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Thermostable β-galactosidases for the synthesis of human milk oligosaccharides

Research Paper

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Human milk oligosaccharides (HMOs) designate a unique family of bioactive lactose-based molecules present in human breast milk. Using lactose as a cheap donor, some β-galactosidases (EC 3.2.1.23) can catalyze transgalactosylation to form the human milk oligosaccharide lacto-*N*-neotetraose (LNnT; Gal-β(1,4)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc). In order to reduce reaction times and be able to work at temperatures, which are less welcoming to microbial growth, the current study investigates the possibility of using thermostable β-galactosidases for synthesis of LNnT and *N*-acetyllactosamine (LacNAc; Gal-β(1,4)-GlcNAc), the latter being a core structure in HMOs. Two hyperthermostable GH 1 β-galactosidases, Ttβ-gly from *Thermus thermophilus* HB27 and CelB from *Pyrococcus furiosus*, were codon-optimized for expression in *Escherichia coli* along with BgaD-D, a truncated version of the GH 42 β-galactosidase from *Bacillus circulans* showing high transgalactosylation activity at low substrate concentrations. The three β-galactosidases were compared in the current study in terms of their transgalactosylation activity in the formation of LacNAc and LNnT. In all cases, BgaD-D was the most potent transgalactosidase, but both thermostable GH 1 β-galactosidases could catalyze formation of LNnT and LacNAc, with Ttβ-gly giving higher yields than CelB. The thermal stability of the three β-galactosidases was elucidated and the results were used to optimize the reaction efficiency in the formation of LacNAc, resulting in 5–6 times higher reaction yields and significantly shorter reaction times.

Introduction

Human milk oligosaccharides (HMOs), which are abundant in human breast milk (present at levels up to 8 g/L) are known to be of major importance for infant health and development [1]. The HMOs share similar structural features: They are all based on a reducing end lactose molecule, which can be substituted with sialic acid or fucose and elongated with *N*-acetylglucosamine (GlcNAc) and galactose (Gal) units; these elongated molecules may in turn also be substituted with sialyl or fucosyl residues [1,2]. Important elongated HMO core structures include lacto-*N*-tetraose (LNT; Gal-β(1,3)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc) and lacto-*N*-neotetraose (LNnT; Gal-β(1,4)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc) [2]. Approaches to synthesize LNT and LNnT include chemical synthesis [3–6], *in vivo* production in *Escherichia coli* [7], the use of glycosyltransferases requiring nucleotide sugars [8], as well as the use of glycosidases with transglycosylation activity [9].

More than a decade ago, it was shown that lacto-*N*-triose II (LNT2; GlcNAc-β(1,3)-Gal-β(1,4)-Glc) could be elongated to LNnT by transgalactosylation catalyzed by the GH 42 β-galactosidase from *Bacillus circulans* using lactose as donor substrate [9]. Using the GH 35 β-galactosidase from *Bacillus circulans* and *p*-nitrophenyl β-galactopyranoside (*p*NP-β-Gal) as donor, LNT (and LNT isomers, most likely LNnT) was also reported to be formed from

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LNT2, but the reaction was not performed with lactose as donor substrate [9]. Similarly, the use of lacto-*N*-biosidase (EC 3.2.1.140) from *Aureobacterium* sp. L-101 which could catalyze the transfer of the lacto-*N*-biose disaccharide to lactose to form LNT also required *p*NP activation of the donor substrate [9].

In the quest for finding routes to HMO production which involve cheap substrates only, we have recently shown that LNT2 can be produced from chitin degradation products and lactose by two novel β-*N*-acetylhexosaminidases at molar yields of 2–8% [10]. Previously, LNT2 synthesis has relied on nucleotide, methyl, or *p*NP-activated donors or chemical synthesis [9,11], all keeping the price of LNT2 too high for a feasible large-scale production. With the possibility of using chitin degradation products as donor substrates and achieving appreciable yields, the β-galactosidase-catalyzed route to production of LNnT with lactose as a cheap donor substrate is becoming increasingly interesting from an economic point of view.

In the only published report on β-galactosidase-catalyzed formation of LNnT from lactose and LNT2, Murata and co-workers [9] reached a molar yield based on LNT2 of 19% by performing the reaction for 36 hours at 40°C. Such reaction conditions are however problematic with regard to microbial safety. In order to speed up the reaction and be able to work at a temperature, which is less welcoming to microbial growth, the current study investigates the possibility of using thermostable β-galactosidases for LNnT synthesis. A β-glycosidase from *Thermus thermophilus* HB27, known as Ttβ-gly or TTP0042, has high thermal stability [12,13] and has been shown to catalyze the transgalactosylation of GlcNAc to form *N*-acetyllactosamine (Gal-β(1,4)-GlcNAc; LacNAc) using *p*NP-β-Gal as donor [13]. β-Glycosidase from *Pyrococcus furiosus*, known as CelB, has even higher thermal stability than Ttβ-gly [14]. This β-galactosidase has not previously been used for transgalactosylation of GlcNAc moieties, but has been shown to efficiently catalyze the formation of galactooligosaccharides at 95°C [15]. Thus, the current work was also undertaken with the objective of comparing these two thermostable β-galactosidases to the benchmark enzyme used for synthesis of LNnT as well as for LacNAc, the GH42 β-galactosidase from *Bacillus circulans* which is found in the popular commercial Biolacta® preparation [9,16]. The Biolacta® preparation contains several different isozymes, but the one used in the current study (BgaD-D) is the one having the highest transgalactosylation activity at low substrate concentrations [17,18]. Ttβ-gly, CelB, and BgaD-D (Table 1) were codon-optimized for expression in *E. coli*, His_6-tagged for purification purposes, and compared in the current study in terms of their transgalactosylation activity in the formation of LacNAc and LNnT at elevated temperatures (Fig. 1). In addition, a sequential reaction with β-*N*-acetylhexosaminidase and β-galactosidase to form LNnT from lactose and the chitin degradation product *N,N′*-diacetylchitobiose ($(GlcNAc)_2$; GlcNAc-β(1,4)-GlcNAc) was tested (Fig. 2).

Materials and methods

Chemicals

Lacto-*N*-triose II (GlcNAc-β(1,3)-Gal-β(1,4)-Glc; LNT2), lacto-*N*-biose (Gal-β(1,3)-GlcNAc), lacto-*N*-tetraose (Gal-β(1,3)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc; LNT), lacto-*N*-neotetraose (Gal-β(1,4)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc; LNnT), and *N,N′*-diacetylchitobiose (GlcNAc-β(1,4)-GlcNAc; $(GlcNAc)_2$) were purchased from Carbosynth Ltd. (Compton, United Kingdom). For analytical confirmation, LNT and LNnT were also purchased from Elicityl SA (Crolles, France). *N*-Acetylglucosamine (GlcNAc), *N*-acetyllactosamine (Gal-β(1,4)-GlcNAc; LacNAc), allo-*N*-acetyllactosamine (Gal-β(1,6)-GlcNAc; allo-LacNAc), *p*-nitrophenyl β-galactopyranoside (*p*NP-β-Gal), β-lactose, and all other chemicals were purchased from Sigma–Aldrich (Steinheim, Germany).

Cloning, expression, and purification of β-galactosidases

Genes encoding the three β-galactosidases BgaD-D, Ttβ-gly, and CelB, each possessing an N-terminal His_6-tag linked *via* a thrombin recognition site, were codon-optimized for expression in *Escherichia coli* (*E. coli*), synthesized and inserted into the vector pJ411 by DNA2.0 (Menlo Park, CA, USA). *E. coli* BL21 (DE3) was transformed with the resulting plasmids and selected for kanamycin resistance.

BgaD-D was expressed as follows: transformants were grown in lysogenic broth (LB) supplemented with 50 μg/ml kanamycin at 37°C. When reaching an OD_{600} of 0.6, the temperature was reduced to 25°C and expression induced by the addition of IPTG to a final concentration of 1 mM. Cells were grown for 15 hours post induction before harvest. Ttβ-gly and CelB were expressed in a similar manner, except that the cells were allowed to grow for 5 hours after induction before being harvested.

Cells were centrifuged and the pellets resuspended in binding buffer (BgaD-D: 20 mM Na-phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7; Ttβ-gly and CelB: 40 mM EPPS buffer, 500 mM NaCl, 24 mM imidazole), followed by sonication to open the cells and centrifugation to remove cell debris. The supernatant containing CelB or Ttβ-gly was pre-purified by incubation for 20 min at 80°C resulting in precipitation of non-thermostable proteins, followed by another centrifugation step.

The supernatant containing the β-galactosidases was passed through a 0.45 μm filter before being loaded onto a 1 ml Ni^{2+}-sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden),

TABLE 1

An overview of the β-galactosidases used in the current work. All three β-galactosidases have an N-terminal His_6-tag and were codon-optimized for expression in *E. coli*.

Trivial name	Organism	Strain	GH family	UniProt entry[a]	Reference
BgaD-D	*Bacillus circulans*	ATCC 31382	GH 42	E5RWQ2 truncated[b]	[18]
Ttβ-gly (TTP0042)	*Thermus thermophilus*	HB27	GH 1	Q746L1/Q9RA61	[12,13]
CelB	*Pyrococcus furiosus*	DSM 3638	GH 1	Q51723	[23]

[a] In addition to the amino acid sequence given in this entry, the enzymes used in the current work have an N-terminal His_6-tag followed by a thrombin cleavage site.
[b] BgaD-D corresponds to amino acids 36-847 in this sequence [18].