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Published in:

Zeitschrift fuer Naturforschung. Section C: A Journal of Biosciences

Link to article, DOI:

[10.1515/znc-2018-0149](https://doi.org/10.1515/znc-2018-0149)

Publication date:

2019

Document Version

Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):

Muschiol, J., & Meyer, A. S. (2019). A chemo-enzymatic approach for the synthesis of human milk oligosaccharide backbone structures. *Zeitschrift fuer Naturforschung. Section C: A Journal of Biosciences*, 74(3-4), 85-89. <https://doi.org/10.1515/znc-2018-0149>

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Rapid Communication

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A chemo-enzymatic approach for the synthesis of human milk oligosaccharide backbone structures

<https://doi.org/10.1515/znc-2018-0149>

Received September 30, 2018; revised November 15, 2018; accepted November 16, 2018

Abstract: The ability of an engineered β -*N*-acetylhexosaminidase to utilize a reactive oxazoline as donor molecule for transglycosylation reaction to synthesize human milk oligosaccharide backbone structures was studied. The human milk oligosaccharide precursor lacto-*N*-triose II and three regioisomers could be synthesized using the oxazoline, which was either in situ-generated resulting in a chemo-enzymatic sequential cascade or was used as a purified compound. The highest observed concentration of overall transglycosylation products in a cascade reaction was 13.7 mM after 18.5 h, whereas the use of purified oxazoline resulted in 25.0 mM of transglycosylation products after 6.5 h. Remarkably, the in situ-generated oxazoline could be used without any further purification and it was shown that the used enzyme tolerated significant amounts of reagents such as triethylamine, which is reported for the first time for an enzyme from the glycoside hydrolase family 20.

Keywords: β -*N*-acetylhexosaminidase; chemo-enzymatic cascade; human milk oligosaccharides; lacto-*N*-triose II; transglycosylation.

1 Introduction

Human milk oligosaccharides (HMOs) are well known for several beneficial effects on infants and their development. For example HMOs have anti-inflammatory and antiadhesive effects within the baby's gut and significantly contribute to the brain's development [1]. However, not every baby can get this precious mother's milk and therefore there is a strong need for high-quality infant

formulations containing HMOs [2]. In previous studies, we already demonstrated the enzymatic synthesis of the simplest HMO trisaccharides (2'- and 3-fucosyllactose as well as 3'- and 6'-sialyllactose) using differently engineered transglycosylases [3–7]. However, because human milk is very complex and also contains significant amounts of longer HMOs (e.g. lacto-*N*-tetraose (LNT)), we wanted to develop synthetic routes toward these molecules as well. For this purpose, two β -*N*-acetylhexosaminidases (HEX1 and HEX2) were discovered in a metagenomic library, which were able to synthesize lacto-*N*-triose II (LNT2) from an unactivated donor molecule (*N,N'*-diacetylchitobiose ((GlcNAc)₂)) and lactose [8]. The two novel enzymes were classified as members of the glycoside hydrolase family 20 (GH20 family) according to the CAZy database (<http://www.cazy.org/>) [9]. Within this family, mainly *exo*-acting activities have been described (e.g. β -*N*-acetylhexosaminidase) catalyzing the hydrolytic cleavage of hexosamines from oligosaccharides. All GH20 enzymes follow a common mechanism for catalysis, which involves neighboring group participation to form the reactive intermediate oxazoline [10, 11]. Attempts to synthesize LNT using these enzymes with galactosidases in a cascade revealed that the wild-type transglycosylation activity of the β -*N*-acetylhexosaminidases was too low to be used in a cascade [12]. To address this issue, we recently described a mutant of HEX1, which had a ninefold higher transglycosylation activity [13]. Because the GH20 mechanism already involves an oxazoline as a reactive intermediate, we wanted to elaborate on the possibility of using the HEX1_{GTEPG} mutant in combination with the well-known one-step aqueous synthesis of GlcNAc oxazoline [14, 15] to synthesize LNT2 enzymatically as depicted in Scheme 1.

2 Materials and methods

2.1 Chemicals

2-Chloro-1,3-dimethylimidazolinium chloride (DMC), 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride

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Scheme 1: Proposed chemo-enzymatic route toward the synthesis of lacto-*N*-triose II.

(CDMBI), 2-methyl-(1,2-dideoxy- α -D-glucopyrano)-[2,1-*d*]-2-oxazoline (GlcNAc oxazoline), and lacto-*N*-triose II (LNT2) were purchased from Carbosynth (Compton, UK). Trisodiumphosphate (Na₃PO₄), *N*-acetylglucosamine, β -lactose, and kanamycin were purchased from Sigma-Aldrich (Steinheim, Germany). Triethylamine $\geq 99.5\%$ (Et₃N) was purchased from Carl Roth (Karlsruhe, Germany).

2.2 Expression and purification of the enzyme

Escherichia coli T7 express lysY harboring the pETM-10-*HEX1*_{GTEPG} plasmid was used for expression. Therefore, a preculture was inoculated into autoinduction [16] medium containing kanamycin (50 μ g/mL) and incubated for 2 h at 37 °C and 180 rpm. Then, the temperature was set to 30 °C and cultivation was continued overnight. The next day, cell harvest was carried out by centrifugation (4400 g, 10 min). The pellets were resuspended in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.8) and subsequently the cells were lysed by ultrasound (0.6 cycle, 100% amplitude on a UP400S, Hielscher Ultrasonics GmbH, Teltow, Germany). The cell debris was removed by centrifugation at 20,000 g for 20 min at 4 °C. The enzyme was purified by immobilized metal affinity chromatography using a HisTrap HP column (GE Healthcare, Brøndby, Denmark) as described previously [13]. Finally, the buffer was exchanged to 10 mM citrate phosphate buffer (pH 8.0) using PD-10 desalting columns (GE Healthcare, Brøndby, Denmark) according to the manufacturer's protocol. Protein concentration was estimated using the BCA assay (Thermo

Fisher Scientific, Roskilde, Denmark) with bovine serum albumin as standard.

2.3 Chemo-enzymatic cascade reactions

Method I: A total of 550 mg of GlcNAc (250 mM) and 3.12 mL of Et₃N (2.25 M) was dissolved in 6.88 mL dH₂O in a 50 mL round-bottomed flask. The resulting solution was cooled to 0 °C–4 °C and agitated with a magnetic stirrer. The reaction was started by the addition of 1.27 g DMC (750 mM) and stirring continued for 15 min. Afterward, the solution was neutralized using HCl (2 M) and the pH was checked to be approximately 8.0 using a pH electrode (SlimTrode, Hamilton, Switzerland). Then, 100 μ L of the neutralized reaction was added to 42.8 mg of β -lactose in a 2 mL tube. The reaction was started by addition of 100 μ L phosphate citrate buffer (10 mM, pH 8.0) and 50 μ L purified enzyme (0.27 mg/mL), so that final concentrations of 100 mM GlcNAc oxazoline and 500 mM β -lactose were reached. The tube was incubated in a thermomixer (Eppendorf AG, Hamburg, Germany) at 25 °C and 950 rpm over 24 h.

Method II: A total of 33.2 mg of GlcNAc (150 mM) and 184 mg of Na₃PO₄ (1.125 M) was dissolved in 0.9 mL dH₂O in a 2 mL tube. The resulting solution was cooled to 0 °C–4 °C and agitated with a magnetic stirrer. To start the reaction, 97.7 mg of CDMBI (450 mM) was added and stirring continued for 2 h at 4 °C. Afterward, the solution was neutralized using HCl (2 M) and the pH was checked as in method I. The precipitated 1,3-dimethyl-1*H*-benzimidazol-3-ium chloride (DMBI) was removed using a centrifugal filter (3K MWCO, VWR, Søborg, Denmark). Then, 166.7 μ L of the neutralized reaction was added to 42.8 mg

of β -lactose in a 2 mL tube. The reaction was started by the addition of 33.3 μ L phosphate citrate buffer (10 mM, pH 8.0) and 50 μ L of purified enzyme (0.27 mg/mL), so that similar final concentrations as described previously were reached. The tube was incubated as described previously.

2.4 Enzymatic transglycosylation using purified GlcNAc oxazoline (benchmark method)

For investigation of the general ability to utilize GlcNAc oxazoline as a substrate by HEX1_{GTEPG}, commercially supplied GlcNAc oxazoline was used in transglycosylation reactions. These were carried out by the addition of 100 μ L GlcNAc oxazoline solution (250 mM in citrate phosphate buffer (10 mM, pH 8.0)) to 42.8 mg of β -lactose in a 2 mL tube. To start the reaction, 100 μ L of citrate phosphate buffer (10 mM, pH 8.0) and 50 μ L of purified enzyme (0.27 mg/mL) was added, so that similar final concentrations as described previously were reached. The tube was incubated as described previously.

All reactions (methods I and II and benchmark method) were done in triplicate. Blanks were prepared as described previously, but instead of purified enzyme, buffer was added. Each reaction was terminated by diluting the samples in 95 °C hot Milli-Q water (1:100). The diluted samples were filtered through a 10 kDa AcroPrep advance 96-well filter plate (Pall, New York, NY, USA). The transglycosylation products were quantified by using high-performance anion exchange chromatography with pulsed amperometric detection as described later by using an LNT2 standard curve in the presence of β -lactose (5 mM). All LNT2 isomers were quantified on the basis of the external LNT2 standard described previously [13].

2.5 High-performance anion exchange chromatography with pulsed amperometric detection

The transglycosylation products were quantified by using an ICS3000 system (Dionex Corp., Sunnyvale, CA, USA) consisting of DP gradient pumps, an ICS5000 electrochemical detector, and a DC chromatography compartment coupled to an AS autosampler (Dionex Corp., Sunnyvale, CA, USA). A total of 10 μ L of the sample was injected. The compounds were separated on a CarboPac PA1 (4 $\times$ 250 mm) analytical column equipped with a

CarboPac PA1 (4 $\times$ 50 mm) guard column (Dionex Corp., Sunnyvale, CA, USA). Elution was performed under isocratic conditions with NaOH (75 mM) in deionized water for 20 min at a flow rate of 1 mL/min. Cleaning of the column was done after each injection with NaOAc (425 mM)/NaOH (75 mM) supplemented with 0.017% NaN₃ for 5 min followed by re-equilibration with NaOH (75 mM) for 5 min. Column and detector temperatures were 30 °C. The following waveforms, that is, pulse potentials and durations, were used for electrochemical detection: 0.1 V for 400 ms (integration for the last 200 ms), ramp to -2.0 V over 10 ms, -2.0 V for 10 ms, ramp to 0.6 V over 10 ms, ramp to -0.1 V over 10 ms, and 0.1 V for 60 ms. The data collection rate was 2.0 Hz. Data was collected and analyzed on a computer equipped with Chromeleon 7.2 software (Dionex Corp., Sunnyvale, CA, USA). LNT2 was quantified from an external standard curve, which was manually computed.

3 Results and discussion

Due to the high pH values (>pH 11) during the chemical reaction, it was necessary to first neutralize the reaction and then add the enzyme for the enzymatic transglycosylation reaction. For method I (utilizing Et₃N as HCl scavenger and DMC as reactant), no further work-up was necessary; the neutralized reaction solution was simply diluted to the desired theoretical concentrations and β -lactose was added as acceptor (in a fivefold excess) together with the enzyme. In method II (utilizing Na₃PO₄ as HCl scavenger and CDMBI as reactant), it was necessary to filter the reaction solution before neutralization, subsequent dilution, and addition of β -lactose and enzyme because the reacted CDMBI formed the insoluble DMBI. However, this phenomenon was already described in the original publication describing CDMBI as a reactant [15].

As depicted in Figure 1A and B, both cascade methods were suitable for transglycosylation reactions with maximum overall product concentrations after 18.5 h of 11.3 $\pm$ 1.5 mM for method I and 13.7 $\pm$ 1.5 mM for method II. As previously reported [8, 13], the reactions produced a product mixture of LNT2 and three other regioisomers of unknown structure with the same molecular weight. We presume the regioisomers are formed due to different binding modes of the acceptor in the active site. In contrast to the in situ generation of the oxazoline, the use of purified GlcNAc oxazoline from a commercial source (benchmark method) resulted in a significantly higher maximum overall product concentration after 6.5 h of

Figure 1: Progress of the different chemo-enzymatic cascade reactions during 24 h. (A) Method I utilizing DMC; (B) method II utilizing CDMBl; and (C) benchmark method utilizing commercially available GlcNAc oxazoline. Each data point represents the average of a triplicate sample. Black squares: LNT2; red circles: LNT2-regioisomer 1; blue upward triangles: LNT2-regioisomer 2; magenta downward triangles: LNT2-regioisomer 3; green diamonds: total concentration of transglycosylation products.

25.0 ± 1.5 mM (Figure 1C). Considering the high initial rate of product formation in the benchmark method (Figure 1C) compared with Figure 1A and B (methods I and II), the point of maximum yield is probably in between the data points of 6.5 and 18.5 h approaching the maximum yield of 30%, as reported in our previous study [13]. Differences in higher reaction rates and the observed secondary hydrolysis of the products could probably be explained by the higher enzyme dosage as compared with our previous work. The fact that the cascade approaches both gave lower product concentrations over the same studied reaction period cannot be explained rationally without further investigations. This might be due to either inhibitory effects by one of the reactants present or due to the fact that the GlcNAc oxazoline formation was not completed in the given amount of time. However, it is remarkable that the enzymatic transglycosylation reaction could be carried out at all in the presence of significant amounts of reagents such as Et_3N , which was previously suspected to deactivate enzymes [15]. Furthermore, to the best of our knowledge, this study is the first successful report of an enzyme from the GH20 family utilizing the GlcNAc oxazoline as a donor molecule for the synthesis of an HMO-like molecule. In general, the use of a reactive oxazoline is more common for glycan remodeling using *endo*-acting β -*N*-acetylglucosaminidases [10, 17] and it seems to be restricted to neutral to basic reaction conditions because fungal GH20 enzymes were described as being inactive on GlcNAc oxazoline due to their low pH optimum [18]. This hypothesis is further supported by another successful report on utilization of the reactive GlcNAc oxazoline for transglycosylation [19].

4 Conclusion

Despite the fact that the cascaded synthesis of HMO-like molecules was not as efficient as the one using the purified oxazoline, we could unequivocally demonstrate that β -*N*-acetylhexosaminidases from the GH20 family can be used for cascade reactions even in the presence of high concentrations of Et_3N . Furthermore, the described reaction also enables the use of *N*-acetylglucosamine as a cheaper and more readily available donor for transglycosylation compared with the previously used *N,N'*-diacetylchitobiose [8, 13]. The lower yields of the cascade reactions compared with the noncascade reaction indicate that further optimization of the reaction conditions is required to increase the yields and also to determine the limiting factors of the current setup, which is all part of ongoing research. The

further development of a one-pot reaction could involve the compartmentalization of the reaction using a pH-sensitive nanofiltration membrane as described for other reactions [20].

Acknowledgments: We are grateful to Birgitte Zeuner for help with the high-performance anion exchange chromatography with pulsed amperometric detection analysis.

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