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Modulation of the Regioselectivity of a *Thermotoga neapolitana* β-Glucosidase by Site-Directed Mutagenesis

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***Thermotoga neapolitana* β-glucosidase (BglA) was subjected to site-directed mutagenesis in an effort to increase its ability to synthesize arbutin derivatives by transglycosylation. The transglycosylation reaction of the wild-type enzyme displays major β(1,6) and minor β(1,3) or β(1,4) regioselectivity. The three mutants, N291T, F412S, and N291T/F412S, increased the ratio of transglycosylation/hydrolysis compared with the wild-type enzyme when *p*NPG and arbutin were used as a substrate and an acceptor, respectively. N291T and N219T/F412S had transglycosylation/hydrolysis ratios about 3- and 8-fold higher, respectively, than that of the wild-type enzyme. This is due to the decreased hydrolytic activity of the mutant rather than increased transglycosylation activity. Interestingly, N291T showed altered regioselectivity, as well as increased transglycosylation products. TLC analysis of the transglycosylation products indicated that N291T retained its β(1,3) regioselectivity, but lost its β(1,4) and β(1,6) regioselectivity. The altered regioselectivity of N291T using two other acceptors, esculin and salicin, was also confirmed by TLC. The major transglycosylation products of the wild type and N291T mutant were clearly different. This result suggests that Asn-291 is highly involved in the catalytic mechanism by controlling the transglycosylation reaction.**

Keywords: Arbutin derivatives, β-glucosidase, regioselectivity, *Thermotoga neapolitana*, transglycosylation

Many of the glycosides that naturally occur in plants have biological activities [17]. These activities are primarily due to the aglycones of the glycosides. The water solubilities of hydrophobic molecules containing one or more hydroxyl groups can be increased by glycosylation, which can influence their physicochemical and pharmacological properties, such as transport through membrane barriers and transport by body fluids [17]. Glycosylation also often reduces the irritation and toxicity caused by the aglycone [17].

Enzymatic synthesis of glycosides has an advantage over chemical synthesis because it can control the regio- and stereospecificities of the reaction [19]. Glycosidases are used in reactions of reverse hydrolysis or transglycosylation [3, 5, 16]. Family 1 glycosyl hydrolase (GH1), which can catalyze the transglycosylation reaction as well as hydrolysis, is ideal for use in the synthesis of glycoside derivatives [22]. Specifically, β-glucosidases have received great interest because of their biosynthetic abilities and their various biological functions [9, 10, 20, 23]. In our early reports, we described the *Thermotoga neapolitana* β-glucosidase-catalyzed synthesis of arbutin derivatives [21]. However, in the previous studies, the yield of the arbutin derivatives was very low. The low yield of the reaction is a demerit of the reaction of GH1 enzymes [14]. Moreover, the presence of undesired regioisomers, which make the purification step difficult, has encouraged us to improve the properties of the enzyme using different strategies. Many review articles have been published about the use of glycoside synthesis for the purpose of increasing the yield and highly regioselective synthesis of glycosides [1, 6, 7, 19, 22]. The β-glycosidase of *Thermus thermophilus* increased its transglycosylation ability to synthesize oligosaccharides through random mutagenesis [11]. The most efficient mutants, F401S and N282T, increased the yield of trisaccharides by 6-fold compared with the wild-type enzyme. Mutations M424K and F426Y also significantly improved (18–40%) the synthesis of galactooligosaccharides from the *Pyrococcus furiosus* β-glucosidase [13].

Changes in the yield of transglycosylation products usually affect the regioselectivity in the enzymatic synthesis of the glycosides. In general, glycosidases exhibit low

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regioselectivity for the hydroxyl linkage of acceptor substrates in the transglycosylation reaction. It is also known that the regioselectivity in the enzymatic synthesis of oligosaccharides using glycosidases is dependent on the origin of the microbe and the selection of acceptors [2, 18]. Therefore, it is important to determine what controls the regioselectivity of glycoside synthesis in the enzyme reaction. Although regioselectivity in the synthesis of glycosides using glycosidases is known to be very low, transglycosylation products were shown to be highly regioselective when glycosidases that showed high specificity were used in the hydrolysis reaction [1].

In this paper, we report our success in isolating BglA mutants that show increased transglycosylation activity compared with the wild-type enzyme. Moreover, one of the BglA mutants was identified as being strongly involved in the regioselectivity of glycoside synthesis. Thus, directed evolution is an efficient approach to the synthesis of various transglycosylation products through the modification of the enzyme regioselectivity. It also acts to improve the yield of the transglycosylation products of glycosidases.

Materials and Methods

Strain and Growth Conditions

Thermotoga neapolitana KCCM 41025 (corresponding to NS-E, ATCC 49049, DSM 4359) was cultivated under anaerobic conditions in a 308 medium as described previously [21]. *E. coli* DH5α and MC1061, which were used as hosts for cloning and expression studies, were grown in Luria-Bertani (LB) medium containing 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl, and supplemented with ampicillin (100 μg/ml).

Enzymes and Chemicals

Restriction endonucleases and modifying enzymes were purchased from Kosco (Sungnam, Republic of Korea) or Takara (Shiga, Japan). A QuikChange II Site-Directed Mutagenesis Kit for mutagenic PCR amplification was purchased from Stratagene (La Jolla, U.S.A.). The QIAquick Gel Extraction Kit, a purification kit for PCR products or DNA restriction fragments, was obtained from Qiagen (Hilden, Germany). A silica gel 60 F_{254} thin-layer chromatography plate from Merck (Haar, Germany) was used for sugar analysis. The determination of β-glucosidase activity was performed with *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and *p*-nitrophenol (*p*NP), both obtained from Sigma (St. Louis, U.S.A.). The arbutin, esculin, and salicin for the transglycosylation experiment were also purchased from Sigma. A glucose oxidase assay kit was purchased from Young Dong Pharmaceuticals (Yongin, Republic of Korea). All other chemicals used in this study were of reagent grade and purchased from Sigma.

Site-directed Mutagenesis and Cloning of Mutants

The p6xHis119-bglAp6 plasmid, which contains the 1.35 kb wild-type *bglA* gene from *T. neapolitana* [21] under the control of the *Bacillus licheniformis* maltogenic amylase promoter in the p6xHis119 vector, was used as the DNA template for site-directed mutagenesis. Mutagenic oligonucleotide primers, N291T-F (5'-GTGACCTGAATAGTAGGTCAGCCCAACGAAGTC-3'), N291T-R (5'-GACTTCGTTGGGCTGACCTACTATTCAGGTCAC-3'), F412S-F (5'-CACGTACACTATACCGGACCTCTTCGAATATCC-3'), and F412S-R (5'-GGATATTCGAA GAGGTCCGGTATAGTGTACGTG-3'), were designed based on the result of directed evolution of *Thermus thermophilus* β-glycosidase [11]. The mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid. Each primer (10 pmol) was mixed with the p6xhis119-bglAp6 plasmid (10 ng) in a 50-μl PCR. The reaction conditions were as follows: 10× *Pfu* reaction buffer, 2.5 mM dNTP, 1.5 mM $MgCl_2$, and 2.5 units of *PfuUltra* HF DNA polymerase. PCR amplication was carried out on a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) with a program consisting of predenaturation for 30 s at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for 6 min. Five μl of each PCR product was analyzed on 0.8% (w/v) agarose gel containing ethidium bromide (0.25 mg/ml). Mutagenized PCR products were digested by a DpnI restriction enzyme at 37°C for 3 h to digest the parental (*i.e.*, nonmutated) double-stranded DNA. Without ligation, digested PCR products were transformed into *E. coli* MC1061 by the heat-shock method. To select for transformants, 100 to 200 μl of the transformation mixture was spread on LB agar plates with ampicillin at a final concentration of 100 μg/ml. The double mutant, N291T/F412S, was constructed by three-piece ligation. The *bgl*A genes of mutants N291T and F412S were digested by NdeI/NcoI and NcoI/HindIII restriction enzymes, respectively. The two digested fragments were ligated with the p6xhis119 vector. DNA sequencing was performed on a DNA auto-sequencer (ABI PRISM 377; Perkin Elmer) at Greengene Biotech (Suwon, Republic of Korea). Oligonucleotides for sequencing were synthesized by Bionics (Seoul, Republic of Korea). Computer analyses of the DNA sequence data and the deduced amino acid sequence were performed with programs available on the Expasy Molecular Biology server (http://www.expasy.ch) on the World Wide Web.

Purification of the Mutant Enzymes

All purification steps of recombinant His_6-tagged BglA mutants were performed at room temperature. *E. coli* harboring the p6xHis119 vector containing a mutated *bglA* gene was cultured at 37°C in a shaking incubator for 18 h, and was then harvested by centrifugation at 10,000 ×*g* for 30 min at 4°C. The cell pellet was thoroughly resuspended in 100 mM Tris-HCl buffer (pH 7.5) and disrupted by sonication on an ice bath. The cell lysate was centrifuged at 16,000 ×*g* for 30 min at 4°C to pellet down the cellular debris. The supernatant was incubated at 80°C for 30 min, and heat-labile proteins of *E. coli* were removed by centrifugation at 16,000 ×*g* for 30 min at 4°C. The supernatant was filtered through a 0.4-μm pore-sized filter and then passed through a nickel-NTA resin (Qiagen). The column was washed twice with two volumes of washing buffer [100 mM Tris-HCl buffer (pH 7.5), 10 mM imidazole], and BglA was eluted with the same buffer containing 250 mM imidazole. Proteins from the eluted fractions were pooled and dialyzed against Tris-HCl buffer (pH 7.5) to remove the excess imidazole, and were then concentrated using the Vivaspin 2 concentrator (Sigma-Aldrich). The purities of the wild-type and mutant enzymes were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% (w/v) polyacrylamide gels. The protein concentration was determined by the Bradford method [4] using a Bradford

reagents kit (Bio-Rad, Hercules, U.S.A.) and bovine serum albumin (BSA) as a standard protein.

Enzyme Assays

β-Glucosidase activity (overall activity) was determined spectrophotometrically in a 30-min assay. Assays were performed in a total of 1 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM *p*NPG and enzyme solution. After the substrate solution was pre-incubated at 80°C for 5 min, the reaction was initiated by the addition of 10 μl (20 ng) of the diluted enzyme solution and continued for 30 min, and was then terminated by the addition of 30 μl of cold stop solution (1:1, 100% ethanol/reaction buffer). The color that developed was read at 405 nm using a microplate reader (Benchmark, Bio-Rad), and translated to micromoles of *p*NP using a standard graph prepared under the same conditions. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per h at 80°C under the above assay conditions. The substrate specificities of the mutant enzymes were determined using various chromogenic substrates, including *p*NPG, as mentioned above.

The transglycosylation activities for the enzyme reaction with *p*NPG and arbutin were determined by measuring the amounts of released *p*NP (overall activity) and glucose (hydrolytic activity). Reaction mixtures (1 ml) containing 20 mM *p*NPG and 20 mM arbutin in a 100 mM sodium phosphate buffer (pH 7.0) were incubated at 80°C. The enzyme concentration was varied depending on the type of enzyme, wild type or mutant. Aliquots (10 μl) of the enzyme reaction mixture were withdrawn at different times. The amount of *p*NP released from *p*NPG was measured as described above, and the amount of glucose remaining after the transglycosylation reaction was measured using a glucose oxidase assay kit. The withdrawn aliquots were mixed with 1.5 ml of glucose oxidase buffer, incubated at 37°C for 5 min, and then read at 505 nm. The activity was calculated using a glucose standard graph prepared under the same assay conditions. Transglycosylation activity was calculated by subtracting the hydrolytic activity from the overall activity.

Purification of the Transfer Products

The transglycosylation reaction was performed at 80°C for 12 h (arbutin and salicin) and 1 h (esculin) with a total of 4 ml of reaction mixture containing the enzyme solution (1.8 μg), 1% cellobiose, and 2% acceptors (arbutin, esculin, or salicin) in a 100 mM sodium phosphate buffer (pH 7.0). Methanol (20%) was also used instead of sodium phosphate buffer in an effort to increase the tranglycosylation products. Transglycosylation products were separated by the recycling preparative high-performance liquid chromatography (HPLC) system equipped with a refractive index detector (JAI, Korea). Approximately 3 ml of the reaction mixture was loaded onto a JAIGEL-W251 (JAI, Korea) column (20×50 cm) and eluted with deionized water at a flow rate of 3.0 ml/min, which resulted in a column pressure of 30 kgf/cm^2. Each separated fraction was verified by thin-layer chromatography (TLC) and then lyophilized for mass spectrometry and nuclear magnetic resonance (NMR) analysis.

Analytical Methods

The molecular mass of the purified enzyme was determined by a Voyager System 6204 mass spectrometer. The detection and identification of hydrolysis and transglycosylation products were carried out by TLC analysis. One-tenth to 1 μl aliquots of the reaction mixture were spotted onto a Silica gel 60 F_{254} plate and developed with a solvent system of *n*-butanol/ethyl acetate/water (5:3:2, v/v/v) in a TLC developing chamber. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood, and was then developed by soaking rapidly in methanol solution containing 20% (v/v) sulfuric acid. The plate was dried and placed in an oven at 110°C for 10 min for visualization of the reaction spots.

RESULTS

Strategy for Obtaining Increased Transglycosylation Activity of BglA

The transferase activity of *T. neapolitana* BglA analyzed by NMR spectroscopy produced three arbutin derivatives with β(1,3), β(1,4), and β(1,6) regioselectivities (Fig. 1) [21]. In order to improve the transglycosylation/hydrolysis ratio of BglA, we performed a site-directed mutagenesis on the *bglA* gene, which we had cloned and overexpressed in a previous study [21]. In recent years, several attempts to increase the transglycosylation activities of natural glycosidases have been made using directed mutagenesis methods [12, 13, 15]. Sequence alignment of BglA with other GH1 enzymes revealed two possible candidate amino acids, Asn-291 and Phe-412. Analysis of the three-dimensional structure of *T. thermophilus* β-glycosidase (Tt-β-Gly) showed that both residues were located just in front of the subsite (−1), which affects the entering acceptor in the subsite (+1) [11]. Both residues were demonstrated

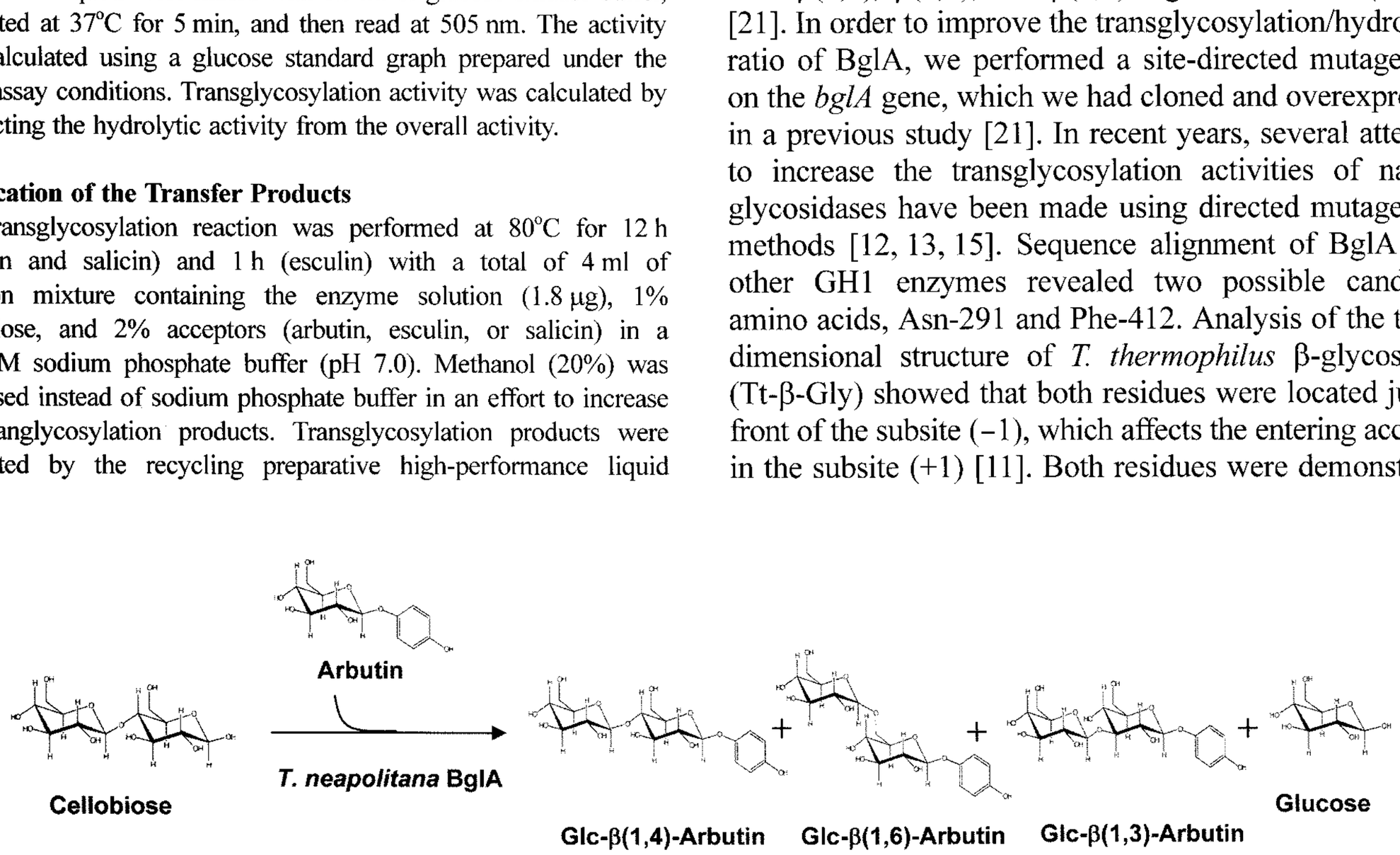

Fig. 1. Transglycosylation reaction catalyzed by the *T. neapolitana* BglA.

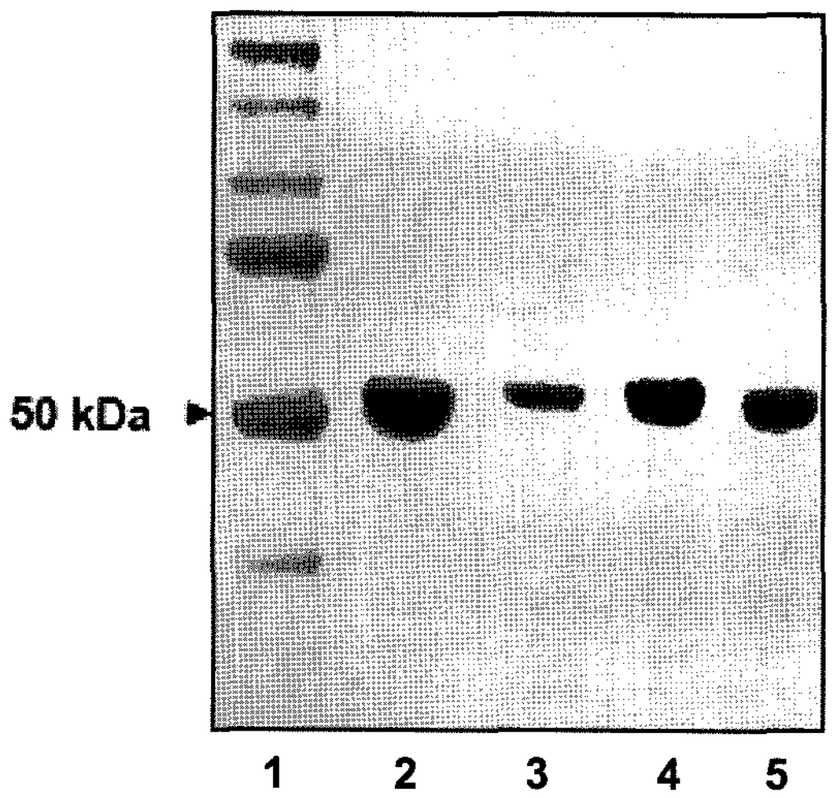

Fig. 2. SDS-PAGE of purified wild-type and mutant enzymes. Lane 1, molecular mass size marker; lane 2, *T. neapolitana* BglA; lane 3, N291T mutant; lane 4, F412S mutant; lane 5, N291T/F412S double mutant.

to be responsible for the strong enhancement of the transglycosylation/hydrolysis ratio by directed evolution of Tt-β-Gly [11].

Expression and Purification of the Wild-Type and Mutant Enzymes

A p6xHis119 vector containing the mutated *bglA* gene, a plasmid carrying a 1.35-kb NdeI and HindIII fragment of the *bglA*, was constructed and successfully expressed in *E. coli* MC1061 as described previously [21]. Heat treatment and nickel-NTA affinity chromatography were used to purify the BglA mutants. Heat treatment of the cell extract at 75°C for 30 min removed most of the *E. coli* proteins. The mutant enzymes were purified 25- to 130-fold, with a recovery yield of 30–80%. SDS-PAGE analysis showed that the purified proteins were homogeneous and the molecular mass of each mutant was the same as that of the wild-type enzyme (Fig. 2). Mass spectrometry analysis showed a molecular mass of 56 kDa, indicating that the enzyme was present as a monomer.

Characterization of the Mutant Enzymes

In order to characterize the reaction properties of the mutants (N291T, F412S, and N291T/F412S), the substrate specificity was measured at 80°C with various substrates. A comparison of the enzyme activities on the aryl-glycoside *p*NP-substrate and other oligosaccharide substrates indicated that *p*NPG was the best substrate for BglA. The specific activities for the wild-type and mutant enzymes with five different *p*NP-glycoside substrates are summarized in Table 1. Four *p*NP-β-glycosides were hydrolyzed efficiently, but the activities for *p*NPαG were relatively very low (2–3.5% for *p*NPG). These findings demonstrated that the wild-type and mutants were specific for β-type substrates. The specific activities of all three mutants showed 11.2% (N291T), 1.5% (F412S), and 0.05% (N291T/F412S) of the activity of the wild-type enzyme with *p*NPG as a substrate. The wild-type enzyme showed similar specific activity for *p*NPG, *p*NPL, and *p*NPC, whereas the mutant enzymes only showed high activity for *p*NPG.

To obtain quantitative information on both transglycosylation and hydrolysis activities, the amounts of *p*NP and glucose released after the enzyme reaction with *p*NPG and arbutin were measured spectrophotometrically (Table 2). Because *p*NPG was a much more efficient substrate than arbutin for the wild-type and mutant enzymes, measurements of the amounts of *p*NP and glucose obtained after the transglycosylation reaction represent the overall activity and hydrolytic activity, respectively. As expected, the transglycosylation/hydrolysis ratio was increased by all three mutants. Most interestingly, N291T and N219T/F412S had transglycosylation/hydrolysis ratios approximately 3-fold and 8-fold higher than that of the wild-type enzyme, which demonstrated the validity and efficiency of the process designed to select for mutants with improved transglycosylation activity. This observation is the result of the decreased hydrolytic activity of the mutant rather than increased transglycosylation activity. Although F412S and N291T/F412S enhanced the transglycosylation/hydrolysis ratio, the activities were too low to efficiently produce the transglycosylation products.

Table 1. Substrate specificity of wild-type and mutant *T. neapolitana* BglA enzymes towards *p*NP-glycosides.

Substrate	WT	N291T	F412S	N291T/F412S
	Sp. act. (U/mg)	Sp act. (U/mg)	Sp act. (U/mg)	Sp act. (U/mg)
*p*NPG	5,220	584	78.7	2.60
*p*NPL	4,760 (91)[a]	92 (16)	4.7 (6)	1.87 (72)
*p*NPC	4,820 (92)	60 (10)	5.3 (7)	2.13 (82)
*p*NPX	1,560 (30)	36 (6)	4.0 (5)	1.93 (74)
*p*NPαG	140 (2)	12 (2)	2.7 (3)	1.80 (69)

Activities were determined by following the release of *p*NP at 405 nm.

[a]Numbers in parentheses indicate the relative activities compared with pNPG substrate. *p*NPG, *p*-nitrophenyl-β-D-glucopyranoside; *p*NPL, *p*-nitrophenyl-β-D-lactoside; *p*NPC, *p*-nitrophenyl-β-D-cellobioside; *p*NPX, *p*-nitrophenyl-β-D-xyloside; *p*NPαG, *p*-nitrophenyl-α-D-glucopyranoside; Sp act, specific activity.

Table 2. Transglycosylation and hydrolysis activities of the wild-type and mutant *T. neapolitana* BglA enzymes.

Enzyme	Transglycosylation[a] (μmol/h·mg)	Hydrolysis[b] (μmol/h·mg)	Overall activity[c] (μmol/h·mg)	Transglycosylation/ hydrolysis (%)
Wild type	183	2,361±73	2,544±10	7.7
N291T	186 (102)[d]	797±18 (34)	983±18	23.3
F412S	2.7 (1.5)	25.3±0.6 (1.1)	28±1	10.7
N291T/F412S	1.5 (0.8)	2.3±0.04 (0.1)	3.8±0.3	65.2

The activities were measured at 80°C using *p*NPG and arbutin.
[a]Transglycosylation activity was calculated by subtracting the hydrolytic activity from the overall activity.
[b]Hydrolytic activity was determined by the amount of glucose released from *p*NPG using a glucose oxidase assay.
[c]Overall activity was determined by the amount of *p*NP released from *p*NPG.
[d]Numbers in parentheses indicate the relative activity compared with the wild-type enzyme.

Transglycosylation Regioselectivity of the Mutant Enzymes

When cellobiose was incubated with the wild-type enzyme in the presence of arbutin, three transglycosylation products were produced; their structures were determined by mass spectroscopy and ^{1}H- and ^{13}C-NMR analyses [21]. The NMR analyses revealed that glucose from a cellobiose donor was transferred to the C-3, C-4, and C-6 in the glucose units of arbutin. The yield of three transglycosylation products by the wild-type enzyme was very similar, which indicates that the wild-type enzyme has broad regioselectivity. To examine the transglycosylation properties of mutant enzymes, the mutant enzymes were incubated with cellobiose and arbutin, and the formation of transglycosylation products was assayed by TLC. Based on the TLC analysis, the overall transglycosylation yield was slightly decreased, although the yield of a certain specific transglycosylation product was increased (Fig. 3). Interestingly, the broad regioselectivity of the wild-type enzyme seems to have changed to a specific β(1,3) regioselectivity in the N291T and F412S mutants (Fig. 3). The relative yield of the transglycosylation products of the wild-type and mutant enzymes is shown in Table 3. The N291T and F412S mutants mainly produced β-D-glucosyl-(1→3)-arbutin, whereas the wild-type enzyme produced β-D-glucosyl-(1→3)-arbutin, β-D-glucosyl-(1→4)-arbutin, and β-D-glucosyl-(1→6)-arbutin at similar amounts. However, in the case of the N291T/F412S double mutant, no detectable transglycosylation products were observed because of the very low enzymatic activity of this mutant, as indicated in Table 2.

To further confirm the change of the regioselectivity by mutation, two arylglycosides (esculin and salicin) were tested as acceptors. The formation of transglycosylation products was examined by TLC. The major transglycosylation products of the wild-type and N291T mutant differed in terms of the presence of either esculin or salicin (Fig. 4). This phenomenon was more apparent with the esculin acceptor, and this change of regioselectivity was also observed regardless of the type of solvent used. The change of the regioselectivity, as well as the yield of the transglycosylation products by mutations, should be affected by the rate of hydrolysis of the transglycosylation product. This rate should be as low as possible to allow for the accumulation of the transglycosylation products.

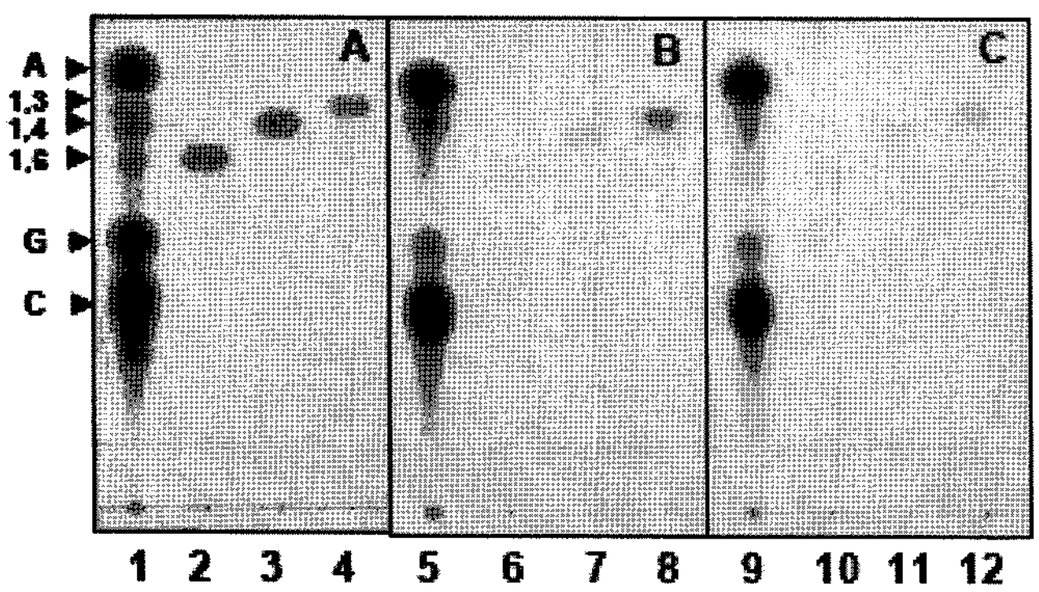

Fig. 3. TLC analysis of the transglycosylation products obtained by transglycosylation reaction with the wild-type (**A**), N291T mutant (**B**), and F412S mutant (**C**) BglA. The enzymatic reaction was carried out with cellobiose and arbutin at 80°C.
Lanes 1, 5, and 9, total transglycosylation products after enzymatic reaction; lanes 2, 6, and 10, purified β(1,6) transglycosylation product; lanes 3, 7, and 11, purified β(1,4) transglycosylation product; lanes 4, 8, and 12, purified β(1,3) transglycosylation product. Locations of regioisomers, β-D-glucosyl-(1→3)-arbutin, β-D-glucosyl-(1→4)-arbutin, and β-D-glucosyl-(1→6)-arbutin, are indicated by the 1,3, 1,4, and 1,6 abbreviations, respectively. C, cellobiose; G, glucose; A, arbutin.

DISCUSSION

The limitation encountered in the transglycosylation reaction occurred in concurrence with hydrolysis of the transfer products, which reduced the final product yield. As a trial experiment to obtain BglA mutants, which have been found to improve transglycosylation activity, the site-directed mutagenesis of BglA was carried out based on the results of directed evolution of Tt-β-Gly [11]. As expected, the mutant enzymes lowered the hydrolysis activity, but the transglycosylation activity was relatively maintained. The relative activities of the mutants, indicated by the transglycosylation activity over the hydrolysis activity, were revealed to be increased compared with that of the wild-type BglA. In a previous study, a few Tt-β-Gly mutants succeeded

Table 3. Transglycosylation properties of the wild-type and mutant *T. neapolitana* BglA enzymes.

Enzyme	Relative yield[a] (%)			Regioselectivity	Total yield[b] by transglycosylation (%)
	β(1,3)	β(1,4)	β(1,6)		
Wild type	25	37	38	β(1,6) ≅ β(1,4) ≅ β(1,3)	2.84
N291T	73	14	12	β(1,3) » β(1,4) ≅ β(1,6)	2.03
F412S	48	28	24	β(1,3) > β(1,4) ≅ β(1,6)	0.91
N291T/F412S	ND[c]	ND	ND	ND	ND

[a]Molar percentages are the yield calculated from integration of the recycling prep HPLC peak area after separation of the transglycosylation products.
[b]Yield based on acceptor is calculated from integration of the peak area of the transglycosylation product.
[c]ND indicates that the value was not determined. The reaction was carried out with cellobiose and arbutin as a donor and an acceptor, respectively.

to prove that the transglycosylation activity increased over hydrolysis activity by random mutagenesis [11]. The N282T mutant of Tt-β-Gly almost completely eliminated hydrolysis activity, whereas it only decreased the transglycosylation activity by 3- to 5-fold compared with the wild-type enzyme. However, in our experiment, although N291T, which is identical to the N282T mutant of Tt-β-Gly, dramatically reduced hydrolysis activity, the transglycosylation activity did not exceed the hydrolysis activity. Interestingly, we found that most of the mutants lost their activities towards the 1,6 and 1,4 linkages, and exhibited a preference for the 1,3 linkage. The shift of the regioselectivity into the 1,3 linkage is mostly due to the reduction of the 1,6 and 1,4 regioselectivities, and not to major improvement of the 1,3 regioselectivity. The reason for the low transglycosylation activity of most of our mutants compared with those from Tt-β-Gly mutants is not yet clear.

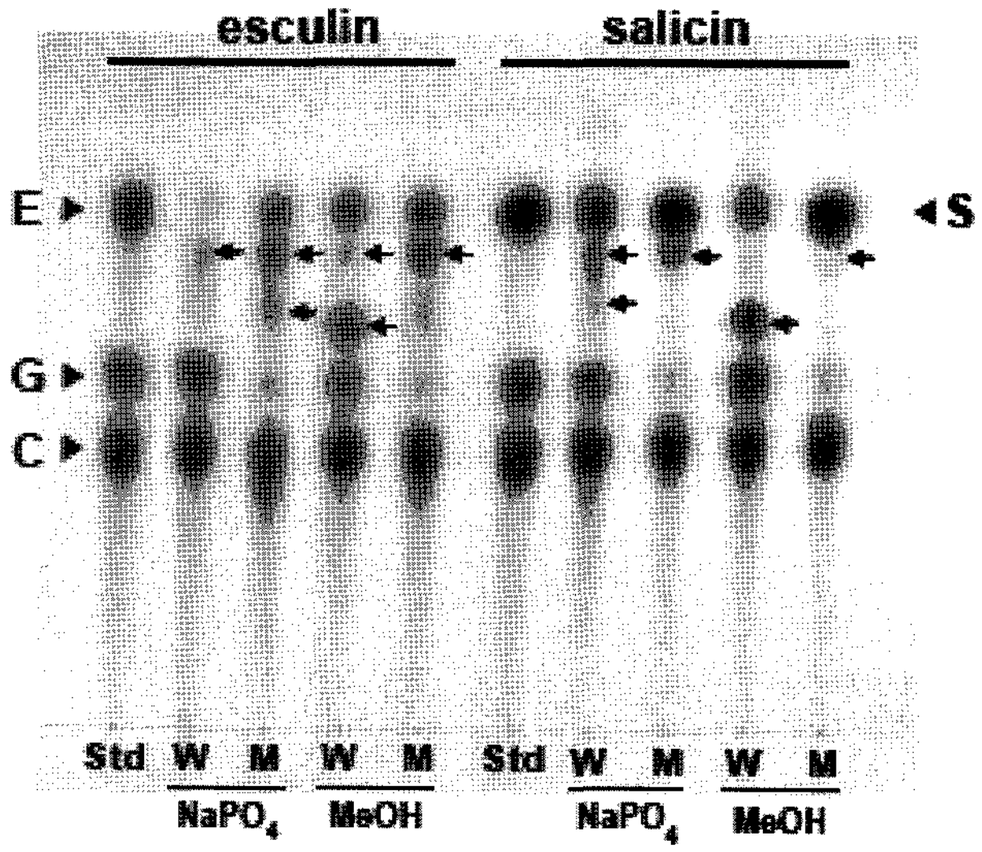

Fig. 4. TLC analysis of transglycosylation products obtained by transglycosylation reaction with the wild-type and N291T mutant BglA. The enzymatic reaction was carried out with cellobiose and esculin or salicin acceptors at 80°C.
Std, standard solution including cellobiose, glucose, and either esculin or salicin. E, esculin; S, salicin; G, glucose; C, cellobiose. W, wild-type enzyme; M, N291T mutant. Arrows indicate the transglycosylation products.

The change of the regioselectivity was also confirmed by using two different acceptors. The major transglycosylation products produced by the enzyme reaction with esculin or salicin (acceptor) and cellobiose (donor) were different between the wild-type and N291T BglA. Although we have not yet identified the transglycosylation products, we can suppose that each product is a regioisomer of β-D-glucosyl esculin and β-D-glucosyl salicin, taking into consideration our previous experience. Otherwise, the production of different transglycosylation products by the mutant may be due to the effect of methanol. The methyl group of methanol will act as an acceptor instead of esculin or salicin. Therefore, the strong spot that appeared in the wild-type enzyme may possibly be a methyl-glucoside. The mutant may affect the transferring ability of the enzyme, thereby resulting in the change of regioselectivity. The effect of positive mutations on transglycosylation activity was confirmed by a molecular modeling study of Tt-β-Gly [11]. The improved transglycosylation activity of N282T was previously explained by a favorable substrate binding docking energy, which is sufficiently significant to allow the changes of transglycosylation activity. In other studies of α-galactosidase (AgaB) from *B. stearothermophilus*, investigators were able to isolate AgaB mutants, in which one mutation resulted in dramatic changes in regioselectivity [8].

In conclusion, we identified BglA mutants that improved transglycosylation efficiency and altered regioselectivity. The analysis of the transglycosylation products obtained from N291T revealed that the Asn-291 residue is important in the control of transglycosylation. The results of this analysis suggest that directed mutagenesis of the glycosidases could be used for fine-tuning of the enzyme properties. Therefore, regioselective engineering of glycosidases will be useful for the efficient synthesis of regiospecific glycosides. Experiments are currently being conducted to broaden the acceptor specificity so that this enzyme can be used in various synthesis reactions in the future.

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