*Phytochemistry*, 1975, Vol. 14, pp. 663-666. Pergamon Press. Printed in England.

# PHENOLIC *O*-METHYLTRANSFERASE FROM *LENTINUS LEPIDEUS* (BASIDIOMYCETE)*

CHI-KIT WAT and G. H. NEIL TOWERS

Department of Botany, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

(*Received* 17 *September* 1974)

**Key Word Index**—*Lentinus lepideus*; Basidiomycetes; methyl *p*-methoxycinnamate; *p*-specific *O*-methyltransferase; carboxyl alkylation enzyme.

**Abstract**—The fungus, *Lentinus lepideus*, produces crystalline methyl *p*-methoxycinnamate in stationary cultures. *O*-methylation and methyl ester formation of hydroxycinnamic acids were examined with enzyme preparations of the fungus. Using *S*-adenosylmethionine-$^{14}CH_3$, it was found that only the methyl esters of the hydroxycinnamic acids are substrates for *O*-methylation and not the free acids. Benzoic acids and their methyl esters are not substrates. The activity of the enzyme is *p*-specific and its specific activity decreases with increasing number of hydroxyl groups in the substrate. The same enzyme preparations catalyze the formation of the methyl ester of cinnamic acid from the free acid.

## INTRODUCTION

Five aromatic metabolites have been isolated from *Lentinus lepideus*, namely methyl cinnamate, methyl *p*-coumarate, methyl *p*-methoxycinnamate, methyl isoferulate and methyl anisate [1–4]. Thus, *L. lepideus* appears to be an organism suitable for study of *para*-directing *O*-methyltransferase (OMT) activity and also for methyl ester formation.

*S*-Adenosylmethionine (SAM) is the methyl donor in a variety of transmethylation reactions [5, 6]. In plants, it provides the methyl group in the formation of the methyl esters of fatty acids [7], pectin [8], magnesium protoporphorin IX [9] and loganic acid [10] as well as the methyl group in the formation of the phenolic *O*-methyl ethers which are ubiquitous in plants. A *meta*-specific OMT has been reported from a number of plants [11, 12]. Shimada *et al.*, in their investigations on the role of OMT in the biosynthesis of angiosperm and gymnosperm lignins purified a "di-function OMT" from bamboo that not only converts caffeate to ferulate but also 5-hydroxyferulate to sinapate [18]. We have purified the same or a similar enzyme from 6-day-old barley roots by $(NH_4)_2SO_4$ fractionation, and treatment with DEAE-cellulose and Sephadex G200 [19]. The specific activity for the formation of sinapic acid is higher than that for ferulic acid with the bamboo enzyme but the specificity of the barley enzyme is the reverse. Ebel *et al.*, have also purified on OMT involved in flavone glycoside biosynthesis in cell suspension cultures of parsley [20]. *Para*-specific OMTs have also been found in plants. For example, an enzyme from peyote was found to transfer the methyl group of SAM to the *m*- and *p*-hydroxyl group of variously hydroxylated phenethylamines [21] and a predominantly *p*-specific OMT was identified in *Nerine bowdenii* [22]. The latter enzyme methylated norbelladine to *N*-isovanillyltyramine (78%) and *N*-vanillyltyramine (3·5%). Again a crude enzyme preparation from *Foeniculum vulgare* has been shown to catalyze the *O*-methylation of *p*-coumaric acid but the specificity of the OMT involved has not been determined [24]. Finkle has reported an enzyme from pampas grass which was completely *m*-specific for caffeic acid and *m*- and *p*-specific for protocatechuic acid [23]. The *meta*-directing activity was heat

* A preliminary report has been presented at the Phytochemical Society of North America 14th Annual Symposium and Meeting at Western Carolina University, August 1974.

labile, being almost completely lost on heating at 50° for 20 min, leaving the *para*-OMT as the active enzyme. Since the separation of these 2 activities has not been achieved in any of these cases the problem of whether there is one or more enzymes is still unresolved.

The biosynthesis of methyl *p*-methoxycinnamate and methyl *iso*ferulate by *L. lepideus* indicates that there could be a *p*-specific methylating system present. We report here the isolation and some of the properties of a *p*-specific OMT obtained from the fungus. The same preparations also contain activity for the alkylation of the carboxyl group of cinnamic acid.

Table 1. $R_f$ values of cinnamic acids and benzoic acids and their methyl esters on TLC with Eastman silica gel chromatograms with fluorescent indicator

| Compound | $R_f \times 100$ Benzene–acetone 9:1 | Bz–HOAc–isooctane 90:10:15 |
|---|---|---|
| Cinnamic acid | 4 | 49 |
| Methyl cinnamate | 55 | 56 |
| *p*-Coumaric acid | 2 | 15 |
| Methyl *p*-coumarate | 35 | 22 |
| *p*-Methoxy cinnamic acid | 3 | 47 |
| Methyl *p*-methoxycinnamate | 57 | 56 |
| Caffeic acid | 0 | 2 |
| Methyl caffeate | 14 | 6 |
| *Iso*ferulic acid | 0 | 27 |
| Methyl *iso*ferulate | 40 | 36 |
| Ferulic acid | 0 | 32 |
| Methyl ferulate | 43 | 41 |
| 3,4-Dimethoxycinnamic acid | 3 | 38 |
| Methyl 3,4-dimethoxy-cinnamate | 52 | 49 |
| 5-Hydroxy ferulic acid | 0 | 6 |
| Methyl 5-hydroxy ferulate | 13 | 12 |
| Sinapic acid | 0 | 26 |
| Methyl sinapate | 32 | 32 |
| 3,4,5-Trimethoxycinnamic acid | 3 | 41 |
| Methyl 3,4,5-trimethoxy cinnamate | 51 | 53 |
| Benzoic acid | 9 | 55 |
| Methyl benzoate | 59 | 60 |
| *p*-Hydroxybenzoic acid | 3 | 14 |
| Methyl *p*-hydroxybenzoate | 39 | 23 |
| *p*-Methoxybenzoic acid | 2 | 49 |
| Methyl *p*-methoxybenzoate | 59 | 57 |
| Protocatechuic acid | 0 | 2 |
| Methyl protocatechuate | 14 | 7 |
| *Iso*vanillic acid | 1 | 27 |
| Methyl *iso*vanillate | 44 | 37 |
| Vanillic acid | 1 | 36 |
| Methyl vanillate | 44 | 47 |
| 3,4-Dimethoxybenzoic acid | 2 | 46 |
| Methyl veratrate | 54 | 54 |
| Gallic acid | 0 | 0 |
| Syringic acid | 0 | 29 |

## RESULTS AND DISCUSSION

After 3–4 weeks of growth, crystalline methyl *p*-methoxycinnamate began to appear under the mycelial mat of the fungus [3, 4]. The pH of the medium at this time had dropped from 6·1 to 4·5. Twenty-four milligrams of the pure compound could be isolated from 1·5 g, dry wt. of mycelium. 2-D PC of an ether extract of the acidified medium (pH2) or the mycelial mat failed to reveal any free hydroxycinnamic acids.

Methyl *p*-methoxycinnamate, the product of the enzyme reaction with methyl *p*-coumarate, is quite volatile and this has to be taken into account in enzyme studies. If the ether extract of the reaction mixture was subjected to TLC with solvent system II (Table 1) and the area corresponding to the methyl *p*-methoxycinnamate was cut from the chromatogram and counted, the activity obtained was only 2/3 of the count calculated from the $Et_2O$ extract. Chromatography by 2-D TLC and autoradiography of the sheets showed that almost all the activity resided in the products expected. In one experiment with methyl *p*-coumarate as substrate, 40 mg of methyl *p*-methoxycinnamate was added to an ether extract of the product and the mixture recrystallized thrice from MeOH–$H_2O$ (1:2). The specific activities of the compound were 3175 dpm/mg, 3162 dpm/mg and 3187 dpm/mg. The results shown in Table 2 are the activities calculated from ether extracts after substracting the value of the control.

Methyl *p*-coumarate was used as substrate in determining the pH optimum (pH 7) of the OMT. The activity decreased rapidly as the pH was increased (50% at pH 7·7) and at a slower rate as the pH was lowered (50% at pH 5·75). $(NH_4)_2SO_4$ fractionation studies showed that most of the activity was recoverable within the range of 20–60% saturation. The enzyme is very unstable, all activity being lost in 24 hr when stored at 4°. In the presence of dithiothreitol (5 mM), only 25% of the activity was retained under the same conditions.

The divalent ion $Mg^{2+}$ has been shown to enhance the OMT from rat liver [22] and the *meta*-specific OMT from plants [25, 26] but no effect was observed on the enzyme from *Lentinus*.

Table 2. Substrate specificity of methylating enzymes from *Lentinus lepideus*

| Substrate | Specific activity* | Product detected |
|---|---|---|
| Cinnamic acid | 1·4 | Methyl cinnamate |
| *p*-Coumaric acid | 0 | |
| Methyl *p*-coumarate | 59·6 | Methyl *p*-methoxycinnamate |
| *p*-Methoxy cinnamic acid | 0 | |
| Caffeic acid | 0 | |
| Methyl caffeate | 14·6 | Methyl *iso*ferulate |
| Ferulic acid | 0 | |
| Methyl ferulate | 14·2 | Methyl 3,4-dimethoxycinnamate |
| *Iso*ferulic acid | 0 | |
| Methyl *iso*ferulate | 0 | |
| 3,4-Dimethoxy cinnamic acid | 0 | |
| 5-Hydroxyferulic acid | 0 | |
| Methyl 5-hydroxyferulate | 3·2 | Methyl 3,4-dimethoxy-5-hydroxycinnamate† |
| Sinapic acid | 0 | |
| Methyl sinapate | 3·3 | Methyl 3,4,5-trimethoxycinnamate |
| Benzoic acid | 0 | |
| *p*-Hydroxybenzoic acid | 0 | |
| Methyl *p*-hydroxybenzoate | | |
| *p*-Methoxy benzoic acid | 0 | |
| Protocatechuic acid | 0 | |
| Methyl protocatechuate | 0 | |
| Vanillic acid | 0 | |
| Methyl vanillate | 0 | |
| *Iso*vanillic acid | 0 | |
| Methyl *iso*vanillate | 0 | |
| 3,4-Dimethoxy benzoic acid | 0 | |
| Gallic acid | 0 | |
| Syringic acid | 0 | |

* Specific activity expressed as the number of nmol of product formed per mg protein per hr.

† No standard available, tentatively identified by $R_f$ value and radioactivity from autoradiography.

The SH-inhibitor, *p*-chloromercuriphenyl sulfonic acid (0·25 and 0·5 mM) inhibited the reaction completely. The same result was also found in the peyote enzyme. The *Nerine* enzyme was reported to be stimulated by $CN^-$ and thiourea. No such effect was observed with the fungal enzyme.

The substrate specificity of the methylating enzyme of *Lentinus* is shown in Table 2. Of all the free acids, only cinnamic acid showed some activity. Methyl *p*-coumarate had the highest activity and decreasing activity was observed with substrates having more hydroxyl groups. The *p*-specificity of this fungal enzyme was clearly demonstrated in its ability to methylate methyl ferulate and not methyl *iso*ferulate and by the formation of only one metabolic product, methyl *iso*ferulate, when methyl caffeate was used as substrate. None of the benzoic acids or their methyl esters were active substrates for these enzyme preparations.

This is unexpected since methyl anisate has been reported to be a metabolite of *Lentinus*. Also the *m*-specific OMT from pine, bamboo and poplar shows activity with protocatechuic aldehyde and the enzymes from pine and bamboo are active with protocatechuic acid [11]. Thus, it would seem that in *Lentinus* the methylating enzyme for benzoic acid derivatives must be a different one to that for cinnamic acids.

The presence of a transmethylating enzyme for the alkylation of carboxyl group with SAM was detected in the fungal enzyme preparation. Only cinnamic acid could act as substrate. The product proved to be methyl cinnamate by 2-D TLC and autoradiography. It would appear that the fungus methylates cinnamic acid once it is produced. There are two observations to support this speculation: the absence of any free hydroxycinnamic acids in the medium and the mycelial mat, and the inability of the OMT to methylate free hydroxycinnamic acids.

## EXPERIMENTAL

*Culture conditions. Lentinus lepideus* was cultured in 200 ml of the following medium: Bacto-malt extract 7 g, Bacto-yeast extract 0·5 g, Bacto-soytone 1 g and $H_2O$ 1 l. All cultures were kept at 25° with 8 hr of fluorescent light per day.

*Chemicals.* All free acids are commercially available, except 5-hydroxyferulic acid. It was synthesized from 5-iodovanillin [28] by the method described by Neish [29]. Methyl esters were prepared from the corresponding acids using anhydrous MeOH with conc $H_2SO_4$ as catalyst.

*TLC* and *PC.* Eastman Si gel chromatogram sheets with fluorescent indicator were used. The $R_f$ values of the various compounds and the solvent systems used are indicated in Table 1. PC was carried out as described by Moore and Towers [30].

*Isolation of methyl p-methoxycinnamate.* The mycelial mat was homogenized with MeOH and filtered. The MeOH was removed and the residue extracted with $C_6H_6$. After the removal of solvent, the residue was redissolved in a small amount of $C_6H_6$ and added to a silicic acid column (2 × 27 cm). The column was developed with $C_6H_6$ and 5 ml fractions collected. The compound appeared in tubes 23–28, mp 87–88° (reported 87–88° [31]). The UV and IR were identical to those of the synthetic compound.

*Extraction of enzyme.* The medium of 3–4-week-old culture was decanted and the mycelial mat washed 3× $H_2O$. The

mycelial mat was frozen in liquid $N_2$ and ground. Polyclar AT was added (5% of mycelial wet wt) and the mixture stired for 20 min in 0·1 M $PO_4^{3-}$ buffer, pH 7, containing 2 mM mercaptoethanol. The suspension was filtered through two layers of cheesecloth and the filtrate centrifuged at 7000 *g* for 15 min. The protein in the supernatant was precipitated between 20–60% $(NH_4)_2SO_4$ saturation. After dissolving the precipitated protein in min. amount of the above buffer, the solution was passed through a column of Sephadex G25. The eluate was used in the enzyme assay. Protein concentration was determined by the method of Lowry *et al.* [32] with BSA as standard.

*Enzyme assay.* The assay mixture consisted of 0·1 ml of substrate (0·1 μmol), 0·1 ml *S*-adenosylmethionine-[$^{14}CH_3$] (0·1 μCi in 0·0525 μmol) and 0·5 ml of the above enzyme. The reaction mixture was incubated at 30° for 1 hr and terminated by the addition of 0·5 ml 5% HCl. The reaction mixture was then extracted with 10 ml $Et_2O$. The ether extract (2·5 ml) was placed in a scintillation vial and the $Et_2O$ allowed to evaporate before adding, 10 ml of toluene–EtOH scintillator [33]. The amount of product formed was calculated from the activity obtained and the sp. act. of SAM.

*Identification of reaction products.* The products were identified by 2-D TLC with reference compounds and by autoradiography using the 7·5 ml of the ether extract remaining from the enzyme assay as described above.

*Acknowledgement*—We thank the National Research Council of Canada for financial support.

## REFERENCES

1. Turner, W. B. (1971) in *Fungal Metabolites*, p. 36. Academic Press, New York.
2. Shibata, S., Natori, S. and Udagawa S. (1964) in *List of Fungal Products*, p. 35. Thomas, Illinois.
3. Nord F. F. and Vitucci, J. C. (1947) *Arch. Biochem.* **14,** 243.
4. Shimazono, H., (1959) *Arch. Biochem. Biophys.* **83,** 206.
5. Shapiro, S. K. and Schlenk, F. (1965) *Transmethylation and Methionine Biosynthesis*, University of Chicago Press, Illinois.
6. Mudd, S. H. (1973) in *Metabolic Conjugation and Metabolic Hydrolysis* (Fishman, W. H., ed.), Vol. 3, pp. 297–350. Academic Press, New York.
7. Akamatsu, Y. and Law, J. H. (1970) *J. Biol. Chem.* **245,** 709.
8. Kauss, H. and Hassid, W. Z. (1967) *J. Biol. Chem.* **242,** 3449.
9. Ellsworth, R. K. and Dullaghan, J. P. (1972) *Biochim. Biophys. Acta* **268,** 327.
10. Madyastha, K. M., Guarnaccia, R., Baxter, C. and Coscia, C. J. (1973) *J. Biol. Chem.* **248,** 2497.
11. Shimada, M., Fushiki, H. and Higuchi, T. (1973) *Mokuzai Gakkaishi* **19,** 13.
12. Finkle, B. J. and Nelson, R. F. (1963) *Biochim. Biophys. Acta* **78,** 747.
13. Finkle, B. J. and Masri, M. S. (1964) *Biochim. Biophys. Acta* **85,** 167.
14. Hess, D. (1964) *Z. Naturforsch.* **19B,** 447.
15. Glass, A. D. M. and Bohm, B. A. (1972) *Phytochemistry* **11,** 2195.
16. Mansell, R. L. and Seder, J. A. (1971) *Phytochemistry* **10,** 2043.
17. Hess, D. (1965) *Z. Pflanzenphysiol.* **53,** 460.
18. Shimada, M., Kuroda, H. and Higuchi, T. (1973) *Phytochemistry* **12,** 2873.
19. Wat, C. K. and Towers, G. H. N., (unpublished results).
20. Ebel, J., Hahlbrock, K. and Grisebach, H. (1972) *Biochim. Biophys. Acta* **269,** 313.
21. Basmadjian, G. P. and Paul, A. G. (1971) *Lloydia* **34,** 91.
22. Mann, J. D., Fales, H. M. and Mudd, S. H. (1963) *J. Biol. Chem.* **238,** 3820.
23. Finkle, B. J. and Kelly, S. H. (1971) *Fed. Proc.* **30,** 1305Abs.
24. Kaneko, K. (1962) *Chem. Pharm. Bull.* **10,** 1085.
25. Shimada, M., Fushili, H. and Higuchi, T. (1972) *Phytochemistry* **11,** 2657.
26. Higuchi, T., Shimada, M. and Ohashi, H. (1967) *Agr. Biol. Chem.* **31,** 1459.
27. Power, D. M., Towers, G. H. N. and Neish, A. C. (1965) *Can. J. Biochem.* **43,** 1397.
28. Banerjee, S. K., Manolopoulo, M. and Pepper, J. M. (1962) *Can. J. Chem.* **40,** 2175.
29. Neish, A. C. (1959) *Can. J. Biochem. Physiol.* **37,** 1431.
30. Moore, K. and Towers, G. H. N. (1967) *Can. J. Biochem.* **45,** 1659.
31. Shimazono, H., Shubert, W. J. and Nord, F. F. (1958) *J. Am. Chem. Soc.* **80,** 1992.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
33. Wat, C. K. and Towers, G. H. N. (1971) *Phytochemistry* **10,** 103.