AN ENDOGENOUS INHIBITOR OF NITRATE REDUCTASE IN RADISH COTYLEDONS

INEKE STULEN, T. KOCH-BOSMA and A. KOSTER

Botanisch Laboratorium, afd. Plantenfysiologie, Rijksuniversiteit, Groningen.

SUMMARY

In nitrate reductase preparations from radish cotyledons an endogenous inhibitor of a non competitive nature was found. A method for calculating the amount of inhibitor present was devised. The amount of inhibitor present in one pair of cotyledons decreased with increasing age of the seedlings, in light as well as in darkness. It is suggested that the inhibitor is of a phenolic nature since the inhibition could be reversed by extraction with polyvinylpyrrolidone.

INTRODUCTION

Upon homogenization of a plant tissue to extract an enzyme, inhibitory substances can be released which are spatially separated from the enzyme in the intact plant. In experiments with a nitrate reductase preparation from radish cotyledons it was found that the relation between enzyme concentration and reaction rate was non-linear, which points to the presence of an endogenous inhibitor in the enzyme preparation (FRIEDENWALD & MAENGWYN-DAVIES 1954). This has not been reported by other workers who have used radish cotyledons (BEEVERS *et al.* 1965, INGLE *et al.* 1966). Since the level of inhibitor may vary due to growth it is clearly important to eliminate this inhibition in experiments with cotyledons of different ages.

Interference by endogenous inhibitors in the assay of various enzymes has been reported by several authors. Interference by phenolic compounds was reviewed by LOOMIS & BATTAILLE (1966) and ANDERSON (1968). After homogenization endogenous phenolics are oxidized by phenol oxidases with the formation of quinones and ultimately of brown pigments, products which are all powerfully inhibitory to enzymes. Phenolic substances can be bound to protein by hydrogen bonding; more stable bonds are formed by copolymerization of quinones with protein.

Inactivation of enzymes by phenolics can be prevented by including reducing agents or polymers in the extraction medium. By using insoluble polyvinylpyrrolidone KLEPPER & HAGEMAN (1969) were able to detect substantial amounts of nitrate reductase in apple leaves, a tissue rich in phenolics.

The objectives of the present experiments were a) to clarify the nature of the inhibition found and b) to determine the amount of inhibitor present in cotyledons of increasing age.

2. MATERIAL AND METHODS

2.1. Radish seedlings

Seeds of *Raphanus sativus* L. var. Cherry Belle were surface sterilized by soaking in 1 % sodiumhypochlorite solution (commercial bleach) for 10 minutes followed by thorough rinsing with tap water. About 200 seeds were sown on a layer of cellulose tissue covering the lid of a petri dish of 15 cm ⌀. The lid was placed in a petri dish of 20 cm ⌀, to which 250 ml of nutrient solution of the following composition was added: KCl 2.5 mM, $CaCl_2$ 2.5mM, $MgSO_4$ 2.0 mM, KH_2PO_4 0.5 mM, Fe^{+++} (as Fe citrate) 0.1 mM, Mo (as Na_2 MO_4) 3.5 μM and A-Z solution of normal strength (HOAGLAND & SNIJDER 1933) except that $Co(NO_3)_2$ was replaced by $CoCl_2$. The pH was adjusted to pH 7.0 with KOH solution. The seeds were germinated in a dark room at 27 °C and a relative humidity of 95–100 %. After 4 days the etiolated seedlings were gently removed from the cellulose tissue and mounted in grooves in the lids of cylindrical perspex vessels to which 100 ml of fresh nutrient solution was added.

On each vessel 20 seedlings were mounted. The nutrient solution was aerated.

2.2. Induction of nitrate reductase

Induction was started by adding substrate solution (half KNO_3, half $Ca(NO_3)_2$) to a final concentration of 10^{-2}M NO_3^- to the nutrient solution in the vessels. For induction in the light, the seedlings were continuously illuminated with 20,000 lux at 25 °C. Induction in the dark was also carried out at 25 °C. Cotyledons were harvested after induction for various lengths of time.

2.3. Extraction and assay

The cotyledons were frozen in liquid nitrogen and pulverized with a mortar and pestle. One gram of pulverized material was ground for 30 seconds with 4.0 ml of extraction medium, consisting of a mixture of 0.01 M potassium phosphate, 0.01 M EDTA and 10^{-4} M cysteine, adjusted to pH 8.2 with KOH solution. The homogenate was centrifuged at 0 °C for 25 minutes at 1800 g, after which the supernatant was used as the enzyme preparation. In some experiments the pellet fraction was also assayed for nitrate reductase activity. In this case the pellet was resuspended in the same volume of extraction medium and the suspension used for enzyme assays. All actions were carried out at 0–4 °C.

The reaction mixture in which the enzyme assay was performed consisted of 3.2 ml 0.1M potassium phosphate (pH 8.2) containing 1.0 μ mol NADH and 4.0μmol KNO_3, 0,2–0,8 ml enzyme preparation and demineralized water to make a final volume of 4.0 ml. After 20 minutes incubation at 27 °C excess NADH was removed by shaking with activated charcoal (STULEN 1970). In 2.0 ml of the filtrate the nitrite formed was determined by adding 1.0 ml 1 % (w/v) sulphanilamide in 1N HCl and 1.0 ml 0,01 % (w/v) N-(1-naphtyl) ethylene diamine dihydrochloride. After 30 minutes the extinction was determined at 540 mμ in a Unicam SP 600 spectrophotometer. Extraction and assay of the enzyme were a modification of the procedure described by BEEVERS *et al.* (1965).

2.4. Extraction with polyvinylpyrrolidone

Polyvinylpyrrolidone (Polyclar AT) was washed with extraction buffer by mixing the powder with the buffer and filtering the slurry by suction. This procedure was repeated twice. To 1.0 g pulverized cotyledons 10.0 g Polyclar AT preparation (25% dry weight) and 4.0 ml extraction buffer were added. The mixture was ground for 30 seconds in a mortar and then centrifuged at 0°C for 20 minutes at 1800 g. All actions were carried out at 0–4°C. The enzyme assay was performed with the supernatant fraction. This extraction procedure was based on the method described by KLEPPER & HAGEMAN (1969).

3. RESULTS

The activity of an enzyme preparation, extracted from cotyledons harvested after an induction period of 6 hours in the light, was determined by adding various amounts of enzyme preparation to the reaction mixture. The apparent activity per ml of enzyme preparation decreased when increasing amounts of enzyme preparation were added to the reaction mixture (*table 1*). It is improbable that this decrease in reaction rate is due to exhaustion of substrate or of

Table 1. The relationship between enzyme concentration and rate of nitrate reductase activity

concentration of enzyme preparation in ml/2.0 ml	nitrate reductase activity in mμ mol NO_2^-/20 min./ml enzyme preparation
0.1	470,9
0.2	385,4
0.3	290,9
0.4	254,4

electron donor, since the maximum amounts of NO_3^- and of NADH used up in the experiment described above were 5% and 20%, respectively. It is likely, therefore, that the enzyme preparation contains an endogenous inhibitor and this can be demonstrated by plotting (E)/V vs (E) where (E) is the enzyme concentration and V the reaction rate at enzyme concentration (E).

When an inhibitor is present, the data will fall on a straight line with an upward slope. By plotting (E)/V against (E) at various substrate concentrations a family of straight lines is obtained, which in the case of non-competitive inhibition will meet at one point on the (E) axis (FRIEDENWALD & MAENGWYN-DAVIES 1954).

The procedure described above was applied to an enzyme preparation from cotyledons which had been induced for 6 hours in the light (*fig. 1*). It appeared that a endogenous inhibitor of a non-competitive nature was present in the enzyme preparation. This was also shown for an enzyme preparation from cotyledons induced for 6 hours in the dark.

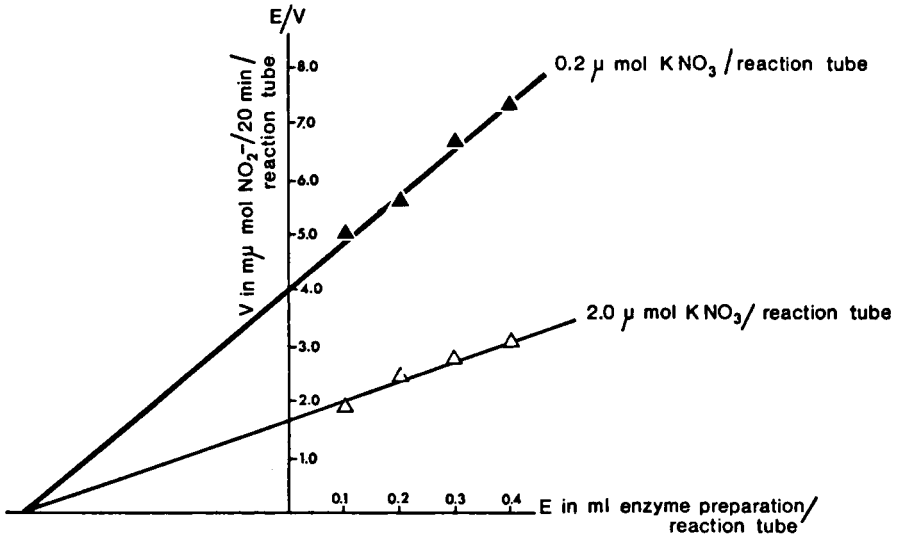

Fig. 1. The relationship between enzyme concentration and observed enzyme activity at two substrate concentrations

A simple derivation[1] of the endogenous inhibition equation was given by SCHIMMER *et al.* (1961). For non-competitive inhibition they gave the following equation:

$$E/V_I = 1/k_1 + \frac{k_2}{k_1 K_I} E \qquad (I)$$

where V_I is the observed enzyme activity at enzyme concentration (E) and k_1, k_2 and K_I are constants. The intercept of this line on the ordinate is $1/k_1$ where k_1 is the specific activity in the absence of inhibitor. Thus it is possible to calculate the specific activity of the enzyme without removing the endogenous inhibitor. It is also possible to calculate the quantity of inhibitor in arbitrary units, present in a fixed amount of enzyme preparation, by dividing the slope of the line

1. For non competitive inhibition:

$$\frac{Vo}{V_I} = 1 + \frac{I}{K_I} \qquad (II)$$

Vo = velocity of enzyme action in absence of inhibitor
V_I = velocity in presence of inhibitor at concentration I
K_I = equilibrium constant of the inhibitor-enzyme complex
Vo = k_1 E and I = k_2 E, where k_1 and k_2 are proportionality constants.
Substitution in equation II gives equation I.

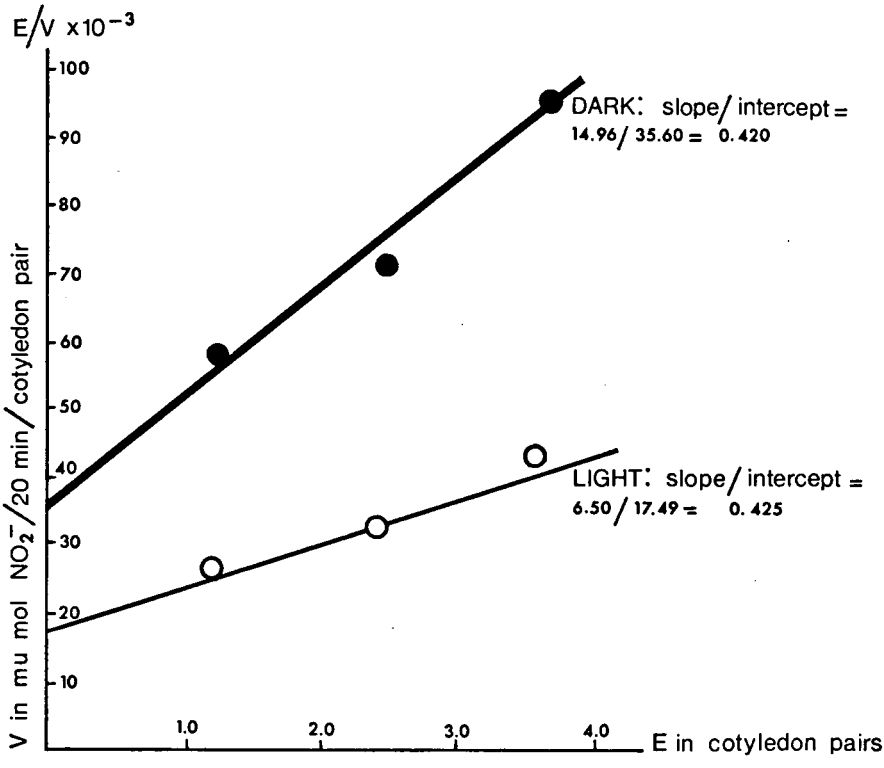

Fig. 2. Calculation of the amount of inhibitor present in one cotyledon pair

by the intercept on the ordinate.[2] This was done for enzyme preparations from cotyledons, induced for 6 hours either in the light or in the dark. The quantity of inhibitor present in an enzyme preparation derived from one pair of cotyledons was computed (*fig. 2*). It is seen that after an induction period of 6 hours, either in light or in darkness, the same quantity of inhibitor is present in one pair of cotyledons. The quantity of inhibitor present in cotyledons harvested at

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2. slope $= \frac{k_2}{k_1 K_I}$

intercept $= 1/k_1$

$I = k_2 E$

By division and substitution we derive:

$$\text{slope/intercept} = \frac{I}{K_I E}$$

for $E = 1$: $\text{slope/intercept} = \frac{I}{K_I}$.

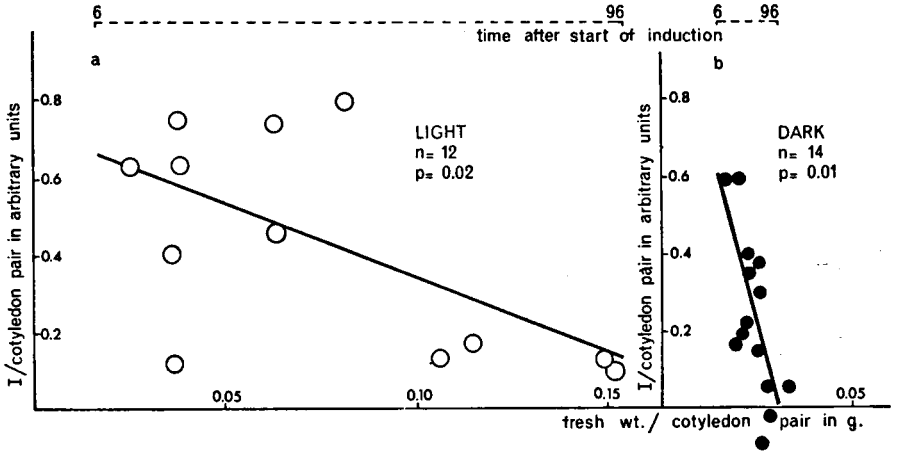

Fig. 3. The relationship between the amount of inhibitor present in one cotyledon pair and increasing age in light (a) and in darkness (b).

various time intervals after the start of induction in light or in darkness was computed. The total quantity of inhibitor in one pair of cotyledons was computed by adding the levels found in the supernatant and in the pellet fraction. The total quantity of inhibitor was plotted against the fresh weight of one pair of cotyledons (*fig. 3*). This procedure was adopted because, despite cultivation under standardized conditions, different batches of seedlings showed different rates of growth, and thus cotyledons fresh weight provided a better basis for expressing inhibitor level than did time per se. From *fig. 3* it is seen that in both light and darkness the amount of inhibitor present decreases as the seedlings grow. Since the amount of inhibitor was computed on a cotyledon basis and not on a fresh weight basis the decrease found is not caused by dilution. Thus it can be concluded that the amount of inhibitor gradually decreases during growth both in light and in darkness.

In order to elucidate the nature of the inhibitor we determined whether the

Table 2. The effect of the use of polyvinylpyrrolidone (PVP) during extraction of nitrate reductase from cotyledons, induced for 6 hours in the light.

concentration of enzyme preparation in ml/2.0 ml reaction mixture	nitrate reductase activity (NRA) in mμ mol	
	NO_2^-/20 min./ml enzyme preparation	
	without PVP	with PVP
0.1	380,6	428,2
0.2	360,0	441,8
0.3	327,2	441,2
0.4	280,2	430,4
	NRA in absence of inhibitor (calculated) 449,0	

inhibition could be reversed by adding polyvinylpyrrolidone to the extraction medium. By adsorption on this polymer phenolics of molecular weight greater than chlorogenic acid (MW 354) can be removed; the optimum pH is 3.5 (ANDERSON 1968). From *table 2* it is seen that the inhibition is completely reversed when polyvinylpyrrolidone is added during the extraction of tissue containing a measurable amount of inhibitor.

4. DISCUSSION

The inhibitor of nitrate reductase, which was mainly found in the youngest seedlings in the current experiments, could be removed by polyvinylpyrrolidone, even at the high pH maintained during the extraction. From this it can be concluded that the inhibitor may be of a phenolic nature, which also agrees with the finding that the inhibition is of a non-competitive nature.

Endogenous phenolics are oxidized, after homogenization, by o-diphenol oxidases to products which can polymerize to yield products of increasing molecular weights. Most plant enzymes are inhibited by the oxidized and polymerised phenolics (ANDERSON 1968). The formation of oxidation products can be prevented by removal of phenolic substrates or by inhibition of o-diphenol oxidase activity. Insoluble polyvinylpyrrolidone can prevent the formation of oxidation products by adsorption of the substrates or possibly also by direct, irreversible inhibition of o-diphenol oxidase (HAREL *et al.* 1964).

According to ANDERSON (1968) the formation of oxidation products can also be prevented in the extraction medium. Reducing agents such as cysteine combine with quinones to form a product which is not further oxidized and does not inhibit enzymes.

Part of the function of cysteine might also be to prevent irreversible oxidation of sulfhydryl groups associated with enzyme activity. Cysteine was included in the extraction medium in the current experiments. The concentration used, 10^{-4}M, was found to be optimal for extracting tissues of different ages: higher concentrations were inhibitory. Inhibition by high concentrations was also reported by HEWITT & NICHOLAS (1964). Despite the inclusion of an optimal concentration of cysteine in the extraction medium inhibitory products were still present in the enzyme preparations.

The decline in amount of inhibitor found in cotyledons of increasing age can be caused by a decline in amount of phenolic substrates. Such a decrease was found in etiolated seedlings of *Helianthus annuus* where the amount of chlorogenic acid present in a given number of seedlings decreased sharply with increasing age. When the etiolated seedlings were placed in the light no new chlorogenic acid was formed. The amount of chlorogenic acid present was not equal for each batch of seedlings; this was attributed to unavoidable differences in the seeds used (RUCKENBROD 1955). In our experiments changes of inhibitor with time closely resemble these changes reported for *Helianthus*. The great variances in amount of inhibitor found in one pair of cotyledons of equal fresh weight (see *fig. 3*) may also be explained by the presence of varying amounts of (precursors of) inhibiting substances in the seeds used.

Since different amounts of inhibitor are present in seedlings of different age, and since a given amount of inhibitor reduces enzyme activity more when enzyme levels are low, it is necessary to eliminate this inhibition in the assay of nitrate reductase. This is particularly important for experiments where enzyme activity is compared for cotyledons of various ages and after various treatments. This can be done either by calculating activity in the absence of inhibitor, from data derived by incubating increasing amounts of extract, or by extraction with polyvinylpyrrolidone.

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