N_2S_2O_3$ per 10 cm^3 0.1 M $NaHCO_3$). Methyl viologen and dithionite solutions were freshly prepared. After incubation the reactions was stopped by agitation of the reaction mixture until the blue color disappeared. The control samples were agitated immediately after addition of the dithionite solution. Further procedure was the same as in NR determination. The activity of NR and NiR were expressed in nmoles NO_2^- formed (NR) or utilized (NiR) per g fresh weight per h.

Reagents: polyvinylpolypyrrolidone (PVPP) and phenylmethyl sulfonylfluoride (PMSF) were obtained from Sigma; methyl viologen (MV) — Koch Light; vitamin-free casein — Fluka AG; sodium dithionite —UCN Brussels; serum albumine defatted with acetone — Biomed Cracow; NADH — Reanal. The remaining reagents were of chemically pure grade from POCH Gliwice.

RESULTS AND DISCUSSION

The results presented in Table 1 indicate a low NR activity in the extracts made with the basic extraction solution containing 5 mM cysteine and 3 mM EDTA. Increasing the cysteine or EDTA concentrations as well as substituting dithiothreitol for cysteine (results not shown) had no effect on the activity of the extracted NR. Introducing 1.5% PVPP, 3% BSA or 0.2 mM PMSF only doubled the enzyme's activity. Higher PMSF concentrations (0.4 and 1mM) only slightly increased its activity. It was therefore shown that, in contrast to the studies by Ferguson (1969) and Vijayaraghavan et al. (1982) done on *S. oligorrhiza*, and the studies

Table 1

Effect of addition of BSA, PVPP, PMSF and casein to the enzyme extraction medium (50 mM pH 7.5 K-phosphate buffer + 5 mM cysteine + 3 mM EDTA) on *on vitro* nitrate reductase activity in 14-day-old *Spirodela polyrrhiza*

Addition to extraction medium	NR activity, nmoles $NO_2^- \times g^{-1}$ fr.wt. $\times h^{-1}$	Stimulation over control, fold
None	10.05	
BSA 3%	17.67	about 2
PVPP 1.5%	18.88	about 2
PMSF 0.2 mM	23.30	about 2
PMSF 0.4 mM	30.25	3
PMSF 1.0 mM	37.25	about 4
BSA 3% + PMSF 0.2 mM	62.84	6
BSA 3% + PMSF 0.4 mM	125.93	12
BSA 3% + PVPP 1.5%	203.81	20
BSA 3% + PVPP 1.5% + PMSF 0.4 mM	427.33	over 40
PVPP 1.5% + casein 3%	1184.33	over 100
Casein 3%	1161.00	over 100

Table 2

Effect of addition of BSA, casein, PVPP and PMSF to the extraction medium (50 mM pH 7.5 K-phosphate buffer + 5 mM cysteine + 3 mM EDTA) on *in vitro* nitrite reductase activity in 14-day-old *Spirodela polyrrhiza*

Addition to extraction medium	NiR activity, nmoles $NO_2^- \times g^{-1}$ fr.wt. $\times h^{-1}$	Stimulation in per cent of control
None	23770	100
BSA 3%	35522	149
PMSF 0.4 mM	23921	101
BSA 3% + PMSF 0.4 mM	30642	129
BSA 3% + PMSF 0.4 mM + PVPP 1.5%	31325	132
Casein 3%	36000	151

by Lakshmi and Maheshwari (1979) on *L. paucicostata,* both PVPP and BSA, as well as increasing the cysteine concentration, were poorly effective in keeping up the NR activity in *S. polyrrhiza* extracts.

The inclusion of 3% BSA + 0.2 mM PMSF or 3% BSA + 0.4 mM PMSF into the extraction medium increased the NR activity 6- and 12-fold respectively. The fact that PMSF which was present in the extraction medium together with BSA clearly prevented the inactivation due to extraction, of NR from *S. polyrrhiza* may suggest the presence of a natural NR inactivator with the properties of a proteolytic enzyme. It is possible that this may be

a serine-protease, which may be suggested by the studies of Gold and Fahrney (1974). These authors showed that PMSF specifically reacts with the active site of α-chymotrypsin, with no side effects. A serine-protease NR inactivator was found in maize roots by Wallace (1973, 1974) and Robin (1979). It was shown that the addition of PMSF to the extraction medium prevented NR inactivation in maize root tips (Wallace 1974) and stabilized the *in vitro* activity in extracts from mature maize roots (Wallace 1975).

It has been recently shown that 3% BSA or 3% casein in extraction solutions greatly increased the *in vitro* NR level and stability in the leaves of two maize genotypes as wel as in tobacco and oat leaves (Schrader et al. 1974), maize roots (Wallace 1975), rice seedlings (Kadam et al. 1974) wheat leaves (Sherrard et al. 1979), barley leaves (Lewis et al. 1982) and rice cell cultures (Yamaya and Ohira 1976, 1977). The studies listed above suggested that BSA and casein, present in the extraction solution, protect NR from specific inhibitors. However, the inactivator isolated from maize roots showed a proteolytic activity (Wallace 1974, 1978) whereas the one isolated from rice cells did not (Yamaya and Ohira 1977).

In our studies the in vitro NR activity increased compared with the control 18 and over 100 times in the presence of 3% BSA +1.5% PVPP or 3% casein, respectively. This data, as well as the fact that 0.4 mM PMSF+3% BSA+1.5% PVPP increased the extracted NR activity by about 40 times, may suggest that the inactivating factor in *S. polyrrhiza* has a proteolytic nature, although other protective mechanisms may function here. Lately Yamaya and Ohira (1977, 1978) showed in rice cell cultures, and Jolly and Tolbert (1978) in soybean leaves, that there is a protein inactivating NR which did not have the properties of a proteolytic enzyme but bound the oxidized form of NR (Yamaya and Ohira 1978).

The activity of nitrite reductase, irrespective of the protective substances used, was very high (Table 2), which indicates that this enzyme does not need any protective substances in order to keep up its activity in tissue extracts from *S. polyrrhiza*. However, a certain increase in NiR activity was found in the presence of 3% BSA and 3% BSA+0.4 mM PMSF as well as in the presence of casein. This increase was not large and did not exceed 50% of the control. It seems then, that NiR in *S. polyrrhiza* extracts is a stable enzyme, and that the slight increase in NiR activity observed in the presence of casein, BSA and PMSF indicates a poor sensitivity of the NiR protein to endogenous inactivators (Wallace 1973, Yamaya and Ohira 1977).

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Występowanie czynnika inaktywującego reduktazę azotanową w ekstraktach z Spirodela polyrrhiza

Streszczenie

NADH-reduktaza azotanowa (NR) wykazywała niską aktywność bezpośrednio po ekstrakcji 14 dniowych kultur *Spirodela polyrrhiza* podstawowym roztworem ekstrakcyjnym (50 mM bufor K-fosforanowy, pH 7,5, 5 mM cysteiny i 3 mM EDTA), natomiast aktywność reduktazy azotynowej (NiR) w tam samym ekstrakcie była bardzo wysoka. Zwiększenie stężenia EDTA czy cysteiny nie miało wpływu na poziom aktywności NR. Wprowadzenie do roztworu ekstrakcyjnego 3% BSA z 0,4 mM fluorku fenylo metylosulfonylu (PMSF) czy 3% BSA z 1,5% polywinylpolypirrolidonem (PVPP) znacznie zwiększyło aktywność NR. Aktywność enzymu wzrosła 40 krotnie w obecności 1,5% PVPP z 3% BSA i 0,4 mM PMSF, jednakże najwyższą aktywność NR (ponad 100 krotną) stwierdzono w obecności 3% kazeiny.