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Research Article

Transglycosylation Properties of a Novel α-1,4-Glucanotransferase from *Bacteroides thetaiotaomicron* and Its Application in Developing an α-Glucosidase-Specific Inhibitor

Hye-Jeong Choi, Dam-Seul Ko, Na-Ri Kim, Woo-Jae Choung, Ye-Seul Koo, Da-Woon Jeong, and Jae-Hoon Shim

Department of Food Science and Nutrition and Center for Aging and Health Care, Hallym University, Hallymdaehak-gil 1, Chuncheon, Gangwon-do 24252, Republic of Korea

Correspondence should be addressed to Jae-Hoon Shim; jhshim@hallym.ac.kr

Hye-Jeong Choi and Dam-Seul Ko contributed equally to this work.

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In this study, α-glucanotransferase from *Bacteroides thetaiotaomicron* was expressed in *Escherichia coli* and characterized. Conserved amino-acid sequence alignment showed that *Bacteroides thetaiotaomicron* α-glucanotransferase (BtαGTase) belongs to the glycoside hydrolase family 77. The enzyme exhibited optimal catalytic activity at 60°C and pH 3.0. BtαGTase catalyzed transglycosylation reactions that produced only glycosyl or maltosyl transfer products, which are preferable for the generation of transglycosylated products with high yield. The 1-deoxynojirimycin (DNJ) glycosylation product G1-DNJ was generated using BtαGTase, and the inhibitory effect of G1-DNJ was analyzed. A kinetic study of inhibition revealed that G1-DNJ inhibited α-glucosidase to a greater extent than did DNJ but did not show any inhibitory effects towards α-amylase, suggesting that G1-DNJ is a potential candidate for the prevention of diabetes.

1. Introduction

Bacteroides thetaiotaomicron is a human colonic gram-negative obligate anaerobe found in high numbers in the human intestine that can ferment a wide diversity of polysaccharides [1]. Members of this genus require carbohydrates as a source of carbon and energy. Polysaccharides are the essential source of carbohydrates for these bacteria in the human intestine [2]. Carbohydrates are fermented by *Bacteroides* and other intestinal bacteria, resulting in the production of volatile fatty acids that are reabsorbed by the large intestine and used by the host as an energy source; these constitute a significant proportion of the host's daily energy requirements. *B. thetaiotaomicron* contains a system that enables the broad utilization of starch and various genes involved in starch binding and application [3]. Wexler reported that its 172 glycohydrolases and 163 homologs of starch-binding proteins enable this organism to utilize a wide variety of dietary carbohydrates available in the gut [4].

The glycoside hydrolase family GH77 is a monospecific family consisting of 4-α-glucanotransferase (α-GTase, EC 2.4.1.25) and defined by an established classification system based on the sequences of all active carbohydrate enzymes from the Carbohydrate-Active Enzyme (CAZy) database [5]. α-GTase catalyzes the transfer of α-1,4-glucan to an acceptor, which is usually the 4-hydroxyl group of another α-1,4-glucan or glucose [6]. In this reaction, hydrolysis of the α-1,4 linkage and subsequent synthesis of a new α-1,4 linkage occur repeatedly within the same glucan molecule or between different molecules [7]. Effective donors of

maltooligosaccharides include amylopectin and soluble starch, which together with glucose also serve as acceptors [8]. These enzymes are found in microorganisms and plants, in which they are involved in maltooligosaccharide metabolism or glycogen and starch metabolism, respectively [6]. While the *Bacteroides thetaiotaomicron* GH77 family contains only one of these enzymes, other GH families contain many carbohydrate-related enzymes.

α-GTase transglycosylation activity is useful in carbohydrate chemistry. Starches that are modified by α-GTases show novel rheological and nutritional properties, such as thermoreversible gelation, fat-replacing properties, and hypocholesterolemic and hypoglycemic effects [9]. α-GTases from different bacteria have successfully modified the properties of various food materials, including increased water solubility, stability, functional effects, and taste [10]. In addition, intramolecular glucan transferase produces cyclic glucans (cycloamyloses) with a higher degree of polymerization compared with cyclodextrins [9]. 1-Deoxynojirimycin (DNJ), an aza-sugar, has structural characteristics similar to those of cyclic monosaccharides; however, the oxygen is substituted with a nitrogen atom. DNJ prevents glucose from entering the bloodstream from the intestines by inhibiting the activities of α-amylase and α-glucosidase [11, 12]. Intestinal α-glucosidase is one of the glucosidases of the small intestinal epithelium [13]. α-Glucosidase hydrolyzes α-1-4-linked D-glucose from the nonreducing end of α-glucoside, which is the last step in the digestion of disaccharides and polysaccharides [14]. Thus, inhibition of intestinal α-glucosidases would prevent the rapid digestion of carbohydrate and, consequently, the sharp postprandial rise in blood glucose [13]. However, antidiabetic drugs that prevent carbohydrate digestion have gastrointestinal side effects, such as abdominal distention and flatulence. These side effects may be caused by the inhibition of α-amylase, which leads to the accumulation of undigested carbohydrates in the intestines [15, 16]. Therefore, antidiabetic drugs that specifically inhibit α-glucosidase are required.

In this study, we cloned a novel α-GTase of the GH77 family from *Bacteroides thetaiotaomicron* (BtαGTase) and examined its reaction pattern using diverse substrates. Additionally, we applied the transglycosylation activity of BtαGTase to DNJ to develop a prospective candidate for the prevention of diabetes.

2. Materials and Methods

2.1. Chemicals and Reagents. *Bacteroides thetaiotaomicron* VPI-5482 was obtained from the Korean Collection for Type Cultures (KCTC). Luria-Bertani (LB) medium was purchased from BD (Franklin Lakes, NJ, USA). NaCl was purchased from GeorgiaChem (Suwanee, GA, USA). Kanamycin monosulfate was purchased from Duchefa Biochemie (Haarlem, the Netherlands). *Escherichia coli* MC1061 (F±, *ara*D139, *rec*A13, D [*ara*ABCleu] 7696, *gal*U, *gal*K, Δ*lac*X74, *rps*L, *thi*, *hsd*R2, and *mcr*B) was used as the parental strain for DNA manipulation and transformation. α-Glucosidase from *Saccharomyces cerevisiae*, α-amylase from porcine pancreas, glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), DNJ, $Zn(C_2H_2O_2)_2$, *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG), and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Soluble starch, $MnCl_2$, and $CuCl_2$ were purchased from Showa Chemical (Showa, Japan). Amylose (potato starch) was purchased from ICN Biomedicals (Tokyo, Japan). $Co(NO_3)_2$ and $CaCl_2$ were purchased from Junsei Chemical (Tokyo, Japan). $FeCl_2$ and $MgCl_2$ were purchased from Shinyo Pure Chemicals (Tokyo, Japan). EDTA was purchased from Promega (Madison, WI, USA). *p*-Nitrophenyl-α-D-maltoside (pNPG2) was purchased from Gold Biotechnology (Olivette, MO, USA).

2.2. Cloning and Sequence Analysis. The gene encoding *bt_2146* was separated from the genomic DNA of *B. thetaiotaomicron*, and the target DNA was amplified by polymerase chain reaction (PCR) using two primers (bt-F, 5′-AAAA*CCATGG*CCACTGTATCATTTAAC-3′ and bt-R, 5′-AAAA*CTCGAG*TTCTTGGGAGCTCTGCC-3′) containing the *Nco*I and *Xho*I restriction enzyme sites, respectively. The PCR conditions were as follows: denaturation for 1 min at 98°C, followed by 30 cycles of 10 s at 98°C, annealing for 30 s at 53°C, and extension for 1 min 30 s at 72°C.

2.3. Expression and Purification of a Recombinant Protein. *E. coli* transformants were cultured in LB medium (10 g/L Bacto Tryptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with kanamycin (50 μg/mL) for 30 h at 30°C with shaking at 150 rpm. The cells were harvested by centrifugation (7,000 ×g, 20 min, 4°C) and suspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl buffer (pH 7.5), and 10 mM imidazole). Cells were disrupted using a sonicator in ice-cold water (output 12, 5 min, 4 times; XL-2000; Qsonica, LLC, Newtown, CT, USA). The cell extract was subsequently centrifuged (7,000 ×g, 20 min, 4°C) and the supernatant collected. The supernatant was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography using washing (0.9 M NaCl, 0.05 M Tris-HCl buffer (pH 7.5), and 0.02 M imidazole) and elution (0.9 M NaCl, 0.05 M Tris-HCl buffer (pH 7.5), and 0.25 M imidazole) buffers. The purified enzyme, BtαGTase, was visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The BtαGTase concentration was determined from 2 μL drops of protein solution using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 280 nm with the appropriate extinction coefficient for BtαGTase (227,128 $cm^{-1}\,M^{-1}$).

2.4. Sequence Analysis. The characterized α-GTase sequences of GH77 family members were obtained from the CAZy database (http://www.cazy.org/). Sequence alignment was performed using the Alignment X software, a component of Vector NTI Suite 5.5 (InforMax, Bethesda, MD, USA). In addition, a phylogenetic tree was constructed using MEGA6 software based on the neighbor-joining tree method (1,000 bootstrap samples) [17].

2.5. Enzymatic Assay. BtαGTase enzymatic activity was measured in an amylose and maltose mixture (0.02% (w/v) amylose solution and 0.05% (w/v) maltose solution in 50 mM citric-NaOH (pH 3.0) at 60°C) as described previously by Lee et al. [7]. After preheating at 60°C for 5 min, the enzyme activity was measured using Lugol's solution. The standard curve consisted of ~0.000391–0.025% (w/v) amylose solution, and one unit of BtαGTase activity was defined as the amount of enzyme that hydrolyzed 1 μg/mL reducing amylose 1 min after transfer to G2. Absorbance was measured at 620 nm using a spectrophotometer (Multiskan FC; Thermo Fisher Scientific).

2.6. Effects of Temperature and pH on Enzyme Activity and Stability. The optimal temperature for BtαGTase activity was determined using amylose and G2 as substrates in 50 mM citric-NaOH (pH 3.0) buffer, and Lugol's solution was used to measure the activity at various temperatures (40–70°C). Similarly, the effect of pH on BtαGTase activity was measured at various pH values (2.5–6) using 50 mM citric-NaOH (pH 2.5–3.5) and 50 mM sodium acetate (pH 3.5–6.0).

2.7. Effects of Metal Ions on Enzyme Activity. To evaluate the effects of metal ions on the activity of the purified enzyme, the reaction mixture was preincubated at 50°C for 10 min at 5 mM final concentrations of $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $ZnCl_2$, and EDTA. The relative activity of BtαGTase was measured under standard conditions (50°C, pH 3.0) using Lugol's solution. The enzyme activity in the absence of metal ions was considered to be 100%.

2.8. Analysis of the Reaction Products Using Thin-Layer Chromatography (TLC). The TLC was performed using a K5F silica-gel plate (Whatman, Maidstone, UK). After the samples were spotted, the silica-gel plate was dried and placed in developing solvent (*n*-butanol:ethanol:water, 5 : 5 : 3, v/v/v). To analyze the reaction products, the TLC plate was dried, saturated in dipping solution (0.33% w/v N-[1-naphthyl]-ethylenediamine and 5% (v/v) H_2SO_4 in methanol), and heated at 110°C for 10 min.

2.9. Preparation of the Transglycosylation Product. For the transglycosylation reaction, a substrate solution containing 1.5% (w/v) DNJ and 1% (w/v) soluble starch was prepared in 50 mM sodium acetate buffer (pH 6.0). BtαGTase was added to this substrate solution and was incubated for 7 h before terminating the enzyme reaction by boiling the solution for 10 min.

2.10. Purification of the Transglycosylation Product Using Preparative-High Performance Liquid Chromatography (Prep-HPLC). The DNJ transglycosylation product was purified using LC-Forte/R preparative-high-performance liquid chromatography (prep-HPLC; YMC Korea, Seongnam, Korea) equipped with a Triart-C18 column (250 × 20 mm; YMC Korea) and an ultraviolet detector (200 and 210 nm). For the isocratic solvent system, 0.1% ammonium in deionized water was used at a flow rate of 12.0 mL/min at room temperature, and 1 mL of the sample was injected.

2.11. High-Performance Anion Exchange Chromatography Analysis (HPAEC). The HPAEC analysis was performed using a CarboPac™ PA1 column (4 × 250 mm; Dionex, Sunnyvale, CA, USA) and a pulsed aerometric detector (ED40; Dionex). For the analysis, 20 μL of the sample was injected and eluted with multiple gradients of 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1 mL/min. The linear gradients of sodium acetate were as follows: 0–2% for 0–20 min, 2–40% for 20–58 min, 40–100% for 58–68 min, 100% for 68–70 min, and 100–0% for 70–78 min.

2.12. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry Analysis of G1-DNJ. The molecular mass of G1-DNJ was determined using the MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Foster City, CA, USA). The sample in methanol was mixed with the matrix, α-cyano-4-hydroxycinnamic acid (CHCA), at a 1 : 1 ratio. The mixture (1 μL) was applied to a MALDI-TOF mass spectrometry probe and dried slowly at room temperature. An accelerating voltage of 20,000 V was used.

2.13. Inhibition Kinetics of α-Glucosidase and α-Amylase. To determine the inhibition mechanism, a modified Dixon plot was produced [18]. The inhibitory mode of DNJ and the DNJ transfer product (G1-DNJ) against α-glucosidase and α-amylase were analyzed using the substrates *p*NPG and *p*NPG2, respectively. The increase in absorption due to the hydrolysis of *p*NPG substrates was observed at 405 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan FC; Thermo Fisher Scientific). The reaction mixture containing 100 μL of the substrate, 50 mM sodium acetate buffer (pH 7.0), 50 mM potassium phosphate buffer (pH 6.9, 40 μL), and various concentrations of inhibitor (0.1–1 μmol) dissolved in 20 μL distilled water was preheated for 5 min before adding the enzyme (40 μL).

3. Results

3.1. Cloning and Expression of Alpha-Glucanotransferase str. IM2 in E. coli. The α-glucanotransferase-encoding gene *bt_2146* was amplified successfully from *B. thetaiotaomicron* using PCR (Figure S1 in Supplementary Data). The amplified gene (~2.7 kb) was ligated into the pTKNd119 vector. The resulting recombinant plasmid, pTKNdBtαgtase, was transformed into *E. coli* MC1061, and the expressed enzyme was subsequently purified by Ni-NTA affinity chromatography. The expression of BtαGTase was analyzed by SDS-PAGE (Figure S2 in Supplementary Data). The predicted size of the expressed enzyme was 105 kDa. Table 1 shows the purification results.

Table 1: Purification of the BtαGTase enzyme.

Step	Volume (mL)	Enzyme activity (U)	Protein concentration (mg/mL)	Protein amount (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell extract	104	3.57	169.44	17621.76	0.20×10^{-3}	100	1.00
Ni-NTA	1.5	2.03	2.26	3.39	0.59	56.8	2953.20

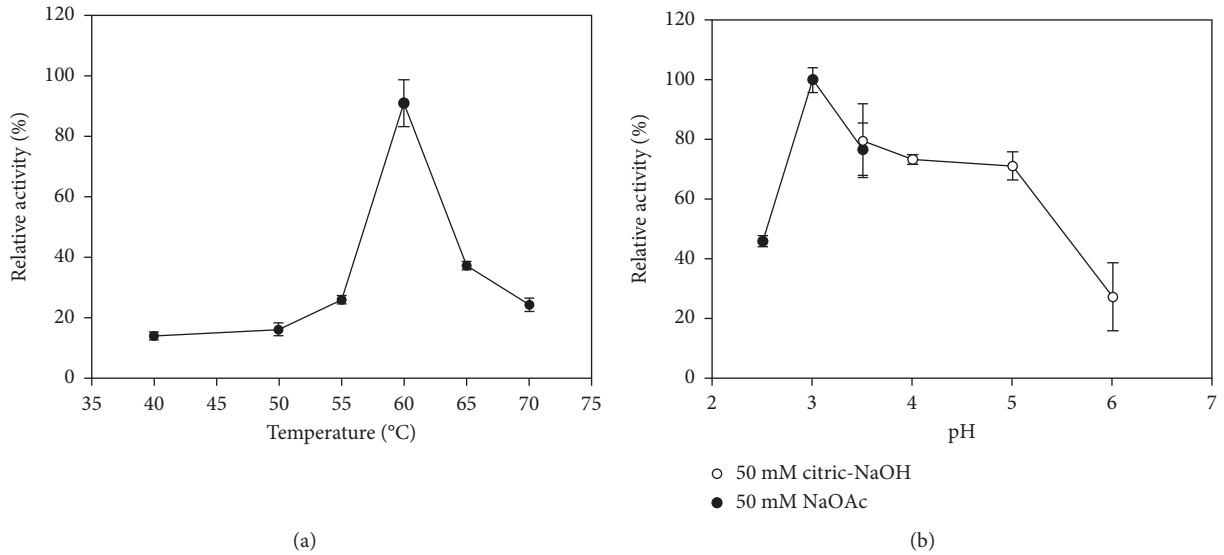


Figure 1: Effect of temperature and pH on *Bacteroides thetaiotaomicron* α-glucanotransferase (BtαGTase) enzyme activity. (a) The optimal temperature and (b) pH were measured using 0.02% amylose and 0.05% maltose as substrates.

3.2. Characterization of Recombinant BtαGTase. The optimal temperature and pH of BtαGTase enzymatic activity, measured using amylose and G2 as substrates, were 60°C and pH 3.0, respectively (Figure 1). BtαGTase showed transglycosylation activity toward α-1,4 linked substrates including G2, G3, G4, G5, G6, and G7 (Figure 2). As shown in Figure 3, some metal ions, including Ca^{2+}, Mg^{2+}, and Cu^{2+}, increased BtαGTase activity; however, other metal ions including Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{3+}, and EDTA decreased BtαGTase activity.

3.3. Preparation of the DNJ Transfer Product. As shown in Figure 4, DNJ and soluble starch were reacted with BtαGTase, and the enzyme reaction was analyzed by HPAEC. The enzyme reaction product was purified using prep-HPLC and analyzed by HPAEC and MALDI/TOF-MS (Figures 5 and 6). With a molecular mass of 349.33 Da, the transfer product was identified as a glucosyl DNJ (G1-DNJ).

3.4. Kinetic Study of the Inhibitory Activity of DNJ and G1-DNJ. The inhibitory effects of DNJ and G1-DNJ against α-glucosidase and α-amylase were analyzed in a kinetic study. As shown in Figure 7(a), both the DNJ and G1-DNJ plots showed a series of lines converging on the same point above the *x*-axis, indicating that DNJ and G1-DNJ have competitive inhibitory effects on α-glucosidase. The K_i value of G1-DNJ was lower than that of DNJ, suggesting that

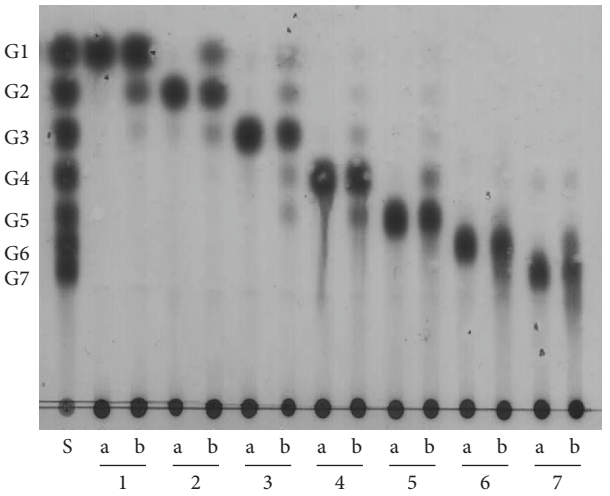


Figure 2: Thin-layer chromatography (TLC) analysis of the transferred products of the BtαGTase reaction. TLC analysis of the hydrolytic products generated by BtαGTase. S, standard (G1–G7); lane 1, glucose (G1); lane 2, maltose (G2); lane 3, maltotriose (G3); lane 4, maltotetraose (G4); lane 5, maltopentaose (G5); lane 6, maltohexaose (G6); and lane 7, maltoheptaose (G7). a, before the reaction; b, after the reaction. Hydrolysis was performed at 60°C at pH 3.0 for 12 h.

G1-DNJ is a better inhibitor of α-glucosidase (Table 2). In the α-amylase inhibition assay, the DNJ plot showed a competitive inhibitory pattern, while the G1-DNJ plot did

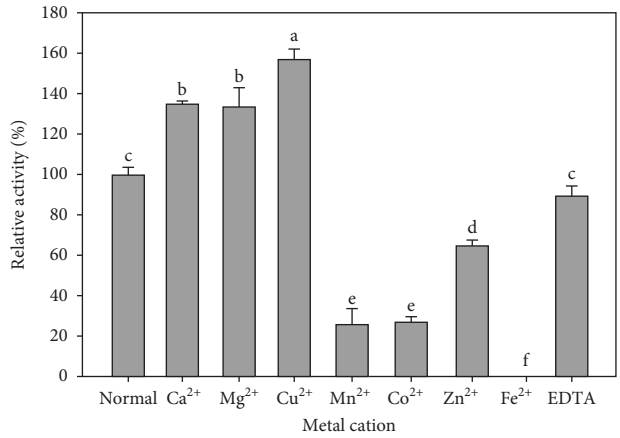


Figure 3: Effect of metal ions on BtαGTase enzyme activity. The purified enzyme was preincubated with various metal ions for 10 min, and the enzyme activity was subsequently measured under standard conditions using G2 and amylose as substrates.

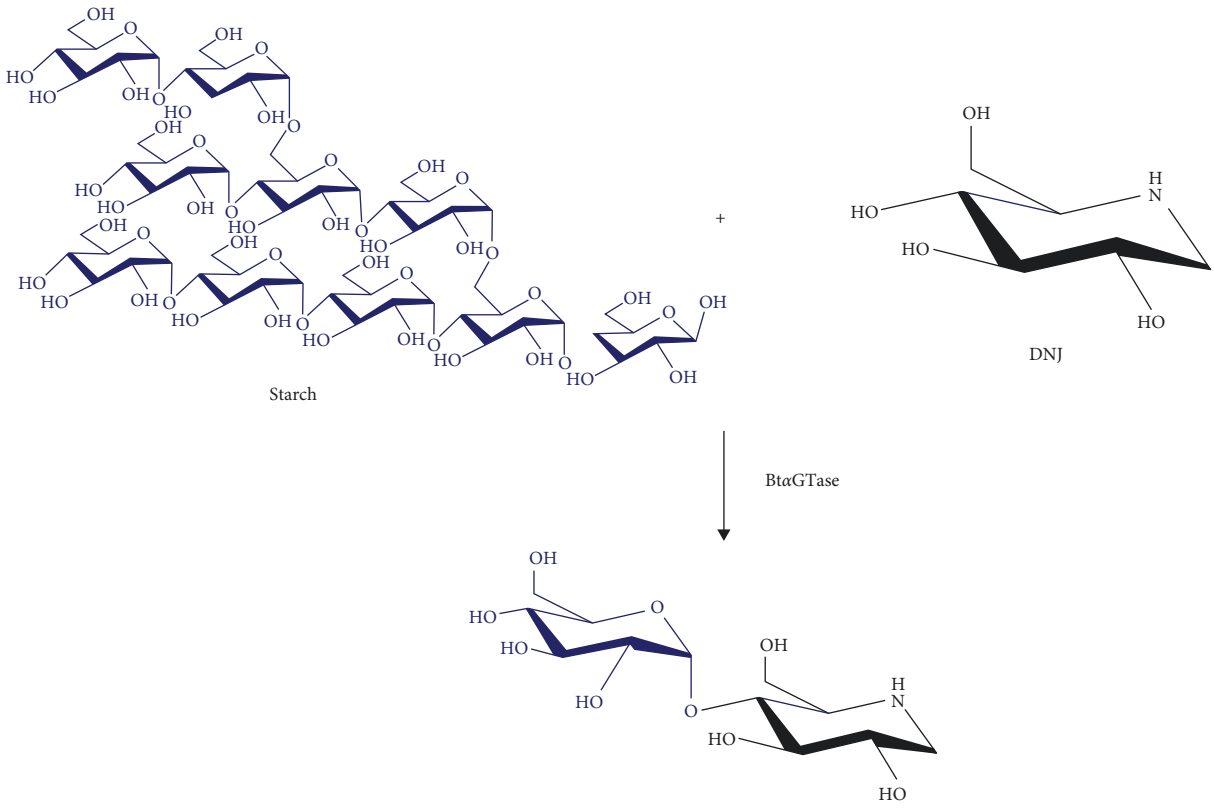


Figure 4: Production of 1-deoxynojirimycin (DNJ) glycosides using BtαGTase from DNJ and starch.

not demonstrate any convergence of lines, indicating that G1-DNJ does not have an inhibitory effect on α-amylase (Figure 7(b)).

4. Discussion

αGTase belongs to the α-amylase superfamily [8, 19]. This enzyme catalyzes disproportionation reactions, which hydrolyze α-glycosidic linkage and subsequently synthesize new α-glycosidic linkage within the same or different glucan molecules [7, 8]. Recently, the use of α-GTase has received considerable attention, specifically for the development of many starch products such as cycloamylose, cyclic cluster dextrin, slowly digestible starch, and thermoreversible starch [20–23]. In our study, a novel α-GTase from *B. thetaiotaomicron* was cloned and characterized. An amino acid sequence alignment indicated that BtαGTase contained conserved regions and three catalytic sites that belong to the GH77 family (Supplementary Data 4). Interestingly, BtαGTase demonstrated low disproportionation activity, resulting in only one or two types of transfer products using various maltooligosaccharides as acceptor molecules

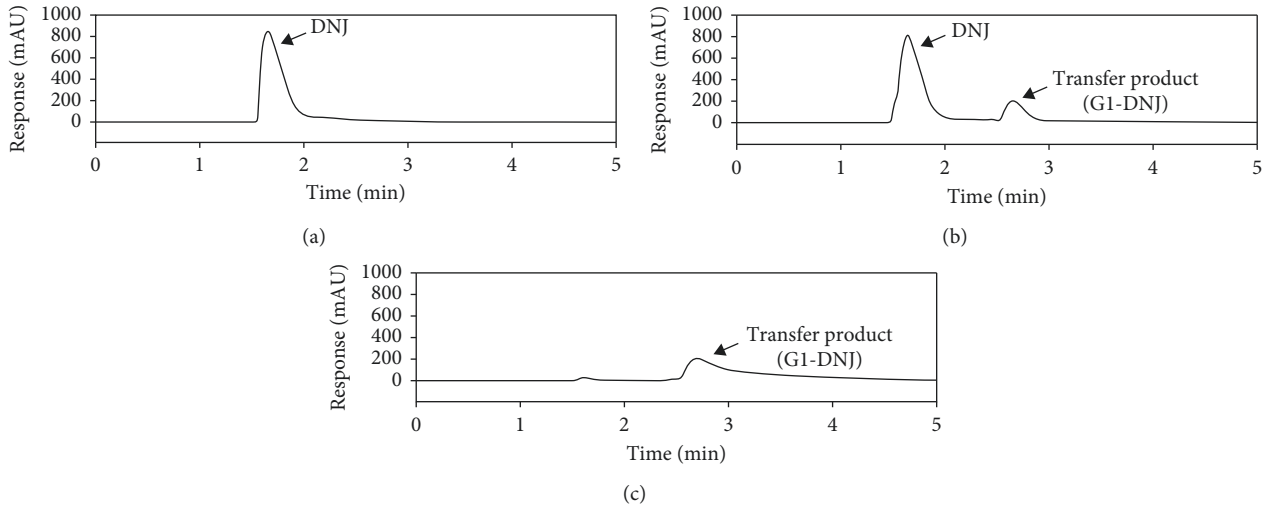


Figure 5: High-performance anion exchange chromatography analysis (HPAEC) chromatograms of DNJ and the transglycosylation product. (a) Before the reaction. (b) After the reaction. (c) The purified transglycosylation product (G1-DNJ).

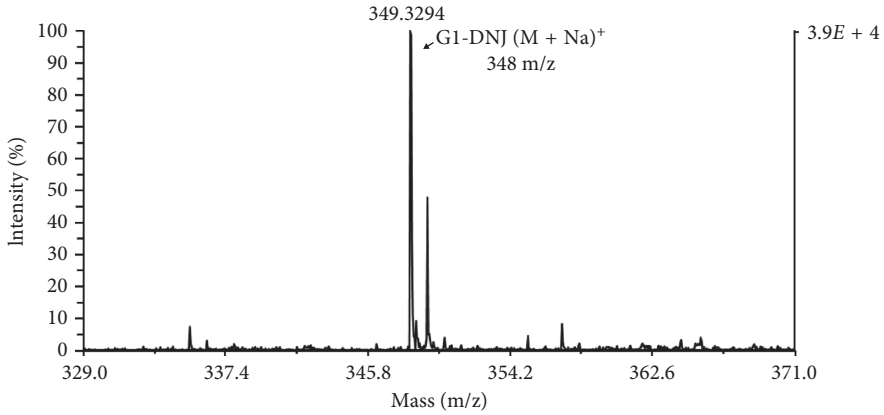
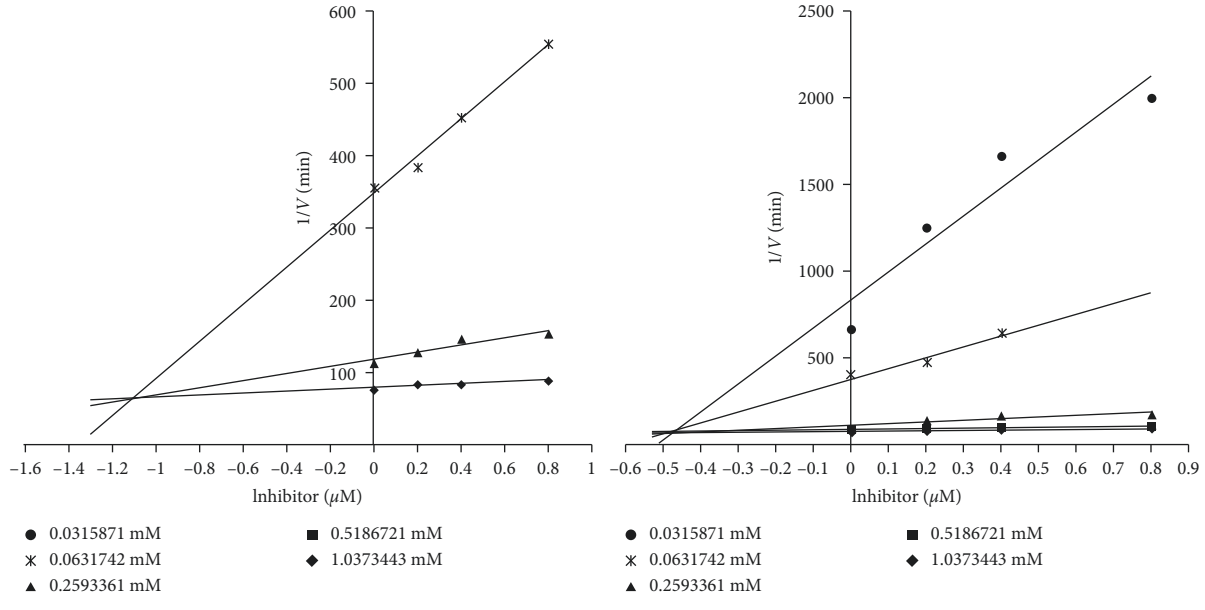


Figure 6: Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of the DNJ transglycosylation product. The purified glycosylation transfer product was determined using MALDI-TOF mass spectrometry. The molecular weight of the glucoside was one glucose residue larger than that of DNJ. G1-DNJ was observed at m/z 348.3294 $(M + Na)^+$.



(a)

Figure 7: Continued.

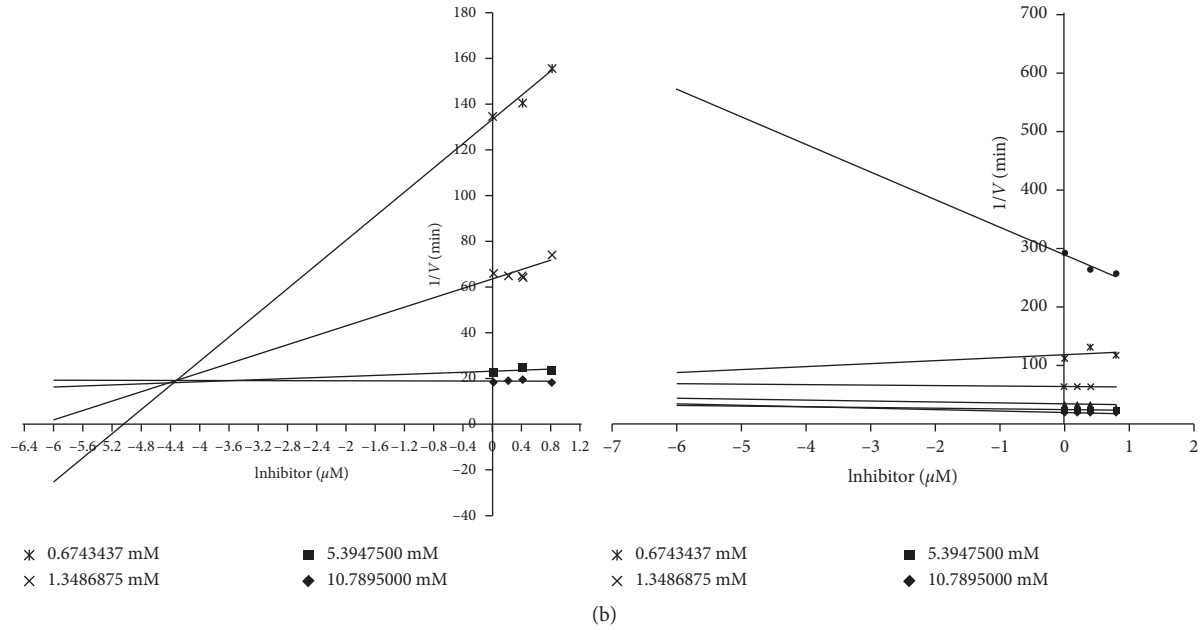


Figure 7: Dixon plots showing the inhibitory effects of the DNJ transglycosylation product on α-glucosidase (a) and α-amylase (b) activity. The reaction rate of the enzyme was measured in the presence of DNJ (left) and G1-DNJ (right).

Table 2: Kinetic study of enzyme inhibition by DNJ and G1-DNJ.

Inhibitor	Enzyme	K_i (μM)	Inhibition type
DNJ	α-Glucosidase	1.1	Competitive
	α-Amylase	4.4	Competitive
G1-DNJ	α-Glucosidase	0.48	Competitive
	α-Amylase	—	—

(Figure 2). Although BtαGTase is not able to elongate several glucosyl units, this low disproportionation activity could be advantageous for the preparation of a single glucosyl-transfer product and to enhance the availability of the acceptor molecule. In addition, the production of various products from an enzyme reaction is not beneficial for the purification of a specific product. Therefore, BtαGTase may be a valuable enzyme for transglycosylation reactions.

Generally, antidiabetic drugs used to treat type II diabetes mellitus, such as acarbose and DNJ, inhibit the activities of α-amylase and α-glucosidase [11]. Inhibition of α-amylase leads to the accumulation of undigested carbohydrates in the intestines, which may cause abdominal distention and flatulence [15, 16]. In this study, the transfer product G1-DNJ inhibited only α-glucosidase (Table 2, Figure 7), which may reduce the side effects of other antidiabetic drugs.

In conclusion, we assessed the properties and industrial applicability of BtαGTase. This enzyme is a novel α-glucanotransferase of the glycoside hydrolase family GH77, and its transglycosylation properties render it efficient in preparing molecules with the transfer of single-glucosyl residues. G1-DNJ, prepared using BtαGTase, showed stronger inhibitory effects than those of DNJ, but it did not affect α-amylase activity, suggesting that this molecule may be a potential drug candidate for the treatment of type II diabetes mellitus.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Figure S1: generation of the DNA construct for BtαGTase expression. (A) The bt_2146 gene was amplified by polymerase chain reaction (PCR). Lane S, DNA marker; lane 1, amplified BtαGTase gene. (B) The pKTNd_bt2146 construct was generated by ligating bt_2146 into the pTKNd vector. Figure S2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of BtαGTase at each purification step. Lane S, protein size standards; lane 1, cellular protein from the crude extract; lane 2, soluble fraction;

lane 3, insoluble fraction; and lane 4, purified BtαGTase. Figure S3: Lineweaver-Burk plots showing the inhibitory effects of DNJ and G1-DNJ on the α-glucosidase (a) and α-amylase (b) activity. Figure S4: sequence alignment of conserved regions between BtαGTase and related glycoside hydrolase family GH77 members. BtαGTase, α-glucanotransferase from *Bacteroides thetaiotaomicron*; SpαGTase, α-glucanotransferase from *Streptococcus pneumoniae*; TsαGTase, α-glucanotransferase from *Thermus scotoductus*; TbαGTase, α-glucanotransferase from *Thermus brockianus*; TaαGTase, α-glucanotransferase from *Thermus aquaticus*; Ss-αGTase, α-glucanotransferase from *Synechocystis* sp.; SmαGTase, α-glucanotransferase from *Streptococcus mutans* UA159; StαGTase, α-glucanotransferase from *Solanum tuberosum*; CbαGTase, α-glucanotransferase from *Clostridium butyricum*; CrαGTase, α-glucanotransferase from *Chlamydomonas reinhardtii*; AtαGTase, α-glucanotransferase from *Arabidopsis thaliana*; Ac-11bαGTase, α-glucanotransferase from *Acidothermus cellulolyticus*; Ecs-k12αGTase, α-glucanotransferase from *Escherichia coli* str. K-12; Tt-hb8αGTase, α-glucosidase from *Thermus thermophilus*; and Sd2-40αGTase, α-glucanotransferase from *Saccharophagus degradans*. Closed circles at the conserved regions III, IV, and V represent the catalytic sites in family GH77 members. (*Supplementary Materials*)

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