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# Characterization of *Fusarium oxysporum* β-1,6-Galactanase, an Enzyme That Hydrolyzes Larch Wood Arabinogalactan▽

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**A type II arabinogalactan-degrading enzyme (FoGal1) was purified from *Fusarium oxysporum* 12S, and the corresponding cDNA was isolated. FoGal1 had high similarity to enzymes of glycoside hydrolase family 5. Treatment of larch wood arabinogalactan with the recombinant enzyme indicated that FoGal1 is a β-1,6-galactanase that preferentially debranches β-1,6-galactobiose from the substrate.**

Type II arabinogalactans in the Western larch consist of a β-1,3-galactan backbone with many side chains at position 6 (15). About half of the main chain galactopyranose residues are replaced by β-1,6-galactopyranobiose, and about a quarter are replaced by single galactopyranose units. The rest of the side chains contain three or more glycosidic residues. Arabinofuranose and arabinopyranose residues exist at the nonreducing ends of both the β-1,3-galactan main chains and the side chains.

Fungi and plants make several type II arabinogalactan-degrading enzymes. These include two endo-β-1,6-galactanases, one acting on grape arabinogalactans from *Aspergillus niger* (2) and one acting on radish arabinogalactans from *Trichoderma viride* (13), a β-1,6-galactanase that acts on acid-treated larch wood arabinogalactan (LWAG) from *A. niger* (10), β-galactosidases from radish seeds (19) and spinach (5) that act on β-1,3 and β-1,6 galactosyl sequences of arabinogalactans but not pectic β-1,4 galactan, and two exo-β-1,3-galactanases (EC 3.2.1.145) from *Irpex lacteus* (22) and *A. niger* (14) that cleave the backbone of type II arabinogalactans in an exo-like manner. The latter-named enzymes are able to bypass the branching points of galactan backbones and to release the intact side chains of type II arabinogalactans and thus are very useful for the structural analysis of polysaccharides.

To analyze the fine structures of polysaccharides enzymatically, recombinant enzymes are preferred, as they exclude the confounding influence of contaminating carbohydrases. However, only four genes encoding type II arabinogalactan-degrading enzymes have previously been characterized: two exo-β-1,3-galactanases from *Phanerochaete chrysosporium* (7) and *Clostridium thermocellum* (6), an endo-β-1,6-galactanase from *T. viride* (9), and a β-galactosidase from radishes (8). In this paper, we describe the isolation and characterization of a β-1,6-galactanase, termed FoGal1, that is produced by *Fusarium oxysporum* 12S. In addition, we report the nucleotide sequence of the gene that encodes FoGal1 (Fo*gal1*) and describe the activity of the recombinant enzyme expressed in *Escherichia coli*.

The *F. oxysporum* 12S strain was isolated from a rotten sugar beet as part of a large-scale screening program (unpublished data). The microorganism is deposited in the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (accession no. FERM P-20380; http://unit.aist.go.jp/ipod/cie/index.html). To purify the FoGal1, the *F. oxysporum* 12S strain was cultivated in 1 liter of LWAG medium consisting of 0.2% $NH_4NO_3$, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4$, and 0.5% LWAG (Sigma-Aldrich) (pH 5) in a 3-liter shaking flask at 30°C for 10 days in a rotary shaker at 100 rpm.

α-L-Arabinofuranosidase and β-L-arabinopyranosidase activities were detected in the culture filtrate. To discriminate FoGal1 from these two enzymes, activities towards LWAG, *p*-nitrophenol (PNP) α-L-arabinofuranoside, and PNP β-L-arabinopyranoside were assayed during the purification steps of FoGal1. LWAG-degrading activity was assayed by incubating a reaction mixture containing 0.1% LWAG and an enzyme sample at 37°C. The release of reducing groups in the reaction mixture was measured using the method of Smogyi, with galactose as the standard (20). One unit of enzyme activity was defined as the amount of enzyme that formed reducing groups corresponding to 1 μmol of galactose in 1 min. Enzyme activity towards PNP glycosides was assayed as described previously (17) except that the concentration of substrates was 0.1%. The culture filtrate (1.4 liters) was adjusted to pH 6 with 0.1 N NaOH and put on a DEAE-Toyopearl 650 M column (Tosoh Corp., Tokyo, Japan) (3 cm inside diameter by 30 cm length) equilibrated with 20 mM sodium phosphate buffer (pH 6). Hardly any fraction exhibiting LWAG-degrading activity bound to this column. The fraction that did not bind to the column was collected, brought up to a 50% saturation of ammonium sulfate by adding pulverized ammonium sulfate crystals, and loaded onto a Butyl-Toyopearl column (Tosoh) (2 cm inside diameter by 30 cm length) equilibrated with the phosphate buffer containing ammonium sulfate at a concentration of 50% saturation. The adsorbed proteins were eluted by a linear gradient of ammonium sulfate (400 ml [from 50% to 0% saturation]). LWAG-degrading activity without any activities toward the two PNP glycosides was detected in this column

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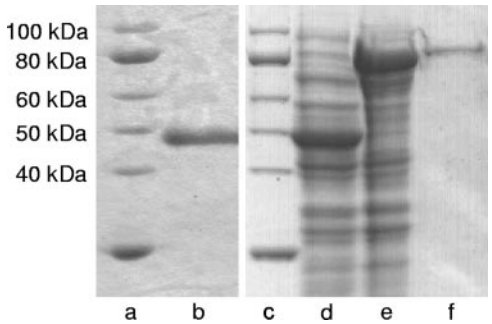


FIG. 1. SDS-PAGE of the native and recombinant FoGal1. a and c, protein standard; b, native FoGal1; d and e, cell extracts of *E. coli* harboring pMAL-c2X and pMAL-fogal1, respectively; f, MBP-FoGal1.

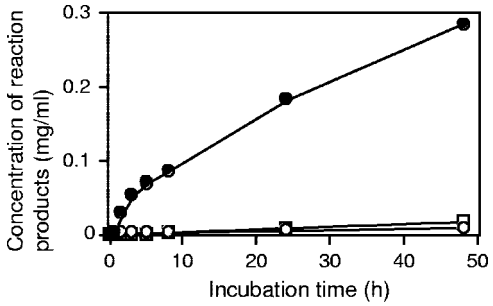


FIG. 3. Progression of products released by FoGal1 on LWAG. Closed circles, unknown compound; open circles, galactose; open squares, arabinose. Concentrations of the unknown compound were determined using the isolated compound as the standard.

chromatography assay. The fractions containing FoGal1 were collected, dialyzed using 20 mM sodium acetate buffer (pH 4), and put on a Mono S HR 5/5 column (Amersham Biosciences) equilibrated with the dialysis buffer. The bound proteins were eluted by a linear gradient of NaCl (40 ml [from 0 to 0.5 M]) in the buffer. The enzyme solutions were concentrated by centrifugal filtration with a 10-kDa cutoff filter (Ultrafree-MC; Millipore) and put on a Superdex 75 HR 10/30 size-exclusion column (Amersham Biosciences) equilibrated with 100 mM NaCl–20 mM sodium acetate buffer (pH 5). Proteins were eluted with the same buffer. This procedure represented an 88-fold purification of FoGal1 with a final yield of 1.4% and specific activity of 1.55 U/mg.

The molecular mass of FoGal1 was estimated to be 47 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The N-terminal amino acid sequence of the enzyme was determined to be AWPNGPFKTEGRWIV NSNG by use of a PSQ-1 protein sequencer (Shimadzu Corp., Kyoto, Japan). To study the effects of pH and temperature on enzyme activity, the enzyme reaction was performed at various pH values by using McIlvaine buffer (pH 3.4 to 8) at 37°C and at various temperatures in 20 mM sodium acetate buffer (pH 5). FoGal1 enzyme activity was highest under conditions of 50°C and pH 5 (Fig. 2). The temperature and pH stabilities were evaluated as described previously (17). After incubation of the enzyme at 60°C for 1 h, more than 80% of the initial enzyme activity remained (Fig. 2). More than 80% of the initial enzyme activity remained after 16 h of incubation at pHs from 4 to 10 at 30°C (Fig. 2). Sensitivity of the enzyme to 15 metals, consisting of $AgNO_3$, $FeSO_4$, and chloride salts of 13 cations, was examined by running the standard assay conditions in the presence of the metal (1 mM). Activity was reduced in the presence of several metals, including $Hg^{2+}$ (80% reduction), $Fe^{2+}$ (65% reduction), $Zn^{2+}$ (60% reduction), $Ag^{+}$ (50% reduction), $Cu^{2+}$ (45% reduction), and $Cd^{2+}$ (40% reduction), and was not affected by the presence of $Ba^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Fe^{3+}$, $K^{+}$, $Mg^{2+}$, $Mn^{2+}$, $Na^{+}$, or $Ni^{2+}$. Adding EDTA to the reaction mixture (1 mM) did not affect the activity, indicating that the enzyme does not require divalent metals for activity. The $K_m$ and $k_{cat}$ values of the purified enzyme acting on LWAG at pH 5 and 37°C were determined to be 0.504 mM and 15.0 $s^{-1}$. The average molecular mass of the substrate was estimated to be 20,000 Da by size-exclusion chromatography using a Superdex 200 HR 10/30 column (Amersham Biosciences).

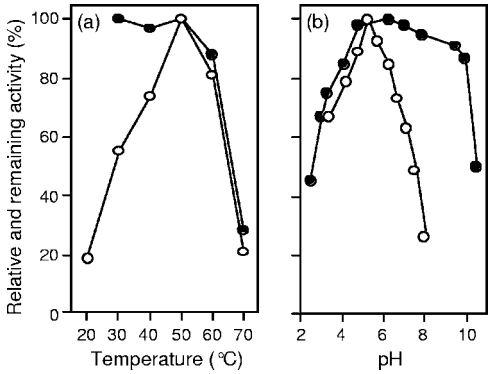


FIG. 2. Effects of temperature (a) and pH (b) on activity (open circles) and stability (closed circles) of FoGal1. Experimental conditions are described in the text.

To analyze reaction products of LWAG with FoGal1, a reaction mixture containing 5 ml of 0.1% LWAG in 20 mM sodium acetate buffer (pH 5) and 6 mU of the enzyme was incubated at 37°C for 30 min to 48 h. The products released into the mixture were quantified by high-performance anion-exchange chromatography (HPAEC) as described previously (18) except that a linear gradient of sodium acetate from 0 to 0.3 M was used. FoGal1 predominantly produced an unknown compound and small amounts of arabinose and galactose (Fig. 3). In order to identify the unknown compound, a large amount of it was isolated as follows. The purified FoGal1 (6 mU) was incubated with 4.5 ml of 20% LWAG in 20 mM sodium acetate buffer (pH 5) for 3 days at 37°C and boiled for 5 min. Three volumes of ethanol were added to the mixture to remove residual polymers. After centrifugation, the supernatant was concentrated under reduced pressure followed by separation of the unknown compound by use of a Bio-Gel P2 column (Bio-Rad) (2.5 cm inside diameter by 48 cm length) equilibrated with 20 mM NaCl. The products in the fractions were analyzed by HPAEC. The sugar composition of the isolated product was determined by HPAEC to consist only of galactose after hydrolysis with 1 M $H_2SO_4$ for 2 h at 100°C. The molecular mass of the compound was estimated to be 341.4 by matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (Kompact PROBE; Shimadzu). The glycosidic

TABLE 1. Chemical shifts of $^1$H- and $^{13}$C-NMR signals for the unknown compound as recorded in $D_2O$ at 80°C

| Sugar residue | $^1$H/$^{13}$C chemical shift(s) (ppm) for spectrum: | | | | | |
|---|---|---|---|---|---|---|
| | 1[a] | 2 | 3 | 4 | 5 | 6 |
| → 6)-α-D-galactopyranose | 5.26 (3.50) | 3.82 | 3.86 | 4.03 | 4.26 | 3.85, 4.03 |
| Reducing end | 95.0 | 71.1 | 71.5 | 72.1 | 72.1 | 71.9 |
| → 6)-β-D-galactopyranose | 4.58 (8.00) | 3.49 | 3.63 | 3.96 | 3.85 | 3.87, 4.04 |
| Reducing end | 99.1 | 74.7 | 75.5 | 71.4 | 76.4 | 71.8 |
| β-D-galactopyranose-(1→ | 4.43 (7.00) | 3.55 | 3.64 | 3.93 | 3.68 | 3.76, 3.77 |
| Nonreducing end | 105.8 | 73.6 | 75.5 | 71.4 | 77.7 | 63.7 |

[a] $J_{H\text{-}1,\ H\text{-}2}$ values are given in hertz in parentheses.

linkage of the unknown compound was confirmed by $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR) spectroscopy. The $^1$H- and $^{13}$C-NMR spectral data of the unknown compound are shown in Table 1. The $^1$H- and $^{13}$C-NMR spectra were recorded on a JEOL 500 MHz (JNM-A500) spectrometer in $D_2O$ at 80°C. NMR chemical shifts of $^1$H- and $^{13}$C-NMR spectra were measured with reference to internal acetone data ($\delta_H$ 2.22 and $\delta_C$ 32.8). Chemical shifts for the $^1$H- and $^{13}$C-NMR spectra were assigned using a $^{13}$C distortionless-enhancement-by-polarization-transfer 135 NMR spectrum, two-dimensional (2D) $^1$H-$^1$H-correlated spectroscopy, 2D total-correlation spectroscopy, 2D $^{13}$C-$^1$H heteronuclear-multiple-quantum-coherence spectroscopy, and previously published data (12, 21). The glycosidic linkage in the unknown compound was determined to be in the β configuration on the basis of the coupling constant ($J$ = 7.0 Hz) of the anomeric protons of the non-reducing-end galactopyranose residues. Signals from C-6 of reducing-end galactopyranose residues (δ 71.8 and 71.9) shifted to much higher fields compared to those of non-reducing-end galactopyranose residues (δ 63.7), demonstrating that the former carbon is involved in the glycosidic linkage. These findings indicate that the unknown compound is β-1,6-galactobiose.

The nucleotide sequence of the Fo*gal1* cDNA gene was determined by a combination of 3′ rapid amplification of cDNA ends and conventional PCR, using the N-terminal amino acid sequence of the native enzyme as a starting point. High-fidelity DNA polymerase (Phusion; Finzymes, Espoo, Finland) was used for amplifying gene fragments throughout the PCR experiments. The coding sequence of the Fo*gal1* gene contains 1,260 bp encoding a protein of 420 amino acids. The N-terminal amino acid sequence of the purified *F. oxysporum* 12S FoGal1 matched residues 21 to 39 of the deduced sequence. The polypeptide consisting of residues 21 to 420 has a calculated molecular mass of 45,240 Da, which was in good agreement with that of FoGal1. The deduced amino acid sequence of the Fo*gal1* gene had highest similarity to hypothetical proteins from *Gibberella zeae* (synonym, *Fusarium graminearum*) (UniProtKB/TrEMBL accession no. Q4HV24; 87% identity), *Chaetomium globosum* (accession no. Q2HBR0; 52% identity) and *Neurospora crassa* (accession no. Q7S526; 52% identity). FoGal1 had high similarity to glycoside hydrolase (GH) family 5 (Pfam accession no. PF00150; E value, 3.8e-5). No carbohydrate-binding module was found in FoGal1. Members of GH family 5 have eight invariant residues making up the active site (3, 4). Comparison of the deduced amino acid sequence of FoGal1 with those of members of GH family 5 suggested that these eight amino acids (R82, H159, N210, E211, H296, Y298, E337, and W371) were conserved in FoGal1. E211 and E337 appear to act as catalytic proton donor and nucleophile, respectively, based on previously published data (1, 11, 16, 23, 24).

To obtain the recombinant FoGal1 in *E. coli*, the DNA fragment encoding the mature enzyme was PCR amplified and ligated to pMAL-c2X vector (New England BioLabs), forming a new plasmid termed pMAL-fogal1. The recombinant FoGal1 expressed from the constructed plasmid was designed to be fused to maltose binding protein (MBP-FoGal1). To produce the recombinant enzyme, 0.1% of the overnight culture of *E. coli* DH5α transformant having pMAL-fogal1 was inoculated to LB medium containing ampicillin (50 μg/ml) and cultured at 37°C for 6 h. Then, isopropyl β-thiogalactopyranoside was added to result in a final concentration of 0.1 mM and incubation was continued at 15°C for 72 h. The cell extract was obtained by sonic treatment on ice. We tried to purify MBP-FoGal1 from the extract by affinity chromatography using an Amylose Resin column (New England BioLabs), but the protein did not bind to this column. Subsequently, the extract was diluted with 4 volumes of 20 mM sodium acetate buffer (pH 5) and loaded onto a CM-Toyopearl 650 M column (Tosoh) equilibrated with the acetate buffer. The bound proteins were eluted by a linear gradient (from 0 to 0.5 M) of NaCl. The active fractions were separated on a Mono S column as described for the purification of the native FoGal1. The purified MBP-FoGal1 showed a single band with molecular mass of 85 kDa by SDS-PAGE (Fig. 1).

Enzymatic characterizations were examined using MBP-FoGal1. Degrading activity of the recombinant enzyme was assayed using polysaccharides, which serve as the substrates for enzymes belonging to GH family 5. The enzyme was active towards LWAG but not towards several other polysaccharides, including carboxymethyl cellulose (Sigma-Aldrich), lupin β-1,4-galactan, wheat arabinoxylan, and galactomannan (Megazyme International Ireland Ltd., Ireland). To study the mode of action of MBP-FoGal1 on LWAG, a reaction mixture containing 0.3 mU of the enzyme and 0.4 ml of 0.5% substrate in 20 mM sodium acetate buffer (pH 5) was incubated at 37°C for 30 min followed by analysis of the products with HPAEC. The enzyme released predominantly β-1,6-galactobiose accompanied by only trace amounts of galactose and arabinose from the substrate (Fig. 4). This result is similar to data obtained with the native FoGal1, suggesting that MBP does not

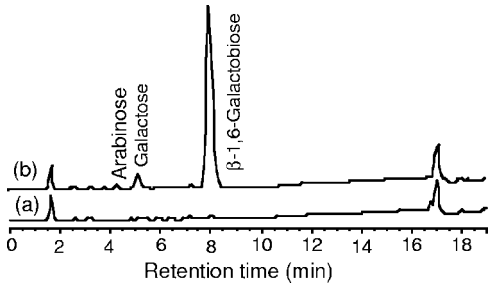


FIG. 4. HPAEC analysis of the enzymatic products of LWAG with MBP-FoGal1 before (a) and after (b) the enzyme reaction.

influence the catalytic property of the enzyme. Based on the structure of LWAG and the release of a large amount of β-1,6-galactobiose by FoGal1 from the substrate, the enzyme appears to preferentially split β-1,6-linkages between galactose residues of the branching points of β-1,6-galactobiose side chains. β-1,6-Galactobiose was slightly degraded by MBP-FoGal1. The enzyme had activity towards PNP β-D-galactopyranoside and α-L-arabinopyranoside, with the ratio of their activities being 1:3, and no activity towards PNP α-L-arabinofuranoside and β-L-arabinopyranoside. The activity on two PNP glycosides is attributed to their ring structures, which are similar.

The FoGal1 purified here was identified as a β-1,6-galactanase, although it remains unclear whether the enzyme can degrade a linear β-1,6-galactan polymer in an endo-like manner, because we were unable to obtain β-1,6-galactan polymer. Three other fungal β-1,6-galactanases have been previously described. *A. niger* endo-β-1,6-galactanase (2) has a molecular mass of 60 kDa and an optimal pH of 3.5 and is thermostable up to 30°C. These properties are quite different from those of FoGal1. *A. niger* β-1,6-galactanase (10) has a molecular mass of 58 kDa. The N-terminal amino acid sequence of the enzyme was determined to be ISSSPLSTSGG(N)IVD (parentheses indicate uncertainty), which is unlike that of FoGal1. The endo-β-1,6-galactanase gene from *T. viride* was sequenced (UniProtKB/TrEMBL accession no. Q76FP5) and was classified as belonging to GH family 5 (9). The deduced amino acid sequence of Fo*gal1* showed no similarity to that of the gene encoding the *T. viride* endo-β-1,6-galactanase. Together, these results suggest that FoGal1 is a new member of the β-1,6-galactanase family.

**Nucleotide sequence accession number.** The coding sequence of the Fo*gal1* gene has been deposited in DDBJ/EMBL/GenBank under accession number AB262781.

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