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Enzymatic production of HMO mimics by the sialylation of galacto-oligosaccharides

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ABSTRACT

Human milk oligosaccharides (HMOs) are a family of structurally diverse unconjugated glycans that exhibit a wide range of biological activities. In this report, we describe an efficient, Multi-Enzyme One-Pot strategy to produce HMO mimics by the sialylation of galacto-oligosaccharides (GOSs), which are often added to infant formula as an inexpensive alternative to HMOs. In this system, the sialyltransferase donor, cytidine-5′-monophospho-N-acetylneuraminic acid (CMP-Neu5Ac), was generated *in situ* using a CMP-sialic acid synthetase. The sialylated GOSs were obtained by one-step purification after digesting CMP using the alkaline phosphatase PhoA to cytidine and inorganic phosphate. Although the synthesized α2,3-, α2,6- and α2,3/8-sialyl-GOSs exhibit different sialylation levels and patterns, all of these mixtures can be fermented by *Bifidobacterium longum* subsp. *infantis* ATCC 15697 but not by *Bifidobacterium adolescentis* ATCC 15703. The sialidase NanH2, which is unique to the former strain, hydrolyzed all of the synthesized HMO mimics.

1. Introduction

Human milk contains more than one hundred structurally distinct oligosaccharides (human milk oligosaccharides, HMOs), in addition to glycoproteins, glycopeptides and glycolipids. HMOs are unique to human milk in terms of their high abundance and structural diversity and are believed to have a wide range of biological activities beyond providing nutrition to the infant.

HMOs comprise neutral and acidic species constructed from 5 monosaccharides: glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Sia), with N-acetylneuraminic acid (Neu5Ac) as the predominant, if not only, form of Sia. HMOs are built on a lactose core at the reducing end, which can be elongated by N-acetyllactosamine units. The lactose or polylactosamine backbone is further fucosylated in α1,2-, α1,3- or α1,4-linkages and/or is sialylated in α2,3- or α2,6-linkages with greater structural diversity produced. HMOs have primarily been recognized for their prebiotic effects. However, since the early 1990s, accumulating evidence has suggested that HMOs protect the breastfed infant against infections and diarrhea by serving as antiadhesive antimicrobials. Many viral, bacterial and protozoan pathogens use lectin-glycan interactions to initiate infection, and HMOs have been shown to block this type of attachment. For example, the Sia components of HMOs can prevent or at least reduce the *in vitro* adhesion of pathogenic bacteria, such as *Escherichia coli* with S-fimbriae, *Salmonella*, *Vibrio cholerae*, *Helicobacter pylori*, *Campylobacter jejuni*, and viruses. (Coppa et al., 2006; Kunz, Rudloff, Baier, Klein, & Strobel, 2000; Ten-Bruggencate, Bovee-Oudenhoven, Feitsma, van-Hoffen, & Schoterman, 2014; Varki, 2008; Yu et al., 2012). In addition, sialyllactose prevents cholera toxin binding (Idota, Kawakami, Murakami, & Sugawara, 1995).

HMOs also function as immune modulators that protect breastfed infants from excessive immune responses. Direct immunological effects of sialylated HMOs have been observed in a recent study (Eiwegger et al., 2010). In addition, new *in vitro* data suggest that HMOs might exhibit glycome-modifying effects. In 2005, Angeloni et al. for the first time demonstrated that the surface expression of α2,3- and α2,6-linked Sia residues on Caco-2 cells is significantly reduced on exposure to 3′-sialyllactose; this change caused a 90% reduction in the adhesion of enteropathogenic *E. coli* (EPEC) compared to control cells (Angeloni et al., 2005).

Although HMOs have potential applications in infants and adults alike, this potential is limited by the difficulty of manufacturing HMOs. No natural sources other than human milk, including

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the milk of farm animals, provide sufficient quantities of the structurally complex HMOs. As an alternative and in an attempt to mimic the prebiotic effects of HMOs, two major classes of non-HMOs, namely galacto-oligosaccharides (GOSs) and fructo-oligosaccharides (FOSs), are currently added to infant formula. GOSs are Gal oligomers with a degree of polymerization (DP) between 3 and 10 and different glycosidic linkages which lead to a variety of different structural isomers (Yanahira et al., 1995). GOSs are synthesized from lactose by a transgalactosylation reaction that is catalyzed by β-galactosidases from bacterium or yeast (Macfarlane, Steed, & Macfarlane, 2008; Park & Oh, 2010). Although both GOSs and HMOs have a lactose core, GOSs are neither sialylated nor fucosylated. However, the carboxyl group of Sia in acidic oligosaccharides (such as sialyllactose) introduces a negative charge that is crucially important for some of the benefits of HMOs.

More recently, several enzymatic approaches have been developed to synthesize sialylated GOSs that are more HMO-like. For example, trans-sialidases from various *Trypanosoma* species were used to produce α2,3-sialylated GOSs (Jers et al., 2014; Sallomons et al., 2013). PmST, a sialyltransferase (SiaT) that is derived from *Pasteurella multocida* subsp. multocida str. Pm70, transfers a Sia moiety from sialoside to GOSs (Guo et al., 2014). However, according to previously reported kinetic data, the trans-sialylation reaction catalyzed by trans-sialidase is less efficient than sialylation catalyzed by sialyltransferase (Table S2). In the PmST-catalyzed trans-sialylation reaction for 6′-sialyllactose production, a mixture of 3′-sialyllactose and 6′-sialyllactose is generated in the early stage, whereas the 3′-sialyllactose can be partially converted to 6′-sialyllactose by prolonging the reaction time.

In this study, we enzymatically and efficiently produced various sialylated products from GOSs using various SiaTs. The sialylation patterns and levels of these HMO mimics were systematically assessed and evaluated. The fermentation of α2,3-, α2,6- and α2,3/8-sialyl-GOSs by various *Bifidobacteria* was also investigated. The specificity of NanH2 (a sialidase that is recruited by *Bifidobacterium longum* subsp. *infantis* ATCC 15697 to metabolize HMOs (Sela et al., 2011)) toward synthesized HMO mimics was also examined *in vitro*.

2. Materials and methods

2.1. Materials

Bifidobacterium adolescentis ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697 were obtained from the American Type Culture Collection. The Histrap FFTM column was purchased from Qiagen (Valencia, CA). The BCA protein assay kit and 96-well culture plates were purchased from Pierce Biotechnology. Neu5Ac and CTP were purchased from Sigma–Aldrich. Pure GOSs ($_p$GOSs) in powder form (GOS-1000-P, purity > 99%) was kindly provided by New Francisco Biotechnology Corporation (NFBC, Guangdong Province, China).

2.2. Expression and purification

nmCSS, *pmST1*, *pd2,6ST*, *cstII* and *pmPpA* were kind gifts from Dr. Chen (University of California-Davis). All enzymes, including CMP-sialic acid synthetase from *Neisseria meningitidis* group B strain MC58 (NmCSS), α2,3-SiaT from *P. multocida* strain Pm70 (PmST1), α2,6-SiaT from *Photobacterium damsela* ATCC 33539 (Pd2,6ST), α2,3/8-SiaT from *C. jejuni* strain OH4384 (CstII) and inorganic pyrophosphatase from *P. multocida* strain P-1059 (PmPpA), were expressed and purified as previously reported (Cheng et al., 2008, 2010; Lau et al., 2010; Muthana et al., 2012; Yu et al., 2005). The full-length codon-optimized (for the *E. coli* expression system) *nanH2* gene was synthesized by GENEWIZ (Suzhou, China) and was inserted in the pET22b(+) vector. *phoA* was cloned from *E. coli* K12 using the primer pair pF1 (5′-AAATCATATGAAACAAA GCACTATTGCACTGGCA) and pR1 (5′-GCGCTCGAGTTTCAGCCCC AGAGCGGCTTTCATG) and inserted into the pET22b(+) vector. The plasmids containing the target genes were then transformed into *E. coli* BL21(DE3) chemically competent cells. The plasmid-bearing strains were cultured in Luria Bertani broth (LB) medium containing ampicillin (100 μg mL^{-1}).

Overexpression of the target proteins was achieved by inducing the *E. coli* culture with 0.1 mM of IPTG at 16 °C for 24 h (180 rpm) when the OD (600 nm) of the culture reached 0.8 with vigorous shaking at 37 °C (250 rpm). The cells were then harvested by centrifugation at 8000 rpm for 10 min, resuspended in lysis buffer (100 mM Tris–HCl pH 8.0 containing 0.1% Triton X-100), and lysed by sonication. After centrifugation (12,000 rpm for 20 min) of the cell lysate, the supernatant was applied onto a Ni^{2+}-NTA affinity column that had been pre-equilibrated with 5 column volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0). Followed by washing with 8 column volumes of washing buffer (40 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0), the target proteins were eluted with elution buffer (200 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0).

2.3. General procedures for the Multi-Enzyme One-Pot sialylation of $_p$GOSs

Enzymatic sialylation of $_p$GOSs was performed in a Multi-Enzyme One-Pot (MEOP) system containing PmPpA, NmCSS and one of the three SiaTs (PmST1; Pd2,6ST; or CstII) with Neu5Ac, CTP and $_p$GOSs (approximately 9.1 mg/mL) as the starting materials (Fig. 1). To optimize the efficiency of the sialylation reaction, various ratios of Neu5Ac and CTP versus $_p$GOSs were tested. In this study, the average molecular weight of $_p$GOSs was estimated to be 479.2 Dalton after the components of DP2 up 6 were separated on a Thin-Layer Chromatography (TLC) plate, which was developed with EtOAc/MeOH/H_2O/HOAc = 4:2:1:0.2 (by volume) and stained with ρ-anisaldehyde sugar stain, and were analyzed by the software Quantity One. The reaction was carried out in 100 mM Tris–HCl buffer (pH 8.0) and incubated at 37 °C for 2 h. In small-scale reactions, 8 μg PmPpA, 14.6 μg NmCSS and either 17.2 μg PmST1; 27.2 μg Pd2,6ST; or 61.6 μg CstII were used with precursor of Neu5Ac and CTP (1, 2.5 or 5 equivalents) and 10 mg $_p$GOSs (approximate 20 mM) as the starting materials in a total volume of 1.1 mL. In large-scale reactions, tenfold enzymes were used with precursor of Neu5Ac and CTP (2.5 equivalents in PmST1 and Pd2,6ST catalyzed reactions, and 5 equivalents in CstII catalyzed reaction) and 100 mg $_p$GOSs (approximate 20 mM) as the starting materials in a total volume of 11 mL. Product formation was monitored using TLC (EtOAc/MeOH/H_2O/HOAc = 4:2:1:0.2). When two of the most abundant components, di- and tri-$_p$GOSs, had reacted completely, the reaction was first heated at 100 °C for 5 min to inactivate the enzymes and then treated with alkaline phosphatase

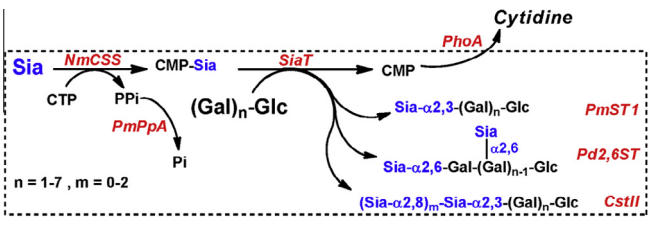

Fig. 1. Schematic representation of sialylation reactions catalyzed by various SiaTs.