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A novel β-galactosidase capable of glycosyl transfer from *Enterobacter agglomerans* B1

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Abstract

A novel transglycosylating β-galactosidase was purified from *Enterobacter agglomerans* B1. It was a homodimer of ~248 kDa. The optimal pH and temperature for *o*NPGal hydrolysis were 7.5–8.0 and 37–40 °C, respectively. The K_m values for *o*NPGal and lactose were 0.06 and 114 mM, respectively. The enzyme produced galacto-oligosaccharides in a 38% yield at the lactose concentration of 12.5% (w/v). When using *o*NPGal as donor, the enzyme was able to catalyze glycosyl transfer to a series of acceptors, including hexose, pentose, β- or α-disaccharides, hexahydroxy alcohol, cyclitol, and aromatic glycosides. This suggested the enzyme to be a potential synthetic tool for preparing galactose-containing chemicals. The gene encoding this enzyme was cloned by degenerate PCR and TAIL-PCR. It revealed an ORF of 3090 nucleotides encoding a 1029 amino-acid protein, which had been expressed in *Escherichia coli*. Transferase activities in both recombinant and natural enzymes were similar.

Keywords: β-Galactosidase; Purification; Gene cloning; Glycosyl transfer; *Enterobacter agglomerans* B1

β-Galactosidases (EC 3.2.1.23) occur in nature very frequently. They are widely distributed in plants and animals, as well as in a wide variety of microorganisms including yeasts, fungi, bacteria, and archaea. These enzymes have attracted particular interest in the industrial applications owing to their hydrolase and transferase activities [1].

During the normal hydrolytic reaction, β-galactosidases hydrolyze lactose and transfer galactose to the hydroxyl group of water, resulting in the liberation of galactose and glucose. The hydrolytic activity has been applied in the food industry for decades for reducing the lactose in milk products, which presents one possibility to decrease the problem of lactose intolerance, prevalent in more than half of the world population. Some β-galactosidases, however, are able to transfer galactose to the hydroxyl groups of the galactose or the glucose moiety in lactose, resulting in the production of galacto-oligosaccharides (GOS). GOS are among the most promising non-digestible prebiotics. When compared with Raftilose P95, Raftiline LS, lactulose, xylo-, isomalto-, and soybean oligosaccharides, GOS cause the largest decrease in harmful *Clostridia*, higher short-chain fatty acid generation and lower gas production [2]. In view of their significant bioactive functions, a lot of reports on GOS synthesis have been published these years [1,3–6].

More recently, interest in β-galactosidases has gained more momentum owing to their biosynthetic abilities of preparing galactose-containing chemicals (GCC). Galactose is an important constituent of the carbohydrate chains of glycoconjugates involved in a variety of biological recognition events. The synthesis of β-galactosyl derivatives is currently receiving a great deal of attention owing to their important roles in many biological processes [7–10]. Strikingly, β-galactosidases exhibit utility in the synthesis of those chemicals, such as Galβ(1–3)GlcNAc and Galβ(1–4)GlcNAc, that are components of blood group determinants of the ABO system and could act as artificial antigens for immunological studies [9,11]. Also β-galactosidases synthesize diverse oligosaccharides, crucial glycoconjugates,

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alkyl-glycosides, and other chemicals that play important roles in the industry of food additives, cosmetics and medicines [12–20].

So far, β-galactosidases from numerous microorganisms have been used to produce prebiotic GOS, but few of them have been applied in synthesizing GCC. Most synthetic work were focused on only two microbial β-galactosidases: one from *Bacillus circulans* and the other from *Aspergillus oryzae* [8–10,13–18].

In this paper, a novel β-galactosidase was first purified and characterized from *Enterobacter agglomerans* B1. It was found to be capable of glycosyl transfer and produced GOS efficiently at low lactose concentrations. Moreover, it tolerated a wide range of glycosyl acceptors and catalyzed transglycosylation to a lot of saccharides that have not been investigated before. These excellent characteristics endowed the enzyme with a high capacity for obtaining novel GCC and would make it an alternative to the current synthetic origins in the future.

Materials and methods

Bacterial strains and media. Enterobacter agglomerans B1 was isolated from the soil and cultured at 30 °C in medium containing 10 g lactose, 5 g peptone, 10 g yeast extract, and 5 g NaCl in 1000 ml of water (pH 7.0).

Enzyme and protein assays. The β-galactosidase activity was measured by adding 50 μl enzyme solution to 450 μl of 2 mM *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal). The reaction was performed at 37 °C for 10 min and then stopped by adding 1 ml of 500 mM Na_2CO_3. The amount of *o*-nitrophenol released was measured at 400 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmol of *o*-nitrophenol per minute under the assay conditions. Assays for the other nitrophenyl glycosides (Sigma, US) were performed under the same conditions. The amount of protein was quantified by the method of Lowry with bovine serum albumin as the standard.

Enzyme purification. All the procedures described below were performed at 4 °C in phosphate buffer at pH 7.0. Cells were harvested from a 2000-ml culture and disintegrated by sonication. The resulting crude enzyme solution was concentrated by ammonium sulfate precipitation (0–45% saturation), followed by desalting, and sequentially applied to a 1.1 × 20-cm DEAE Sepharose Fast Flow column (Amersham, US), a 2.7 × 30-cm Gigapite K-100 S column (Seikagaku, Japan) and a 1.1 × 100-cm Sephadex G-150 column (Amersham, US).

Protein electrophoresis. SDS–PAGE and Native gradient PAGE were performed in 10% (w/v) and 5–10% gels, respectively. Proteins in the gel were visualized by silver staining or by Coomassie brilliant blue (CBB) R-250 staining. The β-galactosidase activity in the native gel was detected by staining with 4-methylumbelliferyl-β-D-galactopyranoside at 37 °C for 10 min. Fluorescent bands were visualized under UV light (365 nm) and photographed.

Biochemical studies. The optimal pH was assayed by incubating the enzyme with *o*NPGal in 50 mM buffers from pH 3.0 to 11.0. The effect of pH on enzyme stability was determined by incubation in the same range at 4 °C for 24 h. The optimal temperature was measured at 20–60 °C for 10 min. Thermal stability was studied by assessing enzyme activity after incubation at the above temperatures for 2 h. To determine the effects of chemicals, enzyme activities were assayed in the presence of 1 mM metal salts or 10 mM additives.

Transglycosylation with different acceptors. Transglycosylation reactions were performed at 50 °C for 8 h by adding 5 μl pure enzyme (20 U/ml), 20 μl of each acceptor (100 mM) and 5 μl *o*NPGal (50 mM) in 50 mM phosphate buffer (pH 7.5). A second group of reactions contained 5 μl of pure enzyme, 20 μl of each acceptor, and 5 μl phosphate buffer. The control reaction contained the enzyme with *o*NPGal in 30 μl phosphate buffer and was incubated under the same conditions. All the reactions were terminated by heating at 100 °C for 10 min.

Table 1
Primers used in the gene cloning

Primers	Nucleotide sequence (5′–3′)
Fbga′	GGSGTKAAYCGNCAYGAR
Rbga′	CKRTCVGGRTAGTTYTC
Fu	ATGATGTTYACVGCNWSNCCNATG
Ru	TTTTCCGGATGGTGCTCATG
Fd1	ATGATATTGGCGTCAGCG
Fd2	CGAAGCCACGCGTATTGA
Fd3	GCAAATGATATTCCGCAGCC
AD1	NTCGASTWTSGWGTT
AD2	NGTCGASWGANAWGAA
AD3	WGTGNAGWANCANAGA
AD4	TGWGNAGWANCASAGA
F1	CAAGGAATTCATGATGTTTACGGCGAGCCC
R3090	CAACAAGCTTATAGTCCTGTCGCCAGCTAA

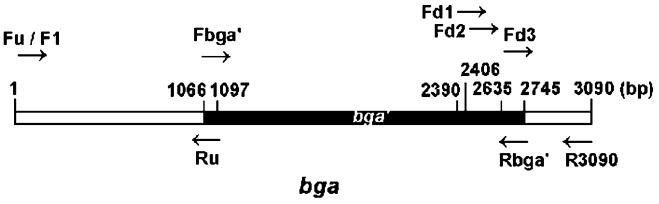


Fig. 1. The location of primers in the *bga* gene.

Oligosaccharide products were separated by thin layer chromatography using butanol–ethanol–water (5:3:2, v/v/v) as the mobile phase. Detection was achieved by spraying with 0.5% (w/v) 3,5-dihydroxytoluene in 20% (v/v) sulfuric acid and heating for 5 min at 120 °C. Novel oligosaccharides were quantified by the software ImageJ v1.28 (http://rsb.info.nih.gov/ij/).

Cloning and expression of the β-galactosidase gene (bga). Primers involved in the gene cloning were shown in Table 1 and Fig. 1, respectively. A fragment within the *bga* gene, designated *bga′*, was amplified by PCR using the degenerate primers Fbga′ and Rbga′, designed based on two conserved regions in other β-galactosidases. Upstream of *bga′* was amplified by the primer Fu, designed according to the *N*-terminal amino acid sequence of the purified enzyme, and the primer Ru, designed from the sequence of *bga′*. Downstream of *bga′* was obtained by thermal asymmetric interlaced PCR (TAIL-PCR). AD1 to AD4 were the arbitrary degenerate primers. The specific primers (Fd1–Fd3) were designed from the sequence of *bga′*. The reaction parameters for TAIL-PCR were referred to the report of Liu et al. [21]. Sequence analysis and multiple alignments were performed by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW program (http://www.ebi.ac.uk/clustalw/), respectively.

The whole gene was amplified by F1 and R3090 primers (the *Eco*RI and *Hin*dIII restriction sites are underlined, respectively). PCR products were sequenced and then cloned into the C-terminal His_6-fusion protein expression vector pET-22 b. A mutation (bold letter) had been introduced in the primer R3090 to avoid the early termination of protein translation. *Escherichia coli* BL21 (DE3) was used for expression. The recombinant enzyme was induced by IPTG and purified by Ni^{2+} chelation chromatography (Qiagen, Germany).

Results

Enzyme purification and its molecular mass

A novel β-galactosidase, designated Bga, was purified about 19-fold from the cell extract with a 1.6% yield. The

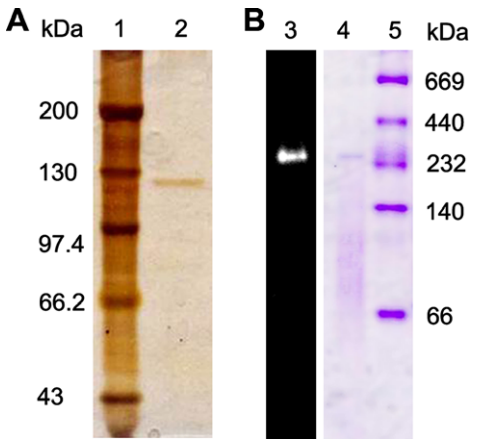


Fig. 2. SDS–PAGE (A) and native-gradient PAGE (B) of Bga: lanes 1 and 5, marker proteins; 2, denatured enzyme with silver staining; 3 and 4, native enzyme with active staining and CBB R-250 staining, respectively.

molecular mass of the enzyme as determined by SDS–PAGE and native-gradient PAGE, were about 120 and 248 kDa, respectively (Fig. 2). This indicated that the enzyme was a dimer with two identical subunits.

N-terminal amino acid sequence

The Bga enzyme was subjected to SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane. The protein band in it was cut out and sequenced by the method of Edman degradation. The N-terminal amino acid sequence was determined as M-F-T-A-S-P-M-S-L.

Characterization of the enzyme

The Bga enzyme was highly active in the pH range of 7.5–8.0 and stable between 7.5 and 10.0. The optimal temperature for enzyme activity was 37–40 °C, and the enzyme was stable below 37 °C. Hg^{2+}, Cu^{2+}, and Ag^{+} completely inhibited the enzyme activity, while Zn^{2+}, imidazole, and EDTA exhibited partial inhibition. Ca^{2+}, Co^{2+}, Na^{+}, K^{+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, and Mn^{2+} increased the activity with 21%, 24%, 44%, 47%, 58%, 94%, 96%, and 164%, respectively. The K_m and V_{max} values for *o*NPGal were calculated as 0.06 mM and 0.43 mM/min, respectively. The K_m value for lactose was estimated to be 114 mM, and the V_{max} value was estimated to be 2.9 mM/min.

The hydrolytic activities of Bga in response to various glycosides were analyzed. The enzyme was highly active when *o*NPGal, a β-D-anomer-linked galactoside, was used. It displayed 56.3% of the *o*NPGal activity when *p*-nitrophenyl-β-D-galactopyranoside was used as the substrate, but it showed less than 3% of the *o*NPGal activity with any of the other substrates tested (Table 2).

Transglycosylation activity

Transglycosylation activity was found when the enzyme was incubated with lactose that may act as both glycosyl donor and acceptor. GOS was produced and reached a high yield of ~38% using 12.5% (w/v) lactose at 50 °C for 12 h.

To further investigate the substrate specificity for transglycosylation, a series of acceptors were selected using *o*NPGal as the donor substrate. As shown in Table 3, the Bga enzyme showed transferase activities toward all the tested acceptors. In reactions without *o*NPGal, there were no novel oligosaccharides products (data not shown), suggesting the tested acceptors could not be self-transferred by the enzyme.

Table 2
Relative hydrolytic activity of various substrates by Bga

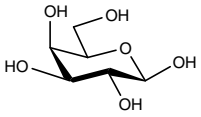
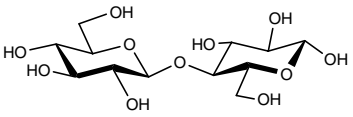
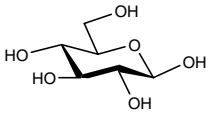
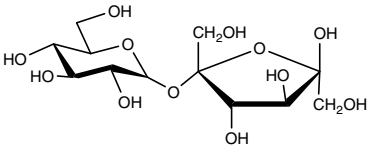
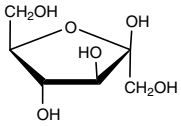
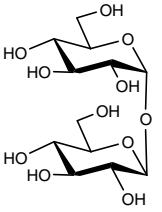
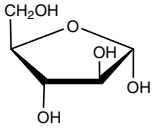
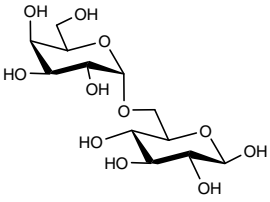
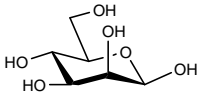
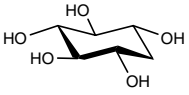
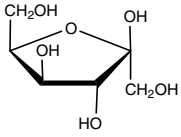
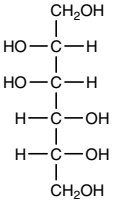
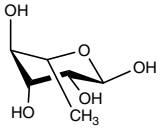
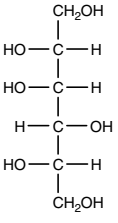
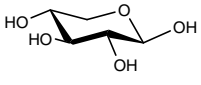
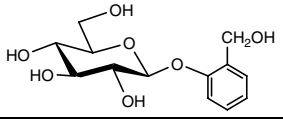
Substrate	Relative activity (%)
o-Nitrophenyl-β-D-galactopyranoside	100
p-Nitrophenyl-β-D-galactopyranoside	56.3
o-Nitrophenyl-α-D-galactopyranoside	0
p-Nitrophenyl-α-D-galactopyranoside	0
o-Nitrophenyl-α-D-glucopyranoside	0
o-Nitrophenyl-β-D-glucopyranoside	0
p-Nitrophenyl-β-D-galacturonide	0
o-Nitrophenyl-*N*-acetyl-β-D-galactosaminide	0
p-Nitrophenyl-*N*-acetyl-β-D-galactosaminide	2.5
p-Nitrophenyl-*N*-acetyl-α-D-galactosaminide	0
p-Nitrophenyl-α-D-fucopyranoside	0
p-Nitrophenyl-β-D-fucopyranoside	0
p-Nitrophenyl-α-mannopyranoside	0
p-Nitrophenyl-β-mannopyranoside	0

Gene cloning and sequence analysis

A 1680-bp fragment (*bga′*) containing part of β-galactosidase gene was amplified by degenerate primers. Upstream of *bga′* (~1.1 kb) was amplified by Fu and Ru primers. Downstream of *bga′* (~1.5 kb) was obtained by TAIL-PCR using the AD3 and the specific primers. Assembly of three fragments yielded a 4081-bp DNA that contained a 3090-bp ORF. The nucleotide sequence has been submitted to the GenBank with Accession No. EF371803. It encodes a protein of 1029 amino acids with a predicted molecular mass of 117 kDa, similar to that of purified enzyme estimated by SDS–PAGE (120 kDa).

The protein sequence showed high identities of 98% and 77% with LacZ from *Enterobacter* sp. 638 and *Enterobacter cloacae* GAO (GenBank Accession Nos.: ZP_01588286 and Q47077), respectively. Also it featured 60–66% identities with β-galactosidases from other genera of *Enterobacteriaceae*, including *Shigella, Escherichia, Serratia, Citrobacter, Salmonella, Erwinia*, and *Yersinia*. As shown in Fig. 3, all aligned β-galactosidase proteins possess residues corresponding to known *E. coli* amino acids essential for enzyme activity (*E. coli* residues: His-357, His-391, Glu-416, His-418, Glu-461, Tyr-503, Glu-537, His-540, Gly-794, and Glu-797) [22–25]. The possible acid/base and nucleophile sites of Bga were estimated to be Glu-464 and Glu-540, respectively.

Table 3
Results of transglycosylation in the presence of *o*NPGal

Acceptors	Structure	Transferase activity	Acceptors	Structure	Transferase activity
Galactose		+++	Cellobiose		++
Glucose		++++	Sucrose		+++
Fructose		++	Trehalose		+++
Arabinose		++	Melibiose		+
Mannose		++	Inositol		++
Sorbose		+++	Mannitol		++
Rhamnose		+	Sorbitol		+++
Xylose		++	Salicin		++++

Novel saccharide yields (%): +, 0–1.0; ++, 1.0–5.0; +++, 5.0–10.0; ++++, 10.0–20.0.

The gene of this enzyme had been successfully expressed in *E. coli* using the vector pET-22b. The recombinant enzyme had been purified and was found to have the same transglycosylation activity as the natural enzyme (data not shown).

Fig. 3. Multiple alignment of the possible acid/base, nucleophile, and other active sites. The function of *E. coli* residues in black boxing regions has already been determined. Their locations in *E. coli* are numbered above. The catalytic sites are indicated by arrows. All the β-galactosidase sequences were available in GenBank and were from *E. coli*, *E. agglomerans* B1 (B1), *E. cloacae* GAO (Enterc), *Shigella dysenteriae* Sd19 (Shiged), *Citrobacter freundii* MF 466 (Citrof), *Salmonella* sp. CDC 156–87 (Salmon), *Serratia* sp. D04 (Serrat), *Erwinia carotovora* subsp. *atroseptica* SCRI104 (Erwina), and *Yersinia pestis* KIM (Yersip), respectively.

Discussion

These days, glycosidases (EC 3.2.1) have been of tremendous utility in the enzymatic synthesis of oligosaccharides [26,27]. Due to availability, stability, organic solvent compatibility, and low cost, glycosidases are more advantageous than glycosyl-transferases (EC 2.4) and non-enzymatic methods in large-scale carbohydrate synthesis [26]. Reactions on synthesis were usually catalyzed by retaining enzymes via a double displacement mechanism involving a glycosylation and deglycosylation step, which is in most instances mediated by two key active residues, the catalytic nucleophile and acid/base [27]. The use of retaining glycosidases is attractive for their strict stereoselectivity. Also glycosidases do have some regioselectivity, and their selectivity may vary with different enzyme source [9], which is facilitated to obtain the desired glycosyl linkages by choosing proper enzymes. Among the class of glycosidases, β-galactosidase is one of the best investigated enzymes. This biocatalyst tolerates a variety of glycosyl acceptors [28]. In this work, a novel β-galactosidase, Bga, from *E. agglomerans* B1 was purified and provided as a new source for oligosaccharide synthesis.

The Bga enzyme was a dimeric protein. Microbial β-galactosidases are often multimeric. They are dimeric in *Sterigmatomyces elviae* and *Lactobacillus reuteri* [6,29] and tetrameric in *E. coli*, *Bacillus macerans*, *Lactobacillus helveticus*, *Penicillium chrysogenum*, and *Bifidobacterium infantis* [30,31]. In some fungi, such as *A. oryzae*, β-galactosidases are monomeric [30].

The enzyme activity was stimulated by a lot of divalent and monovalent ions, such as Mg^{2+}, Mn^{2+}, Na^{+}, K^{+}, and so on. The requirement for Mg^{2+} or/and Mn^{2+} is well-known for a number of different β-galactosidases from such species as *E. coli*, *Bifidobacterium bifidum*, *Kluyveromyces lactis*, *Bacillus* sp. and *L. reuteri*. Na^{+} and K^{+} also activated the Bga activity, similar to that for β-galactosidases from *Lactobacillus casei*, *B. bifidum*, *Streptococcus thermophilus*, and *L. reuter*. Ca^{2+} slightly enhanced the Bga activity whereas it was a known inhibitor of other relevant enzymes [6,31].

Kinetic constants of Bga for the natural substrate lactose and for the chromogenic model substrate *o*NPGal were both determined. The K_m value for *o*NPGal was significantly lower than that for lactose. This is consistent with β-galactosidases from a number of different sources [6,31]. The K_m value for lactose was 114 mM. It is in the range of those of other relevant enzymes from *Bullera singularis*, *K. lactis*, *A. oryzae*, *L. reuter* L103, *Bacillus* sp., and *S. elviae*, which were measured as 580, 500, 49, 13, 5, and 2.4 mM, respectively [5,6,29].

The Bga enzyme catalyzed self-transfer reaction when using lactose as starting material, giving high-yield (38%) of GOS even at a low lactose concentration (12.5%). This property could be applied in the low-lactose products (such as unprocessed milk) treatment, and develop GOS containing milk. Reactions at a lower lactose concentration (5%) by the enzyme were performed (data not shown). The resulting GOS reached a ~20% yield, which was comparable to the relevant enzymes derived from *S. thermophilus* and *Lactobacillus bulgaricus* [32].

It was interesting to note that Bga was able to utilize a wide range of acceptors, including hexose, pentose, β- or α-disaccharides, hexahydroxy alcohol, cyclitol, and aromatic glycosides, in the presence of *o*NPGal. The enzyme showed different efficiency on the various acceptors (Table 3). It preferred to salicin, glucose, galactose, sorbose, trehalose, sucrose, and sorbitol than others. Differences in the structure of the acceptor may influence its interaction with the enzyme and affect the bonding with the glycosyl moiety with unexpected results [11]. Although many galactose-containing chemicals have been synthesized by β-galactosidases [7–20], the glycosyl transfer to such acceptors as fructose, rhamnose, sorbose, arabinose, cellobiose, trehalose, melibiose, sorbitol, mannitol, and salicin have not been reported so far. This suggested that the enzyme was a promising tool for synthesis of novel chemicals, including food ingredients, pharmaceuticals, and other biologically active compounds. Unfortunately, the oligosaccharide yields by the enzyme were modest (Table 3). This was due to the inevitable drawback of glycosidases reactions, in which the products are always substrates for the enzymes and undergo hydrolysis. Traditional optimization of reaction conditions may increase the yields to some extent, but it can not alter the reaction mechanism. The hydrolysis problem would be eventually overcome by molecular evolution. And many glycosidases have been reported to be significantly improved in oligosaccharide yields (60–100%) by directed mutation at the nucleophile or acid/base sites (to generate glycosynthases or thioglycoligases using artificial substrates), random mutagenesis and the like [27,33].

In conclusion, the Bga enzyme derived from *E. agglomerans* B1 showed high transglycosylation activity towards lactose and displayed a wide range of substrate specificity for glycosyl transfer. The enzyme may be useful not only for the efficient synthesis of lactose-derived oligosaccharides in milk and whey, but also for the production of various galactose-containing chemicals. The synthetic β-galactosidase repertoire is expanded and one more glycosidase is available for oligosaccharides synthesis. The gene of Bga was obtained by conventional PCR combined with TAIL-PCR, which would be a straightforward and efficacious method for cloning a new β-galactosidase gene. Since the saccharide yields by the natural enzyme were generally modest, molecular evolution was required to improve the cloned enzyme genetically. Now, work is underway to testify the two catalytic sites of Bga, in order to further convert it into a novel galactosynthase or a thiogalactoligase. Random mutagenesis of Bga was also performed to obtain a transgalactosylase that can efficiently catalyze transglycosylation utilizing natural substrates.

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References

[1] R.R. Mahoney, Galactosyl-oligosaccharide formation during lactose hydrolysis: a review, Food Chem. 63 (1998) 147–154.
[2] C.E. Rycroft, M.R. Jones, G.R. Gibson, R.A. Rastall, A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides, J. Appl. Microbiol. 91 (2001) 878–887.
[3] N. Onishi, A. Yamashiro, K. Yokozeki, Production of galacto-oligosaccharide from lactose by *Sterigmatomyces elviae* CBS8119, Appl. Environ. Microbiol. 61 (1995) 4022–4025.
[4] B.A. Rabiu, A.J. Jay, G.R. Gibson, R.A. Rastall, Synthesis and fermentation properties of novel galacto-oligosaccharides by β-galactosidases from *Bifidobacterium* species, Appl. Environ. Microbiol. 67 (2001) 2526–2530.
[5] Y.J. Cho, H.J. Shin, C. Bucke, Purification and biochemical properties of a galactooligosaccharide producing β-galactosidase from *Bullera singularis*, Biotechnol. Lett. 25 (2003) 2107–2111.
[6] T.H. Nguyen, B. Splechtna, M. Steinbock, W. Kneifel, H.P. Lettner, K.D. Kulbe, D. Haltrich, Purification and characterization of two novel β-galactosidases from *Lactobacillus reuteri*, J. Agric. Food Chem. 54 (2006) 4989–4998.
[7] S. Menzler, H. Seker, M. Gschrey, M. Wiessler, β-Galactosidase catalysed synthesis of branched oligosaccharide analogues, Biotechnol. Lett. 11 (1997) 269–272.
[8] H. Fujimoto, M. Isomura, T. Miyazaki, I. Matsuo, R. Walton, T. Sakakibara, K. Ajisaka, Enzymatic syntheses of GlcNAcβ1-2Man and Galβ1-4GlcNAcβ1-2Man as components of complex type sugar chains, Glycoconj. J. 14 (1997) 75–80.
[9] X. Zeng, R. Yoshino, T. Murata, K. Ajisaka, T. Usui, Regioselective synthesis of *p*-nitrophenyl glycosides of β-D-galactopyranosyl-disaccharides by transglycosylation with β-D-galactosidases, Carbohydr. Res. 325 (2000) 120–131.
[10] X. Zeng, H. Uzawa, Convenient enzymatic synthesis of a *p*-nitrophenyl oligosaccharide series of sialyl *N*-acetyllactosamine, sialyl Le^x and relevant compounds, Carbohydr. Res. 340 (2005) 2469–2475.
[11] J.H. Yoon, K. Ajisaka, The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins, Carbohydr. Res. 292 (1996) 153–163.
[12] O.S. Kuptsova, N.L. Kliachko, A.V. Levashov, Synthesis of alkyl glycosides, catalyzed by β-glycosidases in a reversed micelle system, Bioorg. Khim. 27 (2001) 429–433.
[13] C. Scheckermann, F. Wagner, L. Fischer, Galactosylation of antibiotics using the β-galactosidase from *Aspergillus oryzae*, Enzyme Microb. Technol. 20 (1997) 629–634.
[14] C. Giacomini, G. Irazoqui, P. Gonzalez, F. Batista-Viera, B.M. Brena, Enzymatic synthesis of galactosyl-xylose by *Aspergillus oryzae* β-galactosidase, J. Mol. Catal. B: Enzym. 19 (2002) 159–165.
[15] V. Nieder, S.P. Marx, R.G. Gallego, J.P. Kamerling, J.F.G. Vliegenthart, L. Elling, Synthesis of nucleotide-activated disaccharides with β-galactosidase from *Bacillus circulans* and α-galactosidase from *Bifidobacterium adolescentis*, J. Mol. Catal. B-Enzym. 21 (2003) 157–166.
[16] M. Miyasato, K. Ajisaka, Regioselectivity in β-galactosidase-catalyzed transglycosylation for the enzymatic assembly of D-galactosyl-D-mannose, Biosci. Biotechnol. Biochem. 68 (2004) 2086–2090.
[17] T. Higashiyama, H. Watanabe, H. Aga, T. Nishimoto, M. Kubota, S. Fukuda, M. Kurimoto, Y. Tsujisaka, Enzymatic synthesis of a β-D-galactopyranosyl cyclic tetrasaccharide by β-galactosidases, Carbohydr. Res. 339 (2004) 1603–1608.
[18] R. Shimizu, H. Shimabayashi, M. Moriwaki, Enzymatic production of highly soluble myricitrin glycosides using β-galactosidase, Biosci. Biotechnol. Biochem. 70 (2006) 940–948.
[19] A. Giordano, A. Tramice, G. Andreotti, E. Mollo, A. Trincone, Enzymatic syntheses and selective hydrolysis of *O*-β-D-galactopyranosides using a marine mollusc β-galactosidase, Bioorg. Med. Chem. Lett. 15 (2005) 139–143.

[20] N. Bridiau, S. Taboubi, N. Marzouki, M.D. Legoy, T. Maugard, β-Galactosidase catalyzed selective galactosylation of aromatic compounds, Biotechnol. Prog. 22 (2006) 326–330.
[21] Y.G. Liu, L.N. Mitsukawa, T. Oosumi, R.F. Whittier, Efficient isolation and mapping of *Arabidopis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR, Plant J. 8 (1995) 457–463.
[22] A.G. Bobrov, R.D. Perry, *Yersinia pestis* lacZ expresses a β-galactosidase with low enzymatic activity, FEMS Microbiol. Lett. 255 (2006) 43–51.
[23] N.J. Roth, R.E. Huber, Glu-416 of β-galactosidase (*Escherichia coli*) is a Mg^{2+} ligand and β-galactosidases with substitutions for Glu-416 are inactivated, rather than activated, by Mg^{2+}, Biochem. Biophys. Res. Commun. 219 (1996) 111–115.
[24] M. Martinez-Bilbao, R.E. Holdsworth, L.A. Edwards, R.E. Huber, A highly reactive β-galactosidase (*Escherichia coli*) resulting from a substitution of an aspartic acid for Gly-794, J. Biol. Chem. 266 (1991) 4979–4986.
[25] G. Sutendra, S. Wong, M.E. Fraser, R.E. Huber, β-Galactosidase (*Escherichia coli*) has a second catalytically important Mg^{2+} site, Biochem. Biophys. Res. Commun. 352 (2007) 566–570.
[26] M. Scigelova, S. Singh, D.H.G. Crout, Glycosidases—a great synthetic tool, J. Mol. Catal. B-Enzym. 6 (1999) 483–494.
[27] G. Perugino, A. Trincone, M. Rossi, M. Moracci, Oligosaccharide synthesis by glycosynthases, Trends Biotechnol. 22 (2004) 31–37.
[28] W.H. Binder, H. Kahlig, W. Schmid, Glycosylation by use of β-galactosidase: enzymatic synthesis of disaccharide nucleosides, Tetrahedron-Asymmetr. 6 (1995) 1703–1710.
[29] N. Onishi, T. Tanaka, Purification and properties of a novel thermostable galacto-oligosaccharide-producing β-galactosidase from *Sterigmatomyces elviae* CBS8119, Appl. Environ. Microbiol. 61 (1995) 4026–4030.
[30] Z. Nagy, T. Kiss, A. Szentirmai, S. Biro, β-Galactosidase of *Penicillium chrysogenum*: production, purification, and characterization of the enzyme, Protein Expr. Purif. 21 (2001) 24–29.
[31] M.N. Hung, B.H. Lee, Purification and characterization of a recombinant β-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96, Appl. Microbiol. Biotechnol. 58 (2002) 439–445.
[32] Y. Kobayashi, T. Kan, T. Terashima, Method for producing processed milk containing galactooligosaccharide, US Patent (1990) 4944952.
[33] S.M. Hancock, M.D. Vaughan, S.G. Withers, Engineering of glycosidases and glycosyltransferases, Curr. Opin. Chem. Biol. 10 (2006) 509–519.