CATECHOL *O*-METHYLTRANSFERASES OF TOBACCO: EVIDENCE FOR SEVERAL ENZYMES WITH PARA- AND META-*O*-METHYLATING ACTIVITIES

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1. Introduction

Catechol *O*—methyltransferase (OMT) activity of plants is involved in the biosynthesis of phenylpropanoids [1], flavonoids [2,3], lignins [4–7] and alkaloids [8]. *O*-methylating activity was first detected in plant extracts by Finkle [9,10] and shown to require *S*-adenosyl-methionine (SAM) as methyl donor [9–11]. OMTs (EC 2.1.1.6) are expected to transfer the methyl group of SAM to either the meta- or the para-hydroxyl group of catecholic substrates, depending on the plant material and the substrate. Meta-specific OMTs have been reported in apple tree [9], bamboo [4], poplar [4], pine [5], and suspension cultures of parsley [2], whereas paramethylation of catecholic substrates has been found in pampas grass [10], *Nerine bowdenii* [8] and peyote [12].

Finkle [13] has shown two types of OMT activity in pampas grass. One of these, more labile, attacks substrates at the meta-hydroxyl group with respect to the side chain and is particularly active toward caffeic acid (3,4-dihydroxycinnamic acid). The other, more stable, attacks the para position of some of the same substrates. However, since the para- and meta-*O*-methylating activities have not been separated, it is not known whether these activities arise from a single enzyme having both meta and para capabilities, or from a mixture of OMTs, each one specific for a given ring position.

On the other hand, Poulton [14] found two distinct *S*-adenosyl-*L*-methionine: 3,4-dihydric phenol 3-*O*-methyltransferases in soybean cell suspension cultures. Both enzymes showed meta-directing activity toward all substrates assayed.

We report here the presence in tobacco leaves of three distinct catechol *O*-methyltransferases separable by DEAE-cellulose chromatography. They clearly differed in specificity toward the following substrates: catechol, caffeic acid, chlorogenic acid, esculetin and protocatechuic acid. We observed both para- and meta-directing activities with protocatechuic acid and esculetin as substrates. In contrast, caffeic acid and chlorogenic acid were methylated specifically at the meta position. Para-*O*-methylation of protocatechuic acid arose mainly from one of these enzymes whereas that of esculetin arose from all three. These results suggest that plants contain several ortho diphenol *O*-methyltransferases probably having both meta and para capabilities, but differing greatly in methylation efficiency at a given ring position of the various substrates.

2. Material and methods

2.1. *Radioactive substrate, catecholic substrates, and reference compounds*

S-adenosyl-*L*-Me-^{3}H methionine (SAM) (from the Commissariat à l'Energie Atomique, Saclay, France) was diluted with unlabelled material (Sigma, Saint Louis, Missouri, USA.) to a specific radioactivity of 12 mCi/mmol. Catechol, guaiacol, caffeic acid, ferulic acid (3-methoxy,4-hydroxycinnamic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (3-methoxy,4-hydroxybenzoic acid), chlorogenic acid (3-*O*-caffeoylquinic acid), and quercetin (3,3′,4′,5,7-pentahydroxyflavone) were purchased from Fluka (Buchs, Switzerland); isovanillic acid (3-hydroxy,4-methoxycinnamic acid), from Sigma; and isoferulic acid (3-hydroxy, 4-methoxycinnamic acid), esculetin

(6,7-dihydroxycoumarin), scopoletin (6-methoxy, 7-hydroxycoumarin), and isoscopoletin (6-hydroxy, 7-methoxycoumarin) from Roth (Karlsruhe, Germany).

2.2. *Extraction and separation of OMTs*

As our enzyme source, we used the first two or three fully expanded leaves from the top of 3-month-old tobacco plants *Nicotiana tabacum*, cv Samsun NN, grown in an air-conditioned greenhouse at 22 + 2°C. OMTs were extracted by grinding 150 g of leaves in a Virtis homogenizer in the presence of 450 ml ice-cooled 0.1 M phosphate buffer, pH 7.5, containing 15 mM 2-mercaptoethanol. The mixture was filtered through a double layer of cheesecloth and the filtrate centrifuged at 20 000 × *g* for 20 min. Proteins were precipitated from the supernatant fluid with $(NH_4)_2SO_4$, diluted aqueous NH_4OH being added to maintain the pH at 7.5. The protein fraction that precipitated at 40–75% saturation with $(NH_4)_2SO_4$ was dissolved in 15 ml of 20 mM phosphate buffer, pH 7.5, containing 15 mM mercaptoethanol. This protein fraction was then desalted by gel filtration on Sephadex G-25 (coarse) and was used as the crude extract. This crude extract was chromatographed on a Sephadex G-200 column. The active fractions were pooled and the pH adjusted to 7.7 with 1M NaOH. This protein fraction was then chromatographed on a DEAE-cellulose column, as indicated in the legend of fig.1.

2.3. *Enzyme assays and determination of enzymatic activity*

The standard assay mixture for the methyltransferases consisted of 50 μM tritiated SAM (0.4 μCi per assay), the catecholic substrate (at concentrations ranging from 0.5 mM to 10 mM, see table 1), and 50 μl enzyme solution (crude extract or fractions from

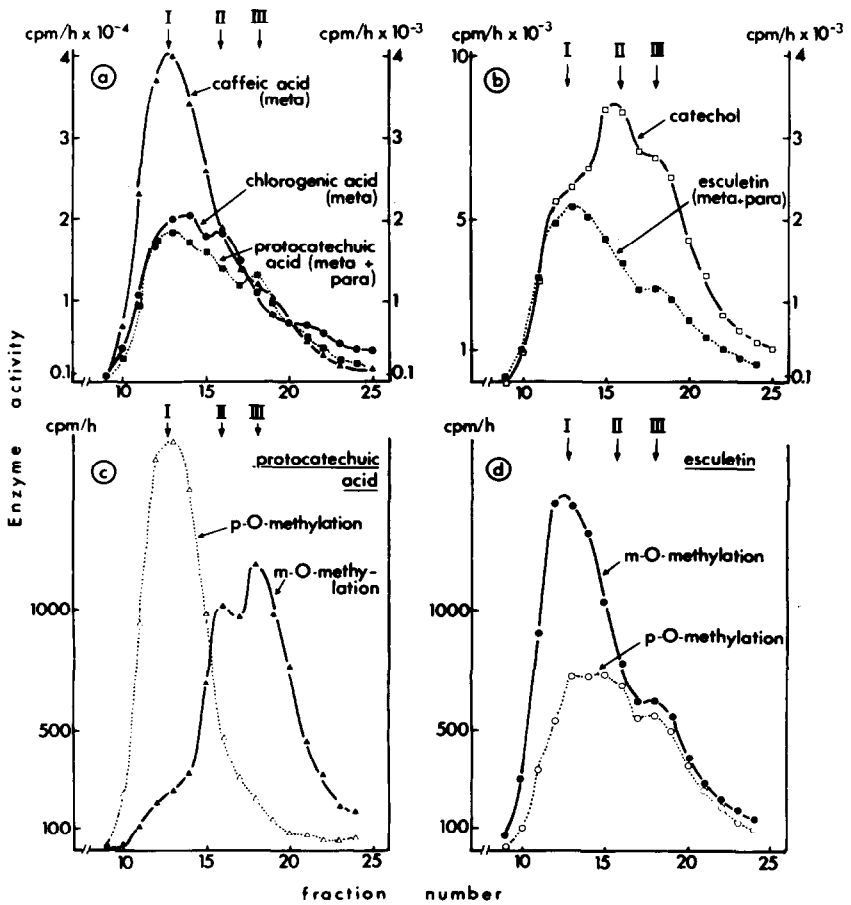

Fig.1

Table 1
Relative rates of methylation of catecholic substrates by crude extracts from tobacco leaves

Substrate	Total methylation (%)	Proportion of	
		Meta–*O*–methylation	Para–*O*–methylation
Catechol 2.5 mM	24	–	–
Caffeic acid 2.5 mM	100	100	0
Chlorogenic acid 10 mM	10	100	0
Protocatechuic acid 10 mM	10	46	54
Esculetin 0.5 mM	12	59	41

The fluid used for enzymatic assays was obtained by fractionation with ammonium sulfate (40–75%) and subsequent chromatography on Sephadex G-25. Conditions of incubation, determination of enzymatic activity, and separation of *p*- and *m*-methylating activities are described in Materials and methods.

Sephadex G-200 chromatography or from DEAE–cellulose chromatography) in a total volume of 0.65 ml. Conditions of incubation, extraction of the reaction products, and measurement of their radioactivity have already been described [1]. When chlorogenic acid was the substrate, the reaction product was hydrolyzed under vacuum with 4 M NaOH before extraction by an organic phase. All assays were duplicated.

2.4. *Separation of para- and meta-methylating activities*

The methylated isomers were separated by thin layer chromatography (TLC) on silica-gel (Merck 60F-254, precoated plates) with the upper phase of C_6H_6–OHAc–H_2O (6:7:3). Carrier compounds added before TLC appeared as dark-absorbing spots under ultraviolet light at 254 nm. The silica gel corresponding to these spots was then scratched from the plates and collected in scintillation vials. After adding 15 ml of Bray's [15] scintillation cocktail, we stirred and then let them stand for 4 h before counting in an Intertechnique SL 32 spectrometer.

3. Results

3.1. *Meta- and para-methylating activities in crude tobacco extracts*

Crude plant extracts contain many ortho-diphenolic compounds that are possible substrates for OMT activity. These were eliminated by ammonium sulfate precipitation and Sephadex G-25 filtration in order to

Fig.1. Separation of the OMTs by column chromatography on DEAE–cellulose. The column (1.3 × 8 cm) was washed with 50 ml of 20 mM phosphate buffer, pH 7.7, and eluted with a 300 ml linear gradient (20–100 mM) of phosphate buffer, pH 7.7. The flow rate was 30 ml/h and the fraction volume was 7.5 ml. All fractions were assayed for OMT activity with ^{3}H-labelled SAM and the five diphenolic substrates (at the concentrations shown in table 1). All incubations were in duplicate: one sample was extracted for determination of total methylating activity (a and b) and one served to differentiate *p*– and *m*– methylating activities (c and d) by means of TLC. Enzymatic activities were measured by the radioactivity (in cpm) in the reaction products(s) per h of incubation. The scales of radioactivity in the extracted compound(s) are as follows: 0–4 cpm/h × 10^{-4} with caffeic acid as substrate; 0–4 cpm/h × 10^{-3} with chlorogenic acid, protocatechuic acid and esculetin; 0–10 cpm/h × 10^{-3} with catechol.

avoid any interference with the substrates to be assayed.

The G-25 filtrates were incubated in the presence of tritiated SAM and a substrate: catechol, caffeic acid, chlorogenic acid, protocatechuic acid, and esculetin. The results are shown in table 1. Free caffeic acid is the best substrate, even better than catechol. Moreover, the methylation efficiency is 10 × greater with this free form than the predominantly occurring bound form chlorogenic acid. Although high concentrations of all these substrates inhibit the enzymatic reaction, the concentrations shown in table 1 saturate but do not inhibit.

The methylation products formed from these different substrates were identified in order to differentiate para- and meta-*O*-methylating activities. Compounds were identified by thin-layer chromatography and comparison with reference samples (see Materials and methods). In contrast to what has been reported previously [13], the *p*-methylated compounds and their *m*-methylated isomers were easily distinguishable by TLC; migrations (in mm) of compounds after 2 h were as follows: ferulic acid (70), was well separated from isoferulic acid (61), vanillic acid (71) from isovanillic acid (57) and scopoletin (29) from isoscopoletin (41). Caffeic acid and its ester, chlorogenic acid, were methylated only at the meta position, whereas esculetin and protocatechuic acid were methylated at either the meta or the para position to the side chain.

3.2. *Evidence that several enzymes are responsible for OMT activity of crude extracts*

The relative rates of *p*- and *m*-methylation of protocatechuic acid and esculetin varied with the batch of leaves used to prepare the crude extracts. For instance, in the experiment reported in table 1, their respective meta/para–methylation ratios were 0.85 and 1.4. In another experiment (1st line of table 2), the respective ratios were 0.39 and 2.0. This is experimental evidence for the occurrence of more than one methylating enzyme in tobacco leaves.

On the other hand, Finkle [13] found that, with protocatechuic acid as substrate, the *p*- and *m*-methylating activities of crude pampas grass extracts were differently sensitive to heat treatment. Using a similar heat treatment (45°C, 20 min) on the crude tobacco leaf extract, we assayed in parallel the two substrates protocatechuic acid and esculetin (table 2). The heat treatment virtually suppressed *p-O*-methylation of protocatechuic acid, but reduced that of esculetin by only 27%. Furthermore, *m*-methylation of protocatechuic acid was almost unaffected whereas that of esculetin was decreased by 66%. Thus, in the case of the tobacco enzymes, *p*-methylating activity toward protocatechuic acid and *m*-methylating activity toward esculetin appear to be very sensitive to heat. In the case of the enzyme(s) from pampas grass, *m*- but not *p*-methylating activity toward protocatechuic acid was lost after heat treatment. The results we show in

Table 2
Effect of heat treatment on para- and meta-methylation of protocatechuic acid and esculetin by crude tobacco leaf extracts

Substrate	Protocatechuic acid		Esculetin	
	Radioactivity (cpm) in reaction products			
	Vanillic acid (meta)	Isovanillic acid (para)	Scopoletin (meta)	Isoscopoletin (para)
Standard assay	2470	6340	8420	4280
Heat treatment	2180	125	3710	3120
Decrease in enzymatic activity (%)	12	98	66	27

Overall conditions were the same as for table 1. The crude extract was heated to 45°C for 20 min.

table 2 suggest the following conclusions: (a) several OMTs are present in the Sephadex G-25 filtrate; (b) the main *p*- and *m*-methylation activities toward protocatechuic acid and esculetin arise from different enzymes; and (c) methylation at a given ring position arises from more than one enzyme.

3.3. *Separation by DEAE-cellulose chromatography of OMTs with different substrate specificities*

The crude extract (Sephadex G-25 filtrate) was first chromatographed on a Sephadex G-200 column and the active peak was again chromatographed on a DEAE-cellulose column as described in Materials and methods and in the legend of fig.1. All fractions collected were assayed for OMT activity with five catecholic substrates: caffeic acid, chlorogenic acid, protocatechuic acid, catechol, and esculetin. For more clarity, the five curves obtained have been drawn on two separate graphs (fig.1a and 1b). Three peaks of OMT activity can be clearly distinguished by differences in relative rates of *p*-*O*-methylation (see section 3.4.) and in substrate specificity (fig.1a and 1b). They correspond to fractions 13, 16, and 18, respectively. Caffeic acid was best methylated by fraction 13 whereas its quinic acid ester, chlorogenic acid, was methylated to a much lower extent but with almost equal efficiencies by fractions 13 and 16. The curves observed with esculetin, protocatechuic acid, and chlorogenic acid as substrates were rather similar. Catechol was best methylated by fraction 16, however, the methylation efficiencies observed with the three enzymatic fractions were of the same order of magnitude. Therefore we conclude that at least three methylation enzymes, OMTs I, II, and III, are present in fractions 13, 16, and 18, respectively.

3.4. *Para- and meta-methylating activities of the three OMTs*

Differentiation of *p*- and *m*-methylating activities was easily achieved by separation of the methylated isomers. This was done by the TLC procedure described in paragraph 3.2. With catechol as substrate, guaiacol was the only possible reaction product. Furthermore, we were able to show that ferulic acid but not isoferulic acid was radioactive when caffeic acid or chlorogenic acid were the substrates, so that the corresponding curves of fig.1a can be related to a specific *m*-methylating activity. The curves obtained with protocatechuic acid and esculetin as substrates corresponded to the sum of *p*- and *m*-methylating activities. These were separated as described in paragraph 3.2., and the results are shown in fig.1c and 1d. The ratios of *m*/*p*-methylation for esculetin were in the range of 2,1, and 1 for OMTs I, II, and III respectively. In contrast, protocatechuic acid was methylated almost exclusively in the para position by OMT I and in the meta position by OMTs II and III. The peak of *m*-*O*-methylation at fraction 18 (fig.1c) observed in all our experiments is important experimental support for the presence of OMT III.

4. Discussion

The presence of at least three methylation enzymes in tobacco leaves was demonstrated by assaying all fractions obtained by DEAE cellulose chromatography in parallel with as many as six substrates: catechol, caffeic acid, chlorogenic acid, protocatechuic acid, esculetin, and quercetin. The data concerning the last substrate, quercetin, are not shown because we have not yet unequivocally identified the reaction product(s). The resulting pattern, however, was very similar to that of catechol (fig.1b). Furthermore, since all three enzymes methylated quercetin much less efficiently than they did caffeic acid, none of them resembles the 3′,4′-dihydroxyflavonoid 3′-*O*-methyltransferase of soybean cell suspension cultures [14]. Surprisingly, both the methylation efficiencies and the patterns observed with caffeic acid and its naturally occurring ester, chlorogenic acid, were very different. Moreover, with esculetin, an internal ester of caffeic acid, *p*-*O*-methylation occurred in addition to *m*-*O*-methylation. This, as reported for the methylase extracted from a fungus [16], specificity toward a given substrate appears to be strongly modified by its esterification.

Finkle [13] concluded that pampas grass, *p*- and *m*-directing activities arose from two different enzymes. This is probably not the case with the tobacco OMTs, however. Every peak of *p*-*O*-methylation of protocatechuic acid and of esculetin coincided with one peak of *m*-*O*-methylation of one or more of the substrates (fig.1). Other experiments (data not presented) have shown that *m*/*p*-methylation ratios in each of fractions 13, 16, and 18 did not vary with the batch of leaves

or after viral infection that produces a strong increase in all three methylation peaks. Therefore, the three methylases of tobacco very likely have both meta and para capabilities.

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References

[1] Legrand, M., Fritig, B. and Hirth, L. (1976) Phytochemistry (in press).
[2] Ebel, J., Hahlbrock, K. and Grisebach, H. (1972) Biochim. Biophys. Acta 269, 313–326.
[3] Hess, D. (1966) Z. Planzenphysiol. 55, 374–386.
[4] Shimada, M., Ohashi, H. and Higuchi, T. (1970) Phytochemistry 9, 2463–2470.
[5] Shimada, M., Fushiki, H. and Higuchi, T. (1972) Phytochemistry 11, 2657–2662.
[6] Karoda, H., Shimada, M. and Higuchi, T. (1975) Phytochemistry 14, 1759–1763.
[7] Glass, A. D. M. and Bohm, B. A. (1972) Phytochemistry 11, 2195–2199.
[8] Mann, J. D., Fales, H. M. and Mudd, S. H. (1963) J. Biol. Chem. 238, 3820–3823.
[9] Finkle, B. J. and Nelson, R. F. (1963) Biochim. Biophys. Acta 78, 747–749.
[10] Finkle, B. J. and Masri, M. S. (1964) Biochim. Biophys. Acta 85, 167–169.
[11] Hess, D. (1965) Z. Pflanzenphysiol. 53, 460–463.
[12] Basmadjian, G. P. and Paul, A. G. (1971) Lloydia 34, 91–93.
[13] Finkle, B. J. and Kelly, S. H. (1974) Phytochemistry 13, 1719–1725.
[14] Poulton, J., Grisebach, H., Ebel, J., Schaller-Hekeler, B. and Hahlbrock, K. (1976) Arch. Biochem. Biophys. 173, 301–305.
[15] Bray, G. A. (1960) Anal. Biochem. 1, 279–285.
[16] Wat, C. K. and Towers, G. H. N. (1975) Phytochemistry 14, 663–666.