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A *Pasteurella multocida* sialyltransferase displaying dual trans-sialidase activities for production of 3′-sialyl and 6′-sialyl glycans

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ABSTRACT

This study examined a recombinant *Pasteurella multocida* sialyltransferase exhibiting dual trans-sialidase activities. The enzyme catalyzed trans-sialylation using either 2-*O*-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid or casein glycomacropeptide (whey protein) as the sialyl donor and lactose as the acceptor, resulting in production of both 3′-sialyllactose and 6′-sialyllactose. This is the first study reporting α-2,6-trans-sialidase activity of this sialyltransferase (EC 2.4.99.1 and 2.4.99.4). A response surface design was used to evaluate the effects of three reaction parameters (pH, temperature, and lactose concentration) on enzymatic production of 3′- and 6′-sialyllactoses using 5% (w/v) casein glycomacropeptide (equivalent to 9 mM bound sialic acid) as the donor. The maximum yield of 3′-sialyllactose (2.75 ± 0.35 mM) was achieved at a reaction condition with pH 6.4, 40 °C, 100 mM lactose after 6 h; and the largest concentration of 6′-sialyllactose (3.33 ± 0.38 mM) was achieved under a condition with pH 5.4, 40 °C, 100 mM lactose after 8 h. 6′-sialyllactose was presumably formed from α-2,3 bound sialic acid in the casein glycomacropeptide as well as from 3′-sialyllactose produced in the reaction. The k_{cat}/K_m value for the enzyme using 3′-sialyllactose as the donor for 6′-sialyllactose synthesis at pH 5.4 and 40 °C was determined to be $23.22 \pm 0.7\ M^{-1}\ s^{-1}$. Moreover, the enzyme was capable of catalyzing the synthesis of both 3′- and 6′-sialylated galactooligosaccharides, when galactooligosaccharides served as acceptors.

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1. Introduction

Human milk oligosaccharides (HMOs) are the third most abundant component (5–10 g/L) in human milk (Bode, 2009). About 180 different HMOs species have been identified recently in a pooled human milk sample from five individuals, and nearly 16% of the total oligosaccharide abundances were found correspond to sialylated oligosaccharides (Ninonuevo et al., 2006). The structural diversity and complexity confer unique biological functions on HMOs. Sialylated HMOs, consisting of *N*-acetylneuraminic acid (Neu5Ac) attached to galactose or *N*-acetylgalactosamine (GalNAc) through α-2,3 or α-2,6 linkage, are known to have both anti-infective and immuno-modulating effects (Bode, 2012). They are also believed to influence brain development and cognitive capacity in the newborn infant (Wang, 2009; Wang et al., 2003). The sialyllactose (3′- and 6′-sialyllactoses) content ranges from 0.4 to 0.8 g/L and the isomers of monosialylated and disialylated lacto-*N*-tetraose are present in lower amounts in human milk (Kunz et al., 2000). In contrast, only trace amounts of sialylated oligosaccharides

Abbreviations: ATP, adenosine-5′-triphosphate; cGMP, casein glycomacropeptide; CMP-NeuAc, cytidine-5′-monophospho-*N*-acetylneuraminic acid; CTP, cytidine-5′-triphosphate; CV, column volume; GalNAc, *N*-acetylgalactosamine; GOS, galactooligosaccharides; HMOs, human milk oligosaccharides; HPAEC-PAD, high-performance anion exchange chromatography; LacMu, 4-methylumbelliferyl β-D-lactoside; LC–MS, capillary liquid chromatography/mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; NDV, Newcastle disease virus; Neu5Ac, *N*-acetylneuraminic acid; N-His6-tag, N-terminal His_6-tag; PEP, phosphoenolpyruvate; PGC, porous graphitic carbon; PmST, *Pasteurella multocida* sialyltransferase; pNP-Neu5Ac, 2-O-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid; RSM, response surface methodology; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sia-GOS, sialylated galactooligosaccharides; TLC, thin-layer chromatography.

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are present in bovine milk, and consequently in the derived infant formula. Therefore, synthesis of sialylated HMOs for supplementation of infant formula has been suggested (Bode, 2009; Espinosa et al., 2007).

A variety of methods based on microbial systems have been developed for synthesis of sialylated HMOs, starting with the synthesis of sialyllactose. Endo and coworkers established a coupled-microbial method using *Corynebacterium ammoniagenes* and three engineered *Escherichia coli* strains overexpressing the genes of cytidine-5′-triphosphate (CTP) synthetase, cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) synthetase and *Neisseria gonorrhoeae* α-2,3-sialyltransferase (SiaT), respectively (Endo et al., 2000). This allowed production of 57 mM 3′-sialyllactose from a 12-h reaction using orotic acid, Neu5Ac and lactose. A single-cell method was established by engineering *E. coli* K12 that enabled production of 4.1 mM 3′-sialyllactose from lactose and Neu5Ac supplemented to the medium (Priem et al., 2002). This strain was further refined to allow *de novo* synthesis of Neu5Ac. Using either *Neisseria meningitides* α-2,3-SiaT or *Photobacterium* sp. α-2,6-SiaT gene led to production of 40.3 mM 3′-sialyllactose (Fierfort and Samain, 2008) or 53.7 mM 6′-sialyllactose (Drouillard et al., 2010), respectively. Despite these promising results, the application of the cell-based systems to a wide range of diverse acceptor molecules might be hampered by the need for efficient uptake or *in vivo* production of the acceptor.

As an alternative, several enzymatic methods have been developed. An interesting approach involved a fusion protein having both CMP-Neu5Ac sythetase and α-2,3-SiaT activities, which was utilized to produce 3′-sialyllactose from lactose, Neu5Ac, phosphoenolpyruvate (PEP), adenosine-5′-triphosphate (ATP) and CMP (Gilbert et al., 1998). When providing CMP-Neu5Ac and lactose as substrates, a *Pasteurella multocida* α-2,3/2,8-SiaT was capable of synthesizing 3′-sialyllactose with a yield of 2.7 mM after a 16-h reaction (Endo and Koizumi, 2004). Besides the use of SiaTs, trans-sialidases/sialidases have also been employed. The *Bacteroides fragilis* sialidase catalyzed trans-sialylation from colominic acid to lactose, resulting in synthesis of both 3′- and 6′-sialyllactoses with a total yield of 0.14% (Tanaka et al., 1995). Using casein glycomacropeptide (cGMP) from dairy streams and lactose as substrates, sialyllactose was produced from either *Arthrobacter ureafaciens* or *Bifidobacterium infantis* sialidase-catalyzed reaction (Mcjarrow et al., 2003). As well, methods for enriching bovine milk with 3′-sialyllactose and 3′-sialylated galactooligosaccharides (3′-Sia-GOS) produced by the *Trypanosoma cruzi* α-2,3-trans-sialidase-catalyzed trans-sialylation were patented (Pelletier et al., 2004; Sallomons et al., 2013).

Previously, a *P. multocida* α-2,3/2,6-SiaT (tPm0188Ph) has been produced in *E. coli* by Yu et al. (2005). The recombinant tPm0188Ph was found to be a multifunctional enzyme, *i.e.*, an α-2,3-SiaT that catalyzes the transfer of a sialic acid residue from CMP-Neu5Ac to galactosides and forms α-2,3 sialyl linkage; an α-2,6-SiaT that forms α-2,6 sialyl linkage much less efficiently; a sialidase that cleaves α-2,3 but not α-2,6 sialyl linkage; a trans-sialidase that transfers sialic acid from α-2,3-sialosides (not α-2,6-sialosides) to galactosides. In this study, a *P. multocida* sialyltransferase (PmST) gene (Genbank accession number AAK02272), which possesses three amino acid differences (N105D, Q135R, and E295G) compared to tPm0188Ph, was overexpressed in *E. coli*. The objective of the work was to examine the PmST-catalyzed trans-sialylation in order to preliminarily assess the efficacy of its trans-sialidase activities for producing sialosides. In a reaction mixture containing cGMP, lactose and recombinant PmST, we observed that 3′-sialyllactose but also 6′-sialyllactose were formed. Thereafter, using response surface methodology (RSM), we examined the effects of pH, temperature and lactose concentration on production of 3′- and 6′-sialyllactoses.

2. Materials and methods

2.1. Chemicals and reagents

Phusion DNA polymerase, T4 DNA ligase, *Nco*I, *Eco*31I and *Kpn*I were purchased from Thermo Scientific (Waltham, USA). Protein standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, USA). 85% orthophosphoric acid and 25% ammonium were obtained from VWR (Denmark). 3′-sialyllactose sodium salt and 6′-sialyllactose sodium salt were purchased from Carbosynth (UK). 2-*O*-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP-Neu5Ac, containing a trace amount of Neu5Ac) was provided by Department of Chemistry, DTU. cGMP was supplied by Arla Foods (Viby, Denmark). The sialic acid content was 0.18 ± 0.011 mmol per gram of cGMP. Before use impurities of low molecular weight in the cGMP solution were removed by filtration through a Ultracel regenerated cellulose membranes (5 kDa cutoff) (Merk Millipore, USA). GOS were purchased from Gulio Gross (Trezzano, Italy). All other chemicals were purchased from Sigma–Aldrich (St. Louis, USA).

2.2. Strain and plasmid

A gene encoding a putative *P. multocida* SiaT lacking the residues 2–25 (Genbank accession number AAK02272) was synthesized by Geneart (Life technologies, Germany) (the nucleotide and amino acid sequences given in Fig. S1). *E. coli* DH5α was used for the maintenance and manipulation of plasmids. The expression host *E. coli* BL21(DE3)plysS (Novagen, USA) and the expression vector pETM-11 (EMBL, Germany) were utilized for the expression of PmST.

2.3. DNA manipulation and strain construction

The gene encoding PmST was PCR amplified using primers 5′-ATCGGGTCTCCCATGAAAACAATCACGCTGTATCTGG and 5′-CGGGGTACCTTACAGCTGTTTCAGGCTATCCCAA (restriction sites underlined). The PCR product was digested with *Eco*31I and *Kpn*I and inserted in pETM-11 between *Nco*I and *Kpn*I in frame with an N-terminal His_6-tag (N-His_6-tag). The resulting recombinant plasmid was used to transform *E.coli* BL21(DE3)plysS.

2.4. Expression and purification of PmST

E. coli BL21(DE3)plysS harboring the recombinant plasmid was cultured in auto-induction medium ZYM-5052 (Studier, 2005) for 24 h with shaking at 30 °C. The cell pellets were harvested by centrifugation (20 min at 5000 g) and re-suspended in binding buffer (20 mM citrate-phosphate buffer, 100 mM NaCl, 15 mM imidazole, pH 7.4). Cells were lysed by sonication and centrifuged at 5000 g for 20 min. The supernatant was subjected to sterile filtration through a 0.45 μm filter and subsequently loaded onto a 5 mL Ni^{2+}-sepharose HisTrap HP column (GE healthcare, UK). The purification was carried out using the method described by Silva et al., 2011. The fractions containing N-His_6-tagged PmST were collected and then desalted using PD-10 desalting columns (GE Healthcare) to remove imidazole. Protein concentrations were determined using the BCA protein assay (Thermo scientific) with BSA as the standard.

2.5. Response surface design of trans-sialylation reactions

A quadratic central composite face centered design was used to generate factor combinations and multiple linear regression models were fitted to the data using MODDE 7.0 (Umetrics AB, Sweden). The investigated factors were as follows: pH 4.4, 5.4 and 6.4; temperatures 30 °C, 40 °C and 50 °C; and lactose concentrations 50 mM, 100 mM and 150 mM. Each experimental design consisted of 17

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