Catalytic Mechanism of a Novel Glycoside Hydrolase Family 16 “Elongating” β-Transglycosylase

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Carbohydrates are complex macromolecules in biological metabolism. Enzymatic synthesis of carbohydrates is recognized as a powerful tool to overcome the problems associated with large scale synthesis of carbohydrates. Novel enzymes with significant transglycosylation ability are still in great demand in glycobiology studies. Here we report a novel glycoside hydrolase family 16 “elongating” β-transglycosylase from Paecilomyces thermophila (PtBgt16A), which efficiently catalyzes the synthesis of higher polymeric oligosaccharides using β-1,3/1,4-oligosaccharides as donor/acceptor substrates. Further structural information reveals that PtBgt16A has a binding pocket around the −1 subsite. The catalytic mechanism of PtBgt16A is partly similar to an exo-glycoside hydrolase, which cleaves the substrate from the non-reducing end one by one. However, PtBgt16A releases the reducing end product and uses the remainder glucosyl as a transglycosylation donor. This catalytic mechanism has similarity with the catalytic mode of amylase, which catalyzes the transglycosylation products gradually extend by one glucose unit. PtBgt16A thus has the potential to be a tool enzyme for the enzymatic synthesis of new β-oligosaccharides and glycoconjugates.

Carbohydrates and their derivatives are ubiquitous in nature and play vital roles in many biological systems (1, 2). Therefore, the synthesis of carbohydrate-based compounds is of considerable interest for both research and commercial purposes (3–6). In organisms, glycosidic linkages are mainly synthesized by Leloir glycosyltransferases. Leloir glycosyltransferases have experienced notable progress as synthetic tools but are still far from being a general preparative methodology due to the difficulties of recombinant expression and purification of these often membrane proteins and their limited stability in cell-free systems (7). Advantageously for chemists, other kinds of synthetic tools named non-Leloir transglycosylases are readily available and stable and have been demonstrated to be effective catalysts for carbohydrate synthesis (8). Nevertheless, non-Leloir transglycosylases are unusual glycoside hydrolases (GHs) as they efficiently catalyze the formation of glycosidic bonds, whereas most GHs favor the mechanistically related hydrolysis of oligo- and polysaccharides (3, 8).

A large number of retaining GHs catalyze both hydrolysis and transglycosylation reactions, but little is known about how the balance between these two activities (transglycosylation/hydrolysis ratio) is determined (9). Some of them display interesting transglycosylation (10, 11). The beneficial properties of transglycosylation by GHs have encouraged researchers to improve these properties via different strategies (12, 13). One of these strategies is based on the basis of substitution of the catalytic nucleophile with a neutral amino acid, which forms new enzymes called glycosynthases (14). They are inactive GHs mutants but efficiently catalyze glycoside bond formation with glycosyl fluoride donors and an acceptor. Another strategy is directed evolution or molecular modification, which modulates the function of transglycosylation of GHs to improve the activity or specificity of transglycosylation (9, 15, 16). Thus, the original starting non-Leloir transglycosylases are particularly important. Novel non-Leloir transglycosylases with high transglycosylation ability and desirable properties are still in great demand.

β-1,3-β-Glucan derivatives (oligo-, polysaccharides, and glycoconjugates) are important carbohydrates in immunology, cellular metabolism, and other physiological functions (17, 18). Thus enzymatic synthesis of β-1,3-glucan derivatives is valuable in glycobiology (13, 19). GH family 16 enzymes are retaining enzymes. They utilize a covalent glycosyl-enzyme intermediate, which is broken-down by glycosyl transfer to water or a carbohydrate acceptor substrate in hydrolysis or transglycosylation

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3 The abbreviations used are: GH, glycoside hydrolase; DP, degree of polymerization; HPAEC, high performance anion exchange chromatography; Pt, P. thermophila; pNP, p-nitrophenol; oNP, o-nitrophenol; HSQC, heteronuclear single quantum correlation; HMBE, heteronuclear multiple bond correlation; Pc, P. chrysosporium; Zg, Z. galactanivorans; Rm, R. marinus.
A Novel GH Family 16 Elongating β-Transglycosylase

reaction, respectively (3). Many GH family 16 members are active toward β-1,4- or β-1,3-glycosidic bonds in various glucans (20–22). Some GH family 16 members indeed are transglycosylases toward β-1,3-glycosidic bonds (named chitin β-1,3/1,6-glucanotransferase) (23). PtBgt16A is a novel GH family 16 member from *Paecilomyces thermophila*. Sequence alignment predicted that PtBgt16A may be active toward β-glucan. However, an enzymatic assay showed that PtBgt16A is a novel “elongating” β-transglycosylate, which exhibited transglycosylation activity to synthesize higher polymeric oligosaccharides. The unique elongating catalytic mechanism was further revealed by structural and functional experiments. To our knowledge, the catalytic properties of PtBgt16A are different from any other transglycosylases in glycoside hydrolases. Thus, these results provide new information about non-Leloir transglycosylases from GHs.

Results

Gene Cloning and Sequence Analysis—The PtBgt16A-full protein was predicted to be anchored in the outer membrane because of the presence of a lipoprotein signal peptide and a transmembrane architecture (Fig. 1A). In the N-terminal region of PtBgt16A-full protein, the signal peptide is followed by a GH family 16 catalytic module (PtBgt16A). The C-terminal region of PtBgt16A-full protein is composed of a transmembrane region (Fig. 1A). Within the GH family 16 members, two conserved glutamates in the pattern EXDX(X)E play the role of the catalytic residues (20–22). In PtBgt16A, the equivalent to the nucleophile is Glu117, whereas the general acid/base is Glu122 (Fig. 1B). To study the enzymatic properties of this GH family 16 catalytic module without potential interference from the signal peptide and the transmembrane region and to facilitate crystallization assays, we decided to clone the nucleotide sequence corresponding to the GH family 16 catalytic module. The recombinant protein (PtBgt16A) was purified by one step of immobilized metal ion affinity chromatography. The recombinant PtBgt16A migrated as a single band with molecular mass of 32 kDa on SDS-PAGE (Fig. 2).

Enzymatic Identification—As PtBgt16A showed about 40% amino acid sequence identity with several GH family 16 β-glucanases (22), we first considered that PtBgt16A was a β-1,3-glucanase or β-1,3-1,4-glucanase. The hydrolytic activity of the purified PtBgt16A was screened by the dinitrosalicylic acid method using different β-glucan substrates, viz. β-1,3-glucans (laminarin, curdlan, and yeast β-glucan), β-1,3-1,4-glucans (lichenin, barley β-glucan, and oat β-glucan), and β-1,4-glucan (carboxymethylcellulose). However, no activity was detected toward all tested β-glucan substrates, which indicated that PtBgt16A was not a β-glucanase. To verify the catalytic ability of PtBgt16A, some natural polysaccharides (locust bean gum, birchwood xylan, pullulan, chitin, and soluble starch) and artificial substrates (pNP-β-xylpyranoside, oNP-β-glactopyranoside, pNP-α-galactopyranoside, pNP-α-glucopyranoside, and pNP-β-glucopyranoside) were further determined. Similarly, no activity was detected toward all tested substrates. In consideration of the sequence similarity, the potential activity of PtBgt16A toward different β-1,3-oligosaccharides (degree of polymerization (DP), 2–6) and β-1,4-oligosaccharides (DP, 2–5) was further investigated (Fig. 3, A and B). Surprisingly, significant activity was detected in the presence of PtBgt16A toward β-1,3-oligosaccharides and β-1,4-oligosaccharides. Thin-layer chromatography (TLC) analysis indicated that PtBgt16A could cleave the β-1,3,1,4 linkage in the oligosaccharides and meanwhile synthesize a series of new higher polymer oligosaccharides. The amount of newly generated oligosaccharide was higher than glucose, which implied that PtBgt16A is a transglycosylase rather than a hydrolase.

Catalytic Property of PtBgt16A—To determine the molecular masses of transglycosidation products, the products from laminaritetaose and cellotetraose were analyzed by matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) (Fig. 3, C and D). Results indicated that PtBgt16A catalyzed the transglycosylation of laminaritetaose and cellotetraose to form a series of oligosaccharides. The main components were hexaose, heptaose and octaose, and the highest DPs of oligosaccharides detected were 16 and 21. The DP of oligosaccharides was continuous, which implied that the transglycosylation products by PtBgt16A may elongate by one glucose residue.

The transglycosylation reaction courses of PtBgt16A were further investigated by analyzing the products from the β-1,3-oligosaccharides, including laminarintriose, laminaritetaose, laminaripteraose, and laminarihexaose (Fig. 4). TLC analysis suggested that PtBgt16A could cleave the β-1,3 linkage in β-1,3-oligosaccharides and form the new linkage at the same time. Whereas the content of substrates decreased quickly, the hydrolytic products were not accumulated. Most of the β-1,3-oligosaccharides substrates were formed into various higher polymeric oligosaccharides after 2-h incubation. The above mentioned results suggested that PtBgt16A was an elongating transglycosylase but not a hydrolase. The unique catalytic property of PtBgt16A is different from the known GH family 16 members and other glycoside hydrolases.

Characterization and Substrate Specificity of PtBgt16A—The effect of pH and temperature on the transglycosylase activity of PtBgt16A was determined using laminaritriose. The enzyme displayed maximal transglycosylase activity at pH 4.5 in 50 mM sodium acetate buffer (Fig. 5A). It was stable within the range of pH 4.5–8.0, retaining more than 80% of its initial activity (Fig. 5B). PtBgt16A exhibited optimal activity at 60 °C and retained over 50% of its initial activity between 55 and 70 °C (Fig. 5C). The enzyme was stable up to 65 °C (Fig. 5D).

The specific activity of PtBgt16A toward different substrates was determined by high performance anion exchange chromatography (HPAEC) (Table 1). PtBgt16A showed significantly higher transglycosylase activity toward the β-1,3-oligosaccharides than other tested oligosaccharides. Among the β-1,3-oligosaccharides tested, PtBgt16A exhibited highest specific activity toward laminariptaose (776.2 units/mg) but showed no activities toward laminarinibiose. PtBgt16A also exhibited low activity toward β-1,4-oligosaccharides (6.2–27.9 units/mg) except cellobiose. No activity was detected toward manno-oligosaccharides, xylo-oligosaccharides, malto-oligosaccharides, chitin oligosaccharides, raffinose, and stachyose. These results suggested that PtBgt16A was a β-transglycosylase. The
FIGURE 1. Sequence analysis of PtBgt16A. A, domain analysis of PtBgt16A-full-length protein. B, structural sequence alignment of some GH family 16 members. Identical residues are shown in white on a red background, and conservative residues are shown in red on a white background. Two catalytic glutamic acid residues, Glu117 and Glu122, are marked by red dots. The key residue, Trp112, of PtBgt16A is marked by a red star. Four disulfide bonds of PtBgt16A are marked by green numbers underneath the relative residues. The sequences of PtBgt16A, P. thermophila β-1,3-1,4-glucanase (PtLic16A; Protein Data Bank code 3WDT), P. chrysosporium β-1,3(4)-glucanase (PcLam16A; Protein Data Bank code 2CL2), Zobellia galactanivorans β-1,3-glucanase (ZgLamCGH16; Protein Data Bank code 4CRQ), Rhodothermus marinus β-1,3-glucanase (RmLamR; Protein Data Bank code 3ILN), and Nocardiopsis sp. β-1,3-glucanase (BglF; Protein Data Bank code 2HYK) were aligned using T-Coffee (43), and the figure was produced using ESPript (44).
products by transglycosylation products. The above-mentioned carbohydrate 1H chemical shift of 83.38.00–85.11 ppm/4.63–4.69 ppm) also existed in the transglycosylation products of laminaritriose by PtBgt16A (Fig. 6B). These results suggested that the transglycosylation products by PtBgt16A were mixed oligosaccharides containing β-1,3 linkages and β-1,4 linkages. Thus, the glycosidic bonds that formed during the transglycosylation reaction by PtBgt16A were mixed β-1,3 linkages and β-1,4 linkages.

Crystal Structure of PtBgt16A—To understand the elongating catalytic mechanism of PtBgt16A, the crystal structure of PtBgt16A was determined. The crystal structure of PtBgt16A was determined at 1.59 Å resolution in space group P121_1. The Rwork/Rfree was 14.87%/17.89% (Table 2). The asymmetric unit contained two monomer models. The protein monomer, with approximate dimensions of 50 × 445 × 35 Å, exhibited single domain architecture and consisted of residues 3–299. The overall structure of PtBgt16A is presented in Fig. 7A. The overall fold of PtBgt16A could be defined as a classical sandwich-like β-jelly roll structure in which all the strands are connected by loops and α-helices. The sandwich was formed by the face-to-face packing of two antiparallel sheets containing seven and eight strands in the order of β1-β18-β8-β14-β15-β16 and β2-β7-β17-β9-β10-β11-β13-β12, respectively. Both β-sheets were twisted and bent, forming a convex and a concave side of the molecule. The protein featured a roughly V-shaped groove with structural homology to other GH family 16 members (22, 24). The catalytic cleft contained the catalytic proton donor and nucleophile (Glu122 and Glu117, respectively) as in previous studies (20, 22). Some hydrophobic residues were found arranged along the inner catalytic cleft. This central groove, ~15 Å in length and 5 Å in depth, lies between α2 and α3 at the N terminus of the protein and between α5 and α6 at its C terminus.

The Unique Binding Pocket and the Active Site—The active site of PtBgt16A was located in a long deep channel at the concave side, allowing binding of oligosaccharides. An inserted loop (109GDTWPPDG116) was found to be present in the one end of the catalytic groove (Fig. 7). Some strong hydrogen bonds are involved in the stability of this loop: N1 atom of Trp112 was stabilized by O2 atom of Glu117 (2.86 Å), O62 of Asp115 was directly hydrogen-bonded to the nitrogen atom of Tyr111 (2.96 Å), oxygen atom of Tyr111 was directly hydrogen-bonded to the nitrogen atom of Asp115 (3.07 Å), and oxygen atom of Pro113 was directly hydrogen-bonded to the nitrogen atom of Gly116 (3.04 Å). This inserted loop formed a bulge region across the catalytic groove of PtBgt16A that blocked the one end of the catalytic groove and thus formed a pocket around the active center (Fig. 7B). It was precisely because of the presence of this unique loop that the catalytic groove of PtBgt16A could only provide a small site in the non-reducing end. To explore the substrate binding of PtBgt16A, laminaritriose ligand was modeled into the putative subsites −1 to +2 according to the structure superposition of complex structures of Phanerochaete chrysosporium GH family 16 laminarinase (PcLam16A) PcLam16A-L2 (Protein Data Bank code 2W39) and PcLam16A-L7 (Protein Data Bank code 2WLQ) (Fig. 7B). According to the superposed complex structures, several key residues were identified to form the active site of PtBgt16A (Fig. 8A). The Nε1 atom of Trp102 formed a direct

FIGURE 2. SDS-PAGE of proteins during purification of the recombinant PtBgt16A by nickel-iminodiacetic acid. Lane M, standard protein molecular weight markers; lane 1, supernatant of lysate cells; lane 2, purified enzyme.

appropriate substrates were β-1,3/1,4-linked gluco-oligosaccharides with a minimum DP of 3.

NMR Analysis of the Reaction Products—TLC analysis showed that the transglycosylation products by PtBgt16A are a series of mixed oligosaccharides. To confirm the generated glycosidic bonds of the transglycosylation products, the reaction products by PtBgt16A toward laminaritriose and cellotriose were analyzed by two-dimensional NMR spectroscopy (HSQC and HMBC) (Fig. 6). The well resolved anomeric correlations (C1/H1: 101.88–103.11 ppm/4.64–4.72 ppm) of the associated carbohydrates were detected in the HSQC spectrum of cellotriose (C/H: 78.03–78.91 ppm/4.40–4.47 ppm) indicated that transglycosylation products of cellotriose contain −1,3 linkages. In the meantime, the characteristic chemical shift at C/H of 84 ppm (Fig. 6A) was identified in the HMBC spectrum, which indicated that β-1,4 linkages also existed in the transglycosylation products of cellotriose by PtBgt16A. Two-dimensional NMR spectroscopy (HSQC and HMBC) of transglycosylation products by PtBgt16A toward laminaritriose showed an analogous phenomenon. The well resolved characteristic chemical shift at C/H of 78.03–78.91 ppm/4.40–4.47 ppm indicated that β-1,4 linkages were produced in the transglycosylation products of laminaritriose by PtBgt16A, although β-1,3 linkages (C/H: 83.38.00–85.11 ppm/4.63–4.69 ppm) also existed in the transglycosylation products of laminaritriose by PtBgt16A (Fig. 6B). These results suggested that the transglycosylation products by PtBgt16A were mixed oligosaccharides containing β-1,3 linkages and β-1,4 linkages.
hydrogen bond to the O6 hydroxyls of the −1 glucose residue. Aromatic residues Trp106 stacked against the glucose units at subsites −1, forming a hydrophobic sugar-binding platform. The O4 hydroxyl of the +1 glucose residue was directly hydrogen-bonded to the side chains of residues Asp119 and His135. Trp252 was involved in hydrophobic interactions with subsite +1.

Two strictly conserved catalytic glutamate residues, Glu117 and Glu122, lay at either end of the core β-sheet (β10), acting as a nucleophile and a proton donor, respectively (Fig. 8A). The importance of these two conserved catalytic residues was corroborated by site-directed mutagenesis in which substitution of each of these residues reduced the activity of the enzyme (Fig. 8C). It is noteworthy that residue Trp112 from the inserted loop played a key role in the formation of the −1 site of binding pocket. It not only provides steric hinderance but also forms a direct hydrogen bond to the O3 hydroxyls of the −1 glucose residue. The importance of residue Trp112 for substrate binding was corroborated by site-directed mutagenesis (Fig. 8C): the mutant W112A exhibited a specific activity of 12.11 units/mg (only 1.88% of the wild type transglycosylation activity).

Structure superposition indicated that PbBgt16A, PCLam16A (25), and a P. thermophila GH family 16 β-1,3,4-glucanase (PtLic16A) (22) have a similar architecture of active sites at −1 and +1 sites (Fig. 7B). Some identified key residues above (Trp102, Trp106, Glu117, Asp119, His135, and Trp252) were conserved in all three enzymes. However, PbBgt16A showed distinct architecture of active sites at +2 and +3 sites. The +2 sites of PtLic16A and PCLam16A have a strict conserved “-16ANA163-” motif (PtLic16A numbering). The main chain of residue Ala and the side chain of residue Asp could form a direct hydrogen bond to the +1 and +2 glucose residues, which take part in the process of substrate recognition. Furthermore, Gln260 in PCLam16A (Asp160 in PtLic16A) in the +3 binding site of this enzyme could form a direct hydrogen bond to the +3 glucose residue; however, the “-ANA-” motif and the +3 binding site are completely absent in PbBgt16A. The catalytic groove of PbBgt16A thus presents an open architecture in +2 site, and the reducing end of the substrate is localized in the solvent region.

Discussion

GH family 16 is a huge glycoside hydrolase family that often has activity toward β-1,4- or β-1,3-glycosidic bonds in various glucans and galactans (26). Most GH family 16 members exhibit glycoside hydrolase activity toward plant and marine polysaccharides, including endo-1,3,β-glucanases (EC 3.2.1.39),
licheninases (EC 3.2.1.73), β-agarases (EC 3.2.1.81), β-porphyrinases (EC 3.2.1.178), κ-carrageenases (EC 3.2.1.83), and xyloglucan-specific endo-β-1,4-glucanase (EC 3.2.1.151). Previous studies revealed that some GH family 16 enzymes could catalyze the hydrolysis of mixed linked β-1,3- and β-1,4-glucan substrates (β-1,3-1,4-glucan). One typical enzyme is licheninase. It is also known as “β-1,3-1,4-glucanase,” which strictly cleaves a β-1,4-glycosidic linkage adjacent to a C3 substituted glucose residue in mixed linked β-1,3-1,4-glucans but is inactive against β-1,4-glucans or β-1,3-glucans (27). Another well known enzyme is β-1,3(4)-glucanase (EC 3.2.1.6), which catalyzes the hydrolysis of a β-1,4- or β-1,3-glycosidic linkage in β-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C3 (28). These two typical β-1,3/1,4-hydrolases both follow an endo-type catalytic mode and often show specificity toward a given substrate motif of β-1,3 and β-1,4 bonds in the vicinity of the bond to be cleaved in mixed linked β-glucan substrates. *PtBgt16A* is a GH family 16 member that shows low identity (∼40%) with *PtLic16A* (22) (Fig. 1B). Catalytic properties indicated that *PtBgt16A* adopts an entirely different catalytic mode compared with known β-1,3/1,4-glycosidase hydrolases. It is noteworthy that some GH family 16 members indeed are transglycosylases, such as xyloglucan:xyloglucosyltransferases (EC 2.4.1.207) (29) and chitin β-1,3/1,6-galactosyltransferase (EC 2.4.1.-) (32). However, *PtBgt16A* displayed unique transglycosylase activity that is different from all reported GH family 16 members. The suitable substrates of *PtBgt16A* were β-1,3/1,4-linked gluco-oligosaccharides, and the minimum DP of substrate was 3. *PtBgt16A* could cleave the β-1,3/1,4 linkage in the oligosaccharides and meanwhile synthesize a large number of higher polymeric oligosaccharides. Moreover, the transglycosidation products were a series of mixed oligosaccharides. There was almost no glucose generating in this reaction.

The overall structure of *PtBgt16A* exhibited a classical GH family 16 sandwich-like β-jelly roll fold (21, 24). However, an inserted loop (109-GDTWPDG116) near the catalytic groove formed a binding pocket around the active center (Fig. 7). All eight residues of this inserted loop were well defined in the electron density maps, and some strong hydrogen bonds are involved in the stability of the loop. Moreover, the B-factors of the atoms composing this additional loop (average B-factors, 20.06 Å²) were not significantly different from the B-factors of other protein atoms (average B-factors, 17.50 Å²) (Fig. 9A). When considering all these elements, this additional loop seems to be stable and not a flexible region. Several glycoside hydrolases go through an open-closed-open conformational transition upon substrate binding and product release by a flexible loop. There is no evidence showing that *PtBgt16A* may follow this mode, and no GH family 16 member exhibited this mode before (26). Sequence alignment showed that this loop region was not conserved in different GH family 16 members (Fig. 9B). In contrast to other GH family 16 hydrolases that have the active site in the bottom of a large cleft, the active site of *PtBgt16A* is at the bottom of a pocket. The −2 subsite is not found in *PtBgt16A* as it is blocked by the additional loop. Thus, this binding pocket may be the structural basis for the unique transglycosylation activity of *PtBgt16A*.

Based on the superposed structures of *PtBgt16A*, *PtLam16A*, and *PcLam16A*, the plausible substrate binding and several key residues of *PtBgt16A* were identified. The detailed transglycosidation catalytic mechanism of *PtBgt16A* was further speculated. The catalytic mechanism of *PtBgt16A* consists of a retaining reaction: a general acid/base catalyst (proton donor; Glu122) works first as an acid and then as a base in two steps (Fig. 8B). In the first step, one oligosaccharide (β-1,3 linkage or β-1,4 linkage; DP ≳ 3) occupies binding sites −1 to +2, and a proton donor (Glu122) facilitates departure of the leaving group by donating a proton to the oxygen atom between glycosyl −1 and +1. As for the unique binding pocket of *PtBgt16A*, the nucleophile (Glu171) only could form an enzyme-sequestered covalent intermediate toward the −1 glucose residue. The other part of the oligosaccharide is then released from the binding pocket as the leaving group. In the second step, another oligosaccharide as acceptor occupies binding sites +1 and +2, and the deprotonated proton donor (Glu122) acts as a general base to activate the C3 or C4 hydroxyl of the acceptor, which then carries out a nucleophilic attack on the glycosyl-enzyme intermediate, leading to the formation of a new transglycosylation product, which adopts a β-1,3 linkage or β-1,4 linkage with the reducing end of glucosyl. The open form in binding sites +3 could lead the acceptor recognition region accepting both β-1,3-linked and β-1,4-linked oligosaccharides. Thus, the formed glycosidic bonds of transglycosylation products were mixed β-1,3 linkage and β-1,4 linkage.

The catalytic mechanism of *PtBgt16A* is partly similar to an exo-glycosidase hydrolyase, which cleaves the substrate from the non-reducing end one by one (11). However, *PtBgt16A* released...
the reducing end product and used the remaining glucosyl as a transglycosylation donor. This characteristic results in the transglycosylation products being gradually extended by one glucose unit. The catalytic property of PtBgt16A may be similar to a member of GH family 13, amylosucrase (EC 2.4.1.4), which catalyzes the synthesis of amylose-like polymers from sucrose (30). The amylosucrase catalyzes the following elongating chemical reaction: sucrose + (1,4-α-D-glucosyl)_n → α-D-fructose + (1,4-α-D-glucosyl)_{n+1}, which generates α-1,4-glucan in a stepwise fashion, releasing fructose from sucrose at each step of extension. The active sites of amylosucrase are also located in the bottom of a pocket, which exhibits an exo-acting enzyme mode (30, 31). Reported -1,3-transglycosylases, often named -1,3-glucanosyltransferases (EC 2.4.1.-), catalyze the transfer of a glycosyl group from a 1,3-linked carbohydrate donor to a suitable (carbohydrate) acceptor (32, 33). -1,3-Glucanosyltransferases are grouped into three GH families: 16, 17, and 72, which function as cross-linking enzymes, branching enzymes, and elongation enzymes, respectively (34). However, to our knowledge, the catalytic modes of PtBgt16A are different from all three of these -1,3-glucanosyltransferases and any other non-Leloir transglycosylases in glycoside hydrolases. Because of its novel elongating catalytic mechanism, PtBgt16A should be named -transglycosylase.

### TABLE 1

| Substrate specificity of PtBgt16A | Specific activity | Relative activity |
|---------------------------------|------------------|------------------|
|                                | units/mg         | %                |
| Laminariobise                  | —                | 0                |
| Laminaritriose                 | 106.7 ± 2.3      | 13.7             |
| Laminaritetractose             | 456.7 ± 15.2     | 58.8             |
| Laminaripentose               | 776.2 ± 26.1     | 100              |
| Laminarihexose               | 574.3 ± 16.8     | 74.0             |
| Cellobiase                        | 6.2 ± 0.16      | 0.8              |
| Cellotriose                     | 24.9 ± 0.77     | 3.2              |
| Cellotetraose                 | 27.9 ± 2.02     | 3.6              |
| Manno-oligosaccharides           | —                | 0                |
| Xylo-oligosaccharides           | —                | 0                |
| Malto-oligosaccharides         | —                | 0                |
| Chitin oligosaccharides         | —                | 0                |
| Raffinose                        | —                | 0                |
| Stachyose                        | —                | 0                |

a Data represent the means ± S.D. of three independent experiments (n = 3).

b Relative activity indicates the ratio between specific activity and highest activity.

* No activity was detected.
anchored in the outer membrane because of the presence of a lipoprotein signal peptide and a transmembrane architecture. Thus, the biological significance of PtBgt16A is most likely involvement in fungal β-glucan metabolism and cell wall rearrangement.

Oligosaccharides, polysaccharides, and glycoconjugates are a relevant part of the bioactive components of the natural products exploited in therapeutics, diagnostics, food additives, and biomaterials (1, 2, 4). Therefore, methods for glycosidic synthesis and modification are urgently needed. Enzymatic synthesis of carbohydrates is still challenging at present due to the low efficiency of glycosyltransferases and complexity of carbohydrates. The catalytic mechanism of non-Leloir transglycosylases is similar to their hydrolytic counterparts, and it is unclear how these enzymes overcome the ubiquity of water, thus avoiding the hydrolytic reaction (3). It is still difficult to create novel non-Leloir transglycosylases using the vast diversity of glycoside hydrolases as protein templates by protein engineering. Although the exact biological role of PtBgt16A is still elusive, we noticed that PtBgt16A has its own unique advantages in enzymatic synthesis of carbohydrates. It has high transglycosylation activity (776.2 units/mg toward laminaripentaose) but nearly no hydrolytic activity. PtBgt16A cleaves the substrate from the non-reducing end by a glucose unit like an exo-glycoside hydrolase and uses this glucosyl as a transglycosylation donor. This unique catalytic mechanism gives

FIGURE 6. Two-dimensional NMR data (HMBC) of transglycosylation products by PtBgt16A. A, the enlarged picture of HMBC spectra of cellotriose before (1) and after (2) reaction. B, the enlarged picture of HMBC spectra of laminaritriose before (1) and after (2) reaction. Characteristic chemical shifts occurred at 84 and 78 ppm, indicating a β-1,3 and β-1,4 linkage in the transglycosylation products, respectively. Transglycosylation reaction (30 ml) was performed with PtBgt16A (1 unit/ml) and 1% (w/v) laminaritriose or cellotriose in 50 mM sodium acetate buffer, pH 5.5, at 50 °C for 2 h.
A Novel GH Family 16 Elongating β-Transglycosylase

PROPANE

Table 2

X-ray data collection and refinement statistics of PtBgt16A

| Data collection statistics | PtBgt16A |
|---------------------------|----------|
| Radiation source          | SSRF-BL19U1 |
| Wavelength (Å)            | 1.59 |
| Temperature of measurements (K) | 100 |
| Resolution (Å)            | 37.17–1.59 (1.65–1.59) |
| Space group               | P121 |
| Unit cell parameters      |                                     |
| a, b, c (Å)               | a = 45.3, b = 91.5, c = 95.5 |
| α, β, γ (°)               | α = 90, β = 93.8, γ = 90 |
| Protein molecules in asymmetric unit | 2 |
| Unique reflections        | 62,905 (6,124) |
| Completeness (%)          | 96.6 (94.1) |
| Rmerge(%)                 | 14.51 |
| Rfree(%)                  | 14.87 (15.34) |
| No. water molecules       | 699 |
| No. atoms                 | 5,297 |
| r.m.s.d.                  | 0.9717 |
| Bond lengths (Å)          | 0.007 |
| Bond angles (°)           | 1.17 |
| Average B-factors (Å²)    | 17.50 |
| Solvent                   | 15.60 |
| Ramachandran              | 25.20 |
| Most favored regions (%)  | 97.17 |
| Additional allowed regions (%) | 2.83 |
| Disallowed regions (%)    | 0 |
| Clashscore                | 2.25 |
| Protein Data Bank code    | 5JVV |

| Refinement statistics     | PtBgt16A |
|---------------------------|----------|
| Resolution (Å)            | 1.59 |
| Rmerge (%)                | 14.51 |
| Rfree (%)                 | 14.87 (15.34) |
| No. residues              | 5,297 |
| No. water molecules       | 699 |
| r.m.s.d.                  | 0.9717 |
| Bond lengths (Å)          | 0.007 |
| Bond angles (°)           | 1.17 |
| Average B-factors (Å²)    | 17.50 |
| Solvent                   | 15.60 |
| Ramachandran              | 25.20 |
| Most favored regions (%)  | 97.17 |
| Additional allowed regions (%) | 2.83 |
| Disallowed regions (%)    | 0 |
| Clashscore                | 2.25 |

PtBgt16A the ability to synthesize glycoconjugates and long-chain oligosaccharides, opening a novel pathway for non-Neloir transglycosylases in glycoside synthesis and modification. Moreover, it provides an excellent protein template for further structure-based protein engineering study in transglycosylases.

Experimental Procedures

Cloning, Expression, and Purification—The thermophilic fungus P. thermophilia J18 was used in this study. The strain has been deposited in the China General Microbiological Culture Center under accession number AS3.6885 (35). Cells were grown as described in a previous study to isolate genomic DNA and total RNA (36). The procedure to clone the target gene from P. thermophila J18 was as described in a previous study with some modifications (36). Degenerate primers DP1 and DP2 (supplemental Table S1) were designed on the basis of the conserved sequences (GEIDIEGV and DTTFCGDWA) of known GH family 16 β-glucanases. The full-length cDNA sequence of the target gene was obtained by 5’ and 3’ rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA amplification kit (Clontech). The obtained PCR product was purified, cloned, and sequenced (designated as PtBgt16A-full). The PtBgt16A-full gene is predicted to be a membrane-anchored protein composed of a GH family 16 catalytic module (PtBgt16A) and a C-terminal transmembrane region. To study the enzymatic properties of this GH family 16 catalytic module, the gene fragments of GH family 16 catalytic module were amplified by PCR using the primers PtBgt16A-Nhel and PtBgt16A-XhoI (supplemental Table S1). The Nhel and XhoI sites (underlined) were added to the forward and reverse primers, respectively. The sequenced PtBgt16A cDNA sequence was deposited in the GenBank nucleotide sequence database under accession number KX234714. The purified PCR products were digested with Nhel and XhoI and subcloned into the pET-28a (+) vector (Novagen). Mutants E117A, E122A, and W112A were generated using the Fast Mutagenesis System site-directed mutagenesis kit (TransGen Biotech, China). The primers used for site-directed mutagenesis are shown in supplemental Table S1. All recombinant plasmids encoding these mutations were sequenced and verified.

The recombinant plasmids were transformed into Escherichia coli BL21(DE3) competent cells for gene expression. Seed cultures of E. coli BL21(DE3) harboring PtBgt16A in the pET-28a (+) vector were prepared by incubation in LB medium containing 50 µg/ml kanamycin at 37 °C on a rotary shaker at 200 rpm for 4 h. When the absorbance of the culture broth at 600 nm reached 0.6–0.8, overexpression of the protein was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside. The cultures were further grown at 30 °C for 12 h, and the cells were harvested by centrifugation at 10,000 × g for 10 min at 4 °C. The recombinant proteins were purified using a nickel- immobilodiacetic acid column (1 × 5 cm) and Sephacryl S-100 HR column (GE Healthcare) as described previously (10). The purified protein fractions were combined and concentrated for subsequent experiments. All mutants were expressed and purified in an identical manner.

Enzyme Assay—Transglycosylation activity was quantitated by HPAEC using different oligosaccharides as the substrates (10). Namely, 2 µl of suitably diluted enzyme and 8 µl of each 10% (w/v) oligosaccharide were mixed with 30 µl of 50 mM sodium acetate buffer, pH 5.5. The reaction mixture was incubated at 50 °C for 5 min and then terminated by boiling for 5 min. The mixture was diluted 500-fold with 100 mM NaOH and analyzed by HPAEC (ICS-5000®, Thermo) with pulsed amperometric detection on a CarboPac PA10 (4 × 250-mm) preparative column (Thermo) with a 0–350 mM sodium acetate gradient in 100 mM NaOH (20 min) at a flow rate of 1 ml/min. One unit of enzyme activity was defined as the amount of enzyme required to consume 1 µmol of oligosaccharides/min. Substrate specificity of PtBgt16A was determined by measuring the activity of the enzyme in the presence of different substrates such as laminarioligosaccharides, cello-oligosaccharides, manno-oligosaccharides, xylo-oligosaccharides, malto-oligosaccharides, chitin oligosaccharides, raffinose, and stachyose. The enzyme activity was determined under standard conditions. Raffinose and stachyose were purchased from Aladdin (China). Other oligosaccharides were purchased from Megazyme (Ireland).

A hydrolytic assay was performed according to the method of Yang et al. (35). Namely, 0.1 ml of properly diluted enzyme solution was added into 0.9 ml of 1% (w/v) polysaccharides (prepared in 50 mM sodium acetate buffer, pH 5.5). The reac-
tion mixture was incubated at 50 °C in a water bath for 10 min. The liberated reducing sugars were quantified using the dinitrosalicylic acid method. One unit of hydrolytic activity was defined as the amount of enzyme liberating 1 mol of mono-saccharide-equivalent reducing sugars/min under the above assay conditions.

**Transglycosylation Properties of PtBgt16A**—Transglycosylation properties of PtBgt16A were investigated by analyzing the products from the laminarioligosaccharides (Megazyme), viz. laminaritriose, laminaritetraose, laminaripentaose, and laminarihexaose. Purified PtBgt16A (1 unit/ml) was added to 1% (w/v) laminarioligosaccharides in 50 mM sodium acetate buffer, pH 5.5, and then incubated at 50 °C for 2 h. Samples withdrawn at different times were immediately boiled for 5 min and then analyzed by TLC (16). Samples were spotted on a TLC plate (Kieselgel 60, Merck), developed in butan-1-ol:acetic acid:water (2:1:1, v/v/v) as solvent, and sprayed with a methanol-sulfuric acid mixture (95:5, v/v). The hydrolysis products were visualized after heating the plate at 130 °C in an oven for a few minutes.

**Characterization of the PtBgt16A**—The optimal pH of purified PtBgt16A was determined by measuring the activity from pH 2.0 to 11.0 using various buffers at 50 mM: McIlvaine buffer (pH 2.0–7.5), sodium acetate buffer (pH 3.5–6.0), Tris-HCl buffer (pH 7.0–9.5), and glycine-NaOH buffer (pH 9.0–11.0). To determine pH stability, residual activity was measured after incubation of the enzyme at 30 °C for 30 min in the aforementioned buffers. The optimal temperature was determined at 40–70 °C in 50 mM sodium acetate buffer, pH 5.5. Enzymatic activity was determined under standard conditions. Thermostability of the enzyme was determined by measuring residual activity after
incubation of the enzyme at different temperatures (40–70 °C) for 30 min in 50 mM sodium acetate buffer, pH 5.5.

Analysis of Transglycosylation Products—To determine the degree of polymerization of transglycosylation products, reaction mixtures derived from laminariotriose and cellotriose were analyzed by MALDI-TOF MS. Enzyme (1 unit/ml) was incubated with 1% (w/v) laminariotriose or cellotriose in 50 mM sodium acetate buffer, pH 5.5, at 50 °C for 2 h (50-uL assay volume). The sample was diluted 100-fold prior to mixing with an equal volume of matrix (2,5-dihydroxybenzoic acid; 10 mg/ml in water). Sample (1 uL) was then spotted onto the MALDI plate and analyzed in an AB SCIEX TOF/TOF™ 5800 system operated in positive ion mode.

For structural analysis of transglycosylation products, a transglycosylation reaction (30 mL) was performed with Ptbgt16A (1 unit/ml) and 1% (w/v) laminariotriose or cellotriose in 50 mM sodium acetate buffer, pH 5.5, at 50 °C for 2 h. The reaction mixtures were deionized by ion exchange resin and then concentrated by rotary evaporation. The purified transglycosylation products (~10 mg) were freeze-dried and dissolved in deuterium oxide (500 mL) prior to recording spectroscopy on a Bruker Avance 500 NMR spectrometer. Two-dimensional HSQC and HMBC spectra were acquired using standard pulse sequences. For the blank control, the same NMR analysis was performed using purified laminariotriose and cellotriose.

Crystallization and Data Collection—Ptbgt16A was concentrated to 10 mg/ml in crystallographic buffer (20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl). Crystallization experiments were performed in 48-well plates by the sitting drop vapor diffusion method at 20 °C, and each sitting drop was prepared by mixing 1 μL each of protein solution and reservoir solution. Optimized crystals suitable for diffraction were grown in drops containing 1 μL of protein solution and 1 μL of reservoir solution.

![Catalytic mechanism of Ptbgt16A transglycosylation.](image)

**FIGURE 8.** Catalytic mechanism of Ptbgt16A transglycosylation. **A**, schematic representation of substrate interactions of Ptbgt16A. The oligosaccharide is drawn as sticks. The hydrogen bonding interactions are shown as dotted lines. **B**, schematic mechanism of the catalysis reaction by Ptbgt16A. **C**, enzyme activity of Ptbgt16A mutants. Data represent the mean ± S.D. of three independent experiments (n = 3).

![B-factor putty (A) and sequence alignment (B) of the unique loop of Ptbgt16A.](image)

**FIGURE 9.** B-factor putty (A) and sequence alignment (B) of the unique loop of Ptbgt16A. The key residue, Tyr112, of Ptbgt16A is marked by a red star. The sequences of Ptbgt16A (Protein Data Bank code 5JVV), Ptlc16A (Protein Data Bank code 3WDT), Pclam16A (Protein Data Bank code 2CL2), ZglamCGH16 (Protein Data Bank code 4CRQ), RmlamR (Protein Data Bank code 3ILN), and BgL (Protein Data Bank code 2HYK) were aligned using T-Coffee (43), and the figure was produced in ESPript (44).
(35% PEG 3350, 0.1 m sodium citrate, pH 4.4) at 20 °C. The rhabdoid crystals were obtained 10 days later.

Crystals were soaked in reservoir solution supplemented with 20% glycerol and then vitrified in liquid nitrogen. Diffraction data for PtBgt16A was collected at 100 K using beamline BL19U1 at Shanghai Synchrotron Radiation Facility (Shanghai, China). All diffraction data were indexed, integrated, and scaled using the program HKL-3000 (37). The X-ray data collection statistics are presented in Table 2.

Phase Determination, Model Building, and Refinement—The structure of PtBgt16A was determined by molecular replacement using the coordinates of P. thermophila GH family 16 β-1,3-1,4-glucanase (Protein Data Bank code 3WDT) as the search model (22) according to their protein sequence alignments. Thereafter, model building and refinement were performed using Coot (38) and Phenix.refine (39). The final models were analyzed and validated with MolProbity (40).

Structural homologs of PtBgt16A were identified using the DALI server (41). The refinement statistics are shown in Table 2. The secondary structural elements were identified with DSSP (42). The schematic depictions of the structures were prepared in PyMOL (version 1.3; Schrödinger LLC). The sequence alignments were created with T-Coffee (43) and ESPript (44). The coordinates and structure factors of PtBgt16A have been deposited in the Protein Data Bank under accession code 5JVV.

Author Contributions—Z. J. designed and supervised the research and revised the manuscript. Z. Q. designed the research, performed experiments, analyzed data, and wrote the manuscript. S. Y., L. Z., and Q. Y. analyzed data and revised the manuscript. X. Y. performed site-directed mutagenesis and measurement of enzymatic activity. All the authors read and approved the manuscript.

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References

1. Cobucci-Ponzano, B., Strazzulli, A., Rossi, M., and Moracci, M. (2011) Glycosynthases in biocatalysis. Adv. Synth. Catal. 353, 2284–2300
2. Díez-Municio, M., Herrero, M., Olano, A., and Moreno, F. J. (2014) Synthesis of novel bioactive lactose-derived oligosaccharides by microbial glycoside hydrolases. Microb. Biotechnol. 7, 315–331
3. Bissaro, B., Monsan, P., Fauré, R., and O’Donohue, M. J. (2015) Glycosynthesis in a waterworld: new insight into the molecular basis of transglycosylation in retaining glycosidase hydrolases. Biochem. J. 467, 17–35
4. Cobucci-Ponzano, B., and Moracci, M. (2012) Glycosynthases as tools for the production of glycan analogs of natural products. Nat. Prod. Rep. 29, 697–709
5. Spaduti, O., Ibatullin, F. M., Peart, J., Gullfot, F., Martinez-Fleites, C., Ruda, M., Xu, C., Sundqvist, G., Davies, G. J., and Brumer, H. (2011) Building custom polysaccharides in vitro with an efficient, broad-specificity xyloglucan glycosynthase and a fucosyltransferase. J. Am. Chem. Soc. 133, 10892–10900
6. Li, T., Tong, X., Yang, Q., Giddens, J. P., and Wang, L. X. (2016) Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. J. Biol. Chem. 291, 16508–16518
7. Pérez, X., Fajies, M., and Planas, A. (2011) Artificial mixed-linked β-glucans produced by glycosynthase-catalyzed polymerization: tuning morphology and degree of polymerization. Biomacromolecules 12, 494–501
8. Siebel, J., Beine, R., Moraru, R., Behringer, C., and Buchholz, K. (2006) A new pathway for the synthesis of oligosaccharides by the use of non-Leloir glycosyltransferases. Biocatal. Biotransformation 24, 157–165
9. Tzee, D., Hendrick, J., Czajek, M., Ropartz, D., Sanejouand, Y. H., Tran, V., Tellier, C., and Dion, M. (2014) Semi-rational approach for converting a GH1 β-glucosidase into a β-transglycosidase. Protein Eng. Des. Sel. 27, 13–19
10. Qin, Z., Yan, Q., Lei, J., Yang, S., Jiang, Z., and Wu, S. (2015) The first crystal structure of a glycoside hydrolase family 17 β-1,3-1,4-glucono-transferase displays a unique catalytic cleft. Acta Crystallogr. D Biol. Crystallogr. 71, 1714–1724
11. Matsuzawa, T., Jo, T., Uchiyama, T., Manninen, J. A., Arakawa, T., Miyazaki, K., Fushinobu, S., and Yaoi, K. (2016) Crystal structure and identification of a key amino acid for glucose tolerance, substrate specificity, and transglycosylation activity of metagenomics β-glucosidase TdF2. FEBS J. 283, 2340–2353
12. Lundemo, P., Karlsson, E. N., and Adlercreutz, P. (2016) Eliminating hydrolytic activity without affecting the transglycosylation of a GH1 β-glucosidase. Appl. Microbiol. Biotechnol. 100, 1765–1776
13. Goodridge, H. S., Wolf, A. J., and Underhill, D. M. (2009) Β-Glucan recognition by the innate immune system. Immunol. Rev. 230, 38–50
14. Magee, A. S., Langslay, R. R., Will, P. M., Daniels, M. E., Wurst, L. R., and Iiams, V. A. (2015) Modification of the degree of branching of a β-(1,3)-glucan affects aggregation behavior and activity in an oxidative burst assay. Biopolymers 103, 665–674
15. Vasur, J., Kawai, R., Jonsson, K. H., Widmalm, G., Engström, A., Frank, M., Andersson, E., Hansson, H., Forsberg, Z., Igarashi, K., Samejima, M., Sandgren, M., and Ståhlberg, J. (2010) Synthesis of cyclic β-glucan using laminarinase 16A glycosynthase mutant from the basidiomycete Phanerochaete chrysosporium. J. Am. Chem. Soc. 132, 1724–1730
16. Laboureil, A., Jam, M., Jeudy, A., Hehemann, J. H., Czajek, M., and Michel, G. (2014) The β-glucanase ZgLamA from Zobellia galactanivorans evolved a bent active site adapted for efficient degradation of algal laminarin. J. Biol. Chem. 289, 2027–2042
17. Cheng, Y. S., Huang, C. H., Chen, C. C., Huang, T. Y., Kuo, T. P., Huang, J. W., Wu, T. H., Liu, J. R., and Guo, R. T. (2014) Structural and mutagenetic analyses of a 1,3-1,4-β-gluco-16A from Paecilomyces thermophila. Biochim. Biophys. Acta 1844, 366–373
18. Blanco, N., Sanz, A. B., Rodríguez-Peña, J. M., Nombeva, C., Farkas, V., Hurtado-Guerrero, R., and Arroyo, J. (2015) Structural and functional analysis of yeast Crh1 and Crh2 transglycosylases. FEBS J. 282, 715–731
19. Ilari, A., Fiorillo, A., Angelaccio, S., Florio, R., Chiaraluce, R., van der Oost, J., and Consalvi, V. (2009) Crystal structure of a family 16 endoglucanase from the hyperthermophile Pyrococcus furiosus—structural basis of substrate recognition. FEBS J. 276, 1048–1058
20. Vasur, J., Kawai, R., Andersson, E., Igarashi, K., Sandgren, M., Samejima, M., and Ståhlberg, J. (2009) X-ray crystal structures of Phanerochaete chrysosporium laminarinase 16A in complex with products from lichenin and laminarin hydrolysis. FEBS J. 276, 3858–3869
A Novel GH Family 16 Elongating β-Transglycosylase