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TWO FORMS OF *O*-METHYLTRANSFERASE IN TOBACCO CELL SUSPENSION CULTURE

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Key Word Index—*Nicotiana tabacum*; Solanaceae; cell suspension culture; *O*-methyltransferase; phenolic biosynthesis; enzyme; *meta*- and *para*-methylation.

Abstract—An *S*-adenosyl-L-methionine: *o*-dihydric phenol *O*-methyltransferase was isolated from tobacco cell suspension culture and was partially purified by $(NH_4)_2SO_4$ precipitation and successive chromatography on DEAE-Sepharose, Sephacryl S-200 and hydroxyapatite columns. It catalysed the *O*-methylation of 3 cinnamic acids, two coumarins and two flavonoids, but to different extents. Results obtained from polyacrylamide gel electrophoresis, *m*-/*p*-methylation ratios and mixed substrate experiments indicated the existence of two forms of the enzyme which were resolved by chromatography on DEAE-cellulose. One form (MW 74000, pI 6.1, opt. pH 7.3) catalysed the *meta*-methylation of caffeic acid, while the other (MW 70000, pI 6.3, opt. pH 8.3) mediated the *para*-methylation of quercetin, though each form exhibited some activity against other substrates.

INTRODUCTION

O-Methylation is an important reaction in the biosynthesis of plant phenolic compounds. *O*-Methyltransferase (OMT) [EC 2.1.1.6.] has been reported to mediate the transfer of methyl groups from *S*-adenosyl-L-methionine to phenylpropanoid [1–11] and flavonoid [9, 12, 13] compounds mainly at the *meta* position, though *para*-*O*-methylation is not uncommon [14–16]. Recently, several workers have observed OMT activity at both *m*- and *p*-hydroxyls of *o*-dihydroxyphenolic compounds [17–20]. However, the problem of whether separate enzymes mediate the reaction at the *meta* and *para* positions has not been unequivocally resolved in any of these cases.

Preliminary work in this laboratory indicated that crude enzyme preparations of tobacco cell culture catalysed both *meta*- and *para*-*O*-methylation of caffeic acid, esculetin, daphnetin and quercetin though with different ratios. We wish to describe the isolation and properties of tobacco culture OMT and to report, for the first time, the separation and characteristics of the *meta*- and *para*-directing forms of the enzyme.

RESULTS

Growth of tobacco cell culture

It was essential to determine the peak of OMT activity in relation to culture growth and the accumulation of phenolic compounds in the cultured cells. Fig. 1 shows that the exponential growth of the cell culture was associated with increasing OMT activity, against both caffeic acid and esculetin, that reached a maximum after 7 days of culture growth. There was also concomitant accumulation of scopolin (scopoletin-7-*O*-glucoside) and its aglucone; both represented the only phenolic metabolites of tobacco cell culture. Scopolin was the major metabolite while scopoletin appeared as a degradation product of late-exponential phase cells.

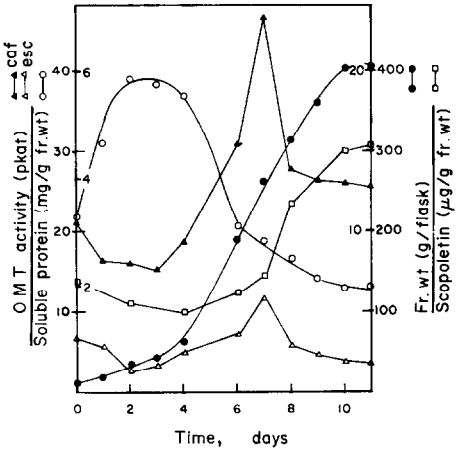

Fig. 1. Time course changes in *O*-methyltransferase activity against caffeic acid (▲) and esculetin (△) as substrates and the accumulation of scopoletin (□) during growth of tobacco culture as measured by cell fr. wt (●) and soluble protein content (○) during a growth cycle.

Purification and general properties of OMT

The enzyme was purified by successive chromatography of the $(NH_4)_2SO_4$ fraction (50–60% satn) on DEAE-Sepharose, Sephacryl S-200 and hydroxyapatite columns (Fig. 2). This procedure resulted in increased sp. act. *ca* 80-, 90- and 45-fold against caffeic acid, esculetin and quercetin as substrates, respectively (Table 1). Whereas the sp. act. ratios of esculetin/caffeic did not change appreciably during enzyme purification, those of quercetin/caffeic exhibited significant fluctuations. The purification data for the enzyme against daphnetin (not

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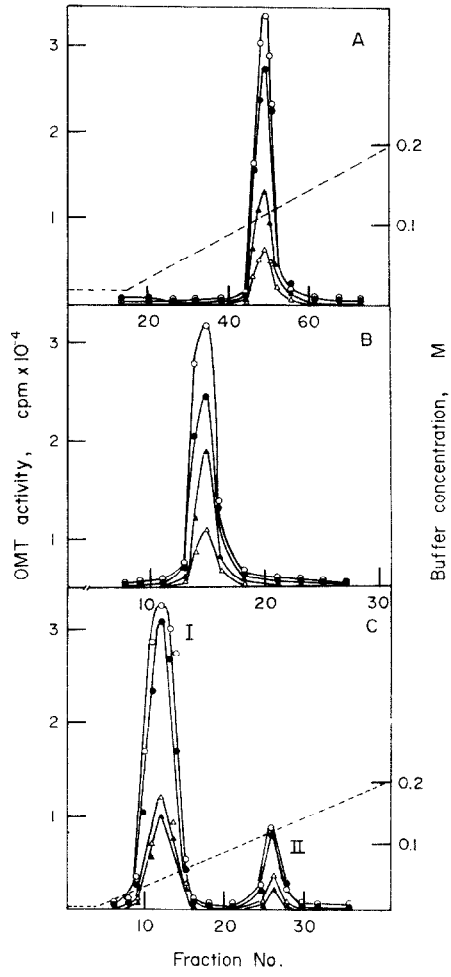

Fig. 2. Elution profiles of *O*-methyltransferase activity against caffeic acid (●), quercetin (○), esculetin (△) and daphnetin (▲). A, DEAE-Sepharose; B, Sephacryl S-200; C, hydroxyapatite.

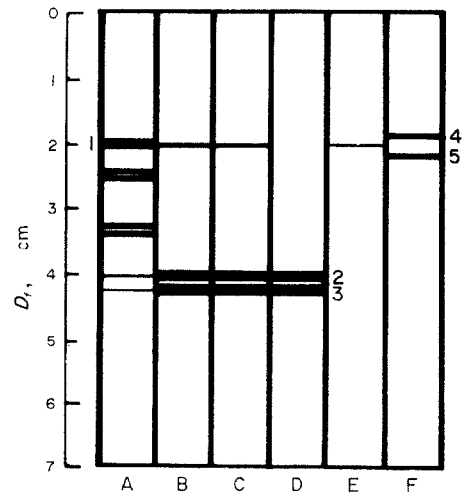

Fig. 3. Polyacrylamide gel protein patterns of *O*-methyltransferase during purification (anode is to the bottom of figure). A, $(NH_4)_2SO_4$ fraction after desalting on Sephadex G-25; B, DEAE-Sepharose eluate; C, Sephacryl S-200 eluate; D, peak I from hydroxyapatite; E, peak II from hydroxyapatite; F, SDS-acrylamide gel of peak I.

shown) were quite similar to those of caffeic acid and resulted in *ca* 85-fold increase in activity as compared with that of the crude extract.

The enzyme activity against the different substrates used was eluted in one single peak (Fig. 2A,B) except for another minor peak of activity which was eluted from hydroxyapatite (Fig. 2C). However, the polyacrylamide gel patterns of the enzyme (Fig. 3) indicate that during the purification process there was a parallel disappearance of the native enzyme (band 1, Fig. 3A) and the appearance of two protein bands (2 and 3, Figs. 3B–D) each of which was active against all the substrates. It was considered, therefore, that the latter bands represented the dissociated form of OMT and band E_1 that of the remaining undissociated enzyme. SDS-acrylamide gel electrophoresis of peak I (Fig. 2C) gave two protein bands (4 and 5, Fig. 3F) indicating two distinct proteins with electrophoretic mobility corresponding to a MW of 70 to 75000.

The effect of divalent cations and SH-group inhibitors on the purified enzyme was tested against caffeic acid, esculetin and quercetin (Table 2). Both Mn^{2+} and Zn^{2+} inhibited the rate of methylation of all substrates through to a different extent, in a manner similar to that reported for the pine [4, 5] and parsley [12] enzymes. Mg^{2+} was not required for OMT activity and resulted in 18–24% inhibition when added at a final concentration of 10 mM. This result is in contrast with those reported for other OMTs which were slightly stimulated [3, 4, 10, 12] or required Mg^{2+} for maximal activity [13]. *p*-Chloromercuribenzoate strongly inhibited the methylation of both caffeic acid and esculetin, but to a lesser extent for quercetin. This inhibition was reduced by the addition of

Table 1. Purification of tobacco culture *O*-methyltransferase*

Fraction	Total protein (mg)	Total activity† (nkat)			Specific activity (nkat/mg)			Purification (fold)			Sp. act. ratio	
		Caf	Esc	Que	Caf	Esc	Que	Caf	Esc	Que	Esc/Caf	Que/Caf
Crude extract	503.8	29.9	8.2	44.3	0.05	0.016	0.09	—	—	—	0.27	1.5
$(NH_4)_2SO_4$ (50–60% satn)	26.3	14.6	3.3	14.2	0.56	0.13	0.55	9.3	8.1	6.1	0.28	0.92
Sephadex G-25	22.8	13.2	3.6	13.2	0.58	0.16	0.57	9.7	10.0	6.3	0.27	0.95
DEAE-Sepharose	10.9	11.3	3.1	12.4	1.04	0.28	1.14	17.3	17.5	12.7	0.28	1.10
Sephacryl S-200	2.3	3.8	1.1	5.2	1.65	0.48	2.26	27.5	30.0	26.1	0.29	1.40
Hydroxyapatite (peak I)	0.27	1.3	0.4	1.2	4.81	1.48	4.07	81.4	92.5	45.8	0.30	0.85

* Details of the purification steps and assay conditions are described in the Experimental section.

† Enzyme activity assayed against caffeic (Caf), esculetin (Esc), quercetin (Que) and daphnetin (not shown).

Table 2. Effects of divalent cations and SH-group inhibitors on *O*-methyltransferase activity

Reagent and concentration	Activity relative to control = 100*		
	Caffeic	Esculetin	Quercetin
Divalent cations†			
$MgCl_2$ (1.0 mM)	94	98	90
(10 mM)	82	86	76
$MnCl_2$ (0.5 mM)	60	56	16
(1.0 mM)	13	8	4
$ZnCl_2$ (0.5 mM)	67	60	50
(1.0 mM)	32	26	18
p-Chloromercuribenzoate (1 mM)	22	27	68
plus 5 mM β-mercaptoethanol	67	62	85
Iodoacetate (20 mM)	73	59	20

* The standard assay was used as described in the Experimental section. Activities of the control were (cpm): caffeic acid, 15500; esculetin, 3940; quercetin, 18700.

† Assayed in absence of EDTA.

Table 3. Substrate specificity of tobacco culture *O*-methyltransferase*

Substrate†	Activity relative to control = 100
Caffeic acid	100
5-Hydroxyferulic acid	122
3,4,5-Trihydroxycinnamic acid	44
Caffeic-4-*O*-glucoside	9.2
Chlorogenic acid	8.0
Daphnetin	40
Esculetin	26
Esculin	6.4
Protocatechuic acid	10
o-Pyrocatechuic acid	8.2
Gallic acid	8.9
Quercetin	121
Quercetrin	12
Rutin	16
Luteolin	24

* The potential substrates were supplied at final concn of 0.1 and 0.2 mM and radioactivity, based on total methylation, was determined as described in the Experimental section. Activity of methylated glycosides was determined after acid hydrolysis.

† The following substrates were not methylated or gave values less than 6% of control: monohydroxybenzoic and monohydroxycinnamic acids; umbelliferone; ferulic and isoferulic acids; scopoletin and isoscopoletin; cyanidin, kaempferol, apigenin, naringenin or genistein.

β-mercaptoethanol indicating the requirement of SH-group for enzyme activity. Iodoacetate, on the other hand, inhibited the methylation of quercetin more effectively than either that of caffeic acid or esculetin.

Substrate specificity

Of the variety of phenolic compounds that were tested for their methyl acceptor ability (Table 3), caffeic acid, 5-hydroxyferulic acid (5HFA) and quercetin were the best substrates. 3,4,5-Trihydroxycinnamic acid, daphnetin, esculetin and luteolin were also *O*-methylated but to a lesser extent. Monohydroxy compounds as well as dihydroxybenzoic acids were poor methyl acceptors. There was no significant methylation of ferulic, isoferulic, scopoletin or isoscopoletin, indicating possible steric hindrance of OMT by the OMe group. Esterification of the -COOH (as in chlorogenic acid) or glucosylation (as in caffeic-4-*O*-glucoside, esculin, quercetrin or rutin) markedly decreased the enzyme activity, suggesting that the latter reaction is a final step in phenolic biosynthesis.

Kinetic constants and product inhibition

The kinetic constants of the purified enzyme when tested against different substrates (Table 4) gave K_m values of the same order of magnitude, though the V_{max} were different. When calculated on the basis of *m*-methylation, the V_{max}/K_m ratios showed the following order of affinity for the enzyme, namely: caffeic/5HFA > daphnetin > quercetin > esculetin. The K_m for *S*-adenosyl-L-methionine was 4 to 4.4 μM.

S-Adenosyl-L-homocysteine, the second product of the reaction, exhibited strong competitive inhibition of OMT activity and gave K_i values of 2.5 to 2.8 μM against the different substrates tested (Table 4). Ferulic acid, sinapic acid, scopoletin and rhamnetin (at 0.2 mM final concentration) inhibited OMT activity when tested

Table 4. Kinetic constants and product inhibition of tobacco culture OMT against five substrates

Characteristic	Caffeic	5HFA	Esculetin	Daphnetin	Quercetin
V_{max}, total methylation (pkat)	170	231	33.1	60	191
V_{max}, *meta*-methylation (pkat)	153	153	18.7	60	37.7
m-/*p*-methylation*	9.4	1.9	1.3	∞	0.33
K_m, substrate (μM)	100	100	45	45	45
$10^{-6} \times V/K_m$ (pkat/M)	1.5	1.5	0.4	1.3	0.8
K_m, *S*-adenosyl-L-methionine (μM)	4	nd	4	4	4.4
K_i, *S*-adenosyl-L-homocysteine (μM)	2.5	nd	2.5	2.5	2.8
Product inhibition†					
Ferulic acid	31	27	24	26	25
Sinapic acid	35	43	48	56	41
Scopoletin	30	33	35	26	22
Rhamnetin	38	26	42	43	26

* Reaction products were chromatographed in different solvents and the activity in *m*- and *p*-methylation products was determined as described in the Experimental section. Value for 5HFA represents ratio of *m*-/total methylation; daphnetin was methylated at *m*-position only.

† The assay mixture contained 0.1–0.2 mM substrate to which 50 nmol of potential inhibitors were added. Values represent activities relative to the control of each substrate = 100.

Table 5. *O*-Methyltransferase activity against mixed substrates*

Substrate and concentration	Activity in products (cpm)† 0.0 mM	0.08 mM	0.2 mM
Quercetin	16500	23500	34200
	(100)	(143)	(207)
Esculetin	16700	11600	9630
	(100)	(70)	(58)
Daphnetin	16900	13700	10800
	(100)	(83)	(64)

* Peak I fraction eluted from hydroxyapatite column. The standard assay contained 0.2 mM caffeic acid to which the indicated substrate concentrations were added.

† Values in parentheses are relative to control = 100.

against the major substrates (Table 4). Strong inhibition was observed for caffeic acid, 5HFA and esculetin in the presence of their corresponding methylated products.

Meta- *and* para-*methylation*

Purified tobacco culture OMT catalysed the methylation of a number of *o*-dihydroxyphenolic substrates at both *meta* and *para* positions. The products were separated by TLC in different solvent systems and were identified by co-chromatography with reference compounds. Caffeic acid gave ferulic and isoferulic acids; 5HFA gave sinapic acid and another unidentified product; esculetin gave scopoletin and isoscopoletin; daphnetin gave exclusively 7-OH-8-OMe-coumarin possibly due to its highly nucleophilic 8-OH group; quercetin gave isorhamnetin and rhamnetin. The *m*-/*p*-methylation ratios (Table 4) seem to indicate the presence of two forms of OMT; one catalysed the *O*-methylation of caffeic acid almost predominantly at the *meta* position and the other methylated quercetin to a large extent at the *para* position.

Further evidence for the existence of *meta*- and *para*-directing forms of the enzyme was obtained from mixed-substrate experiments (Table 5). The additive effect observed when quercetin was added to caffeic acid is in sharp contrast with the decreased activity obtained after the addition of either esculetin or daphnetin. This result is in agreement with the kinetic theory [21] which demonstrates that at near saturating substrate concentration, a higher activity is observable in the presence of two substrates than with either one alone if the system contains separate enzymes mediating the reaction of both substrates.

Table 6. *Meta* and *para* activity ratios of the two forms of tobacco culture OMT eluted from DEAE-cellulose and their characteristic properties*

Property	Peak I			Peak II		
MW	74000 ± 5%			70000 ± 5%		
pI	6.1			6.3		
pH Optimum†	7.3			8.3		
Methylation ratio‡						
	Caf	Esc	Que	Caf	Esc	Que
Meta/para	11.2	1.6	0.15	3.3	0.7	0.07
Para/meta	0.09	0.6	6.8	0.3	1.5	14.8

* Fractions 52 and 57, respectively.

† Against caffeic acid and quercetin using 0.1 M Tris, Pi and glycine buffers.

‡ Assay mixtures contained saturating concentration of caffeic acid (Caf), esculetin (Esc) or quercetin (Que) and the *meta*- and *para*-methylation products were separated by TLC as described in the Experimental section.

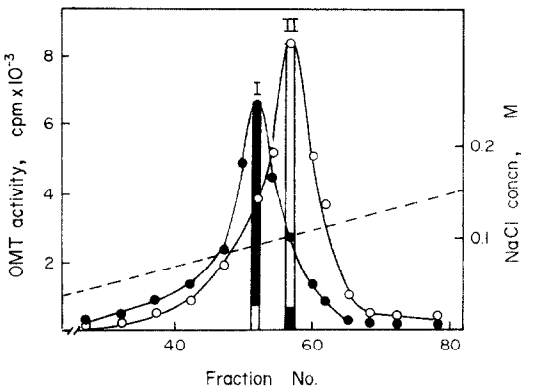

Fig. 4. Methylation of caffeic acid (●) and quercetin (○) by *O*-methyltransferase eluted from DEAE-cellulose column. Peaks I and II represent *meta*- (solid column) and *para*- (open column) methylation of both substrates, respectively.

A partial, but reasonable separation of the two forms of enzyme activity was achieved by chromatography of the purified enzyme on DEAE-cellulose (Fig. 4) and elution with a linear gradient between 0.02 to 0.15 M NaCl in 5 mM Pi buffer, pH 7.5. Two peaks of activity were observed when the eluates were assayed against caffeic acid and quercetin as substrates. Peak I catalysed the *meta*-methylation of caffeic acid while peak II mediated the *para*-methylation of quercetin, though either peak exhibited some activity towards the other substrates (Table 6). The two forms of the enzyme had almost similar MWs (70–74000) and pI values (6.1–6.3) but differed in their pH optima. The MW of tobacco culture OMT appears similar to that of the pine enzyme [5] and is intermediate among those of *Ruta* [10], parsley [12] and *Cicer* [14] cell cultures.

DISCUSSION

To our knowledge, this is the first reported instance where the *meta*- and *para*-directing forms of an *S*-adenosyl-L-methionine: *o*-dihydric phenol *O*-methyltransferase were isolated and characterized. Despite small differences observed in their MWs and pI values, there are several properties indicative of the existence of two distinct enzymes acting at the *meta* and *para* positions of *o*-dihydroxyphenolic compounds. (a) The elution profile of enzyme activity from DEAE-cellulose was resolved into two peaks; one catalysed the *O*-methylation of caffeic acid at the *meta* position and the other, quercetin at the *para* position, though they did not differ significantly in substrate specificity. (b) The differences observed in the degree of enzyme purification against caffeic acid, daphnetin and esculetin (80- to 90-fold) on the one hand and that of quercetin (45-fold) on the other hand, tends to suggest that the para-directing enzyme was less stable than its *meta* counterpart, though both forms were heat-labile contrary to earlier reports of differential thermal stability [17, 20]. The failure of sp.

act. ratios of quercetin/caffeic to remain constant as compared with those of esculetin/caffeic supports the above view. Moreover, there were significant differences observed in the effects of divalent cations and SH-group inhibitors on enzyme activity, as well as the difference in pH optima of the two forms of the enzyme. (c) SDS-acrylamide gel electrophoresis of the purified enzyme (Fig. 3F) indicates the presence of two distinct proteins each of which appears as a single unit. Indirect evidence strongly suggests that protein bands 2 and 3 (Fig. 3D), and bands 4 and 5 (Fig. 3F) on the one hand, and peaks I and II (Fig. 4) on the other hand, correspond respectively to the *meta*- and *para*-directing forms of the enzyme. (d) More conclusive evidence for the existence of two discrete enzymes came from mixed-substrate experiments (Table 5) indicating that each enzyme was acting on its own substrate. Very recently, Thompson *et al.* [10] reported the presence in *Ruta graveolens* cell culture of two distinct enzymes which mediated the *O*-methylation of linear furanocoumarins at the *ortho* and *meta* positions. Our finding, together with the latter report, add to our knowledge of methylation at the different hydroxyls of phenolic compounds.

Tobacco culture OMT exhibited a fairly wide range of substrate specificity as compared with those reported from other sources which were specific to cinnamic acids [3, 4, 7–9, 15], coumarins [10, 19] and flavonoids [9, 12–14]. The tobacco enzyme catalysed the *O*-methylation of 3 cinnamic acids, two coumarins and two flavonoids. The efficiency with which it methylated caffeic acid and 5HFA suggests that this enzyme is involved in the sequence of reactions leading to lignin formation as was shown with other OMTs [3–5, 8, 22]. Both daphnetin and esculetin were readily methylated but to different extents, possibly due to the difference in nucleophilicity of the 6- and 8-OH groups of coumarins. This indicates, however, that *O*-methylation of coumarins may proceed after lactone ring formation. Earlier work based on isotopic evidence suggested that scopoletin biosynthesis in tobacco leaves [23, 24] and tobacco callus tissue [25] proceeded via ferulic acid glucoside or its glucose ester. Our results tend to suggest an alternative pathway whereby caffeic acid → esculetin → scopoletin → scopolin. The failure of tobacco culture OMT to methylate phenolic glucosides or esters supports the proposed pathway and agrees with the view that glucosylation/esterification is a final step in phenolic biosynthesis [26]. Furthermore, the recent discovery in tobacco cell culture of a glucosyltransferase which mediates the transfer of glucose from UDPG to scopoletin with the formation of scopolin (unpublished results) is consistent with the above proposal which has yet to be demonstrated.

The *O*-methylation of quercetin at both *meta* and *para* positions also indicates that this reaction occurs after flavonoid ring formation. However, it is interesting to note that the latter reaction involved the 7-position rather than the 4′-position. We observed a similar situation with luteolin which gave chrysoeriol and the 7-OMe derivative. Enzymic *O*-methylation of ring A of flavonoid compounds is not uncommon; Poulton *et al.* [13] reported the efficient methylation of texasin (6,7-diOH-4′-OMe-isoflavone) by a purified soybean culture OMT, though the exact position of methylation remains to be determined. More recently, Brunet *et al.* [27] demonstrated the stepwise methylation of quercetin by cell-free extracts of citrus tissues to rhamnetin, isorhamnetin and rhamnazin, therefore suggesting the presence of *meta*- and *para*-directing OMTs in that tissue. The common occurrence in plants of 7-OMe derivatives of quercetin and luteolin would be consistent with the physiological role of this enzyme.

Except for the parsley enzyme [12], there is no information on the extent of OMT inhibition by its methylated products. Tobacco culture OMT was inhibited by 22 to 50% in the presence of ferulic acid, sinapic acid, scopoletin or rhamnetin. Considering the fact that tobacco cell cultures do not accumulate any of the intermediates of the pathway of scopoletin biosynthesis, this end-product inhibition may act as a mechanism for the regulation of OMT activity *in vivo* and hence, the biosynthesis of lignin precursors or other end metabolites.

EXPERIMENTAL

Plant material. Tobacco (*Nicotiana tabacum* L. cv Wisc. #38) cell suspension culture was initiated from the stem pith and its growth was maintained on a salt-nutrient medium [28] containing 3% sucrose, 2 μM IAA and 0.1 μM kinetin. Batch cultures were agitated on gyrotary shakers (150 rpm) in diffuse light at 24 ± 1° and were subcultured at weekly intervals using 10% inoculum.

Growth parameters. Triplicate culture samples were taken at daily intervals for the determination of fr. wt, soluble protein, phenolic content and OMT activity. Cell fr. wt was determined after filtering the cells through a fritted glass funnel using suction. Soluble protein was estimated on aliquots of the buffer extracts (0.1 M Pi buffer, pH 7.5) of fresh cells using the method of ref. [29]. Investigation of the phenolic compounds in the cell culture indicated that scopolin was the main metabolite with small amounts of scopoletin. Total scopoletin was estimated at 340 nm after acid hydrolysis of alcoholic extracts. OMT activity was determined with Pi buffer extracts, pH 7.5, using caffeic acid and esculetin as substrates and the standard assay described below.

Enzyme extraction and purification. Unless stated otherwise, all procedures were carried out at 2–4° and all buffers contained 5 mM EDTA. The filtered cells were homogenized with Polyclar AT and 2 vol. of 0.1 M Pi buffer (pH 7.5), then centrifuged for 15 min at 20000 *g*. The supernatant was stirred with Dowex 1X2 and filtered. The filtrate was fractionated with solid $(NH_4)_2SO_4$ while maintaining the pH at 7.5 and the protein which precipitated between 50 and 60% satn was collected by centrifugation and resuspended in the minimum vol. of 5 mM Pi buffer (pH 7.5). It was desalted on a column of Sephadex G-25, previously equilibrated, and then eluted with the same buffer. The protein extract was applied onto a column of DEAE-Sepharose (Pharmacia, 1.5 × 30 cm) and washed with one bed vol. of the same buffer. The protein was eluted with a linear salt gradient between 0.01 and 0.2 M NaCl in 5 mM Pi buffer (pH 7.5). Fractions (4 ml) were collected (15 ml/hr) and assayed for enzyme activity. The fractions with highest activity were pooled and concd by ultrafiltration under N_2 pres. The concd protein was carefully applied onto a Sephacryl S-200 column (Pharmacia, 1.5 × 65 cm) previously equilibrated with 5 mM Pi buffer (pH 7.5). Fractions (3 ml) were eluted (12 ml/hr) using the same buffer and were assayed for activity. The most active fractions were combined, concd on an ultrafilter, then applied onto a column of hydroxyapatite (1 × 7 cm) previously equilibrated with 5 mM Pi buffer (pH 7.5). The column was washed with 10 ml of the same buffer followed by a linear gradient between 0.01 and 0.2 M Pi buffer (pH 7.5). Fractions (2 ml) were collected

and assayed for enzyme activity. For the separation of the two forms of OMT activity, peak I from hydroxyapatite was loaded onto a column of DEAE-cellulose (1 × 15 cm, Sigma, fine grade) and eluted with a linear salt gradient between 0.01 and 0.15 M NaCl in 5 mM Pi buffer (pH 7.5). Fractions (1 ml) were collected and assayed for activity against caffeic acid and quercetin as substrates. Soluble protein was determined by the method of ref. [29] or spectrophotometrically [30].

O-Methyltransferase assay and product identification. During enzyme purification, OMT activity was assayed against caffeic acid, esculetin, daphnetin and quercetin. The standard assay mixture consisted of 50 nmol substrate (in 10 µl DMSO), 7 nmol *S*-adenosyl-L-[$^{14}CH_3$]-methionine (containing 0.02 µCi), 1.4 µmol β-mercaptoethanol and 10–100 µg enzyme protein (in Pi buffer, pH 7.5) in a total vol. of 250 µl. The mixture was incubated for 30 min at 35° and the reaction was terminated by the addition of 20 µl of 6 M HCl. The methylated products were extracted 2 × 1 ml of Et_2O (for phenylpropanoids) or EtOAc (for flavonoids). The organic phase was transferred to scintillation vials, evapd and counted for total radioactivity in a toluene-based scintillation fluid. For product identification and determination of *m*-/*p*-methylation ratios, the products were separated by TLC using the following adsorbents and solvent systems. Caffeic acid gave ferulic and isoferulic acids (R_f 0.25 and 0.37) on MN-cellulose using *n*-BuOH–NH_4OH–EtOH–C_6H_6 (5:3:1:1); 5HFA gave sinapic acid (R_f 0.27) and an unidentified product (R_f 0.34) on cellulose-Avicel using the latter solvent system; esculetin gave scopoletin and isoscopoletin (R_f 0.47 and 0.63) on MN-silica gel in *n*-BuOH–NH_4OH–EtOH–C_6H_6–CCl_4 (5:3:5:4:1); daphnetin gave only 6-OMe-7-OH-coumarin (R_f 0.75) on cellulose-Avicel using C_6H_6–HOAc–H_2O (2:2:1, org. layer); quercetin gave rhamnetin and isorhamnetin (R_f 0.75 and 0.83) on Eastman–Kodak Si gel plates in C_6H_6–Py–HCO_2H (86:19:5). The identity of *m*- and *p*-methylation products was confirmed by co-chromatography with reference compounds, autoradiography and visualization in UV-light (366 nm). Individual compounds were scraped off the plates, mixed with Cab-O-Sil and counted by liquid scintillation.

Polyacrylamide gel electrophoresis and isoelectric focusing. The purity of active fractions from column eluates was monitored by the use of standard 7.5% polyacrylamide gels (pH 8.9) prepared according to the method of ref. [31] but without sample gel. Electrophoresis was conducted at 5 mA/gel for 45–60 min. The gels were stained with 1% Amido-black in 7% aq. HOAc for 1 hr and were destained electrophoretically using 7% aq. HOAc. SDS-gels were prepared by the method of ref. [32] and run for 4–5 hr at 4 mA/gel. They were stained with 0.25% Coomassie blue in 50% MeOH–10%HOAc for 6 hr and destained with 5% MeOH in 7.5% HOAc. Isoelectric focusing was performed in polyacrylamide gel columns (10 cm long) using carrier ampholite soln (pH 5–7, LKB Ltd.) and following the method of ref. [33]. The focused gels were fixed for 18 hr in a soln containing 0.5% $CuSO_4$ and 0.01% Coomassie blue in 10% HOAc–27% EtOH, stained with 0.05% Coomassie blue in 10% HOAc–25% EtOH for 6 hr then destained with 10% HOAc–10% EtOH. The gels were sliced to 0.5 cm segments, extracted with 1 ml double dist. H_2O and the pH measured.

MW determination. MWs of the two forms of OMT were determined by calculating the elution vol. from a calibrated Sephacryl S-200 column (1.5 × 65 cm) using 5 mM Pi buffer (pH 7.5) and were confirmed by TLG filtration [34] on Sephadex G-150 (superfine) using proteins of known MWs.

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