Evidence for the Existence of *meta* and *para* Directing O-Methyltransferases in Tobacco Cell Cultures

Yun-Fuk Tsang and Ragai K. Ibrahim

Department of Biological Sciences, Concordia University, Sir George Williams Campus, Montreal, Québec

Z. Naturforsch. **34 c**, 46–50 (1979); received November 17, 1978

O-Methyltransferase, Enzyme, *meta* and *para* Methylation, Phenols, Tobacco Cell Suspension Culture, *Nicotiana tabcaum*

The O-methyltransferase of tobacco cell culture was resolved to its *meta* and *para* directing forms by chromatography on DEAE-cellulose. Despite similarities in molecular weights and pI values of the two forms, however, evidence from pH optima, SH-group inhibitors, methylation ratios, SDS-acrylamide gels and mixed substrate experiments indicates the existence of two discrete enzymes acting at the *meta* and *para* positions of caffeic acid and quercetin, respectively; though the latter enzyme was less substrate specific than its *meta* counterpart.

Introduction

O-Methyltransferase (OMT) has been detected in several plant species involving the biosynthesis of phenylpropanoid [1–11] and flavonoid [9, 12–14] compounds. In the presence of S-adenosyl-L-methionine, OMT transfers the methyl group predominantly at the *meta* hydroxyls of catecholic substrates [15, 16], though the methylation of *p*-hydroxy compounds has also been reported [14, 17, 18]. Recently, several workers have observed OMT activity at both *meta* and *para* hydroxyls of *o*-dihydroxy phenolic compounds [19–22]; though it is not known whether one or more enzymes catalyze such methylation. We report here, for the first time, on the isolation and some properties of two forms of OMT from tobacco cell culture. One form mediated the O-methylation of caffeic acid almost predominantly at the *meta* position, and the other, quercetin at the *para* position, but not to the exclusion of other substrates.

Experimental

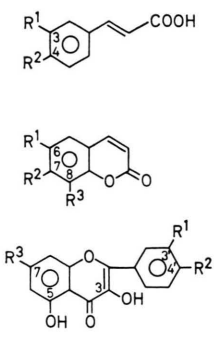
Culture conditions

Tobacco cell culture (*Nicotiana tobacum* cv. Wisconsin 38) was initiated and maintained on a salt-nutrient medium [23] containing 3% sucrose, 2 μM indoleacetic acid and 0.1 μM kinetin. Batch cultures were agitated on a gyrotary shaker (150 rpm) in diffuse light at 24 °C and were subcultured at weekly intervals using 10% inoculum.

Requests for reprints should be sent to Dr. R. K. Ibrahim, Department of Biological Sciences, Concordia University, Montreal H3G 1M8, Canada.

Enzyme purification

All procedures were carried at 2–4 °C and buffers were made of potassium phosphate, pH 7.5 containing 5 mM EDTA in the following concentrations: (I) 0.1 M, (II) 5 mM, (III) linear gradient between 10–200 mM. Filtered cells were homogenized with Polyclar-AT and 2 volumes buffer I, then centrifuged for 20 min at 20,000 *g*. The supernatant was stirred for 20 min with Dowex 1 ×2 and filtered. The filtrate was fractionated with solid ammonium sulphate and the protein which precipitated between 50–60% saturation was collected by centrifugation, dissolved in the minimum amount of buffer II then desalted on a Sephadex G-25 column. It was chromatographed successively on DEAE-sepharose, sephacryl S-200 and hydroxyapatite columns and the protein was eluted with a linear gradient (10–200 mM) of NaCl in buffer II, buffer II and buffer III, respectively. OMT activity of the different fractions was assayed against caffeic acid, esculetin, daphnetin and quercetin as substrates (Fig. 1) and the purity of active fractions was monitored by acrylamide gel electrophoresis. Sepataion of *m*- and *p*-directing activities was achieved by chromatography of the Sephadex G-25 fraction on DEAE-cellulose column and elution with a linear gradient (10–150 mM) NaCl in buffer II. The fractions collected were assayed against caffeic acid and quercetin as substrates.



	R^1	R^2	R^3
Caffeic acid	OH	OH	
Ferulic acid	OMe	OH	
Isoferulic acid	OH	OMe	
Esculetin	OH	OH	H
Scopoletin	OMe	OH	H
Isoscopoletin	OH	OMe	H
Daphnetin	H	OH	OH
Hydrangetin	H	OH	OMe
Quercetin	OH	OH	OH
Rhamnetin	OH	OH	OMe
Isorhamnetin	OMe	OH	OH

Fig. 1. Structural formulae for substrates and products used with O-methyltransferase assays.

O-Methyltransferase assay

The standard assay mixture consisted of 50 nmol substrate (in 10 µl DMSO), 7 nmol S-adenosyl-L-[$^{14}CH_3$]methionine (0.02 µCi), 1.4 µmol β-mercaptoethanol and 10–100 µg protein in a total volume of 250 µl. The mixture was incubated at 35 °C for 30 min and the reaction was terminated by the addition of 20 µl of 6 N HCl. Assays were performed in duplicates and the methylated products were extracted twice with diethyl ether (for phenylpropanoids) or ethyl acetate (for quercetin). The products of one assay were counted for total radioactivity in a toluene-based scintillation fluid.

Identity of reaction products

The products of the other assay were separated by TLC using different supports and solvent systems (Table 1) followed by autoradiography. The identity of *m*- and *p*-methylation products was confirmed by cochromatography with reference compounds and visualization in UV-light (366 nm). Individual compounds were scraped off the TLC plates, mixed with Cab-O-Sil and counted by liquid scintillation.

Other determinations

The molecular weights of the two forms of OMT were determined by gel filtration on a calibrated Sephadex G-100 column [25] and by thin-layer gel filtration on Sephadex G-150 [26] using standard proteins. Isoelectric focusing was performed in 10-cm long acrylamide gel columns containing carrier ampholite, pH 3–10 [27].

Results and Discussion

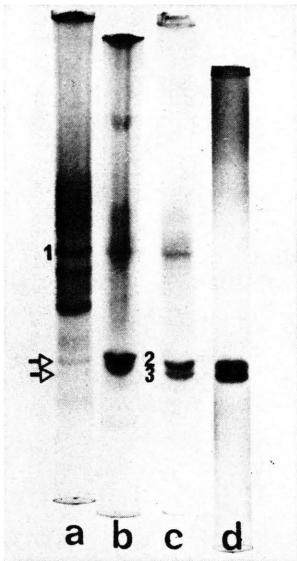
Table 1 shows the *meta* and *para* methylation products of the purified enzyme preparation (hydroxyapatite fraction) when assayed against four substrates. Daphnetin was methylated exclusively at the *meta* position, possibly due to its highly nucleophilic 8-OH group. The ratios of *m*-/*p*-methylation of the other substrates, however, seem to indicate the presence of two forms of OMT as has been suggested with the citrus cell-free extracts [28]. One form methylated caffeic acid almost predominantly at the *meta* position and the other form methylated quercetin to a large extent at the *para* position.

Whereas the procedure used for enzyme purification did not separate the two forms of OMT activity, examination of the acrylamide gel protein patterns

Table I. O-Methyltransferase activity of the hydroxyapatite fraction against four substrates and identification of the reaction products [a].

Substrate [b]	Products and R_f values		*m*-/*p*-ratio [c]	TLC characteristics	
	Meta	*Para*		Solvent system [d]	Support [e]
Caffeic acid	Ferulic 0.25	Isoferulic 0.37	9.4	A	MNC
Daphnetin	Hydrangetin 0.52	—	∞	B	CA
Esculetin	Scopoletin 0.47	Isoscopoletin 0.63	1.3	B	MNS
Quercetin	Isorhamnetin 0.83	Rhamnetin 0.75	0.33	C	EKS

[a] Structural formulae of substrates and products are shown in Fig. 1.
[b] Supplied at final concentration of 0.2 mM.
[c] Determined after TLC separation of *m*- and *p*-methylation products.
[d] Solvent systems used: A, *n*-BuOH-NH_4OH-EtOH-C_6H_6 (5:3:1:1); B, *n*-BuOH-NH_4OH-EtOH-C_6H_6-CCl_4 (5:3:5:4:1); C, C_6H_6-pyridine-HCOOH (36:18:5).
[e] TLC supports used: MNC, MN-cellulose; CA, cellulose-Avicel; MNS, MN-silica gel; EKS, Eastman Kodak silica plates.



(Fig. 2) shows parallel disappearance of protein band 1 and the appearance of two other proteins (bands 2 & 3, gels a – d). These bands were active against all substrates and the latter two bands were considered to represent the dissociated form of the enzyme. A partial, but fair separation of the two forms of OMT activity was achieved by chromatography on DEAE-cellulose (Fig. 3), and their characteristic properties listed in Table II. Despite the similarities observed in their molecular weights and pI values of the two forms of OMT, there are several properties indicative of the existence of two distinct enzymes acting at the *meta* and *para* positions: (a) The elution profile from DEAE-cellulose was

◀ Fig. 2. Acrylamide gel protein patterns of O-methyltransferase during purification steps: (a) $(NH_4)_2SO_4$ pellet after desalting on Sephadex G 25; (b) DEAE-sepharose; (c) Sephacryl S-200; (d) hydroxyapatite.

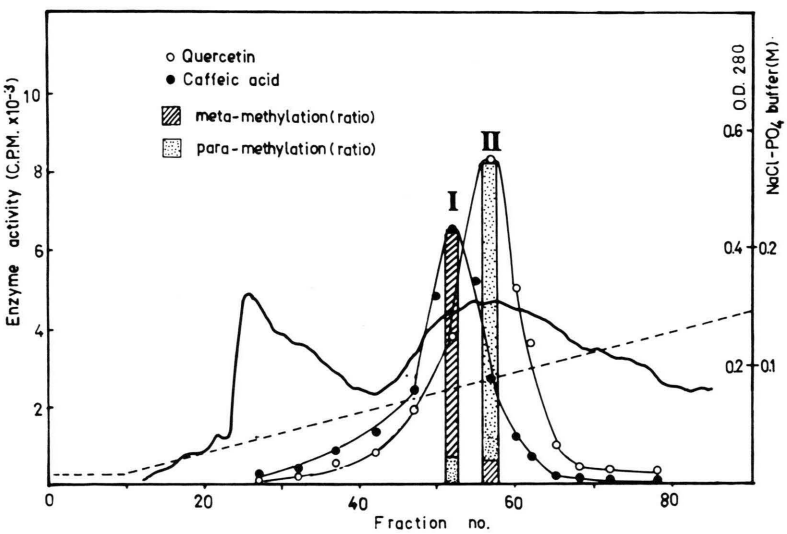


Fig. 3. Elution profile of O-methyltransferase from DEAE-cellulose and *meta* and *para* methylating activity of peaks I and II against caffeic acid and quercetin.

Table II. Some characteristics of the two forms of tobacco culture O-methyltransferase [a].

Characteristics	Fraction I			Fraction II		
Molecular weight	74,000 ± 7%			70,000 ± 6%		
pI	6.1			6.3		
pH optimum [b]	7.3			8.3		
Inhibition by PCMB (1 mM) [c]	22			68		
Iodoacetate (20 mM)	73			20		
Methylation ratios	Caffeic	Esculetin	Quercetin	Caffeic	Esculetin	Quercetin
Meta/para	11.13	1.59	0.15	3.32	0.68	0.07
Para/meta	0.09	0.63	6.78	0.30	1.46	14.80

[a] Fractions No. 52 and 57, respectively (Fig. 3).
[b] For caffeic acid and quercetin, respectively.
[c] Values are percent of control; assayed in absence of EDTA.

resolved into *m*-directing (*m*-/*p*-ratio 11.1) and *p*-directing (*p*-/*m*-ratio 14.8) peaks against caffeic acid and quercetin respectively; though the latter was less substrate specific than its *meta* counterpart to the extent of being able to catalyze significant methylation of esculetin and caffeic acid. (b) There were significant differences between the two forms of the enzyme in their pH optima and their behaviour towards SH-group inhibitors (Table II). The *meta* directing OMT was more sensitive to *p*-chloromercuribenzoate inhibition than its *para* counterpart, while the reverse was observed with iodoacetate. (c) SDS-acrylamide gel electrophoresis of bands 2&3 (not shown) seemed to indicate that the latter are not charge isomers or isoenzymes, but two distinct proteins with electrophoretic mobilities correspondong to 70–75 K range and appear to be intermediate among those reported for other OMT's [5, 10, 12, 14]. (d) More conclusive evidence for the existence of two discrete enzymes was obtained from mixed substrate experiments using purified OMT preparation (hydroxyapatite fraction). The results shown in Table III indicate that 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 kinetic theory [29] which demonstrates that at near saturating substrate concentrations, a higher activity is observable in the presence of two substrates than with either one alone, if the system contains separate enzymes mediating the reactions of both substrates.

Table III. Methylating activity of the hydroxyapatite eluate against mixed substrates [a].

Substrate and concentration	Relative activity [cpm/assay] [b]		
	0.0 mM	0.08 mM	0.2 mM
Quercetin	16 485 (100%)	23 540 (143%)	34 150 (207%)
Esculetin	16 670 (100%)	11 640 (70%)	9 630 (58%)
Daphnetin	16 850 (100%)	13 650 (83%)	10 750 (64%)

[a] The standard assay contained 0.2 mM caffeic acid (control = 100%) to which the indicated substrate concentrations were added and assayed as described in the Methods section.

[b] Counts represent total methylating activities.

To our knowledge, this is the first reproted instance where the *meta* and *para* directing OMT's have been isolated and partially characterized. This finding together with the report on the presence in *Ruta graveolens* cell culture of two distinct enzymes catalyzing the O-methylation of linear furanocoumarins at the *ortho* and *meta* positions [10] add to our knowledge of O-methylation at the different positions of phenolic rings.

Financial support by the Natural Science & Engineering Research Council of Canada and by University funds is gratefully acknowledged.

[1] R. U. Byerrum, J. H. Flokstra, L. J. Dewey, and C. D. Ball, J. Biol. Chem. **210,** 633 (1954).
[2] B. J. Finkle and R. F. Nelson, Nature **197,** 902 (1963).
[3] M. Shimada, T. Ohashi, and T. Higuchi, Phytochemistry **9,** 2463 (1970).
[4] M. Shimada, H. Fushiki, and T. Higuchi, Phytochemistry **11,** 2627 (1972).
[5] H. Kuroda, H. Shimada, and T. Higuchi, Phytochemistry **14,** 1759 (1975).
[6] J. E. Poulton and V. S. Butt, Biochim. Biophys. Acta **403,** 301 (1975).
[7] J. E. Poulton, H. Grisebach, J. Ebel, B. Schaller-Hekler, and K. Hahlbrock, Arch. Biochem. Biophys. **173,** 301 (1976).
[8] J. E. Poulton, K. Hahlbrock, and H. Grisebach, Arch. Biochem. Biophys. **176,** 449 (1976).
[9] R. Sütfeld and R. Wiermann, Biochem. Physiol. Pflanzen **172,** 111 (1978).
[10] H. J. Thompson, S. K. Sharma, and S. A. Brown, Arch. Biochem. Biophys. **188,** 272 (1978).
[11] D. Hess, Z. Pflanzenphysiol. **53,** 460 (1965).
[12] J. Ebel, K. Hahlbrock, and H. Grisebach, Biochim. Biophys. Acta **269,** 313 (1972).
[13] J. E. Poulton, K. Hahlbrock, and H. Grisebach, Arch. Biochem. Biophys. **180,** 543 (1977).
[14] H. Wengenmayer, J. Ebel, and H. Grisebach, Eur. J. Biochem. **50,** 135 (1974).
[15] J. Axelrod, Science **126,** 400 (1957).
[16] J. Axelrod and R. Tomchick, J. Biol. Chem. **233,** 702 (1958).
[17] C. K. Wat and G. H. N. Towers, Phytochemistry **16,** 663 (1975).
[18] J. D. Mann, H. M. Fales, and S. H. Mudd, J. Biol. Chem. **238,** 3820 (1963).
[19] B. J. Finkle and S. H. Kelly, Phytochemistry **13,** 1719 (1974).
[20] D. Müller-Enoch, E. Seidel, and H. Thomas, Z. Naturforsch. **31 c,** 280 (1976).
[21] D. Müller-Enoch, H. Thomas, W. Streng, W. Wildfeuer, and O. Haferkamp, Z. Naturforsch. **31 c,** 509 (1976).

[22] M. Legrand, B. Fritig, and L. Hirth, FEBS Letters **70,** 131 (1976).
[23] T. Murashige and F. Skoog, Phyisol. Plant. **15,** 473 (1962).
[24] R. J. Davis, Ann. N. Y. Acad. Sci. **121,** 404 (1964).
[25] P. Andrews, Biochem. J. **96,** 595 (1965).
[26] B. J. Radola, J. Chromatography **38,** 61 (1968).
[27] S. N. Vindogradov, S. Lowenkvon, H. R. Andonian, J. Bagshaw, K. Felgenhauer, and S. J. Pak, Biochem. Biophys. Res. Commun. **54,** 501 (1973).
[28] G. Brunet, N. A. M. Saleh, and R. K. Ibrahim, Z. Naturforsch. **33 c,** 1308 (1978).
[29] M. Dixon and E. C. Webb, Enzymes, p. 84, Academic Press, New York 1964.