Identification of a phenolic 3-O-methyltransferase in the lignin-degrading fungus *Phanerochaete chrysosporium*

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A methyltransferase enzyme catalysing the 3-O-methylation of isovanillic acid (3-hydroxy-4-methoxybenzoic acid) by S-adenosylmethionine (SAM) was identified in *Phanerochaete chrysosporium* and purified. Gel filtration indicated an $M_r$ of 71 000 and SDS-PAGE showed that the enzyme was composed of two subunits of $M_r$ approximately 36 000. Substrate utilization studies demonstrated that the enzyme was highly specific, displaying an exclusive preference for the methylation of the 3-hydroxyl group of several substituted benzoic acids. 3-Hydroxybenzoic acids with a methoxyl or hydroxyl substituent in the 2 or 4 position were the best substrates with isovanillic and 3,4-dihydroxybenzoic acids showing the highest rates of methylation. The 3-O-methyltransferase enzyme was induced later in the growth cycle than the 4-O-methyltransferase previously isolated from this fungus, which is believed to have a role in the 4-O-methylation of lignin degradation products. However the function of this meta-specific enzyme, the first phenolic 3-O-methyltransferase isolated from a fungus, remains unclear. The combined activities of the 3- and 4-O-methyltransferase enzymes satisfactorily account for the pattern of SAM-dependent methylating activity shown by whole mycelia to phenolic substrates.

**Keywords:** O-methyltransferase, *Phanerochaete chrysosporium*, phenolic acids, S-adenosylmethionine, lignin degradation

**INTRODUCTION**

Information in the literature on the nature and function of O-methyltransferase enzymes in white rot fungi is comparatively limited. 4-O-Methylation of lignin degradation products such as vanillic and syringic acids has been reported in whole mycelia of *Phanerochaete chrysosporium* and other white rot fungi (Chen et al., 1982; Eriksson et al., 1984) and it has been suggested that this process may serve as a detoxification mechanism in such species. The degradation of chlorinated phenols and dioxins in *P. chrysosporium* apparently proceeds via methoxylated intermediates (Valli & Gold, 1991; Valli et al., 1992) and methylation in this case serves to regenerate a substrate capable of being attacked by lignin peroxidase. The role of methylation, if any, in the wider context of lignin degradation is, however, unclear.

Methylation reactions can be involved in the biosynthesis of secondary metabolites by wood rotting fungi. Thus an O-methyltransferase that catalyses the methylation of p-hydroxy groups of esters of hydroxycinnamic acids has been isolated from the fungus *Lentinus lepideus* (Wat & Towers, 1975). This S-adenosylmethionine (SAM)-dependent enzyme participates in the biosynthesis of the natural product methyl p-methoxycinnamate by the fungus. Enzymic methylation stages in which chloromethane (CH$_3$Cl) rather than SAM acts as methyl donor have been identified in the biosynthesis of veratryl alcohol [3,4-dimethoxybenzyl alcohol] by *P. chrysosporium* (Harper et al., 1990) and in the formation of methyl esters and anisoles by *Phellinus pomaceus* (Harper et al., 1989). Isotopic labelling experiments reported by Coulter et al. (1993a), in which the methylation of the synthetic substrate acetovanillone by whole mycelia of *P. chrysosporium* was examined, provided evidence for the presence of two biochemically distinct systems in the organism, one utilizing SAM as a methyl donor and the
other utilizing CH₃Cl as a methyl donor. All attempts to isolate such CH₃Cl-dependent enzyme systems involved in methyl transfer have to date proved fruitless.

However, Coulter et al. (1993b) succeeded in isolating and characterizing a SAM-dependent 2,4-disubstituted phenol O-methyltransferase from *P. chrysosporium*. The enzyme was strongly para-specific, methylating the 4-hydroxy groups in 3-methoxy- and 3,5-dimethoxy-substituted 4-hydroxybenzaldehydes, 4-hydroxybenzoic acids and 4-hydroxyacetophenones and appears to be responsible for the 4-O-methylation of lignin degradation products observed by earlier workers (Chen et al., 1982; Eriksson et al., 1984). Recent work by Harper et al. (1996), in which the relative rates of methylation of several phenolic substrates by CH₃Cl and [methyl-²H₃]-labelled SAM by whole mycelia of *P. chrysosporium* were measured, indicated that isovanillic acid (3-hydroxy-4-methoxybenzoic acid) was rapidly methylated by both CH₃Cl- and SAM-dependent systems. The existence of SAM-dependent isovanillic acid methylating activity in this fungus suggests the presence of a SAM-dependent meta-specific O-methyltransferase in *P. chrysosporium* in addition to the para-specific enzyme isolated by Coulter et al. (1993b).

In this paper we describe the purification and properties of this meta-specific enzyme from *P. chrysosporium* and compare its activity at different stages of the fungal growth cycle with that of the para-specific enzyme isolated by Coulter et al. (1993b). We also demonstrate that the combined activities of the meta- and para-specific enzymes isolated from the fungus satisfactorily account for the pattern of SAM-dependent methylating activity exhibited by whole mycelia towards hydroxylated aromatic compounds.

**METHODS**

**Organism and fungal culture.** *Phanerochaete chrysosporium* Burds INA-12 (CNCM 1-398) is a strain previously used by Harper et al. (1990, 1991) and Coulter et al. (1993b). The fungus was grown as described by Coulter et al. (1993a) without agitation at 37 °C in 2 litre conical flasks containing 200 ml medium. Cultures were harvested by filtration after 70 h of growth. Superficial moisture was removed by pressing mycelia gently between pieces of filter paper before freezing at −20 °C until required. A yield of approximately 0.4 g (wet wt) mycelia per litre of culture medium was obtained.

**Preparation of cell extracts.** Frozen mycelia (100 g) were suspended in 0.1 M potassium phosphate buffer (pH 6.5, 100 ml) containing 1 mM dithiothreitol and the cells were macerated at 4 °C with a tissue grinder composed of a 50 ml glass mortar tube with a polytetrafluoroethylene-headed pestle attached to a power drill rotated at maximum speed for 10 min. The resulting homogenate was centrifuged at 30000 × g for 30 min at 4 °C and the clear supernatant (150 ml) was decanted and subjected to the enzyme purification procedure.

**Enzyme and protein assays.** O-Methyltransferase activity was assayed by measuring the production of veratric acid (3,4-dimethoxybenzoic acid) on incubation of the preparation with isovanillic acid (3-hydroxy-4-methoxybenzoic acid) and SAM. The standard assay was conducted at 37 °C in triplicate 25 ml screw-cap vials sealed with poly(tetrafluoroethylene)-coated silicone discs. The vials contained, in a total volume of 2 ml, potassium phosphate buffer pH 7.5, 50 mM, isovanillic acid (0.5 mM), SAM (1 mM) and the enzyme preparation to be assayed (0.05 ml). After incubation for 3 h the reaction was terminated by quickly freezing the mixture at −20 °C and storing it at this temperature until extraction and determination of veratric acid. Protein was assayed by the Lowry method with bovine serum albumin as the standard.

**Extraction and determination of veratric acid.** After thawing, the contents of each vial were washed into ground-glass-stoppered test tubes with 15 ml water. The solution was adjusted to pH 2.0 with 2 M HCl and 3,4,5-trimethoxybenzoic acid (0.1 ml of a 50 μg ml⁻¹ solution in acetone) was added as an internal standard. The mixture was extracted with chloroform (3 × 15 ml) and the bulked extract was evaporated with saturated NaCl solution (5 ml). The extract was dried over anhydrous MgSO₄ and evaporated to 1 ml under reduced pressure. An aliquot of the extract (0.1 ml) was then placed in a 0.25 ml glass vial and the veratric acid was derivatized by addition of 0.1 ml N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA). The solution (1 μl) was injected splitless onto an HP 5890 Series II gas chromatograph equipped with a flame ionization detector and fitted with a fused silica wall-coated open tubular column (25 m × 0.32 mm) with a bonded phase of cross-linked methyl silicone (0.17 μm). Helium was utilized as carrier gas at a flow rate of 1.5 ml min⁻¹. The oven temperature was held initially at 100 °C for 1 min and then programmed to increase at 10 °C min⁻¹ to 300 °C. Veratric acid was quantitated by comparison of peak areas at the retention times of the MSTFA derivatives of veratric acid and the internal standard 3,4,5-trimethoxybenzoic acid with those of standards of known concentrations of these compounds. Results are expressed as means of triplicate samples.

**Extraction and determination of methylated products other than veratric acid.** Methoxylated benzoic, phenylacetic and cinnamic acids were determined by gas chromatography–mass spectrometry (GC-MS) as described by Coulter et al. (1993b) after extraction and derivatization with MSTFA as described above for veratric acid. 3,4,5-Trimethoxybenzoic acid was used as an internal standard in the quantification of the methoxylated products from vanillic and 3,4-dihydroxybenzoic acid, and 3-methoxybenzoic acid was used for products formed from all other acids apart from 3-hydroxybenzoic acid for which 4-methoxybenzoic acid was employed.

Vanillin, isovanillin and veratraldehyde were determined by GC-MS as described by Coulter et al. (1993b) using p-methoxycetophenone as internal standard. Vetrolal alcohol was assayed in a similar manner using 3,4,5-trimethoxybenzyl alcohol as internal standard. Methoxylated products from 1,3-dihydroxybenzene were determined by the same techniques using 1,3-dihydroxybenzene and acetovanillone as internal standards whilst products from 1,4-dihydroxybenzene were determined using 1,3-dihydroxybenzene and acetovanillone as internal standards.

**Protein purification.** A Pharmacia FPLC system was employed. Columns were connected to a UV-M monitor linked to an LCC-500 liquid chromatography controller which regulated the flow of buffers from two P-500 pumps. Fractions were collected with a FRAC-100 fraction collector.

**SDS-PAGE.** A Pharmacia Phastsystem gel electrophoresis unit was employed with commercially prepared gels of 12.5% polyacrylamide. Samples were prepared for application by the addition of 5% SDS and 10% mercaptoethanol followed by heating at 100 °C for 30 s and mixing with an equal volume of bromophenol blue (0.02%). Electrophoresis was performed
with Phastgel SDS buffer strips containing 0.2 M N-tris(hydroxymethyl)methylglycine, 0.2 M Tris and 0.55% SDS at pH 8.0 and gels were stained with Coomassie blue R. The following proteins were used in calibration (M, of subunit in parentheses): rabbit muscle myosin (205 000), Escherichia coli β-galactosidase (116 000), rabbit muscle phosphorylase b (97 000), bovine serum albumin (66 000), ovalbumin (45 000), rabbit glyceraldehyde-3-phosphate dehydrogenase (36 000), bovine erythrocyte carbonic anhydrase (29 000), bovine pancreatic trypsinogen (24 000), soybean trypsin inhibitor (20 100) and bovine milk α-lactalbumin (14 200).

Isoelectric focusing. Isoelectric focusing on polyacrylamide gel [Phastgel IEF (3–9) 0.35 mm thick] was conducted using a Pharmacia Phastsystem. Enzyme protein was dissolved in 25 mM phosphate buffer pH 7.5 and an aliquot (2 μl) containing approximately 100 μg protein ml⁻¹ was run alongside a calibrating mixture of proteins of known pI ranging from 3–10. Protein bands were located by staining gels with 0.02% Coomassie blue R in methanol/acetic acid/water (30:10:60, by vol.) containing 0.1% CuSO₄. Gels were washed and destained with methanol/acetic acid/water (30:10:60) and fixed in 20% trichloroacetic acid.

**Determination of M,** The M, of the intact enzyme was determined by gel filtration of the pure 3-O-methyltransferase on a Pharmacia Superdex 200 HR 10/30 column in 100 mM phosphate buffer (pH 6.5): containing 1 mM dithiothreitol. The column had previously been calibrated with the following reference proteins (M, in parentheses): β-amylose (200 000), aldolase (145 000), bovine serum albumin (67 000), peroxidase (40 000), carbonic anhydrase (29 000) and myoglobin (17 000). The M, values of enzyme subunits were determined by SDS-PAGE as described above.

**Chemicals.** Isovanillic acid, veratric acid and all substituted benzoic acids, benzaldehydes, benzyl alcohols and cinnamic acids were obtained from Aldrich, and MASTFA reagent was acquired from Pierce. S-Adenosylmethionine p-toluenesulphonate salt and S-adenosylhomocysteine (SAH) together with proteins used for calibration in M, determinations were purchased from Sigma.

**RESULTS AND DISCUSSION**

**Purification of 3-O-methyltransferase**

All stages of purification were conducted at approximately 4 °C. The cell-free extract prepared as described in Methods was concentrated to approximately 10 ml using an Amicon Centriprep 30 000 ultrafiltration unit. The concentrated enzyme preparation (2 ml) was applied to a Pharmacia FPLC Mono Q 10/10 anion-exchange column equilibrated with 100 mM MOPS buffer (pH 7.5) containing 1 mM dithiothreitol. The column was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM MOPS buffer (pH 7.5) containing 1 mM dithiothreitol. Fractions (1 ml) were collected and tested for 3-O-methyltransferase activity using the standard assay. Enzyme activity emerged as a single discrete peak confined to fractions eluting at NaCl concentrations between 0.3–0.5 and 0.325 mM (Fig. 1). 3-O-Methyltransferase activity was well resolved from the 4-O-methyltransferase activity previously characterized in this fungus by Coulter et al. (1993b). The latter enzyme which was assayed using acetoacetilone as substrate as previously described (Coulter et al., 1993b) eluted at NaCl concentrations between 0.334 and 0.372 mM at this pH. Those fractions with highest 3-O-methyltransferase activity were pooled and applied to a Pharmacia FPLC Sepharose 12 gel filtration column equilibrated with 100 mM phosphate buffer (pH 6.5) containing 1 mM dithiothreitol. The enzyme eluted from the column at an elution volume of 1.99 relative to the void volume of the column (20.6 ml) and was subjected to a final purification by passage through a Pharmacia Superdex 200 HR 10/30 gel filtration column equilibrated with 100 mM phosphate buffer (pH 6.5) containing 1 mM dithiothreitol. The pure enzyme eluted at an elution volume of 2.47. Isoelectric focusing of the purified enzyme on polyacrylamide gel yielded a single band with pI 4.6 confirming the homogeneity of the preparation. Purification by this procedure was 256-fold and the overall yield was 3.2%. The results of a typical enzyme purification procedure are summarized in Table 1.

**Enzyme properties**

Preparations from step 4 or 5 of the purification procedure (Table 1) had a half-life of approximately 30 h in 100 mM phosphate buffer (pH 7.5) containing 1 mM dithiothreitol at 4 °C. Freezing solutions of the enzyme from these stages, either alone or with the addition of 10% glycerol, resulted in complete loss of activity. However, preparations from step 3 had a half-life of 12 d at 4 °C and could be frozen at −15 °C for several weeks without significant loss of activity. In view of the lability of step 4 and 5 preparations and the low yield of the purification procedure, enzyme characterization was conducted mainly with enzyme preparations from step 3.

Under standard assay conditions (0.5 mM isovanillic acid, 0.1 mM SAM) the rate of reaction was linear with respect to time over at least 6 h at 37 °C. The velocity under these conditions was directly proportional to enzyme concentration at protein concentrations between 80 and 400 μg ml⁻¹.

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**Fig. 1.** Elution profile of concentrated cell-free extracts from *P. chrysosporium* on a Mono Q 10/10 anion exchange column. Relative absorbance at 254 nm; --- phenolic O-methyltransferase activity, ——.

1977
**Table 1. Purification of isovanillic acid 3-O-methyltransferase from P. chrysosporium**

The results shown are of a typical enzyme purification as described in the text.

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage of purification</th>
<th>Vol (ml)</th>
<th>Total enzyme activity (mU)*</th>
<th>Yield (%)</th>
<th>Protein (mg ml⁻¹)</th>
<th>Sp. activity [mU (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell extract</td>
<td>13.5</td>
<td>343</td>
<td>100</td>
<td>4.6</td>
<td>0.55</td>
</tr>
<tr>
<td>2</td>
<td>Concentrate after ultrafiltration</td>
<td>9</td>
<td>253</td>
<td>74</td>
<td>43.0</td>
<td>0.65</td>
</tr>
<tr>
<td>3</td>
<td>Anion-exchange chromatography</td>
<td>2.9</td>
<td>121</td>
<td>35</td>
<td>3.4</td>
<td>12.3</td>
</tr>
<tr>
<td>4</td>
<td>Gel filtration (Sepharose 12)</td>
<td>0.9</td>
<td>33</td>
<td>9.6</td>
<td>0.31</td>
<td>118</td>
</tr>
<tr>
<td>5</td>
<td>Gel filtration (Superdex 200)</td>
<td>0.6</td>
<td>11</td>
<td>3.2</td>
<td>0.13</td>
<td>141</td>
</tr>
</tbody>
</table>

*a* One milliunit (mU) of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 nmol veratric acid min⁻¹ at 37 °C under the conditions of the standard assay in 50 mM phosphate buffer (pH 7.5) with 0.5 mM isovanillic acid and 1 mM SAM.

The effect of pH on 3-O-methyltransferase activity was measured under standard conditions in 50 mM 2,2-dimethylsuccinate, phosphate, Tris and sodium carbonate buffers spanning a pH range of 4-10. A relatively broad pH optimum between pH 6.5 and 8.5 was observed with maximum activity being displayed at pH 7.5. The effect of temperature on 3-O-methyltransferase activity was determined at temperatures between 15 and 55 °C under standard assay conditions. Maximal activity under such conditions was exhibited at 37 °C. Significant denaturation of the enzyme occurred at 50 °C and above. The activation energy of the enzyme reaction under the conditions of the standard assay in 50 mM phosphate buffer (pH 7.5) was 69.6 kJ mol⁻¹.

The rate of methylation was measured at concentrations of isovanillic acid of 0·066-1 mM whilst the concentration of SAM was held at a number of fixed values between 0·2 and 1 mM and the usual double reciprocal plot for two substrate reactions was obtained (Dixon & Webb, 1979). Kᵢ values were determined by plotting intercepts of the primary plots against the reciprocal of substrate concentration for each substrate. The Kᵢ values of the enzymes were 76 µM for isovanillic acid and 136 µM for SAM [111 µM if a correction is made for the 18% unnatural S-(+)-isomer present in commercial SAM]. With 3,4-dihydroxybenzoic acid, the only other substrate readily utilized by this enzyme (see Table 2), Kᵢ values of 108 µM for 3,4-dihydroxybenzoic acid and 130 µM for SAM [107 µM if corrected for the S-(+)-isomer present] were recorded.

On gel filtration on Superdex 200 HR 10/30, 3-O-methyltransferase activity emerged as a single peak at a relative elution volume corresponding to an Mₑ of 71000. SDS-PAGE of the purified enzyme indicated an Mₑ of 36000 suggesting the presence of two subunits in the native enzyme.

Most transmethylation reactions involving SAM are strongly inhibited by low concentrations of the demethylated product, SAH (Poulton, 1981). The effect of SAH concentrations of 0·0-0·135 mM on the velocity of 3-O-methyltransferase activity was determined at SAM concentrations of 0·1, 0·3 and 0·5 mM whilst holding the concentration of isovanillic acid at 0·5 mM. SAH was a powerful competitive inhibitor of the enzyme reaction: the Kᵢ derived from a Dixon plot (Dixon, 1953) was 24 µM revealing the enzyme to have a very much higher affinity for SAH than for SAM.

The effect of metal ions, thiol blocking agents and other possible inhibitors on enzyme activity in the standard assay was investigated after dialysis of the enzyme against 50 mM phosphate buffer (pH 7.5). Although the enzyme was not affected by either 0·01 mM N-ethylmaleimide or 0·5 mM iodoacetamide, the thiol blocking agent phenylmercuriacetate reduced activity by 52%. Activity was completely abolished by heavy metal ions such as 0·05 mM Hg²⁺ and Ag⁺. These findings suggest the presence of a thiol group at the active site. Certain O-methyltransferases of plant and animal origin show maximal activity in the presence of a bivalent ion such as Mg²⁺ (Poulton, 1981). However, 1 mM Mg²⁺, Fe²⁺ or Mn²⁺ had no significant effect on the enzyme from P. chrysosporium and the failure of the chelating agent EDTA (1 mM) to affect activity indicated the absence of an obligatory metal ion requirement.

**Substrate specificity**

The rates of methylation of a number of different phenolic substrates by the 3-O-methyltransferase are compared in Table 2. The enzyme was highly specific displaying an exclusive preference for the methylation of the 3-hydroxyl group of several substituted benzoic acids. m-Hydroxybenzoic acids with a methoxyl or hydroxyl substituent in the o- or p-position appeared to be the best substrates, with isovanillic and 3,4-dihydroxybenzoic acids exhibiting high rates of 3-O-methylation. 3,4,5-Trihydroxybenzoic acid was also attacked to some extent with 3,5-dimethoxy-4-hydroxybenzoic acid being the sole product formed. The failure to detect the monomethylated product, 3-methoxy-4,5-dihydroxybenzoic acid is significant, implying that it must be an excellent substrate for the enzyme as its rate of methylation obviously far exceeds...
Table 2. Substrate specificity of 3-O-methyltransferase

No methylation of the following compounds was observed: catechol, resorcinol, hydroquinone, guaiacol, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 3-hydroxy-4-methoxycinnamic acid (isofelutic acid) and 3-methoxy-4-hydroxycinnamic acid (ferulic acid).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate of methylation*</th>
<th>Substrate</th>
<th>Rate of methylation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxy-4-methoxybenzoic acid (isovanillic)</td>
<td>100</td>
<td>3,4-Dihydroxybenzoic acid</td>
<td>2‡</td>
</tr>
<tr>
<td>3-Methoxy-4-hydroxybenzoic acid (vanilllic)</td>
<td>&lt;1</td>
<td>2,3-Dihydroxybenzoic acid</td>
<td>37§</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td>77†</td>
<td>3,4,5-Trihydroxybenzoic acid (gallic acid)</td>
<td>19‖</td>
</tr>
<tr>
<td>3,5-Dimethoxy-4-hydroxybenzoic acid (syringic)</td>
<td>&lt;1</td>
<td>3-Hydroxy-4-methoxybenzaldehyde</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2-Hydroxybenzoic acid</td>
<td>14</td>
<td>3,4-Dihydroxybenzaldehyde (isovanillin)</td>
<td>1</td>
</tr>
<tr>
<td>3-Hydroxybenzoic acid</td>
<td>&lt;1</td>
<td>3-Hydroxy-4-methoxybenzyl alcohol</td>
<td>1</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>&lt;1</td>
<td>(isovanillyl alcohol)</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxy-4-methylbenzoic acid</td>
<td>14</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Rate of methylation on a molar basis relative to that with isovanillic acid as substrate in 50 mM phosphate buffer (pH 7.5) with 1 mM SAM and 1 mM phenolic substrate at 37 °C. Absolute rate of methylation of isovanillic acid was 13 nmol min⁻¹ (mg protein)⁻¹.
† Product composed solely of vanilllic acid.
‡ Product composed of 65% 3-methoxy-5-hydroxybenzoic acid and 35% 3,5-dimethoxybenzoic acid.
§ Product composed solely of 2-hydroxy-3-methoxybenzoic acid.
‖ Product composed solely of 3,5-dimethoxy-4-hydroxybenzoic acid.

Comparison of 3- and 4-O-methyltransferase activities at different stages of growth

Mycelia were harvested after periods of growth ranging from 48 to 98 h and cell-free extracts were prepared from each batch of mycelia. Each of the resulting extracts was separately concentrated by ultrafiltration and subjected to anion-exchange chromatography as described above. Fractions eluting at NaCl concentrations of 0.25-0.40 mM were assayed both for 3-O-methyltransferase activity using the standard assay with isovanillic acid as substrate and for 4-O-methyltransferase activity using vanillic acid as substrate as described by Coulter et al. (1993b). The activity of each enzyme, expressed as mU (g fresh wt mycelia)⁻¹, at various stages of the growth cycle is shown in Fig. 2.

Fig. 2. SAM-dependent 3- and 4-O-methyltransferase activities in P. chrysosporium mycelia at different stages of growth. Cell-free extracts were prepared from mycelia, concentrated and subjected to anion exchange chromatography. Appropriate fractions were assayed for 3-O-methyltransferase activity (○) as described in Methods and for 4-O-methyltransferase activity (■) as described by Coulter et al. (1993b) using 3-methoxy-4-hydroxycetophenone as substrate. Where the SD exceeds the size of the symbol, error bars indicate ±SD.
Table 3. SAM-dependent O-methylating activity in whole mycelia of 72 h cultures of *P. chrysosporium* compared to that of individual SAM-dependent enzymes isolated from the organism

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SAM-dependent O-methylating activity</th>
<th>Whole mycelia*</th>
<th>Purified enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-O-Methyltransferase†</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Isovanillic acid</td>
<td>43</td>
<td>1</td>
<td>44‡</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>88</td>
<td>91</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>4</td>
<td>8</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>3-Hydroxybenzoic acid</td>
<td>6</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Acetovanillone</td>
<td>90</td>
<td>102</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Isovanillyl alcohol</td>
<td>&lt; 1</td>
<td>&lt; 0.8</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>4-Hydroxybenzyl alcohol</td>
<td>&lt; 1</td>
<td>&lt; 0.2</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>4-Hydroxybenzaldehyde</td>
<td>7</td>
<td>2</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

*Relative activities in whole mycelia at 72 h given in Harper et al. (1996).
†Relative 4-O-methyltransferase activities given in Coulter et al. (1993b). ND, Not determined.
‡Activity with isovanillic acid as substrate was assigned a value of 44 based on the relative activities of 4-O-methyltransferase and 3-O-methyltransferase at 72 h given in Fig. 2.

O-Methyltransferase activity rose rapidly at an early stage in the growth cycle between 50 and 65 h, attaining a maximum after about 70 h. By contrast, only a relatively small increase in 3-O-methyltransferase activity occurred prior to 65 h but after that time activity increased substantially to reach a peak at about 75 h. Thus, the two methyltransferases seem to be induced sequentially in the growth cycle with the 4-O-methyltransferase appearing during the early exponential phase whilst 3-O-methyltransferase activity develops during the mid-exponential phase.

Substrate specificity of SAM-dependent O-methylating activity in whole mycelia compared with that of individual enzymes isolated from the organism

The relative activities on various phenolic substrates of the SAM-dependent O-methylation system in whole mycelia after 72 h as given by Harper et al. (1996) are shown in the first column of Table 3. The second and third columns show the substrate specificities of, respectively, the 4-O-methyltransferase isolated by Coulter et al. (1993b) and the 3-O-methyltransferase described in this paper. The activity of the latter enzyme has been normalized with respect to 4-O-methyltransferase activity to reflect the relative amounts of the two enzymes present in mycelia after 72 h growth as shown in Fig. 2. It is clear that the pattern of SAM-independent methylating activity on the different substrates in whole mycelia closely corresponds to the pattern produced by the combined and normalized 3- and 4-O-methyltransferase activities of the purified enzymes isolated from the mycelia. Bearing in mind that activities measured in whole mycelia are subject to some uncertainty due to variables which are more rigorously controlled in isolated enzyme assay, e.g. SAH concentration, the degree of consistency observed between the results is surprisingly good. This implies that all SAM-dependent methylating activity observed with these substrates in whole mycelia is probably accounted for by the two methyltransferase enzymes that have been isolated from *P. chrysosporium.*

Concluding remarks

Unlike the strongly para-specific O-methyltransferase isolated from *P. chrysosporium* by Coulter et al. (1993b), the enzyme described in this paper displays an absolute specificity for meta-hydroxyl groups. Moreover, whilst the 4-O-methyltransferase readily attacked hydroxybenzoic acids, hydroxybenzaldehydes and hydroxyacetophenones and even showed some activity against hydroxybenzyl alcohols, the 3-O-methyltransferase exhibited a very restricted substrate range with *m*-hydroxybenzoic acids being the only compounds methylated. In this respect the latter enzyme would appear to bear similarities to some of the O-methyltransferases involved in lignin biosynthesis, which attack the *m*-hydroxyl group of phenylpropanoid substrates such as caffeic, 5-hydroxyferulic and 3,4,5-trihydroxycinnamic acids (Poulton, 1981). However, the 3-O-methyltransferase from *P. chrysosporium* is not capable of utilizing substituted cinnamic acids as substrates.

In contrast to the 4-O-methyltransferase produced by *P. chrysosporium* for which, on the basis of its broad substrate specificity, a role has been suggested in catabolism of the products of lignin degradation (Coulter,
1993b), it is difficult to delineate a function for the 3-O-methyltransferase. The high degree of specificity shown by the enzyme towards its substrates tends to rule out a role similar to that proposed for the 4-O-methyltransferase. The induction of the enzyme at a later stage of the growth cycle than that of the 4-O-methyltransferase may indicate a role in secondary metabolism. Jensen et al. (1994) have demonstrated that biosynthesis of veratryl alcohol in *P. chrysosporum* proceeds from phenylalanine via benzoate or benzaldehyde. It is possible to envisage either of the two pathways. Although superficially the participation of the 3-O-methyltransferase, i.e. isovanillic acid or 3,4-dihydroxybenzoic acid as intermediates in such a pathway. Although superficially the participation of the 3-O-methyltransferase in veratryl alcohol biosynthesis is an attractive hypothesis it would seem highly unlikely in the light of the findings of Harper et al. (1990, 1996) that CH$_2$Cl acts as the methyl donor of the methoxyl carbons of both the 3 and 4 positions of veratryl alcohol formed *de novo* by *P. chrysosporum* mycelia and that SAM does not serve as a significant methyl source for either of these carbon atoms *in vitro.* Also militating against such a role for the enzyme is the observation that veratryl alcohol is not present in the culture medium under these growth conditions until after 90 h incubation (Harper et al., 1991), 20 h after the production of the enzyme has peaked. So the function of this enzyme, the first phenolic 3-O-methyltransferase to be isolated from a fungus, remains to be established.

REFERENCES


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