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Efficient and regioselective synthesis of globotriose by a novel α-galactosidase from *Bacteroides fragilis*

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Abstract Globotriose (Galα1–4Galβ1–4Glc) is an important cell surface epitope that acts as the receptor for Shiga-like toxins, and it is also the core structure of Globo H and SSEA4 that are tumor-associated glycans. Hence, the enzymatic synthesis of globotriose would be necessary for the development of carbohydrate-based therapeutics for bacterial infections and cancers. Here, a novel GH27 α-galactosidase gene (*agaBf*3S), a 1521-bp DNA encoding 506 amino acids with a calculated molecular mass of 57.7 kDa, from *Bacteroides fragilis* NCTC9343 was cloned and heterogeneously expressed in *Escherichia coli*. The recombinant enzyme Aga*Bf*3S preferentially hydrolyzed *p*-nitrophenyl-α-D-galactopyranoside (*p*NPαGal) in all tested nitrophenyl glycosides. It showed maximum activity at pH 4.5 and 40 °C, and it was stable at pH 4.0–11.0 below 40 °C and metal-independent. The K_m and k_{cat} values for *p*NPαGal, melibiose, and globotriose were 1.27 mM and 172.97 S^{-1}, 62.76 mM and 17.74 S^{-1}, and 4.62 mM and 388.45 S^{-1}, respectively. Aga*Bf*3S could transfer galactosyl residue from *p*NPαGal to lactose (Galβ1–4Glc) with high efficiency and strict α1–4 regioselectivity. The effects of initial substrate concentration, pH, temperature, and reaction time on transglycosylation reaction catalyzed by Aga*Bf*3S were studied in detail. Aga*Bf*3S could synthesize globotriose as a single transglycosylation product with a maximum yield of 32.4 % from 20 mM *p*NPαGal and 500 mM lactose (pH 4.5) at 40 °C for 30 min. This new one-enzyme one-step synthetic reaction is simple, fast, and low cost, which provides a promising alternative to the current synthetic methods for access to pharmaceutically important Galα1–4-linked oligosaccharides.

Keyword α-Galactosidase · *Bacteroides fragilis* · Transglycosylation · Regioselectivity · Globotriose

Introduction

Oligosaccharides or their analogs are potential therapeutics, but their use has been limited because the large-scale production of these biomolecules still remains difficult. Enzymatic synthesis of oligosaccharides and glycosides has been a highly attractive approach as it possesses the advantages of high stereo/regioselectivity which can be achieved only through many protecting group manipulations in chemical synthesis (Sears and Wong 2001). Glycosyltransferases and glycosidases are two classes of enzyme which have been applied for oligosaccharide synthesis in one-step reactions. Glycosyltransferases, the natural enzymes responsible for the synthesis of oligosaccharides and polysaccharides, catalyze stereo/regioselective reactions effectively, but their use has been hampered by the requirement of costly nucleotide diphosphate glycosyl donors (Weijers et al. 2008). Glycosidases, the enzymes that normally hydrolyze carbohydrates, can catalyze reverse hydrolysis or transglycosylation reactions for glycoside formation using simple sugar donors (Trincone and Giordano 2006). These properties make glycosidases attract worldwide attentions for low-cost, large-scale synthesis of oligosaccharides.

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α-Galactosidases (EC 3.2.1.22) are an important class of glycosidases that catalyze the hydrolysis of terminal galactose from various oligosaccharides and glycoconjugates (Ademark et al. 2001). Based on the protein sequence similarities, α-galactosidases have been classified into glycosyl hydrolase families GH4, GH27, GH36, GH57, GH97, and GH110 (http://www.cazy.org/). Transglycosylation activities of some α-galactosidases have been investigated and applied in the enzymatic synthesis of important α-galacto-oligosaccharides or α-galactosides. Most of the known α-galactosidases form Galα1–3 or/and Galα1–6 linkages (Hashimoto et al. 1995; Vic et al. 1996, 1997; Singh et al. 1999; Spangenberg et al. 2000; Shabalin et al. 2002; Puchart and Biely 2005; Merceron et al. 2012; Bobrov et al. 2013); only very few enzymes are able to form Galα1–4 linkage (Kato et al. 1982; Zhao et al. 2008; Wang et al. 2014).

α-Galacto-oligosaccharides are widely distributed in nature and have very important functions and applications. For examples, there are many kinds of non-digestible α-galacto-oligosaccharides in soymilk, and their structural analogs, usually containing Galα1–6 linkages, can be used as prebiotics for selective stimulation of the beneficial gut microbiota (Peterbauer and Richter 2001; Tzortzis et al. 2004). α-Gal epitope oligosaccharides bearing the Galα1–3 linkages are present as the major carbohydrate antigens in non-primate mammals, prosimians, and New World monkeys, resulting in the immune processes when it is recognized by the nature antibody anti-Gal in human. Thus, the α-Gal epitope oligosaccharides and their derivatives can be used in xenotransplantation and vaccine development for cancer therapy (Galili 2013). Another important α-galacto-oligosaccharide, globotriose (Galα1–4Galβ1–4Glc), exists on human cells. It acts as the receptor for binding the Shiga toxins produced by *Shigella dysenteriae* or enterohemorrhagic *Escherichia coli.* The resulting invasion of Shiga toxins is dangerous and could finally result in lethal hemolytic uremic syndrome (HUS). The artificially synthesized globotriose and its derivatives can mimic the receptor structure and then bind the toxins and thus can be used as an affinity inhibitors for the toxins in the prevention and treatment of related diseases (Okochi et al. 2013; Ryu et al. 2013; Li et al. 2012). Globotriose is also the core structure of Globo H and SSEA4 existing in many cancer cells such as brain, breast, lung, ovary, stomach, and small-cell lung tumor cells (Tsai et al. 2013); thus, it can be used as a building block in the synthesis of the carbohydrate-related cancer antigens for anti-cancer vaccine development (Hakomori and Zhang 1997; Zhang et al. 1997; Hakomori 2008; Lou et al. 2014; Gilewski et al. 2001; Danishefsky et al. 2015). Additionally, recent reports showed that cellular or soluble P^k/Gb3 histo-blood group antigen containing galabiose (Galα1–4Gal) terminal structure and its analogs can resist HIV-1 infection by probably inhibiting the fusion of the HIV-1 envelope to the cell target membrane (Branch 2010). These results suggested that globotriose and its derivatives might be developed as a novel therapeutic approach for the prevention of HIV/AIDS.

In our previous work, an engineered GH 36 α-galactosidase from *Bifidobacterium breve* 203 was found to be the first glycosidase capable of synthesizing globotriose derivative from a donor of *p*NPαGal and an acceptor of methyl lactoside in one step (Zhang et al. 2015). However, that enzyme displayed relaxed regioselectivity toward methyl lactose and simultaneously produced another regio-isomer of Galα1–3 linkage, which not only reduced the yield of the methyl globotrioside (16 % yield based on the donor) but also required extra procedure for isolation of the isomer products.

In this work, a novel GH 27 α-galactosidase gene (named *agaBf*3S) was cloned from *Bacteroides fragilis* NCTC9343 and expressed in *E. coli* LMG194. The recombinant enzyme (named Aga*Bf*3S) was purified and characterized. It was found that Aga*Bf*3S displayed strict α1–4 regioselectivity toward lactose; thus, it produced globotriose as a single transglycosylation product in high efficiency using *p*NPαGal and lactose as substrates. This enzyme was the first glycosidase that showed strict α1–4 regioselectivity toward lactose. Its use in the synthesis of globotriose would be of practical meaning as it has advantages of low cost and short reaction time when compared to the current synthetic methods.

Materials and methods

Materials

Standard globotriose, *p*NPαGal, and other nitrophenyl glycosides were purchased from Sigma (St. Louis, USA). Ni^{2+} Sepharose High Performance was the product of GE Healthcare (Uppsala, Sweden). Bio-Gel P2 was purchased from Bio-Rad Laboratories (Hercules, USA). Other chemicals used were of analytical grade.

Microorganisms

Bacteroides fragilis NCTC9343 was grown anaerobically at 37 °C, in medium containing (L^{-1}) 5 g yeast extract, 20 g proteose peptone, 5 g NaCl, 60 g glucose, 5 mg hemin, and 0.5 mg vitamin Kl (pH 7.2) (Pantosti et al. 1991).

E. coli LMG194 and plasmid pBAD/His A were purchased from Invitrogen, Carlsbad, CA, USA. The strain LMG194 was grown in RM plus glucose medium containing (L^{-1}) 20 g casamino acids, 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 100 μmol thiamine, 95.21 mg $MgCl_2$, and 2 g glucose (pH 7.4).

Gene cloning and heterogeneous expression

There is a putative GH27 α-galactosidase (BF0227, GenBank No. CAH06003) from *Bacteroides fragilis* NCTC9343 in CAZy database. This enzyme gene is located from nucleotides 246,933 to 248,525 in *Bacteroides fragilis* NCTC9343 genome and contained a 1593-bp ORF encoding a 530-amino-acid protein with a putative signal sequence of 24 amino acids at *N*-terminus as analyzed by SignalP 4.1. The gene encoding the truncated protein devoid of signal sequence was amplified by PCR. It consisted of 1521 nucleotides corresponding to the sequence from 247,005 to 248,525 in *Bacteroides fragilis* NCTC9343 genome and designated as *agaBf*3S. Primers for the gene cloning were 5′-GGCCGCTAGCCAAAACACAAATACTCCCAT-3′ and 5′-GGCCCTCGAGTTAACGACCTTTGATGATTT-3′, respectively (*Nhe* I and *Xho* I restriction sites are underlined). The genome of *Bacteroides fragilis* NCTC9343 was extracted by TIANamp Bacteria DNA Kit from Tiangen Biotech (Beijing, China) and used as template for PCR. The PCR conditions were as follows: a hot start at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 1.5 min, and a final step at 72 °C for 10 min. The PCR product was purified, treated with *Nhe* I and *Xho* I, and cloned into the expression vector pBAD/His A. The resulting recombinant plasmid was subsequently transformed into *E. coli* LMG194. The proper transformant was first grown at 37 °C in RM plus glucose medium containing ampicillin (50 μg/mL). When the cell density reached 0.6–0.8 at 600 nm, L-arabinose (2 g/L) was added for enzyme induction and the temperature was decreased to 28 °C for cell growth. After continuous cultivation for 12 to 14 h, the cells were harvested and disrupted by ultrasonic treatment. The resulting lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was subjected to enzyme purification by nickel affinity chromatography.

Enzyme assays

The α-galactosidase activity was measured by adding 1 μL of enzyme solution to 79 μL of 2 mM *p*NPαGal in 50 mM acetate buffer (pH 4.5). The reaction was performed at 37 °C for 10 min and then stopped by adding 120 μL of 0.2 M sodium borate buffer (pH 10.5). One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol per min under the assay conditions. Assays for the other nitrophenyl glycosides (Sigma) were performed under the same conditions. Protein was determined by NanoDrop 2000 calibrated with the extinction coefficient predicted by ExPASy (http://web.expasy.org/protparam/).

Protein electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10 % (*w*/*v*) gel. Proteins in the gel were visualized by Coomassie brilliant blue R-250 staining.

Biochemical studies

The hydrolytic substrate specificity in response to various nitrophenyl glycosides was detected in the same way as the enzyme assay condition. The optimal pH was determined by assaying the enzyme activity with *p*NPαGal at pH values ranging from 3.0 to 12.0 in 30 mM buffers containing citric acid, KH_2PO_4, boric acid, and barbitone and using NaOH to adjust the pH. The pH stability was determined by incubating the enzyme in the presence of the above different pH buffers at 4 °C for 12 h and assaying the residual activity under standard reaction conditions. The optimal temperature for enzyme reaction was determined by assaying the enzyme activity with *p*NPαGal for 10 min at 30–70 °C. Thermo-stability was determined by assaying residual enzymatic activity after incubating the enzyme for 30 min at 0–50 °C. To determine the effects of chemicals, enzyme activities were assayed in the presence of 1 mM metal salts or additives. K_m and k_{cat} values of the enzyme were determined by GraphPad Prism 5 using different concentrations of *p*NPαGal, melibiose, and globotriose as substrates.

Synthesis of globotriose by the recombinant Aga*Bf*3S

For preliminary determination of transglycosylation, the synthesis reaction was performed at 40 °C for 20 min in a mixture (100 μL) containing 0.06 U of enzyme, 400 mM lactose, and 15 mM *p*NPαGal in 100 mM acetate buffer (pH 4.5). The reaction was stopped by heating at 100 °C for 10 min. Sugars were analyzed by the thin-layer chromatography (TLC) and high-performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) as described below.

To achieve the maximum yield, the reaction conditions including substrate concentration, pH, and temperature were investigated in detail. The effects of variations in acceptor concentration were determined by incubating 0.06 U enzyme with 15 mM *p*NPαGal at 40 °C for 20 min, in the presence of lactose ranging from 100 to 600 mM. The effects of donor concentration were determined by incubating the enzyme with 500 mM lactose at 40 °C for 20 min, in the presence of *p*NPαGal from 5 to 30 mM. The effects of pH were examined by incubating the enzyme with 500 mM lactose and 20 mM *p*NPαGal at 40 °C for 20 min in 100 mM buffers at different pH values (acetate buffer at 4.0, 4.5, 5.0, and 6.0 and phosphate buffer at 6.0, 7.0, and 8.0). The effects of temperature and reaction time were investigated by incubating the enzyme with 500 mM lactose and 20 mM *p*NPαGal in pH 4.5 at six

different temperatures (30, 37, 40, 50, 60, and 70 °C) followed by interval sampling within 90 min. All the reactions were stopped by heating at 100 °C for 10 min, and the resulting products were analyzed by HPEAC. The yield of transglycosylation product was based on *p*NPαGal added.

Isolation of the oligosaccharide product

The oligosaccharide product was separated by a Bio-Gel P2 column (1.6 × 90 cm) with distilled water as the eluent. The fractions were eluted at a flow rate of 0.2 mL/min and collected at 1 mL per tube. The resulting samples were subjected to sugar determination by TLC. The fractions with identical sugar compositions were combined and lyophilized to dry powder.

TLC and HPAEC analysis

TLC was performed by loading samples on Silica gel 60 F254 plates (Merck, Germany). The developing solvent was a mixture of n-butanol/ethanol/water (5:3:2, *v*/*v*/*v*). Sugars on the TLC plate were visualized by spraying with a solvent containing 20 % sulfuric acid and 0.5 % 3,5-dihydroxytoluene, followed by incubation at 120 °C for 5 min.

HPAEC-PAD was performed using a Dionex CarboPacTM PA-100 column (4 × 250 mm) with temperature maintained at 30 °C. Samples were diluted 50 times before filtered through a 0.22-μm polypropylene filter, and the mobile phase NaOH (60 mM) was degassed in an ultrasonic bath before use. The samples were eluted at a flow rate of 1 mL/min and detected by an ED50 detector. The data was analyzed by Chromeleon 6.80 SR8 Build 2623.

MS and NMR analysis

Mass spectra were recorded on a Shimadzu LCMS-IT-TOF instrument (Kyoto, Japan) equipped with an ESI source in positive ion mode at a resolution of 10,000 full width at half-maximum. The NMR data were collected on an Agilent DD2 600 MHz instrument at room temperature in D_2O at 600 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts were given in ppm downfield from internal TMS of D_2O. The oligosaccharide produced by the enzyme and the commercial standard globotriose were both analyzed by recording the ^{1}H NMR, ^{13}C NMR, and heteronuclear single quantum coherence (HSQC). The data were processed using VnmrJ 4.0 and MestReNova software.

Results

Gene cloning and heterogeneous expression of *agaBf*3S

The gene (*agaBf*3S) encoding a putative α-galactosidase (Aga*Bf*3S) without predicted signal peptide was cloned from *Bacteroides fragilis* NCTC9343 by PCR. It was 1521 nucleotides encoding a protein consisting of 506 amino acids with a predicted molecular mass of 57.7 kDa. According to the BLAST results of amino acid sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the deduced protein sequence of Aga*Bf*3S had sequence identity of 46–99 % to top 100 Blast hits. Most of the top 100 Blast hits were putative α-galactosidases from various strains of *Bacteroides fragilis* and *Bacteroides* sp.; only 13 % the top 100 Blast hits were putative α-galactosidases from other bacteria, such as *Tannerella* sp. CAG:118 (52 % identity), *Coprobacter secundus* (52 % identity), and *Parabacteroides* sp. CAG:2 (49 % identity). In CAZy database, there are more than ten kinds of GH27 α-galactosidases from eukaryota which have been characterized, with only four from bacteria. The deduced protein sequence of Aga*Bf*3S revealed low homology to four bacterial GH27 α-galactosidases from *Cellvibrio mixtus* ATCC 12120 (GenBank No. AAS19696), *Clostridium cellulolyticum* ATCC 35319 (GenBank No. ACL75593), *Clostridium josui* FERM P-9684 (GenBank No. BAB83765), and *Saccharopolyspora erythraea* NRRL 2338 (GenBank No. AAC99325) with 35, 26, 38, and 35 % identities, respectively. Aga*Bf*3S also showed low homology to two fungal GH27 α-galactosidases with transglycosylation activity from *Aspergillus fumigatus* IMI 385708 (GenBank No. ACO72591) and *Talaromyces leycettanus* JCM12802 (GenBank No. AJF11712) with 28 and 26 % identities, respectively (Puchart and Biely 2005; Wang et al. 2016). Additionally, Aga*Bf*3S showed very low homology to the reported α-galactosidases which can synthesize α1–4 linkage from *Bifidobacterium breve* 203 (GenBank No. ABB76662) and *Lactobacillus reuteri* 100–16 (GenBank No. AGT37392) with 9 and 13 % identities, respectively (Zhao et al. 2008; Wang et al. 2014). Figure 1 shows a multiple sequence alignment of the deduced amino acid sequences of Aga*Bf*3S and four bacterial GH27 α-galactosidases analyzed by DNAMAN. The characteristic consensus motif of α-galactosidase ([LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-D-x-[WY]) (Fridjonsson et al. 1999) was found in Aga*Bf*3S as [Y]-GY-[V]-N-[I]-D-D-G-[Y], from Y42 to Y51. The conserved domain of GH27 (residues 6–277) and the predicted catalytic aspartate residues (D135 and D191) were concluded based on the BLAST result from NCBI.

The *agaBf*3S gene was subsequently cloned into pBAD/His A and transformed into *E. coli* LMG194. The recombinant enzyme Aga*Bf*3S was expressed and purified to electrophoretic purity. The subunit molecular mass as determined by SDS-PAGE (Fig. 2) was about 59.2 kDa, consistent with the predicted molecular mass deduced from its nucleotide sequence.

Characterization of the recombinant Aga*Bf*3S

The substrate specificity of recombinant Aga*Bf*3S toward nitrophenyl glycosides were examined and compared with the

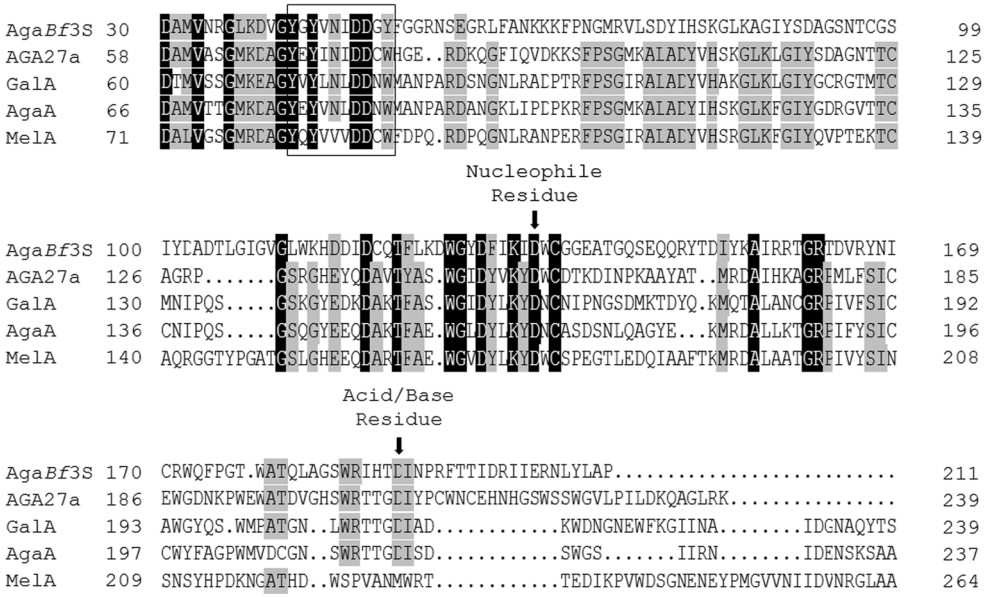

Fig. 1 Multiple alignment of the amino acid sequences of α-galactosidase. Aga*Bf*3S from *B. fragilis* NCTC9343, Aga27A from *Cellvibrio mixtus* ATCC 12120, GalA from *Clostridium cellulolyticum* ATCC 35319, AgaA from *Clostridium josui* FERM P-9684, and MelA from *Saccharopolyspora erythraea* NRRL 2338. Identical residues are shaded *black*. Residues conserved in at least four out of the five sequences are *gray*. The characteristic consensus motif of α-galactosidase is *boxed*. The *arrows* indicate the predicted catalytic aspartate residues

hydrolysis of *p*NPαGal. Aga*Bf*3S was highly active toward *p*NPαGal. It displayed 37.6 % of the *p*NPαGal activity when *o*NPαGal was used as the substrate, but it showed no activity toward *m*NPαGal and the other substrates with β-linkages or without galactose in the glycon moieties, including *o*NPβGal, *p*NPβGal, *p*NPα- or β-D-glucopyranoside, *p*NPα-L-fucopyranoside, *p*NPβ-D-Fuc, *p*NPα- or β-D-mannopyranoside, *p*NP-2-acetylamino-2-deoxy-α- or β-D-galactopyranoside, and *p*NP-2-acetylamino-2-deoxy-β-D-glucopyranoside.

Aga*Bf*3S was highly active at pH 4.5 and stable at pH 4.0–11.0. The optimal temperature for enzyme activity was 40 °C, and it was stable below 40 °C (Supplementary Fig. S1). Hg^{2+} and Ag^{+} completely inactivated the enzyme activity, whereas Ca^{2+} exhibited a little promoting effect on enzyme activity; Fe^{3+}, Cu^{2+}, and Fe^{2+} partly inhibited the enzyme activity by 81.06, 38.47, and 40 %, respectively. NH_4^{+}, K^{+}, Na^{+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and EDTA slightly affected the enzyme activity (less than 19.06 %) (Supplementary Fig. S2).

The K_m and k_{cat} values of Aga*Bf*3S for *p*NPαGal, melibiose, and globotriose were determined to be 1.27 mM and 172.97 S^{-1}, 62.76 mM and 17.74 S^{-1}, and 4.62 mM and 388.45 S^{-1}, respectively. Thus, the k_{cat}/K_m values of Aga*Bf*3S for *p*NPαGal, melibiose, and globotriose were 135.88, 0.28, and 84.12 $mM^{-1}S^{-1}$, respectively.

Synthesis of globotriose by the recombinant Aga*Bf*3S

The results of preliminary determination of transglycosylation of Aga*Bf*3S were shown in Fig. 3a. Using *p*NPαGal and lactose (Galβ1–4Glc) as the substrates, Aga*Bf*3S displayed transglycosylation activity, and a novel spot of oligosaccharide product (named as P_{Lac}) appeared below the spot of lactose. Besides P_{Lac}, a by-product, galactose, also appeared as a result of hydrolysis of *p*NPαGal catalyzed by the enzyme. The P_{Lac} had the same migration distance with the standard globotriose on the TLC plate. The result of HPAEC analysis also displayed that the peak of P_{Lac} shared identical retention time to the standard globotriose (Fig. 3b). These results suggested the P_{Lac} formed by Aga*Bf*3S might be globotriose.

The influences of various factors on the yields of transglycosylation product P_{Lac} formed by Aga*Bf*3S were further evaluated, including substrate concentration, pH, reaction time, and temperature. Figure 4a showed the influences of acceptor concentration on the product yields. The yield of

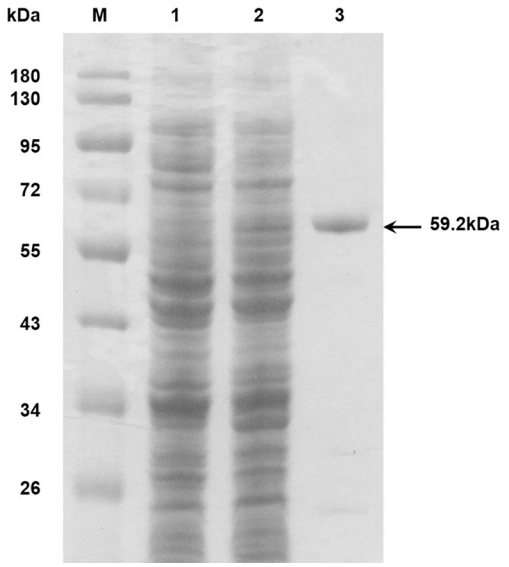

Fig. 2 SDS-PAGE of recombinant Aga*Bf*3S. Lane M, marker proteins; lanes 1 and 2, cell-free extract of recombinant strain before and after induction, respectively; lane 3, purified enzyme

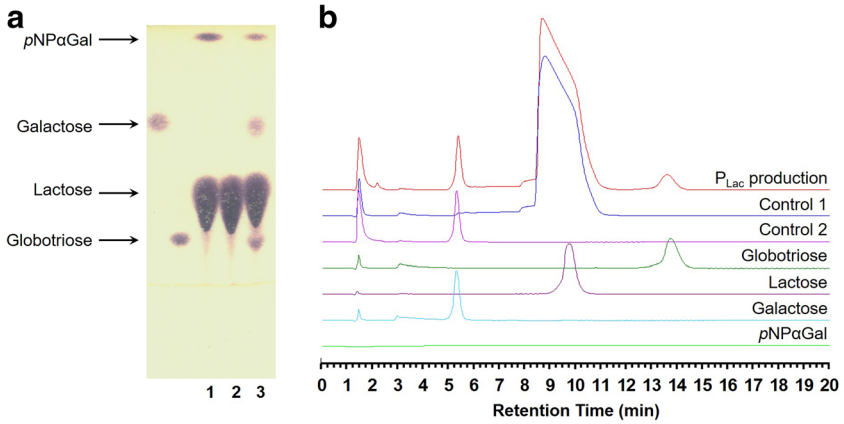

Fig. 3 Analysis of transglycosylation reaction mixture of Aga*Bf*3S by TLC (**a**) and HPAEC (**b**). **a** Lane 1, control reaction containing *p*NPαGal, lactose, and inactivated Aga*Bf*3S; lane 2, control reaction containing lactose and Aga*Bf*3S; lane 3, reaction containing *p*NPαGal, lactose, and Aga*Bf*3S. **b** P_{Lac} production, the same as lane 3 in (**a**); Control 1, the same as lane 1 in (**a**); Control 2, reaction containing *p*NPαGal and Aga*Bf*3S. The retention times of standard galactose, lactose and globotriose were 5.5, 10, and 14 min, respectively. The donor substrate of *p*NPαGal was invisible in HPAEC analysis. Peaks at 1.5 min result from the glycerol in enzyme samples

P_{Lac} was increased when the lactose concentrations were raised from 100 to 500 mM, and then it decreased when the lactose was changed to 600 mM (Fig. 4a). The maximum product yield of 17.10 % occurred at 500-mM acceptor. Thus, the subsequent reactions were performed at 500 mM lactose. Figure 4b showed the influences of donor concentration on the product yields. The yield improved when *p*NPαGal concentrations were increased from 5 to 20 mM. The product yield was 7.34 % at 5 mM donor and it reached a maximum yield of 19.69 % at 20 mM donor. Continuous increase of donor concentration from 20 to 30 mM led to the decrease of product yields. Thus, the subsequent reactions were performed by using 500 mM lactose and 20 mM *p*NPαGal.

According to Fig. 4c, the effects of pH on transglycosylation product synthesis showed the similar trend with that for hydrolysis of *p*NPαGal by Aga*Bf*3S. The yield reached the maximum (19.74 %) at pH 4.5 and dropped outside the pH range. The influences of temperature and reaction time are shown in Fig. 4d. The reaction temperature affected product formation remarkably. When the temperature was increased from 30 to 40 °C, the product yields reached the peak values quickly. When the reaction temperatures were elevated to 50 and 60 °C, the rates changed slightly after the yields achieved the maximum, and the peak values decreased. All the maximum yields between 37 and 60 °C reached peak values for about 30 min. The products formed between 30 and 40 °C showed higher peak values, but when the reaction time was prolonged, the yields gradually decreased as a result of secondary hydrolysis. In contrast, the maximum yields at 50–70 °C kept stable during the tested time. Accordingly, the optimal conditions for transglycosylation product synthesis by Aga*Bf*3S were an initial concentration of 20 mM *p*NPαGal and 500 mM lactose, at pH 4.5 and incubation at 40 °C for 30 min. As a whole, the highest yield of P_{Lac} achieved 32.4 % at these conditions.

Under the optimal conditions, the transglycosylation reaction was performed in a scale of 10 mL. The resulting transglycosylation product was purified and analyzed by MS and NMR spectroscopy. The positive-ion ESI-mass spectrum of the oligosaccharide product showed a peak of $[M + Na]^+$ ion at *m*/*z* 527.155 (Supplementary Fig. S3), which is consistent with the molecular mass of galactosyl lactose (504). The ^1H NMR and ^1H, ^{13}C-HSQC spectra of the product were fully in agreement with those of the standard globotriose (Fig. 5). Combining these results with the results of TLC and HPAEC analyses (Fig. 3), the transglycosylation product formed by Aga*Bf*3S was completely confirmed as globotriose. The enzymatic synthesis process and the product structure were shown in Fig. 6.

Discussion

Development of an efficient route to the large-scale synthesis of oligosaccharides is necessary for the study of the biological function of glycoconjugates and the development of potential therapeutic carbohydrate agents. Currently, the α-galactosidases have showed great potential in the enzymatic synthesis of functional α-galacto-oligosaccharides and glycomimetics. However, the known α-galactosidases mainly form Galα1–3 or/and Galα1–6 linkages but rarely form Galα1–4 linkages.

Bacteroides fragilis is a common anaerobic bacterium in human intestine. This gut microbe produced a wide range of hydrolytic enzymes for degradation of the host-derived glycan (Macfarlane and Gibson 1991). There are eight α-galactosidase genes in *Bacteroides fragilis* NCTC9343 genome (http://www.cazy.org/), two of which have been studied. Gal110A (GenBank No. CAH09922) and Gal110B (GenBank No. CAJ33351) are two GH110 α1–3-galactosidases that have

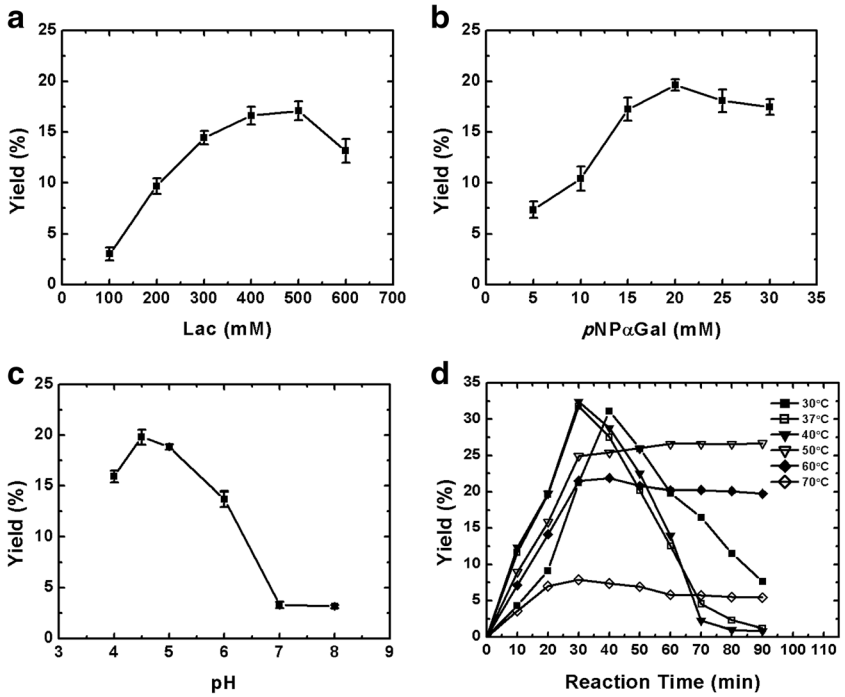

Fig. 4 The effects of substrate concentrations (**a**, **b**), pH (**c**), and temperature (**d**) on the yields of P_{Lac}. **a** The acceptor concentrations from 100 to 600 mM were tested at pH 4.5 and 40 °C in the presence of 15 mM *p*NPαGal for 20 min; **b** the donor concentrations were tested by incubation with 500 mM lactose at pH 4.5 and 40 °C, in the presence of *p*NPαGal from 5 to 30 mM for 20 min; **c** in pH tests, the reactions were carried out at 40 °C by incubating the enzyme with 500 mM lactose in the presence of 20 mM *p*NPαGal for 20 min in buffers from pH 4.0 to 8.0; **d** temperature and reaction time were tested at pH 4.5 by incubation of the enzyme with 500 mM lactose and 20 mM *p*NPαGal at 30 to 70 °C and detected within 90 min. Data points represent the means ± SD of three replicates

exquisite substrate specificity for the terminal α1–3-galactose residues, but their transglycosylation activities have not been studied (Liu et al. 2008). The other six α-galactosidases, three putative GH27 enzymes (BF0227, GenBank No. CAH06003; BF1418, GenBank No. CAH07128; BF4189, GenBank No. CAH09862) and three putative GH36 enzymes (BF0233, GenBank No. CAH06009; BF0498, GenBank No. CAH06254; BF0803, GenBank No. CAH06547), have not yet been studied. In this work, the gene (*agaBf*3S) encoding a putative GH27 α-galactosidase (Aga*Bf*3S, BF0227 without

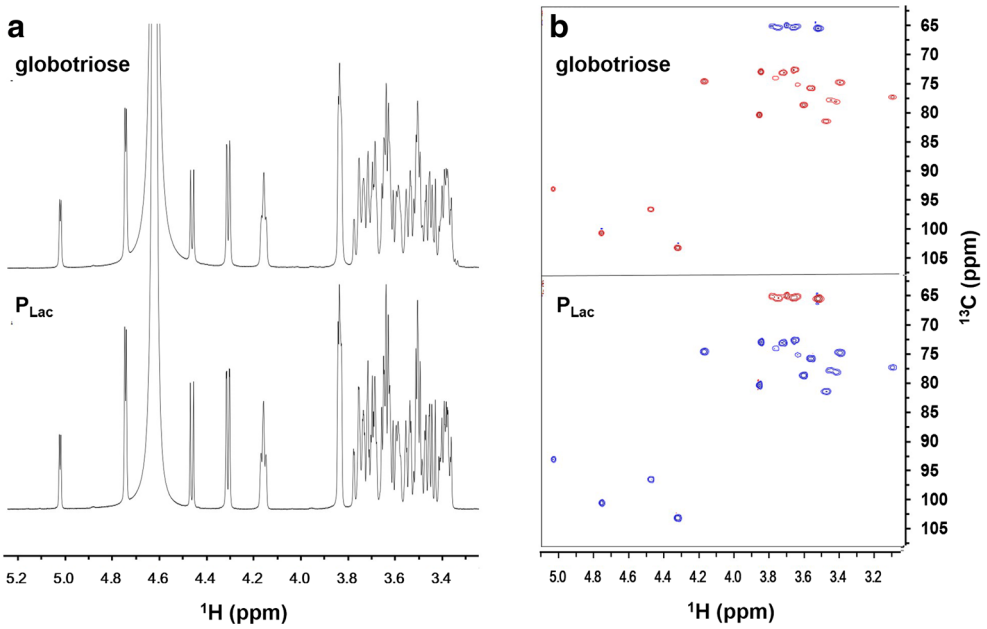

Fig. 5 ^{1}H NMR (**a**) and ^{1}H, ^{13}C-HSQC (**b**) of standard globotriose and P_{Lac} synthesized by Aga*Bf*3S

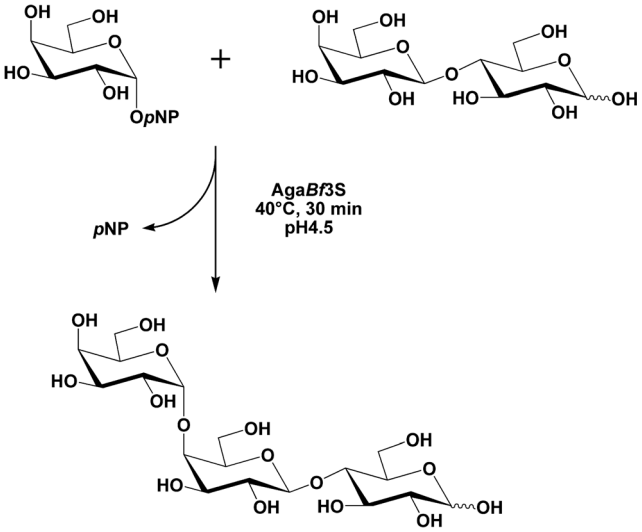

Fig. 6 Synthesis of globotriose by the recombinant Aga*Bf*3S

predicted signal peptide) of *Bacteroides fragilis* NCTC9343 was successfully cloned, heterogeneously expressed, and characterized for the first time. It was also first found that Aga*Bf*3S exhibited efficient transgalactosylation activity with strict α1–4 regioselectivity toward lactose; thus, globotriose could be synthesized as a single transglycosylation product. This enzyme would be a powerful and practical tool for synthesis of this important trisaccharide.

Generally, fungal and yeast α-galactosidases show maximum activities and stabilities in the acidic pH range (pH 4.5–5.5), whereas the bacterial α-galactosidases are maximally active and stable in the neutral pH range (Katrolia et al. 2014). In this work, it was found that Aga*Bf*3S showed maximum enzyme activity for *p*NPαGal hydrolysis at pH 4.5 and the activity dramatically decreased at pH 7.0. Aga*Bf*3S also preferred pH 4.5 for transglycosylation. This result was similar to the enzymes capable of synthesizing Galα1–4-linked products, such as those from *Stachys affinis* (pH 5.2 for synthesis of Galα1–4Gal) (Kato et al. 1982), *Bifidobacterium breve* 203 (pH 5.5 for synthesis Galα1–4-melibiose) (Zhao et al. 2008), and *L. reuteri* 100–16 (pH 4.7 for synthesis of Galα1–4-fucose and Galα1–3-fucose) (Wang et al. 2014).

The transglycosylation products would be accumulated if the donor substrate is more reactive than the product, and the donor with low K_m could minimize the hydrolysis of product (Zeuner et al. 2014). The α-galactosidases always show a lower K_m value for the artificial model substrate *p*NPαGal than the natural substrates, such as the K_m values of Aga2 from *Bifidobacterium breve* 203 for *p*NPαGal and melibiose of 0.27 and 4.3 mM, respectively (Zhao et al. 2008), and the K_m values of AgaB from *Geobacillus stearothermophilus* KVE39 for *p*NPαGal and raffinose of 0.83 and 200 mM, respectively (Merceron et al. 2012). In this work, Aga*Bf*3S also showed a lower K_m value for *p*NPαGal (1.27 mM) than the common natural substrate melibiose (62.76 mM). Considering that glycosidases usually catalyze the hydrolysis of transglycosylation products, the kinetic constants of the enzyme for globotriose was also determined to compare with the donor substrates in this work. The catalytic efficiencies (k_{cat}/K_m) of Aga*Bf*3S toward *p*NPαGal (135.88 mM^{-1} S^{-1}), globotriose (84.12 mM^{-1} S^{-1}), and melibiose (0.28 mM^{-1} S^{-1}) were sequentially decreased, which indicated that the *p*NPαGal would be a better donor substrate than melibiose for synthesis of globotriose and the enzyme would prefer *p*NPαGal to globotriose for hydrolysis.

It has been reported that the acceptor concentration could reduce water activity, which tends to favor the transglycosylation reaction over the hydrolysis reaction (Zeuner et al. 2014). In this work, using *p*NPαGal as donor, the yields of transglycosylation product were improved with the lactose increase from 100 to 500 mM, but the yield dropped at 600 mM lactose. The phenomenon showed that the molecular crowding effect caused by excessive amount of lactose might partly inhibit the reaction (Kim and Yethiraj 2009).

To the best of our knowledge, Aga*Bf*3S was the first GH27 α-galactosidase that could synthesize Galα1–4 linkage toward lactose with strict regioselectivity. It is well known that the regioselectivity of glycosidases not only depends on the enzyme source but also correlates with the acceptor structure which may interact each other (Zeuner et al. 2014), and the enzymes capable of synthesizing Galα1–4 linkage are very rare. So far, only four α-galactosidases have been reported to show α1–4 regioselectivity, three of which are microbial GH36 α-galactosidases: One enzyme is from *Bifidobacterium breve* and two are from *L. reuteri* (Zhao et al. 2008; Wang et al. 2014). The fourth enzyme is from plant *Stachys affinis*, and its ability to form Galα1–4 linkage was reported in 1982 and no information of the enzyme gene has been published (Kato et al. 1982). The transglycosylation acceptors of those α1–4 selective enzymes were limited in galactose, melibiose, fucose, and methyl β-lactoside. Concerning the natural lactose, Galα1–6 products can be synthesized by the enzymes from *L. reuteri* 100–16 and *Candida guilliermondii* H-404 (Wang et al. 2014; Hashimoto et al. 1995), and Galα1–3 linkage can be formed by the enzymes from *Penicillium multicolor* (Singh et al. 1999), but no Galα1–4 product has been reported. Glycosidases usually catalyze transglycosylation with relaxed regioselectivity. Enzymes from *Bacillus stearothermophilus* KVE39, *Thermus brockianus* ITI360, *Aspergillus niger*, and *Aspergillus oryzae* synthesize Galα1–3 and Galα1–6 products (Spangenberg et al. 2000; Vic et al. 1996, 1997), and enzyme from *Thermotoga maritima* MSB8 forms Galα1–2, Galα1–3, and Galα1–6 products by self-condensation of *p*NPαGal (Bobrov et al. 2013). Even the engineered α-galactosidase Aga2V564N from *Bifidobacterium breve* 203 still forms a mixture of α-Gal epitope (Galα1–3 linkage) and globotriose (Galα1–4 linkage) derivatives from *p*NPαGal and methyl β-lactoside. The relaxed regioselectivity of glycosidase in transglycosylation results in a

relatively low yield of desired product. Additionally, the resulting isomer products share the similar chemical or physical properties and thus are hard for isolation from each other. Hence, the strict α1–4 regioselectivity of Aga*Bf*3S using *p*NPαGal as donor and lactose as acceptor was valuable as it just formed globotriose as a single transglycosylation product which was easy for purification by gel filtration based on the difference of molecular mass of the products.

More importantly, Aga*Bf*3S-catalyzed synthesis of globotriose provided a novel one-step method to produce the Galα1–4-linked trisaccharide. The reported methods for the synthesis of Galα1–4 linkage are mostly through chemical or glycosyltransferase-mediated process. The chemical approach needs 11-step reactions for the synthesis of globotrioside from methyl D-galactopyranoside and *p*NPβ-lactoside (Dohi et al. 1999), and the synthesis of an aminooxy derivative of globotriose in 11 steps just achieves only 15 % overall yield (Ghosh and Andreana 2014). The glycosyltransferase is able to synthesize globotriose in one step using UDP-galactose (UDP-Gal) and lactose as substrates, but the glycosyl donor UDP-Gal is highly expensive (Weijers et al. 2008). Recently, multi-enzyme systems for one-pot synthesis of globotriose have been studied for the regeneration of UDP-Gal donor to reduce the production cost. Using trehalose, UDP-glucose, and lactose as substrates, coupling a trehalose synthase and a UDP-galactose 4′-epimerase for synthesizing UDP-Gal followed by incubation with a glycosyltransferase, globotriose was synthesized successfully (Ryu et al. 2013). Combining a galactokinase, a UDP-glucose pyrophosphorylase, a pyrophosphatase, and a α1–4-galactosyltransferase, the synthesis of globotriose from galactose, ATP, UTP, and lactose was also achieved (Zhao et al. 2014). However, these multi-enzyme processes are complex and time-consuming as they require multiple steps and more than 24 h for total reactions. The Aga*Bf*3S-catalyzed synthesis of globotriose in this work seemed more advantageous as it utilized low-cost donor substrate and accomplished in one step within 0.5 h.

In conclusion, a novel α-galactosidase from *Bacteroides fragilis* NCTC9343 was found to be a powerful synthetic tool which was able to transfer galactosyl from *p*NPαGal to lactose to form globotriose as a single transglycosylation product. This new one-enzyme one-step synthetic reaction has the advantages of low cost, strict regioselectivity, and ease of operation, which would potentially be implemented at an industrial scale to prepare pharmaceutically important Galα1–4-linked oligosaccharides in the future.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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