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Loop engineering of an α-1,3/4-L-fucosidase for improved synthesis of human milk oligosaccharides

Birgitte Zeuner, Marlene Vuillemin, Jesper Holck, Jan Muschiol, Anne S. Meyer*

Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Building 229, DK-2800 Kgs. Lyngby, Denmark

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ABSTRACT

The α-1,3/4-L-fucosidases (EC 3.2.1.111; GH29) *Bb*AfcB from *Bifidobacterium bifidum* and *Cp*Afc2 from *Clostridium perfringens* can catalyse formation of the human milk oligosaccharide (HMO) lacto-*N*-fucopentaose II (LNFP II) through regioselective transfucosylation of lacto-*N*-tetraose (LNT) with 3-fucosyllactose (3FL) as donor substrate. The current work exploits structural differences between the two enzymes with the aim of engineering *Bb*AfcB into a more efficient transfucosidase and approaches an understanding of structure-function relations of hydrolytic activity vs. transfucosylation activity in GH29. Replacement of a 23 amino acids long α-helical loop close to the active site of *Bb*AfcB with the corresponding 17-aminoacid α-helical loop of *Cp*Afc2 resulted in almost complete abolishment of the hydrolytic activity on 3FL (6000 times lower hydrolytic activity than WT *Bb*AfcB), while the transfucosylation activity was lowered only one order of magnitude. In turn, the loop engineering resulted in an α-1,3/4-L-fucosidase with transfucosylation activity reaching molar yields of LNFP II of 39 ± 2% on 3FL and negligible product hydrolysis. This was almost 3 times higher than the yield obtained with WT *Bb*AfcB (14 ± 0.3%) and comparable to that obtained with *Cp*Afc2 (50 ± 8%). The obtained transfucosylation activity may expand the options for HMO production: mixtures of 3FL and LNT could be enriched with LNFP II, while mixtures of 3FL and lacto-*N*-neotetraose (LNnT) could be enriched with LNFP III.

1. Introduction

Human milk oligosaccharides (HMOs) are soluble complex glycans which are present in human milk at concentrations of 5–15 g/L, often exceeding the concentration of protein thus being the third most abundant component in human milk. In the human colostrum, concentrations are even higher [1]. In contrast, HMOs are virtually absent from bovine milk, which is the basis of infant formula. Approx. 1% of the HMOs are adsorbed, while the majority is either metabolized by the gut microbiota of the infant or excreted in the faeces [2,3]. HMOs function as prebiotics and antimicrobial agents in the gut of breastfed infants, and they further protect the infant against pathogens by functioning as soluble decoy receptors for pathogen adhesion as well as through a number of immunomodulating effects. HMOs may also be involved in infant brain development [1]. No single HMO has all these effects at once, suggesting why as many as 200 different HMO structures have been identified in human milk [4,5].

Five monosaccharides, i.e. galactose (Gal), glucose (Glc), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (Sia) derivative *N*-acetyl-neuraminic acid, form the HMO building blocks. All HMO molecules have lactose (Gal-β1,4-Glc) at the reducing end can be elongated in β1,3- or β1,6-linkages by two different disaccharide moieties, namely Gal-β1,3-GlcNAc (type 1) or Gal-β1,4-GlcNAc (type 2). The HMO backbone can be further modified with Sia and/or Fuc substitutions [1,5]. Among them, the fucosylated species are the most abundant − at least in breastmilk of approx. 80% of the population since the degree and type of HMO fucosylation are linked to the secretor and Lewis blood status group of the mother [6].

Several routes to production of HMOs *in vitro* exist, one of them being the use of glycosidases catalysing transglycosylation [7–9]. Advantages of glycosidases include easy expression, robustness, and the option of using inexpensive naturally occurring donor substrates. Indeed, the α-1,3/4-L-fucosidase *Bi*AfcB from *Bifidobacterium longum* subsp. *infantis* (EC 3.2.1.111) was recently shown to catalyse transfucosylation of the HMO core structure lacto-*N*-tetraose (LNT) with another HMO, namely 3-fucosyllactose (3FL), as fucosyl donor leading to formation of lacto-*N*-fucopentaose II (LNFP II) [10] (Fig. 1). For this WT enzyme, transfucosylation yields on 3FL ranged from 6% to 12%

Abbreviations: 2'FL, 2'-fucosyllactose; 3FL, 3-fucosyllactose; A:D, acceptor-to-donor ratio; *Bb*AfcB, α-1,3/4-L-fucosidase from *Bifidobacterium bifidum*; *Bi*AfcB, α-1,3/4-L-fucosidase from *Bifidobacterium longum* subsp. *infantis*; *Cp*Afc2, α-1,3/4-L-fucosidase from *Clostridium perfringens*; Fuc, fucose; GH, glycoside hydrolase; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HMO, human milk oligosaccharide; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNFP, lacto-*N*-fucopentaose; Sia, sialic acid; WT, wild type

* Corresponding author.
E-mail address: am@kt.dtu.dk (A.S. Meyer).

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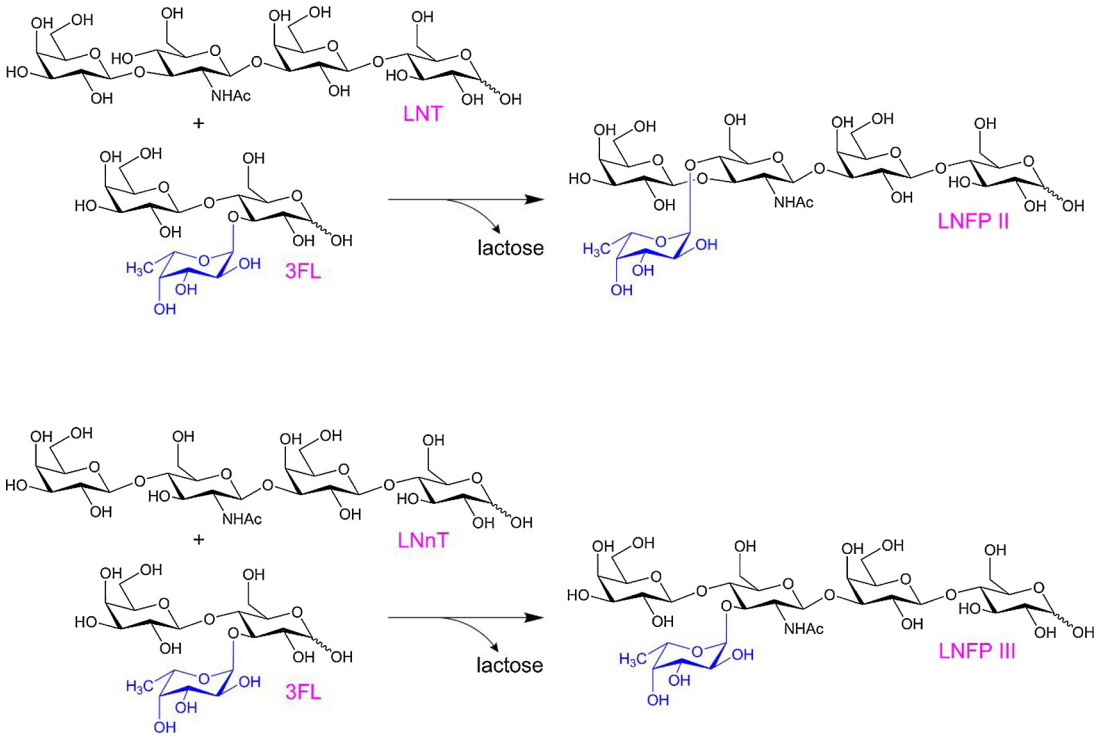

Fig. 1. Transfucosylation reactions catalysed by α-L-1,3/4-fucosidases. Top: Formation of lacto-*N*-fucopentaose II (LNFP II) from 3-fucosyllactose (3FL) and lacto-*N*-tetraose (LNT). Bottom: Formation of LNFP III from 3FL and lacto-*N*-neotetraose (LNnT). The fucosyl moieties are shown in blue.

depending on the substrate concentration, but through elaborate engineering of *Bi*AfcB mutants transfucosylation yields on 3FL of up to 60% were achieved [10,11]. We recently studied two WT α-L-1,3/4-fucosidases (EC 3.2.1.111) – *Bb*AfcB from *Bifidobacterium bifidum* and *Cp*Afc2 from *Clostridium perfringens:* In terms of their transfucosylation potential *Cp*Afc2 was superior to the *Bb*AfcB producing molar transfucosylation yields of up to 39% (on the donor 3FL) in production of LNFP II [12]. These α-L-fucosidases belong to the glycoside hydrolase family 29 subfamily B (GH29B) which comprises retaining α-L-fucosidases with high specificity for branched α-1,3/4-fucosylations [13]. In turn, GH29B α-1,3/4-L-fucosidases have also been shown to possess high regioselectivity in transfucosylation [10,12].

When comparing the transfucosylation and hydrolytic activities of *Bb*AfcB and *Cp*Afc2 it was evident that *Cp*Afc2 had markedly higher transfucosylation activity than *Bb*AfcB using 3FL and LNT for formation of LNFP II, and that BbAfcB in turn had significantly higher hydrolytic activity on 3FL than *Cp*Afc2 [12]. Unfortunately, *C. perfringens* is a potential pathogen, so the use of an enzyme derived from this organisms for production of HMOs for infant formulae may be controversial. In contrast, *B. bifidum* is considered a beneficial probiotic microbe. Consequently, the current work set out to exploit any structural differences between the two GH29B α-1,3/4-L-fucosidases with the aim of engineering of *Bb*AfcB for improving its transfucosylation activity and/or reducing its hydrolytic activity. Comparison of homology models of *Bb*AfcB and *Cp*Afc2 with the crystal structure of the well-studied *Bi*AfcB, reveal that *Bb*AfcB and *Cp*Afc2 appear similar in terms of active site structure and substrate-interacting residues. However, the models of the active site region suggest that an α-helical loop on the side of the active site constitutes a major difference between *Bb*AfcB and *Cp*Afc2. Since the transsialylation yield of a sialidase from *Trypanosoma rangeli* could be significantly improved by replacing a loop near the active site with the corresponding loop of a native transsialidase from *T. cruzi* [14], a similar approach was pursued here: The identified α-helical loop in *Bb*AfcB was replaced with that of *Cp*Afc2, hypothesizing that the shape of the loop in *Cp*Afc2 provide better shielding of the active site from the aqueous environment and that this feature could be transferred to *Bb*AfcB by loop engineering.

2. Materials & methods

2.1. Chemicals

The fucosylated oligosaccharides 3-fucosyllactose (3FL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), and lacto-*N*-fucopentaose V (LNFP V) were purchased from Elicityl Oligotech (Crolles, France). All other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Homology modelling

Structure homology models were prepared using the *Homology Modeling* function of YASARA Structure, version 16.9.23 (YASARA Biosciences GmbH, Vienna, Austria) [15]. The program automatically identified the following template structures in structural databases: 4ZRX, 3UES, 3MO4, 2BER, 2 × 0Y for *Bb*AfcB and 4ZRX, 5K9H, 3EYP, 3RB5, 2OZN for *Cp*Afc2. For each of the templates up to 5 homology models were prepared by the program and ranked automatically. Based on the ranking a hybrid model was prepared by the program, and the model was equilibrated by molecular dynamics simulation using the YASARA macro md_refine without changes [16]. The refined models with the lowest energy were quality checked using QMEAN4 [17] and subsequently used for comparison with the *Bi*AfcB structure 3UET. All figures showing 3D structures were prepared using PyMol (The PyMOL Molecular Graphics System, Version 1.1 Schrödinger, LLC). Sequence alignments were made with MUSCLE 3.8 [18].

2.3. Cloning, expression, and purification of α-L-fucosidases

The sequence of α-L-1,3/4-fucosidase *Bb*AfcB from *Bifidobacterium bifidum* JCM 1254 (GenBank BAH80310.1; EC 3.2.1.111) was slightly truncated and codon-optimized for expression in *Escherichia coli* previously [12]. The resulting pET22b+/*bbafcb* plasmid was used as a template for deletion of the encoded loop 759-AAYNDGVDKVSLKPG-QMAPDGKL-781 and introduction of the new loop