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EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE ACTIVITY OF *O*-METHYLTRANSFERASE IN CARROT CELL CULTURE

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; cell culture; *O*-methyltransferase; auxin.

Abstract—The activity of caffeic acid-*O*-methyltransferase (OMT) in carrot cells was greatly affected by the amount of 2,4-dichlorophenoxyacetic acid (2,4-D) supplemented to the culture medium. The OMT fraction was purified by $(NH_4)_2SO_4$ followed by ultrafiltration and gel filtration or DEAE-Sephadex chromatography after cells were cultured in the medium containing [2-^{14}C]-2,4-D. Thus, this purified fraction revealed high OMT activity and was still radioactive. The OMT activity was about eight-fold higher (or more) in cells cultured at 0.05 ppm 2,4-D than in those at 1.0 ppm 2,4-D. The ratio of radioactivity to OMT activity was about four-fold higher in cells cultured at 1.0 ppm 2,4-D than those at 0.05 ppm 2,4-D. On the other hand, the OMT fraction was separated into two radioactive protein fractions by DEAE-Sephadex chromatography. The radioactive fractions became Et_2O-soluble after HCl hydrolysis, but not after salt–urea treatment. From these results, it was concluded that 2,4-D is covalently bound to proteins in the OMT fraction. Such 2,4-D protein conjugates may play a role in the regulation of OMT activity.

INTRODUCTION

In earlier studies [1–6], we reported that the formation of secondary metabolites (e.g. flavonoids, phenolic acids and carotenoids) in carrot-cultured cells was greatly affected by the change in amount of 2,4-D added to the culture medium. Our attention was thus focused on how this growth regulator affects the biosynthesis of secondary metabolites. The present paper describes the effect of 2,4-D *in vivo* on the enzyme caffeic acid-*O*-methyltransferase (OMT), which catalyses the synthesis of ferulic acid and is regarded as a key enzyme of lignin biosynthesis.

RESULTS AND DISCUSSION

In carrot cells in suspension culture, three phenolic acids, caffeic, ferulic and *p*-hydroxybenzoic, are present at all growth phases [3,7]. However, certain features of the synthesis of these phenolic acids, that is, the changes in PAL activity and the incorporation of phenylalanine into the acids, and so on, were found to be greatly dependent on the growth phase and the amount of 2,4-D added to the culture medium [3,6,7]. In the present paper, the activity of the enzyme OMT was investigated in relation to changes in the amounts of the growth hormone. OMT

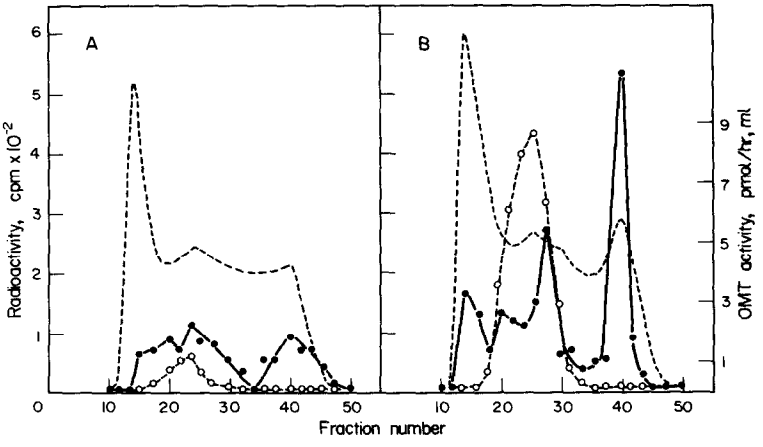

Fig. 1. Elution profile of the OMT fraction on a column (1.5 × 24 cm) of Sephadex G-200. Fraction volume, 1 ml. ----, monitored at 280 nm; ○---○, OMT activity; ●—●, radioactivity. A and B, samples prepared from cells cultured in media with 1.0 and 0.05 ppm 2,4-D, respectively.

Table 1. Radioactivity of the OMT fraction in carrot cells cultured at 0.05 or 1.0 ppm 2,4-D

Concentration of 2,4-D (ppm)	A OMT activity (fkat*/mg protein)	B Radioactivity (cpm/mg protein)	B/A
0.05	5.06	1020	202
1.0	0.61	550	902

*femtokatals.

was extracted from cells cultured in the medium containing [2-^{14}C]-2,4-D and purified by $(NH_4)_2SO_4$ precipitation followed by ultrafiltration and gel filtration or DEAE-Sephadex chromatography.

The elution profile of gel filtration on a column of Sephadex G-200 is shown in Fig. 1. The OMT fractions (20–30) were collected, pooled and filtered through a membrane of UM-10. This fraction was still radioactive. The OMT activity was about eight-fold higher in cells cultured at 0.05 ppm 2,4-D than in those at 1.0 ppm 2,4-D. The ratio of radioactivity to OMT activity was about four-fold higher in cells cultured at 1.0 ppm 2,4-D than those at 0.05 ppm 2,4-D (Table 1). Estimating from the dilution of ^{14}C (1 μCi/150 ml culture; specific activity of 2,4-D, 20–40 mCi/mmol) in the medium containing 1.0 ppm 2,4-D, this ratio would be even higher. DEAE-Sephadex chromatography revealed that the OMT fraction could be separated into two radioactive protein fractions (Fig. 2).

In order to obtain more detailed information on the presence of ^{14}C in the OMT fraction, it was subjected to HCl hydrolysis or salt–urea treatment. After acid hydrolysis at 110°, 50% of the radioactive fraction was Et_2O-soluble in OMT from cells cultured in media with 0.05 ppm 2,4-D, and 75% for the OMT from those with 1.0 ppm 2,4-D treatment. However, 16% of the total radioactivity was found in the Et_2O-soluble fraction of the OMT of cells cultured at 0.05 ppm 2,4-D and 18% for those at 1.0 ppm 2,4-D even by acid hydrolysis at 0°. Thus, the ratio of net radioactivity in the Et_2O-soluble fraction to total OMT activity by acid hydrolysis at 110° was

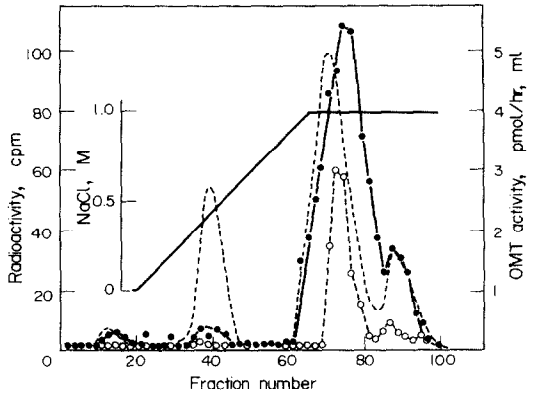

Fig. 2. Elution profile of the OMT fraction on a column (1.5 × 24 cm) of DEAE-Sephadex A-50. Fraction volume, 1 ml. ----, monitored at 280 nm; ○---○ OMT activity; ●—●, radioactivity. The sample applied to the column was prepared from cells cultured in media containing 0.05 ppm 2,4-D.

Table 2. Radioactivity of the Et_2O-soluble fraction after HCl hydrolysis of the OMT fraction in carrot cells cultured at 0.05 or 1.0 ppm 2,4-D

Concentration of 2,4-D (ppm)	Radioactivity* (cpm/mg protein) A	B	B-A/OMT activity
0.05	160	516	70
1.0	97	412	516

*In Et_2O-soluble fraction after hydrolysis in glass-distilled HCl. A, 0° for 22 hr; B, 110° for 22 hr (see Table 1 for total value).

Table 3. Radioactivity of the OMT fraction in carrot cells after NaCl–urea treatment

OMT fraction*	Radioactivity (cpm/mg protein) Et_2O	NaCl–urea
1	1	968
2	0	948

*Cells were cultured in media containing 0.05 ppm 2,4-D and both fractions had OMT activity of 5.06 fkat/mg protein. 1, Stirred in 2 M NaCl–5 M urea–20 mM Tris–5 mM 2-mercaptoethanol (pH 7.5); and 2, stirred in 20 mM Tris–5 mM 2-mercaptoethanol (pH 7.5).

about seven-fold higher at cells cultured at 1.0 ppm 2,4-D than those at 0.05 ppm 2,4-D (Table 2). On the contrary, no radioactivity was detected in the Et_2O-soluble fraction after salt–urea treatment (Table 3).

Several workers have already reported the metabolism of 2,4-D in plant tissues or cells [8,9]. They showed that the Et_2O-soluble fraction contains mainly unmetabolized 2,4-D and amino acid conjugates, whereas water-soluble metabolites were mainly ring-hydroxylated glycosides and the glucose ester of 2,4-D. The biochemical responses to 2,4-D in plant tissues or cells also have been investigated widely. However, almost all these investigations concerned the effect of this growth regulator on primary metabolism or the behaviour of nucleic acids [10–13]. There have been few reports so far on changes in secondary metabolism to 2,4-D or other auxins in relation to the level of protein or enzymes. In the present work, the OMT fraction was prepared by $(NH_4)_2SO_4$ precipitation followed by ultrafiltration and further gel filtration or ion-exchange chromatography. Since the preparation was radioactive after growth in labelled 2,4-D, it must consist of protein(s) which bind covalently to the hormone. Such 2,4-D protein conjugates may play a role in the regulation of OMT activity in carrot cells in suspension culture.

EXPERIMENTAL

Cultured cells. Carrot cell line GD-2, used in these experiments, was derived from the storage root of red carrot cv Kintoki [1,2] and maintained in the liquid medium of Murashige and Skoog

[14] containing 1 ppm 2,4-D in place of IAA and kinetin. The detailed procedure of obtaining suspension cultures was as described previously [15]. Where required, [2-^{14}C]-2,4-D was added at 1 μCi/150 ml culture. Cultured cells were collected at the late logarithmic phase when the level of OMT activity was minimal [7].

Extraction and purification of OMT. Cultured cells were washed with H_2O and then 100 mM KH_2PO_4–5 mM 2-mercaptoethanol (pH 7.5) on a Buchner funnel. Washed cells were disrupted with quartz sand in the above buffer and centrifuged at 10000 *g*. The ppt. with 30–70% saturation of $(NH_4)_2SO_4$ was dissolved in 20 mM potassium phosphate (pH 7.5) and ultrafiltered through a membrane of Amicon UM-10 (above 10^4) with excess buffer. The ultrafiltrate was added to a column of Sephadex G-200 and eluted with 20 mM KH_2PO_4–5 mM 2-mercaptoethanol (pH 7.5). Alternatively, the ultrafiltrate was chromatographed on a column of DEAE-Sephadex A-50 with a gradient of 0–1.0 M NaCl containing 20 mM Tris and 5 mM 2-mercaptoethanol (pH 7.5). The OMT fraction was pooled, desalted and concd on a membrane of UM-10. This fraction was referred to as the purified OMT fraction.

Assay of OMT activity. The reaction mixture contained 10 μmol cysteine, 0.5 μmol caffeic acid, 1 μmol $MgCl_2$, 0.1 μCi [^3H_3C]-*S*-adenosylmethionine and the enzyme (300 μg protein) in 1 ml 100 mM potassium phosphate–5 mM 2-mercaptoethanol (pH 7.5). The reaction proceeded for 1 hr at 30° and was stopped by the addition of HCl and ferulic acid. After extraction of mixture with Et_2O, the organic layer was washed with H_2O and evapd to dryness. The radioactivity of the residue was counted in a soln of toluene–EtOH PPO POPOP with a liquid scintillation counter.

Acid hydrolysis of OMT fraction. The purified OMT fraction was lyophilized, dissolved in glass-distilled HCl and left for 22 hr at 0° or refluxed at 110° under N_2 gas for 22 hr. The hydrolysate was extracted with Et_2O, the top layer washed with glass-distilled HCl, and evapd to dryness. The radioactivity of the residue was counted as described above.

Treatment of OMT fraction with salt and urea. The purified OMT fraction was lyophilized, suspended and stirred in 2 M NaCl–5 M urea–20 mM Tris (pH 7.5) for 3 hr. The acidified suspension was extracted with Et_2O. The radioactivities of the Et_2O and NaCl–urea layers were counted as described above.

Other procedures. All procedures were carried out at 2–4° unless stated otherwise. The protein fraction was monitored at 280 nm or assayed by the method of Lowry *et al.* [16].

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