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PURIFICATION AND PROPERTIES OF *O*-METHYLTRANSFERASE INVOLVED IN THE BIOSYNTHESIS OF GYMNOSPERM LIGNIN

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Key Word Index—*Pinus thunbergii*; Pinaceae; gymnosperm; *O*-methyltransferase; competitive inhibition; lignin biosynthesis; conifer lignin; guaiacyl units.

Abstract—An *O*-methyltransferase which catalyzes the formation of the methoxyl groups of guaiacyl lignin was extracted from Japanese black pine seedlings. The enzyme was purified 90-fold by ammonium sulfate precipitation, and by chromatography on DEAE-cellulose and Sephadex G100. The enzyme preparation mainly catalyzed the methylation of caffeic acid to give ferulic acid, although the methylation of 5-hydroxyferulic acid to sinapic acid, which was competitively inhibited by caffeic acid, was slightly catalyzed. The V_{max} for caffeic acid was 25 times higher than that for 5-hydroxyferulic acid, and the corresponding K_m values were $5{\cdot}11 \times 10^{-5}$ and $2{\cdot}77 \times 10^{-4}$ M, respectively. The provisional MW of the enzyme was estimated to be $6{\cdot}7 \times 10^4$ by gel filtration chromatography. Magnesium ions were not an absolute requirement but increased enzymic activity. These results explain the preponderance of guaiacyl units in conifer lignin.

INTRODUCTION

Several workers have reported the role and regulation of *O*-methyltransferase (OMT) in the biosynthesis of phenolic compounds in plants [1–9], especially on the important function of *meta*-OMT in lignin biosynthesis [10–16]. It has been shown that angiosperm OMT catalyzes the formation of both ferulic acid (FA) from caffeic acid (CA) and sinapic acid (SA) from 5-hydroxyferulic acid (5-HFA), but that gymnosperm OMT can hardly affect the latter methylation [15]. These findings well explain the fact that angiosperm lignins, generally, contain both guaiacyl and syringyl units, while gymnosperm lignins consists almost entirely of guaiacyl units [17].

Thus, OMT is believed to be a key enzyme which explains the phylogenic difference in the formation of guaiacyl and syringyl units of lignins. A previous investigation of purified bamboo OMT indicated that the formation of FA and SA is catalyzed by the same enzyme and that the formation of the former acid is inhibited by feedback control of 5-HFA which is postulated to be a precursor of syringyl lignins [16]. However, gymnosperm OMT has not been purified and examined in the same detail [13]. The present paper describes the properties of the purified pine (*Pinus thunbergii*) OMT which is responsible for the methylation step in the biosynthesis of gymnosperm lignin.

RESULTS

Purification of OMT from Pinus thunbergii

Pine (*Pinus thunbergii*) OMT was purified about 90-fold in a yield of 22% with respect to the starting crude homogenate (Table 1). FA- and SA-activities of the final enzyme preparation could not be resolved even by polyacrylamide gel electrophoresis (Table 2), although as expected the SA-activity was very much lower than FA-activity. It can be seen, however, that the ratio of SA- and FA-activity (SA/FA ratio) of the enzyme became smaller during purification (0·065

Table 1. Purification of *O*-methyltransferase from *Pinus thunbergii* seedlings

Purification procedure	Total protein (mg)	Specific activity (units/mg) FA	SA	Recovery (%) FA	Purification FA	SA/FA ratio
Homogenate	5330	0·18	0·012	100	1·0	0·065
0–60% $(NH_4)_2SO_4$	1079	0·62	0·041	70	3·5	0·066
DEAE-cellulose	321	1·82	0·093	61	10	0·051
Sephadex G100	23	14·6	0·609	36	82	0·042
DEAE-cellulose	13	15·9	0·588	22	89	0·037

to 0·037), suggesting the removal of some SA-OMT activity contained in crude enzyme preparation. In fact, a minor fraction, which gave a higher SA/FA ratio, was detected in the 0–60% ammonium sulfate precipitate (step 2) by polyacrylamide gel electrophoresis (Table 2) and by gel filtration chromatography on Sephadex G100. This minor fraction was more mobile in gel electrophoresis and eluted faster in gel filtration chromatography than the major one. However, the overall ratio in the enzyme preparation at step 2 was calculated to be 0·17 by the results shown in Table 2, a value higher than that expected. A similar discrepancy between the calculated and found ratios was also observed in the fractions separated by gel filtration chromatography but its cause remained obscure. In any event, the changes of the ratio during purification is at least partially explained by the removal of the minor fraction.

Metal requirement and other properties of pine OMT

It was previously reported that the activity of crude pine OMT (ammonium sulfate precipitate) was almost completely inhibited by 5 mM of EDTA, and that Mg^{2+} might stimulate the activity [13]. Ebel *et al.* reported that the activity of parsley OMT was completely inhibited by 1·0 mM of EDTA but activated by Mg^{2+}, but not by Ca^{2+} [4]. In contrast with these results, Table 3 shows that the activity of finally purified enzyme was not only inhibited but also activated by 0·5 mM EDTA or NaF in the absence of Mg^{2+}. It thus appears that Mg^{2+} is not an absolute requirement for the enzymic activity. However, Mg^{2+}, Ca^{2+} and Mn^{2+} are useful for keeping high enzymic activity. These results show that the properties of the pine enzyme were similar to those of purified catechol-*O*-methyltransferase from human liver which is stabilized by 0·2 mM $MgCl_2$ and EDTA [18].

Other experiments showed that addition of glycerol was effective against denaturation of OMT during freeze-thawing and the presence of 10 mM of *iso*-ascorbate, cystein and 2-mercaptoethanol was necessary for the full enzymic methylation. The provisional MW of the major fraction of the enzyme preparation at step 2 was estimated to be $6·7 \times 10^4$ by the gel filtration on Sephadex G100.

Kinetic studies of OMT

The K_m value of parsley OMT for CA was reported to be 1·6 mM [4], which is hundred times higher than that of the pine OMT. These differences are perhaps related to the fact that the parsley enzyme is involved in the methylation of flavonoid compounds whereas pine OMT is only responsible for the biosynthesis of guaiacyl lignin. The kinetic constants summarized in Table 4

Table 2. The SA/FA ratio of the major and minor fractions of OMT preparations after disc electrophoresis on polyacrylamide gel

OMT fraction	$(NH_4)_2SO_4$ Precipitate (step 2) Relative activity FA	SA	Ratio SA/FA	Final step ratio SA/FA
Major	1	0·05	0·05	0·03
Minor	0·30	0·18	0·6	*

* Minor fraction was not observed.

Table 3. Effect of metal ions on purified O-methyltransferase of *Pinus thunbergii*

Additions	Concn. (mM)	Methylation rate of CA (10^3 cpm/0·1 ml/hr)	Relative rate (%)
None*		7·8	80·1
NaF	0·5	9·8	99·8
EDTA	0·5	9·6	97·9
$MgCl_2$	1·0	9·8	100·0
$MgCl_2$	0·5	9·3	95·0
$CaCl_2$	0·5	9·5	96·9
$MnCl_2$	0·5	9·3	94·4
(Less NaN_3)†		9·9	100·1

The total volume of reaction mixture was 1·0 ml, and the methylation rate was evaluated by comparison of that of the complete reaction mixture as described in the text.

* None consists of the complete system without $MgCl_2$.

† Removal of NaN_3 from the complete system.

show that the affinity of the enzyme for CA is higher than 5-HFA in contrast with angiosperm OMT [16].

The Lineweaver–Burk plots for the methylation of 5-HFA to SA in the presence or absence of CA gave typical competitive inhibition pattern, indicating that the SA-formation was greatly inhibited by CA. The K_i value, which should be equal to the K_m value for 5-HFA, and other kinetic constants obtained by these plots were in good agreement with the values shown in Table 4. Further experiments indicated that SA-formation from 5-HFA ($2·5 \times 10^{-4}$ M) is completely inhibited by $2·3 \times 10^{-5}$ M of added CA.

DISCUSSION

The substrate specificity of gymnosperm OMT was found to be completely different from that of the angiosperm enzyme since the SA/FA ratio, which is a good indicator of the substrate specificity, was very much smaller; for example, the SA/FA ratio for a variety of crude preparations from gymnosperms (*Ginkgo biolba* 0·1, *Pinus strobus* 0·4, *P. teada* 0·3, *P. densiflora* 0·1 and *P. thunbergii* 0·1) averaged 0·2 while that from dicotyledons (*Populus nigra* 3·0, *Magnolia grandiflora* 3·0 and *Pisum sativum* 2·7) and monocotyledons (Gramineae: *Phyllostachys pubescens* 1·3, *Triticum aestivum* 1·0 and *Oryza sativa* 0·9) averaged 2·9 and 1·1, respectively. No evidence that inhibitors or activators were present was observed in *Pinus thunbergii* OMT during purification, and hence it is likely that the low SA/FA ratio is a property of the pine OMT itself and explains the presence of only low amounts of syringyl units in pine lignin.

The role of OMT has been discussed with respect to the regulation of phenolase, and phenylalanine and cinnamates were examined as the regulating factors of OMT [1,5], the effects of illumination on OMT activity has also been investigated [3, 6, 7]. On the other hand, it was suggested that for bamboo OMT feedback inhibition by 5-HFA might operate at the FA-formation stage in favor of the formation of syringyl lignin in angiosperms [16]. In contrast with these results, the kinetic constants of pine OMT, the SA-activity of which is competitively inhibited by CA, are in good agreement with the formation of guaiacyl lignin in conifers. These results clearly explain the phylogenic difference between gymnosperms and angiosperms in the distribution of guaiacyl and syringyl units of lignins. It was recently suggested that the enzymes responsible for the hydroxylation and reduction of *p*-hydroxycinnamic acids were also important in determining the phylogenic difference of lignins [15, 19]. Furthermore, it was anticipated that pine OMT should be inhibited by *S*-adenosyl-L-homocysteine, because this compound is known as an inhibitor of the OMT of animals [20] and of pea OMT [21].

Table 4. Kinetic constants of O-methyltransferase from *Pinus thunbergii*

Substrate	K_m (μM)	Relative value of $1/K_m$	V_{max} (cpm/ng/hr)	Relative value of V_{max}
Caffeic acid	51·1	5·4	66·1	24·8
5-Hydroxyferulic acid	277	1·0	2·7	1·0
S-adenosyl-L-methionine	40·6	6·8	69·9	26·2

It is necessary to ascertain whether or not the minor fraction of OMT in the crude enzyme preparation is a naturally occurring enzyme and is in any way concerned with the OMT activity in *Pinus strobus* and *teada* to give relatively high SA/FA ratios. However, possible conformational changes of binding site, known in a number of other enzymes [22], might also explain the elimination of SA-activity during purification. It is noteworthy that the rate of FA-formation catalyzed by *Populus* OMT becomes progressively slower during incubation while the SA-formation by the same enzyme increases lineally [21].

In anion exchange chromatography, the gymnosperm OMT was eluted by lower KCl concentrations than in the case of bamboo OMT, and the mobility of the pine OMT on polyacrylamide gel electrophoresis was smaller than that of the bamboo OMT. These results suggest that the pine OMT has a lower negative charge than the bamboo enzyme, which may be important in determining the specificity of lignin biosynthesis.

EXPERIMENTAL

Plant material. Pine (*Pinus thunbergii*) seedlings were grown at 25° on wet vermiculite in 12 hr light for 16 days. The SA/FA ratios of various plants were observed in the seedlings except that in *Ginkgo biloba*, *Populus nigra*, *Magnolia grandiflora* and *Phyllostachys pubescens* the shoots were used.

Purification procedures. All purification procedures were performed at 4°. Pine seedlings (545 g) were homogenized in the presence of polyclar AT (50 g) with equal wt of 0·1 M K Pi buffer (pH 7·5), containing 5 mM of each 2-mercaptoethanol, cysteine, NaN_3 and *iso*-ascorbate. The homogenate was filtered through cheese-cloth and centrifuged at 17000 g for 30 min. The supernatant (920 ml) was adjusted to 60% saturation with solid $(NH_4)_2SO_4$ in the presence of 5 mM EDTA, and centrifuged at 7000 *g* for 30 min. The ppt. was dissolved in 0·02 M phosphate buffer (pH 7·4, 25 ml) and was passed through Sephadex G25 column (42 × 3 cm). Desalted enzyme solution (108 ml) thus obtained was applied to a DEAE-cellulose column (3 × 7 cm) and eluted by 0·02 M K Pi buffer with step-wise increases in ionic strength using KCl. Proteins eluted by 0·1 and 0·15 M KCl (344 ml) were precipitated by the addition of solid $(NH_4)_2SO_4$ with 5 mM EDTA and CA (500 mg, anticipated to be a stabilizer of bamboo OMT). The ppt was dissolved in the buffer (3·5 ml) and the soln. then applied to a Sephadex G100 column (2·7 × 100 cm; upward-flow rate 8·2 ml/hr; void vol. 166 ml). The eluate (total vol. 42 ml) was finally applied to a DEAE-cellulose column (3 × 1·3 cm) with a linear gradient of 0–0·2 M of KCl-buffer soln.

Assay of OMT activities. The reaction mixture (total vol. 1 ml) for assay contained 0·1 ml of enzyme soln., 0·1 M of cysteine, 2-mercaptoethanol and *iso*-ascorbate, 0·01 M $MgCl_2$, 0·2 ml of CA or 5-HFA (0·5 μmol), 0·5 M K Pi buffer (pH 7·5). This reaction mixture without *S*-adenosyl-L-methionine-^{14}Me (SAM; 0·25 μmol, 0·025 μCi) was preincubated for 5 min, the SAM was then added and the whole incubated for 0·5–1·0 hr at 30°. Extraction and measurement of the radioactivity were carried out as described previously. 10^4 cpm was equivalent to 53 nmol of product formed. Protein content was determined by the method of Lowry *et al.* [23].

Disc electrophoresis. Disc electrophoresis was performed in a polyacrylamide gel according to Davis [24]. After electrophoresis the gel was cut lengthwise and then into 5 mm thick discs. Each disc was crushed in the above reaction mixtures, and FA- and SA-activities assayed as before. The FA- and SA-activities of the discs at the purification step 2 was assayed for 3 hr at 30°, using the same reaction mixture described above except the concentration of the substrates was changed as follows, SAM (0·25 μCi), and CA (5×10^{-4} M) for FA-activity; SAM (0·1 μCi), and 5-HFA ($2{\cdot}5 \times 10^{-3}$ M) for SA-activity. Furthermore, the enzyme at step 5 was incubated for 2 hr with SAM (0·1 μCi), and substrates (5×10^{-4} M). The SA/FA ratios were calculated by subtracting 300 cpm in blank assay (minus substrate), except the case of SA-activity of the disc at the step 2 was calculated by the following equation; SA-activity = (found act.−300 × 2·5)/2·5 × 1·9, where, 2·5 is the coefficient obtained from the specific activity of SAM, and 1·9 is the coefficient of the substrate concentration calculated from the Lineweaver-Burk plots, respectively.

Molecular weight of the OMT. The MW was estimated by determining the elution volume on a calibrated Sephadex G100 column (1·4 × 88 cm; flow rate 8·5 ml/hr; K Pi buffer 0·05 M, pH 7·5), using the enzyme preparation at step 2.

Kinetic studies of the OMT. Competitive inhibition patterns were obtained from the reaction rate, when the concentration of 5-HFA was changed from 8×10^{-4}–5×10^{-3} M in the presence or absence of 5×10^{-5} and 10^{-4} M of CA. The enzyme assay was performed for 30 min, minimizing influence of the enzyme denaturation and product inhibition during the assay. When CA and 5-HFA were incubated at the same time, the acids formed were separated by PPC developed in toluene–HOAc–H_2O (4:1:5, upper layer, descending method).

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