Biochemical characterization of a novel β-galactosidase from *Lacticaseibacillus zeae* and its application in synthesis of lacto-N-tetraose

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ABSTRACT

Lacto-N-tetraose (LNT) is one of the most important components of human milk oligosaccharides, which has various beneficial health effects. β-Galactosidase is an important enzyme used in dairy processing. The transglycosylation activity of β-galactosidases offers an attractive approach for LNT synthesis. In this study, we reported for the first time the biochemical characterization of a novel β-galactosidase (LzBgal35A) from *Lacticaseibacillus zeae*. LzBgal35A belongs to glycoside hydrolases (GH) family 35 and shared the highest identity of 59.9% with other reported GH 35 members. The enzyme was expressed as soluble protein in *Escherichia coli*. The purified LzBgal35A displayed optimal activity at pH 4.5 and 55°C. It was stable within the pH range of 3.5 to 7.0 and up to 60°C. Moreover, LzBgal35A could catalyze the synthesis of LNT via transferring the galactose residue from o-nitrophenyl-β-galactopyranoside to lacto-N-triose II. Under optimal conditions, the conversion rate of LNT reached 45.4% (6.4 g/L) within 2 h, which was by far the highest yield of LNT synthesized through a β-galactosidase-mediated transglycosylation reaction. This study demonstrated that LzBgal35A has great potential application in LNT synthesis.

Key words: lacto-N-tetraose, β-galactosidase, transglycosylation, human milk oligosaccharides

INTRODUCTION

Human milk oligosaccharides (HMO) are a series of unique oligosaccharides that exist naturally in human milk (Wang et al., 2022). They are the third nutritional components of human milk after lactose and lipids, and they show many biological functions for the health of newborns (Chen, 2015; Masi and Stewart, 2022). To date, more than 200 types of HMO have been identified, and about 100 types of HMO have been structurally characterized (Sarkozy et al., 2021). Lacto-N-tetraose (Galβ1-3GlcNAcβ1-3lactose; LNT) is a tetrasaccharide formed by linking a molecule of galactose with β-1,3-glycosidic bond at the nonreducing end of lacto-N-triose II (GlcNAcβ1-3lactose, LNT2; Zhu et al., 2021). As one of relatively abundant components of HMO (6%, wt/wt), LNT is an attractive additive in infant formulas and other dietary supplements that have been authorized by the European Union and United States (EFSA Panel on Nutrition, Novel Foods and Food Allergens et al., 2019). However, the large-scale production of LNT still remains a challenge.

Extraction from breast milk, chemical synthesis, whole-cell synthesis, and enzymatic synthesis are the main methods for LNT production (Faijes et al., 2019). Among these methods, whole-cell and enzymatic synthesis of LNT are suitable for food fields, clinical applications, and large-scale production (Bode et al., 2016). Whole-cell synthesis reaches a relatively high LNT yield by introducing necessary metabolic genes and removing unfavorable metabolic genes through molecular biology technology (Baumgärtner et al., 2015; Zhu et al., 2022). However, some problems remain, such as a long fermentation period, some side products, and multiple purification steps (Lu et al., 2021). Enzymatic synthesis of LNT in vitro shows a short reaction time and extracellular products that are more easily purified, compared with whole-cell synthesis (Liu et al., 2009). Moreover, an enzymatic method will facilitate large-scale synthesis of LNT in as short a pathway as possible.

Generally, enzymatic synthesis of LNT is performed by 3 types of enzymes: β-1,3-galactosyltransferase, lacto-N-biosidase, and β-galactosidase. β-1,3-
Galactosyltransferase catalyzed the transfer of galactose to LNT2 to synthesize LNT using UDP-Gal as glycosyl donor and LNT2 as glycosyl acceptor (Zhu et al., 2021). However, UDP-Gal is expensive, which limits its application in LNT synthesis in vitro (Nidetzky et al., 2018). Lacto-N-biosidases can hydrolyze LNT and release lacto-N-biose from its nonreducing end. Some lacto-N-biosidases also have transglycosidic activity and can synthesize LNT by directly coupling lacto-N-biose and lactose through the β-1,3-glycosidic bond (Murata et al., 1999; Schmölzer et al., 2019). As the transglycosylation activities of lacto-N-biosidases are low, protein engineering has been used to enhance the synthetic capabilities, with maximum yields of about 30% (Castejón-Vilatbarsana et al., 2021; Vuillemin et al., 2021). β-Galactosidases (E.C.3.2.1.23) are exoglycosidases that hydrolyze β-galactose residues from oligosaccharides and polysaccharides (Lu et al., 2020). In addition to hydrolysis, some β-galactosidases show transglycosylation activity, which can synthesize many prebiotic oligosaccharides, such as galactooligosaccharides and several kinds of HMO, through the transfer of galactosyl units onto the specific glycosylated receptors (Miyazaki et al., 2010; Gao et al., 2019). Synthesis of LNT by β-galactosidases is of great interest owing to its simple reaction, high selectivity, and inexpensive donor (Zheng et al., 2022). At present, only one β-galactosidase from Bacillus circulans ATCC31382 has been reported to synthesize LNT using o-nitrophenvl-β-galactopyranoside (oNPG) as donor and LNT2 as acceptor, and the conversion ratio of LNT was only 20% (Murata et al., 1999). Thus, new β-galactosidases exhibiting high transglycosylation efficiency and capacity of LNT synthesis are highly desirable.

In our previous study, a β-N-acetylhexosaminidase (HaHex74) from Haloferula sp. that displayed high transglycosylation activity has been successfully expressed in Pichia pastoris, and an efficient strategy for the preparation of LNT2 from chitin by enzyme cocktails was developed (Liu et al., 2020). Given the significant physiological role of LNT, herein, a further enzymatic route to synthesize LNT from LNT2 was explored. Herein, we cloned and expressed a novel β-galactosidase (LzBgal35A) from Lactaseibacillus zeae. The biochemical properties of LzBgal35A were studied. Subsequently, the potential application of LzBgal35A in preparing LNT from LNT2 was investigated.

### Materials and Methods

Because no human or animal subjects were used, this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.
BL21 (DE3) by chemical transformation for cultivation (Luria–Bertani medium, 37°C, 220 rpm). When the optical density (at 600 nm) of culture solution reached 0.6 to 0.8, isopropyl-β-D-galactopyranoside was added at a final concentration of 1 mM for protein expression. After induction, the cells were harvested by centrifugation (10,621 × g, 10 min). The crude enzyme was dialyzed and loaded on a Ni-IDA affinity column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM imidazole and 300 mM NaCl at 0.5 mL/min. Then, the proteins were eluted with a 20 to 200 mM linear gradient imidazole dilution by 20 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl at 1.0 mL/min. The fractions with β-galactosidase activity were collected, dialyzed overnight in 20 mM phosphate buffer (pH 7.0), and then concentrated for subsequent analysis. The molecular weight and purity of LzBgal35A were checked using SDS-PAGE.

**Purification of LzBgal35A**

The β-galactosidase activity of LzBgal35A was determined by the oNPG method. The reaction mixture containing 225 µL of oNPG (5 mM) in 50 mM citrate buffer (pH 5.0) and 25 µL of LzBgal35A solution was incubated at 55°C for 10 min. The reaction was terminated by adding 750 µL of 2 M sodium carbonate. The liberated oNP was determined by measuring the absorbance at 410 nm (OD_{410}). One unit of activity was defined as the amount of enzyme required to release 1 µmol oNP per minute under standard conditions (55°C, 10 min).

**Enzyme Activity Assay of LzBgal35A**

The optimal pH of LzBgal35A was determined by measuring the enzyme activity in different buffers (50 mM), including citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 7.0–9.0). The optimal temperature of LzBgal35A was determined by measuring the enzyme activity in the temperature range of 25 to 70°C in 50 mM citrate buffer (pH 4.5). The final concentration of LzBgal35A was 0.3 mg/mL, and enzyme activity was measured using the oNPG method, as mentioned above. The maximal enzyme activity was defined as 100%, and the relative enzyme activities under different pH and temperatures were calculated.

To determine pH stability, LzBgal35A (1.0 mg/mL) was incubated in the above buffers at 40°C for 30 min. For thermostability, LzBgal35A (1.0 mg/mL) was incubated at different temperatures (25–65°C) in 50 mM citrate buffer (pH 4.5) for 30 min. The half-lives of LzBgal35A at different temperatures (55, 60, and 65°C) were determined by incubating the enzyme (1.0 mg/mL) in 50 mM citrate buffer (pH 4.5) for 6 h. The residual enzyme activities were measured using the oNPG assay. The activity of untreated enzyme solution was defined as 100%, and the relative enzyme activities under different pH and temperatures were calculated.

The effects of different metal ions and chemicals on the enzyme activity of LzBgal35A were investigated by measuring the residual enzyme activities after incubation of the enzyme (1.0 mg/mL) in 50 mM citrate buffer (pH 4.5) with 1 mM metal ions (Na+, K+, Mg2+, Ca2+, Mn2+, Co2+, Zn2+, Ni2+, Fe2+, Cu2+, Ag+, Ba2+, Cr3+, and Fe3+) and chemicals (SDS, EDTA, β-mercaptoproethanol, hexadecyl trimethyl ammonium bromide (CTAB), and dithiothreitol) at 40°C for 30 min. The activity of untreated enzyme solution was defined as 100%, and the relative enzyme activities under different metal ions and chemicals were calculated.

**Substrate Specificity of LzBgal35A**

The substrate specificity of LzBgal35A (0.3 mg/mL) was determined by measuring the enzyme activities in 50 mM citrate buffer (pH 4.5) at 55°C using various synthetic substrates (oNPβGal, pNPβGal, pNPβfuc, pNPβGlu, pNPβMan, pNPβXy, pNPβGlcNAc, pNPoGal, pNPoGlu) and lactose. For synthetic substrates, the enzymatic activity was assayed by the oNPG method. For lactose, the enzyme activity was measured by the glucose oxidase-peroxidase method, and the released glucose was determined by the glucose oxidase kit. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of oNP or glucose per minute under the assay conditions.

**Biochemical Characterization of LzBgal35A**

The optimal pH of LzBgal35A was determined by measuring the enzyme activity in various buffers (50 mM), including citrate buffer (pH 3.0–6.0), phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 7.0–9.0). The optimal temperature of LzBgal35A was determined by measuring the enzyme activity in the temperature range of 25 to 70°C in 50 mM citrate buffer (pH 4.5). The final concentration of LzBgal35A was 0.3 mg/mL, and enzyme activity was measured using the oNPG method, as mentioned above. The maximal enzyme activity was defined as 100%, and the relative enzyme activities under different pH and temperatures were calculated.

**Optimization of LNT Synthesized by LzBgal35A**

The enzymatic synthesis of LNT was performed by incubating LzBgal35A with oNPG as the glycosyl donor and LNT2 as the glycosyl acceptor. The LNT2 was prepared according to the method described in our previous study (Liu et al., 2020). The reaction temperatures, reaction time, enzyme dosage, and concentration of LNT2 were optimized in terms of the LNT conversion rate. All reactions were incubated in 50 mM citrate buffer (pH 4.5) for 30 min. The residual enzyme activities were measured using the oNPG assay. The activity of untreated enzyme solution was defined as 100%, and the relative enzyme activities under different pH and temperatures were calculated.
phosphate buffer (pH 8.0). The effects of temperature on the synthesis of LNT were investigated at different temperatures (25–65°C) using 0.1 U/mL LzBgal35A with 20 mM oNPG and 100 mM LNT2 for 4.0 h. The effects of reaction time were determined for different times (0.5–7.0 h) at 55°C using 0.1 U/mL LzBgal35A with 20 mM oNPG and 100 mM LNT2. The effects of enzyme dosage (0.01–2 U/mL) were determined at 55°C using 20 mM oNPG and 100 mM LNT2 for 2.0 h. The effects of LNT2 content (50–700 mM) were investigated at 55°C using 0.2 U/mL LzBgal35A and 20 mM oNPG and 100 mM LNT2 for 2.0 h. The reactions were stopped by incubating at 100°C for 10 min to inactivate the enzyme. The LNT contents of the samples were determined via HPLC.

Separation and Identification of LNT Synthesized by LzBgal35A

The synthesized products were loaded onto an activated carbon column (50 × 1.6 cm) equilibrated with 70% ethanol and deionized water and then eluted linearly with 0 to 30% ethanol at a flow rate of 1 mL/min. The eluents exhibiting high product concentration were collected and concentrated by vacuum in a rotary evaporator at 75°C. The concentrated product was further purified by HPLC, and the corresponding eluent was collected and concentrated and lyophilized for subsequent identification. The molecular weight and structure of the product were determined by electrospray ionization (ESI) MS and nuclear magnetic resonance (NMR) analysis.

Analytical Methods

We performed HPLC analysis on an Agilent 1260 Infinity II (Agilent Technologies) equipped with a refractive index detector. An XBridge BEH Amide column (4.6 × 250 mm, Waters) was used at 45°C. The mobile phase was acetonitrile-water solution (72:28, vol/vol) at a flow rate of 0.6 mL/min. The LNT conversion rate (%) was calculated as follows: LNT conversion rate (%) = LNT synthesized (mM)/oNPG (mM) × 100%. For thin-layer chromatography analysis, the silica gel plate loaded with the samples was developed in a solvent containing butanol/acetic acid/water (2:2:1, vol/vol/vol), visualized by dipping into sulfuric acid/methanol (95:5, vol/vol) reagent and heating at 130°C for a few seconds. We performed ESI-MS analysis on an ultrafleXtreme MALDI-TOF mass spectrometer. The 13C spectrum was analyzed using an Agilent DD2 500 MHz NMR spectrometer. The sample (10 mg) was dissolved in 0.5 mL of D2O, and 4,4-dimethyl-4-silipentane-1-sulfonic acid (DSS, 5 mM) was added as an internal standard.

Data Processing

Data statistics were carried out by Origin 8.0 (Origin-Lab) with 3 replicates.

RESULTS

Sequence Analysis of LzBgal35A

The β-galactosidase gene (LzBgal35A, GenBank no. WP_138117930.1) is 1,797 bp and encodes a protein with 598 amino acids. The predicted molecular weight and isoelectric point of LzBgal35A were 68.0 kDa and 5.08, respectively. There was a Glyco_hydro_35 domain (Asp9-His327) in LzBgal35A. LzBgal35A showed the highest identity of 59.9% with the GH35 β-galactosidase (BgaC) from Streptococcus pneumoniae (GenBank, CAP80500.1) in the CAZy database (http://www.cazy.org/), suggesting that LzBgal35A should be a novel GH 35 member. Multiple sequence alignment (Figure 1) showed that 3 aromatic residues (viz. Trp240, Trp243, and Tyr458) in LzBgal35A were highly conserved. These were previously demonstrated to be crucial for both substrate specificity and hydrolysis activity (Cheng et al., 2012). Moreover, the important catalytic residues such as Tyr 52, Ile95, Cys96, Ala97, Glu98, Asn155, Glu156, Glu238, Tyr275, and Tyr305 were also conserved.

Expression and Purification of LzBgal35A

The β-galactosidase gene (LzBgal35A) was successfully expressed in E. coli (Figure 2). LzBgal35A was purified to electrophoretic homogeneity with a recovery yield of 73.8% and a purification fold of 3.3 (Table 1). Our SDS-PAGE analysis showed a single and clear band with a molecular mass of about 64.1 kDa (Figure 2).

Biochemical Characterization of LzBgal35A

LzBgal35A was optimally active at pH 4.5 in 50 mM citrate buffer (Figure 3A) and exhibited good stability within a pH range of 3.5 to 7.0 (Figure 3B). The optimal temperature of LzBgal35A was determined to be 55°C (Figure 3C). As shown in Figure 3D, LzBgal35A retained more than 80% of its initial activity after incubation at 60°C for 30 min. Under a high temperature (65°C), LzBgal35A was not stable, due to the residual enzyme activity remaining only 6.4% after 30-min incubation. The thermal denaturing half-lives of LzBgal35A at 55, 60, and 65°C were determined to be 754, 223, and 3 min, respectively (Figure 3E). The effects of metal ions and chemicals on the activities of LzBgal35A are shown in Table 2. Most metal ions and chemicals slightly
affected the enzyme activity of LzBgal35A. LzBgal35A was obviously activated by Fe$^{2+}$ (25.3%) and hexadecyl trimethyl ammonium bromide (29.8%). Additionally, LzBgal35A was strongly inhibited by Ag$^{+}$, with only 19% activity remaining after 30-min incubation.

Substrate Specificity of LzBgal35A

The hydrolysis activities of LzBgal35A toward different substrates are shown in Table 3. LzBgal35A showed the highest specific activity toward oNPG (16.6 U/mg), followed by pNPG (7.2 U/mg) and pNPFuc (0.67 U/mg), but no hydrolysis activity toward other artificial substrates. In addition, LzBgal35A showed low activity toward lactose (0.32 U/mg).

High-Level Synthesis of LNT by LzBgal35A

The conversion rates of LNT at different reaction conditions are shown in Figure 4. The optimal temperature was determined to be 55°C. Under the optimal temperature, the conversion rate of LNT reached the
maximum within 2 h. Furthermore, the conversion rate of LNT increased gradually with the increase of the enzyme dosage, and the maximal LNT conversion rate was obtained at 0.2 U/mL. An increase in LNT2/oNPG ratio from 2.5 to 15 dramatically increased the conversion rate of LNT from 8.5% to 45.4%. In summary, the optimal conditions for LNT synthesis were 20 mM oNPG and 300 mM LNT2, with an enzyme dosage of 0.2 U/mL at 55°C for 2 h. The maximum conversion rate of LNT under the optimal conditions was 45.4% with a yield of 6.4 g/L.

Furthermore, the synthesized product was identified. The positive-ion ESI mass spectrum of purified product showed a peak of [M+H]+ and [M+Na]+ ions at m/z 708.1 and 730.0, respectively (Figure 5A), which was consistent with the molecular weight of LNT (707.6). Also, the 13C NMR spectra of LNT was identical to that of other studies (Figure 5B; McArthur et al., 2019).

DISCUSSION

Lacto-N-tetraose has gained much attention due to its applications in food industry, especially in the field of infant formula. β-Galactosidase is one of the most important enzymes used in dairy processing. Most studies of β-galactosidases have been focused on hydrolysis of lactose or synthesis of galactooligosaccharides, with little attention paid to LNT synthesis. In this study, a novel β-galactosidase (LzBgal35A) from Lacticaseibacillus zeae was expressed, biochemically characterized, and applied to synthesize LNT in vitro.

β-Galactosidases from different organisms show varying properties. The majority of bacterial β-galactosidases have a molecular mass of more than 100 kD. However, the molecular weight of LzBgal35A was 64.1 kDa (Figure 1), which is different from those of GH 35 β-galactosidases from Akkermansia muciniphila (74 kDa; Guo et al., 2018) and Arthrobacter sp. (52 kDa; Gutshall et al., 1997). LzBgal35A displayed maximum activity at pH 4.5 (Figure 3A), which is same as β-galactosidase from Aspergillus oryzae (pH 4.5; Yin et al., 2017) but higher than that of β-galactosidase from Guehomyces pullulans 17-1 (pH 4.0; Song et al., 2010). However, the optimal pH of LzBgal35A is much lower than those of β-galactosidases from Alteromonas sp. (pH 8.0; Yao et al., 2019), Halomonas sp. S62 (pH 7.5; Wang et al., 2013), and Lactobacillus kefiranofaciens ZW3 (pH 7.0; He et al., 2016). LzBgal35A exhibited good stabilities within a pH range of 3.5 to 7.0 (Figure 3B), which

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Purification factor (fold)</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>396.5</td>
<td>78.0</td>
<td>5.1</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>292.8</td>
<td>17.6</td>
<td>16.6</td>
<td>3.3</td>
<td>73.8</td>
</tr>
</tbody>
</table>

Ni-NTA = nickel nitrilotriacetic acid.
is wider than some other bacterial β-galactosidases, such as those from Alteromonas sp. ML52 (pH 7.0–8.5; Sun et al., 2018), Lactobacillus helveticus DSM 20075 (pH 5.5–8.0; Kittibunchakul et al., 2019), and Pyrococcus furiosus DSM 3638 (pH 4.0–6.5; Park et al., 2011). So far, some bacterial β-galactosidases, such as β-galactosidases from Alteromonas sp. ML117 (30°C; Yao et al., 2019) and Alteromonas sp. ML52 (35°C; Sun et al., 2018), are defined as mesophilic enzymes, and their optimal temperatures are less than 50°C. However, the optimal temperature of LzBgal35A was determined to be 55°C (Figure 3C), which is the same as that of β-galactosidase from Lactobacillus helveticus DSM 20075 (55°C; Kittibunchakul et al., 2019) and much

**Figure 3.** Biochemical properties of the purified novel β-galactosidase from Lacticaseibacillus zeae (LzBgal35A). (A) Optimal pH, (B) pH stability, (C) optimal temperature, (D) temperature stability, (E) thermal denaturing half-lives. Symbols for optimal pH (A) and pH stability (B): (■) citrate buffer (pH 3.0–6.0), (●) phosphate buffer (pH 6.0–8.0), (▲) Tris-HCl buffer (pH 7.0–9.0). Symbols for thermal inactivation (E): (■) 55°C, (●) 60°C, (▲) 65°C. All experiments were performed with 3 replicates. Error bars indicate SD.
lower than those of β-galactosidases from *Alicyclobacillus vulcanalis* (70°C; Park et al., 2011) and *Pyrococcus furiosus* DSM 3638 (102–105°C; Murphy et al., 2020).

The β-galactosidases from *Lactobacillus* species have been previously reported to synthesize lacto-N-biose and galacto-N-biose (Bidart et al., 2017). In the present study, we found that LzBgal35A could not use lactose as the glycosylated donor to synthesize LNnT, but instead utilized oNPG as the glycosylated donor to synthesize LNT. This may be related to the structure-based catalytic mechanism of β-galactosidase. The structural insights into the substrate specificity of β-galactosidase BgaC, a GH-35 β-galactosidase of specific activity toward β(1,3)-linked galactosyl bonds, were explored (Cheng et al., 2012). Through simulation of a putative substrate entrance tunnel and modeling of a complex structure with Galβ(1,3)NAG, 3 aromatic residues (viz. Trp-240, Trp-243, and Tyr-458) determined the substrate specificity of BgaC. This may be appropriate for LzBgal35A, because LzBgal35A shared 60.64% of structural identities with BgaC (PDB: 4e8d.1.A). Furthermore, Trp-240, Trp-243, and Tyr-458 in LzBgal35A were also highly conserved.

A potential disadvantage of β-galactosidases is that β-galactosidase-catalyzed transglycosylation takes place in competition with hydrolysis, which may reduce the yield of the target product (Karimi Alavijeh et al., 2021). To overcome this obstacle, some strategies have been used to alleviate the hydrolysis reaction and improve transglycosylation activity (Abdul Manas et al., 2018). In this study, the yields of LNT were optimized through controlling the reaction conditions, including temperature, time, enzyme concentration, and acceptor concentration (Figure 4). The optimal temperature for synthesis of LNT was relatively high (55°C), which may improve the solubility and binding of oNPG.

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**Figure 4.** Effects of reaction temperature (A), reaction time (B), enzyme dosage (C), and acceptor (lacto-N-triose II, LNT2) concentration (D) on the conversion rate of lacto-N-tetraose (LNT) synthesized by a novel β-galactosidase from *Lacticaseibacillus zeae* (LzBgal35A). All reactions were incubated in 50 mM phosphate buffer (pH 8.0). The effects of temperature on the synthesis of LNT were investigated at different temperatures (25–65°C) using 0.1 U/mL LzBgal35A with 20 mM o-nitrophenyl-β-galactopyranoside (oNPG) and 100 mM LNT2 for 4.0 h. The effects of reaction time were determined for different times (0.5–7.0 h) at 55°C using 0.1 U/mL LzBgal35A with 20 mM oNPG and 100 mM LNT2. The effects of enzyme dosage (0.01–2 U/mL) were determined at 55°C using 20 mM oNPG and 100 mM LNT2 for 2.0 h. The effects of LNT2 content (50–700 mM) were investigated at 55°C using 0.2 U/mL LzBgal35A and 20 mM oNPG for 2.0 h. Error bars indicate SD.
Figure 5. Identification of the transglycosylation product, lacto-N-tetraose (LNT). (A) MS spectrum of the purified product; (B) $^{13}$C spectrum of the purified product.
et al., 2010). Furthermore, we speculate that excessive concentration of LNT2 induced more nucleophilic attacks and improved the transglycosylation reaction of LzBgal35A (Zeuner et al., 2014).

In the only report on glycoside hydrolases-catalyzed synthesis of LNT, a mixtures of LNT and its β(1,6) isomer were synthesized by β-galactosidase from Bacillus circulans ATCC31382, with a conversion rate of 20% using oNPG as the glycosyl donor and LNT2 as the glycosyl acceptor (Murata et al., 1999). To improve the

Table 3. Substrate specificity (± SD) of the novel recombinant β-galactosidase from Lacticaseibacillus zeae (LzBgal35A)1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U/mg)</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>β-oNPGal</td>
<td>16.60 ± 0.17</td>
<td>100.0</td>
</tr>
<tr>
<td>β-pNPGal</td>
<td>16.20 ± 0.48</td>
<td>97.2</td>
</tr>
<tr>
<td>β-MG</td>
<td>14.49 ± 0.97</td>
<td>86.9</td>
</tr>
<tr>
<td>β-Mn</td>
<td>15.53 ± 0.20</td>
<td>93.2</td>
</tr>
<tr>
<td>Ba</td>
<td>16.77 ± 0.02</td>
<td>100.6</td>
</tr>
<tr>
<td>Na+</td>
<td>14.74 ± 0.61</td>
<td>88.4</td>
</tr>
<tr>
<td>K+</td>
<td>15.60 ± 0.61</td>
<td>93.5</td>
</tr>
<tr>
<td>Cu2+</td>
<td>16.11 ± 0.30</td>
<td>96.7</td>
</tr>
<tr>
<td>Ar2+</td>
<td>3.05 ± 0.07</td>
<td>18.3</td>
</tr>
<tr>
<td>Cr3+</td>
<td>14.32 ± 0.24</td>
<td>85.9</td>
</tr>
<tr>
<td>Zn2+</td>
<td>16.58 ± 0.02</td>
<td>99.5</td>
</tr>
<tr>
<td>Co2+</td>
<td>14.46 ± 0.44</td>
<td>86.7</td>
</tr>
<tr>
<td>Fe3+</td>
<td>20.89 ± 1.14</td>
<td>125.3</td>
</tr>
<tr>
<td>Fe2+</td>
<td>16.38 ± 0.18</td>
<td>98.3</td>
</tr>
<tr>
<td>Cu2+</td>
<td>14.55 ± 0.04</td>
<td>87.3</td>
</tr>
<tr>
<td>SDS</td>
<td>12.76 ± 0.42</td>
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<td>EDTA</td>
<td>15.31 ± 0.07</td>
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<tr>
<td>β-ME</td>
<td>16.68 ± 0.07</td>
<td>100.1</td>
</tr>
<tr>
<td>CTAB</td>
<td>21.63 ± 1.23</td>
<td>129.8</td>
</tr>
<tr>
<td>DTT</td>
<td>15.78 ± 0.15</td>
<td>94.7</td>
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</table>

1β-ME = β-mercaptoethanol; CTAB = hexadecyl trimethyl ammonium bromide; DTT = dithiothreitol.

Table 4. Comparison of biosynthesis of lacto-N-tetraose (LNT) (LzBgal35A)1

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<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Substrate</th>
<th>Reaction conditions</th>
<th>Conversion rate (%)1</th>
<th>LNT yield (mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Galactosidase</td>
<td>Bacillus circulans ATCC31382</td>
<td>oNPG</td>
<td>0.25 U, 50°C, pH 5.2; 8 h</td>
<td>99</td>
<td>16.25</td>
<td>Murata et al., 1999</td>
</tr>
<tr>
<td>Lacticaseibacillus zeae</td>
<td>oNPG</td>
<td>LNT2</td>
<td>0.2 U, 50°C, pH 4.5; 2 h</td>
<td>45.4</td>
<td>6.4</td>
<td>This study</td>
</tr>
<tr>
<td>Lacto-N-biosidase</td>
<td>Aureobacterium sp. L-101</td>
<td>LNB-pNP</td>
<td>20 U, 40°C, 5 h</td>
<td>3.7</td>
<td>0.47</td>
<td>Murata et al., 1999</td>
</tr>
<tr>
<td>Bifidobacterium bifidum</td>
<td>Lactose</td>
<td>LNB-oxa</td>
<td>—</td>
<td>30</td>
<td>—</td>
<td>Schmölzer et al., 2019</td>
</tr>
<tr>
<td>β-1,3-Galactosyltransferase (in vitro)</td>
<td>Chromobacterium violaceum</td>
<td>UDP-Gal</td>
<td>pH 8.0, 37°C, 30 h; 100 rpm</td>
<td>99</td>
<td>130</td>
<td>McArthur et al., 2019</td>
</tr>
<tr>
<td>β-1,3-Galactosyltransferase (in vivo)</td>
<td>Escherichia coli O55:H7</td>
<td>UDP-Gal</td>
<td>LNT2; 24 h</td>
<td>0.2</td>
<td>3</td>
<td>150</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>0.8</td>
<td>3</td>
<td>12.74</td>
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<td></td>
<td></td>
<td>40</td>
<td>3</td>
</tr>
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<td>77.75</td>
<td>76.470</td>
</tr>
</tbody>
</table>

1Conversion rate (%) was calculated as follows: LNT conversion ratio (%) = LNT synthesized (mM)/oNPG (mM) × 100%.

2Not available.

3Shake-flask fermentation.

4Fed-batch fermentation.

oNPG = o-nitrophenyl β-D-galactopyranoside; UDP-Gal = UDP-galactose; pNP = p-nitrophenol; LNT2 = lacto-N-triose II; LNB = lacto-N-biose; oxa: 1,2-oxazoline.
purity and yield of LNT, a β-galactosidase from \textit{E. coli}, which has a high specificity for Galβ-1-6-linkages, was used to hydrolyze the regio-isomer (Gal3[1, 6GlcNAc31, 3Gal31, 4Glc]) in the presence of a co-solvent (10% DMF), and the conversion rate of LNT reached up to 22% (Miyazaki et al., 2010). LzBgal35A displayed a maximal conversion rate of 45.4% (Table 4), which is the highest conversion rate among β-galactosidase-mediated synthesis of LNT so far (Murata et al., 1999; Miyazaki et al., 2010). Although β-1,3-glycosyltransferases-mediated synthesis of LNT in vivo or in vitro showed higher conversion rates of LNT (Table 4), enzymatic synthesis of LNT using LzBgal35A has some advantages, such as short operation time (only 2 h) and easy isolation of LNT. Thus, LzBgal35A has great application potential in enzymatic synthesis of LNT. Rational design of LzBgal35A to modify its substrate selectivity and further excavation of new β-galactosidases with high activity toward natural substrates are the main priorities for future study.

CONCLUSIONS

A novel β-galactosidase (LzBgal35A) from \textit{Lactocaseibacillus zea} was expressed, biochemically characterized, and used to synthesize LNT in vitro. LzBgal35A showed optimal activity at pH 4.5 and 55°C. LzBgal35A could efficiently catalyze the synthesis of LNT from αNPG and LNT2. Under optimal conditions, the conversion rate of LNT reached 45.4% (6.4 g/L) within 2 h. LzBgal35A may be an ideal enzyme for efficient synthesis of LNT in vitro.

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