Characterization of a Novel β-Glucosidase from a Compost Microbial Metagenome with Strong Transglycosylation Activity*

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The β-glucosidase encoded by the td2f2 gene was isolated from a compost microbial metagenomic library by functional screening. The protein was identified to be a member of the glycoside hydrolase family 1 and was overexpressed in Escherichia coli, purified, and biochemically characterized. The recombinant β-glucosidase, Td2F2, exhibited enzymatic activity with β-glucosidic substrates, with preferences for glucose, fucose, and galactose. Hydrolysis occurred at the nonreducing end and in an exo manner. The order of catalytic efficiency for glucodisaccharides and cellooligosaccharides was sophorose end and in an exo manner. The order of catalytic efficiency for cellotetraose > cellotriose > laminaribiose > cellobiose > cellobiose > cellobiose > laminaribiose, respectively. Intriguingly, the p-nitrophenyl-β-D-glucopyranoside hydrolisis activity of Td2F2 was activated by various monosaccharides and sugar alcohols. At a D-glucose concentration of 1000 mM, enzyme activity was 6.7-fold higher than that observed in the absence of D-glucose. With 31.3 mM D-glucose, Td2F2 catalyzed transglycosylation to generate sophorose, laminaribiose, cellobiose, and gentiobiose. Transglycosylation products were detected under all activated conditions, suggesting that the activity enhancement induced by monosaccharides and sugar alcohols may be due to the transglycosylation activity of the enzyme. These results show that Td2F2 obtained from a compost microbial metagenome may be a potent candidate for industrial applications.

β-Glucosidases (β-glucoside glucohydrolases, EC 3.2.1.21) are widely distributed in bacteria, fungi, plants, and animals and play vital roles in many biological processes, such as biomass conversion by microbes and insects (1, 2), biogenesis of various functional molecules (e.g. terpenols and flavonoids) from glycoside precursors (3, 4), and cyanide-based biological defense mechanisms (the release of cyanide from cyanoglucosides) in plants (5). Recent interest in β-glucosidases arose from their involvement in the biological conversion of cellulose. β-Glucosidases are essential for efficient cellulolysis systems and are important for the degradation of cellooligosaccharides, particularly the breakdown of cellobiose to yield glucose. In addition, β-glucosidases also release product inhibition on cellobiohydrolases and endoglucanases, which are two major enzymes responsible for the degradation of cellulose. Both of these catalytic functions of β-glucosidases are critical to various biorefinery processes, such as bioethanol production (1). However, most β-glucosidases are very sensitive to the presence of glucose, a primary product of β-glucosidase catalysis (6–8). The search for a β-glucosidase that is insensitive to product inhibition by glucose has been newly reinvigorated.

Recently, due to their ability to activate glycosidic bonds, β-glucosidases have been considered as promising biocatalysts for the synthesis of stereo- and regiospecific glycosides or oligosaccharides, which are in turn are potentially useful as functional materials, nutraceuticals, or pharmaceuticals because of their biological recognition, signaling mechanisms, and antibiotic properties (1). In addition to hydrolytic activities, β-glucosidases may catalyze glycosidic bond formation via either a thermodynamically controlled reverse hydrolysis or a kinetically controlled transglycosylation (9). Although glycosyltransferases are highly efficient in syntheses of glycosidic linkages, their application to industrial processes is limited by expensive nucleotide sugar precursors, narrow substrate specificity, and low enzyme availability. In contrast, β-glucosidases are attractive for large scale applications because they are more abundant, commercially available, and exhibit a relatively broad acceptor-substrate specificity with simple substrates (10). However, their use in glycoside synthesis is limited by lower yields to industrial applications.

The need for more suitable enzymes has fueled the search for novel β-glucosidases. Here, we report the cloning, heterologous expression, and detailed enzymatic characterization of a glyco-

Background: There is an ongoing search for a β-glucosidase that has insensitivity to product inhibition.

Results: A β-glucosidase, Td2F2, was isolated from a metagenomic library and has high glucose tolerance, and its activity is enhanced by various monosaccharides.

Conclusion: The high glucose tolerance of Td2F2 is related to high transglycosylation activity.

Significance: This is the first report of a β-glucosidase having high glucose tolerance and high transglycosylation activity.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) HV538882.

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The abbreviations used are: GH1, glycoside hydrolase family 1; pNP, p-nitrophenol; pNPGLc, p-nitrophenyl-β-D-glucopyranoside; pNPFuc, p-nitrophenyl-β-D-fucopyranoside; pNPGal, p-nitrophenyl-β-D-galactopyranoside; HPAE-PAD, high performance anion exchange chromatography with pulsed amperometric detection; CAD, charged aerosol detector.

**β-Glucosidase with Strong Transglycosylation Activity**

side hydrolase family 1 (GH1) protein, named Td2F2, from a compost microbial metagenome. According to our results, Td2F2 exhibits the highest potential for use not only in cellulosic ethanol production but also in the synthesis of stereo- and regiospecific glycosides, which is afforded by insensitivity to glucose inhibition and a relatively high transglycosylation activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases, DNA ligase, and DNA polymerase were purchased from Takara Bio (Shiga, Japan). A QIAquick Gel Extraction kit was used to purify DNA restriction fragments and polymerase chain reaction (PCR) products and was obtained from Qiagen (Hilden, Germany). All chromogenic substrates were purchased from Sigma-Aldrich. Cellooligosaccharides, laminarioligosaccharides, xylolbiose, and chitobiose were acquired from Seikagaku Kogyo (Tokyo, Japan). Gentiobiose was purchased from Tokyo Chemical Industry (Tokyo, Japan). Isoprimeverose was obtained from Megazyme (Wicklow, Ireland). Curdlan was purchased from Wako Pure Chemical Industries (Osaka, Japan). Phosphoric acid-swollen cellulose was prepared from Avicel (Wako Pure Chemical Industries) as described previously (11). Other mono- and disaccharides, including β-glucoronolactone and deoxyxojirimycin, were purchased from Sigma-Aldrich.

**Construction of Metagenomic Library and Screening**—High molecular weight metagenomic DNA was prepared from compost according to the protocol of Uchiyama et al. (12). We prepared a compost in which bark and humus were mixed and fermented for 3 years, a fermentation that was accelerated by inoculation with *Tricoderma* strains. Metagenomic DNA was partially digested with Sau3AI and separated by gel electrophoresis. DNA fragments of 5 kb were recovered and ligated into a BamHI-digested p18GFP vector (12), and the product was transformed into *Escherichia coli* DH10B (Invitrogen). The screening of the clones with β-glucosidase activity was performed on LB agar plates supplemented with 0.1 mM isoprimeverose (pH 7.4) with BugBuster (Novagen) and Benzonase (Novagen). The crude extract was incubated for 30 min at room temperature. Cell debris was removed by centrifugation at 4 °C and 15,000 × g for 20 min. The supernatant was filtered and run through a Ni²⁺-nitrilotriacetic acid affinity column (Novagen) to purify the recombinant enzyme, Td2F2. Multiple washes with washing buffer (20 mM sodium phosphate buffer (pH 7.4), 0.5 mM NaCl, 25 mM imidazole) were performed, and the enzyme was eluted with a linear gradient of 20–500 mM imidazole. Enzyme fractions were concentrated using an ultrafiltration membrane (Amicon Ultra, Millipore (Billerica, MA)). The concentrated enzyme solutions were then applied to a GE Healthcare gel filtration column (HiLoad 16/600 Superdex 200 prep grade) and eluted with 50 mM sodium phosphate buffer (pH 6.0) containing 0.5 mM NaCl at a flow rate of 1.0 ml min⁻¹. The enzyme fractions were again concentrated using an ultrafiltration membrane (Amicon Ultra). The purity of the resulting Td2F2 was verified by SDS-PAGE, and the enzyme was stored at 4 °C. Protein concentration was estimated from the solution absorbance at 280 nm using a molar extinction coefficient calculated from the amino acid composition (13).

**Molecular Mass Estimation of Td2F2**—The molecular mass of native state Td2F2 was estimated by gel filtration through a GE Healthcare gel filtration column (HiLoad 16/600 Superdex 200 prep grade) in 50 mM sodium phosphate buffer (pH 6.0) containing 0.5 mM NaCl at a flow rate of 0.8 ml min⁻¹. The molecular weight standards were aldolase from rabbit muscle (158 kDa), conalbumin from chicken egg white (75 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), ribonuclease A from bovine pancreas (13.7 kDa), and aprotinin from bovine lung (6.5 kDa).

**Effects of pH and Temperature on Enzymatic Activity**—The optimal pH for Td2F2 activity with p-nitrophenyl-β-D-glucopyranoside (pNPGLc) was evaluated at 75 °C for 5 min in McIlvaine’s buffer (14) over a pH range of 3.0–8.5. pH stability was determined by measuring residual enzyme activity after incubation at 25 °C for 30 min in appropriate buffers. The effect of temperature on enzyme activity for pNPGLc was measured from 20 to 85 °C while incubating in buffer at pH 5.5 for 5 min. The thermostability of Td2F2 was determined by incubating the enzyme at various temperatures (20–85 °C) in 20 mM Tris-base (pH 7.4), 0.5 mM NaCl, 25 mM imidazole, and 1 ampicillin. Positive clones were detected by the formation of blue colonies following incubation at 37 °C for 14 h. The colonies were then incubated at 4 °C for 1 week. The resulting clones were reexamined for β-glucosidase activity using the same type of indicator plates.

**Cloning, Expression, and Purification of the β-Glucosidase Td2F2**—A potential open reading frame of a β-glucosidase gene (termed *td2f2*) was identified in the inserted DNA fragments. The *td2f2* gene was amplified by PCR using the primer pair 5'−TTTTTGAATATGGCAGGAGAACGATTCCCG−3' and 5'−AAAAAAAAAGCTTATGCCGCCGTTCGGCGCATG−3'. This introduced Ndel and HindIII sites, respectively (underlined), at each end of the gene. The PCR product was then purified and digested with Ndel and HindIII. The digested fragment was ligated into pET-15b (Novagen, Madison, WI), which was then subjected to the same digestion treatment to generate the plasmid pJTtd2f2 with six histidine residues attached to the N-terminal amino acid of Td2F2. *E. coli* BL21(DE3) harboring pJTtd2f2 was grown in 1 liter of Overnight Express Instant LB Medium (Novagen), supplemented with 100 μg ml⁻¹ ampicillin, at 37 °C. Growth was allowed for 24 h. Afterward, the cells were harvested by centrifugation at 4 °C and 5000 × g. The resulting cell pellet was suspended in 20 mM sodium phosphate buffer (pH 7.4) with BugBuster (Novagen) and Benzonase (Novagen). The crude extract was incubated for 30 min at room temperature. Cell debris was removed by centrifugation at 4 °C and 15,000 × g for 20 min. The supernatant was filtered and run through a Ni²⁺-nitrilotriacetic acid affinity column (Novagen) to purify the recombinant enzyme, Td2F2. Multiple washes with washing buffer (20 mM sodium phosphate buffer (pH 7.4), 0.5 mM NaCl, 25 mM imidazole) were performed, and the enzyme was eluted with a linear gradient of 20–500 mM imidazole. Enzyme fractions were concentrated using an ultrafiltration membrane (Amicon Ultra, Millipore (Billerica, MA)). The concentrated enzyme solutions were then applied to a GE Healthcare gel filtration column (HiLoad 16/600 Superdex 200 prep grade) and eluted with 50 mM sodium phosphate buffer (pH 6.0) containing 0.5 mM NaCl at a flow rate of 1.0 ml min⁻¹. The enzyme fractions were again concentrated using an ultrafiltration membrane (Amicon Ultra). The purity of the resulting Td2F2 was verified by SDS-PAGE, and the enzyme was stored at 4 °C. Protein concentration was estimated from the solution absorbance at 280 nm using a molar extinction coefficient calculated from the amino acid composition (13).

**Assays of β-Glycosidase Activity**—The activity of Td2F2 was assayed for 5 min at 75 °C in 100 mM sodium acetate buffer (pH 5.5) using substrates containing the nonphysiological chromogenic aglycone p-nitrophenol (pNP) or natural oligosaccha-
rides. The total reaction volume when using the chromogenic substrates was 20 μl and contained 0.05 μg of the enzyme. To stop the reaction, 50 μl of 1.0 M sodium bicarbonate was added to the reaction, and the concentration of pNP was determined by measuring the solution absorbance at 405 nm (Infinite M200 PRO, Tectec (Zurich, Switzerland)). For oligosaccharides, the total reaction volume was 10 μl and contained 0.5 μg of the enzyme. The reaction was stopped by heating the sample to 98 °C for 10 min. The amount of glucose, liberated as a product of the enzymatic reaction, was measured using a glucose oxidase-peroxidase assay (Amplex Red Glucose/Glucose Oxidase Assay Kit, Invitrogen) in accordance with the manufacturer’s protocol. In all assays, spontaneous hydrolysis of the substrate was accounted for by assay of blank mixtures, which lacked the enzyme. All measurements were performed in triplicate.

**Kinetic Analysis of Td2F2** — The kinetic parameters of Td2F2 were determined using pNPGlc, p-nitrophenyl-β-D-fucopyranoside (pNPFuc), p-nitrophenyl-β-D-galactopyranoside (pNPGal), cellobiosaccharides (di-, tri-, tetra-, and penta-), sophorose, laminarigosaccharides (di-, tri-, tetra-, and penta-), gentiobiose, and lactose as substrates. The reaction conditions and the methods used to detect enzymatic activity are described above. The reaction velocity was determined at seven different substrate concentrations from 0.5 to 5.0 × K_{m} where possible for each substrate. The kinetic constants K_{m} and k_{cat} were calculated by a nonlinear regression of the Michaelis-Menten equation using GraphPad PRISM version 5.0 (GraphPad Software, La Jolla, CA).

**Effects of Inhibitors and Additives** — The inhibitory effects (K_{i} values) of δ-glucosonolactone and deoxynojirimycin on the reaction of the enzyme with pNPβGlc were determined at three substrate concentrations (2.5, 5, and 10 mM) and five concentrations of inhibitor (5.0–40 mM δ-glucosonolactone and 1.6–25 mM deoxynojirimycin). δ-Glucosinolate solutions were always applied within 5 min of preparation. K_{i} values were determined from Dixon plots. The effects of additives were evaluated by measuring enzyme activity in the presence of various metals, EDTA, ethanol, and DMSO and co-incubating the enzyme solution and 5 mM pNPβGlc at 75 °C and pH 5.5 for 5 min following the measurement of pNP levels.

**Analysis of Cellulose Degradation** — Cellulose degradation was analyzed using a high performance anion exchange chromatograph with pulsed amperometric detection (HPAE-PAD; ICS-3000, Dionex (Sunnyvale, CA)). The column was precolumn with CarboPac PA-1 (4 × 250 mm; Dionex). Solvents A (100 mM NaOH), B (100 mM NaOH, 1 M sodium acetate), and C (water) were used as eluents (flow rate 1 ml min^{-1}). The eluent program was 10–80% A/(A + C) from 0 to 15 min in gradient mode, 0–2% B/(A + B) from 15 to 20 min in gradient mode, 2–10% B/(A + B) from 20 to 45 min in gradient mode, and 10% B/(A + B) from 45 to 50 min in linear mode. Cellulose was identified and quantitated by comparing its retention time and peak area with those of an authentic standard.

**Kinetic Analysis of Td2F2 Activity with Monosaccharides** — The kinetic parameters of Td2F2 with monosaccharides were determined using 140 or 1000 mM D-glucose, D-fucose, D-galactose, L-arabinose, D-mannose, and D-xylene with pNPβGlc as a substrate. The reaction conditions and the detection of enzymatic activity are described above. The reaction velocity was determined at 6–9 pNPβGlc concentrations (0.5–5.0 × K_{m}). The calculation of kinetic constants is described above.

**Analysis of Transglycosylation Products** — Transglycosylation products were analyzed by high performance liquid chromatography (LC-2000 series; Jasco, Tokyo, Japan) using a Corona® charged aerosol detector (Corona® CAD, ESA Biosciences (Chelmsford, MA)) and a prepacked Shodex Asahipak NH2P-50 column (4.6 × 250 mm; Showa Denko K. K. (Kanagawa, Japan)). The eluent was water with a linear gradient of CH_{3}CN from 80 to 74% over 30 min. The column was kept at 40 °C and eluted at 1.0 ml min^{-1}. Glucosidascardases were identified and quantitated by comparing retention times and peak areas with those of authentic standards. The reactants were pretreated for 16 h at 37 °C with glucose oxidase from *Aspergillus niger* (Wako Pure Chemical Industries) to degrade excess D-glucose prior to analysis by Corona® CAD.

**RESULTS**

**Screening of Clones with β-Glucosidase Activity from the Metagenomic Library** — Many positive clones exhibiting β-glucosidase activity were screened from the metagenomic library. The plasmid DNA for each positive clone was retransformed into *E. coli*. The transformants exhibited β-glucosidase activity, indicating that the enzyme was encoded in the plasmid inserts. One of these positive clones, designated pt2d2f2, was selected for further detailed characterization due to its relatively high β-glucosidase activity (pNPβGlc degradation) activated by excess D-glucose.

**Sequence Analysis of Td2F2** — The insert in pt2d2f2 was sequenced and revealed one open reading frame (named td2f2) of 1338 bp with a GC content of 67.2%. Td2F2 consisted of 445 amino acids with a predicted molecular mass of 49,343 Da. Comparisons with the BLAST database revealed that Td2F2 was similar to enzymes belonging to GH1 of a class of carbohydrate-active enzymes in the CAZy database. Td2F2 exhibited the highest identity (61%) with β-glucosidase of *Meiothermus ruber* DSM 1279 (UniProt accession number D3PLV5), which was revealed from whole genome sequencing and had not been biochemically characterized. The highest identity (52%) with an enzyme with experimentally evidenced β-glucosidase activity was found with GlyB from *Alicyclobacillus acidocaldarius* (GenBank™ accession number DQ092439) (15). The alignment of the amino acid sequence of Td2F2 with highly homologous GH1 enzymes is shown in Fig. 1. The region containing the catalytic acid, Asn^{165}-Glu^{166}, is well conserved. The region containing the catalytic nucleophile Glu^{352} in Td2F2 differs only slightly from the consensus pattern of (L/I/V/M/F/S/T/C)(L/I/V/F/Y/S)/(L/I/V)/(L/I/V/M/S/T)E^{352}NG(L/I/V/M/F/A/R)(C/S/A/G/N) as described by the PROSITE motif PS00572, which is well conserved among the GH1 enzymes. In Fig. 1, Td2F2 contains a Cys^{355} residue after the well conserved catalytic nucleophile region E^{352}NG. This feature is unique to Td2F2; other GH1 proteins have an Ala residue at this position. The amino acid residues related to subsites −1 and +1 are also shown in Fig. 1. These residues were identified from structural analyses of *Clostridium cellulosolvens* β-glucosidase, BglA (GenBank™ accession number AY268940) (16). Whereas sub-
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Recombinant Production of Td2F2—Recombinant Td2F2 enzyme was efficiently expressed in a soluble protein fraction in E. coli BL21(DE3). N-terminal His-tagged Td2F2 enzyme was purified to homogeneity from the soluble protein fraction by affinity chromatography and gel filtration (Fig. 2). The last purification step yielded about 2.4 mg of pure protein per liter of E. coli culture.

Gel filtration with suitable molecular weight standards revealed that the recombinant Td2F2 existed as a monomer of about 52 kDa under native conditions (Fig. 3). A molecular mass of 52 kDa agrees well with the predicted size of Td2F2 (49.3 kDa) plus the His tag and polylinker regions (2.2 kDa).

Properties of Td2F2—Td2F2 exhibited an optimal activity at pH 5.5 (Fig. 4A) when assayed with 1 mM pNPGlc at 75 °C. The last purification step yielded about 2.4 mg of pure protein per liter of E. coli culture.

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pNPGlcr, chromogenic substrates, not only pNPGlc but also pNPFu, was measured to determine substrate specificity. Among the temperatures lower than 65 °C.

Stability experiments indicated that the enzyme was stable at temperatures from 20 to 85 °C and exhibited substantial activity at temperatures higher than 85% of its original activity after incubation. Td2F2 showed substantial activity at temperatures from 20 to 85 °C and exhibited substantial activity at temperatures lower than 65 °C.

The hydrolytic activity of Td2F2 toward various substrates was measured to determine substrate specificity. Among the chromogenic substrates, not only pNPGlc but also pNPFu, pNPGal, p-nitrophenyl-β-D-celllobioside, and p-nitrophenyl-β-D-lactopyranoside were good substrates of Td2F2 (Table 1).

The enzyme showed very little activity with p-nitrophenyl-β-D-mannopyranoside, p-nitrophenyl-β-D-xylopyranoside, and p-nitrophenyl-α-L-arabinopyranoside, even after prolonged incubation for 1 h. No activity was observed with p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, p-nitrophenyl-β-L-arabinopyranoside, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-α-D-xylopyranoside, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-α-L-fucopyranoside, and p-nitrophenyl-α-L-rhamnopyranoside, even

| Substrate* | Linkage of glycosyl group | Relative activity % |
|------------|---------------------------|--------------------|
| pNPGlc     | β-Glucose                 | 100 ± 6.7b         |
| pNPFu      | β-Fucose                  | 198 ± 3.1          |
| pNPGal     | β-Galactose               | 197 ± 7.2          |
| p-Nitrophenyl-β-D-celllobioside | β-Cellobiose | 243 ± 0.3 |
| p-Nitrophenyl-β-D-lactopyranoside | β-Lactose | 46.8 ± 5.5 |

**Oligosaccharides (5 mM)**

| Oligosaccharide | Linkage of glycosyl group | Relative activity % |
|-----------------|---------------------------|--------------------|
| Cellobiose      | β (1,4)-Glucose           | 100 ± 4.3c         |
| Cellotriose     | β (1,4)-Glucose           | 175 ± 5.4          |
| Celloctaose     | β (1,4)-Glucose           | 145 ± 8.4          |
| Celloctaose     | β (1,4)-Glucose           | 847 ± 53           |
| Celloctaose     | β (1,4)-Glucose           | 47.7 ± 6.2         |
| Laminaritriose  | β (1,3)-Glucose           | 112 ± 3.1          |
| Laminaritriose  | β (1,3)-Glucose           | 116 ± 10           |
| Laminaritriose  | β (1,3)-Glucose           | 23.6 ± 2.9         |
| Laminaritriose  | β (1,3)-Glucose           | 72.1 ± 7.6         |
| Laminaritriose  | β (1,3)-Glucose           | 154 ± 1.6          |
| Laminaritriose  | β (1,3)-Glucose           | 129 ± 1.7          |
| Laminaritriose  | β (1,3)-Glucose           | 137 ± 8.6          |

* No activity or poorly activity was detected with p-nitrophenyl-β-D-mannopyranoside, p-nitrophenyl-β-D-xylpyranoside, p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, p-nitrophenyl-β-L-arabinopyranoside, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-α-D-xylopyranoside, p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-α-L-fucopyranoside, and p-nitrophenyl-α-L-rhamnopyranoside, even

| Substrate | Relative activity (%) |
|-----------|----------------------|
| pNPGal    | 12.1 ± 0.6           |
| pNPFu     | 1.6 ± 0.2            |
| pNPGal    | 2.9 ± 0.1            |
| p-Nitrophenyl-β-D-celllobioside | 6.7 ± 0.3 |
| p-Nitrophenyl-β-D-lactopyranoside | 84.7 ± 5.5 |

**FIGURE 3.** Plot of the molecular weights of protein markers (solid circles) and the elution volume/void volume ratio. Td2F2 was eluted at 52 kDa (open circle).

**FIGURE 4.** The enzymatic activity (solid circles) and stability (open circles) of Td2F2 are shown at various pH values (A) and temperatures (B). Error bars, S.D.
after prolonged incubations. The enzyme acted on \( \beta \)-glycosidic linkages, such as those in cellooligosaccharides (cellbiose to cellohexaose), sophorose, laminarioligosaccharides (laminaribiose to laminarintriaose), gentiobiose, and lactose. Td2F2 exhibited a very low activity with laminarhexaose after a prolonged incubation of 1 h. No enzymatic activity was observed with trehalose, kojibiose, nigerose, maltose, and isomaltose, which have \((1,1), (1,2), (1,3), (1,4), \) and \((1,6)\) glycosidic linkages, respectively. Insoluble \( \beta \)-glucans, such as phosphoric acid-swollen cellulose \((\beta(1,4)-\text{glucan})\) and curdlan \((\beta(1,3)-\text{glucan})\) were likewise not substrates of Td2F2. Analysis of degradation products by HPAE-PAD revealed that sucrose, isopentulose, gentiobiose, and xylobiose were also not substrates of Td2F2. Only cellotriose and glucose were produced during a time course HPAE-PAD analysis of cellotetraose hydrolysis. The enzyme showed a greater hydrolytic efficiency for pNPFuc and pNPGlc. Among the glucodisaccharides, sophorose \((\text{1,2})\), cellobiose \((\beta(1,4)-\text{glucan})\) and curdlan \((\beta(1,3)-\text{glucan})\) were likewise not substrates of Td2F2. Analysis of degradation products by HPAE-PAD revealed that sucrose, isopentulose, gentiobiose, and xylobiose were also not substrates of Td2F2. Only cellotriose and glucose were produced during a time course HPAE-PAD analysis of cellotetraose hydrolysis. This confirms an exo type of activity for Td2F2 (data not shown). A comparison of enzymatic activity with various substrates confirmed that Td2F2 is specific for \( \beta \)-glycosidic substrates, with preferences for the nonreducing ends of glucose, fucose, and galactose.

**Kinetic Constants of Td2F2**—The steady-state kinetic parameters of Td2F2 were measured for those \( \beta \)-glycosides shown to be good substrates. Table 2 summarizes these data for three aryl \( \beta \)-glycosides, a variety of cellooligosaccharides, and laminarioligosaccharides, sophorose, gentiobiose, and lactose. The enzyme showed a greater hydrolytic efficiency for pNPFuc and pNPGlc. Among the glucodisaccharides, sophorose \((\beta(1,2)-\text{linked glucose})\) was the most easily hydrolyzed. Enzymatic activity decreased with the other substrates in the following order: laminaribiose \((\beta(1,3))\), celllobiose \((\beta(1,4))\), and gentiobiose \((\beta(1,6))\). A different efficacy was observed with cellooligosaccharides, with cellotetraose being the most easily degraded. Activity then decreased in the following order: cellotriose, -biose, and -pentaose. The observed hydrolizing efficacy was again slightly different for laminarioligosaccharides. The laminaritriose was the most easily degraded, with activity decreasing in the following order: laminaribiose, -pentaose, and -tetrose.

**Effects of Inhibitors, Metals, and Other Reagents on Td2F2 Activity**—The kinetics of Td2F2 inhibition were determined using pNPGlc as a substrate and \( \delta \)-gluconolactone and deoxynojirimycin as inhibitors. Dixon plots indicated competitive inhibition for both inhibitors with \( K_i \) values of 4.56 ± 0.64 and 32.5 ± 1.09 mM, respectively.

The effects of various metals and regents on Td2F2 activity were also investigated (Table 3). Significant inactivation was observed with ZnCl₂ and FeCl₃. In contrast, enzyme activity was stimulated by MnCl₂. The addition of 10% (v/v) ethanol resulted in a slight enhancement in activity, whereas 25% (v/v) ethanol dramatically reduced enzyme activity. The addition of 10% or 25% (v/v) DMSO resulted in a slight activity enhancement. EDTA did not affect enzyme activity.

**D-Glucose Activation of Td2F2**—The activity of Td2F2 was enhanced with increasing concentrations of D-glucose (Fig. 5). With 1000 mM D-glucose, and 10 mM pNPGlc as substrate, the hydrolysis rate of pNPGlc was 6.7 times that of the control, which did not contain D-glucose (Fig. 5A). This same phenomenon was also observed with 10 mM cellbiose as the substrate. In this case, 1000 mM D-glucose increased the hydrolysis activity by a factor of 2.4 over the control (Fig. 5B).

Transglycosylation products, namely gentiobiose, laminaribiose, and sophorose (data not shown), were detected by HPAE-PAD as the products of Td2F2 enzymatic activity with 1.25, 5, or 10 mM cellbiose and 125–1000 mM D-glucose. In contrast, when Td2F2 was incubated with only cellbiose, the major product was D-glucose, and transglycosylation products were not detected.

**Kinetic Analysis of Td2F2 Activity with Monosaccharides**—The effects of 140 and 1000 mM D-glucose, D-fucose, D-galactose, L-arabinose, D-mannose, and D-xylose on Td2F2 activity were evaluated using pNPGlc as a substrate (Table 4). The addition of monosaccharides increased both \( K_m \) and \( k_{\text{cat}} \). The most significant change in \( K_m \) was observed with 1000 mM D-fucose, which raised \( K_m \) by a factor of 14.8. The most significant change in \( k_{\text{cat}} \) was observed with the addition of 1000 mM D-glucose, which increased \( k_{\text{cat}} \) by a factor of 10.8.

The ratio of \( k_{\text{cat}} \) to \( K_m \) was significantly different for each monosaccharide and concentration (Table 4). The addition of 1000 mM L-arabinose, D-mannose, and D-xylose increased \( k_{\text{cat}}/K_m \) by factors of 1.8, 1.4, and 1.3-fold, respectively, relative to \( k_{\text{cat}}/K_m \) values observed with 140 mM of monosaccharide. However, no significant changes were observed between the \( k_{\text{cat}}/K_m \) values for 140 and 1000 mM D-glucose and D-galac-
The effects of sugar addition for pNPGlc degradation efficiencies of the recombinant Td2F2 are summarized in Table 4. The addition of D-glucose, L-glucose, D-glucosamine, D-glucuronic acid, and D-galacturonate, respectively, reduced pNPGal degradation to 53, 41, 66, 62, and 72%. The addition of D-glucosamine also reduced the enzymatic degradation of pNPGlc and pNPFuc to 49 and 71%, respectively. This amino saccharide is a common inhibitor of the enzymatic activity of Td2F2.

The effects of various monosaccharides and their derivatives were examined by HPAE-PAD and TLC. The products included not only glucose but also the transglycosylation products of D-glucose, L-fucose, D-arabinose, L-arabinose, D-mannose, D-galactose, L-sorbose, L-rhamnose, D-xylene, xylitol, glucitol, mannitol, and arabitol (data not shown).

Transglycosylation Activity of Td2F2—Transglycosylation activity was demonstrated using 10 mM pNPglc as a substrate and 0–1000 mM D-glucose. Corona® CAD analyses revealed that the reaction of Td2F2 with pNPglc plus 31.3 mM D-glucose resulted in the transglycosylation products laminaribiose, cellobiose, sophorose, and gentiobiose (Fig. 7). When Td2F2 was incubated with only pNPglc, no transglycosylation products were detected. When the D-glucose concentration was elevated, the dominant transglycosylation product was sophorose, followed by laminaribiose.
β-Glucosidase with Strong Transglycosylation Activity

DISCUSSION

We report here the molecular cloning, expression in E. coli, and functional characterization of the product of the td2f2 gene from the compost microbial metagenome. Amino acid sequencing showed that Td2F2 belongs to GH1, with all of the conserved regions of GH1 represented. Substrate specificity analyses suggested that Td2F2 hydrolyzes aryl-β-glycosides, β-linked disaccharides, and β-linked short chain oligosaccharides but not aryl-α-glycosides, α-linked disaccharides, and long chain β-glucons. Td2F2 exhibited a broad specificity for β-glucosidases, which are the most commonly observed (1).

Kinetic analyses of Td2F2 revealed higher \( k_{cat}/K_m \) ratios for pNPfuc and pNPgal than for natural oligosaccharides. Several GH1 enzymes exhibit high specificity toward aryl-β-glycosides, such as chromogenic β-glucosides or arbutin, and salicin (15, 17, 18), in which the aglycone group is aromatic. Aromatic β-glucosides are found in plants, and the presence of specific enzymatic systems against such substrates suggests a specific

1000 mM D-glucose was used as a substrate without the addition of pNPgal, no transglycosylation or hydrolysis products were observed, indicating that transglycosylation is coordinated with pNPgal hydrolysis.
interaction of the corresponding microorganism with living plants. Because Td2F2 was cloned from a compost microbial metagenome, its enzyme action probably degrades complex polysaccharides from plant residues.

The $k_{cat}/K_m$ for hydrolysis of cellotetraose is 3.2- and 1.5-fold greater than that of cellobiose and cellotriose, respectively. This increase is due to the low $K_m$ of cellotetraose. This phenomenon is consistent with the kinetic results of GH1 $\beta$-glucosidase from *Sulfolobus solfataricus* (19). The crystal structure of $\beta$-glucosidase from *S. solfataricus* suggests that cellotetraose has a stronger interaction with a tandem array of subsites in the enzyme compared with cellobiose and cellotriose. This hypothesis supports the low $K_m$ of cellotetraose (20). The $k_{cat}/K_m$ for the hydrolysis of laminaripentaose is 6-fold greater than that for laminaritetraose. This increase is due to the high $k_{cat}$ of laminaripentaose, which may be due to steric hindrance at the active site and inhibition of laminaritetraose hydrolysis. Kinetic analyses of Td2F2 showed that the catalytic efficiency for $\beta$-linked glucosidases decreased in the order of sophorose > laminaribiose > cellobiose > gentiobiose, which indicates that a glycosyl group could be accommodated in several ways at the +1 subsite of Td2F2.

Two well characterized $\beta$-glucosidase inhibitors, $\delta$-gluconolactone and deoxyxojirimycin, inhibited Td2F2, which suggests that Td2F2 is a $\beta$-glucosidase rather than an exo-$\beta$-glucanase. The calculated $K_i$ values of 4.56 and 32.5 mM, respectively, suggest that Td2F2 would have a high resistance to these inhibitors (21). Various chemical reagents also affected the activity of Td2F2. Ten percent (v/v) ethanol and DMSO stimulated Td2F2 activity. Alcohol activation of some $\beta$-glucosidases due to glycosyltransferase activities has been observed (7, 22). Likewise, activation by organic solvents such as DMSO has been reported (22). The chelating agent EDTA did not inhibit Td2F2, indicating that divalent cations are not required for enzyme activation. However, the activity of Td2F2 was enhanced by the addition of Mn$^{2+}$. In previous studies, strong activation by Mn$^{2+}$ was observed with other GH1 $\beta$-glucosidases (16), which suggests that Mn$^{2+}$ ions might assist catalytic function. Td2F2 activity was strongly inhibited by Zn$^{2+}$ and Fe$^{3+}$. These metal ions may hinder substrate binding or inhibit the catalytic reaction at the active site.

Activity inhibition by glucose is a common characteristic of $\beta$-glucosidases (6–8). Most of the $\beta$-glucosidases that have been studied are competitively inhibited by their end product, glucose. However, several $\beta$-glucosidases are tolerant to, or may actually be stimulated by, glucose (17, 23–25). Using pNPGlC as a substrate, the enzymatic activity of this type of $\beta$-glucosidase was enhanced by factors of 1.2–2.6 in the presence of 50–200 mM glucose. This activity was followed by a gradual inhibition at higher glucose concentrations (17, 23–25). Td2F2 is not only particularly resistant to glucose inhibition, but its enzymatic activity is significantly activated by glucose with no inhibition at higher concentrations. This particular characteristic of Td2F2 is a result of its high transglycosylation activity (Fig. 8). Td2F2 can preferentially utilize glucose rather than water as an acceptor for the glycosyl moiety during the catalytic degradation of pNPGlC and cellobiose, thereby resulting in elevated reaction rates. Td2F2 produced a series of $\beta$-linked glucodisaccharides from pNPGlC with $d$-glucose via transglycosylation. Under these conditions, sophorose was the primary transglycosylation product. Kinetic studies on the degradation of various substrates agreed with this result; the $k_{cat}/K_m$ for the hydrolysis of sophorose was higher than those for the hydrolysis of other $\beta$-linked glucosidases.

Kinetic analyses of Td2F2 activity with excess monosaccharides in the presence of pNPGlC showed that the addition of monosaccharides increases both $K_m$ and $k_{cat}$. These increases in $K_m$ reflect a decreased affinity of pNPGlC for Td2F2 caused by the excess monosaccharides. Increases in $k_{cat}$ may be due to the transglycosylation activity of the enzyme. Differences in the $k_{cat}/K_m$ ratios revealed that $d$-fucose behaved as an inhibitor at a concentration of 1000 mM. $d$-Glucose and $d$-galactose were comparatively poor inhibitors. However, $l$-arabinose, $d$-mannose, and $d$-xylose each stimulated enzyme activity. These results suggest that $d$-glucose, $d$-fucose, and $d$-galactose have a greater affinity for docking into the $-1$ subsite of the enzyme than $l$-arabinose, $d$-mannose, and $d$-xylose. The lower affinity of the latter three sugars for the $-1$ subsite was demonstrated by the weak activity of Td2F2 with $p$-nitrophenyl-$\alpha$-$l$-arabinopyranoside, $p$-nitrophenyl-$\beta$-$d$-mannopyranoside, and $p$-nitrophenyl-$\beta$-$d$-xylopyranoside as substrates.

Various monosaccharides and sugar alcohols also stimulated the activity of Td2F2 for the degradation of pNPGlC, pNPFuc, and pNPGal. However, this activity stimulation was not observed with the additions of amino sugars or sugar acids. In fact, $d$-glucosamine inhibited Td2F2 activity. These results indicate that the OH-2 of a monosaccharide may be important for stimulating Td2F2 and that a COOH-5 group may hinder transglycosylation.

The activation of Td2F2 by $d$-glucose was due to the transglycosylation activity of the enzyme. Notably, the combined production rates of sophorose and laminaribiose always exceeded pNP production rates when the reactants included 3.1–1000 mM $d$-glucose. This phenomenon may be the result of the enzyme catalyzing the reverse reaction of pNPGlC hydrolyase, reproducing pNPGlC from pNP and $d$-glucose. This reproduced pNPGlC might then be consumed by the transglycosylation activity of the enzyme to produce glucodisaccharides. These experimental results show that Td2F2 may be defined as a $\beta$-glucosidase with remarkable transglycosylation activity. Analyses of the x-ray crystal structure of Td2F2 are currently under way. The resulting three-dimensional model of Td2F2 may provide valuable clues as to the presence of glucosyl-binding subsites and may offer an explanation not only for the enzyme’s ability to hydrolyze a broad range of dimeric substrates but also for its high transglycosylation activity.

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