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Conversion of 1′-Deoxy-^{2}H-ABA to ^{2}H-ABA in Cell-Free Extracts from *Cercospora rosicola*

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Abstract. Abscisic acid (ABA) biosynthesis in the fungus *Cercospora rosicola* has been studied previously by feeding potential intermediates to growing cultures and examining the products. The present work describes the isolation and partial characterization of an enzyme system which catalyzes the last step in this pathway, the conversion of 1′-deoxy-^{2}H-ABA to ^{2}H-ABA. Enzyme extracts were prepared from cold-pressed mycelia of actively growing *C. rosicola* in tricine buffer containing sucrose and β-mercaptoethanol. Low-speed supernatants and resuspended microsomal preparations were active in converting 1′-deoxy-^{2}H-ABA to ^{2}H-ABA. Optimum conditions for the reactions were established at 10 μM substrate, 300 μl enzyme extract in a total volume of 1 ml. Reaction products were chromatographed by reverse-phase high-performance liquid chromatography (HPLC). The presumptive ABA fractions were collected and the ^{2}H-ABA was measured by combined gas chromatography-mass spectrometry (GC-MS) with an ^{2}H-ABA standard. Most of the enzyme activity was found in the microsomal fraction. Typical reaction rates were on the order of 1.5 pmol ^{2}H-ABA formed/min/mg protein. The reaction required NADPH and was enhanced by FAD. Triarimol, a substituted pyrimidine, which inhibits ABA synthesis in *C. rosicola*, had no effect on the conversion of 1′-deoxy-^{2}H-ABA to ^{2}H-ABA.

Abscisic acid (ABA) is a sesquiterpenoid plant growth regulator with multiple physiological roles in higher plants. ABA biosynthesis has been studied in higher plants and in phytopathogenic fungi, including *Cercospora rosicola* Passerini which accumulates relatively large amounts of ABA (Assante et al. 1977). Evidence is building that the ABA biosynthetic pathway in higher plants differs from that in *C. rosicola*. In higher plants ABA has been presumed to arise from preexisting oxygenated carotenoids (Gage et al. 1989, Li and Walton 1987, 1990, Zeevaart et al. 1989), whereas in *C. rosicola* it is probably derived directly from farnesyl pyrophosphate (Bennett et al. 1984, Neill and Horgan 1983, Neill et al. 1981, 1982a,b, and 1984).

Most of the work on the biosynthetic pathway in fungi has involved metabolism of presumed substrates in growing cultures. In feeding experiments with liquid cultures of *C. rosicola*, (*2Z,4E*) forms of α-ionylideneethanol and α-ionylideneacetic acid were converted to the corresponding form of 4′-hydroxy-α-ionylideneacetic acid, 1′-deoxy-ABA, and ABA (Horgan et al. 1983, Neill and Horgan 1983, Neill et al. 1982a,b, 1984, and 1987, Norman et al. 1985). (*2Z,4E*)-1′-deoxy-ABA is metabolized to (*2Z,4E*)-ABA, and is thought to be the immediate precursor of ABA in *C. rosicola* (Neill et al. 1981, 1982a,b).

The goal of this study was to establish a cell-free enzyme system capable of catalyzing one or more of the reactions in ABA biosynthesis. We describe the development of a cell-free system from *C. rosicola* extracts which converts 1′-deoxy-^{2}H-ABA to ^{2}H-ABA.

Methods

Organism and Culture Methods

Strain no. 138.35 of *C. rosicola* (from R. Bennett, USDA-ARS, Pasadena, CA, USA) was cultured at room temperature on a reciprocal shaker at 90 rpm for 5 days in a chemically defined liquid medium described by Norman et al. (1981).

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Enzyme Extracts

Cultures were harvested by filtration under vacuum, and the mycelial mat was rinsed with water (~60 ml/g fresh wt) followed by an equal volume of 50 mM tricine buffer, pH 8.0. The mat was then frozen in liquid nitrogen, crushed in a Sager press at 15,000 psi, and suspended in 50 mM tricine buffer, pH 7.8, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol. The suspension was centrifuged for 10 min at 10,000 *g*. The supernatant (S_{10}) was used as a source of enzyme in some of the experiments. In other experiments, S_{10} was recentrifuged for 60 min at 100,000 *g*. Microsomes in the 100,000 *g* pellet were resuspended in the same buffer (3 ml/g fresh wt of original mycelial mat). The high-speed supernatant (S_{100}) and the resuspended microsomal fraction (P_{100}) were frozen in liquid nitrogen to be used later as an enzyme source.

Enzyme Assay

We used the cell-free extracts to test the effects of several variables on synthesis of ABA *in vitro*. The variables tested were substrate concentration, enzyme concentration, and time of incubation. The effects of NADPH, FAD, and 10^{-7} M triarimol on the conversion of 1′-deoxy-^{2}H-ABA to ^{2}H-ABA were also tested.

Unless otherwise indicated, reaction mixtures contained 300 µl enzyme extract, 10 µM 1′-deoxy-^{2}H-ABA, 1 µM $MgCl_2 \cdot 6H_2O$, and 1 mM NADPH. The volume of the reaction mixture was adjusted to 1 ml with 50 mM tricine buffer containing 0.25 M sucrose at pH 7.8. Reaction mixtures were incubated for 7–20 min in a shaking water bath at 30°C and 200 rpm. Boiled enzyme or zero-time reactions were included in each experiment as controls. Typically, reactions were run in duplicate, and experiments were repeated several times. Reactions were stopped by addition of 1 ml acetone.

Product Analysis

Mixtures were extracted with 1 ml benzene:acetone, 3:1. The aqueous residue was acidified to pH 3.0 with 1 M HCl and extracted three times with 1 ml of EtOAc. The organic fraction was dried under vacuum, redissolved in 20 µl MeOH, and analyzed by high-performance liquid chromatography (HPLC) followed by gas chromatography-mass spectrometry (GC-MS). HPLC and GC-MS columns, elution solvents, and gradient programs were described earlier (Al-Nimri and Coolbaugh 1990). A standard of (*2Z,4E*)-ABA was chromatographed before each set of samples to monitor the constancy of ABA retention time.

HPLC fractions containing presumptive ^{2}H-ABA were collected and dried under vacuum. The dried residue was redissolved in 250 µl MeOH, transferred to conical vials where it was dried again, and methylated with ethereal diazomethane. The samples were dried under stream of nitrogen, redissolved in 10 µl MeOH, and reduced to 2–3 µl for injection into the GC-MS. Selected ion monitoring (SIM) method was used in ^{2}H-ABA detection unless otherwise indicated. The major ions detected by this procedure were at m/z 193 (base peak), 165, 137, 128, and 94 which are the trideuterated forms of m/z 190, 162, 134, 125, and 91, the characteristic ions of ^{1}H-ABA.

Source and Purity of Reagents

Triarimol [α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol] was technical grade from Eli Lilly and Co. (Greenfield, IN, USA). 1′-Deoxy-^{2}H-ABA (trideuterated in the side chain methyl) was prepared by Horgan et al. (1983) and provided by D. Walton (SUNY, Syracuse, NY, USA).

Results

Active enzyme preparations were obtained from low-speed supernatants (S_{10}) of 5- to 6-day-old fungal cultures. ^{2}H-ABA was detected in active enzyme reaction mixtures analyzed by GC-MS analysis of HPLC fractions containing ABA. Deuterated product was not found in the boiled enzyme controls. In an attempt to localize the site of the enzymatic activity, the S_{10} was further fractionated into a high-speed supernatant (S_{100}) and resuspended microsomes (P_{100}). Most of the activity was in the resuspended microsomal pellet and not in the supernatant (Fig. 1). The effect of 1′-deoxy-^{2}H-ABA concentration on its conversion into ^{2}H-ABA is shown in Fig. 2. Activity peaked at 15 µM and declined at higher concentrations. The relation between ^{2}H-ABA production and enzyme concentration was linear up to 400 µl P_{100} per reaction (Fig. 3). The time course for converson of 1′-deoxy-^{2}H-ABA to ^{2}H-ABA is shown in Fig. 4. Under the conditions of this assay, ^{2}H-ABA production was reasonably linear through 10 min.

The effects of NADPH, FAD, and 10^{-7} M triarimol on the converson of 1′-deoxy-^{2}H-ABA to ^{2}H-ABA are shown in Table 1. NADPH is clearly necessary for this reaction to occur. Triarimol had no inhibitory effect on this conversion using S_{10} as an enzyme source. FAD was not necessary for the reaction to take place, but enzymatic activity was enhanced in its presence.

Discussion

The development of a cell-free system to convert 1′-deoxy-^{2}H-ABA to ^{2}H-ABA has been our goal for some time to gain a better understanding of the ABA biosynthetic pathway in *C. rosicola*. The first cell-free system related to ABA biosynthesis was developed by Milborrow (1974) who used ^{14}C-MVA with a preparation of lysed chloroplasts isolated from ripening avocado fruit in reaction mixtures containing ATP, FAD, FMN, NAD, NADH, NADP, and NADPH. Cell-free systems for study of ABA biosynthesis in plants were not reported again until Sindhu and Walton (1987) described the incorporation of xanthoxin into ABA in a cell-free extract from *Phaseolus vulgaris* leaves. In these extracts, xanthoxin was incorporated into ABA in an NAD/NADP-dependent reaction. The enzymatic

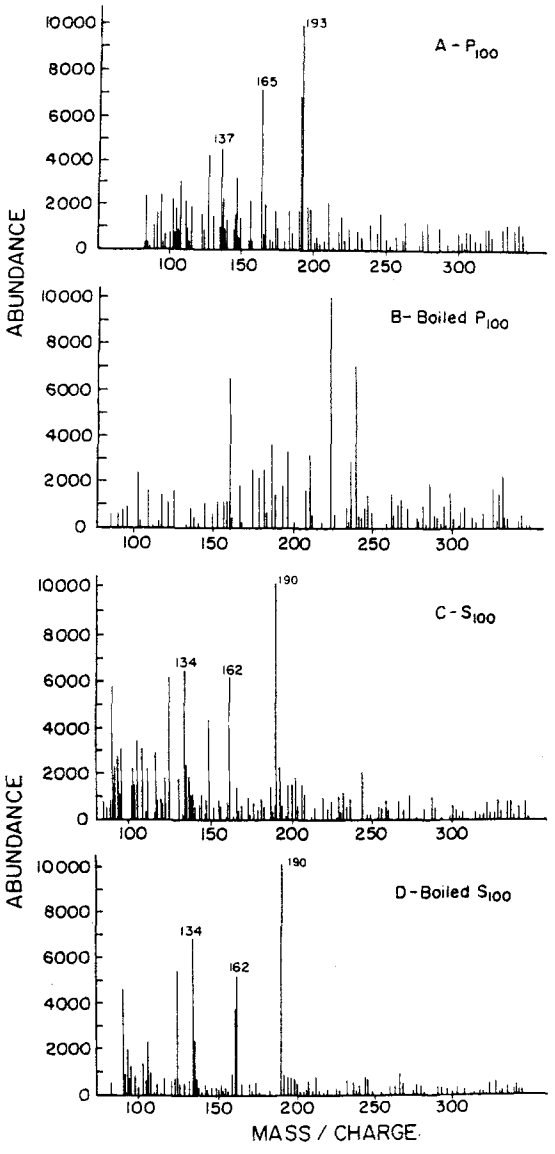

Fig. 1. Mass spectra of methylated ABA fractions from reaction mixtures containing cell-free enzyme extracts: **(A)** P_{100}, **(B)** boiled P_{100}, **(C)** S_{100}, and **(D)** boiled S_{100} as an enzyme source from *C. rosicola* preparations. Prominent native Me-ABA ions are at m/z 190, 162, and 134. Prominent deuterated Me-ABA ions are at m/z 193, 165, and 137.

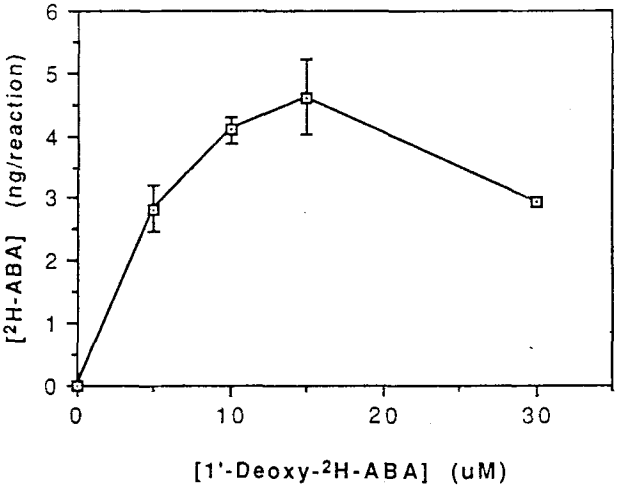

Fig. 2. Effect of 1'-deoxy-^{2}H-ABA concentration on ^{2}H-ABA production in a cell-free enzyme extract from *C. rosicola*. Reactions incubated 7 min. ^{2}H-ABA value at 30 μM 1'-deoxy-^{2}H-ABA represents one reaction without replication. Bars represent standard errors.

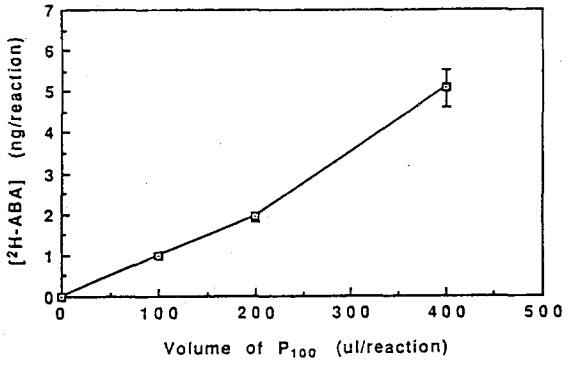

Fig. 3. Effect of the microsomal (P_{100}) enzyme concentration on conversion of 1'-deoxy-^{2}H-ABA into ^{2}H-ABA in cell-free extracts from *C. rosicola*. Reactions incubated 7 min. The protein concentration in the microsomal enzyme preparation was 1.50 mg/ml.

activity appeared to be cytosolic. More recently, Sindhu et al. (1990) have shown abscisic aldehyde is an intermediate in the two-step reaction from xanthoxin to ABA in the system from bean leaves.

In our study, a number of experiments were conducted to characterize the cell-free system from *C. rosicola*. Most of the enzymatic activity was found in the microsomal fraction, indicating that the hydroxylase enzyme is membrane-bound. In subsequent experiments with the microsomal fraction as an enzyme source, 10 μM 1'-deoxy-^{2}H-ABA was converted to ^{2}H-ABA at an average rate of 1.5 pmol ^{2}H-ABA mg protein^{-1} min^{-1}. The results indicate that NADPH is necessary for the reaction. When S_{10} was used for these studies ^{2}H-ABA was produced in the presence and absence of NADPH, but to a lesser extent in reactions with no NADPH. Microsomal fractions did not produce ^{2}H-ABA in the absence of NADPH. These results indicate the presence of some endogenous reducing potential in S_{10}, but not in the microsomal fraction.

Triarimol inhibits kaurene oxidase (a mixed function oxidase) in the gibberellin biosynthetic path-

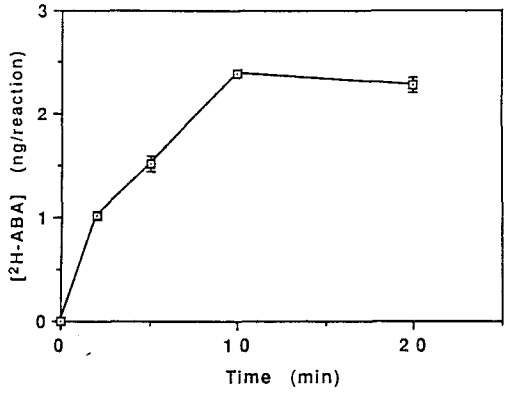

Fig. 4. Time course for the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA in cell-free extracts from *C. rosicola*.

Table 1. Effects of NADPH, FAD, and the inhibitor triarimol on the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA (±SE) in cell-free extracts from *C. rosicola*.

	ng ^{2}H-ABA formed			
Enzyme	Complete	−NADPH	−FAD	+Triarimol
S_{10}	2.66 ± 0.03	1.08 ± 0.32	ND[a]	2.16 ± 0.67
S_{10}	2.99 ± 0.24	ND	ND	3.63 ± 1.00
P_{100}	0.51 ± 0.19	0.0	0.25 ± 0.05	ND
P_{100}	0.80 ± 0.2	0.0	0.58 ± 0.03	ND
P_{100}	2.72 ± 0.89	0.0	2.01 ± 0.17	ND
P_{100}	6.37 ± 0.69	0.0	ND	ND

Reaction mixtures contained 10–30 μM substrate, 50 mM tricine buffer containing 0.25 M sucrose at pH 7.8, 100 μl S_{10} or 300 μl P_{100} enzyme extract, 1 μM $MgCl_2$, ±1 mM NADPH, ±4.7 μM FAD, and $\pm 10^{-7}$ M triarimol in a total volume of 1 ml. Reactions were incubated 7 min at 30°C.
[a] ND, not determined.

way in the fungus *Gibberella fujikuroi* (Coolbaugh et al. 1982). This inhibitor also blocks endogenous ABA production in liquid cultures of *C. rosicola*, but does not inhibit the conversion of 1'-deoxy-^{2}H-ABA to ^{2}H-ABA (Al-Nimri and Coolbaugh 1990). The present results from studies with a cell-free system confirm our previous conclusion that the site(s) of triarimol inhibition is prior to 1'-deoxy-ABA in the ABA biosynthetic pathway. We are now characterizing the enzyme responsible for 1'-deoxy-ABA oxidation.

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