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MEMBRANE-BOUND *O*-METHYLTRANSFERASE OF DOUGLAS-FIR CALLUS

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Key Word Index—*Pseudotsuga menziesii*; Pinaceae; Douglas-fir; callus; *O*-methyltransferase; lignins; tannins; flavonoids.

Abstract—A lignin-specific *O*-methyltransferase (OMT) was localized in the cell wall fraction of Douglas-fir needle callus homogenates. The OMT was released from wall-associated membrane by digitonin and partially purified by salt fractionation. Further purification proved to be unfeasible, due to the high tannin content of the callus. The K_m values of the partially purified OMT for caffeic acid and *S*-adenosylmethionine (SAM) were 250 and 8.0 μM, respectively. Substrate inhibition as well as inhibition by *S*-adenosylhomocysteine (SAH) was observed. Coupled with low levels of caffeic acid found in the callus, 65 μM at maximum with a mean of 11.5 μM throughout a subculture period, the properties of this OMT should account in large part for the high tannin and low lignin content characteristic of this cultured tissue.

INTRODUCTION

O-Methylation is an important step in the biosynthesis of lignin [1]. *O*-Methyltransferase (OMT) specificities have been shown to account for the difference between angiosperm lignin, which contains both guaiacyl- and syringyl-type units, and gymnosperm lignin, which contains predominantly guaiacyl-type units [2]. Two types of OMT have been isolated from different plant sources: a lignin-specific OMT and a flavonoid-specific OMT [3]. Functioning of the former enzyme leading to ferulic acid would appear to commit phenylpropanoid precursors largely to lignin rather than to tannin production; therefore, if this OMT were limiting, any coumaric or caffeic acid formed in a plant would tend to be directed into the flavonoid pathway.

Phloroglucinol staining of Douglas-fir callus grown in tissue culture reveals little lignin, whereas the tannin content, as determined by the butanol–HCl procedure, is extremely high, up to 60% of the dry weight. This investigation was undertaken to determine whether these observations were due to a deficiency of lignin specific OMT resulting from *in vitro* culture as callus.

RESULTS

The OMT was found in a membrane-bound state associated with the cell wall fraction of the callus homogenate, presumably bound to the plasmalemma (Table 1). The cell wall fraction shows several times more total activity than any other fraction; it is not clear if the lesser activity in the other fractions is OMT liberated from the cell wall fraction upon homogenization.

After solubilization with digitonin and fractionation with ammonium sulphate the OMT resisted further purification. With DEAE cellulose and AH Sepharose 4B-linked ferulic acid columns, the OMT could not be eluted, remaining bound to the tops of the columns and still active. Other column materials (Sephadexes, hydroxyapatite and C-γ alumina) and electrophoresis, among other procedures, failed to yield fractions with increased specific activity. The ammonium sulphate fraction used as partially purified enzyme had a specific activity of 14 pkat/mg.

Table 1. Intracellular distribution of OMT activity

Fraction	Ferulic acid (nmol/hr/g fr. wt)
Cell wall	11.14
Chloroplasts	2.94
Mitochondria	3.58
Microsomes	1.64
Cytoplasm	2.66

The best yield of OMT was obtained by direct buffer extraction with 5 mM each of EDTA, reduced glutathione, and ascorbic acid plus 10% insoluble PVP (based on the tissue wet weight). The enzyme was inactivated during the preparation of acetone powders, but was completely stable for at least nine months when frozen. Storage at 0–4° led to a slow decrease in activity over one month.

The OMT was very specific for caffeic acic (Table 2). The flavonoids were virtually unmethylated, as were the other compounds tested. Exogenous Mg^{2+} was not essential, but 5 mM Mg^{2+} increased the activity by up to 35%.

The kinetic constants were determined for both the membrane-bound and membrane-free forms of the OMT.

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Table 2. Substrate specificity of callus OMT

Substrate	Product (cpm/hr)	Activity (%)
Caffeic acid	1190	100
Caffeic acid + 5 mM $MgCl_2$	1600	135
Taxifolin	13	1.1
Quercetin	56	4.9
Catechin	12	1.0
Epicatechin	0	0.0
Aesculetin	23	2.0
p-Coumaric acid	0	0.0
Catechol	13	1.1

In animal OMT the kinetic constants for these forms can vary significantly [4]. The membrane-bound OMT gave K_m values of 333 μM for caffeic acid and 9.6 μM for SAM. The V_{max} value was 8.3 pkat/mg. The partially purified OMT gave K_m values of 250 μM for caffeic acid and 8.0 μM for SAM with a V_{max} of 11.7 pkat/mg. In contrast to this, a Douglas-fir seedling homogenate yielded a caffeic acid K_m of 90 μM and this OMT did not sediment with the wall fraction. SAH was a noncompetitive inhibitor with a K_i of 15.1 μM. SAM was found to be a noncompetitive inhibitor.

The endogenous free cinnamic acids, catechin and procyanidin, were isolated and quantified as a function of time from subculture. The steady-state level of caffeic acid was found to be very low, reaching only 65 μM at the maximum 12 days after subculture. The catechin and procyanidin concentrations were one and two orders of magnitude higher, respectively.

DISCUSSION

All previous investigators report that plant OMT is found in the supernatant after centrifugation of cell homogenates at 9000 to 30000 *g* [5–15]. Generally this has been taken to support the idea that the OMT is a soluble enzyme, although Stafford has found microsomal forms as well [16]. It was shown here that Douglas-fir needle callus has a cell wall-associated, membrane-bound form of OMT. It was also found that most of the OMT from Douglas-fir seedlings is in a soluble form corresponding to that reported by others for other plant species. The differences in the intracellular location for seedling versus callus OMT raise interesting questions as to (a) origin or, alternatively, (b) whether the membrane-bound form in callus is due to a mutation or an epigenetic change in the cultured tissue.

The purification difficulties encountered probably stem from the very high levels of phenolics (especially procyanidins) found in this callus (up to 60% of its dry weight was procyanidin). It is likely that this tannin would bind the OMT during homogenization and in turn then bind onto column support material, thus immobilizing the OMT. That the immobilized OMT remained active may relate to its role *in vivo*.

The OMT was definitely shown to be lignin-specific; almost no activity toward the more prevalent flavonoids was found. The kinetic properties of this OMT are unusual in that the K_m for caffeic acid is high, even though this is a lignin-specific OMT. It was found that the caffeic acid concentration never begins to approach the K_m of 333 μM. The OMT also shows substrate inhibition at saturating substrate levels as predicted by Sharma and Brown [17] for the ordered Bi-Bi mechanism that they found. SAH was not a particularly potent inhibitor of the OMT. The K_i value was more than 50% greater than the K_m value for SAM.

It was concluded that there is no deficiency of OMT in callus, but the enzyme is associated with membrane and has a high K_m for caffeic acid. Coupled with the low endogenous substrate levels found in callus, it is not surprising that tannin accumulation predominates, since the lignin OMT catalysis rate would be minimal.

EXPERIMENTAL

Biological material. Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needle callus was initiated and grown under a 16-hr daylight cycle at 200–220 ft candles on a modified Murashige and Skoog medium with 5 mg/L NOAA and 0.1 mg/L BAP. Based upon an investigation of OMT activity as a function of time in culture, callus 10 days after subculture was used to maximize the recovery of OMT. Seedlings were grown under greenhouse conditions in peat and vermiculite. They were harvested 30 days after shoots appeared.

Enzyme extraction and purification. The enzyme was extracted at 4° with 2 vols of 0.1 M K-Pi buffer, pH 7.6, containing 10% Polyclar AT (based on the tissue fr. wt), and 5 mM each of EDTA, reduced glutathione and ascorbic acid. The tissue was homogenized with a Brinkman Polytron. The homogenate was filtered through four layers of cheesecloth, and the filtrate was used for the OMT homogenate assay.

The OMT was partially purified by adding 1% (w/v) digitonin to the homogenate and stirring for 2–4 hr at 4°. The resulting slurry was then centrifuged at 20000 *g* for 20 min and the supernatant was dialysed against 20 vols of buffer. The enzyme soln was then fractionated with solid $(NH_4)_2SO_4$. The protein precipitating between 20 and 35% saturation was collected by centrifugation and resuspended in a minimum of buffer. This was then dialysed against 20 vols of buffer and stored in 5 ml lots at −20°.

OMT assay. The general reaction mixture consisted of a 1 ml enzyme-buffer soln to which was added 100 μl caffeic acid soln and 5 μl [Me − ^{14}C] SAM soln to give final substrate concns of 1 mM caffeic acid and 40 μM SAM. The reaction mixtures were incubated for 1 hr at 30° and stopped by the addition of 200 μl 5 N HCl immediately following the addition of 1 μmol ferulic acid as a carrier. The labeled ferulic acid was extracted into Et_2O (3 × 5 ml) and was isolated by TLC on silica gel G with C_6H_6–HOAc–H_2O (5:5:1, organic layer) as the solvent system. The ferulic acid spot was scraped off and counted in a scintillation counter. Protein was determined by the Bradford Coomassie blue procedure [18].

Intracellular distribution of OMT. Four-month-old seedlings were harvested, washed and the needles were removed. The bark was scraped from the stem and treated separately from the xylem of the stem. Callus was taken 10 days after subculture. All three tissue types were hand-ground with a pestle in a cooled mortar in 2 vols 0.1 M K-Pi buffer, pH 7.6, containing 30 mM sucrose and 5 mM each of EDTA, reduced glutathione and ascorbic acid. The homogenate was centrifuged at 20000 *g* for 20 min and the supernatant was removed for assay. This supernatant was centrifuged at 100000 *g* for 2 hr, and the pellet was resuspended for assay as the microsomal fraction. The pellet from the 20000 *g*

centrifugation was resuspended in 5 ml buffer, thoroughly mixed, and centrifuged at 1000 *g* to leave the mitochondria in suspension. The resulting pellet was again resuspended in 5 ml buffer and then centrifuged at 200 *g* to leave the chloroplasts in suspension. The resulting pellet was resuspended in 5 ml buffer for assay as the cell wall fraction. Fractions comprising mitochondria and chloroplasts were sonicated for 15 sec prior to assay.

Isolation of cinnamic acids, catechins and procyanidin. The cinnamic acids and catechins were isolated by acidifying a buffer extract of the callus with HCl and then extracting with 3 × 10 ml Et_2O. After the Et_2O was evaporated under N_2 the extract was spotted on a TLC plate which was then developed in the above solvent system. The individual spots were removed and eluted with MeOH and the absorbances at the appropriate UV wavelengths were measured. All concentrations were normalized per g fr. wt.

The procyanidin was estimated by the procedure of Stafford and Cheng [19] using a standard curve generated in this study for this tissue.

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