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Detection and localization of a new enzyme catalyzing the β-aryl ether cleavage in the soil bacterium (*Pseudomonas paucimobilis* SYK-6)

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Cleavage of the arylglycerol-β-aryl ether linkage is the most important process in the biological degradation of lignin. We determined the activity of the enzyme cleaving the β-aryl ether linkage in membranes of *Pseudomonas paucimobilis* SYK-6. This enzyme was tightly associated with the cellular membrane and catalyzed the unique and reductive cleavage of compound II but not cleavage of compound I. This enzymatic activity was stimulated by addition of NADH. On the basis of this evidence, we present a model of the specific cellular assimilation of β-aryl ether by *P. paucimobilis* SYK-6.

Lignin metabolism; Arylglycerol-β-aryl ether linkage; Aryl ether cleaving enzyme, β-; Cell-free lysate; Membrane enzyme; (*Pseudomonas paucimobilis* SYK-6)

1. INTRODUCTION

Lignins are the most abundant aromatic material in plants and are constructed with various intermonomer linkages between phenylpropanes, of which the arylglycerol-β-aryl ether linkage is the most abundant (approx. 50%). The cleavage of this linkage is essential to the biological degradation of lignin.

Katayama and Fukuzumi [1,2] first reported that β-hydroxypropiovanillone (V) was produced as an intermediate from the guaiacylglycerol moiety of guaiacylglycerol-β-coniferyl ether by the soil bacterium *Pseudomonas* FK-2 strain, and that this reaction was probably catalyzed by a β-aryl ether cleaving enzyme of the bacterium. However, enzymes catalyzing this reaction have not yet been detected.

In a previous study [3], we described the metabolism of DDVA (biphenyl structure in lignin) and the mechanism of obtaining metabolic energy from lignin in *P. paucimobilis* SYK-6. Here, we report the first successful detection of a high activity of the β-aryl ether cleaving enzyme in the cellular membrane of this bacterium and show its substrate specificity. On the basis of these facts, we discuss a functional model of the enzyme for assimilation of the arylglycerol-β-aryl ether type by this bacterium.

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Abbreviations: DDVA, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl; MEGA-8, octanoyl-*N*-methylglucamide; MEGA-9, nonanoyl-*N*-glucamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio] - 1 - propanesulfonate; HAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate; NADH, reduced nicotinamide adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Substrates and chemicals*

Compounds I–IV were synthesized according to Adler and

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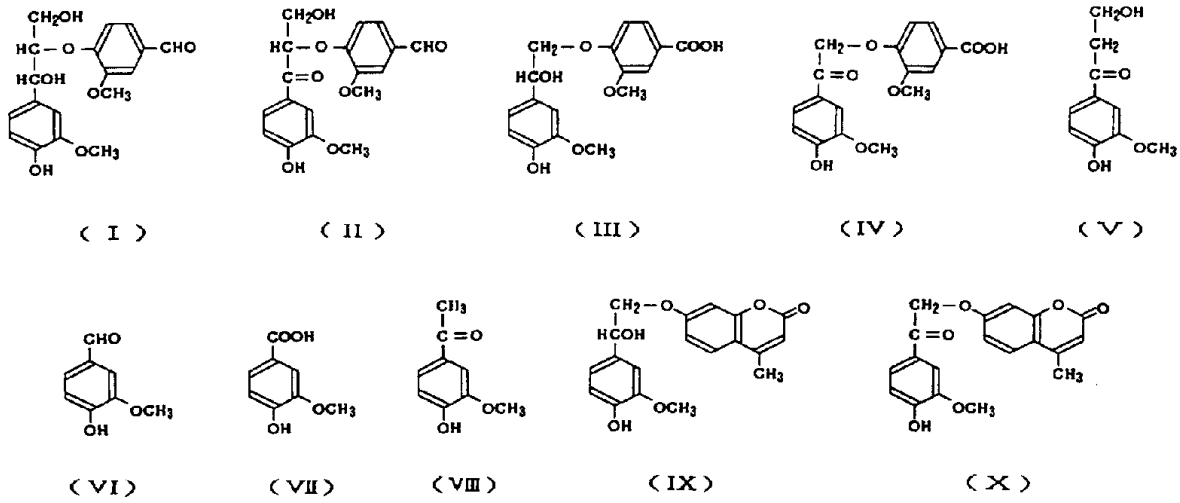

Fig.1. Structures of the model compounds used in this study.

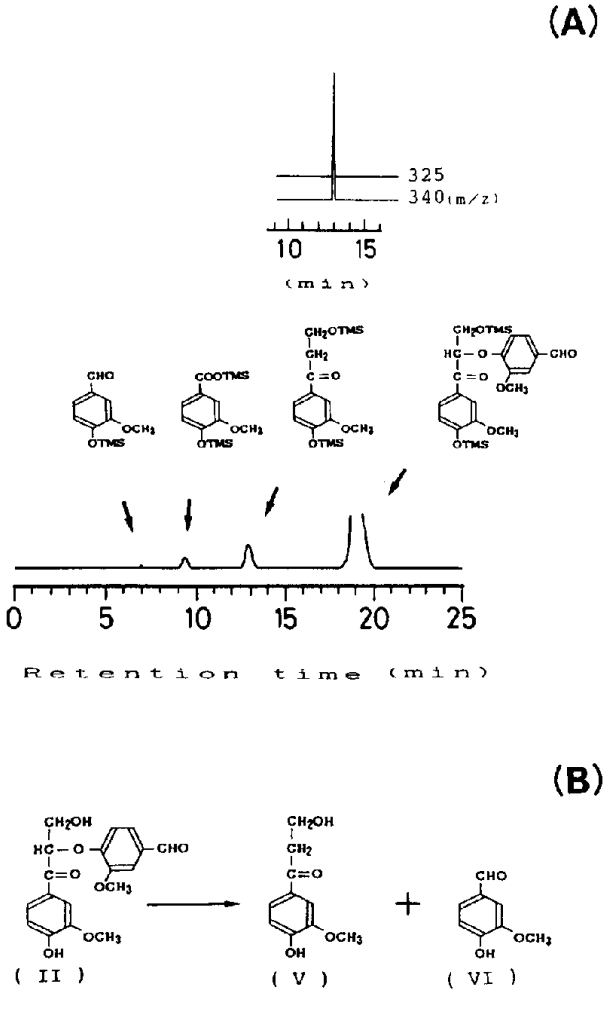

Fig.2. (A) Gas chromatogram and mass chromatogram of degradation products from compound II by *Pseudomonas paucimobilis* SYK-6. (B) Degradation pathway of compound II by *P. paucimobilis* SYK-6.

Eriksoo [4]. Compounds IX and X were prepared as described by Weinstein and Gold [5]. β-Hydroxypropiovanillone (V), of which the isolation was reported previously [1], was used as an authentic compound. Vanillin (VI), vanillate (VII) and acetovanillone (VIII) were purchased from Tokyo Kasei Co. Fig.1 shows the chemical structures of compounds I–X. Detergents (MEGA-8, MEGA-9, CHAPS, CHAPSO, *n*-octyl-β-D-glucose, *n*-heptyl-β-D-thioglucoside, *n*-octyl-β-D-thioglucoside, Tween-20, Triton X-100) were purchased from Wako Chemical Co.

2.2. *Identification of metabolites by GC-MS analysis and culture conditions*

P. paucimobilis SYK-6, described in [3], was able to grow well in medium containing compound I or II as sole carbon source. The methods of cultivation and identification of metabolites were similar to those in [3].

2.3. *Preparation of cell-free lysate and cellular membrane fraction*

Cells of *P. paucimobilis* SYK-6 (1 g) were mixed with 5 ml of 10 mM Tris-HCl (pH 7.5, containing 1 mM DTT) and 1 g of aluminum oxide. They were homogenized using a mortar and pestle for 5 min on ice. The mixture was centrifuged at 15000 rpm for 10 min to obtain the supernatant and used as the cell-free lysate. The cell-free lysate was centrifuged at 70000 × g for 60 min and the resultant sediment was suspended in the same buffer and used as the cellular membrane fraction. The cytoplasmic fraction was the supernatant from centrifugation at 70000 × g. Protein was determined according to Bradford [6].

2.4. *Assay of β-aryl ether cleaving enzyme*

Enzyme activity was measured in a total volume of 1 ml in-

cluding 10 mM Tris-HCl buffer (pH 7.5), 1 mM DTT, 1% detergent (MEGA-8), 0.1 mM substrate (compound IX or X dissolved in DMSO was added) and enzyme preparation (about 8 mg protein). The enzymic reaction started with the addition of substrate and the reaction mixture was incubated at 28°C for 30 or 60 min. The reaction was terminated by addition of 0.1 ml of 500 mM glycine-NaOH buffer (pH 10). Fluorescence of 4-methylumbelliferone released from the substrate was measured with excitation at 360 nm and emission at 450 nm using an Ajinomoto Fluororead Model 200.

3. RESULTS

3.1. *Cleavage of the β-aryl ether linkage by* P. paucimobilis *SYK-6*

The change in the UV absorption spectra of the culture medium during culture showed that compound II was rapidly degraded by *P. paucimobilis* SYK-6. This phenomenon was also observed in compounds I, III and IV. From the results of GC-MS analysis, it was clear that β-hydroxypropiovanillone (V) was produced as a common intermediate in the metabolism of compounds I and II as shown in fig.2A and B. In the case of compounds III and IV, acetovanillone (VIII) and vanillate (VII) were common intermediates (not shown). These results indicated that the enzyme of this bacterium could catalyze cleavage of the β-aryl ether linkages of two structural types such as those of compounds I and II and those of other compounds (III and IV) in a similar manner.

3.2. *Detection of β-aryl ether cleaving enzyme activity in cell-free lysate of* P. paucimobilis *SYK-6*

No β-aryl ether cleaving enzyme activity was detected in the cell-free lysate of *P. paucimobilis* SYK-6 grown with compound I or II. However, when the cell-free lysate was treated with detergent (MEGA-8) for 1 h on ice, high activity of the β-aryl ether cleaving enzyme was noted. However, the activity was not detected in the cell lysate treated with the other detergents tested. Although MEGA-8 and MEGA-9 have similar properties, only MEGA-8 was effective in detecting the enzyme activity in the cell-free lysate.

3.3. *Cleavage reaction of the β-aryl ether linkage of compound II in the cell-free lysate*

Compound II was incubated for 2 h at 28°C with the cell-free lysate treated with MEGA-8 (from cells grown with DDVA). GC-MS analysis of the reaction products showed accumulation of β-hydroxypropiovanillone (V) (fig.3A). The kinetic patterns obtained via plots of $[S]_0/v$ vs $[S]_0$ with the cell-free lysate showed that cleavage of the

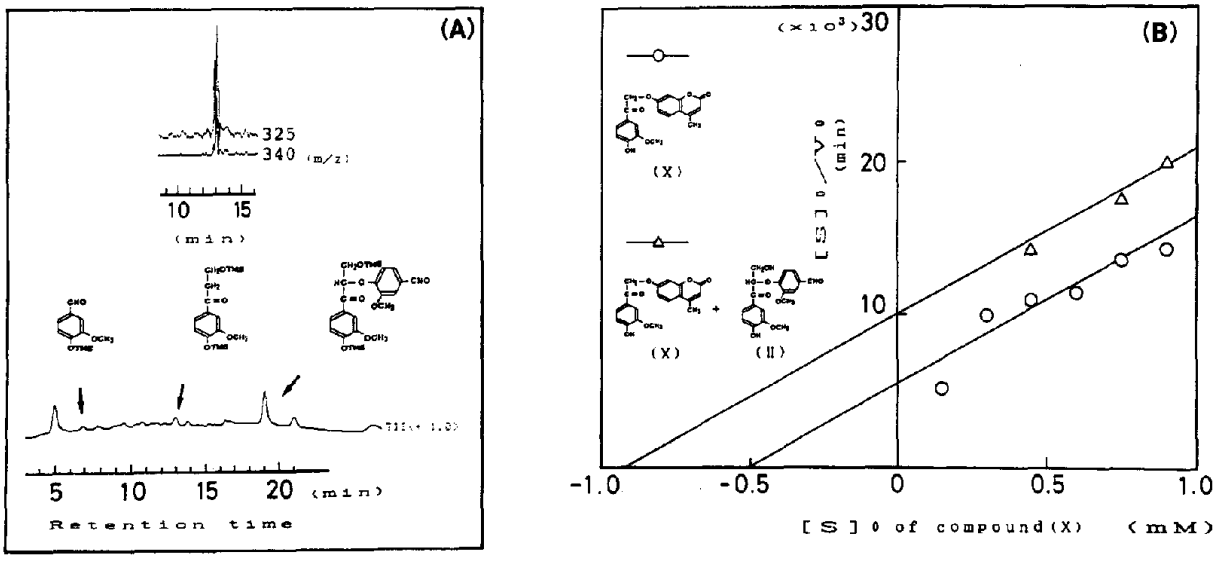

Fig.3. (A) Gas chromatogram and mass chromatogram of reaction products from compound II by the cell-free lysate treated with MEGA-8. (B) Competitive inhibition of cleaving activity of the β-aryl ether linkage in substrate X by compound II. Reaction mixture contained various concentrations of compound X and 0.3 mM (△) or 0 mM (○) compound II.

Table 1

Cellular localization and substrate specificity of β-aryl ether cleaving enzyme

Substrate	Fraction	Increase of fluorescence intensity (%)
Compound X	membrane	39
	membrane[a]	50
	cytoplasm	1
Compound IX	membrane	1

[a] 0.3 μmol NADH added

β-aryl ether linkage in the fluorescent substrate X and compound II competitively inhibited each other (fig.3B).

3.4. *Subcellular distribution and substrate specificity of the β-aryl ether cleaving enzyme*

The activity of the β-aryl ether cleaving enzyme was detected in the membrane fraction but not the cytoplasmic fraction (table 1). This enzymic activity was detectable for compound X but only barely so for compound IX, and that of compound X was stimulated about 20% by addition of 0.3 μmol NADH (table 1).

4. DISCUSSION

Katayama and Fukuzumi [1,2] first described the metabolism of guaiacylglycerol-β-coniferyl ether via β-hydroxypropiovanillone (V) by *Pseudomonas* FK-2 strain. Recently, Samejima et al. [7] and Vicuna et al. [8] obtained similar results in some bacterial strains. In *P. paucimobilis* SYK-6, we also confirmed the same metabolic pathway for guaiacylglycerol-β-guaiacyl ether via β-hydroxypropiovanillone (V) (fig.2B). Although this pathway is generally observed in some soil bacteria, the enzymes catalyzing this process have not yet been characterized.

Here, we provide the first report of the detection of high activity of the β-aryl ether cleaving enzyme

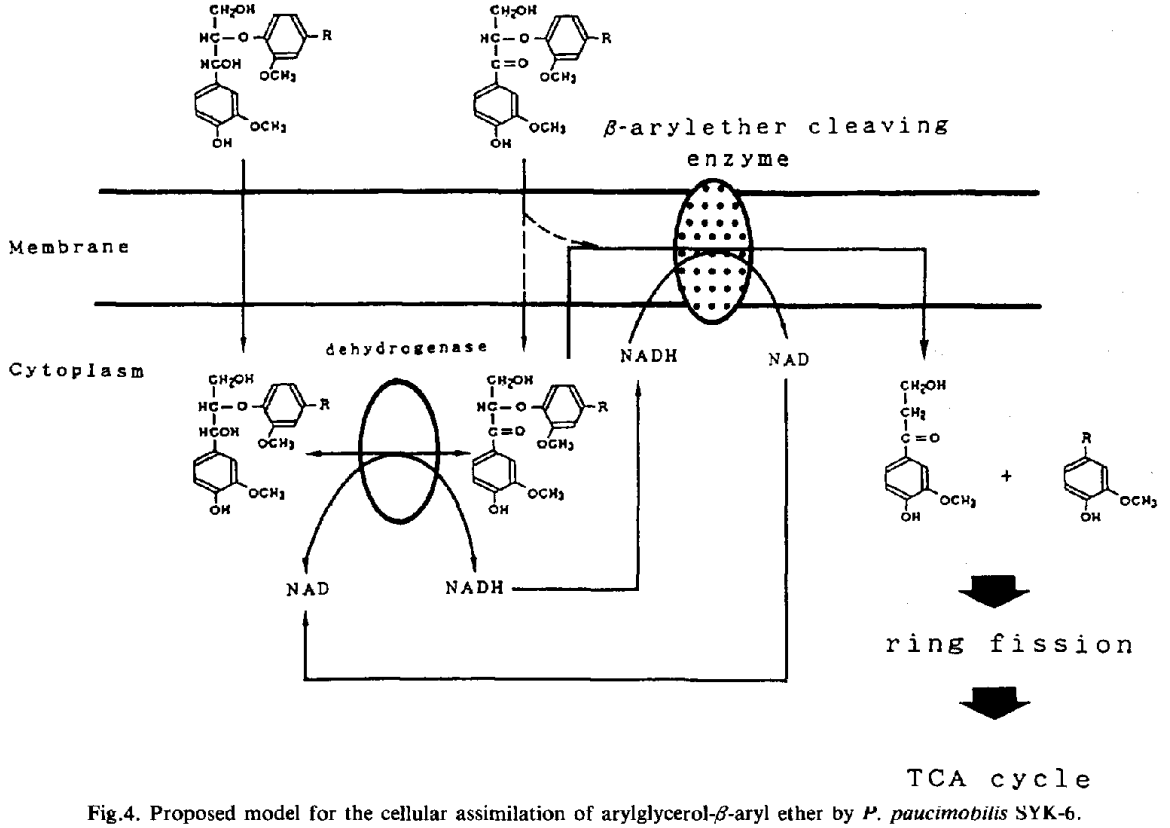

Fig.4. Proposed model for the cellular assimilation of arylglycerol-β-aryl ether by *P. paucimobilis* SYK-6.

in the cell-free lysate of *P. paucimobilis* SYK-6, and of the cellular distribution of the activity. The ,β-aryl ether cleaving enzyme of this bacterium was detected only in the cell-free lysate treated with detergent (MEGA-8). These results indicated that the β-aryl ether cleaving enzyme was tightly associated with the cellular membrane and that only MEGA-8 could expose the enzyme from the membrane-enzyme complex without destruction of the possible tertiary structure essential for enzymatic activity.

The cell-free lysate treated with MEGA-8 catalyzed cleavage of the β-aryl ether linkage of compound II to accumulate β-hydroxypropiovanillone (V) (fig.3A). Cleavage of the β-aryl ether linkage in the fluorescent assay substrate X was competitively inhibited by compound II (fig.3B). These results showed that cleavage of the β-aryl ether linkage in compounds II and X was catalyzed in the same manner at an identical position, i.e. the active site of the enzyme. Therefore, we were able to use compound X as the substrate in a sensitive fluorescent assay. Using this method, the β-aryl ether cleaving enzyme in this bacterium was observed to be clearly induced (about 10-fold) by lignin model compounds (not shown).

This inducible β-aryl ether cleaving enzyme was localized only in the cellular membrane of this bacterium (table 1). Enzymatic activity in this membrane was highly specific for substrate X, and was clearly stimulated by addition of NADH (table 1). These results showed that the β-aryl ether cleaving enzyme catalyzed the unique and reductive cleavage of compounds II and X in the cellular membrane, which probably required NADH as cofactor.

Pelmont et al. [9] and Habu et al. [10] isolated NAD-linked dehydrogenase from the cytoplasmic fractions of a number of soil bacteria. Here, we have also detected the dehydrogenase activity specifying oxidation of compounds I and IX in the cytoplasmic fraction of *P. paucimobilis* SYK-6, and observed cleavage of the β-aryl ether linkage in compound IX by the cell-free lysate containing the cytoplasmic and membrane fractions (not shown).

In this report, we have been able to gain a better understanding of the function and cellular localization of the enzyme specifying the β-aryl ether cleavage which is the most important process in lignin degradation. The facts outlined above have allowed us to postulate the specific model for the assimilation of arylglycerol-β-aryl ether by *P. paucimobilis* SYK-6 shown in fig.4. The arylglycerol-β-aryl ether is initially taken up into the cell and oxidized by NAD-linked dehydrogenase to produce NADH and the specific substrate of the β-aryl ether cleaving enzyme. Thereafter, the β-aryl ether cleavage of the resulting substrate is catalyzed by the membrane enzyme with consumption of NADH. The reaction products from this enzyme are then further metabolized to proceed along the pathway described in the previous report [3].

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