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BIOSYNTHESIS OF DIHYDROISOCOUMARIN BY EXTRACTS OF ELICITOR-TREATED CARROT ROOT

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Key Word Index—*Daucus carota*; Umbelliferae; phytoalexin; polyketide biosynthesis; 6-methoxymellein; dihydroisocoumarin.

Abstract—Cell-free extracts from carrot root disks treated with 2-chloroethylphosphonic acid catalysed an NADPH-dependent conversion of acetyl-CoA and malonyl-CoA to 6-hydroxymellein, the immediate precursor of the carrot phytoalexin 6-methoxymellein. In the absence of NADPH, neither 6-hydroxymellein nor 3,4-dehydro-6-hydroxymellein, another possible intermediate, was formed. These findings suggest that 6-hydroxymellein serves, *in vivo*, as the substrate of the *O*-methyltransferase for 6-methoxymellein synthesis, and that the dihydroisocoumarin is formed from a reduced polyketide intermediate.

INTRODUCTION

Phytoalexins are antimicrobial compounds which are synthesized by higher plants in response to the invasion of microorganisms. Recently, the biosynthetic pathways of some pterocarpan phytoalexins have been extensively investigated, and some of the enzymes characterized [1–6]. However, there is little information on the biosynthetic enzymes of phytoalexins other than pterocarpan derivatives.

The carrot phytoalexin, 6-methoxymellein, accumulates in carrot cells which have been treated with uronide elicitor [7] or some other stress factor [8]. From ^{13}CNMR studies, it has been proposed [9] that 6-methoxymellein is synthesized from acetyl-CoA and malonyl-CoA via a pentaketide, and 3,4-dehydro-6-hydroxymellein has been considered as a key intermediate which would be converted to 6-methoxymellein (**3**) (Fig. 3). We have recently demonstrated [8] that an *O*-methyltransferase activity which specifically methylates the hydroxyl group at the 6-position of isocoumarin is induced in elicitor-treated carrot slices. Since this enzyme methylates 3,4-dehydro-6-hydroxymellein at the same rate as 6-hydroxymellein (**2**), it remains uncertain whether the reduction of the double bond at the 3,4-position of the possible intermediate, 3,4-dehydro-6-hydroxymellein, occurs before *O*-methylation or the modification of the isocoumarin proceeds in the reverse sequence. The aim of the present study was to elucidate the process of isocoumarin synthesis in elicitor-treated carrot tissue in more detail. A change in the enzyme activity for isocoumarin synthesis in elicitor-treated carrot cells is also reported.

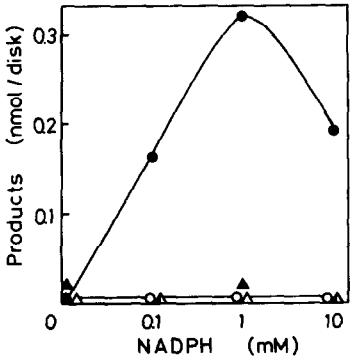

Fig. 1. Activity of NADPH-dependent 6-hydroxymellein synthesis in elicitor-treated carrot slice extract. Carrot disks were incubated in the presence of 2-CEPA for three days, and the biosynthetic activity was tested in the presence of *S*-adenosyl-L-homocysteine at various concentrations of NADPH. ●—●, 6-hydroxymellein; ○—○, 6-methoxymellein; ▲—▲, 3,4-dehydro-6-hydroxymellein; △—△, 3,4-dehydro-6-methoxymellein.

RESULTS

In the presence of NADPH, cell extracts prepared from 2-chloroethylphosphonic acid (2-CEPA)-treated carrot disks catalysed the synthesis of isocoumarin from acetyl-CoA and malonyl-CoA. 6-Hydroxymellein was produced as a major product, and a trace amount of 6-methoxymellein was also observed. *O*-Methylation of the dihydroisocoumarin was almost completely inhibited by the addition of 2 mM *S*-adenosyl-L-homocysteine, and 6-hydroxymellein (**2**) was accumulated as a sole product (Fig. 1). Another possible intermediate, 3,4-dehydro-6-hydroxymellein, was not detected. This reaction was markedly affected by the concentration of NADPH, and the most efficient incorporation of the acyl-CoAs into dihydroisocoumarin was achieved at 1 mM NADPH. In the absence of NADPH, neither 6-hydroxymellein nor 3,4-dehydro-6-hydroxymellein was detected in the reaction mixture.

In the next experiment, we examined the change in the activity of 6-hydroxymellein biosynthesis in carrot disks treated with 2-CEPA (Fig. 2). The synthetic activity began to increase about a day after the addition of elicitor. Maximum activity was observed after 3–4 days,

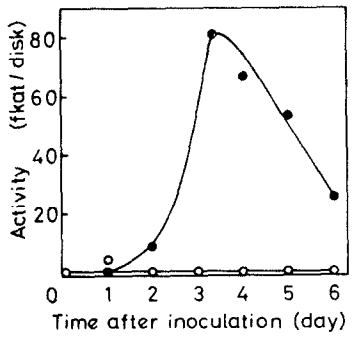

Fig. 2. Change in the biosynthetic activity of 6-hydroxymellein in 2-CEPA-treated carrot slice extract. ●—●, activity in treated cells; ○—○, controls which received sodium acetate buffer instead of the elicitor.

and it declined thereafter. Activity of the enzyme in control disks was almost negligible during the experimental period.

The effect of some reducing co-factors other than NADPH and of divalent cations on the 6-hydroxymellein synthetic activity was tested with dialysed enzyme preparations, and the results are summarized in Table 1. NADPH could not fully be replaced by NADH, and, in the presence of FMN and FAD, the acyl-CoAs were incorporated only slightly into the product. Enhancement of the activity was not observed even by the combined use of NADPH and FMN or FAD. Addition of divalent cations, as far as tested, also did not show any significant effect on the enzyme activity.

DISCUSSION

Upon treatment with 2-CEPA, carrot slices accumulate the isocoumarin phytoalexin, 6-methoxymellein [8]. Concomitant with phytoalexin formation, the biosynthetic activity of 6-hydroxymellein from acetyl-CoA and malonyl-CoA could be demonstrated in the cell extracts prepared from treated slices (Figs 1, and 2). The situation was very similar to the induced formation of 6-hydroxymellein-*O*-methyltransferase, which catalyses the final step of 6-methoxymellein biosynthesis, in elicitor-treated carrot slices reported previously [8]. The product of the reaction was solely 6-hydroxymellein when *O*-methyltransferase activity was inhibited by the addition of *S*-adenosyl-L-homocysteine (Fig. 1), and this process was found to be an NADPH-dependent reaction. In our previous study, it was shown [8] that the *O*-methyltransferase involved in 6-methoxymellein synthesis does not recognize the double bond at the 3,4-position of isocoumarin *in vitro*, and reacts with 3,4-dehydro-6-hydroxymellein as well as 6-hydroxymellein. Therefore, the sequence of the modifications of the biosynthetic intermediate of 6-methoxymellein (reduction and *O*-methylation) could not be deduced from the substrate

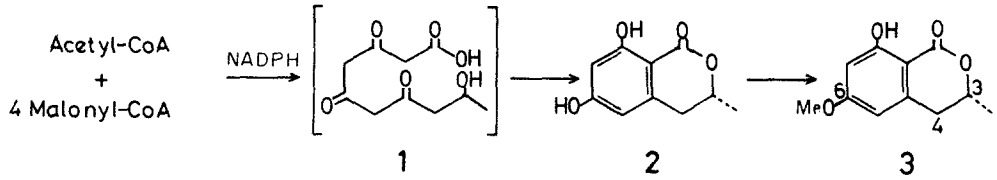

Fig. 3. Possible pathway for the biosynthesis of 6-methoxymellein.

Table 1. Effect of reducing co-factors and divalent cations on the synthetic activity of 6-hydroxymellein

Additions	Concentration (mM)	Relative activity (%)*
NADPH	1	100
NADH	1	40
FMN	0.05	21
FAD	0.05	11
NADPH + FMN	1 + 0.05	77
NADPH + FAD	1 + 0.05	93
$MgCl_2$†	1	90
$MnCl_2$†	1	85
$FeCl_2$†	1	114
$CaCl_2$†	1	102

*Relative activity expressed as percentage of the rate of 6-hydroxymellein synthesis in the presence of 1 mM NADPH. Data are the means obtained from two independent experiments.

†Divalent cations were added in the presence of 1 mM NADPH.

specificity of the *O*-methyltransferase. The fact that 6-hydroxymellein but not 3,4-dehydro-6-hydroxymellein is synthesized in the presence of *S*-adenosylhomocysteine, suggests that 6-hydroxymellein is the substrate for the *O*-methyltransferase *in vivo*, and that *O*-methylation takes place after reduction.

Figure 3 shows a possible biosynthetic pathway for 6-methoxymellein formation. The process requires NADPH as a co-factor, and we assume that the reduction of the terminal carbonyl group of polyketomethylene chain occurs before ring closure. Another possibility, the completion of isocoumarin skeleton followed by reduction of the double bond at the 3,4-position, seems less likely. If the reduction occurs after formation of isocoumarin skeleton, 3,4-dehydro-6-hydroxymellein would have been detected in the absence of NADPH as an intermediate. Moreover, we did not find any evidence for a reductase which converts 3,4-dehydro-6-hydroxymellein to its saturated form [8]. In 6-methylsalicylic acid biosynthesis of *Penicillium patulum*, it was shown [10] that NADPH-dependent reduction of polyketomethylene chain followed by dehydration occurs before the final condensation with malonyl-CoA. Coxon *et al.* [11] reported that 6-methoxymellein and 6-hydroxymellein were isolated as stable metabolites from ethylene-treated carrot roots, but the occurrence of 3,4-dehydro-melleins in carrot tissues has not been reported. These findings also support our hypothesis (Fig. 3) that 3,4-dehydro-6-hydroxymellein is not involved as an intermediate in 6-methoxymellein biosynthesis.

6-Methylsalicylic acid synthetase is a multi-functional complex [10]. However, in 6-hydroxymellein synthesis, it is not clear at present whether the condensation of the acyl-CoAs and the reduction are catalyzed by a single catalytic unit or by separable components. Purification and further characterization of the enzyme(s) for 6-hydroxymellein synthesis are now underway.

EXPERIMENTAL

Chemicals. 6-Methoxymellein was isolated from infected carrot roots [12], and 3,4-dehydro-6-methoxymellein was obtained by TLC from culture filtrates of *Ceratocystis fimbriata* [13]. 6-Hydroxymellein and 3,4-dehydro-6-hydroxymellein were prepared by demethylating the respective methoxylated compounds with BBr_3 in dry CH_2Cl_2 as described previously [8, 14].

Plant material. Carrot roots were purchased from a local market, and disks of the root tissues (1.8 cm diameter and 2 mm thickness) were prepared as described previously [8]. They were placed in Petri dishes and a 50 μl-aliquot of filter-sterilized 2-CEPA soln (10 mg/ml in 100 mM NaOAc buffer, pH 5.2) was dropped onto each of the disks. Control disks received the buffer instead of the elicitor. The disks were incubated at 26°, and at regular intervals, 4 were harvested to determine the activity of isocoumarin synthesis.

Enzyme preparation. Carrot disks were immediately homogenized in a Waring Blendor in 2.5 ml of 200 mM Na-Pi buffer (pH 7.5) containing 0.2% mercaptoethanol and 0.2 g polyvinylpolypyrrolidone. The homogenate was filtered through a double layer of gauze, and centrifuged at 10000 g for 20 min. The resultant supernatants were mixed with 0.2 g AG 1-X8 resin (Bio-Rad) and stirred for 3 min. They were then passed through a glass fibre filter paper, and the vol. adjusted to 3 ml with homogenization buffer. The preparation was immediately used for the enzyme assay.

Enzyme assay. The enzyme assay mixture contained, in a total vol. of 250 μl, 100 mM Na-Pi buffer (pH 7.5), 125 μl enzyme preparation, 0.1 mM acetyl-CoA, 0.4 mM [2-^{14}C]malonyl-CoA (sp. act. 41.1 mCi/mmol, 0.1 μCi), 2 mM *S*-adenosyl-L-homocysteine, 1 mM NADPH unless indicated otherwise, and 0.1% mercaptoethanol. The reactions were run for 1 hr at 37°, and terminated by the addition of 50 μl of 6 M HCl. Products were extracted with 200 μl EtOAc by blending, and 100 μl-aliquots were applied to a TLC plate along with authentic 6-methoxymellein, 3,4-dehydro-6-methoxymellein, 6-hydroxymellein and 3,4-dehydro-6-hydroxymellein (R_fs 0.76, 0.87, 0.35 and 0.48, respectively). The plate was developed in hexane–EtOH (5:1) and the gel at the positions corresponding to each of these compounds was scraped off and assayed for radioactivity. Co-migration of authentic 6-hydroxymellein and the radioactivity was further confirmed by 2D-TLC (hexane–EtOH, 5:1 followed by C_6H_6–MeOH, 50:1).

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