Expression and Substrate Range of *Streptomyces* Vanillate Demethylase

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Vanillate is converted to protocatechuate by an O-demethylase consisting of VanA and VanB in *Streptomyces* sp. NL15-2K. In this study, vanillate demethylase from this strain was functionally expressed in *Escherichia coli*, and its substrate range for vanillate analogs was determined by an in vivo assay using recombinant whole cells. Among aromatic methyl ethers, vanillate, syringate, m-anisate, and veratrater were good substrates, whereas furulate, vanillin, and guaiacol were not recognized by *Streptomyces* vanillate demethylase. After vanillate, 4-hydroxy-3-methylbenzoate was a better substrate than m-anisate and veratrater, and the 3-methyl group was efficiently oxidized to a hydroxymethyl group. These observations suggest that the combination of a carboxyl group on the benzene ring and a hydroxyl group in the para-position relative to the carboxyl group may be preferable for substrate recognition by the enzyme. 1H-NMR analysis showed that the demethylation product of veratrater was isovanillate rather than vanillate. Therefore, it was concluded that O-demethylation of veratrater by *Streptomyces* vanillate demethylase occurred only at the meta-position relative to the carboxyl group.

Key words vanillate demethylase; *Streptomyces*; expression; substrate range

Lignin is a major component of plant biomass, and partial decay of lignin yields numerous aromatic compounds, such as vanillate and catechol, that have many applications in the cosmetics, food, pharmaceutical, and chemical industries. To develop a bioconversion system for lignin-related aromatic compounds, we have focused on the enzymology and genetics of this strain.1–4) The enzymatic pathway of the initiation codon ATG in *A.*2) The enzymatic pathway of the initiation codon ATG in *A.*

Vanillate demethylase is responsible for the conversion of vanillate to protocatechuate in the degradation of lignin-related aromatic compounds in bacteria. This enzyme from *Streptomyces* NL15-2K is a two-component monooxygenase (class I) consisting of oxygenase and reductase components encoded by vanA and vanB, respectively, and is assumed to convert vanillate to protocatechuate via hydroxylation of the O-methyl group, thereby forming an unstable hemiacetal that spontaneously decomposes into protocatechuate and formaldehyde.5,6) The *Streptomyces* vanA (1071bp) and vanB (936bp) genes are organized in a cluster and co-transcribed from a putative promoter that is located approximately 61bp upstream of the initiation codon ATG in vanA.2) The enzymatic characteristics of vanillate demethylases from *Pseudomonas* spp. and *Acinetobacter* sp. have been determined in studies using cell-free extracts or recombinant *Escherichia coli* cells transformed with the vanillate demethylase gene.7,9) Vanillate demethylase from *P. testosteroni* has a broad substrate range and shows activity towards m- and p-anisate in addition to vanillate,7) whereas the enzyme from *P. fluorescens* is inactive toward p-anisate.8) Similarly, although vanillate demethylase from *Acinetobacter* sp. has a wide substrate range, including m-anisate, veratrater, and 3,4,5-trimethoxybenzoate, it does not attack the 4-methoxy group relative to the carboxyl group.9) Additionally, all of these enzymes have not been purified because their demethylation activity is very sensitive to dilution and to oxidation by air.5,7,9) In a preliminary experiment, when a lysate was prepared from *Streptomyces* sp. NL15-2K cells exhibiting vanillate demethylase activity, activity of the enzyme was not detected in the lysate regardless of supplementation with cofactors such as reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). Therefore, *Streptomyces* vanillate demethylase was also assumed to be unstable. The other characteristics of this enzyme have not been elucidated. Here, we describe the construction of a plasmid for expression of the *Streptomyces* vanillate demethylase gene in *E. coli* and present data on the substrate range, which was determined using an in vivo assay with recombinant *E. coli* cells.

MATERIALS AND METHODS

**Bacterial Strains, Vector, Cultivation Media, and Chemicals** *Streptomyces* sp. NL15-2K was used for isolation of chromosomal DNA, which was extracted according to Hopwood et al.10) *E. coli* DH5α was used as the host strain for recombinant plasmid preparation. *E. coli* BL21(DE3) and pET-28a(+) (Novagen, U.S.A.) were used for protein expression. *E. coli* strains were routinely grown in LB broth or on LB agar. M9 medium was used for an in vivo assay with recombinant *E. coli* cells. When necessary, kanamycin (30 μg/mL) was added to the media. All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

**Plasmid Construction** The *Streptomyces vanA* and vanB genes (GenBank accession no. AB252870) were separately amplified to replace their own Shine-Dalgarno (SD) sequences with the pET-28a vector-derived SD sequence and inserted into pET-28a. Polymerase chain reaction (PCR) amplification was performed with PrimeSTAR®GXL DNA polymerase (TaKaRa Bio, Shiga, Japan). The primers used in this study are listed in...
Table 1. List of Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Restriction site</th>
<th>Specific use</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA-F1</td>
<td>CCCACATGTCCACATGACCGCC</td>
<td>PciI</td>
<td>PCR for vanA</td>
</tr>
<tr>
<td>vanA-F2</td>
<td>CCCGCTACGCTAACATTGTTTAC</td>
<td>Nhel</td>
<td>PCR for vanA with pET-derived SD sequence</td>
</tr>
<tr>
<td>vanA-R</td>
<td>AGCGCTACCTGACGTCGTCCT</td>
<td>SacI</td>
<td>PCR for vanA with pET-derived SD sequence</td>
</tr>
<tr>
<td>vanB-F</td>
<td>AGTCATAGCCGATGCTCAGAAGCC</td>
<td>BspHI</td>
<td>PCR for vanB</td>
</tr>
<tr>
<td>vanB-R</td>
<td>TTCATATGACCTAGCAGATCCAGATCCAGC</td>
<td>Ndel</td>
<td>PCR for vanB</td>
</tr>
</tbody>
</table>

Underlined sequences indicate restriction sites.
Safe Coomassie Stain (Bio-Rad, U.S.A.).

**NMR Analyses of Metabolites** Conversion of veratrulate, syringate, and 4-hydroxy-3-methylbenzoate was also analyzed by $^1$H-NMR spectroscopy. Each substrate was incubated with *E. coli* cells harboring pVDM-B/A according to the *in vivo* assay technique described later, except that the cell concentration, substrate concentration, and incubation time were changed from $1.6 \times 10^8$ cells/mL to $9.6 \times 10^9$ cells/mL, $0.36 \text{mm}$ to $3.6 \text{mm}$, and 60 min to 21 h, respectively. After incubation, the bacterial pellet was removed by centrifugation and the supernatant was acidified with dilute HCl aqueous solution. The mixture of residual substrate and metabolites was extracted with ethyl acetate and concentrated under reduced pressure to dryness. The $^1$H-NMR spectra (CD$_3$OD, Bruker Daltonics) with methanol (3.30 ppm) as an internal standard.

**In Vivo Assay for Recombinant Vanillate Demethylase Activity** Overnight cultures of *E. coli* BL21(DE3) harboring pVDM-B/A or pET-28a were diluted 1:100 into LB broth (25 mL in a 125-mL baffled flask) supplemented with kanamycin, and incubated at 30°C. When the OD$_{600}$ reached 0.8, IPTG (25 mL in a 125-mL baffled flask) supplemented with kanamy was added at 0.5 mM and the incubation was continued. After 4 h of incubation, the culture fluid was centrifuged at 8000×g for 5 min. The bacterial pellet was recovered and washed with M9 medium supplemented with 0.5% glucose, 0.5 mM IPTG, and kanamycin. The pellet was then suspended (1.6×10$^9$ cells/mL) in the same medium supplemented with 0.36 mM vanillate or other substrates and incubated at 30°C for 60 min. The residual substrates and their products in the culture supernatant were quantified by HPLC under the same conditions described above, except that 35% methanol–0.1% phosphoric acid was used for elution of m-anisate, m-hydroxybenzoate, m-toluolate, and 3,5-dimethoxybenzoate. Vanillate, veratrulate, vanillin, ferulate, m-anisate, 4-hydroxy-3-methylbenzoate, m-toluolate, guaiacol, 3,5-dimethoxybenzoate, and syringate were used as substrates for the *in vivo* assay. Compounds were identified by comparison with the retention times of authentic standards.

**RESULTS AND DISCUSSION**

To express the *Streptomyces* vanillate demethylase gene in *E. coli*, we constructed the plasmid pVDM-B/A, in which vanA and vanB were positioned to be co-transcribed from the T7lac promoter, and the pET-derived SD sequence was inserted just upstream of each gene (Fig. 1A). To evaluate the plasmid, *E. coli* cells harboring pVDM-B/A or pET-28a were incubated with 1 mM IPTG and 3.6 mM vanillate for 16 h. Two polypeptides of 40 kDa and 33 kDa, which were assumed to correspond to VanA and VanB products, respectively, were observed in the lysate of *E. coli* harboring pVDM-B/A (Fig. 1B). However, when the amounts of vanillate and protocatechuate in the culture supernatant were measured by HPLC, the percentage of vanillate converted to protocatechuate was 32.4% (Fig. 2B), which seemed to be slightly low relative to the amount of vanillate used in the experiment.

![Fig. 2. HPLC Analyses of Vanillate Demethylation by Recombinant *E. coli* Harboring pET-28a (A) or pVDM-B/A (B)](image)

Conversion of vanillate to protocatechuate by vanillate demethylase expressed in recombinant *E. coli* strains was monitored by HPLC. The weak peak at 4.6 min in chromatogram A was derived from the protocatechuate present as a trace impurity in the vanillate used in the experiment.

Veratrulate has two methoxy groups at the 3- and 4-positions of its benzoate and is demethylated by vanillate demethylase from strain NL15-2K. To identify the position where demethylation occurs, $^1$H-NMR analysis was performed. After incubation of *E. coli* BL21(DE3) harboring pVDM-B/A with 3.6 mm veratrulate, the veratrulate metabolites in the culture supernatant were extracted and analyzed by $^1$H-NMR spectroscopy. The extract included the residual veratrulate and a single product (44:56 molar ratio). The spectral data for veratrulate are δ 3.86 (3H, s, OCH$_3$), 3.89 (3H, s, OCH$_3$), 7.01 (1H, d, $J=8.4$ Hz, 5-H), 7.55 (1H, d, $J=1.8$ Hz, 2-H), 7.62 (1H, dd, $J=8.4$, 1.8 Hz, 6-H). The data for the product were δ 3.91 (3H, s, 4-OCH$_3$), 6.97 (1H, d, $J=8.4$ Hz, 5-H), 7.43 (1H, d, $J=1.8$ Hz, 2-H), 7.54 (1H, dd, $J=8.4$, 1.8 Hz, 6-H). The spectral data were comparable with those of authentic veratrurate and isovanillate. Because vanillate was not detected, demethylation by *Streptomyces* vanillate demethylase was concluded to occur only at the 3-position of veratrurate and not at the 4-position. Similarly, the products formed from syringate and 4-hydroxy-3-methylbenzoate were analyzed by $^1$H-NMR. In the extract of the culture supplemented with syringate, three molecular species, namely residual syringate, 3,4-dihydroxy-5-methoxybenzoate, and gallate, were identified; the respective molar ratio was determined to be approximately 50:39:11 from the spectra (Fig. 3). The $^1$H-NMR spectral data for syringate were δ 3.88 (6H, s, 3-OCH$_3$ and 5-OCH$_3$), 7.32 (2H, s, 2-H and 6-H). The data for 3,4-dihydroxy-5-methoxybenzoate were δ 3.87 (3H, s, 5-OCH$_3$), 7.17 (1H, d, $J=1.8$ Hz, 2-H or 6-H), 7.18 (1H, d, $J=1.8$ Hz, 2-H or 6-H). The data for gallate...
were \( \delta \) 7.05 (2H, s, 2-H and 6-H). We confirmed that the spectral data of syringate and the two demethylated products were comparable with those of authentic samples. This observation indicates that one of the two methoxy groups in the meta-position of syringate was demethylated first, and then the second methoxy group was demethylated at a slower rate. In contrast, the reaction with 4-hydroxy-3-methylbenzoate gave an oxidized product at the 3-position: 4-hydroxy-3-(hydroxymethyl)benzoate. The product structure was identified by \(^1\)H-NMR analysis: \( \delta \) 4.65 (2H, s, \( \text{C}_2\text{H}_2\text{OH} \)), 6.80 (1H, d, \( J=8.4 \text{ Hz} \), 5-H), 7.79 (1H, dd, \( J=8.4, 2.4 \text{ Hz} \), 6-H), 8.01 (1H, d, \( J=2.4 \text{ Hz} \), 2-H). Residual substrate and contaminants were not observed. Thus, Streptomyces vanillate demethylase is confirmed to also oxidize the 3-methyl group, yielding a hydroxymethyl group.
Next, we investigated the substrate range of vanillate demethylase for vanillate and its analogs using an in vivo assay. Vanillate was the best substrate among 10 aromatic compounds examined; the amount of vanillate decreased by 94% over 60 min (Fig. 4). After vanillate, 4-hydroxy-3-methylbenzoate was a better substrate than veratrate and m-anisate, suggesting that the combination of a carboxyl group on the benzene ring and a hydroxyl group in the para-position relative to the carboxyl group may be preferable for substrate recognition. This speculation is supported by the lack of detectable activity for guaiacol, vanillin, and ferulate, which do not have carboxyl groups on their benzene rings. Although syringate was a good substrate, it was demethylated at a lower rate than vanillate, indicating that the two methoxy groups in the meta-position may interfere with access to the enzyme active site. Furthermore, trace activity was detected towards 3,5-dimethoxybenzoate (2%) and m-toluate (1%). These results indicate that the vanillate demethylase from \textit{Streptomyces} sp. NL15-2K is different from homologous enzymes from \textit{P. fluorescens} and \textit{Acinetobacter} sp. with regard to substrate preference.

In this study, we expressed vanillate demethylase from \textit{Streptomyces} sp. NL15-2K in \textit{E. coli} and identified its substrate range for vanillate analogs using recombinant \textit{E. coli} whole cells. Because \textit{E. coli} is generally a more convenient and effective host for protein expression than \textit{Streptomyces}, the recombinant strain prepared in this study may also be potentially valuable as a whole-cell biocatalyst for bioconversion of aromatic methy ethers or methyl compounds. However, for this purpose, it is indispensable to improve the expression of recombinant vanillate demethylase in \textit{E. coli} cells. Control of inclusion body formation by heat-shock treatment is now under investigation in our laboratory.

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REFERENCES


