APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1993, p. 438–441
0099-2240/93/020438-04$02.00/0

Vol. 59, No. 2

Xylosylation of Phenolic Hydroxyl Groups of the Monomeric Lignin Model Compounds 4-Methylguaiacol and Vanillyl Alcohol by *Coriolus versicolor*

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Received 1 October 1992/Accepted 2 December 1992

When 4-methylguaiacol (MeG), a phenolic lignin model compound, was added to a culture that was inoculated with *Coriolus versicolor*, it was bioconverted into 2-methoxy-4-methylphenyl β-D-xyloside (MeG-Xyl). The phenolic hydroxyl group of vanillyl alcohol was much more extensively xylosylated than the alcoholic hydroxyl group. When a mixture of MeG and commercial UDP-xylose was incubated with cell extracts of mycelia, transformation of UDP-xylose into MeG-Xyl was observed. This result suggested that UDP-xylosyltransferase was involved in the xylosylation of phenolic hydroxyl groups of lignin model compounds.

Lignin, a complex and heterogeneous aromatic biopolymer and one of the major components of wood, can be degraded by some microorganisms, of which the basidiomycetes *Phanerochaete chrysosporium* and *Coriolus versicolor* are currently well characterized (10). Much effort has been expended towards understanding these biochemical conversions at the molecular level. The influence of carbohydrates on lignin degradation has been investigated by several researchers. Some articles have shown a possibility for interaction between the degradation of lignin and that of carbohydrates (1, 5, 18, 27, 30).

A number of papers have reported on the bioformation of a wide variety of glucosides with aglycones, such as pyridoxine in bacteria (20), panthothenic acid in yeasts (7), riboflavin in both yeasts (29) and molds (26), and esculetin in molds (19). These glucosides are considered to have been formed by the transglucosylating activities of glucosidases occurring in these microorganisms. We have reported the formation of glycosides of lignin model compounds in cultures of wood-rotting fungi growing on cellobiose (11, 13) and on holocellulose and xylan (14).

We have also found that lignin model compounds, including monomeric models (veratryl alcohol, veratraldehyde, and veratric acid) and a dimer model (4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether), were bioconverted to their xylosides, veratryl β-D-xyloside (VE-Xyl), and 3-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)-propyl β-D-xyloside, respectively, in glucose media that were inoculated with *C. versicolor* (16). Mechanistic investigation suggested that UDP-xylose and UDP-xylosyltransferase were involved in this xylosylation reaction (22). Recently, we showed that the xyloside formation activity of *C. versicolor* was activated by the exogenous addition of phenolic compounds, in which guaiacol had the best stimulating effect of all tested compounds (17). In this report, we demonstrate that the phenolic hydroxyl group of lignin model compounds is xylosylated in cultures of *C. versicolor* and that the phenolic hydroxyl group is much more extensively xylosylated than the alcoholic hydroxyl group.

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MATERIALS AND METHODS

Organism. *C. versicolor* (L.:Fr.) Quél. (IFO-6482) was obtained from the Institute for Fermentation, Osaka, Japan.

Culture conditions. The basal liquid medium used was described previously (13); it contained 2% glucose as the carbon source and 1 mM veratryl alcohol. To prepare holocellulose medium, 2.5 g of holocellulose prepared by sodium chlorite treatment of birch wood meal was added to 10 ml of liquid medium without glucose. Stationary cultures in 100-ml Erlenmeyer flasks containing 10 ml of liquid medium or holocellulose medium were inoculated with 5-mm-diameter plugs of *C. versicolor* grown on potato dextrose agar culture; the inoculated stationary cultures were grown at 30°C.

Isolation and identification of lignin model xylosides. After 7 days of incubation, 4-methylguaiacol (MeG) in 70% aqueous ethanol (200 μl) was added to the culture to give a final concentration of 1 mM. Thirty flasks were used under the same conditions. After an additional 24 h of incubation, 30 cultures were combined and filtered. The filtrate was lyophilized and dissolved into ethanol. From the ethanol extracts, a bioconversion product was isolated by preparative high-performance liquid chromatography (HPLC), which was conducted with a Waters 208D chromatograph by using a μBondasphere C_{18} column (19.0 by 150 mm) with a mobile phase consisting of acetonitrile-water (20:80, vol/vol) at a flow rate of 7 ml/min, eluting a bioconversion product from the column from 22 to 24 min. The absorbance detector was a Waters Lambda-Max model 481 set at 280 nm.

Determinations of amounts of substrates and metabolites. The amounts of substrates and metabolites in culture filtrates were determined by HPLC with a μBondasphere C_{18} column (3.9 by 150 mm) at a flow rate of 0.8 ml/min. The mobile phase was a mixture of acetonitrile and water containing small amounts of trifluoroacetic acid (pH 3.0). The ratios of CH_3CN to water containing trifluoroacetic acid were 17/83 for MeG (retention time, 33.4 min) and 2-methoxy-4-methylphenyl β-D-xyloside (MeG-Xyl) (retention time, 15.8 min) and 7/93 for vanillyl alcohol (VA) (retention time, 10.4 min), vanillyl β-D-xyloside (VA-Xyl-Al) (retention time, 21.4 min) and 2-methoxy-4-hydroxymethylphenyl β-D-xyloside (VA-Xyl-Ph) (retention time, 8.8 min).

Incubation under a nitrogen atmosphere. After *C. versicolor* was incubated under air for 7 days, guaiacol (1 mM)

was added to the culture to stimulate xyloside formation (17). After a 24-h incubation, MeG (1 mM) was added, and the culture was purged thoroughly with 100% nitrogen. After an additional 24-h incubation, the amounts of MeG and MeG-Xyl were determined by HPLC.

Incubation in the presence of laccase inhibitors. After a 7-day incubation, L-cysteine (50 mM) or L-ascorbic acid (50 mM) was added to the cultures along with MeG (1 mM). After a 24-h incubation, the amounts of MeG and MeG-Xyl were determined by HPLC.

Bioconversion of VA. MeG (1 mM) was added to the culture on day 7, and VA (1 mM) and ascorbic acid (50 mM) were added on day 8. After further incubation for 24 h, the culture was filtered, and the filtrate was analyzed by HPLC.

Treatment of MeG and MeG-Xyl with *C. versicolor*. *C. versicolor* was incubated with MeG (1 mM) or MeG-Xyl (1 mM) in glucose medium. At various intervals after inoculation (indicated in Fig. 2), the cultures were filtered, and the recovery of the substrates was determined by HPLC.

MeG-Xyl. MeG-Xyl was prepared from 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide and MeG by the method of Enoki et al. (2). Colorless crystals, mp 118 to 120°C; field desorption-mass spectrometry (FD-MS) *m/z*, 270 (M^+, 100%), 138 (3.7%), 71 (3.9%); ^{13}C-nuclear magnetic resonance (NMR) (in CD_3COCD_3) δ ppm, 150.6 (aglycone C-2), 145.1 (C-1), 133.5 (C-4), 121.9 (C-5), 118.3 (C-6), 103.1 (xylose C-1′), 76.5 (C-3′), 73.8 (C-2′), 70.6 (C-4′), 65.9 (C-5′), 56.3 (OCH_3), 21.0 (CH_3).

VA-Xyl-Al. VA-Xyl-Al was synthesized enzymatically. A reaction mixture containing 300 mg of VA, 7 g of xylan, 880 mg of commercial Meicelase P-1 (Meiji Seika Kaisha, Ltd.), and 70 ml of 0.1 M acetate buffer, pH 4.5, was incubated with shaking for 2 days at 40°C. After sugars were removed, the reaction mixture was fractionated on a silica gel column packed with Wakogel C-200 by using chloroform-methanol (4:1) as an eluting solvent. Colorless crystals, mp 158 to 160°C; ^{13}C-NMR (in CD_3OD) δ ppm, 149.8 (aglycone C-3), 148.2 (C-4), 131.1 (C-1), 123.1 (C-6), 116.6 (C-5), 114.0 (C-2), 104.5 (xylose C-1′), 78.6 (C-3′), 75.7 (C-2′), 72.7 (C-7), 72.1 (C-4′), 67.8 (C-5′), 57.2 (OCH_3).

VA-Xyl-Ph. VA-Xyl-Ph was prepared from vanillin and 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide, followed by reduction with $NaBH_4$ and deacetylation with a methanolic sodium methoxide solution. Colorless crystals, mp 142 to 144°C; ^{13}C-NMR (in CD_3OD) δ ppm, 151.9 (aglycone C-2), 147.6 (C-1), 138.8 (C-4), 121.3 (C-5), 119.1 (C-6), 113.4 (C-3), 104.3 (xylose C-1′), 78.2 (C-3′), 75.4 (C-2′), 71.8 (C-4′), 67.6 (C-5′), 65.7 (C-7), 57.4 (OCH_3).

Intracellular enzyme preparation. After *C. versicolor* was incubated in glucose medium for 7 days, guaiacol (1 mM) was added to the culture. After further incubation for 24 h, the liquid culture was filtered, and the mycelium was washed with water. Ten flasks were used under the same conditions. Collected mycelia were homogenized with a Waring blender in 5 ml of 0.1 M phosphate buffer (pH 7.0), and cell extracts of the mycelia were obtained by centrifugation.

Enzyme reactions. A reaction mixture containing 500 μl of cell extract, 1 mM of MeG, 50 mM of ascorbic acid, and 10 mM glucose or UDP-xylose was incubated at 37°C. Transformation of MeG to MeG-Xyl was determined by HPLC.

RESULTS

Bioconversion of MeG into MeG-Xyl. MeG was incubated with *C. versicolor* grown in a glucose medium. From the culture filtrate, a metabolite was obtained as a colorless

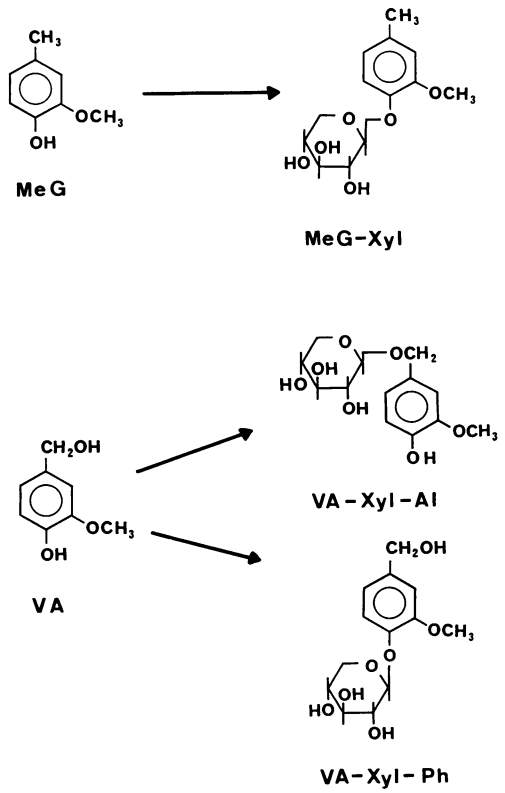

FIG. 1. Bioconversion of MeG and VA by *C. versicolor*.

powder. The FD-MS gave the M^+ at an *m/z* of 270, confirming the molecular weight. The ^{13}C-NMR spectrum indicated that the product was MeG-Xyl. The C-1 resonance for the xylose residue occurred at 103.1 ppm, which, by comparison with the chemical shifts of C-1 signals of methyl α-D-xylopyranoside and methyl β-D-xylopyranoside (4), suggested that the xylose was a β anomer. This was confirmed by a comparison with an independently synthesized authentic sample. Thus, it was found that a phenolic compound was bioconverted into its xyloside in the glucose medium by *C. versicolor* as shown in Fig. 1. Formation of MeG-Xyl in holocellulose medium was also observed.

To avoid rapid consumption of MeG by laccase in the culture medium, cultivation was performed under a nitrogen atmosphere or by addition of L-cysteine and L-ascorbic acid. Table 1 shows the yield of MeG-Xyl and the recovery of MeG after a 24-h incubation. By avoiding oxidation of MeG by laccase, the bioconversion of MeG into MeG-Xyl increased significantly. In particular, 40% of MeG was trans-

TABLE 1. Formation of MeG-Xyl and recovery of MeG in cultures of *C. versicolor*[a]

Condition	MeG-Xyl yield (mol% MeG)	MeG recovered (%)
Control	3	0
N_2 substitution	28	0
Addition of L-cysteine	28	15
Addition of L-ascorbic acid	40	20

[a] Cultures were incubated for 24 h under a nitrogen atmosphere or in the presence of laccase inhibitors.

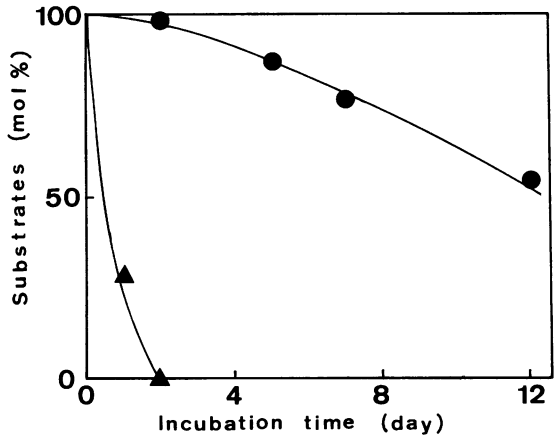

FIG. 2. Time course of consumption of MeG (▲) and MeG-Xyl (●) in cultures of *C. versicolor*.

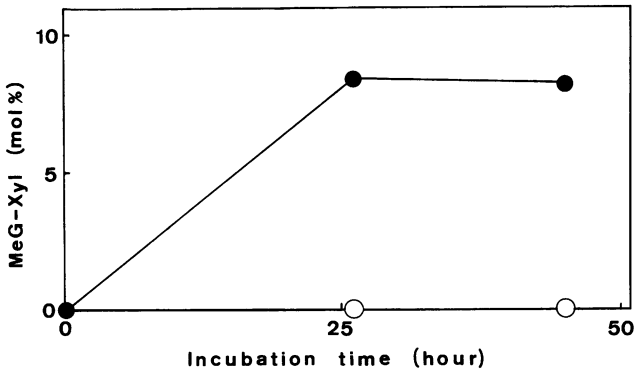

FIG. 3. Formation of MeG-Xyl by mycelial extracts. Incubation mixtures: ●, MeG, UDP-xylose, ascorbic acid, and cell extracts; ○, MeG, glucose, ascorbic acid, and cell extracts.

formed to MeG-Xyl in the presence of ascorbic acid in the culture in 24 h of incubation.

Bioconversion of VA. To elucidate whether the alcoholic or phenolic hydroxyl group would be xylosylated more easily by *C. versicolor*, bioconversion of VA was measured. The formation of two xylosides, VA-Xyl-Al and VA-Xyl-Ph, was confirmed by a comparison with the synthesized authentic samples by HPLC (Fig. 1). After a 24-h incubation, the yields of both xylosides were 20.7% for VA-Xyl-Ph and 1.6% for VA-Xyl-Al, and 75.9% of VA was recovered. The results showed that the phenolic hydroxyl group was much more extensively xylosylated than the alcoholic hydroxyl group.

Treatment of MeG and MeG-Xyl by *C. versicolor*. *C. versicolor* was inoculated into glucose medium containing MeG or MeG-Xyl. The rates of consumption of these two compounds are shown in Fig. 2. During growth in the presence of MeG, the color of the medium turned to yellow because of phenoloxidase activity. MeG disappeared completely after 2 days of incubation. However, the color of the medium containing MeG-Xyl did not change, and MeG-Xyl was relatively stable, as about 50% of the MeG-Xyl remained in the medium after a 12-day incubation.

Xylosylation of MeG in cell extracts. After *C. versicolor* was incubated in glucose medium for 8 days, the liquid culture was filtered, and the mycelium was washed with water. The mycelium was homogenized with a phosphate buffer (pH 7.0), and cell extracts of the mycelium were obtained by centrifugation. The mixture of MeG, a glycosyl donor (glucose or UDP-xylose), ascorbic acid, and cell extracts was incubated, and formation of MeG-Xyl was determined by HPLC (Fig. 3). Formation of MeG-Xyl was observed only when MeG was incubated with UDP-xylose and cell extracts. This result suggested that UDP-xylosyltransferase was involved in the xylosylation of the phenolic hydroxyl group of lignin model compounds.

DISCUSSION

In previous articles, we reported the formation of glucosides and xylosides of lignin model compounds in cultures of wood-rotting fungi growing on cellobiose (11, 13) and on holocellulose or xylan (14). When VA was placed in cellobiose liquid medium, VA was metabolized by *Tyromyces palustris* to vanillyl glucoside [1′-*O*-(β-D-glucosyl)vanillyl alcohol] but formed no phenolic glucoside [4-*O*-(β-D-glucosyl)vanillyl alcohol] (14). These glycosides were considered to have been formed by the transglycosylating activities of glycosidases excreted from wood-rotting fungi. Recently, Shinoyama et al. reported that xylosides were produced from xylobiose and either guaiacol or hydroquinone by the transfer reaction of *Aspergillus niger* β-xylosidase (23).

On the other hand, we have also found that monomeric and dimeric lignin model compounds were bioconverted to their xylosides in glucose media that were inoculated with *C. versicolor* (16). The formation of these xylosides could not be attributed to transxylosylating activities of xylanases and xylosidases but were attributed to the action of a xylosyltransferase, since the xylosides were formed in the glucose media. In the present study, we demonstrated that the phenolic hydroxyl group of MeG and VA was xylosylated in cultures of *C. versicolor* growing on glucose and holocellulose media.

The key detoxification reaction of organic compounds in plants is glycoside formation, which is triggered by a glycosyltransferase enzyme in the presence of energy-rich UDP-glycose as a cofactor. Foreign compounds containing phenolic groups are detoxified directly as glycosides. Tabata et al. showed that plant cell cultures can glucosylate various foreign phenolics which are not originally present in the cultured cells, although the efficiency of glucosylation varies with the culture strain, depending on the chemical structure (28). Recently, a strain of *Rhizoctonia solani* was shown to be able to biotransform anthracene to the following xyloside conjugates of anthracene: 1-*O*-(2-hydroxy-*trans*-1,2-dihydroanthryl)-β-D-xylopyranoside, 2-*O*-(1-hydroxy-*trans*-1,2-dihydroanthryl)-β-D-xylopyranoside, and 1-*O*-anthryl-β-D-xylopyranoside (25). On the other hand, there are very few reports of glycosylation of phenolic groups by white rot fungi. Sutherland et al. showed that a part of phenanthrene is bioconverted to 9-phenanthryl β-D-glucopyranoside, which may be considered a detoxification product of phenanthrene, by the white rot fungus *P. chrysosporium* (24). However, to our knowledge, our present work is the first study to demonstrate the conversion of lignin model compounds to xylosides with phenolic hydroxyl groups by the white rot fungus *C. versicolor*.

C. versicolor is known to produce laccase (3), a copper-containing phenoloxidase which oxidizes phenols and polyphenols by one-electron abstraction, yielding radicals which can subsequently polymerize (8), while reducing oxygen to water. Oki et al. showed that formation of polymers from guaiacylglycerol-β-guaiacyl ether in a polyphenoloxidase-

rich crude enzyme solution prepared from a culture of *C. versicolor* was inhibited remarkably by the addition of KCN, NaN_3, L-cysteine, and L-ascorbic acid (21). When the cultivation was performed under a nitrogen atmosphere to prevent the action of laccase from oxidation of phenolic compounds, the xylosylation of phenolic compounds was increased significantly. The culture might have become metabolically inactive while it was cultivated under nitrogen for 24 h. Therefore, we assume that the xyloside was produced by the preexisting enzyme. The presence of L-cysteine and L-ascorbic acid enhanced the xylosylation of phenolic groups of model compounds remarkably (Table 1).

C. versicolor did not extracellularly secrete an enzyme having xylosyltransferase action for phenolic compounds during the entire course of cultures; that is, the culture filtrate from *C. versicolor* did not form a xylosyl transfer product when either MeG or VA was incubated with filtrate containing glucose, xylose, or UDP-xylose. In contrast, when MeG was incubated with UDP-xylose and cell extracts of the mycelium, the formation of MeG-Xyl was observed. Thus, we suggest that the phenolic xylosides isolated in this study were formed by the action of a β-xylosyltransferase which was present in the surface of cells and had high specificity for the phenolic hydroxyl group, because much more VA-Xyl-Ph than VA-Xyl-Al was formed when VA was incubated in the culture.

Several explanations for the role of the formation of glycosides in the degradation of lignin by wood-rotting fungi are possible (15): (i) detoxification of phenolic compounds by the formation of their xylosides, (ii) conversion to hydrophilic lignin, and (iii) inhibitory effects on the polymerization of phenolic compounds by a phenol-oxidizing enzyme. Kirk and Chang reported that protolignin did not seem to undergo coupling polymerization during the degradation of wood by *C. versicolor* and *Polyporus anceps* (9). This finding conflicts with the results obtained by in vitro polymerization of milled wood lignin (6) and synthetic dehydrogenative polymer (12). In vivo, therefore, there must exist a system that prevents polymerization. We suggest that formation of xylosides of phenolic compounds may be one of the major candidates for a means of preventing repolymerization of lignin fragments modified by extracellular enzymes excreted from *C. versicolor*.

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