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NOTE

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Specific degradation of β-aryl ether linkage in synthetic lignin (dehydrogenative polymerizate) by bacterial enzymes of *Sphingomonas paucimobilis* SYK-6 produced in recombinant *Escherichia coli*

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Abstract *Sphingomonas paucimobilis* SYK-6 produces unique and specific enzymes, such as β-etherases, *O*-demethylases, and ring fission dioxygenases, for lignin degradation. Cleavage of arylglycerol-β-aryl ether linkage is the most important process in the lignin metabolic pathway of *S. paucimobilis* SYK-6. We reported the genes (*ligD*, *ligE*, *ligF*) for enzymes that cleaved β-aryl ether linkage of dimeric compounds in previous studies. In this study we synthesized the fluorescent high-molecular-weight lignin (UBE-DHP) by dehydrogenative polymerization. We investigated the β-aryl ether cleavage ability of these enzymes produced in recombinant *Escherichia coli*. When UBE-DHP was incubated with LigF, 4-methylumbeliferone was released as a result of β-aryl ether cleavage of α-*O*-methylumbelliferyl-β-hydroxypropiovanillone (compound III) incorporated in UBE-DHP. Here, we report that β-etherase of *S. paucimobilis* SYK-6 can be expressed in *E. coli* and is able to cleave the β-aryl ether linkage in synthetic high-molecular-weight lignin.

Key words Lignin biodegradation · β-Etherase · Specific β-aryl ether cleavage

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Introduction

Lignin is one of the major components of plant cell wall and the most abundant aromatic compound in the biosphere. The degradation of lignin is a significant step in the global carbon cycle. *Sphingomonas paucimobilis* SYK-6 can grow on several dimeric compounds of lignin with linkages such as β-aryl ether, biphenyl, phenylcoumarane, pinoresinol, and diarylpropane. The ability of *S. paucimobilis* SYK-6 to grow on several low-molecular-weight lignin compounds as a sole source of carbon and energy indicates that *S. paucimobilis* SYK-6 strain produces unique and specific enzymes, such as β-etherases, *O*-demethylases, and ring fission dioxygenases. We have identified several genes and characterized the function of these genes involved in this pathway (Fig. 1).[1–12]

In the lignin metabolic pathway of this bacterium, cleavage of β-aryl ether is the most important process because it is the major linkage (approximately 50%).[13] The β-aryl ether cleavage reaction in the lignin metabolic pathway of *S. paucimobilis* SYK-6 is catalyzed by LigD (Cα-dehydrogenase),[3] LigE, and LigF,[2,4] as reported previously (Fig. 1). The β-aryl ether compound containing Cα-hydroxyl (compound I) is oxidized to Cα-carbonyl (compound II) structure by LigD. The substrate specificities of LigE and LigF were restricted to the Cα-carbonyl structure. LigE and LigF each had β-etherase activity, catalyzing reductive cleavage of arylglycerol-β-aryl ether.[2,4] The β-etherase of *S. paucimobilis* SYK-6 could cleave the β-aryl ether linkage specifically in various β-aryl ether compounds.[1] In these studies, we used the fluorescent substrate α-*O*-methylumbelliferyl-acetovanillone, including 4-methylumbelliferone (4-MU) in the Cα position of acetovanillone, as a substrate for identifying the β-etherases. 4-MU is an analogue of lignin that fluoresces when free.

The kinetic patterns obtained in the previous study showed that cleavage of the β-aryl ether linkage in the fluorescent substrate and compound II competitively inhibited each other.[1] This meant that cleavage of β-aryl ether

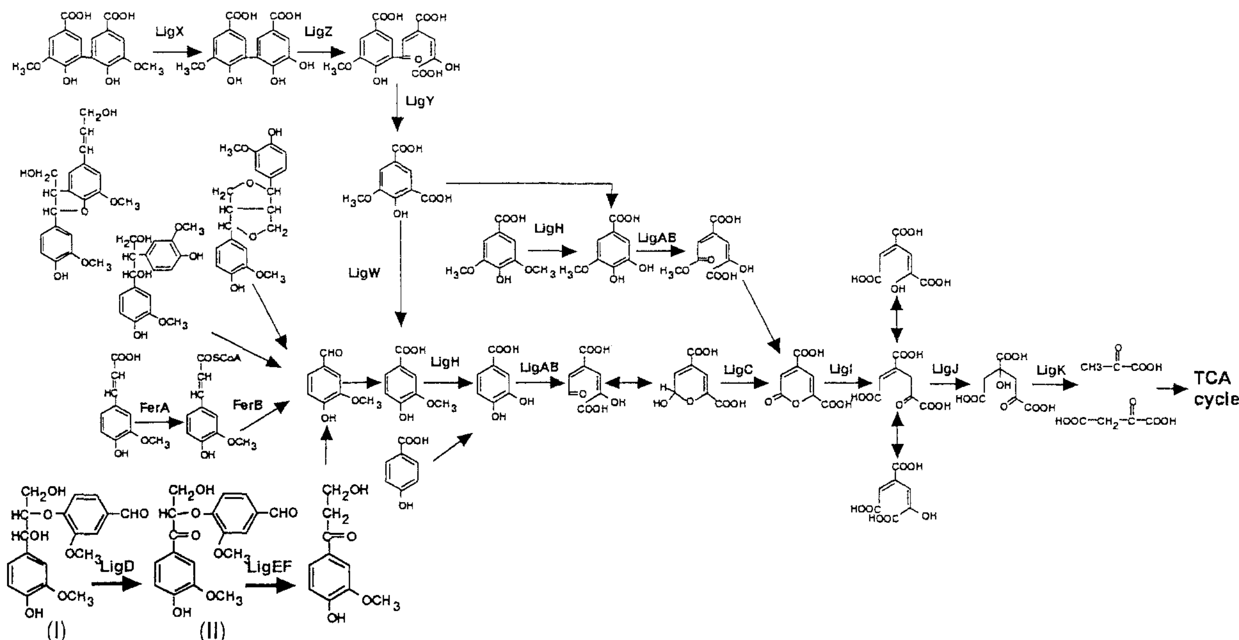

Fig. 1. Metabolic pathway of various lignin compounds in *S. paucimobilis* SYK-6. *LigD*, Cα-dehydrogenase;[4] *LigE*, β-etherase;[3,5] *LigX*, DDVA (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl) *O*-demethylase;[10] *LigZ*, OH-DDVA (2,2′,3′-trihydroxy-3-methoxy-5,5′-divanillic acid) dioxygenase;[7] *LigY*, OH-DDVA meta-cleavage compound hydrolase;[8] *LigW*, 5-carboxy vanillate decarboxylase; *LigH*, the enzyme essential for vanillate and syringate *O*-demethylations;[6] *LigAB*, protocatechuate 4,5-dioxygenase;[2] *LigC*, 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase;[12] *LigI*, 2-pyrone-4,6-dicarboxylate hydrolase;[9] *LigJ*, 4-oxalomesaconate hydratase;[11] *LigK*, 4-carboxy-4-hydroxy-2-oxoadipate aldolase; *FerA*, Ferulate: CoA ligase; *FerB*, feruloyl-CoA hydoratase/lyase

linkage in the fluorescent substrate and compound II was catalyzed at the same active site in β-etherase. Hence, the fluorescent substrate was useful as a substrate for assaying the β-aryl ether cleavage incorporated in high-molecular-weight lignin with high sensitivity.

In this study, we synthesized fluorescent dehydrogenative polymerizate (UBE-DHP) to determine how β-etherases of *S. paucimobilis* SYK-6 could cleave the β-aryl ether linkage in high-molecular-weight lignin. We assayed the cleavage ability of the enzymes essential for degradation of β-aryl ether compound in *S. paucimobilis* SYK-6. We prepared cell-free extracts of *S. paucimobilis* SYK-6 and assessed the β-etherase activity in high-molecular-weight lignin. Furthermore, we introduced the *ligD*, *ligF*, and *ligE* genes, involved in β-aryl ether cleavage in *S. paucimobilis* SYK-6 strain, into the expression vector of *Escherichia coli*. Using the cell-free extracts of the recombinant *E. coli* strains, we assayed the degradation ability of high-molecular-weight lignin. Here, we report that the β-etherase of *S. paucimobilis* SYK-6 has the ability to cleave the β-aryl ether linkage in high-molecular-weight lignin.

Materials and methods

Substrates

The substrates used in this study were synthesized. α-*O*-Methylumbelliferyl-β-hydroxy-propiovanillone (compound III) (see Fig. 5) was prepared according to our previous study.[2] Dehydrogenative polymerizate of compound III and conyferyl alcohol, designated UBE-DHP, was prepared as follows: Acetone (50 ml) containing 0.2% compound III and 0.2% conyferyl alcohol, respectively, and 20 ml of 3% H_2O_2 were dropped into 430 ml of 100 mM potassium phosphate pH 6.0 containing 30% acetone and 6 mg horseradish peroxidase (44 U/mg) (Sigma, St. Louis, MO, USA) over 14 h with stirring at 20°C. Peroxidase (4 mg) was added and the reaction mixture was stirred for an additional 12 h. The resultant precipitate was collected and washed three times with 10 ml water and centrifuged at 4000 *g* for 10 min. The precipitate was completely dried over phosphorus (V) oxide. A dioxane/water (9:1) solution dissolved the crude UBE-DHP and the solution was poured into 300 ml of diethyl ether for recrystallization. The precipitate was washed four times with diethyl ether and dispersed in distilled water. Lyophilization was performed to give the fluorescent-labeled synthetic lignin (UBE-DHP). UBE-DHP was fractionated by gel permeation chromatography on an Asahipack GS310 column (500 mm length × 7.6 mm diameter). *N,N*-Dimethylformamide containing 0.1 M lithium chloride was used as eluent at a flow rate of 0.5 ml/min. The molecular weight (MW) was estimated by calibration with polystyrene standard (MW 175 000, 9000, 4000, 2000, 800). The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra of UBE-DHP were analyzed using a JEOL-GX270 NMR spectrometer (solvent DMSO-*d6*). For the Cα position reduction of compound III in UBE-DHP, 1 g each of UBE-DHP and sodium borohydride were dissolved in dioxane-methanol (4:1) and stirred for 12 h at 4°C. A large excess of water was added and the resulting precipitate was

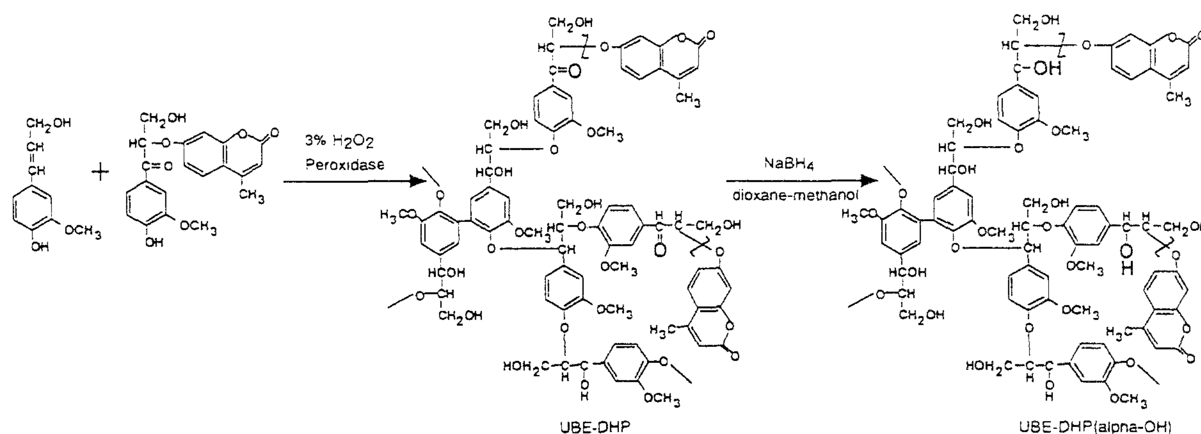

Fig. 2. Synthetic pathway of the fluorescent lignin [ubiquitin-activating enzyme–dehydrogenative polymerizate (*UBE-DHP*) and *UBE-DHP* (*α-OH*)]

collected as UBE-DHP (α-OH). All of the substrates used for enzyme assay in this study were dissolved in dimethylsulfoxide.

Enzyme assay of DHP degradation

The cell-free extracts of the recombinant strains *Escherichia coli* strain MV1190, harboring pUBX7 and pUBX78 (see Fig. 5) were prepared as shown in our previous study.[2,4] The extracts were assayed using a protein assay kit (Bradford-type reagent) purchased from Bio-rad Laboratories (Richmond, CA, USA). The enzyme activities of the cell-free extracts against compound III were measured as described in our previous report.[4] The fluorescence of 4-MU released from compound III was measured with excitation at 360nm and emission at 450nm with a fluorometric analyzer (Fluororead model 200; Ajinomoto, Japan). The degradation of each substrate by LigDFE and LigF was measured as follows: aliquots of 50mM KH_2PO_4-NaOH buffer (1.0ml, pH 7.4) containing 3mg of protein from the cell-free extracts, 2mM of glutathione (GSH), 1mM of NAD (except for LigF), and 0.3mg of each substrate [UBE-DHP UBE-DHP (α-OH)] were incubated at 28°C. After 24h, the fluorescence intensity was measured with a fluorometric analyzer.

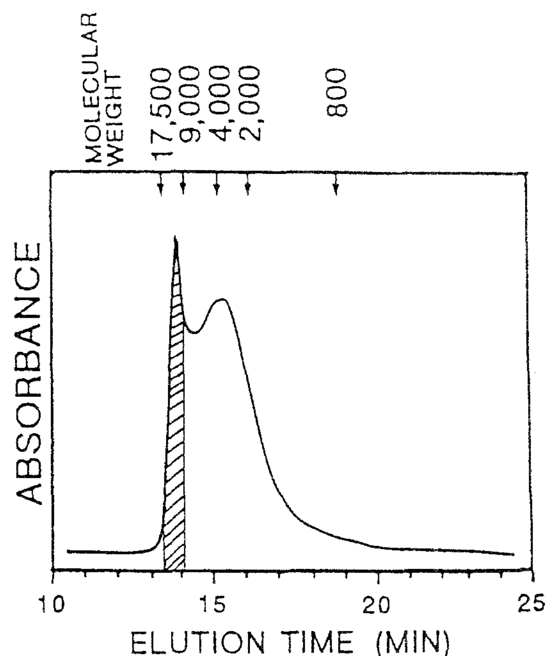

Fig. 3. Gel permeation chromatogram of UBE-DHP. Fractions used in this study are *hatched*. The molecular mass was calibrated with polystyrene standard series (MW 175000, 9000, 4000, 2000, 800)

Results

Synthesis of fluorescent lignin model compounds

In our previous study, we synthesized [β-^{13}C]DHP and confirmed that β-aryl ether, phenylcoumarane, pinoresinol, and other typical intramonomer linkages were included in synthetic [β-^{13}C]DHP, as seen by ^{13}C-NMR spectrometry.[14] To assay the specific cleavage ability of the β-etherase of *S. paucimobilis* SYK-6 in high-molecular-weight lignin, we synthesized the fluorescent lignin model compound UBE-DHP (Fig. 2), applying previously described methods[14] (see Materials and methods). The synthesized UBE-DHP was fractionated by gel permeation chromatography (Fig. 3). We used a mixture that contained 9000–17000MU UBE-DHP. The ^{1}H-NMR spectrum of acetylated UBE-DHP was analyzed (Fig. 4). A signal at 3.8ppm was assigned to the methoxyl group (OCH_3) of the guaiacyl structure in coniferyl alcohol and compound III. The signal at 2.2ppm was assigned to the methyl group (CH_3) originated from 4-MU in compound III. The integration ratio between the signals at 3.8ppm and 2.2ppm was calculated to be 10:1. It was thought that UBE-DHP contained coniferyl alcohol and compound III in a ratio of 9:1. UBE-DHP(α-OH) was prepared by Cα position reduction of compound III in UBE-DHP (see Materials and methods). These two kinds of UBE-DHP were used to determine how β-etherase of *S.*

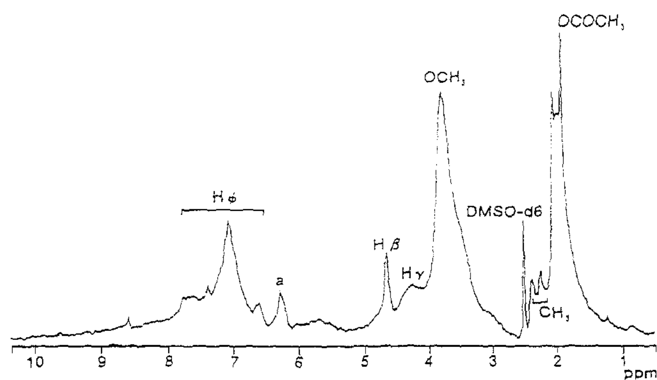

Fig. 4. ^{1}H-NMR spectrum of acetylated UBE-DHP

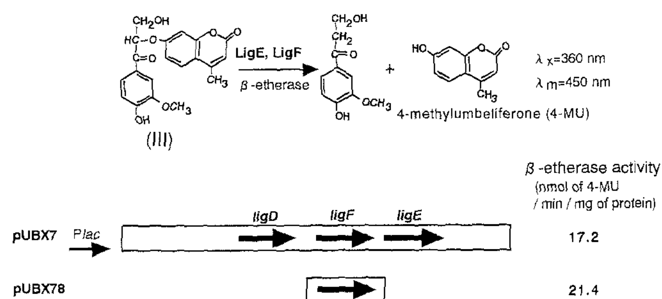

Fig. 5. Detection of β-etherase activity in the cell-free extracts of *Escherichia coli* recombinant strains

paucimobilis SYK-6 cleaved the β-aryl ether linkage in high-molecular-weight synthetic lignin.

β-Etherase activity in UBE-DHP

Using UBE-DHP and UBE-DHP(α-OH) synthesized as high-molecular-weight lignin model compounds, the β-etherase activity for high-molecular-weight lignin of *S. paucimobilis* SYK-6 strain was assayed by measuring fluorescence intensity (FI). *S. paucimobilis* SYK-6 could not cleave the β-aryl ether linkage in UBE-DHP in vivo. However, the cell-free extracts of SYK-6 strain had β-aryl ether cleavage activity in both UBE-DHP and UBE-DHP (α-OH). To investigate the function of the gene products of *ligD*, *ligE*, and *ligF* on high-molecular-weight lignin, we constructed two kinds of plasmid: pUBX7 and pUBX78 (Fig. 5). Each of these plasmids was introduced into *E. coli* strain MV1190 (Takara Shuzo, Kyoto, Japan) and cell-free extracts of the recombinant strains were prepared. The cell-free extracts of MV1190/pUBX78 containing LigF showed high β-etherase activity against compound III and released 4-MU (21.4 nmol/min/mg protein) (Fig. 5). We succeeded in achieving high production of β-etherase in the active state in *E. coli*. Using the extracts with high β-etherase activity against compound III, we investigated the ability of β-etherase to degrade the florescent synthetic lignin. The cell-free extracts of MV1190/pUBX78 containing LigF released 0.969 nmol of 4-MU/mg of protein from 0.3 mg of UBE-DHP (Table 1). These results revealed that the β-etherase

Table 1. β-Etherase activity in the cell-free extracts of *Escherichia coli* recombinant strains

Enzyme	β-Etherase activity (nmol 4-MU/mg protein)	
	UBE-DHP	UBE-DHP(α-OH)
LigF (pUBX78)	0.969	0.025
LigDFE (pUBX7)	0.744	0.224

Each value shows the quantity of released 4-MU calculated from the difference in fluorescence intensity (ΔFI) between sample and control (without cell-free extracts), after 24 h

(LigF) of *S. paucimobilis* SYK-6 has the ability to recognize and cleave the β-aryl ether linkages in high-molecular-weight lignin. However, the extracts containing LigF showed less activity on UBE-DHP(α-OH) (0.025 nmol of 4-MU/mg of protein). On the other hand, the cell-free extracts of MV1190/pUBX7 containing LigDFE also showed β-etherase activity against compound III (17.2 nmol of 4-MU/min/mg of protein) (Fig. 5). The extracts containing LigDFE released 0.744 nmol of 4-MU/mg of protein from UBE-DHP and 0.224 nmol of 4-MU/mg of protein from UBE-DHP(α-OH) (Table 1). These results indicated that Cα-dehydrogenase (LigD) of *S. paucimobilis* SYK-6 also could recognize the Cα-hydroxyl structure in synthetic high-molecular-weight lignin and dehydrogenate the carbonyl structure.

Discussion

Sphingomonas paucimobilis SYK-6 has specific β-aryl ether cleavage capability in the arylgrycerol-β-aryl ether structure (Fig. 1, compound I). This bacterium reductively cleaved β-aryl ether linkage in compound II by the specific activity of LigE and LigF (Fig. 1). The Cα-hydroxyl structure is converted to Cα-carbonyl structure by LigD activity. *S. paucimobilis* SYK-6 can grow well in various lignin dimeric compounds, but it cannot grow on high-molecular-weight lignin because of membrane permeability. It has not yet been clarified how enzymes essential for lignin degradation in *S. paucimobilis* SYK-6 can degrade high-molecular-weight lignin. In this report, we investigated the degradation ability of β-etherases (especially LigF) of *S. paucimobilis* SYK-6 in high-molecular-weight lignin using fluorescent synthetic lignin (UBE-DHP). In previous studies we reported on the β-etherases (LigE, LigF) of *S. paucimobilis* SYK-6 that could cleave β-aryl ether in compound II,[2,4] and LigF had about 100-fold higher activity than LigE in compounds II and III.

We synthesized UBE-DHP by dehydrogenative polymerization of compound III and coniferyl alcohol (incorporation rate 1:9) that included 4-MU in its β-aryl ether linkages (Fig. 2). If the β-aryl ether of compound III incorporated in UBE-DHP was cleaved, 4-MU was released and emitted fluorescence at 450 nm after excitation at 360 nm. The cell-free extracts of *E. coli* recombinant strain containing LigF released 2.829 nmol of 4-MU (0.969 nmol of

4-MU/mg of protein) from 0.3 mg of UBE-DHP. This indicates that 58% of the β-aryl ether linkage of compound III in UBE-DHP was cleaved. Although LigF could release 4-MU from UBE-DHP, it showed less activity in UBE-DHP(α-OH). LigF and LigE had substrate specificity for the Cα-carbonyl structure, so LigF did not catalyze β-aryl ether cleavage in UBE-DHP(α-OH). As expected, the cell-free extracts of *E. coli* recombinant strain containing LigDFE released 4-MU from both UBE-DHP (corresponding to 54%) and UBE-DHP(α-OH) (18%) as a result of cleaving the β-ether linkage in UBE-DHP. This resulted in Cα-hydroxyl structures in UBE-DHP(α-OH) being dehydrogenated by catalysis of LigD to Cα-carbonyl structure; then LigF could cleave the β-aryl ether linkage. The β-etherases of *S. paucimobilis* SYK-6 itself could recognize and cleave β-aryl ether linkage in the synthetic high-molecular-weight lignin. Furthermore, Cα-dehydrogenase exhibited enzymatie activity in high-molecular-weight lignin.

Conclusions

In this study we investigated the degradation ability of β-etherases of *S. paucimobilis* SYK-6 toward β-aryl ether linkages in synthetic high-molecular-weight lignin. The enzymes LigD, LigE, and LigF, essential for β-aryl ether cleavage, could be expressed in *E. coli* while its activity was retained. LigF, expressed in *E. coli*, could cleave β-aryl ether linkages in high-molecular-weight lignin; and LigD, expressed in *E. coli*, also could dehydrogenate Cα carbonyl to Cα alcohol structure in DHP, similar to the cell-free extracts of *S. paucimobilis* SYK-6. The β-etherases of *S. paucimobilis* SYK-6 could recognize and cleave β-aryl ether linkages not only in the β-aryl ether dimer but also in high-molecular-weight lignin, and thereby depolymerizing lignin. These results are important to our understanding of lignin degradation, including specific lignin depolymerization and lignin modification. We are now working on the characterization of LigE and LigF, which will be reported in detail in the near future.

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