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PURIFICATION AND PROPERTIES OF THE THREE *o*-DIPHENOL-*O*-METHYLTRANSFERASES OF TOBACCO LEAVES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; leaves; enzyme; *O*-methyltransferases; phenylpropanoid metabolism; lignification.

Abstract—Three *o*-diphenol-*O*-methyltransferases (OMTs I, II and III) which catalyse the monomethylation of various *o*-diphenols using *S*-adenosyl-L-methionine as methyl donor were isolated and purified about 210-, 70-, and 70-fold, respectively, from leaves of *Nicotiana tabacum* cv Samsun NN. They had slightly different MWs (93 000, 90 000 and 100 000 for OMTs I, II and III respectively) and slightly different pIs (5.21, 4.80 and 4.74). The activities of all three enzymes were very stable when stored at 0° but they had different sensitivities to ultrafiltration and to heat treatment (45°). In none of the enzymes was there any change in reaction rate when Mg^{2+} ions or EDTA were added. The three enzymes exhibited very high and similar affinities towards the substrate *S*-adenosylmethionine and the reaction product *S*-adenosylhomocysteine, but they differed markedly in specificities towards the various *o*-diphenolic substrates. Relative methylation efficiencies were estimated from the calculation of the V/K_m ratios that led to the following decreasing order of best substrates: 5-hydroxyferulic acid > caffeic acid > homocatechol > esculetin > protocatechuic aldehyde > catechol > hydrocaffeic acid > chlorogenic acid, for OMT I, and: homocatechol > catechol > protocatechuic aldehyde > esculetin ≈ caffeic acid > 5-hydroxyferulic acid, for both OMTs II and III. Most of the *o*-diphenols assayed were methylated exclusively in the *meta* position, but all three tobacco OMTs showed both *para*- and *meta*-directing activities with protocatechuic acid, protocatechuic aldehyde and esculetin. Since K_m values towards the two positions of methylation were always found to be identical, we conclude that each enzyme bears only one catalytic site.

INTRODUCTION

O-Methylation is an important reaction in the biosynthetic process of lignification. The infection of plants by viruses [1–3] or by other pathogens [4–6] has been reported to lead to an increased lignification that is apparently correlated with host resistance. We have already shown that in tobacco-mosaic-virus (TMV)-infected hypersensitive tobacco plants, the phenylpropanoid pathway is highly activated [7]. Furthermore, if this TMV-induced activation of the phenylpropanoid pathway is inhibited by treatment with α-aminooxyacetate, a competitive inhibitor of phenylalanine ammonia-lyase, then the hypersensitive resistance to the virus weakens [8]. Stimulated activities of several enzymes of this pathway, particularly caffeic acid *O*-methyltransferases, appear to be very good biochemical markers of the hypersensitive resistance of tobacco plants to several strains of TMV [9]. In previous publications [10, 11] we reported that the *o*-diphenol-*O*-methyltransferase (OMT) activity of *N. tabacum* cv Samsun NN plants is due to three enzymes that have clearly different stabilities and substrate specificities. In TMV-infected leaves, however, although overall OMT activity was due to the same three enzymes, activities increased to varying extents: in heavily infected leaves, the percentages of increase in the activities of OMTs II and III can be as much as 5 times higher than that of OMT I, the major enzyme of healthy leaves [11]. This finding raised the question of the role of the preferentially stimulated OMTs II and III and their exact substrate specifities. In the present paper, we describe the purification and some physicochemical properties of the three OMTs. We also indicate the kinetic constants (K_m or K_i, V/K_m) of each enzyme towards various *o*-diphenolic substrates, *S*-adenosylmethionine, and the reaction product *S*-adenosylhomocysteine.

RESULTS

Purification of the O-*methyltransferases*

We have shown previously that the activities of all three OMTs of tobacco plants are drastically increased after infection by TMV [11]. Therefore highly infected tobacco leaves were used as the source of enzyme material. The purification procedure is illustrated in Table 1. It appears that the first steps of the extraction ($(NH_4)_2SO_4$ fractionation and Sephadex G-25 and G-100 chromatography) are the most critical for the preservation of enzymatic activity. In the experiment illustrated in Table 1, nearly 80% of the activity was lost during the first steps. The loss was less severe when the purification was carried out on smaller batches, enabling the Sephadex G-25 extract to be obtained more quickly. The first chromatography on DEAE-cellulose resulted in a large increase in total OMT activity (Table 1), indicating that an inhibitor had been removed. The apparent loss of

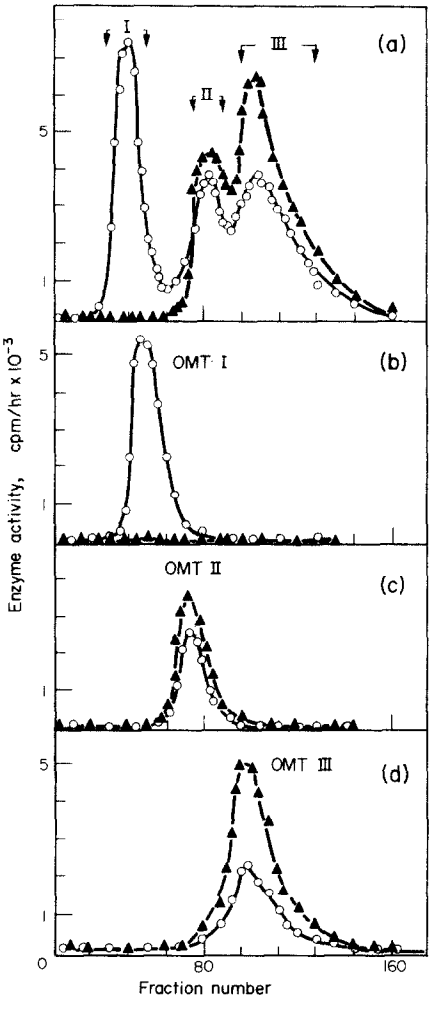

Fig. 1. Separation of the three OMTs of tobacco leaves by two successive chromatographies on DEAE-cellulose. The conditions of the runs and of enzyme assays are described in the Experimental. The *o*-diphenols used as substrates were 2.5 mM caffeic acid (○) and 0.1 mM catechol (▲). After the first run (a), the fractions corresponding to the three peaks of activity were pooled as indicated by the vertical arrows (Nos. of fractions indicated in the text) and were submitted to second runs, the profiles of which are illustrated by (b), (c) and (d), in order to isolate OMTs I, II and III, respectively.

activity between the two runs on the DEAE-cellulose column arose from the fact that we deliberately pooled and rechromatographed only a part of the active fractions obtained after the first run. Indeed, the enzyme activities were very stable at this stage of purification.

Fig. 1(a) shows the activity profiles obtained after the first run on a DEAE-cellulose column. The activity towards two substrates was eluted in three peaks, peaks II and III shouldering each other. Fractions Nos. 28–50 (peak I), 74–90 (peak II) and 100–130 (peak III) were pooled and rechromatographed. All three enzymes efficiently methylated caffeic acid (2.3 mM), whereas only enzymes II and III methylated catechol (0.1 mM). No activity towards catechol was detected in peak I, suggesting that very little of enzymes II and III contaminated peak I (Fig. 1(a)). This was confirmed by the activity profile after the second run of enzyme I on DEAE-cellulose (Fig. 1(b)): only one peak of activity appeared with caffeic acid as substrate, and no activity at all was detected throughout the gradient of elution when catechol was used as the substrate. The fractions that were likely to be the least contaminated (the top of peak II and the second half of peak III) were rechromatographed on DEAE-cellulose. The elution profiles (Fig. 1(c) and (d)) revealed the presence of one single peak of activity towards both substrates, demonstrating that each enzyme was free of contamination by the two others. The most active fractions were pooled and used for all studies described below. By taking into account the relative proportions of enzymes I, II and III after separation on DEAE-cellulose (first run), we evaluated the contribution of each enzyme to the total activity of the crude extract and, hence, calculated the purification factor of the procedure. This factor was *ca* 210 for OMT I and 70 for OMTs II and III (Table 1).

Stability of the OMTs

The activities of the three OMTs after various periods of storage at 0° were studied. The enzyme solutions were maintained at 0° in ice, and every 2 weeks 2-mercaptoethanol and NaN_3 were added, to final concentrations of 15 mM and 0.02%, respectively. Under these conditions, 65%, 72%, and 85% of the initial activity of enzymes I, II, and III respectively, remained after 5 months of storage.

The stability of the three OMTs was also tested at 45°. Fig. 2 shows the curve of residual activity of each enzyme vs the time of pre-incubation at 45°. Enzyme I appears to have been much less heat-stable than enzymes II and III: only 5% of the activity of enzyme I remained after 5 min of heating, whereas 30% and 50% of the initial activities of enzymes II and III respectively were recovered after 2 hr. Enzyme I was also the only one that lost activity rapidly during dialysis and isoelectric focusing. Moreover, all the activity of OMT I was lost in ultrafiltration, while 50% of

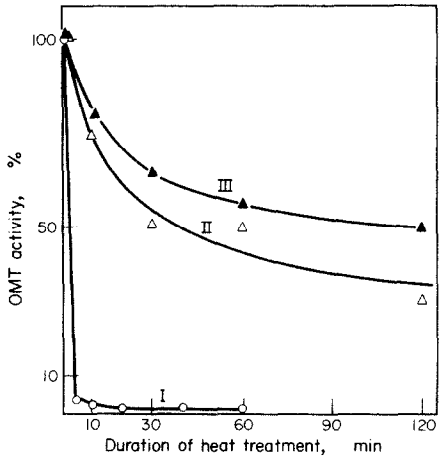

Fig. 2. Differential thermal stability of the three OMTs of tobacco leaves. After heating at 45° for various periods, assays of enzyme activities were performed at the usual incubation temperature (37°) for 2 hr with either 2.5 mM caffeic acid (OMT I) or 0.1 mM catechol (OMTs II and III) as diphenolic substrates.

Table 1. Purification of the OMTs

Fraction	Total activity (nkat)	OMT	Activity of separated enzymes (nkat)	Protein (g)	Specific activity (nkat/g)	Purification factor
Crude extract	15	—	—	8.5	1.7	—
$(NH_4)_2SO_4$ fractionation + Sephadex G-25 chromatography	4.6	—	—	2.3	2.0	1.2
Sephadex G-100 chromatography	2.7	—	—	1.2	2.3	1.4
DEAE-cellulose chromatography:						
1st run	7.5	I	3.0	0.15	20	33
		II	1.8	0.08	22	44
		III	2.7	0.11	24	41
DEAE-cellulose chromatography:						
2nd run	5.3	I	2.5	0.02	125	210
		II	1.1	0.03	36	70
		III	1.7	0.04	42	70

OMT activity was assayed under standard conditions with 2.3 mM caffeic acid as the diphenolic substrate. The contribution of each enzyme to the total activity of the crude extract was evaluated from the relative activities of OMTs after the first run on DEAE-cellulose and was used for calculating the purification factor for each OMT.

the activities of OMTs II and III was retained. The activities of all three OMTs were completely lost after freezing.

Time linearity of the enzymatic reaction

The rates of methylation of the substrate by the OMTs were linear for various periods of incubation depending on the substrate and the enzyme. For example, with caffeic acid and catechol as substrates, 2 hr was the maximum time of linearity for all the three enzymes, but with the other *o*-diphenolic substrates tested, the periods of linearity varied between 1 and 4 hr depending on the enzyme. They were always shorter for enzyme I than for enzymes II and III (data not shown). In the studies of the properties of the enzymes, incubation periods never exceeded time linearity in each particular case.

Effect of Mg^{2+}

In some cases, this divalent cation is required for the methylation catalysed by OMTs from plants [12, 13]. In other plant materials the *O*-methylation activity was only slightly stimulated by the addition of Mg^{2+} to the incubation mixture [14–16]. The activity of each OMT from tobacco plants was tested in the presence of $MgCl_2$ (1 mM) or EDTA (1 mM). No change in the methylation activity towards caffeic acid, catechol, or protocatechuic aldehyde was observed. We concluded that OMTs from tobacco leaves do not require Mg^{2+} for maximum activity, a finding that is in agreement with the results reported for OMTs from spinach leaves [17] and from suspension cultures of soybean cells [18] and tobacco cells [19].

MW determinations

MWs were evaluated by comigration of each enzyme with the MW markers on a Sephadex G-100 column (see Experimental). MWs were 93 000 ± 4000 for OMT I, 90 000 ± 4000 for OMT II, and 100 000 ± 4000 for OMT III. The standard error was estimated by at least three determinations for each enzyme. Mixtures of either enzymes I and III or enzymes II and III were chromatographed on the same column. The collected fractions were assayed with two substrates methylated with differential efficiencies by the two enzymes of each pair. In both cases, distinct maxima of activity were detected, demonstrating the higher MW of enzyme III.

Isoelectric point determinations

The isoelectric points of the purified enzymes were determined by isoelectric focusing in liquid media; they were 5.21 ± 0.03 for OMT I; 4.80 ± 0.03 for OMT II, and 4.74 ± 0.03 for OMT III. These values are the averages of three determinations.

Densities of OMTs

The densities of OMTs were evaluated by isopycnic equilibrium centrifugation on a highly resolutive salt, potassium formate. Their values were virtually identical and were 1.277 ± 0.001 kg/l.

Kinetic constants

The kinetic constants of the purified enzymes were measured against various *o*-diphenolic substrates at a saturating concentration of *S*-adenosylmethionine (SAM). The methylation rates were evaluated after the

Table 2. Kinetic constants of OMTs towards nine *o*-diphenolic substrates

Substrate		OMT I		OMT II		OMT III	
		K_m	V/K_m	K_m	V/K_m	K_m	V/K_m
Caffeic acid		100	52	250	1.7	200	3.2
5-Hydroxyferulic acid		65	73	330	0.5	700	0.3
Hydrocaffeic acid		230	0.16	500	0.8	160	0.9
Chlorogenic acid		2500	0.1	1300	0.12	1400	0.6
Catechol		2000	1.8	15	406	25	564
Homocatechol		40	34	20	430	10	1245
Protocatechuic acid	*meta*-methylation	2500	0.02	20000	0.03	20000	0.03
	para-methylation	2500	0.12	—	—	—	—
Protocatechuic aldehyde	*meta*-methylation	700	9	250	31	250	27
	para-methylation	700	0.3	250	0.7	250	0.7
Esculetin	*meta*-methylation	30	11	200	2.5	200	2.5
	para-methylation	30	5	200	2.5	200	2.5

The enzymes were assayed after the second run on a DEAE-cellulose column. Assay conditions and the extraction and purification of reaction products are described in the Experimental. V and K_m values were determined by the Lineweaver–Burk method. V is expressed in pkat/kg fr. wt and K_m in μM.

reaction product(s) had been extracted and identified. In a double reciprocal plot these values were always on a straight line when the concentration of substrate was less than $2 \times K_m$ (data not shown). For the higher concentrations of diphenols the $1/V$ values fell either under the straight line (indicating an increase of the reaction velocity) or above it (indicating an inhibition of the reaction by high concentration of substrate), depending on the substrate. The K_m and V values were determined by the Lineweaver–Burk method for the linear part of the plot $1/V = f(1/s)$, since the lowest concentrations of substrates are the ones that are most likely to be present in the plant cell. V values were expressed as pkat/kg fr. wt so that they would take into account the differential loss of activity of the OMTs between the two runs on DEAE-cellulose and the relative proportions of the enzymes in tobacco leaves as indicated by the first run on DEAE-cellulose. The V/K_m ratios reflect the efficiencies of each enzyme towards the different substrates (Table 2).

For OMT I, the phenylpropanoids caffeic acid and 5-hydroxyferulic acid were the best substrates, that is, had the highest V/K_m ratios. The double bond of the phenylpropane structure appears to be required for high activity of OMT I: the V/K_m value for hydrocaffeic acid was clearly lower than that of caffeic acid. The poor methylation of chlorogenic acid, the quinic acid ester of caffeic acid, by enzyme I indicates that the equilibrium between the bound and the free forms of caffeic acid might determine the rate of methylation of caffeic acid by enzyme I *in vivo*. Cyclization of caffeic acid into esculetin markedly decreased methylation efficiency; it also resulted in large changes in the specificity of the enzyme, since OMT I catalysed *para*-methylation of esculetin at a higher rate than it did *meta*-methylation, whereas the phenylpropanoid-type substrates were methylated exclusively in the *meta* position. In the case of substrates methylated in both positions (esculetin and protocatechualdehyde), the calculated K_ms were the same for the two positions suggesting that there is only one catalytic site for the *o*-diphenolic substrate. The importance of the lateral chain for *meta*- or *para*-specificity is indicated by the differences of methylation rates in each position for protocatechuic acid (exclusively *para*-methylation) and protocatechualdehyde (mainly *meta*-methylation).

Enzymes II and III very efficiently catalysed the methylation of catechol and homocatechol. They methylated the phenylpropanoid-type substrates at a lower rate than did enzyme I, 5-hydroxyferulic acid being a particularly poor substrate for enzymes II and III. The finding of large differences in methylation efficiencies of the three OMTs towards the *para* or *meta* position of esculetin, protocatechualdehyde, and protocatechuic acid confirmed results previously reported [10, 11].

K_m values for the second substrate, *S*-adenosyl-L-methionine (SAM), and K_i values for the second product, *S*-adenosyl-L-homocysteine (SAH), vs SAM or an *o*-diphenol are presented in Table 3. K_m values for SAM

Table 3. Affinities of OMTs for SAM and SAH

Affinity constant (μM)	OMT I	OMT II	OMT III
K_m, SAM	4.5	3	3
K_i, SAH vs SAM	5	5	5
K_i, SAH vs *o*-diphenol	6	12	12

Affinity constants were evaluated as described in the Experimental.

were of the same order of magnitude for all three enzymes. SAH exhibited strong competitive inhibition of the activities of all three enzymes against both SAM and the phenolic substrate.

DISCUSSION

In the past few years, several authors have reported the presence of more than one *O*-methylating enzyme in various plant materials [13–22]. There were always two enzymes present and in some cases [13, 16, 18, 19] these were separated and their properties were examined. Tobacco leaves represent the first case reported so far where three distinct enzymes contribute to the *O*-methylating activity [10, 11]. These enzymes have been purified and separated by two successive runs on DEAE-cellulose columns, and their physicochemical and kinetic properties are described here.

The MW of OMT III (100 000) appears to be slightly higher than those of the two other enzymes (93 000 and 90 000 for OMTs I and II respectively). The values found are higher than those reported for OMTs from tobacco-cell suspension cultures [19] and from tulip anthers [16]. The three enzymes of tobacco leaves exhibited similar high stability upon storage at 0°, in contrast with the behaviour of OMTs isolated from soybeans [22] and tulips [16]. OMT I differed from the other two in heat lability and in pI value. However, the most pronounced differences between OMTs were observed in substrate specificities. OMT I exhibited the highest V/K_m values for substrates of the phenylpropanoid-type such as caffeic acid and 5-hydroxyferulic acid. Since these two compounds are known as intermediates in lignin biosynthesis, OMT I appears to be specialized in that pathway. The best substrates for OMTs II and III were catechol and homocatechol and, to a lesser extent, protocatechualdehyde. In agreement with previous reports [10, 11], the three OMTs methylated most of the substrates exclusively in the *meta* position, but the enzymes showed *para*-methylating capabilities with protocatechuic aldehyde, protocatechuic acid, and esculetin as substrates.

The infection of tobacco leaves by TMV triggers a sharp increase of the activities of enzymes involved in phenylpropanoid metabolism, particularly of OMT activity [9]. The three OMTs are not stimulated to the same extent: OMTs II and III increase preferentially in infected leaves [11]. It is very likely that the increased activity of OMT I will participate in an increased biosynthesis of the normal monomers of lignins [1]. Furthermore, the preferentially stimulated OMTs II and III could be involved in the production of other aromatic compounds with hydroxy- and methoxy-substitution (such as guaiacol and 2-methoxy-4-methylphenol, which are the methylated products of catechol and homocatechol). All these substances bearing hydroxy–methoxy groups on the aromatic ring can produce free radicals which can polymerize in the presence of peroxidases, the activities of which are also enhanced by infection [9]. We believe that this increased biosynthesis of lignins and/or lignin-like polymers is involved in the mechanism of virus localization in hypersensitive plants. Work is now in progress to test this hypothesis.

EXPERIMENTAL

Plant material. The experiments were performed with 3-month-old tobacco plants *Nicotiana tabacum* cv Samsun NN grown in a greenhouse at 22 ± 2°. The first three fully expanded leaves of each plant were inoculated with an aq. suspension of highly purified TMV (common strain) in the presence of the abrasive Celite. We used the virus at 100 μg/ml in order to induce 600–800 local lesions/leaf. The infection was allowed to develop in a growth chamber at 22 ± 1° for 60 hr. Leaves were harvested, the mid-ribs were removed, and each half leaf was frozen in deep liquid nitrogen and stored at −70° until use.

Extraction and separation of the OMTs. The extraction procedure was as described earlier [11], amplified 10× and modified as follows: 850 g of leaves were ground in a Waring blender in 2.5 l. of Na–Pi buffer, pH 7.5, containing 2-mercaptoethanol (15 mM) in the presence of 8.5 g activated charcoal. The protein fraction that precipitated between 40 and 75% saturation of ammonium sulphate was dissolved in 50 ml Na–Pi buffer. The sizes (in cm) of the columns were 30 × 4.5 for Sephadex G-25, 80 × 4.5 for Sephadex G-100, and 15 × 4.5 for DEAE-cellulose. The DEAE-cellulose column was eluted with Pi buffer, pH 7.7, in a linear gradient from 20 to 100 mM (1.5 l. at both concns) at a flow rate of 60 ml/hr. Each enzyme was chromatographed a second time on a DEAE-cellulose column under the same conditions. The fractions from the first run were pooled as described in the text and applied on the column after 2-fold dilution with distilled water. Soluble protein was determined by the method of Lowry *et al.* [23].

Substrates and reference compounds. *S*-Adenosyl-[methyl-^{3}H]methionine was purchased from New England Nuclear. Unlabelled SAM and phenolic substrates were obtained or prepared as previously described [11].

Assay of enzyme activity. Standard assay. The standard assay mixture for the OMTs consisted of 50 μM tritiated SAM (0.18 μCi/assay), an *o*-diphenolic substrate (at a concn depending on the substrate), and 50 μl of enzyme soln (crude extract, or fractions after chromatography on Sephadex G-25, Sephadex G-100, or DEAE-cellulose), made up to a final vol. of 0.65 ml with a 20 mM Pi buffer. After 1 hr of incubation at 37°, the reaction was stopped with 0.1 ml 3 N H_2SO_4. The reaction products were extracted by the addition of 1.25 ml H_2O and 2 ml of a 1:1 mixture of Et_2O and cyclohexane. Each tube was shaken for 15 sec. 1 ml of the organic phase was mixed with 10 ml of scintillation cocktail (Beckman Ready-Solv GP) for counting in a Beckman LS 9000 liquid scintillation spectrometer.

Assay used for the kinetic studies. The methylation of substrate by the purified enzyme was assayed by measuring the rate of formation of the radioactive product(s) purified by TLC. The conditions of incubation were as described above except that the vol. of enzyme and the time of incubation differed depending on the substrate. The reaction was stopped with 150 μl of 4 N HCl, and 100 μg of the reaction product(s) (40 μg when esculetin was the substrate) was added as carrier. After the addition of 1.25 ml H_2O, the incubation medium was treated twice with 1 ml Et_2O. The organic phases were pooled and evapd under vacuum, and the residue was dissolved in EtOH and chromatographed as already described [10]. Methylated compounds were detected under UV, scraped off, and counted as described above. The background radioactivity was determined by incubating the enzyme soln under the same conditions but omitting the *o*-diphenolic substrate; the reaction mixture was treated as for the assays and the measured radioactivity was taken as background.

Identification of methylated products. The products were identified by TLC, as previously described [10].

MW determinations. The MWs of the three OMTs were determined by molecular sieving on a Sephadex G-100 column (80 cm × 2.5 cm) eluted with a 20 mM Pi buffer, pH 7.5, containing 2-mercaptoethanol (1.5 mM). For every MW determination the enzyme soln was comigrated with monomeric

(65000) and dimeric (135000) forms of BSA, egg albumin (45000), and cytochrome *c* (14000). Enzyme soln (2 ml) containing 10 mg of BSA, 10 mg of egg albumin, and 5 mg of cytochrome *c* was applied on the G-100 column and eluted at a flow rate of 17 ml/hr. The MW markers were detected by *A* at 230 nm and the enzyme by its activity towards caffeic acid (for enzyme I) or catechol (for enzymes II and III). Elution vols were evaluated by weight, and MWs were determined on the experimental curve MW = $f(V_e)$. All determinations were made × 3.

5-Hydroxyferulic acid (2 mM) and catechol (0.1 mM) were used as substrates for the cochromatography of enzymes I and III, and quercetin (0.05 mM) and catechol (0.1 mM), for that of II and III.

Isoelectric focusing (IEF). The pIs of the three OMTs were determined by IEF in liquid media. Ampholines pH 4–6 (800 μl of a 40% soln) and pH 5–8 (100 μl of a 40% soln) were added to form the pH gradient, which was stabilized by a vertical sucrose gradient (5–50%) in a 60 ml column. The electrode solns were prepared as suggested by Vesterberg [24]. Before electrophoresis, each enzyme soln was dialysed against 1% glycine (3 × 2 l.) for 24 hr at 4° for OMTs II and III and 8 hr in an ice-cold bath for OMT I. Electrophoresis was carried out at 3° for 8 hr for OMT I and 24 hr for OMTs II and III, at a constant power of 2 W. Fifteen-drop fractions were collected; 200 μl of each was incubated with caffeic acid as substrate for OMT I and with catechol for OMTs II and III. The pH of each fraction was measured at 3°. Under these conditions the pH gradient was linear between 4.5 and 5.7 and the pH corresponding to the top of the activity curve was the pI of the enzyme.

Densities of native OMTs. These determinations were done by isopycnic equilibrium centrifugation using HCOOK as the support. We used a double layer of HCOOK, of densities 1.4000 and 1.1513 kg/l. for the lower and upper layers, respectively. OMTs and β-galactosidase (density-marker enzyme) were included in the upper layer. Centrifugation was at 68000 rpm (70 Ti fixed-angle rotor) for 88 hr. Of every three successive fractions, the second was used for the assay of OMT activity and the third for the measurement of refractive index. The detailed method will be published elsewhere.

Kinetic constants. The K_m values were determined towards 9 *o*-diphenolic substrates, on a Lineweaver–Burk plot. Each K_m value is the average of 3 determinations, made with about 10 different concns of the phenolic substrate (in the range $0.1\text{–}10K_m$). A pre-incubation with the lowest substrate concn enabled us to adjust the incubation time and the vol. of enzyme so as to convert a maximum of 20% of the substrate. The incubation time never exceeded the maximum time of linearity. K_m values towards SAM and K_i values towards SAH were determined using caffeic acid (2.3 mM) as *o*-diphenolic substrate for OMT I and catechol (0.1 mM) for OMTs II and III. For these expts the various solns of SAM were prepared by dilution from a 325 μM soln (0.58 μCi/ml). The exact concns of the diluted solns were determined by counting the radioactivity. The incubations were carried out with 7 different concns of SAM, from 0.5 to 50 μM. K_is for SAH against SAM were evaluated by Dixon's method after incubation in the presence of SAM (10, 25 or 50 μM), and of various concns of SAH, ranging from 1 to 150 μM. K_is of SAH against the *o*-diphenolic substrate were measured by assaying OMT I with 0.1, 0.17 and 2.3 mM caffeic acid and OMTs II and III with 15, 20 and 200 μM catechol. In every case the range of SAH concns was the same as above and a Dixon plot was used for the calculation of the K_i values.

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REFERENCES

1. Kimmins, W. C. and Wuddah, D. (1977) *Phytopathology* **67,** 1012.
2. Faulkner, G. and Kimmins, W. C. (1975) *Phytopathology* **65,** 1393.
3. Wu, J. H. (1973) *Virology* **51,** 474.
4. Friend, J. (1976) in *Biochemical Aspects of Plant Parasite Relationships* (Friend, J. and Threlfall, D. R., eds.) p. 291. Academic Press, London.
5. Ride, J. P. (1975) *Physiol. Plant Pathol.* **5,** 125.
6. Vance, C. P. and Sherwood, R. T. (1976) *Plant Physiol.* **57,** 915.
7. Fritig, B., Legrand, M. and Hirth, L. (1972) *Virology* **47,** 845.
8. Massala, R., Legrand, M. and Fritig, B. (1980) *Physiol. Plant Pathol.* **16,** 213.
9. Legrand, M., Fritig, B. and Hirth, L. (1976) *Phytochemistry* **15,** 1353.
10. Legrand, M., Fritig, B. and Hirth, L. (1976) *FEBS Letters* **70,** 131.
11. Legrand, M., Fritig, B. and Hirth, L. (1978) *Planta* **144,** 101.
12. Ebel, J., Hahlbrock, K. and Grisebach, H. (1972) *Biochim. Biophys. Acta* **269,** 313.
13. Poulton, J. E., Hahlbrock, K. and Grisebach, H. (1977) *Arch. Biochem. Biophys.* **180,** 543.
14. Shimada, M., Fushiki, H. and Higuchi, T. (1972) *Phytochemistry* **11,** 2657.
15. Kuroda, H., Shimada, M. and Higuchi, T. (1975) *Phytochemistry* **14,** 1759.
16. Sutfeld, R. and Wiermann, R. (1978) *Biochem. Physiol. Pflanz.* **172,** 111.
17. Poulton, J. E. and Butt, V. S. (1975) *Biochim. Biophys. Acta* **403,** 301.
18. Poulton, J., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **176,** 449.
19. Tsang, Y. F. and Ibrahim, R. K. (1979) *Phytochemistry* **18,** 1131.
20. Finkle, B. J. and Kelly, S. H. (1974) *Phytochemistry* **13,** 1719.
21. Thompson, H. J., Sharma, S. K. and Brown, S. A. (1978) *Arch. Biochem. Biophys.* **188,** 272.
22. Poulton, J., Grisebach, H., Ebel, J., Schaller-Hekeler, B. and Hahlbrock, K. (1976) *Arch. Biochem. Biophys.* **173,** 301.
23. Lowry, O. H., Rosebrough, N. J., Farr, H. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
24. Vesterberg, O. (1971) *Methods Enzymol.* **22,** 389.