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Characterization of the *meta*-Cleavage Compound Hydrolase Gene Involved in Degradation of the Lignin-Related Biphenyl Structure by *Sphingomonas paucimobilis* SYK-6

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Sphingomonas paucimobilis **SYK-6 has the ability to transform a lignin-related biphenyl compound, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl (DDVA), to 5-carboxyvanillic acid (5CVA) via 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl (OH-DDVA). In the 4.9-kb *Hin*dIII fragment containing the OH-DDVA *meta*-cleavage dioxygenase gene (*ligZ*), we found a novel hydrolase gene (*ligY*) responsible for the conversion of the *meta*-cleavage compound of OH-DDVA to 5CVA. Incorporation of ^{18}O from $H_2{}^{18}O$ into 5CVA indicated there was a hydrolytic conversion of the OH-DDVA *meta*-cleavage compound to 5CVA. LigY exhibited hydrolase activity only toward the *meta*-cleavage compound of OH-DDVA, suggesting its restricted substrate specificity.**

The complex aromatic polymer lignin comprises about 25% of the land-based biomass on earth, and its recycling is a vital component of the earth's carbon cycle. The study of the biochemical and enzymatic processes involved in lignin biotransformation can supply a variety of catalytic reactions useful for the production of valuable aromatic chemicals. Lignin is composed of various intermolecular linkages between phenylpropanes, including guaiacyl, syringyl, and *p*-hydroxyphenyl, and contains biphenyl nuclei (2). Biphenyl linkages are one of the key connections between phenylpropane units. The biphenyl structure is so stable that its decomposition should be the rate-limiting step in lignin degradation.

Sphingomonas paucimobilis SYK-6 was isolated from a kraft pulp effluent and degrades and grows on a lignin-related biphenyl compound, 2,2′-dihydroxy-3,3′-dimethoxy-5,5′-dicarboxybiphenyl (DDVA) (8). DDVA is transformed to a diol compound, 2,2′,3-trihydroxy-3′-methoxy-5,5′-dicarboxybiphenyl (OH-DDVA), by demethylation (7) (Fig. 1). A *meta*-ring cleavage of OH-DDVA is catalyzed by a *meta*-cleavage dioxygenase encoded by the *ligZ* gene, which resides in a 4.9-kb *Hin*dIII fragment of SYK-6 (16). A metabolite, 5-carboxyvanillic acid (5CVA), was observed during the degradation of DDVA by SYK-6, and is expected to be generated from a *meta*-ring cleavage compound of OH-DDVA.

In this study, we focused on the gene involved in the transformation of a *meta*-ring cleavage compound of OH-DDVA to elucidate the DDVA metabolic pathway in SYK-6. Hydrolysis of a *meta*-cleavage compound following *meta*-ring cleavage by a dioxygenase is a well-known metabolic sequence in aromatic compound metabolism, including those of toluene, xylene, naphthalene, and biphenyl (6, 9, 12, 13, 20). Here we characterized the *ligY* gene whose product catalyzes the hydrolysis of a *meta*-cleavage compound of OH-DDVA.

When OH-DDVA was incubated with the crude extract of *Escherichia coli* MV1190 cells containing plasmid pFK09 carrying the *ligZ* gene, a *meta*-cleavage compound of OH-DDVA accumulated (Fig. 2, lane 2), which showed an absorption maximum at 455 nm and presented the yellow color common to *meta*-cleavage compounds (3). When OH-DDVA was incubated with the crude extract of *E. coli* cells, which harbored pTE491 carrying the 4.9-kb SYK-6 *Hin*dIII fragment containing the *ligZ* gene and its adjacent region, OH-DDVA was converted to 5CVA. A 20-μl portion of 50 mM OH-DDVA was added to the crude extract (0.8 mg of protein) in 1 ml of 20 mM Tris-HCl (pH 7.5), and this reaction mixture was incubated for 10 min at 25°C. It was acidified with hydrochloric acid and extracted with 400 μl of ethyl acetate. The organic phase was dried in vacuo and dissolved in 20 μl of ethyl acetate. The resulting sample was analyzed by thin-layer chromatography (TLC) by using silica gel 60 F254 (E. Merck, Darmstadt, Germany). The developing solvent was chloroform-ethyl acetate-formic acid (10:8:3 [vol/vol/vol]). Compounds were visualized under UV light (at 254 nm). The metabolite 5CVA was provisionally identified by comparing the R_f value on TLC with that of authentic 5CVA (Fig. 2, lanes 3 and 4). Authentic 5CVA was synthesized according to the method of Profft and Krause (18). Its identification was confirmed by gas chromatography-mass spectrometry (GC-MS) analysis. The metabolite was methylated by using trimethylsilyldiazomethane (Wako Chemical Industries, Ltd., Tokyo, Japan). The resultant methyl ester was analyzed by GC-MS (model 5971A; Hewlett-Packard Co., Palo Alto, Calif.) by using an Ultra-2 capillary column (50 m by 0.2 mm; Hewlett-Packard Co.). The injection and detector temperatures were 250 and 280°C, respectively. The sample was chromatographed by using a temperature program which began at 60°C, was raised to 150°C at 20°C/min, and then was raised to 300°C at 3°C/min. The retention time and mass spectrum of the methyl ester are fully equivalent to those of authentic 5CVA (see Fig. 5). The mass spectrum of the methyl ester derivative of 5CVA had a molecular ion (M) at an m/z of 254 and a major fragment ion at an m/z of 223. Since the hydrolysis products of a *meta*-cleavage compound of OH-DDVA are presumed to be 5CVA and 4-carboxy-2-hydroxypenta-2,4-dienoic acid (Fig. 1A), these results indicated that the 4.9-kb SYK-6 *Hin*dIII fragment carrying the *ligZ* gene contains a *meta*-cleavage compound hydrolase gene. In these

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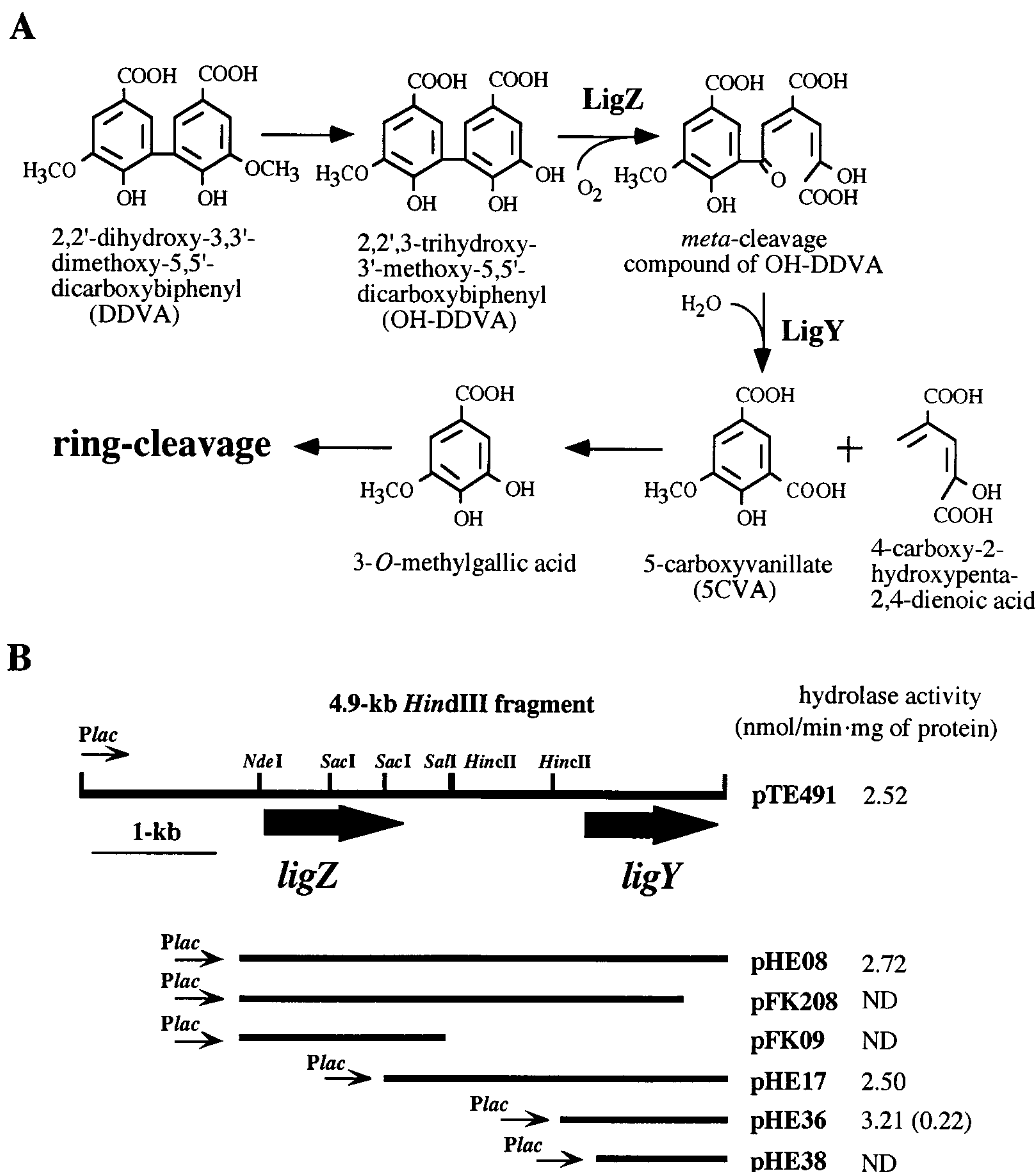

FIG. 1. (A) Proposed metabolic pathway for DDVA in *S. paucimobilis* SYK-6. LigZ, OH-DDVA dioxygenase; LigY, OH-DDVA *meta*-cleavage compound hydrolase. (B) Deletion analysis to locate the *meta*-cleavage compound hydrolase gene (*ligY*). The direction of transcription from the vector-located promoter (P*lac*) is depicted by a thin arrow. The large arrows represent the coding regions of *ligZ* and *ligY* genes. The hydrolase activities of *E. coli* strains containing each plasmid are presented on the right. ND, no product detected. The value in parentheses represents the activity obtained in the reaction in which the crude LigY was added 1 min after the incubation of LigZ with OH-DDVA.

experiments, 4-carboxy-2-hydroxypenta-2,4-dienoic acid was not observed. It might have been metabolized in *E. coli* cells.

A series of deletion derivatives of the 4.9-kb *Hin*dIII fragment of pTE491 were constructed to limit the region encoding an OH-DDVA *meta*-cleavage compound hydrolase by using restriction enzymes and *E. coli* exonuclease III (Takara Shuzo Co., Ltd., Kyoto, Japan). The production of 5CVA from OH-DDVA was then catalyzed by sequential actions of LigZ, and an OH-DDVA *meta*-cleavage compound hydrolase was examined. The crude extracts of deletion clones and a *ligZ* recombinant clone (0.8 mg of protein each) were incubated with OH-DDVA, and the 5CVA formed from OH-DDVA was evaluated by GC-MS, as described above. A deletion clone, pHE36, contained the minimum fragment which conferred hydrolase activity toward the *meta*-cleavage compound of OH-DDVA (Fig. 1B). The 1.3-kb insert in pHE36 was subjected to nucleotide sequencing. The determination of the nucleotide sequence was performed by the dideoxy termination method (19) with an ALFred DNA sequencer (Pharmacia, Milwaukee, Wis.). Analysis of nucleotide sequence was done with GeneWorks software (Intelligenetics, Inc., Mountain View, Calif.).

A 996-bp open reading frame (ORF) was found in the 1.3-kb insert (Fig. 3). The 5′- and 3′-terminal parts of this ORF were deleted in pHE38 and pFK208, respectively, both of which lacked the hydrolase activity (Fig. 1B). These results indicated that this ORF encodes an OH-DDVA *meta*-cleavage compound hydrolase. This ORF was designated *ligY*. The *ligY* gene encoded a protein of 332 amino acid residues, whose molecular mass was estimated to be 37,280 Da. Its G+C content was 63%, which was almost equivalent to those of the proteins

FIG. 2. Thin-layer chromatogram of the reaction products of OH-DDVA with *E. coli* crude extracts. Lanes: 1, synthetic OH-DDVA; 2, *E. coli* (pFK09); 3, *E. coli* (pTE491); 4, synthetic 5CVA.

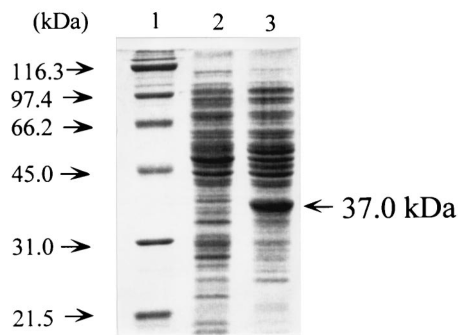

FIG. 4. SDS-PAGE of LigY hydrolase produced in *E. coli*. Proteins (10 μg each) were separated on an SDS–12% PAGE gel and stained with Coomassie brilliant blue. Lanes: 1, molecular mass standard proteins; 2, *E. coli* MV1190 (pBluescript II KS+); 3, *E. coli* MV1190(pHE36).

encoded by the other lignin-degradative genes of SYK-6 (11, 14–16). A putative ribosome binding sequence, 'AAGGGGA', was present in the upstream region of the start codon for *ligY* (Fig. 3). The deduced amino acid sequence of *ligY* showed no similarity to those of other aromatic compound hydrolases involved in benzene, toluene, xylene, and biphenyl metabolism, and there was little identity with previously reported enzymes, indicating that *ligY* encodes a novel aromatic compound hydrolase. These aromatic hydrolases contain a Gly-X-Ser-X-Gly motif constituting an active site, which is shared by serine hydrolases (1, 5). The deduced LigY amino acid sequence did not contain this motif, suggesting that LigY does not belong to serine hydrolase family. In addition, LigZ has little similarity to the deduced amino acid sequences of *meta*-ring cleavage dioxygenases for benzene, toluene, xylene, and biphenyl degradation. The *ligZ* and *ligY* genes seem to have evolved from separate ancestors of the genes coding for *meta*-ring cleavage dioxygenases and *meta*-cleavage compound hydrolases for benzene, toluene, xylene, and biphenyl degradation.

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GGTCACTGGATGCGCACCGGCAAGGACCGGGTGCAGGTGGAAGTCACCAATTGCCCCGTCAACATCGGTGGCGCC   75
CGTGTGGCGCCGGCGACATATTACGCGGCGACCCGGACGGCGTGATCGTCATCCCGCAAGCGCATGAGGAGGCGG  150
TGCTCGATGCCGCCGAGGAGATCCACGCCGCCGAGGAAAGTATCCGTGCCGCCTGCCGCACCGGCATGCGCCTCG  225
                                                           ligY →
ATGAGGCGCGCAAACAGTTCAAATATCACAGCCTTCAGACACGTCAGAAATAAGGGGATCACCCGATGATCATCG  300
                                                    RBS        M  I  I  D
ACTGCCATGGTCACGTCAGCGCTCCGGTGGAGCTTTGGGCCTATAAGGCGAGCCTGCTCGCGCATCGCGGCTCGC  375
 C  H  G  H  V  S  A  P  V  E  L  W  A  Y  K  A  S  L  L  A  H  R  G  S  H
ATGGGCGCGGGGGCGTCAAGGTGACGGACGAGCAGATCATTGCCGCCGCGCACCACAAGGAGACCTGGCCGGACG  450
 G  R  G  G  V  K  V  T  D  E  Q  I  I  A  A  A  H  H  K  E  T  W  P  D  G
GGCACATCGAGCTGCTGCACAATCACGGCACGGACATGCAGCTCATCTCGCCGCGCACGTTCCAGATGATGAACT  525
 H  I  E  L  L  H  N  H  G  T  D  M  Q  L  I  S  P  R  T  F  Q  M  M  N  S
CGGCCAAGCCCGCCCGCGTCGTCCACTGGTTCTGCGAGGAAGTGAACACGCTCATCCACCGCCAGTGCACGCTGA  600
 A  K  P  A  R  V  V  H  W  F  C  E  E  V  N  T  L  I  H  R  Q  C  T  L  I
TCCCGGAGATGTTCATCCCGGTCGCCGGCCTGCCGCAGGTGGCGGGCGAGCCCATCGAGAATGTGTTTGCGGAGA  675
 P  E  M  F  I  P  V  A  G  L  P  Q  V  A  G  E  P  I  E  N  V  F  A  E  M
TGGACCGCTGCGTCTCCATGGGCTTCAAGGGCTTCCTGCTGAACCCGGACCCCTATGAGAATGGCGCCGAGGAAG  750
 D  R  C  V  S  M  G  F  K  G  F  L  L  N  P  D  P  Y  E  N  G  A  E  E  A
CCCCGCCACTGGGCGACCGCTACTGGTATCCGCTCTACGAGAAGCTGTGCGAGCTGGACCTGCCCGCCCATATCC  825
 P  P  L  G  D  R  Y  W  Y  P  L  Y  E  K  L  C  E  L  D  L  P  A  H  I  H
ACGCCACCGGCTCCCAGTCCGAGCGCTCGCCTTATTCGCTGCACTTCATCAATGAAGAGACGATCGCCACCTATA  900
 A  T  G  S  Q  S  E  R  S  P  Y  S  L  H  F  I  N  E  E  T  I  A  T  Y  N
ACCTCTGCACCTCATCGGTGTTCGATGATTTTCCGCAGCTCAAGGTGGTGGTGAGCCATGGCGGCGGGGCCATCC  975
 L  C  T  S  S  V  F  D  D  F  P  Q  L  K  V  V  V  S  H  G  G  G  A  I  P
CCTATCAGCTCGGCCGCTTTGAATCCCAGTCGCGCCGCAGCAAGCACCTCTTCTCCGAGCGGATGGCCAAGCTCT 1050
 Y  Q  L  G  R  F  E  S  Q  S  R  R  S  K  H  L  F  S  E  R  M  A  K  L  Y
ATTTCGATACCGTGCTCTACACCGAGGGCGCGCTGCGGCTGCTCATCGAGACCGTCGGCCCCGAGCGCTGCCTGT 1125
 F  D  T  V  L  Y  T  E  G  A  L  R  L  L  I  E  T  V  G  P  E  R  C  L  F
TCGGCTCCGAATGCCCCGGCGTCGGCTCCACCATCGATCCCGCCACCGGCAAGCAGATGGACCACATCGCGCCCT 1200
 G  S  E  C  P  G  V  G  S  T  I  D  P  A  T  G  K  Q  M  D  H  I  A  P  F
TCATCCAGAAATTCGACTTCCTGAGCGACGCCGACAAGAAGCTGATTTTTGAGGATAATGCGCGGAAGGTGTTTA 1275
 I  Q  K  F  D  F  L  S  D  A  D  K  K  L  I  F  E  D  N  A  R  K  V  F  N
ATTTGGAGGTTTGAGGATCTAGTAGGTCTGATCATCGACTGGGAGAGACGTAAACTAATTCAGAATCCTAAACGG 1350
 L  E  V  *
CTAAGGGCGAAAAGCTT                                                          1367
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FIG. 3. Nucleotide and deduced amino acid sequences of the hydrolase gene (*ligY*) from *S. paucimobilis* SYK-6. The putative ribosome binding sequence (RBS) for *ligY* is underlined. A stop codon is indicated by an asterisk. The deduced amino acid sequence of *ligY* is presented below the nucleotide sequence.

The LigY hydrolase was overproduced in *E. coli* MV1190 under the control of the *lac* promoter in pHE36. A 37-kDa polypeptide was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) with 12% (wt/vol) polyacrylamide, and its molecular mass is in good agreement with that estimated from the deduced amino acid sequence of LigY (Fig. 4).

When the crude LigY prepared from the *E. coli* cells harboring pHE36 was added together with the crude LigZ to the reaction mixture containing OH-DDVA, OH-DDVA was transformed to 5CVA. However, the crude LigY was added 1 min after the reaction of LigZ with OH-DDVA, *meta*-cleavage compound of OH-DDVA remained, and a small amount of 5CVA was produced (Fig. 1B). These results suggested that the *meta*-cleavage compound of OH-DDVA would be so unstable that the sequential actions of LigZ and LigY are required. The close interaction between LigZ and LigY might be needed for the efficient transformation of OH-DDVA to 5CVA. The close physical association between *meta*-cleavage pathway enzymes has been reported. Aldehyde dehydrogenase (acylating) is associated with the preceding enzyme, 4-hydroxy-2-ketovalerate aldolase, in the pathway from *Pseudomonas* sp. strain CF600 (17), and 2-oxopent-4-enoate hydratase is associated with the preceding enzyme, 4-oxalocrotonate decarboxylase, from *Pseudomonas putida* (4). In the latter case, the close association between two enzymes is supposed to ensure efficient transformation of the unstable intermediate by avoiding the conversion of the enol form to the keto form. Assuming a conversion between the enol and keto forms of the *meta*-cleavage compound of OH-DDVA, the association between LigZ and LigY seems to be advantageous. Further investigations are needed to address this notion.

We examined the activity of LigY on the *meta*-cleavage compounds formed during benzene, toluene, and biphenyl degradation. The substrates were enzymatically produced from catechol, 3-methylcatechol, and 2,3-dihydroxybiphenyl by using 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) of *Pseudomonas* sp. strain KKS102 (9). The crude enzyme preparations of BphC, LigZ, and LigY were added to the reaction mixture containing 100 μM substrate in 1 ml of 20 mM Tris-HCl (pH 7.5), and this mixture was then incubated for 30 min at 25°C.

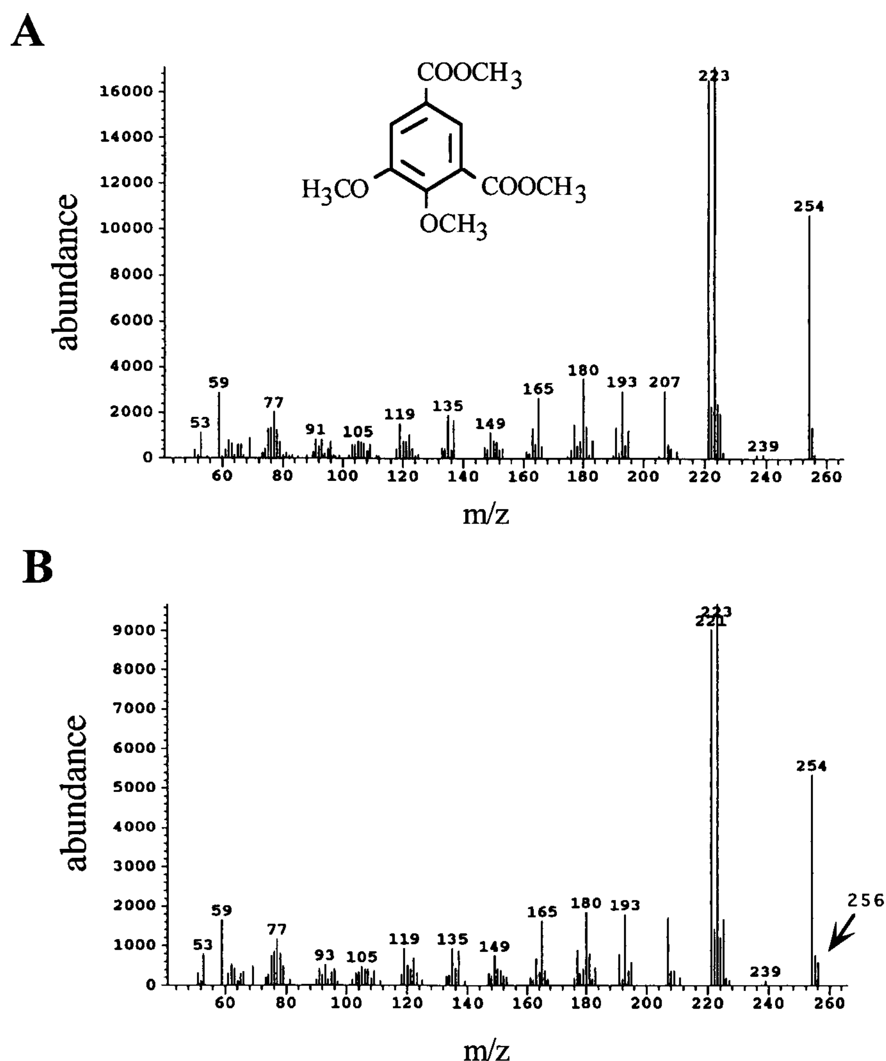

FIG. 5. (A) The mass spectrum of the methyl ester derivative of the product 5CVA. The inset shows the chemical structure of the 5CVA methyl ester derivative. (B) The mass spectrum of the methyl ester derivative of 5CVA produced in the presence of $H_2{}^{18}O$. The molecular ion that originated from ^{18}O-containing 5CVA is indicated by an arrow.

The absorbance spectrum of this reaction mixture was measured with a DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) to evaluate the transformation of a *meta*-cleavage compound. LigY did not exhibit hydrolase activity toward any of these *meta*-cleavage compounds, suggesting that the substrate specificity of LigY was restricted.

To confirm the hydrolysis reaction of a *meta*-cleavage compound of OH-DDVA catalyzed by LigY hydrolase, the incorporation of ^{18}O into 5CVA from $H_2{}^{18}O$ was examined. The reaction mixture of crude LigZ and LigY containing 9% $H_2{}^{18}O$ (Aldrich Chemical Company, Milwaukee, Wis.) was incubated with OH-DDVA for 3 h at 25°C, and the methyl ester derivative of metabolite 5CVA was analyzed by GC-MS. A molecular ion peak at an *m/z* of 256 was observed and is specific to the reaction with $H_2{}^{18}O$ (Fig. 5). Its abundance is about 8% of the molecular ion at an *m/z* of 254, which is mostly equivalent to the proportion of $H_2{}^{18}O$ in the reaction mixture. These results indicate that this molecular ion originated from 5CVA, in which ^{18}O was incorporated from $H_2{}^{18}O$, and prove the hydrolysis of a *meta*-cleavage compound of OH-DDVA catalyzed by LigY.

Nucleotide sequence accession number. The nucleotide sequence of *ligY* has been deposited in the DDBJ, EMBL, and GenBank sequence databases under accession no. AB018415.

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