Phytochemistry, Vol. 20, No. 12, pp. 2635–2639, 1981.
Printed in Great Britain.

0031–9422/81/122635–05 $02.00/0

CHARACTERIZATION OF A LIGNIN-SPECIFIC *O*-METHYLTRANSFERASE IN ASPEN WOOD

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(*Received* 6 *October* 1980)

Key Word Index—*Populus euramericana*; Salicaceae; aspen; secondary xylem; lignin biosynthesis; angiosperm-type *O*-methyltransferase; guaiacyl and syringyl lignin.

Abstract—*O*-Methyltransferases were extracted from the differentiating xylem of 10-yr-old *Populus euramericana*. The enzymes were partially purified by ammonium sulfate precipitation, and column chromatography on DEAE-cellulose, Sephadex G200 and hydroxyapatite. The enzymes were resolved into two peaks by DEAE-cellulose chromatography, and the MWs of the respective enzymes were estimated to be 72 000 and 75 000 by gel filtration chromatography. The enzyme corresponding to the latter peak was unstable and thus only the former peak enzyme was characterized completely. Magnesium ions had no effect, EDTA moderately stimulated and heavy metals and SH group inhibitors strongly inhibited enzyme activity. K_m values for caffeate and 5-hydroxyferulate were estimated to be 3.8×10^{-4} and 3.1×10^{-4} M, respectively. The ratio of V_{max}/K_m for 5-hydroxyferulate was 5.4 times greater than that for caffeate. The enzyme(s) catalysing the formation of ferulate from caffeate and of sinapate from 5-hydroxyferulate were not separated during the purification or by the disc electrophoresis using polyacrylamide gel. Quercetin, cyanin and catechin were not methylated by the enzyme preparation. The *O*-methyltransferase of aspen wood, where the phenolic metabolism is almost exclusively directed to lignin biosynthesis, catalyses the methylation of both guaiacyl and syringyl lignin precursors, with preferential utilization of the latter substrate. These findings lead to the conclusion that the enzyme is a typical angiosperm-type *O*-methyltransferase related to guaiacyl and syringyl lignin biosynthesis in aspen wood.

INTRODUCTION

In relation to lignin biosynthesis, we have reported that plant *O*-methyltransferases (OMTs) can be roughly classified into three groups: i.e. gymnosperm-type OMT, angiosperm-type OMT and bamboo-type OMT [1–3]. The first type catalyses the formation of guaiacyl units but scarcely that of syringyl units [3]. The second catalyses the formation of both but the rate of syringyl group production is *ca* 3 times higher than that of guaiacyl types. Most dicotyledon OMTs belong to this group. The bamboo-type OMT is similar to the angiosperm-type except that the rate of formation is almost equal [2]. These findings explain why gymnosperm lignins are mainly composed only of guaiacyl units while angiosperm lignins contain both guaiacyl and syringyl units.

Several OMTs have been characterized and their functions discussed in relation to the biosynthesis of lignin [1–8], flavonoids [9–12], furanocoumarins [15] and *m*- or *p*-*O*-methylation [11, 14, 18, 22]. The source of OMTs were buds [11], leaves [13], roots [7], young shoots [2], seedlings [3] and callus [4–6, 9, 10, 12, 14, 15]. In addition to lignin precursors, various methylated phenolics often occur in the same tissues and multiple forms of OMTs have been reported [6, 11, 14, 15, 18]. To characterize the OMTs preferentially responsible for lignin biosynthesis, it is desirable to use the tissue which exclusively produces lignified cells. Tree trunks are suitable for this purpose. However, the isolation of enzymes from tree trunks have rarely been reported [17] because of the difficulty of extraction.

The present paper deals with the properties of OMTs from differentiating xylem tissues isolated from 10-yr-old trunks of *Populus euramericana* (aspen). The role of the angiosperm-type OMT will be discussed in relation to the formation of guaiacyl and syringyl lignin precursors in dicotyledons.

RESULTS

Evaluation of aspen wood as a source of OMT

O-Methyltransferase was extracted from the differentiating xylem of 10-yr-old trunks of *P. euramericana* as described in Experimental. This preparation catalyses the *O*-methylation of both caffeate and 5-hydroxyferulate. The crude juice obtained from the phloem and young shoots rapidly turned brown even after addition of Polyclar AT presumably due to a high concentration of polyphenol oxidase. However, the sp. act. of the OMT in the crude juice was reasonably high (10.6 pkat/mg protein for 5-hydroxyferulate) compared with that reported for other plant materials before purification (between 1 and 100 pkat/mg protein [2–4, 14]). Although the recovery of the enzyme could be improved by means of grinding the tissues with sea sand the procedure described, using a Wiley mill on frozen meal in the presence of liquid nitrogen, proved effective on a large scale.

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Partial purification

The apparent polymorphism of aspen OMT was observed when the crude preparation, fractionated by ammonium sulfate, was subjected to DEAE-cellulose chromatography (Fig. 1). The first peak eluted was unstable in subsequent purification steps. Addition of cysteine or EDTA had no effect on its stability, and a complete purification was not possible.

Table 1 summarizes one experiment in the purification of aspen OMT. FA- and SA-activities are the formation of ferulate from caffeate and of sinapate from 5-hydroxyferulate, respectively. It was found that neither of these activities in each peak obtained in DEAE-cellulose chromatography could be resolved. Also they were not separable by polyacrylamide gel electrophoresis (PAGE) using the final preparation (step 5, Table 1). The ratio of the two activities remained constant (*ca* 3) during purification (Table 1) and the two peaks on the DEAE-cellulose chromatogram (Fig. 1) showed almost the same SA/FA ratio (peak I 3.2; peak II 3.0).

Properties

The MWs of peaks I and II (Fig. 1) were almost the same, and were estimated to be 72000 and 75000, respectively, by gel filtration chromatography. The optimum pH of the enzymes for FA- and SA-activities at step 2 was *ca* 8 (a mixture of peaks I and II). The optimum pH in the final preparation was not measured because the amount of the enzyme preparation was insufficient.

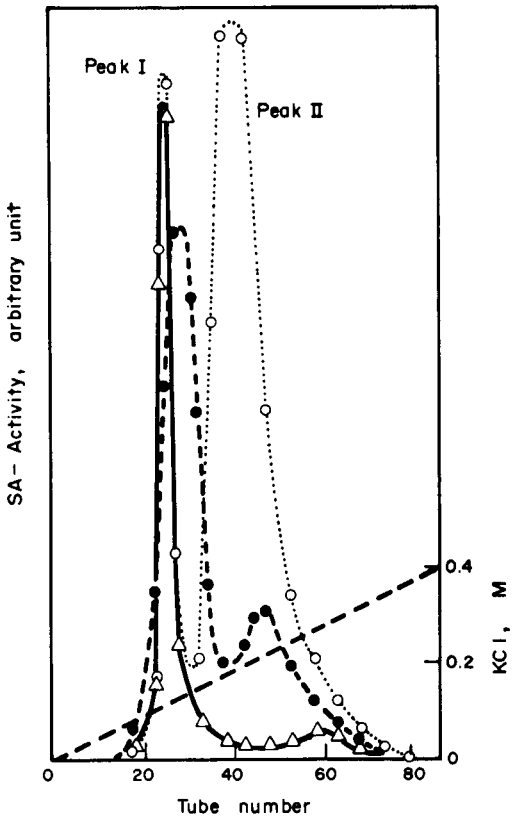

Fig. 1. Elution profiles of aspen OMTs from DEAE-cellulose column chromatography. ○ ··· ○, A crude juice corresponding to step 1. ● — ● Step 3 in Table 1. △—△ Re-chromatography after step 3 (kept in a refrigerator for more than 2 weeks).

Table 1. Partial purification of aspen OMT

Purification step*	Protein (mg)	Specific activity (nkat/mg) SA	FA	Ratio SA/FA	Recovery (%)
Step 1	461	10.6	3.38	3.14	100
Step 2	159	21.5	6.68	3.22	69.9
Step 3	31.3	82.0	25.1	3.27	52.5
Peak I	12.9	175	53.0	3.30	
Peak II	18.4	16.8	5.56	3.02	
Step 4	12.4	135	42.2	3.20	34.1
Peak I	5.80	224	68.9	3.25	
Peak II	6.60	56.1	18.5	3.04	
Step 5	3.71	183	57.9	3.16	13.9

* Step 1: Crude extract; step 2: $(NH_4)_2SO_4$ fractionation; step 3: DEAE-cellulose chromatography; step 4: Sephadex G200 chromatography; step 5: hydroxyapatite chromatography (see also Experimental).

Table 2 shows the effect of metal ions on the FA- and SA-activities in the final preparation. Addition of Mg^{2+} (1 and 10 mM) had no effect on the enzyme activities. Other divalent metal ions (1 mM) also showed no effect. Addition of EDTA, NaF and NaN_3 (1, 0.1, 10 mM, respectively) did not inhibit the enzyme but, in fact, showed moderate activation. The fact that aspen OMT was not inhibited by EDTA and NaF shows that there is no requirement for Mg^{2+} ions. Some heavy metal ions inhibited the methylation rates (Table 2) and caused more than 90% inhibition when cysteine was omitted from the reaction mixture (data not shown).

An SH group is essential for optimal catalytic activity (Table 3). Thiourea and iodoacetate inhibited the activity, while cysteine and mercaptoethanol were needed for optimal methylation rates. It should also be noted that the ratio of SA/FA activities were kept almost constant in these experiments.

Table 2. Effect of metal ions on aspen OMT

System*	Relative FA	SA	Ratio SA/FA
Standard	100	100	3.15
$MgCl_2$	100.2	100.6	3.16
$CaCl_2$	101.4	96.8	3.01
$MnCl_2$	88.8	83.0	2.95
$BaCl_2$	104.8	102.1	3.07
$NiCl_2$	83.4	77.2	2.91
$ZnCl_2$	63.7	63.1	3.12
$CoCl_2$	72.8	67.0	2.90
$HgCl_2$	73.9	72.0	3.07
NaN_3	113.2	113.4	3.16
NaF	117.8	117.3	3.14
EDTA	114.5	107.8	2.97

* The system contained a standard reaction mixture (see Experimental) except that the added chemicals substituted for Mg^{2+}. When NaF was added to the system, KPi buffer was used instead of Tris. All salts were added at 1 mM except NaN_3 (10 mM) and NaF (0.1 mM).

Table 3. Participation of the SH group in the enzymic methylation

System*	SA (pkat/mg)	FA	Ratio SA/FA	Relative SA
Standard	171	54.8	3.12	100
Minus $MgCl_2$ MESH CySH	36.4	11.8	3.08	21.3
Minus MESH CySH	37.4	11.9	3.14	21.9
Minus $MgCl_2$ MESH	172	55.3	3.11	100.6
Minus $MgCl_2$ CySH	146	47.9	3.05	85.4
Plus *p*CMB (0.1 mM)	3.59	2.79	1.29	2.10
Plus thiourea (0.1 mM)	32.1	11.2	2.87	18.8

* MESH = 2-mercaptoethanol, CySH = cysteine, *p*CMB = *p*-mercuric benzoate.

Substrate specificity

Table 4 shows the kinetic data of purified OMT (step 5, Table 1). The K_m values for protocatechualdehyde, 5-hydroxyvanillin, caffeate and 5-hydroxyferulate were found to be of the order of 10^{-4} M. The V_{max}/K_m ratio for 5-hydroxyferulate was 5.4 times greater than that for caffeate, and even protocatechualdehyde was a better substrate than the latter acid. Other *o*-diphenolic substrates examined (quercetin, cyanin, catechin, protocatechuic acid and pyrocatechol) showed rates less than 2% of that for caffeate. Almost no methylation was observed in the case of all other phenols examined (apigenin, kaempferol, 3,5-, 2,4-, 2,6-, 2,5-dihydroxybenzoates, saligenin, salicin, salicylaldehyde, arbutin, *o*-, *p*-coumarate, ferulate, *iso*-ferulate and sinapate).

DISCUSSION

Multiple forms of plant OMTs have been reported by several authors [6, 11, 14, 15, 18]. It was shown that *Populus* glandular tissue contains several OMTs [11], as we have observed in the differentiating xylem in aspen wood. It is of interest to consider this point in relation to the finding that differentiating xylem cells contain many lysosome-like vesicles and developing vacuoles. It seems likely that proteases occur in such organelles at the differentiating stage, so as to redistribute nitrogen from lignified xylem cells to cambium in tree trunks. Such a phenomenon has been reported during seed development in wheat [19]. It is assumed that proteases are the probable cause of the lability of aspen OMT when the tissue was extracted and subjected to time-consuming enzyme purification processes. Such an assumption is in accord with the fact that the MW of peak II is slightly smaller than that of peak I (Fig. 1). It must be kept in mind that multiple forms of the OMTs might be produced during extraction and purification as was reported in proteins present in tobacco cell suspensions [14]. It is assumed, however, that the polymorphism of OMTs observed is not an artifact but reflects some physiological role *in vivo*. At present, there is no direct evidence to show the multiplicity of aspen OMTs and further research is needed to clarify this problem.

Table 4. K_m, relative V_{max} and V_{max}/K_m values of aspen OMT

Substrates	K_m (10^{-4} M)	Relative V_{max}	Relative V_{max}/K_m
Caffeate	3.8	1.0	1.0
Protocatechualdehyde	2.6	2.6	3.8
5-Hydroxyvanillyl alcohol	3.4	3.7	4.1
5-Hydroxyvanillin	4.8	3.5	2.8
5-Hydroxyferulate	3.1	4.4	5.4

It is difficult to find general correlations between the biosynthetic role and properties of plant OMTs. The properties of OMTs in different plants are not always consistent even when they play similar biosynthetic roles. Purified preparations do not always show the same properties as the crude extract and this makes it difficult to describe the general properties of OMTs accurately. However, plant OMTs might be roughly characterized as follows: the optimum pH of caffeate-specific OMT ranges from pH 6.5 to 8.0 [2–4, 7, 20]; lignin-specific OMTs generally do not require Mg^{2+} ions [3, 4] except in the case of that from bamboo [21]. This is in marked contrast to flavonoid-specific OMTs in parsley and soybean cell suspension cultures [9, 10], which have an absolute requirement for the Mg^{2+} ion. Sulfhydryl group inhibitors generally inhibit plant OMTs to various extents [3, 12, 14, 15, 21] except flavonoid-specific parsley OMT, which was not affected by such compounds [9]. The effect of EDTA on the OMTs is not always the same: some OMTs showed no effect (e.g. lignin- [4] and furanocoumarin- [15] specific OMTs) but others were moderately activated (lignin-specific OMT [3]) or inhibited (flavonoid-specific OMT [10]) by EDTA. The MWs of plant OMTs lie between 40 000 and 110 000 [3, 9, 12, 14, 15]. The aspen OMT described here thus fills the general properties of lignin-specific OMTs.

It is noteworthy that catechols with *meta* side-chains of aldehyde or alcohol are as good substrates, as is caffeate. This finding indicates that the enzyme might also be involved in the methylation of C_6–C_1 diphenols. However, virtually no methylation of protocatechuic acid was observed even though vanillic and syringic acids are widely distributed in aspen. Such substrate specificity of the OMT seems to be affected by the functional groups of the side-chains. This might be expanded to the diphenolic C_6–C_3 lignin precursors. It has been demonstrated that cinnamate derivatives are reduced to the corresponding alcohols via CoA-esters and aldehydes during lignification [16]. The C_6–C_3 *o*-diphenolic substrates carrying such side-chains might be methylated by a lignin-specific OMT, although caffeoyl-CoA was not methylated [4]. A cell-free system involved in the reduction of sinapate to sinapyl alcohol has been demonstrated, but the general process of syringyl lignin formation is still obscure [25]. A further survey of the substrate specificity for the OMT is desirable to clarify the biosynthetic pathway of lignin, especially the formation of the syringyl units.

The results in the present investigation can be summarized as follows. First, aspen OMT is obviously concerned in lignin biosynthesis, because of the phenolic metabolism of the xylem tissues and substrate specificities

of the enzyme. The cambium of aspen tree trunks differentiates into secondary xylem where polymers are present along with a small amount of lignin precursors. Since the phenolic metabolism of the differentiating xylem tissues is considered to be directed towards lignin formation exclusively, the OMT in these xylem cells is probably only involved in the methylation of lignin precursors. The substrate specificities of plant OMTs have been discussed from the standpoint of lignification, flavonoid biosynthesis and *o*- or *p*-*O*-methylation. It has been shown that aspen OMT does not methylate flavonoids or the *p*-position in any of the phenolic substrates surveyed. This strongly suggests that the OMT is solely concerned in lignin biosynthesis. Secondly, aspen OMT probably operates as a 'fine adjustment' enzyme in guaiacyl and syringyl lignin formation. This hypothesis arises from the finding that the same OMT catalyses the formation of both guaiacyl and syringyl nuclei albeit with preferential formation of the latter [26]. If caffeate and 5-hydroxyferulate are formed at the same site in xylem cells, the latter will be preferentially methylated by the OMT until it is reduced to a level which does not interfere with the methylation of the former. Fine and coarse adjustments appear to be operated to yield an increasing ratio of syringyl to guaiacyl nuclei during the development of xylem in dicotyledonous plants [8, 27]. The coarse adjustment might also be operated by enzymes participating in the reduction of *p*-hydroxycinnamates to the corresponding alcohols. Lastly, the substrate specificities of aspen OMTs explain in part why aspen lignin contains more syringyl nuclei compared with gymnosperms. This property appears to be generally applicable to angiosperm OMTs which relate to lignin biosynthesis. Interesting examples have been reported in swede root [7], mistletoe [24] and *Erythrina* [23]. The swede root OMT, which shows inferior SA-activity to FA-activity, is the only exception in the dicotyledonous OMTs so far described.

EXPERIMENTAL

Materials. *S*-Adenosyl-L-methionine-$^{14}CH_3$ (SAM: 45.9 mCi/mmol) was purchased from New England Nuclear, and diluted with unlabelled SAM. The diluted SAM was purified by PC using 5% HOAc in 80% EtOH at 4–6° (sp. act. of the SAM: 0.3 mCi/mmol). A kit for the calibration of MWs and unlabelled SAM were obtained from Boehringer. Hydroxycinnamates were synthesized from the corresponding benzaldehydes and malonic acid [28]. 5-Hydroxyvanillin was prepared as described in ref. [29]. 5-Hydroxyvanillyl alcohol was obtained from Mr. Kutsuki of our laboratory.

Two 10-yr-old aspen trees (*P. euramericana*) were chopped down in June and the trunks, free of bark, used as the enzyme source. The differentiating secondary xylem was scraped off and immediately frozen in liquid N_2. The frozen material was milled in a Wiley mill with the continuous addition of liquid N_2, and stored at −20°.

Chromatographic methods. The methylated products formed were co-chromatographed on paper with the solvent systems: (1) toluene–HOAc–H_2O (4:1:5, organic layer), (2) $CHCl_3$–HOAc–H_2O (2:1:1, organic layer) and on TLC (Si gel) with MeOH–*i*-propyl ether (1:10).

Enzyme assay. The standard reaction mixture contained phenolic substrate (0.2 μmol), $MgCl_2$ (2 μmol), Tris buffer (pH 8, 10 μmol), enzyme soln (up to *ca* 50 mg equivalent fr. wt) and SAM (0.03 μCi/0.1 μmol). SAM was added after pre-incubation for 5 min (total vol 200 μl). The mixture was incubated for 10, 20 and 30 min at 30° and the reaction terminated by the addition of 2 M HCl (20 μl). The products were extracted with either $CHCl_3$ (for cinnamates) or EtOAc (for flavonoids). The methylation rate was determined from the radioactivity in the extract measured by scintillation spectrometry.

Extraction and purification. The wood meal (615 g) was first homogenized with 1.7 l. of 0.2 M KPi buffer (pH 7.7) containing 5 mM of both 2-mercaptoethanol and NaN_3. All procedures of the extraction and purification were performed in a cold room at 4–6°. The homogenate was squeezed through four layers of gauze and the filtrate centrifuged at 9000 rpm for 20 min. The supernatant (1.2 l.) was treated with solid $(NH_4)_2SO_4$ with the pH maintained at 7.7 and the protein pptd. between 20 and 55% satn was collected by centrifugation. The ppt. was resuspended in a min vol of KPi buffer and dialysed for 8 hr against the same buffer (total vol. 77 ml). The desalted soln was applied to a DEAE-cellulose column previously equilibrated with KPi buffer. Proteins were eluted with a linear salt gradient between 0 and 0.5 M KCl in KPi buffer after washing with 50 ml of the buffer. Peaks I and II were eluted at *ca* 0.1 and 0.2 M KCl, respectively. The two peaks were concd by the addition of $(NH_4)_2SO_4$ (90% satn.) and the ppted proteins dissolved in a min. amount of KPi buffer. Each soln was applied to a Sephadex G200 column (1.55 × 89 cm) which was equilibrated with KPi buffer. The MWs of the enzymes were calibrated using this column. The combined fractions (peaks I and II) were absorbed on a hydroxyapatite column buffered with 2 mM KPi (pH 6.7) containing 5 mM 2-mercaptoethanol. The proteins were eluted with a linear gradient between 2 and 200 mM KPi buffer containing 5 mM of 2-mercaptoethanol. Fractions of 40 drops were collected. OMT activity was not resolved by this procedure but a shoulder of the elution profile was observed.

Proteins were determined by the method of ref. [30].

Disc electrophoresis was carried out by the method of ref. [31]. The gel after electrophoresis, was cut into 2-mm-thick slices. Half of each slice was directly assayed for FA- and SA-activities, respectively.

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