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A semi-multifunctional sialyltransferase from *Bibersteinia trehalosi* and its comparison to the *Pasteurella multocida* ST1 mutants

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ABSTRACT

Sialic acids are well known for their crucial roles in many physiological and pathological processes. Improvement in the efficacy of protein drugs, an increase in the anti-inflammatory activity of intravenous immunoglobulin, preparation of infant milk and the diagnosis of diseases are examples of why there is a need for efficient *in vitro* sialylation. Sialyltransferases are crucial enzymes for the synthesis of sialo-oligosaccharides. Here, we introduce a new α2,3-sialyltransferase from bacteria *Bibersteinia trehalosi* (BtST1), which is homological to sialyltransferase from *Pasteurella multocida* (PmST1), *Pasteurella dagmatis* (PdST1) and *Haemophilus ducreyi* (Hd0053). BtST1 is active in a wide pH range and shows considerable acceptor flexibility. Very good specific activities have been detected with lactose and LacNAc as acceptors, and these activities were comparable to those of efficient multifunctional PmST1 and higher than PdST1, Hd0053 and also PmST1 M144D which was constructed to decrease the high sialidase activity of PmST1. Testing of PmST1 mutant forms revealed that mutations that included S143 caused only the restriction of sialyltransferase activity, whereas mutations including G142 resulted in the loss of activity with lactose. BtST1 possesses only low sialidase and trans-sialidase activities that are comparable to mutant PmST1 M144D, which are detected only in the presence of CMP. The combination of large acceptor flexibility, high activity for lactose and LacNAc and naturally low sialidase activity make BtST1 an attractive enzyme for biotechnological applications.

1. Introduction

Protein-based therapeutics have an increasing market due to their great clinical importance and efficient recombinant production. For example, intravenous immunoglobulin (IVIg) is extensively used in the treatment of many immune-mediated diseases (Elvin et al., 2013 Séïté et al., 2015). There is great interest in improving the therapeutic efficacy of protein drugs by engineering their physicochemical properties (e.g., molecular stability) and pharmacological properties (e.g., dose/response profiles). One of the most promising approaches to improving both is a rational design for the glycosylation of glycoproteins. Over the past 45 years, a generalized role of sialic acid in determining the survival of glycoproteins in the circulation has been confirmed many times and is generally accepted (Morell et al., 1971; Kontermann, 2011). The mammalian glycosylation machinery is quite complex and sophisticated; therefore, improvement of the glycosylation profiles in cell lines, such as in the Chinese hamster ovary, has limitations in capping each terminal galactose by sialic acid at 100%; i.e., "oversialylation". To overcome this limitation, the *in vitro* sialylation of therapeutic proteins by sialyltransferases (STs) has attracted the interest of the pharmaceutical industry because it offers the opportunity to use concentrations of substrates unreachable in the host cells producing the recombinant proteins. Additionally, the *in vitro* production of sialo-oligosaccharides offers the possibility of using structurally modified sialic acids to generate sialoconjugate diversity (Fessner et al., 2014) for modification of the physicochemical and pharmacological properties of therapeutic proteins. Additionally, the in vitro synthesized sialo-oligosaccharides have potential for the development of "biochips", or microarrays that can be used for profiling protein-sialoglycan interactomes in the diagnosis of various diseases (Deng et al., 2013; Padler-Karavani et al., 2012). In a third line, biotechnological *in vitro* sialylation of milk oligosac-

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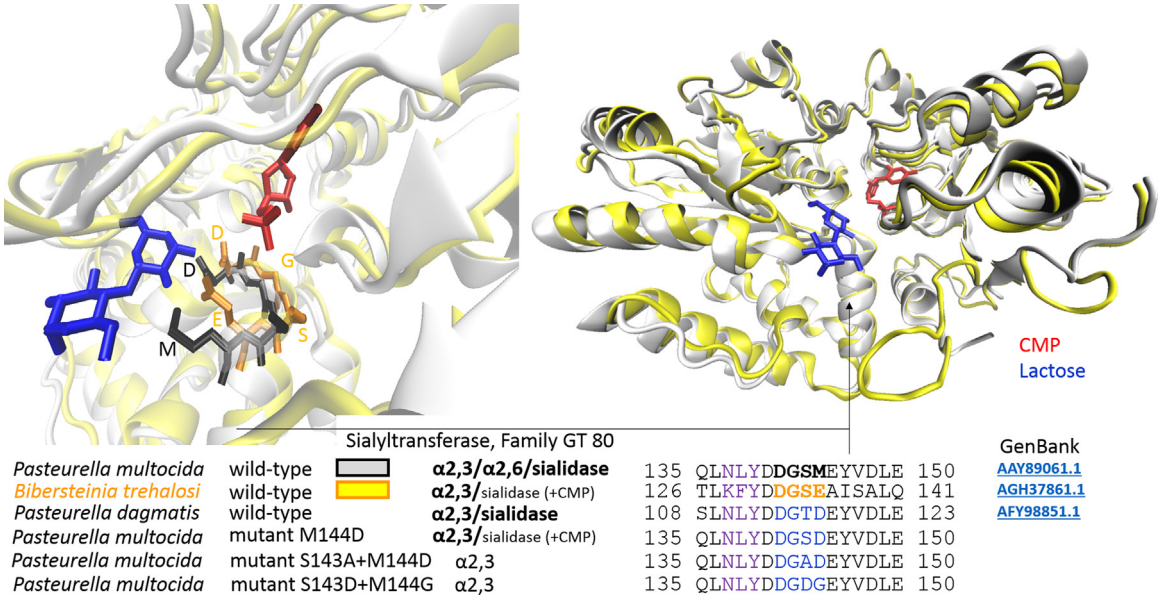

Fig. 1. Structural comparison between model of ST from *Bibersteinia trehalosi* (BtST1, yellow) and ST1 from *Pasteurella multocida* (PmST1, grey, PDB code 2ILV). Differences between BtST1 and mutants of PmST1 are shown in the alignment of the substrate-binding site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

charides offers attractive possibilities for the enrichment of infant milk preparations (Guo et al., 2014; Wang et al., 2015).

Human STs are a family of at least 20 glycosyltransferases, mainly used for α 2,3 and α 2,6 sialylation of the terminal galactose using CMP-*N*-acetylneuraminic acid as the donor substrate (ST3 Gal and ST6 Gal; CMP-Neu5Ac). Human infective bacteria use their own STs to synthetize sialoglycan mimicry on the cell surface to escape from the host immune system (Talafová and Nahálka, 2012). Over the past decade, a number of STs from human pathogens have been biochemically characterized (Yu et al., 2005; Li et al., 2007; Lee et al., 2011; Schmölzer et al., 2013). Based on their biochemical data, a multifunctional ST (α 2,3/α 2,6/sialidase/trans-sialidase activity) from *Pasteurella multocida* (PmST1) showed the best biotechnological potential for sialylation *in vitro* (Yu et al., 2005). A truncated PmST1 lacking the N-terminal 2–25 amino acid residues was highly soluble and could be expressed in high yields in *Escherichia coli* (100 mg of purified PmST1 from a 1 L *E. coli* culture) with high specific activity (60 U/mg protein). Additionally, PmST1 showed excellent donor and acceptor substrate flexibility, which was valuable for the synthesis of complex carbohydrates and glycoconjugates (Yu et al., 2005). However, the significant drawback of PmST1 was high sialidase activity and donor hydrolysis. This problem was solved by a single M144D mutation that reduced sialidase activity and decreased donor hydrolysis. The single mutation was directly in the catalytic centre (see Fig. 1). An adverse effect of this mutation was a decrease in α2,3-sialyltransferase activity with the lactose acceptor, although sialylation activity with a poor fucose-containing acceptor was not significantly affected (Sugiarto et al., 2012).

Characterization of ST from *Bibersteinia trehalosi* (BtST1) is introduced in this work. The enzyme resembles previously characterized PmST1 and PdST1 (*Pasteurella dagmatis*; Schmölzer et al., 2013). BtST1 shares 30% protein sequence identity and 52% positives (similar amino acids) to PmST1 (blast.ncbi.nlm.nih.gov). Compared with the PmST1 M144D mutant, BtST1 showed approximately 4-times higher activity for lactose (Lac) and *N*-acetyl-D-lactosamine (LacNAc), and even lower sialidase activity. Compared with PdST1, BtST1 showed approximately 10-times higher activity for Lac and LacNAc. BtST1 had sialidase and trans-sialidase activities only in the presence of free CMP and had no α 2,6 activity.

2. Materials and methods

2.1. Bacterial strains, plasmids, and materials

Chemically competent *E. coli* BL21(DE3)T1R were supplied by Sigma–Aldrich (St. Louis, USA). Synthetic gene encoding potential sialyltransferase from *B. trehalosi* (BtST1) and primers were purchased from Generi Biotech (Hradec Králové, Czech Republic). Synthetic gene encoding sialyltransferase from *P. multocida* PmST1 M144D and double mutant forms of this enzyme containing mutations S143A + M144D and S143D + M144G were supplied by GenScript (Piscataway, USA) in the vector pET-52b(+). Vector pET-51b(+) Ek/LIC, T4 DNA polymerase (LIC qualified), BugBuster Protein Extraction Reagent and Strep-Tactin SpinPrep Kits were supplied by Novagen (Madison, WI) and dATP (100 mM) by Jena Bioscience (Jena, Germany). HotStarTaq Master Mix, the QIAquick PCR Purification Kit and QIAprep Spin Miniprep Kit were purchased from Qiagen (Hilden, Germany). Bensonase Nuclease (25 U) was purchased from Sigma–Aldrich (St. Louis, USA). CMP-Neu5Ac was self-produced as described by Nahálka and Pätoprstý (2009).

2.2. Cloning, expression and purification of proteins

Genes encoding BtST1 and PmST1 mutants underwent codon optimizing for *E. coli* (http://eu.idtdna.com/CodonOpt) and were commercially synthesized. The BtST1 gene was delivered in plasmid pUC-BtST1 that served as a template for PCR to amplify the target gene. The target gene with LIC extensions was purified, treated with T4 DNA polymerase for preparation of overhangs, and annealed with the linearized pET-51b(+) Ek/LIC. Chemically competent cells were then transformed with the resulting construct and with pET-52b(+) plasmids containing genes for various mutants of PmST1, respectively. Isolated plasmids were confirmed by DNA sequencing. *E. coli* clones containing the desired plasmid constructs were cultured in LB medium (1% peptone, 0.5% yeast extract and 0.5% NaCl) supplemented with ampicillin (100 μg/mL) for 24 h at 37 °C, and then were inoculated into new LB medium with ampicillin. After 4 h of cultivation at 37 °C, IPTG was added (400 μM) and cells were incubated for 24 h at 19 °C. Cultures were harvested by centrifugation (3000 × *g*, 20 min, 4 °C)