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RAPID COMMUNICATION

Tomonori Sonoki · Yuichiro Otsuka · Seiichiro Ikeda
Eiji Masai · Shinya Kajita · Yoshihiro Katayama

Close association between the enzymes involved in the lignin metabolic pathway of *Sphingomonas paucimobilis* SYK-6: interaction of *O*-demethylase (LigX) and ring fission dioxygenase (LigZ)

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Key words Lignin biodegradation · Protein–protein interaction · Biphenyl structure

Lignin is 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, a bacterium that can grow on 5,5′-dehydrodivanillic acid (2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl) (DDVA) as a sole carbon source, was isolated from pulp-bleaching wastewater in Japan. This bacterium can also grow on several dimeric model compounds of lignin such as β-arylether, 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 SYK-6 strain produces unique and specific enzymes, such as *O*-demethylases, β-etherases, and ring fission dioxygenases. We have identified several genes and characterized the function of these genes involved in this pathway[1] (Fig. 1). SYK-6 has several enzymatic systems for getting energy from lignin. Do such enzymes in a single microorganism exist apart from each other? If they are apart from each other, the energy efficiency for metabolism of carbon source is low. In cellulolytic microorganisms such as *Ruminococcus albus*[2] and *Clostridium cellubolyticum*,[3] it is well known that the many enzymes (e.g., endoglucanases and xylanases) involved in cellulose degradation form multienzyme complexes called cellulosome. Therefore, cellulosome may be the system for efficient cellulose metabolism. Similar to lignin biodegradation, the existence of multienzyme complexes consisting of various enzymes are expected in *S. paucimobilis* SYK-6.

In the DDVA metabolic pathway of SYK-6, DDVA is first demethylated by LigX (DDVA *O*-demethylase)[4]; then the benzene ring of the reaction product, OH-DDVA (2,2′,3′-trihydroxy-3-methoxy-5,5′-dicarboxybiphenyl), is cleaved by LigZ (OH-DDVA dioxygenase),[5] as shown in Fig. 1. In this study we identified the close association of *O*-demethylase (LigX) with ring fission dioxygenase (LigZ) in the DDVA metabolic pathway using antisera against LigX and LigZ, respectively.

To investigate the DDVA metabolic pathway, we isolated four DDVA *O*-demethylation-deficient mutants of *S. paucimobilis* SYK-6 following nitrosoguanidine mutagenesis. One of these mutants (NT-1) had no DDVA *O*-demethylation activity, and another mutant (NT-23) showed no OH-DDVA ring cleavage activity in addition to DDVA *O*-demethylation activity. We already reported that the *ligX* gene, as DDVA *O*-demethylase, was able to complement the DDVA *O*-demethylation of strain NT-1.[4] However the 1.5-kbp DNA fragment containing the *ligX* gene did not complement the DDVA *O*-demethylation activity of strain NT-23. These results suggested that the NT-23 mutation did not come from the *ligX* gene and its gene product, and that the other mutation in the genome of NT-23 would affect DDVA *O*-demethylation activity. Further characterization of NT-23 revealed that the 4.8-kbp DNA fragment harboring OH-DDVA dioxygenase (*ligZ*) and OH-DDVA *meta*-cleavage compound hydrolase (*ligY*) genes[6] was able to complement not only OH-DDVA ring cleavage activity but also DDVA *O*-demethylation of strain NT-23. Following the deletion analysis, the 1.5-kbp DNA fragment containing the *ligZ* gene could complement both the DDVA *O*-demethylation and OH-DDVA ring fission activities of the mutant NT-23 (Fig. 2). The mutant strain NT-23 had interesting features: It lost both LigX and LigZ activities as a result of *ligZ* mutation. These results indicated that LigX was active so long as LigZ activity existed.

Does LigX stably appear in the mutant NT-23? If LigX cannot be detected in NT-23, it suggests that LigZ is an essential factor for transcription of the *ligX* gene or stabili-

T. Sonoki (✉) · Y. Otsuka · S. Ikeda · S. Kajita · Y. Katayama
Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
Tel. +81-42-388-7364; Fax +81-42-388-7364
e-mail: tomosono@cc.tuat.ac.jp

E. Masai
Department of Bioengineering, Nagaoka University of Engineering, Kamitomioka, Nagaoka, Nigata 940-2188, Japan

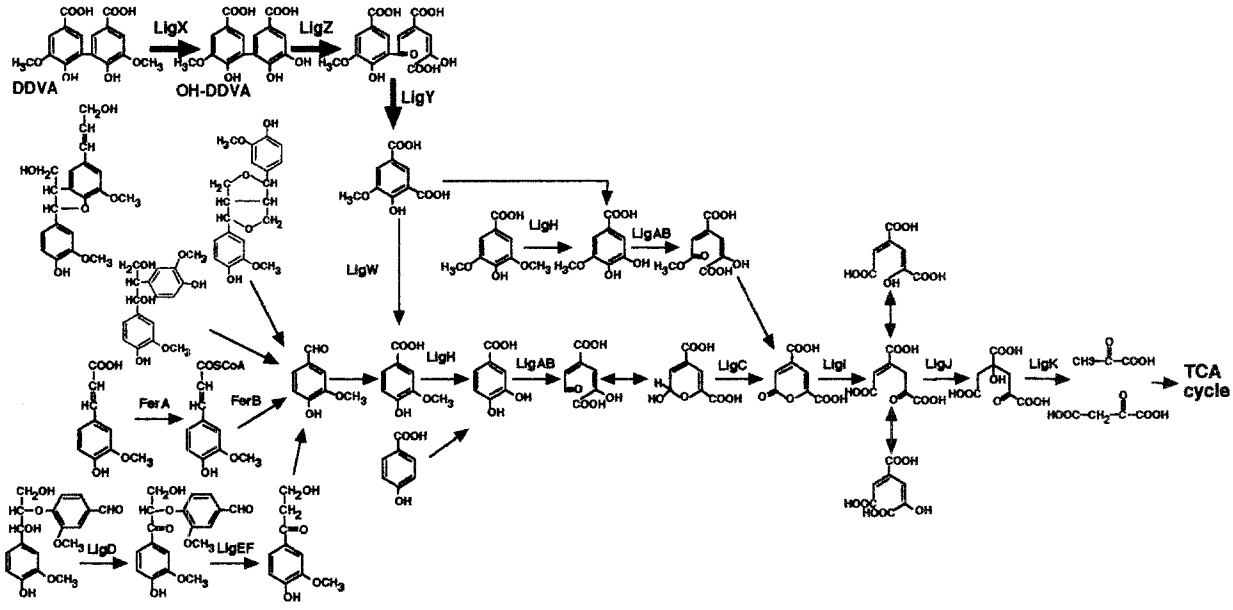

Fig. 1. Lignin metabolic pathway of *Sphingomonas paucimobilis* SYK-6

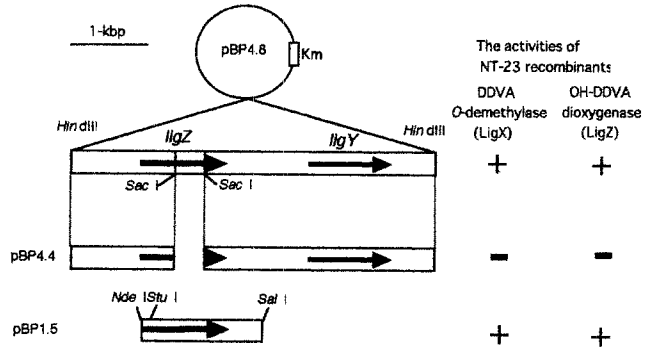

Fig. 2. Deletion analysis of complementation ability for DDVA *O*-demethylation of strain NT-23

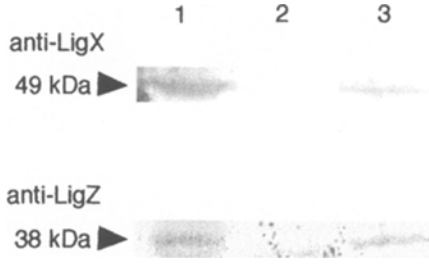

Fig. 3. Immunological detection of the *ligX* gene and *ligZ* gene products. Immunological detection of LigX (49 kDa) and LigZ (38 kDa) in the cell-free extracts. Lanes: *1*, *S. paucimobilis* SYK-6; *2*, DDVA *O*-demethylation and OH-DDVA ring fission-deficient mutant NT-23; *3*, recombinant strain NT-23/pBP1.5 (harboring *ligZ* gene)

zation of LigX. To investigate the action of the LigX and LigZ proteins in cells of the mutant strain NT-23, we prepared antibodies against LigX and LigZ, respectively. The cell-free extracts of *S. paucimobilis* SYK-6 and mutants were prepared as described in our previous study.[4] DDVA *O*-demethylase and OH-DDVA dioxygenase activities were detected by measuring substrate-dependent oxygen consumption with a galvanic cell electrode (Iijima Denshi, Aichi, Japan), as described previously.[4,5] For Western blot analysis, the proteins were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (12% polyacrylamide). After SDS-PAGE, the separated proteins were transferred onto PVDF membranes (Bio-rad Laboratories, Richmond, CA, USA). Immunocomplexes were detected with a biotin-streptoavidin-alkaline phosphatase system. In the cell-free extracts of wild-type strain SYK-6, both LigX [molecular weight (mw) about 49 kDa] and LigZ (mw about 38 kDa) were detected (Fig. 3, lane 1). Both LigX and LigZ activities were also detected (oxygen uptake rates were 4.58 and 18.3 nmol/min/mg of protein, respectively); no activities were seen in the extracts of mutant NT-23 (Fig. 3, lane 2). Neither LigX nor LigZ activity was detected in the cell-free extracts of NT-23. However, LigX and LigZ activities were detected in the cell-free extracts of recombinant strain NT-23/pBP1.5 (harboring the *ligZ* gene) (Fig. 3, lane 3). Their enzyme activities were approximately equal to the value observed for SYK-6 (oxygen uptake rates were 5.03 and 17.5 nmol/min/mg of protein, respectively). We then investigated the expression level of the *ligX* gene by RNA slot blot analysis (Fig. 4). The transcripts of *ligX* and *ligZ* genes were detected in the wild-type strain. The expression level of the *ligX* gene in mutant NT-23 was no different from that of the wild-type. Regardless of the existence of LigZ protein, the *ligX* gene was transcripted.

Furthermore, we constructed the expression vector containing histidine-tagged *ligZ* gene, which encoded LigZ fusing six His residues at its N-terminal, and introduced in into the SYK-6 strain. After preparing cell-free extracts of the recombinant strain SYK-6/pKQZ01, crosslinking was carried out using glutaraldehyde (Wako, Saitama, Japan). The

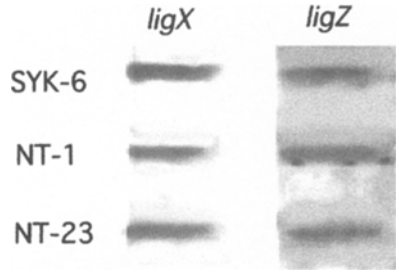

Fig. 4. RNA slot blot analysis of *ligX* and *ligZ* genes

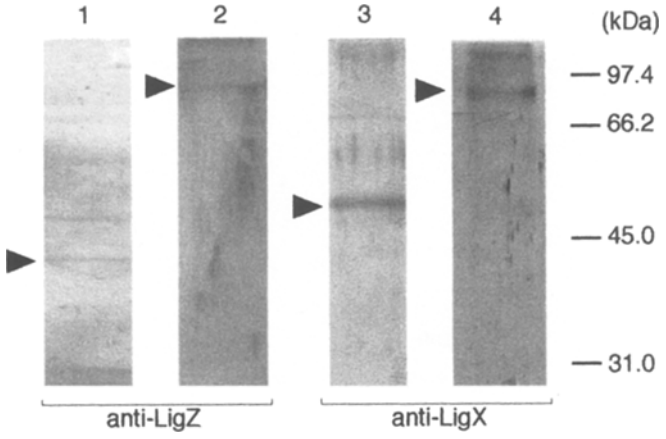

Fig. 5. Immunological detection of glutaraldehyde crosslinking of His-tagged LigZ and LigX. Lanes: *1* and *3*, cell-free extracts not treated with glutaraldehyde; *2* and *4*, purified protein using an Ni-NTA column after glutaraldehyde treatment

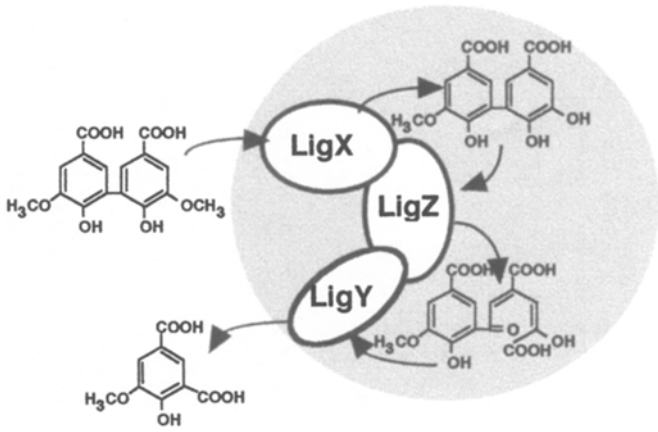

Fig. 6. Proposed protein interaction model in the DDVA transformation by *S. paucimobilis* SYK-6

assembled proteins were crosslinked with glutaraldehyde.[7] Proteins were purified by affinity chromatography using an Ni-NTA affinity column (Qiagen, Valencia, CA, USA). Purified proteins were electrophoresed and analyzed by Western blotting (Fig. 5). LigZ was detected at the position of about 38 kDa in the wild-type strain. The molecular mass of LigZ increased after crosslinking. Interestingly, LigX antiserum also reacted at the same position where LigZ antiserum reacted as a result of crosslinking. These data revealed that LigX and LigZ had a close association in a cell of *S. paucimobilis* SYK-6.

Sphingomonas paucimobilis SYK-6 has the ability to degrade various lignin-related compounds. This bacterium has various enzyme systems for the mineralization of lignin. In the DDVA metabolic pathway, the DDVA *O*-demethylase (LigX) has a close association with OH-DDVA dioxygenase (LigZ). LigX forms a protein complex with LigZ for efficient transformation of DDVA. The association between these two enzymes is advantageous as a sequential reaction system. Furthermore, it was observed in our previous study that LigY loses its activity when LigZ is not present.[6] LigY may also have a close association with LigZ, which may have a chaperon-like function in addition to OH-DDVA ring cleavage activity. We first reported the close association with protein in the lignin-degrading bacterium *S. paucimobilis* SYK-6 using antisera. It is thought that an enzyme complex such as cellulosome is formed during lignin metabolism for efficient degradation of lignin in *S. paucimobilis* SYK-6. Recently, investigations have focused on the possibility that the protein–protein interaction is important for understanding the biological steps. It is as important that we understand the interactions between proteins in the lignin metabolic pathway as it is to understand cellulose degradation. Further studies will clarify the protein interaction in the lignin metabolic pathway.

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