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ACTIVATION OF AN NADPH OXIDASE FROM MAIZE BY DIPICOLINIC ACID

VIJAI V. S. TYAGI, R. R. HENKE and W. R. FARKAS*

Department of Botany and Department of Environmental Practice*, University of Tennessee, Knoxville, TN 37996, U.S.A.

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Key Word Index—NADPH oxidase; dipicolinic acid; maize.

Abstract—An NADPH oxidase has been partially purified from maize kernels. The activity of this enzyme, as measured by NADPH disappearance or oxygen consumption, was enhanced by dipicolinic acid (pyridine 2,6-dicarboxylic acid). Dipicolinic acid was not consumed in this reaction indicating that it was an activator rather than a substrate of this enzyme.

INTRODUCTION

The committed step in lysine biosynthesis in bacteria [1] and in plants [2] is the condensation of aspartic semialdehyde and pyruvate to form a molecule which cyclizes to dihydrodipicolinic acid by forming an internal Schiff's base between the α-keto group and the ε-amino group. A reductase then catalyses the reduction of dihydrodipicolinic acid to tetrahydrodipicolinic acid [3–5]. Dipicolinic acid (DPA) is a potent competitive inhibitor of plant and bacterial dihydrodipicolinic acid reductase [4, 5] and could prove useful in studies where it is necessary to inhibit lysine biosynthesis. However, nothing is known about the metabolism of DPA in plants. In order to determine if DPA was converted to dihydrodipicolinic acid in maize we added DPA to maize extracts along with NADPH and observed a marked increase in the rate of NADPH disappearance over control samples that did not contain DPA. An assay for DPA revealed that its concentration did not change during the reaction. In this report, we show that maize kernels contain an NADPH oxidase that is activated by DPA.

RESULTS AND DISCUSSION

Stimulation of NADPH oxidase by dipicolinic acid

The rate of NADPH and NADH oxidation in crude maize kernel extract, as determined by decrease in absorbance at 340 nm, was found to be linear with protein concentration. The rate of NADH oxidation was about one-third the rate of NADPH oxidation. The rate of NADPH oxidation increased up to two times its basal rate on addition of DPA to the reaction mixture at a final concentration of 2 mM. This indicated that either DPA was a substrate and was being reduced by NADPH or that DPA activated the NADPH oxidase in maize extract.

The enzyme fraction that was eluted from DEAE cellulose at 0.07 m KCl (see Experimental) showed high enzymatic activity for the oxidation of NADPH. After dialysis against phosphate buffer (pH 7.0; 0.01 M) to remove KCl, it was used in the experiment to demonstrate the effect of DPA on the rate of NADPH oxidation. As shown in Fig. 1 the presence of 2.2 mM DPA doubled the rate of NADPH oxidation.

In order to determine if DPA was reacting directly with NADPH or was stimulating the enzyme-catalysed reaction of NADPH with oxygen we studied the effect of DPA on oxygen uptake. Figure 2 shows that the rate of oxygen consumption in the reaction mixture doubled in the presence of 2 mM DPA.

Dipicolinic acid is not a substrate during NADPH oxidation

The possibility that the increased rate of disappearance of NADPH in the presence of DPA was due to an NADPH-dependent reduction of DPA to dihydrodipicolinic acid was considered. Dihydrodipicolonic acid is a known intermediate in the pathway for the biosynthesis of lysine in maize [5].

Table 1 shows that the concentration of DPA did not change during the reaction that consumed NADPH, thus giving further proof that DPA was stimulating the oxidation of NADPH and was not acting as a substrate for an NADPH-dependent DPA reductase.

Dipicolinic acid is a major constituent of bacterial spores. Halvorson *et al.* [6] reported that DPA stimulated the rate of oxygen uptake during glucose oxidation by extracts of *Bacillus cereus* spores. Dipicolinic acid also stimulated NADPH–cytochrome *c* reductase and NADPH oxidase activities. Since bacterial spores contain large amounts of DPA which is not utilized during germination, it was suggested that DPA acts as a regulatory molecule during bacterial spore germination by stimulating the aforementioned enzymes. However, DPA has not been observed in any biological source other than the bacterial spore [7]. Since DPA does not occur in plants, it is unlikely that it has a regulatory role in maize. Nevertheless, the degree of stimulation of maize NADPH oxidase by a given concentration of DPA is remarkably similar to that observed for the NADPH oxidase of the bacterial spore. Possibly, the ability of NADPH oxidases to be stimulated by DPA has been conserved during

Abbreviation: DPA, dipicolinic acid (pyridine 2,6-dicarboxylic acid).

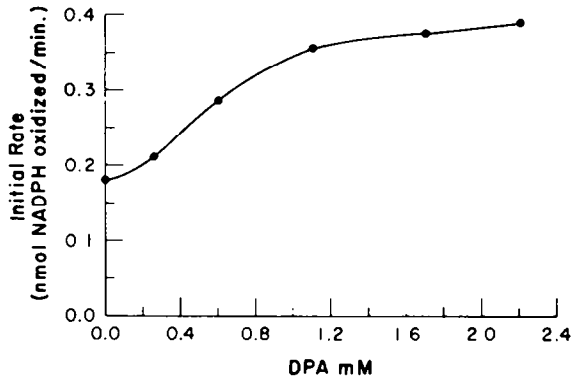

Fig. 1. Rate of oxidation of NADPH vs concentration of dipicolinic acid. The reaction mixture contained 0.05 μmol NADPH, 10 μg NADPH oxidase (DEAE-cellulose column fraction), 0.18 mmol phosphate buffer, pH 7.0, containing 13 nmol mercaptoethanol, in a final volume of 1.8 ml. Initial rate was calculated from the decrease in absorbance at 340 nm for the first 5 min of the reaction.

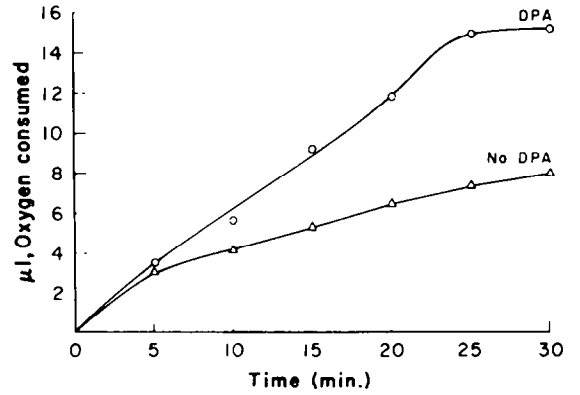

Fig. 2. Stimulation by DPA of oxygen uptake by NADPH oxidase. The reaction mixture contained 0.16 μmol NADPH, 32 μg NADPH oxidase (DEAE-cellulose column fraction), 0.6 mmol phosphate buffer (pH 7.0), 33 μmol DPA, in a final volume of 6 ml.

evolution or it is a general property of these oxidases to be stimulated by DPA.

EXPERIMENTAL

Spectrophotometric assay of NADPH oxidase. NADPH oxidation was measured at room temp. in a reaction mixture containing the indicated amount of enzyme, 0.18 mmol Pi buffer (pH 7.0), 13 nmol 2-mercaptoethanol and 0.05 μmol NADPH in 1.8 ml. The disappearance of NADPH was followed at 340 nm.

Assay of NADPH oxidase by determination of oxygen consumption. The NADPH oxidase activity was also measured by determining the uptake of O_2. An O_2 monitoring probe (model DO–166, Lazar Research Laboratory, Los Angeles, CA) was used to measure the rate of O_2 consumption. The probe was connected to a standard pH meter set to read voltage, and the membrane end was lowered into the reaction mixture. The composition of this reaction mixture is given in the legend to Fig. 2. The reaction mixture was stirred at a constant rate by a magnetic stirrer during the O_2 measurement. The concentration of O_2 as a function of time was calculated according to the calibration provided by the manufacturer of the probe. A steady decline in voltage with time indicating O_2 uptake was observed.

Table 1. Dipicolinic acid is not consumed while NADPH is oxidized

Reaction time (hr)	Amount of DPA in reaction mixture (μmol) 1	2	3
0	4.0	4.0	4.0
2	3.8	3.8	3.9

1. Complete reaction mixture: NADPH 0.1 μmol; DPA 4.0 μmol; enzyme 20.0 μg (DEAE-cellulose column fraction); potassium phosphate 0.36 mmol (pH 7.0); 26 nmol of 2-mercaptoethanol; glucose-6-phosphate, 12 μmol and glucose-6-phosphate dehydrogenase 10 units in a final vol. of 3.5 ml.

2. Reaction mixture as in 1 without enzyme.

3. Reaction mixture as in 1 without NADPH.

The colourimetric assay was used for the determination of DPA. The data in this table were confirmed using the assay in which DPA was extracted into diethyl ether (see Experimental).

Assay for dipicolinic acid disappearance. In order to determine if dipicolinic acid was being reduced in a reaction with NADPH, we felt it important to keep the concentration of NADPH comparable to the NADPH concentration in Figs 1 and 2. Therefore, an NADPH generating system consisting of glucose 6-phosphate and glucose 6-phosphate dehydrogenase was used. See legend to Table 1.

Two different assays for dipicolinic acid were used. The method of Janssen *et al.* [8] monitors the yellow colour (440 nm) that develops as a result of the reaction between DPA and ferrous ammonium sulphate. Freshly prepared reagent (0.99 ml), consisting of a soln containing 1% ferrous ammonium sulphate and 1% ascorbic acid in 0.5 M sodium acetate buffer, pH 5.5, was added to 3.5 ml of a soln containing dipicolinic acid. The yellow colour developed instantly at room temp.

The second assay consisted of extracting the reaction mixture with Et_2O and determining the absorbance of the extracted DPA [9]. One and a half ml of the reaction mixture was withdrawn at the beginning and at the end of the reaction and added to 5 ml Et_2O, containing 0.5 ml H_2O. After vigorous shaking the Et_2O layer was separated and its absorbance at 269 nm (λ_{max} of DPA) measured against an Et_2O blank. The concentration of DPA was determined from standard calibration curves in both assays.

Purification of NADPH oxidase: preparation of crude extract of maize kernels. Maize (*Zea mays* cv pioneer 3145) was field grown [10]. The ears were harvested 3 weeks after pollination and the kernels removed and stored at −20°. To prepare the extract, 300 g of kernels were soaked overnight at 4° in 500 ml 0.05 M Tris–Cl, pH 8.0. The kernels were collected and then homogenized for 1 min at 4° with a Waring blender containing 300 ml of 7.3 mM 2-mercaptoethanol–0.05 M Tris–Cl, pH 8.0, (buffer A). The resulting suspension was filtered through 4 layers of

Table 2. Purification of NADPH oxidase

	Total amount of protein (mg)	Specific activity nmol NADPH oxidized/mg protein/min	Fold purification	Total amount of enzyme (units)
Crude extract	62	0.4	—	25
Ammonium sulphate (0–90%)	47	0.7	1.8	33
Heat treatment	22	3.4	8.5	75
DEAE-cellulose	6	18.5	46	111

One unit = 1 nmol of NADPH oxidized/min.

cheesecloth and then centrifuged at 27000 *g* for 15 min.

$(NH_4)_2SO_4$ *fractionation.* The supernatant was brought to 90% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 27000 *g* and was resuspended in 50 ml of buffer A. The precipitate did not dissolve completely and the suspension was dialysed against 4 l. of the above buffer overnight at 4°. After dialysis, the suspension was centrifuged at 186000 *g* for 1 hr to remove the undissolved material. The clear supernatant, which was yellowish brown, was brought to a concentration of 10% glycerol and was stored at −80° until further purification.

Heat treatment of the $(NH_4)_2SO_4$ *fraction.* The partially pure enzyme fraction was kept at temperatures ranging from 35° to 85° in a thermostatically controlled water bath for 10 min. After cooling to room temp., the denatured protein was removed by centrifugation and enzyme activity was determined by the disappearance of NADPH. The fraction that had been treated for 10 min at 62° was further purified by DEAE-cellulose chromatography.

DEAE-cellulose chromatography. Thirty ml of the heat-treated enzyme extract containing 30 mg of protein was loaded onto a DEAE-cellulose column (1.5 × 6 cm) equilibrated with buffer A. The column was eluted batchwise with 25 ml aliquots of the same buffer containing 0.01, 0.04, 0.07 and 0.1 M KCl. Two and a half ml fractions were collected at a flow rate of 0.3 ml/min. The NADPH oxidase activity was eluted at 0.07 M KCl. This material was stored at −80° in 10% glycerol.

The results of the enzyme purification are summarized in Table 2. Note that the total activity as well as the specific activity increased during the purification, presumably due to removal of an inhibitor(s).

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