Carbohydrate Polymers 99 (2014) 39–46

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Transglycosylation of engineered cyclodextrin glucanotransferases as *O*-glycoligases

Chao Li [a], Hee-Jeong Ahn [a], Jin-Hyo Kim [b], Young-Wan Kim [a,*]

[a] Department of Food and Biotechnology, Korea University, Sejong, 339-700, Republic of Korea
[b] Chemical Safety Division, National Academy of Agricultural Sciences, Rural Development Administration, Suwon, 441-701, Republic of Korea

ARTICLE INFO

Article history:
Received 11 July 2013
Received in revised form 20 August 2013
Accepted 22 August 2013
Available online 27 August 2013

Keywords:
O-Glycoligase
Cyclodextrin glucanotransferase
Saturation mutagenesis
Transglycosylation
O-α-Glycosidic linkage

ABSTRACT

An *O*-glycoligase is a hydrolytically impaired mutant of a retaining α-glycosidase in which the catalytic acid/base has been removed, but which can still perform transglycosylation when incubated with activated glycosyl fluoride donor sugars. In this paper, we describe another example, wherein a cyclodextrin glucanotransferase mutant (CGT-E284A) with an alanine residue at its general acid/base catalyst position (Glu284), was constructed. This mutant was hydrolytically inactive, but exhibited significant transglycosylation activity using α-maltosyl fluoride (αG2F) as donor, and either 4-nitrophenyl glucosides or maltosides as acceptors. To improve transglycosylation activity, a site-saturation mutagenesis library at Glu284 was created. Through a thin-layer chromatography-based screening process, two mutants were identified; (1) a mutant with a glycine residue at Glu284 (CGT-E284G) exhibiting improved transglycosylation activity compared with the original alanine mutant and (2) a mutant with a serine residue with residual hydrolytic activity. Kinetic analysis revealed that 4-nitrophenyl maltosides were better acceptors than 4-nitrophenyl glucosides. Transglycosylation activities of CGT-E284A and CGT-E284G were inhibited at high concentrations (>0.8 mM) of the acceptor sugars. In contrast, typical saturation kinetic behavior was observed upon varying the donor (αG2F) concentration at a fixed acceptor concentration (0.8 mM). The catalytic efficiencies (apparent k_{cat}/K_M) of CGT-E284G were generally three- to sixfold higher than those of CGT-E284A. Due to the rate at high concentrations of the acceptors, higher transglycosylation yields were achieved at a low concentration of the acceptors (69–84% at 1 mM) compared to those at a higher concentration (22–36% at 10 mM).

1. Introduction

Enzymatic transglycosylation, a process used to produce oligosaccharides and glycosides, is one of the oldest topics in enzymology related to carbohydrate chemistry. Since the rapid emergence of glycomics, which focuses on the relationship between biological function and structures of carbohydrates, many synthetic pathways for formation of glycosidic bonds have been developed (Boltje, Buskas, & Boons, 2009; McKay & Nguyen, 2012). However, in many cases, these chemical reactions yield a mixture of two stereoisomers, α- and β-anomers. Although several chemical synthesis routes have been developed to address the regio- and stereo-selectivity of complex structures of carbohydrates, the optimization for each case always introduces relatively time-consuming and laborious process, such as multi-step protection–deprotection procedure (Demchenko, 2003). Therefore, biocatalysis has become an important alternative to conventional chemical methods in oligosaccharide synthesis due to its highly specific stereoselectivity and regioselectivity (Blanchard & Thorson, 2006; Bojarová & Kren, 2009).

Retaining glycosidases hydrolyze glycosidic bonds by a double displacement mechanism in which two key catalytic residues are involved (Zechel & Withers, 2000). A covalent glycosyl enzyme intermediate is first formed by the action of a catalytic nucleophilic residue to attack the anomeric center of the substrate with the assistance of a catalytic general acid/base residue to protonate the departing leaving group. Next, the glycosyl moiety of the intermediate is transferred to a hydroxyl group of a water molecule that is deprotonated by the action of the general acid/base residue as a base catalyst (hydrolysis in Fig. 1A). However in transglycosylation the hydroxyl group of a productively bound acceptor sugar attacks in place of the water, resulting in the formation of a new glycosidic linkage (transglycosylation in Fig. 1A). Although a variety of retaining α-glycosidases have been successfully applied to synthesis of α-linked oligosaccharides and glycosides (Cheng et al., 2012; Choi et al., 2010; Prade, Mackenzie, & Withers, 1997), their industrial application remains challenging due to subsequent hydrolysis of the transfer products by the glycosidase used. To overcome

* Corresponding author. Tel.: +82 44 860 1436; fax: +82 44 860 1586.
E-mail address: ywankim@korea.ac.kr (Y.-W. Kim).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.08.056

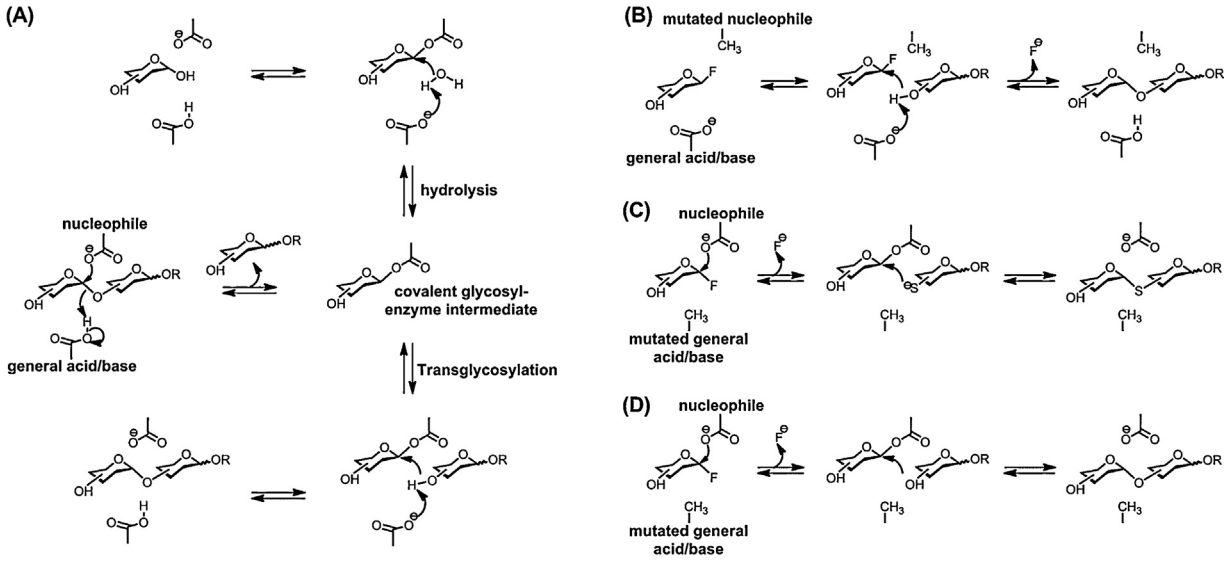

Fig. 1. Mechanisms of transglycosylation catalyzed by a retaining α-glycosidase (A) and engineered mutant glycosidases: α-glycosynthase (B), α-thioglycoligase (C) and *O*-glycoligase (D) derived from a retaining α-glycosidase. Glycosynthases use a glycosyl fluoride with anomeric stereochemistry opposite to its original substrate to mimic the glycosyl enzyme intermediate. Thioglycoligase and *O*-glycoligase use a glycosyl fluoride with the same anomeric stereochemistry as its original substrate.

the subsequent hydrolysis problem, the Withers group developed a revolutionary strategy to engineer retaining glycosidases termed glycosynthases and thioglycoligases (Mackenzie, Wang, Warren, & Withers, 1998; Okuyama, Mori, Watanabe, Kimura, & Chiba, 2002; Yamamoto & Davis, 2012). Glycosynthase mutants with non-nucleophilic residues at catalytic nucleophile positions can catalyze the formation of a new glycosidic linkage with the same anomeric stereochemistry as the original substrates using fluoride glycosides (with stereochemistry opposite to the original substrates) as sugar donors (Fig. 1B). Although this strategy has been applied to various retaining glycosidases (Cobucci-Ponzano & Moracci, 2012; Cobucci-Ponzano, Strazzulli, Rossi, & Moracci, 2011; Hancock, Vaughan, & Withers, 2006), few successful attempts to generate α-glycosynthases derived from retaining α-glycosidases have been reported (Cobucci-Ponzano et al., 2009, 2011b; Okuyama et al., 2002; Sakurama et al., 2012; Wada et al., 2008). In contrast, thioglycoligases are retaining glycosidase mutants at their general acid/base catalytic residue, thus producing non-hydrolyzable *S*-glycosidic linked disaccharides (Armstrong, Reitinger, Kantner, & Withers, 2010; Jahn, Marles, Warren, & Withers, 2003; Kim et al., 2006). The mechanism of action of thioglycoligases is formation of glycosyl enzyme intermediates using highly reactive substrates, such as dinitrophenyl glycosides and glycosyl fluorides, followed by acceleration of the turnover of the intermediate by the addition of proper thio-sugar acceptors bearing a thiol group, resulting in the formation of an *S*-glycosidic linkage (Fig. 1C). For the synthesis of α-thio-linked disaccharides, two α-thioglycoligases have been reported (Kim et al., 2006). Recently, it was determined that an α-thioglycoligase derived from a retaining α-xylosidase was able to transfer a xylosyl moiety of α-D-xylosyl fluoride not only to a thiol group of thiosugar acceptors, but also to a hydroxyl group of normal sugar acceptors (Kim, Zhang, Chen, & Withers, 2010). This glycosidase mutant category comprises acid/base mutants of a retaining α-glycosidase, which catalyzes the synthesis of *O*-α-glycosidic linkage, was termed *O*-glycoligase (Fig. 1D). This approach was proposed as an alternative to the glycosynthase strategy for enzymatic synthesis of *O*-α-glycosidic linkages. Unfortunately, since the first report of *O*-glycoligase in 2010, no other successful example has been reported.

Cyclodextrin glucanotransferases (CGTases, EC2.4.1.19), one of retaining glycosidases belonging to glycoside hydrolase family 13, catalyze not only the degradation of starch but also the transglycosylation to produce cyclomaltodextrins via the double-displacement mechanism involving a covalent maltooligosaccharyl-enzyme intermediate (Mosi, He, Uitdehaag, Dijkstra, & Withers, 1997). CGTases also possess disproportionation activity to transfer maltooligosaccharides from the non-reducing end of one maltooligosaccharide molecule to the non-reducing end of another. Their inherent transglycosylation activity of the wild-type CGTases has been applied to produce maltooligosaccharyl glycosides (Qi & Zimmermann, 2005; Shimoda & Hamada, 2010). Herein, we describe successful conversion of a CGTase from *Bacillus* sp. I-5 (CGT-I5, Genbank accession code AAR32682) (Shim et al., 2004) to an *O*-glycoligase. A mutant with an alanine residue at its general acid/base catalytic residue (CGT-E284A) exhibited *O*-glycoligase activity using α-maltosyl fluoride (αG2F) as the sugar donor. In addition, screening of an improved mutant enzyme from a library generated by site-directed saturation mutagenesis of the acid/base residue demonstrated that a glycine residue was better for *O*-glycoligase activity than alanine.

2. Materials and methods

2.1. General experiments

Escherichia coli TOP10 [F-, *mcr*A, Δ(*mrr-hsdRMS-mcrBC*), φ80*lacZ*ΔM15, *ΔlacX*74, *nupG*, *recA*1, *araD*139, Δ(*ara-leu*)7697, *galE*15, *galK*16, *rpsL*(*StrR*), *endA*1, λ-] was used as the host for DNA manipulation, and *E. coli* BL21(DE3) [F-, *dcm*, *ompT*, *hsdS*(r_B^- m_B^-), *gal*, λ(DE3)] for protein production. *Pwo* polymerase was purchased from Roche (Mannheim, Germany) and restriction enzymes from Fermentas (Opelstrasse, Germany). αG2F was synthesized as described previously (Damager, Numao, Chen, Brayer, & Withers, 2004). 4-Nitrophenyl α-D-glucopyranoside (pNPαG1), 4-nitrophenyl α-maltoside (pNPαG2), 4-nitrophenyl β-D-glucopyranoside (pNPβG1), 4-nitrophenyl β-maltoside (pNPβG2), and silica gel (200–425 mesh) for flash chromatography were purchased from Sigma–Aldrich Co. (St. Louis, USA). High-performance liquid chromatography (HPLC) was carried out using a YL9100 HPLC system (Younglin, Anyang, Korea) equipped with a Nova-Pak® C18 column (3.9 × 150 mm, Waters, Milford, USA). The products were eluted at 0.8 mL/min with acetonitrile–water

(10:90, v/v) and analyzed using an ultraviolet (UV) detector at 254 nm. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a 300 MHz spectrometer from Varian Technologies Ltd. Mass spectra for transfer products were recorded using a LTQ XL linear ion trap mass spectrometer (Thermo Scientific Inc., Waltham, USA). Thin-layer chromatography (TLC) was performed on aluminum-backed sheets of silica gel $60F_{254}$ (Whatman, Maidstone, UK) of 0.2 mm thickness using *n*-butanol/acetic acid/water = 3:1:1 (v/v/v). The plates were visualized using UV light (254 nm) and/or by exposure to 10% sulfuric acid in methanol followed by charring.

2.2. Construction of the CGT-E264A mutant

First, the gene for mature CGTase was amplified using pR2CGT-I5 (Shim et al., 2004) as the template. To remove an internal *Nde*I site, the rear part of the gene for CGT-I5 was first amplified using ExTaq polymerase (Takara Bio Inc., Otsu, Japan) with a CGTΔNd-fw primer (5′-GCGGTCAAGCACATGCCATT C-3′, the mutated sequence to remove the *Nde*I site is underlined) and the following M13 reverse primer (30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, 30 cycles). Polymerase chain reaction (PCR) products were purified using a MEGA-spin™ agarose gel extraction kit (Intron Co., Seongnam, Korea), and subsequently used as megaprimers to obtain full-length genes for mature CGT-I5 along with the CGTΔ27-Nd-fw primer (5′-CTTGAGCCCGGTGCATATGGCCCCGGATACCT-3′, a *Nde*I site for cloning is underlined). Final PCR products were digested with *Nde*I and *Hind*III and extracted from the agarose gel using an agarose gel extraction kit (Intron Co., Seongnam, Korea). To fuse the N-terminus of CGTase with a 6xHis tag, the PCR product was ligated with pBL6xHT (Lee & Kim, 2013), which had previously been digested with the corresponding restriction enzymes. The resulting plasmid, pBL6xHΔCGT, was used as a template for the mutation of CGT-I5 in a four-primer method. Two synthetic primers, ATGSEQ primer (5′-ATCGACTTTGTAGGGT-3′) and CGT-E284-rev primer (5′-GCCGAAGGTGAAGACCGGCTT-3′), were used for amplification of the front part of the gene fragment, whereas CGT-E284A-fw primer (5′-AAGCCGGTCTTCACCTTCGGCGCATGGTTCCTT-3′, the mutated codon for E284A is underlined) and the T7 terminator primer were used for the rear gene fragment. The two PCR products were separately purified with the agarose gel extraction kit. Twenty nanograms of each PCR product were combined for the next primer-less assembly PCR. After seven cycles using ExTaq polymerase, the ATGSEQ and T7 terminator primers were added to the reaction mixture at a final concentration of 50 μM each, and the PCR continued for an additional 25 cycles. The resulting PCR product was digested with *Nde*I and *Hind*III, followed by subcloning into the pET29a vector (Novagen, Darmstadt, Germany) to generate pET29-6xHΔCGT-E284A.

2.3. Saturation mutagenesis at the catalytic acid/base residue of CGT-I5

The plasmid pBL6xHΔCGT was used as a template for the construction of a saturation mutagenesis library of CGT-I5 in a four-primer method. Two synthetic primers, ATGSEQ primer and CGT-E284-rev, were used for amplification of the front part of the gene fragment, while the CGT-E284X-fw (5′-AAGCCGGTCTTCACCTTCGGCNNKTGGTTCCTT-3′, the mutated codon for the saturation mutagenesis at Glu284 is underlined) and T7 terminator primers were used for the rear part. Two PCR products were purified separately using the agarose gel extraction kit. Assembly PCR was conducted, and the resulting PCR product was digested with *Nde*I and *Hind*III followed by subcloning into the pET29a vector (Novagen).

2.4. Screening of CGT-I5 variants showing O-glycoligase activity

The ligation mixture was first transformed into *E. coli* TOP10 cells, and colonies (~3000) were collected using a scraper. Plasmids from the collected colonies were then prepared using a DNA-spin™ Plasmid DNA Purification Kit (Intron Co.). Five hundred nanograms of the recombinant plasmid mixture were transformed into *E. coli* BL21 (DE3) cells. From the library plates, a total of 100 colonies (threefold larger than the size of the library, NNK = 32) were selected randomly and cultured in 3-mL Luria–Bertani (LB) broth medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 0.5% [w/v] NaCl) supplemented with kanamycin (20 μg/mL) at 37 °C. After 4 h, 6 μL of 0.2 M isopropyl β-D-1-thiogalactoside (IPTG) solution was added to each tube to induce the targeted DNA expression at 20 °C overnight. After centrifugation at 8000 × *g* at 4 °C, the cell pellet was resuspended in 50 μL of 50 mM sodium-phosphate buffer (pH 7.5). An equal volume of BugBuster™ protein extraction reagent (Novagen) with 0.1% (w/v) lysozyme (Sigma–Aldrich Co.) was then added to each sample, and the mixtures were incubated for 2 h at room temperature to lyse the cells. Ten microliters of cell lysates were added to 40 μL of 100 mM sodium-phosphate buffer (pH 7.5) containing 20 mM αG2F and 20 mM pNPβG2 as the substrates, and the reaction mixtures were incubated at 25 °C for 24 h. The mixtures were then subjected to thin-layer chromatography (TLC).

2.5. Expression and purification of CGT-I5 variants

The recombinant *E. coli* carrying a recombinant pET29a vector containing a gene for one of CGTase variants was cultured overnight in 1 L of LB broth supplemented with kanamycin (20 μg/mL) at 37 °C until the optical density at 600 nm reached 0.5, and then 0.2 mM IPTG was added to culture broth to induce protein expression. After induction for 20 h at 20 °C with shaking, the cells were harvested, resuspended in 50 mM Tris–HCl buffer (pH 7.5) and then disrupted by sonication. The crude extract supplemented with $CaCl_2$ (final 5 mM) was heated at 60 °C for 20 min and clarified by centrifugation. Then, the heat-treated crude enzyme solution was subjected to Ni-affinity chromatography using an AKTA prime™ plus (GE Healthcare, Uppsala, Sweden) equipped with a His-Trap® column (GE Healthcare) as previously described (Lee & Kim, 2013). The eluted target proteins were dialyzed against 50 mM Na-phosphate buffer (pH 7.5). The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard.

2.6. Transglycosylation reaction catalyzed by CGT-I5 variants

To investigate substrate specificity, 20 mM αG2F and 20 mM a sugar acceptor (either pNPαG1, pNPαG2, pNPβG1, or pNPβG2) in 0.2 M sodium-phosphate buffer (pH 7.5), were treated with CGT-E284A (5 mg/mL final). The mixture was then incubated at 25 °C for 24 h. Reaction mixtures were subjected to TLC or high-performance liquid chromatography (HPLC). For time-course analyses, 20-μL aliquots of the reaction mixtures were removed at the indicated time points, and then added to 80-μL acetonitrile to stop the reaction, followed by HPLC analysis. To quantify the transfer product using HPLC, a standard curve was generated using pNPβG2 as the standard.

2.7. Kinetic analysis of transglycosylation catalyzed by CGT-I5 variants

All kinetic studies were performed at 25 °C in 0.2 M phosphate buffer (pH 7.5). Concentrations of either αG2F (10 mM) or an acceptor sugar (0.8 mM) were fixed, and that of the counterpart was

varied to allow apparent K_M and k_{cat} determinations. Every 60 min, 20 μL of the reaction mixture were removed and added to 80 μL acetonitrile to stop the reaction. The initial rates were determined by measuring production of the transfer product per minute using HPLC. A standard curve for the transfer products was determined by HPLC using pNPβG2 as the standard. Apparent K_M and k_{cat} values were determined by fitting the initial velocity curves to the Michaelis–Menten equation using non-linear regression in GraFit, version 7.0 (Erithacus Software Ltd., East Grinstead, UK). Apparent k_{cat}/K_M values of the CGTase mutants for pNP glycosidases were determined from the slopes of the plots of v versus [S] ($V_o = k_{cat}E_o[S]/K_M$), measured at three or four pNP glycoside concentrations at a fixed concentration of αG2F (10 mM).

2.8. Assay of transglycosylation rates in the presence of external transfer product

The reactions were conducted at fixed donor (αG2F, 10 mM) and acceptor (pNPαG2, 0.8 mM) concentrations in the presence of various concentrations of an external transfer product (pNPβG4) ranging from 0 to 0.25 mM. The reactions were performed with 5 mg/mL CGT-E284G at 25 °C in 0.2 M phosphate buffer (pH 7.5). Every 1 h, 20 μL of the reaction mixture were removed and added to 80 μL acetonitrile to stop the reaction. The initial rates were determined by measuring production of the transfer product per minute using HPLC.

2.9. Preparative scale reaction and purification of transfer products

A mixture of αG2F (17.2 mg, 50 μmol) and pNPβG2 (23.2 mg, 50 μmol) in phosphate buffer (5 mL of 0.1 M, pH 7.5) was treated with CGT-E284G (25 mg), and the mixture was then incubated for 24 h at 25 °C. Aryl glycoside products were purified using a C18 SEP PAK cartridge (Waters), and the solvent was evaporated under reduced pressure. Transfer products were isolated by flash silica gel chromatography using solvent gradient elution (*n*-butanol/acetic acid/water = 8:1:1→ 3:1:1).

p-Nitrophenyl β-D-glucopyranosyl-(1,4)-α-D-glucopyranosyl-(1,4)-α-D-glucopyranosyl-(1,4)-α-D-glucopyranoside (13.4 mg, 34%) white powder, ^{1}H NMR (D_2O, 300 MHz): 8.35 (m, 2H, Ph-H), 7.32 (m, 2H, Ph-H), 5.53 (d, 1H, J = 4.2 Hz, H-1′), 5.48 (d, 1H, J = 1.5 Hz, H-1″), 5.46 (d, 1H, J = 1.8 Hz, H-1‴), 5.37 (d, 1H, J = 7.8 Hz, H-1), 4.05–3.65 (m, 24H, the rest protons of sugar ring). ^{13}C NMR (D_2O, 75 MHz): 164.59, 145.80, 129.01 (2C), 119.36 (2C) (Ph-C); 104.17, 102.60 (2C), 101.47, 78.74, 78.45, 78.28, 75.69, 75.50 (2C), 75.22, 74.64, 74.51, 74.38 (2C), 73.89, 73.77, 73.55, 73.31, 73.13, 63.33 (2C), 63.08, 63.01. ESI-MS: calc. for $C_{30}H_{45}NO_{23} + Na^+$ = 810.4; found 810.4.

3. Results

3.1. O-Glycoligase activity of CGT-E284A

CGT-E284A was homogeneously purified through heat treatment followed by Ni-immobilized column chromatography (Fig. S1 in supplementary information). Upon employment of αG2F and one pNP glycoside (pNPαG1, pNPαG2, pNPβG1, or pNPβG2) as the donor and acceptor (final 20 mM each), respectively, a single transfer product spot in each reaction was detected by TLC after an overnight reaction (Fig. 2A). HPLC analysis revealed that the transfer products were pNP maltooligosaccharides, whose degree of polymerization increased by two (Fig. 2B). It is worth noting that all the four transfer products bear α-linkages including the formed glycosidic bonds except for the linkage to pNP group. Reaction yields of the transfer products by HPLC were 15, 17, 22, and 25%, for reactions using pNPαG1, pNPβG1, pNPαG2, and pNPβG2 as acceptors, respectively, based on the quantities of subjected acceptors.

The transfer product expected to be pNP β-D-maltotetraoside (pNPβG4) was purified using flash column chromatography and subjected to structural investigation by ESI-MS/MS, ^{1}H, and ^{13}C NMR. The mass of the transfer products was determined by ESI-MS to be 810.4 Da (the mass for Na^+-adducted pNPβG4). In the secondary MS/MS spectrum, all fragments of the peak with a mass corresponding to pNPβG4 were the oxocarbenium ion forms of maltooligosaccharides ranging from maltotetraose (671.4 Da) to maltose (347.1 Da) (Fig. S2). These results suggested that the maltose moiety from αG2F was transferred to the acceptor (pNPβG2). ^{1}H NMR data indicated that all monosaccharide residues have α-anomeric configurations based on the spin-spin coupling values for the anomeric hydrogen atoms ($J_{1,2} \approx 1.5$–4.2 Hz), except the pNP linked sugar moiety, which displayed β-anomeric configuration ($J_{1,2}$ = 7.8 Hz). In addition, signals of the phenyl group appeared from 110 to 170 ppm on ^{13}C NMR spectra, whereas a chemical shift of sugar rings was exhibited from 60 to 110 ppm at high-field. Three peaks of ^{13}C-signals at 78.74, 78.45 and 78.28 ppm unambiguously revealed the presence of three α-(1→4)-linkages in the transfer product (Hansen et al., 2008). Further, the absence of chemical shifts at ca. 80–85 ppm and 65–70 ppm, which typically correspond to C-3 and C-6 of the glucose rings in α-1,3-linked and α-1,6-linked maltooligosaccharides (Corzana et al., 2004; Grun et al., 2005), respectively, confirmed that no α-(1→3)- or α-(1→6)-linkage was formed, thereby indicating that CGT-E284A exclusively forms α-(1→4)-linkages.

3.2. Screening of CGTase variants with O-glycoligase activity from a saturation mutagenesis library

To optimize the residue at the acid/base catalyst position for *O*-glycoligase, saturation mutagenesis at the catalytic general acid/base residue of CGT-I5 was conducted and crude enzymes were prepared. The transglycosylation reactions were carried out as described in experimental section. Based on analysis of products by TLC, the control reaction using crude CGT-E284A prepared in the same fashion did not produce a detectable transfer product under the screening condition (Fig. S3, lane 1). In contrast, five clones produced a single transfer product, expected to be pNPβG4 (Fig. S3, lane 2). In addition, 21 clones produced a series of pNP glycosides ranging from pNPβG1 to pNPβG4, suggesting that they possessed transglycosylation activity as well as hydrolysis (Fig. S3, lanes 3 and 4). Therefore, the former five clones should possess improved transglycosylation activity compared to CGT-E284A, the original *O*-glycoligase, and the latter 21 clones were believed to be the wild-type enzyme. DNA sequencing analysis revealed that all five clones had a glycine residue at the general acid/base catalyst position of CGT-I5 (the mutant is termed CGT-E284G hereafter). Interestingly, of 21 clones that showed both transglycosylation and hydrolysis activities, six were mutants whose general acid/base catalyst was replaced with a serine residue (the mutant is termed CGT-E284S hereafter), while the rest were identified as the wild-type enzyme, as expected.

3.3. Catalytic properties of CGTase mutants

To compare the catalytic properties of CGT-I5 mutants, three acid/base mutants (CGT-E284A, CGT-E284G and CGT-E284S) and the wild-type enzyme were purified using Ni-NTA chromatography. Reaction mixtures catalyzed by the CGT-I5 variants were analyzed by TLC (Fig. 3). As seen during the screening stage, CGT-E284G showed only transglycosylation activity using αG2F and pNPβG2 as substrates without hydrolysis toward the product and

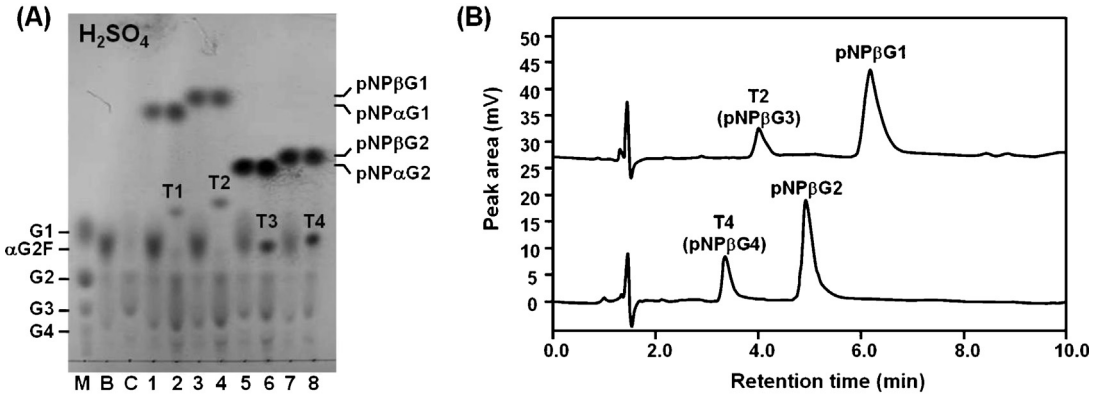

Fig. 2. (A) TLC analysis of reaction mixtures by CGT-E284A using various acceptors. Reaction conditions were 20 mM αG2F, 20 mM acceptor and CGTase-E284A (5 mg/mL) in 0.2 M sodium-phosphate buffer (pH 7.5) at 25 °C for 24 h. Lane M, G1–G4 standard; lane B, αG2F standard; lane C, reaction mixture with only αG2F; lanes 1, 3, 5, and 7, blank reactions without CGT-E284A using pNPαG1, pNPβG1, pNPαG2, and pNPβG2 as acceptors, respectively; lanes 2, 4, 6, and 8, reactions using pNPαG1, pNPβG1, pNPαG2, and pNPβG2 as acceptors, respectively. T1, T2, T3, and T4 indicate pNPαG3, pNPβG3, pNPαG4, and pNPβG4, respectively. (B) HPLC analysis of reaction mixtures using pNPβG1 (upper) and pNPβG2 (lower) as acceptors.

acceptor, whereas CGT-E284G exhibited greater transglycosylation activity than CGT-E284A (Fig. 3, lines 1 and 3). In contrast, the Ser mutant (CGT-E284S) possessed not only transglycosylation activity, but also significant hydrolysis activity, resulting in the production of pNPβG4 as the transfer product, as well as pNPβG1 and pNP β-D-maltotrioside (pNPβG3) as hydrolysis products from the transfer product (Fig. 3, line 2). The HPLC time-course analysis showed that CGT-E284G exhibited dramatically higher catalytic activity than CGT-E284A and CGT-E284S (Fig. 4), consistent with TLC results (Fig. 3). Although CGT-E284S produced more transfer product in the early stage than CGT-E284A, its final reaction yield of pNPβG4 was the lowest among these transglycosylations due to the hydrolysis of the transfer product by CGT-E284S.

3.4. Kinetic analysis of the transglycosylation reaction catalyzed by CGTase mutants

In terms of the kinetics of transglycosylation of CGTase variants, there are two chemical steps during the transglycosylation reaction, the formation of the glycosyl-enzyme intermediate and the nucleophilic attack of the acceptor to the intermediate. This reaction mechanism will give two rate constants, and the overall rate constant would depend on which step is rate-limiting. Here, we simply calculated apparent k_{cat} and K_M values by fitting the initial rates to Michaelis–Menten equation at a fixed concentration of either donor or acceptor, followed by the comparison of the apparent k_{cat}/K_M values of the variants.

To investigate the effect of mutations on acceptor specificity of *O*-glycoligases derived from CGT-I5 (CGT-E284A and CGT-E284G), kinetic parameters were determined for various acceptor sugars using a fixed donor sugar concentration (αG2F, 10 mM). At high acceptor concentrations (>1 mM), severe inhibition was observed. Unfortunately, the fit of the initial rate to the substrate inhibition equation was poor (curved dash lines in Fig 5A and Fig. S4A–C). For this reason, k_{cat}/K_M values were calculated using data obtained using a low substrate concentration, which exhibited a linear relationship between the acceptor concentrations (0.2–0.6 mM) and corresponding rates (linear solid lines in Fig 5A and Fig. S4A–C). The k_{cat}/K_M values of CGT-E284G for all acceptor sugars increased more than threefold relative to that of CGT-E284A (Table 1). Of the acceptors, pNPαG1 was the worst for both variants, but it is worth mentioning that the greatest improvement of the catalytic efficiency (k_{cat}/K_M) was achieved (6.3-fold compared to those of CGT-E284A). Meanwhile, the kinetic results indicated that two variants have similar substrate specificities (maltosides > glucosides).

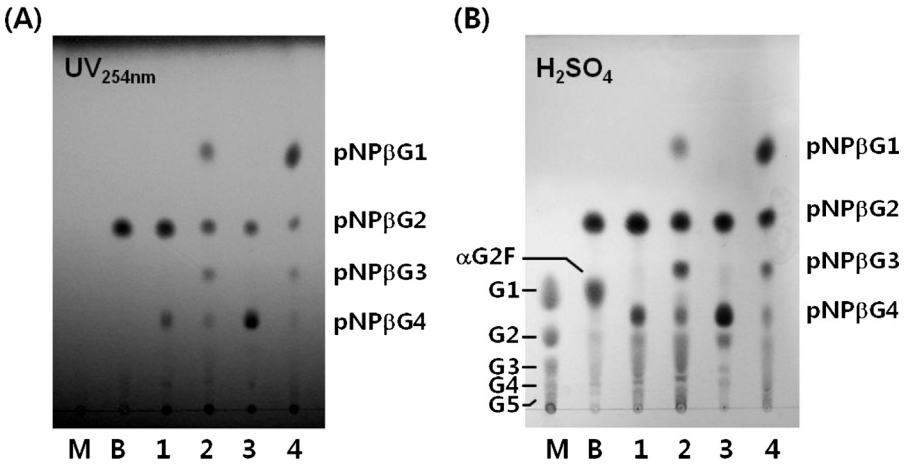

Fig. 3. TLC analysis of reaction mixtures by CGT-I5 wild-type and its variants. TLC plate was visualized under UV light (A) and by H_2SO_4 (B). Reaction conditions were 20 mM αG2F, 20 mM pNPβG2 and each CGTase mutant (5 mg/mL) in 0.2 M sodium-phosphate buffer (pH 7.5) at 25 °C for 24 h. Lane M, maltooligosaccharides standard from glucose (G1) to maltopentaose (G5); lane B, blank reaction; lane 1, reaction mixture of CGT-E284A; lane 2, reaction mixture of CGT-E284S; lane 3, reaction mixture of CGT-E284G; lane 4, reaction mixture of CGT-I5 wild-type.

Table 1
Comparison of kinetic parameters for transglycosylation of various substrates by CGT-E284A and CGT-E284G.

Mutant	Variable substrate	Fixed substrate[a]	k_{cat}[b] (min^{-1})	K_M[b] (mM)	k_{cat}/K_M[b] (min^{-1} mM^{-1})	Fold
CGT-E284A	pNPβG1	αG2F	–[c]	–[c]	$(4.52 \pm 0.55) \times 10^{-2}$	1.0
	pNPβG2	αG2F	–[c]	–[c]	$(1.16 \pm 0.08) \times 10^{-1}$	1.0
	pNPαG1	αG2F	–[c]	–[c]	$(2.65 \pm 0.34) \times 10^{-2}$	1.0
	pNPαG2	αG2F	–[c]	–[c]	$(9.36 \pm 0.76) \times 10^{-2}$	1.0
	αG2F	pNPβG1	$(1.83 \pm 0.28) \times 10^{-2}$	2.53 ± 0.47	0.72×10^{-2}	1.0
	αG2F	pNPβG2	$(2.06 \pm 0.33) \times 10^{-2}$	2.07 ± 0.61	1.00×10^{-2}	1.0
CGT-E284G	pNPβG1	αG2F	–[c]	–[c]	$(2.52 \pm 0.12) \times 10^{-1}$	5.6
	pNPβG2	αG2F	–[c]	–[c]	$(3.67 \pm 0.06) \times 10^{-1}$	3.2
	pNPαG1	αG2F	–[c]	–[c]	$(1.67 \pm 0.05) \times 10^{-1}$	6.3
	pNPαG2	αG2F	–[c]	–[c]	$(3.67 \pm 0.08) \times 10^{-1}$	3.9
	αG2F	pNPβG1	$(7.52 \pm 0.49) \times 10^{-2}$	3.86 ± 0.48	1.95×10^{-2}	2.7
	αG2F	pNPβG2	$(9.78 \pm 0.59) \times 10^{-2}$	3.12 ± 0.35	3.13×10^{-2}	3.1

[a] Concentrations of αG2F and pNP glycosides were fixed at 10 and 0.8 mM, respectively.
[b] Values are apparent parameters for the variable substrates at the co-substrate concentration indicated.
[c] Not determined.

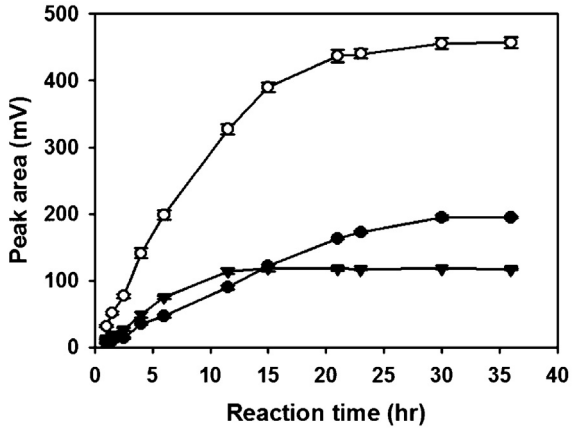

Fig. 4. Time-course analysis of transglycosylation product produced by CGT-I5 variants. Reaction conditions were 20 mM αG2F, 20 mM pNPβG2 and CGT-I5 mutants (5 mg/mL) in 0.2 M sodium-phosphate buffer (pH 7.5) at 25 °C. CGT-E284A (●), CGT-E284G (○) and CGT-E284S (▼).

Due to substrate inhibition by the acceptors at varying concentrations of the donor αG2F, the rates were measured at a fixed acceptor concentration (0.8 mM). Under these conditions, both enzymes showed standard saturation kinetics (Fig. 5B and Fig. S4D). Using pNPβG2 as the acceptor, the k_{cat} for αG2F of CGT-E284G (0.098 min^{-1}) increased by 4.7-fold relative to that of CGT-E284A (0.021 min^{-1}). The catalytic efficiency (k_{cat}/K_M) of αG2F increased 3.1-fold due to decreased affinity of CGT-E284G for αG2F (K_M values for CGT-E284A and CGT-E284G were 2.07 and 3.12 mM, respectively). The trends of improvement of CGT-E284A and CGT-E284G for pNPβG1 were consistent with those of pNPβG2 (4.1-fold improved k_{cat} and 2.7-fold improved k_{cat}/K_M, etc.; Table 1).

The rate reduction at high acceptor concentrations may be due to substrate inhibition caused by binding of the acceptors to glycone subsites of the CGTase mutants in non-productive binding mode. If not, product inhibition may reduce the reaction rate, as the transfer products with longer degree of polymerization than the subjected acceptors may bind well to both aglycone and glycone subsites of the enzymes. In order to investigate the product inhibition, the initial rates were measured using 10 mM αG2F and 0.8 mM pNPαG2 as the donor and the acceptor sugars, respectively, in the presence of different concentrations of pNPβG4 as an inhibitor. At 0.05 mM of pNPβG4 which was less than 10% of the subjected acceptor (0.8 mM pNPαG2) the initial rate slowed down to 83% of the rate without the external product and kept decreasing as the acceptor concentration increased (Fig. 5C). This result allows us to know that the transfer products, such as trisaccharides and tetrasaccharides, would have higher affinity than the corresponding acceptors, and the reduction of the reaction rates at high acceptor concentration may be resulted from production of more inhibitor molecules (the transfer products).

3.5. Time-course analysis of transglycosylation catalyzed by CGT-E284G

In order to investigate the effect of inhibition of CGT-E284G at a high acceptor concentration on transfer product yield, a

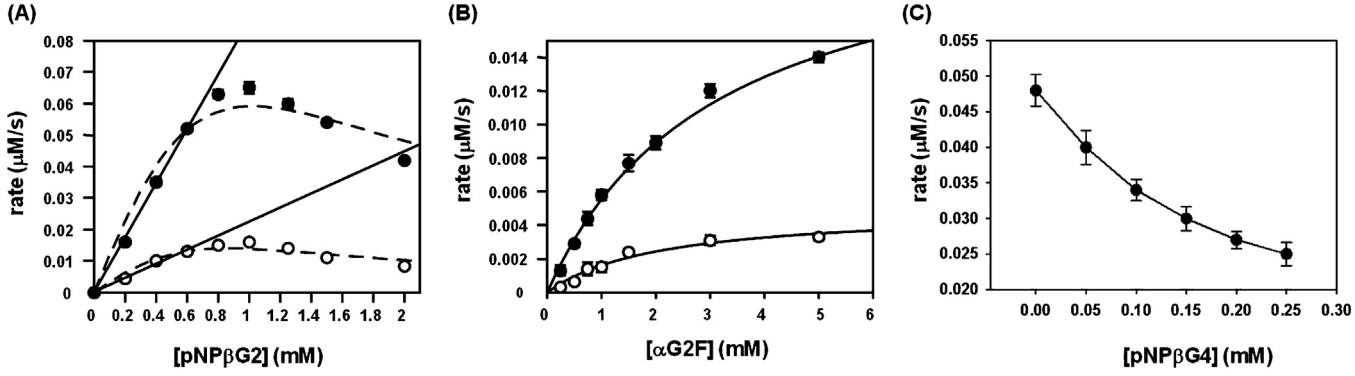

Fig. 5. Comparisons of transglycosylation kinetics with CGT-E284A and CGT-E284G for pNPβG2 using αG2F as a donor fixed at 10 mM (A) and for αG2F using pNPβG2 as an acceptor fixed at 0.8 mM (B). Panel C represents the effect of pNPβG4 as an inhibitor on the transglycosylation rate of CGT-E284G using 10 mM αG2F and 0.8 mM pNPαG2 as the substrates. The dashed curves and linear solid lines in panel (A) represent the results of the fitting data into the substrate inhibition kinetics equation and those of the fitting data at the low concentrations of the substrates into the linear regression equation to get slopes giving the values for v_{max}/K_M. The solid curved lines in panel (D) represent the results of the fitting data into the Michaelis–Menten kinetics equation.

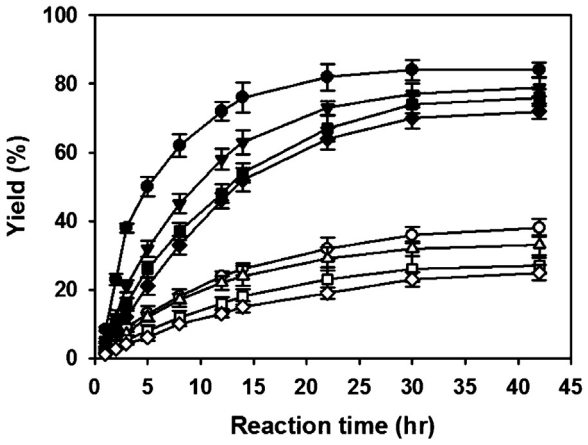

Fig. 6. Time-course analysis of transglycosylation catalyzed by CGT-E284G. Reactions were carried out in 0.2 M sodium-phosphate buffer (pH 7.5) with 5 mg/mL CGT-E284G at 25 °C for 24 h. Concentrations of the donor and acceptor are as indicated. *Y*-axis represents the relative yields that were calculated by dividing the amount of transfer product by that of the subjected acceptor, as determined by HPLC. Open symbols represent the reaction at 1 mM of each substrate, whereas closed symbols represent the reaction at 10 mM of each substrate. The acceptors were pNPβG2 (circle), pNPαG2 (triangle), pNPβG1 (square), and pNPαG1 (diamond).

Table 2
Relative transglycosylation yields of the reactions by CGT-E284G[a].

Concentration	Donor	Acceptor	Yield (%)[b]
1 mM	αG2F	pNPαG1	69 ± 1.9
		pNPβG1	72 ± 3.3
		pNPαG2	76 ± 2.1
		pNPβG2	84 ± 2.9
10 mM	αG2F	pNPαG1	22 ± 1.5
		pNPβG1	28 ± 2.4
		pNPαG2	32 ± 2.7
		pNPβG2	36 ± 2.3

[a] Concentrations of the donor and acceptors in reactions for this table were fixed as indicated.
[b] Yields were calculated based on the amounts of subjected acceptor sugars.

time-course analysis of reactions using 1 and 10 mM acceptors was conducted (Fig. 6). Transglycosylation yields, determined by HPLC, of the reaction using 10 mM αG2F and pNP glycosides as substrates with CGT-E284G (5 mg/mL), were 22–36%. In contrast, at 1 mM donor and acceptors, relative reaction yields increased by more than twofold (69–84%, Table 2) compared to those with use of 10 mM substrates. The preference order of the acceptor was pNPβG2 > pNPαG2 > pNPβG1 > pNPαG1, which is consistent with the kinetic analysis.

4. Discussion

Applications and engineering of glycosidases for glycoside synthesis, and the improvement of transglycosylation properties, are interesting topics in carbohydrate chemistry. Glycosynthase methodology is a powerful enzymatic tool for glycosidic bond synthesis, and has been applied to various retaining glycosidases (Hancock et al., 2006). Although application of the glycosynthase strategy to α-glycosidases is limited compared with β-glycosidases, the repertoire of α-glycosynthases has expanded to GH27, GH31 and GH97 (Cobucci-Ponzano et al., 2009, 2011b; Okuyama et al., 2002; Sakurama et al., 2012; Wada et al., 2008). Thus far, all α-glycosynthases have been derived from exo-type α-glycosidases, not endo-type enzymes.

Recently, it was reported that an α-xylosidase mutant (YicI-D482A) with an alanine residue at its catalytic acid/base residue, efficiently catalyzes the synthesis of not only a *S*-linked α-(1,6)-xylosidic linkage as a thioglycoligase, but also an *O*-linked α-(1,6)-xylosidic linkage (Kim et al., 2006, 2010). Although the reaction mechanism is not clearly understood, the reactive β-skew boat conformation of the glycosyl enzyme intermediate of the α-xylosidase mutant (Lovering, Lee, Kim, Withers, & Strynadka, 2005) could drive transglycosylation without the assistance of the general acid/base catalyst. The mutant, *O*-glycoligase, produced transfer products at near-quantitative yields. Therefore, expansion of the *O*-glycoligase strategy to other α-glycosidases is important for the synthesis of various α-glycosidic linkages. Unfortunately, attempts to generate *O*-glycoligases using an α-amylase from *Bacillus halodurans* (GH13), α-glucosidase from *Sulfolobus solfataricus* (GH31), and α-galactosidase from *S. solfataricus* (GH36), were unsuccessful. Additionally, these mutants only hydrolyzed α-glycosyl fluorides used as the donor sugar (unpublished data), raising the possibility that the inherently high transglycosylation activity of the wild-type enzyme is more important than the conformation of the intermediate. Indeed, the wild-type enzyme (YicI) of the first *O*-glycoligase showed strong transglycosylation activity (Kang et al., 2007). Therefore, we chose a CGTase, one of the most powerful enzymes for transglycosylation reactions, as a candidate for the second *O*-glycoligase. CGTase mutants transferred an α-maltosyl moiety to pNP glycosides as acceptors, albeit at slow rates (k_{cat}/K_M values ranged from 0.02 to 0.03 min^{-1} mM^{-1} for αG2F, and 0.16 to 0.37 min^{-1} mM^{-1} for pNP glycosides, Table 1). Therefore, the effect of binding of acceptor sugars on transition state stabilization of the transglycosylation step of the parent enzymes is likely critical for generating active *O*-glycoligases. The importance of the powerful transglycosylation activity of parent enzymes for glycosynthases has also been discussed (Ducros et al., 2003).

In the development of engineered glycosidase mutants such as *O*-glycoligases, as well as glycosynthases and thioglycoligases, the choice of the residue for mutation at the key catalytic residue is of the highest importance. Extensive work on glycosynthases indicates that Ala, Ser, and Gly residues are candidates for mutation at the nucleophilic position (Cobucci-Ponzano et al., 2011a; Hancock et al., 2006). Of these, the Gly residue is often the most appropriate mutation (Cobucci-Ponzano et al., 2009, 2011b; Mayer et al., 2001; Wada et al., 2008; Yamamoto & Davis, 2012), but not always (Ducros et al., 2003). In the case of thioglycoligases, Gln as the most appropriate mutation for a thioglycoligase derived from *Agrobacterium* sp. β-glycosidase (Müllegger, Jahn, Chen, Warren, & Withers, 2005), whereas optimal substitutions of a thioglycoligase from *Bacillus circulars* xylanase (Bcx) were small, preferably polar amino acids such as Thr, Cys, and Ser (Armstrong et al., 2010). In this study, we screened for the most appropriate mutation of the CGTase variant as the *O*-glycoligase for the first time. Interestingly, our screening findings indicated that the identity of mutation in *O*-glycoligase were the Ala, Gly and Ser residues at the acid/base position, and that the Gly mutant (CGT-E284G) was the most appropriate of the CGTase variants (Figs. 3 and 4 and Table 1), which is consistent with the previous works for glycosynthases (Ducros et al., 2003; Hancock et al., 2006). Although the Ser mutant (CGT-E284S) exhibited higher *O*-glycoligase activity than CGT-E284A, the yield for CGT-E284S decreased after 12 h due to hydrolysis of the transfer product (Fig. 4), which is consistent with the case of Bcx thioglycoligases possessing polar amino acids (threonine, serine, and cysteine) in place of the acid/base residue (Armstrong et al., 2010).

In terms of the kinetics of transglycosylation of both CGT-E284A and CGT-E284G, reaction rates decreased as the acceptor concentration increased. The presence of 0.05 mM of pNPβG4 reduced the reaction rate of CGT-E284G under the condition that no inhibition was observed; 0.8 mM pNPαG2 as the acceptor. This result implies that the transfer product can bind well to the active site in

non-productive binding mode, leading to severe product inhibition. Such product inhibition resulted in modest reaction yields under the 10 mM substrate condition (<40%, Table 2 and Fig. 6). A decrease in substrate concentration to reduce the effect of product inhibition elevated relative reaction yields by approximately twofold (up to 80%, Table 2 and Fig. 5). Although relative reaction yields at 1 mM were higher than those at 10 mM the absolute quantity of product (pNPβG4) in the reaction at 10 mM was greater than that at 1 mM. Therefore, generation of greater quantities of inhibitors (transfer products) under high-substrate-concentration conditions would markedly reduce transglycosylation rates at an early stage of the reaction, leading to lower yields. For the first *O*-glycoligase (YicI-D482A), near-quantitative yields were achieved due to the low K_M values of the acceptors (10–20 μM), which would be expected to overcome strong product inhibition (Kim et al., 2010). More importantly no by-product such as that produced in the reactions catalyzed by wild type CGTases due to their hydrolysis, cyclodextrin forming and disproportionation activities was observed. In the reaction conditions for transglycosylation using CGT-E284G, there was neither detectable cyclodextrin-forming activity (Data not shown) nor production of by-products (Lane 3 in Fig. 3 and Fig. S5). Although the catalytic power of the improved CGTase variant (CGT-E284G) is still not enough for industrial applications, further engineering through directed evolution would likely lead to discovery of mutations that improve *O*-glycoligase activity. In addition, the superiority of Gly compared to Ala as the *O*-glycoligase mutation in this study may facilitate the conversion of other retaining α-glycosidases to active *O*-glycoligases.

Acknowledgment

This work was supported by a grant for research fellowship from Korea University (for C. L.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.056.

References

Armstrong, Z., Reitinger, S., Kantner, T., & Withers, S. G. (2010). Enzymatic thioxyloside synthesis: Characterization of thioglycoligase variants identified from a site-saturation mutagenesis library of *Bacillus circulans* xylanase. *Chembiochem*, *11*(4), 533–538.

Blanchard, S., & Thorson, J. S. (2006). Enzymatic tools for engineering natural product glycosylation. *Current Opinion in Chemical Biology*, *10*(3), 263–271.

Bojarová, P., & Kren, V. (2009). Glycosidases: A key to tailored carbohydrates. *Trends in Biotechnology*, *27*(4), 199–209.

Boltje, T. J., Buskas, T., & Boons, G. J. (2009). Opportunities and challenges in synthetic oligosaccharide and glycoconjugate research. *Nature Chemistry*, *1*(8), 611–622.

Cheng, W., Wang, L., Jiang, Y. L., Bai, X. H., Chu, J., Li, Q., et al. (2012). Structural insights into the substrate specificity of *Streptococcus pneumoniae* β(1,3)-galactosidase BgaC. *Journal of Biological Chemistry*, *287*(27), 22910–22918.

Choi, C. H., Kim, S. H., Jang, J. H., Park, J. T., Shim, J. H., Kim, Y. W., et al. (2010). Enzymatic synthesis of glycosylated puerarin using maltogenic amylase from *Bacillus stearothermophilus* expressed in *Bacillus subtilis*. *Journal of the Science of Food and Agriculture*, *90*(7), 1179–1184.

Cobucci-Ponzano, B., Conte, F., Bedini, E., Corsaro, M. M., Parrilli, M., Sulzenbacher, G., et al. (2009). β-Glycosyl azides as substrates for alpha-glycosynthases: Preparation of efficient alpha-L-fucosynthases. *Chemistry and Biology*, *16*(10), 1097–1108.

Cobucci-Ponzano, B., & Moracci, M. (2012). Glycosynthases as tools for the production of glycan analogs of natural products. *Natural Products Reports*, *29*(6), 697–709.

Cobucci-Ponzano, B., Strazzulli, A., Rossi, M., & Moracci, M. (2011). Glycosynthases in biocatalysis. *Advanced Synthesis & Catalysis*, *353*(13), 2284–2300.

Cobucci-Ponzano, B., Zorzetti, C., Strazzulli, A., Carillo, S., Bedini, E., Corsaro, M. M., et al. (2011). A novel α-D-galactosynthase from *Thermotoga maritima* converts β-D-galactopyranosyl azide to α-galacto-oligosaccharides. *Glycobiology*, *21*(4), 448–456.

Corzana, F., Motawia, M. S., Penhoat, C. H., van den Berg, F., Blennow, A., Perez, S., et al. (2004). Hydration of the amylopectin branch point. Evidence of restricted conformational diversity of the α-(1–>6) linkage. *Journal of the American Chemical Society*, *126*, 13144–13155.

Damager, I., Numao, S., Chen, H., Brayer, G. D., & Withers, S. G. (2004). Synthesis and characterisation of novel chromogenic substrates for human pancreatic α-amylase. *Carbohydrate Research*, *339*(10), 1727–1737.

Demchenko, A. V. (2003). Stereoselective chemical 1,2-*cis* *O*-glycosylation: From 'Sugar Ray' to modern techniques of the 21st century. *Synlett*, 1225–1240.

Ducros, V. M., Tarling, C. A., Zechel, D. L., Brzozowski, A. M., Frandsen, T. P., von Ossowski, I., et al. (2003). Anatomy of glycosynthesis: Structure and kinetics of the Humicola insolens Cel7B E197A and E197S glycosynthase mutants. *Chemistry and Biology*, *10*(7), 619–628.

Grun, C. H., Hochstenbach, F., Humbel, B. M., Verkleij, A. J., Sietsma, J. H., Klis, F. M., et al. (2005). The structure of cell wall alpha-glucan from fission yeast. *Glycobiology*, *15*, 245–257.

Hancock, S. M., Vaughan, M. D., & Withers, S. G. (2006). Engineering of glycosidases and glycosyltransferases. *Current Opinion in Chemical Biology*, *10*(5), 509–519.

Hansen, P. I., Larsen, F. H., Motawia, S. M., Blennow, A., Spraul, M., Dvortsak, P., et al. (2008). Structure and hydration of the amylopectin trisaccharide building blocks—Synthesis, NMR, and molecular dynamics. *Biopolymers*, *89*(12), 1179–1193.

Jahn, M., Marles, J., Warren, R. A. J., & Withers, S. G. (2003). Thioglycoligases: Mutant glycosidases for thioglycoside synthesis. *Angewandte Chemie International Edition*, *42*(3), 352–354.

Kang, M. S., Okuyama, M., Yaoi, K., Mitsuishi, Y., Kim, Y. M., Mori, H., et al. (2007). Aglycone specificity of *Escherichia coli* α-xylosidase investigated by transxylosylation. *FASEB Journal*, *274*(23), 6074–6084.

Kim, Y. W., Lovering, A. L., Chen, H., Kantner, T., McIntosh, L. P., Strynadka, N. C., et al. (2006). Expanding the thioglycoligase strategy to the synthesis of α-linked thioglycosides allows structural investigation of the parent enzyme/substrate complex. *Journal of the American Chemical Society*, *128*(7), 2202–2203.

Kim, Y. W., Zhang, R., Chen, H., & Withers, S. G. (2010). *O*-Glycoligases, a new category of glycoside bond-forming mutant glycosidases, catalyse facile syntheses of isoprimeverosides. *Chemical communications (Cambridge)*, *46*(46), 8725–8727.

Lee, J. I., & Kim, Y. W. (2013). Characterization of amine oxidases from *Arthrobacter aurescens* and application for determination of biogenic amines. *World Journal of Microbiology and Biotechnology*, *29*(4), 673–682.

Lovering, A. A., Lee, S. S., Kim, Y. W., Withers, S. G., & Strynadka, N. C. J. (2005). Mechanistic and structural analysis of a family 31 α-glycosidase and its glycosyl-enzyme intermediate. *Journal of Biological Chemistry*, *280*, 2102–2125.

Mackenzie, L. F., Wang, Q., Warren, R. A., & Withers, S. G. (1998). Glycosynthases: Mutant glycosidases for oligosaccharide synthesis. *Journal of the American Chemical Society*, *120*, 5583–5584.

Mayer, C., Jakeman, D. L., Mah, M., Karjala, G., Gal, L., Warren, R. A., et al. (2001). Directed evolution of new glycosynthases from *Agrobacterium* β-glucosidase: A general screen to detect enzymes for oligosaccharide synthesis. *Chemistry and Biology*, *8*(5), 437–443.

McKay, M. J., & Nguyen, H. M. (2012). Recent advances in transition metal-catalyzed glycosylation. *ACS Catalysis*, *2*(8), 1563–1595.

Mosi, R., He, S., Uitdehaag, J., Dijkstra, B. W., & Withers, S. G. (1997). Trapping and characterization of the reaction intermediate in cyclodextrin glycosyltransferase by use of activated substrates and a mutant enzyme. *Biochemistry*, *36*(32), 9927–9934.

Müllegger, J., Jahn, M., Chen, H., Warren, R. A., & Withers, S. G. (2005). Engineering of a thioglycoligase: Randomized mutagenesis of the acid-base residue leads to the identification of improved catalysts. *Protein Engineering, Design and Selection*, *18*, 33–40.

Okuyama, M., Mori, H., Watanabe, K., Kimura, A., & Chiba, S. (2002). Alpha-glucosidase mutant catalyzes "alpha-glycosynthase"-type reaction. *Bioscience, Biotechnology, and Biochemistry*, *66*(4), 928–933.

Prade, H., Mackenzie, L. F., & Withers, S. G. (1997). Enzymatic synthesis of disaccharides using *Agrobacterium* sp. β-glucosidase. *Carbohydrate Research*, *305*(3–4), 371–381.

Qi, Q., & Zimmermann, W. (2005). Cyclodextrin glucanotransferase: From gene to applications. *Applied Microbiology and Biotechnology*, *66*(5), 475–485.

Sakurama, H., Fushinobu, S., Hidaka, M., Yoshida, E., Honda, Y., Ashida, H., et al. (2012). 1,3-1,4-α-L-Fucosynthase that specifically introduces Lewis a/x antigens into type-1/2 chains. *Journal of Biological Chemistry*, *287*(20), 16709–16719.

Shim, J. H., Kim, Y. W., Kim, T. J., Chae, H. Y., Park, J. H., Cha, H., et al. (2004). Improvement of cyclodextrin glucanotransferase as an antistaling enzyme by error-prone PCR. *Protein Engineering, Design and Selection*, *17*(3), 205–211.

Shimoda, K., & Hamada, H. (2010). Production of hesperetin glycosides by *Xanthomonas campestris* and cyclodextrin glucanotransferase and their anti-allergic activities. *Nutrients*, *2*(2), 171–180.

Wada, J., Honda, Y., Nagae, M., Kato, R., Wakatsuki, S., Katayama, T., et al. (2008). 1,2-α-L-Fucosynthase: A glycosynthase derived from an inverting alpha-glycosidase with an unusual reaction mechanism. *FEBS Letters*, *582*(27), 3739–3743.

Yamamoto, K., & Davis, B. G. (2012). Creation of an α-mannosynthase from a broad glycosidase scaffold. *Angewandte Chemie International Edition*, *51*(30), 7449–7453.

Zechel, D. L., & Withers, S. G. (2000). Glycosidase mechanisms: Anatomy of a finely tuned catalyst. *Accounts of Chemical Research*, *33*(1), 11–18.