Analytical Biochemistry 444 (2014) 67–74

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A plate-based high-throughput activity assay for polysialyltransferase from *Neisseria meningitidis*

Ching-Ching Yu [a], Tara Hill [b], David H. Kwan [b], Hong-Ming Chen [b], Chun-Cheng Lin [a], Warren Wakarchuk [c], Stephen G. Withers [b,*]

[a] Department of Chemistry, National Tsing Hua University, Hsinchu 30013, Taiwan
[b] Centre for High-Throughput Biology and Department of Chemistry, University of British Columbia, Vancouver, BC V6T 1Z1, Canada
[c] Department of Chemistry and Biology, Ryerson University, Toronto, ON M5B 2K3, Canada

ARTICLE INFO

Article history:
Received 3 July 2013
Received in revised form 25 September 2013
Accepted 27 September 2013
Available online 10 October 2013

Keywords:
Polysialyltransferase
Polysialic acid
GFP
Endosialidase
High-throughput screening
Click chemistry

ABSTRACT

Polysialyltransferases (PSTs) assemble polysialic acid (PSA) and have been implicated in many biological processes. For example, certain bacteria such as neuroinvasive *Neisseria meningitidis* decorate themselves in a PSA capsule to evade the innate immune system. Identifying inhibitors of PSTs therefore represents an attractive therapeutic goal and herein we describe a high-throughput, robust, and sensitive microtiter-plate-based activity assay for PST from *N. meningitidis*. A trisialyl lactoside (GT3) serving as the acceptor substrate was immobilized on a 384-well plate by click chemistry. Incubation with PST and CMP-sialic acid for 30 min resulted in polysialylation. The immobilized PSA was then directly detected using a green fluorescent protein (GFP)-fused PSA-binding protein consisting of the catalytically inactive double mutant of an endosialidase (GFP-EndoNF DM). We report very good agreement between kinetic and inhibition parameters obtained with our on-plate assay versus our in-solution validation assay. In addition we prove our assay is robust and reliable with a Z' score of 0.79. All aspects of our assay are easily scalable owing to optimization trials that allowed immobilization of acceptor substrates prepared from crude reaction mixtures and the use of cell lysates. This assay methodology enables large-scale PST inhibitor screens and can be harnessed for directed evolution screens.

Polysialic acid (PSA)[1] is a linear homopolymer of the negatively charged nine-carbon sugar sialic acid (SA). Variations in the linkages and length of the homopolymer are found in nature, with several bacterial species such as *Neisseria meningitidis* [1], *Escherichia coli* K1 [2], *Moraxella nonliquifaciens* [3], and *Mannheimia haemolytica* A2 [4] producing α2,8-linked PSA and with other bacteria producing α2,9-linked or a mixed copolymer of α2,8/α2,9-linked PSA [5]. These PSA structures are found on the cell surface, as capsular polysaccharides, and mimic PSA structures present in mammals. This mimicry is a means for the bacteria to evade the innate immune system by preventing uptake or degradation by the host immune system, implicating PSA as an important factor in the pathogenicity of these bacteria [6,7]. Mammalian PSA, exclusively an α2,8-linked homopolymer, is mostly found on the brain-specific neural cell adhesion molecule (NCAM protein) [8], on CD36 [9], and on the recently described SynCAM [10]. The NCAM molecule has been implicated in numerous normal and pathological processes, including mammalian development, neuronal plasticity, and tumor metastasis [11]. With the prominence of PSA in both the pathogen and the host, the enzymes responsible for the biosynthesis of PSA represent potential therapeutic targets and attractive enzymes to study.

The biosynthesis of PSA is carried out by enzymes known as polysialyltransferases (PSTs), which catalyze the successive addition of sialic acid residues from the activated donor sugar CMP-sialic acid (CMP-Neu5Ac; CMPSA) to the nonreducing end of the growing PSA chain. Mammalian and bacterial PSTs share no significant sequence similarity despite catalyzing the same reaction, albeit in different cellular environments and onto varied acceptor substrates. Mammalian PSTs, along with all STs for mammals, belong to CAZy family GT-29 [12]. Bacterial PSTs are grouped into

* Corresponding author. Fax: +1 (604) 822 8869.
E-mail address: withers@chem.ubc.ca (S.G. Withers).

[1] *Abbreviations used:* PSA, polysialic acid; SA or Sia, sialic acid (*N*-acetylneuraminic acid; Neu5Ac); NCAM, neural cell adhesion molecule; CMPSA, CMP-sialic acid; PST, polysialyltransferase; CAZy, carbohydrate active enzymes database; GT, glycosyltransferase; PSTNm, polysialyltransferase from *Neisseria meningitidis*; STD NMR, saturation transfer difference nuclear magnetic resonance; MBP, maltose-binding protein; DP, degree of polymerization; FCHASE, 6-(fluorescein-5-carboxamido) hexanoic acid N-hydroxysuccinimidyl ester; HPLC, high-performance liquid chromatography; GFP, green fluorescent protein; BTTES, 2-[4-{(bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]ethyl hydrogen sulfate; NADH, reduced nicotinamide adenine dinucleotide; TLC, thin-layer chromatography; GM3, αNeu5Ac(2-3)βGal(1-4)βGlc; GD3, αNeu5Ac(2-8)αNeu5Ac(2-3)βGal(1-4)βGlc; GT3, αNeu5Ac(2-8)αNeu5Ac(2-8)αNeu5Ac(2-3)βGal(1-4)βGlc; LB, lysogeny broth; IPTG, isopropyl-β-D-1-thiogalactopyranoside; BCA, bicinchoninic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; OSu, *O*-succinimidyl; S/N, signal-to-noise ratio; BSA, bovine serum albumin.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.09.030

their own CAZy family, GT-38, with those responsible for making polysialic acid capsules in *N. meningitidis* (PSTNm), *E. coli*, and *Ma. haemolytica* being the most attractive therapeutic targets [13]. GT-38 PSTs are membrane-associated proteins, making study challenging because of their low expression and solubility as well as poor stability. As a result, very little detailed structural, functional, or mechanistic information is available to guide inhibitor development. In recent years, however, STD NMR studies along with bioinformatics approaches have enabled some insightful structural information [14], though a GT-38 crystal structure remains elusive. All studies done to date indicate that PSTNm requires at least a disialylated oligosaccharide for PST priming [5], and with regard to free sialic acid acceptor, at least a trimer (DP3) is required [15]. Soluble PST–maltose binding protein (PST–MBP) fusions have been expressed, purified, and studied [5]. However, the lack of stable, soluble native enzyme impedes crystallization attempts and thus rational inhibitor design.

To our knowledge, no specific, small-molecule inhibitors of bacterial PSTs have been reported. Research efforts in this vein therefore center on screening large libraries of small molecules, but a hurdle facing this goal is the lack of straightforward, scalable activity assays. Challenges included the constantly changing acceptor composition throughout the reaction, the difficulty in directly detecting the polymeric product, as well as a supply of sufficient quantities of active PST. To date, activity assays have relied on labor-intensive synthetic oligosaccharides such as $(Sia)_{2\text{-}3}$-lactose-FCHASE as acceptor substrates analyzed in capillary electrophoresis systems [5], detection of PSA produced in whole cells via immunoblotting, and infrared fluorescence detection [16] or, more recently, an HPLC-based assay using a fluorescent acceptor [17]. It is also possible to assay PST activity by a standard glycosyltransferase coupled assay, which couples the release of CMP to the oxidation of NADH, monitoring the decrease in absorbance at 340 nm [18,19]. All these approaches suffer from problems of scale, but herein we describe an assay that addresses these concerns and works well in a 384-well format. Highlights of our approach involve immobilizing the appropriate acceptor substrate(s) onto microtiter plates via click chemistry, carrying out PSA elongation on the plates using stable, crude cell lysates, and directly detecting the product with a catalytically inactive double mutant of the endosialidase from K1F bacteriophage [20,21] fused with GFP [22] (GFP-EndoNF DM). All aspects of our screen are easily scalable with the appropriate automated instrumentation. Importantly we established through Z' analysis that crude preparations of the azido-modified acceptor can be immobilized and used for assay with statistical similarity to data using purified oligosaccharides. We also demonstrated that crude cell lysates can be used and that these are more stable than purified protein. This is important with respect to use of the screen for directed evolution. Further, we demonstrate that inhibition parameters can be accurately measured by comparing kinetic parameters measured on-plate to those obtained in-solution via the coupled assay. Challenges studying PSTs were overcome with this high-throughput PST activity assay, which can be used to screen large libraries and identify both potent and modest inhibitors therein.

Material and methods

Materials

CMPSA for initial work was graciously provided by Neose Technologies, Inc. (Horsham, PA, USA). BTTES was synthesized according to [23]. Unless otherwise stated, all other reagents were purchased from commercial suppliers such as Sigma–Aldrich, VWR, and Fischer.

Azido oligosaccharide synthesis and purification

6-Azidohexanyl-β-lactoside (**1**, Lac C6N3) was synthesized according to [24] and used as an acceptor for enzymatic elongations with sialyltransferases Cst-I or Cst-II based on already described procedures ([19,25] respectively), with minor modifications. CMPSA was prepared as already described [26] and purified based on the differential precipitation method as previously described [27]. Briefly, Lac C6N3 (13 mM) was dissolved in 50 mM Hepes/20 mM $MgCl_2$ buffer, pH 7.5, along with CMPSA (1.5 mol equivalents for Cst-I reaction to produce monosialylated product and 5 mol equivalents for Cst-II reaction for oligosialylation) and incubated at 37 °C in the presence of Cst-I (2 mg/ml, 1% v/v) or Cst-II (17 mg/ml, 1% v/v) and alkaline phosphatase (130 U/μl, 2 μl) for 4 h. If the products were to be purified, the reaction was quenched with an equal volume of ethanol and centrifuged (10,000*g*, 10 min) to remove proteins and insoluble precipitates. The supernatant was filtered (0.45-μm filter; Millipore), concentrated, and passed through a BioGel P-2 gel (packed in Bio-Rad column 737–1576, 1.5 × 75 cm) with water (10 ml/h) to obtain the desired product. Product-containing fractions were identified by TLC analysis, pooled, and lyophilized. The mixture of oligomers obtained was purified by C18 reverse-phase silica chromatography (Sep-Pak Vac tC18 cartridge 20 cc/5 g 37–55 μm; Waters). Products started to elute at 15% methanol (MeOH). Yield of GM3 C6N3 (**2**) from the Cst-I reaction was 85%. In order of elution, yields for the Cst-II reaction were as follows: GT3 C6N3 (**3**) (36%), GD3 C6N3 (**4**) (39%), and GM3 C6N3 (**2**) (6%).

If the products from the enzymatic elongation were to be used in large crude batches for plate fabrication, the reaction was quenched with ice-cold MeOH (60% v/v of reaction buffer), vortexed (2 min), and centrifuged (10,000*g*, 10 min) to precipitate and remove proteins and other insoluble materials. The supernatant was removed and stored at −20 °C until use in plate fabrication. The concentration of Sia_xLac C6N3 was estimated by assuming 80% conversion from the starting material Lac C6N3 along with careful measurement of the supernatant volume.

Microtiter plate fabrication

Nunc Immobilizer Amino (ThermoScientific) 384-well flat black plates were prepared by initially incubating the amine-reactive plates with propargylamine (50 mM) in sodium carbonate buffer (100 mM, pH 9.6) at 37 °C for 16 h, followed by thorough washing with water to yield the alkyne-modified plates. These could be stored at 4 °C for at least 1 month without noticeable degradation. The alkyne-modified plates were then incubated with one of the azido-containing sialyl oligosaccharides (500 μM; **1**, **2**, **4**, and **3**) or the crude mixture of Sia_xLac C6N3 (500 μM) as well as azido ethanol (500 μM) and a mixture of click reagents, $CuSO_4$ (10 mol%), BTTES [23] (10 mol%), and sodium ascorbate (40 mol%), in 30 μl deionized water at room temperature for 12 h. The modified plate containing triazole-linked acceptor oligosaccharides was then extensively washed with deionized H_2O and then used to assay activity of PSTNm.

Preparation of recombinant PST

The MBP–PSTNm fusions were expressed and purified as previously described [5] with minor modifications. Briefly, plasmid DNA was transformed into BL21 cells and grown in LB at 37 °C until cells reached OD_{600} 0.6, at which point the cultures were cooled to 20 °C, induced with 0.5 mM IPTG, and allowed to grow overnight. Cells were harvested and frozen until lysis. The cells were resuspended in 25 mM Tris, 150 mM NaCl, pH 7.5, buffer (2.5% volume of initial culture), lysed using a french press cell in the presence