Journal of Biotechnology 209 (2015) 50–57

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Novel enzymatic synthesis of spacer-linked P^k trisaccharide targeting for neutralization of Shiga toxin

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ARTICLE INFO

Article history:
Received 20 March 2015
Received in revised form 10 June 2015
Accepted 16 June 2015
Available online 20 June 2015

Keywords:
α1,4-Galactosyltransferase
Shiga toxin
Globotriose
Neisseria gonorrhoeae
Cellulase-mediated condensation

ABSTRACT

A novel alkyl spacer-conjugated derivative of P^k trisaccharide (P^k), one of the active receptors of Shiga toxins (Stxs; Stx1 and Stx2) produced by pathogenic *Escherichia coli* (STEC), was designed and synthesized by a combination of cellulase-mediated condensation from *Trichoderma reesei* and α1,4-galactosyltransferase (LgtC) from *Neisseria gonorrhoeae*. The specific activity of *N. gonorrhoeae* LgtC was 66 U/mg, which was 13-fold higher than that from *N. meningitidis* expressed in *E. coli*. 5-trifluoroacetamidopentyl-β-P^k (TFAP-P^k) was synthesized (yield of 86%, based on the amount of TFAP-lactose added) and its binding to Stx1a-B and Stx2a-B was evaluated. The dissociation constants (K_Ds) of Stx1a-B and Stx2a-B to the spacer-linked P^k, immobilized on a CM5 sensor chip, were 6.8×10^{-6} M ($k_{on} = 4.1 \times 10^1$ M^{-1} S^{-1}, $k_{off} = 2.8 \times 10^{-4}$ S^{-1}) and 2.2×10^{-5} M ($k_{on} = 3.9 \times 10^2$ M^{-1} S^{-1}, $k_{off} = 8.6 \times 10^{-3}$ S^{-1}), respectively. This result suggests that the monovalent P^k-derivative, conjugated to a pentylamino group, represents a promising Stx-neutralizing agent. This cellulase-mediated condensation using cellulase and glycosyltransferase is a valuable tool for the synthesis of spacer-linked oligosaccharide.

1. Introduction

Cell surface glycans are involved in many physiological phenomena, including cell differentiation, cell development, signal transduction, virus and pathogen infection, and cancer metastasis. Glycan-binding proteins (GBPs) have crucial roles in these phenomena by recognizing and binding specific glycans. This GBP-glycan interaction is comparatively weak, compared to protein–protein interactions, but is never negligible biologically, because glycans are very important molecules together with proteins and nucleic acids (van Kooyk and Rabinovich, 2008). In addition, synthetic glycans and oligosaccharides have been investigated for the detection and prevention of virus infection (Ogata et al., 2007; Schofield et al., 2007).

To analyze the binding properties of GBPs, various glycan arrays have now been developed, in which chemically synthesized glycans containing an amine or other functional group are arrayed on *N*-hydroxysuccinimide (NHS)- or epoxy-activated glass slides (Blixt et al., 2004). Such a glycan array was previously utilized as a glycan library to investigate various GBP-binding parameters (Song et al., 2008). Several glycans and oligosaccharides have been synthesized by chemical and enzymatic reactions to investigate their characteristic properties and GBP specificity (Hsu et al., 2011; Lepenies et al., 2010). In chemical synthesis of glycan, many tedious steps required to protect and deprotect hydroxyl groups (Pazynina et al., 2002, 2003) can be circumvented by stereo- and region-specific reaction by glycosyltransferases (Palcic, 2011). From a practical point, the use of glycosyltransferases is attractive for glycan synthesis, because it is highly regioselective for specific hydroxyl groups. On the other hand, glycosidases, which normally hydrolyze glycosidic bonds, catalyze two types of reactions, transglycosylation (Yamamoto, 2013) and condensation (Yasutake et al., 2003), and

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http://dx.doi.org/10.1016/j.jbiotec.2015.06.403
0168-1656/

the condensation reaction has been used in the synthesis of spacer-*O*-linked glycans.

In the present study, a novel P^k trisaccharide (P^k)-conjugated derivative with an alkyl spacer, a sugar unit monomer in the chemical structure, was designed and synthesized by the combination of cellulase-mediated condensation, by *Trichoderma reesei* glycosidase, and glycan transfer, using the *Neisseria gonorrhoeae* α1,4-galactosyltransferase (LgtC). This P^k would be expected to bind *E. coli* O-157 Shiga-like toxins 1a (Stx-1) and 2 (Stx-2). Shiga toxin-producing *E. coli* (STEC) produces Shiga toxin (Stx), which belongs to the AB_5 family of protein toxins, composed of one A subunit and five B subunits (Bergan et al., 2012). The A subunit has RNA *N*-glycosidase activity that causes cell death by inhibiting protein synthesis. The B subunit is non-toxic and functions to bind P^k to the surface of eukaryotic cells, allowing the toxin to enter the cell. Each B subunit has three P^k-binding sites, thus totaling 15 P^k binding sites per Stx molecule. We showed that this monovalent P^k-derivative, conjugated with a pentylamino group, showed strong binding activity to both Stx-1 and Stx-2, and thus represents a promising new candidate Stx-neutralizing agent.

2. Materials and methods

2.1. Expression of N. gonorrhoeae LgtC and Stxs B subunits

A partially deleted *lgtC* gene (1–858 bp) of *N. gonorrhoeae* F62 was synthesized by Eurofins MWG Operon (Tokyo, Japan). This synthesized *lgtC* gene was codon-optimized for expression in *E. coli* with its C-terminal 25 amino acids deleted. To attach a spacer (GGGGSGGGGS) and 6× His tag, the *lgtC* gene was amplified by PCR using A4GalT-frw and A4GalT-21-rev primers (Table 1). In addition, the sequence of the spacer and 6× His tag was prepared using GS-H6 as a PCR template for GS-H6(-21)-frw and GS-H6-rev PCR primers. The *lgtC* gene attached to the spacer and 6× His tag was PCR-amplified using the *lgtC* gene and DNA fragment of the spacer and 6× His tag as templates and the A4GalT-frw and GS-H6-rev primers (Table 1). The amplified gene was then inserted into a pET32b vector by In-Fusion technology (CLONTECH, Mountain View, CA, USA). Linearized pET32b was prepared by PCR using pET32-frw and pET32-rev as primers. The recombinant pET32b construct was transformed into *E. coli* BL21 (DE3) cells. Expression of 6× His-tagged LgtC was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) in the culture of this transformant in LB medium supplemented with 100 μg/ml of ampicillin.

DNA fragments composed of the coding sequences for the Stx1a-B and Stx2a-B subunits were synthesized by Eurofins MWG Operon, Inc. To attach the sequence of a spacer (GGGGSGGGGS) and 6× His tag, each B subunit gene was amplified by PCR using Stx1a-B-frw and Stx1a-B-rev primers or Stx2a-B-frw and Stx2a-B-rev primers (Table 1), respectively. The sequence of the spacer and 6× His tag was also prepared using GS-H6 as a PCR template and GS-H6-1aB-frw or GS-H6-2aB-frw and GS-H6-rev as PCR primers. The sequence containing the spacer and 6× His tag was added to each gene by PCR, and the amplified genes inserted into pET32b vectors in the same manner as the *lgC-His* gene, using In-Fusion technology. Each constructed vector was then transformed into *E. coli* BL21 (DE3) cells, and the expression of each His-tagged B subunit carried out in the same manner as LgtC expression.

2.2. Purification of LgtC and Stx B subunits

Purification of LgtC, Stx1a-B-His and Stx2a-B-His was performed using His60 Ni Super flow (CLONTECH) or TALON affinity gel column chromatography. Pelleted cells were suspended in 50 mM Tris–HCl (pH 7.8) containing 150 mM NaCl (Buffer A) and disrupted by sonication. The homogenate was then centrifuged at 5000 × g and the supernatant collected and loaded onto a TALON resin affinity column, and this column was washed by Buffer A containing 40 mM imidazole. Each protein was eluted with Buffer A containing 300 mM imidazole. For size-exclusion chromatography, a Superdex 200 10/300 GL column (GE Healthcare Japan, Tokyo, Japan) was used. Buffer A was used as a running buffer. One milliliter of purified Stx1a-B (0.05 mg/ml) or Stx2a-B (0.4 mg/ml) was used for this size-exclusion chromatography.

2.3. SDS-PAGE

Recombinant protein samples were subjected to SDS-PAGE on 10 or 12% polyacrylamide gels using the Mini-protean II system (Bio-Rad, Hercules, CA, USA). For Stx-Bs, Tris-Tricine SDS-PAGE was adopted. Total proteins on SDS-PAGE gels were detected by Coomassie Brilliant blue R-250 or silver staining. Protein concentrations were measured by BCA Protein Assay-Reducing Agent Compatible (Thermo Fisher Scientific, Rockford, IL, USA).

2.4. 5-Trifluoroacetamido-1-pentanol (TFAP)-linked P^k (TFAP-P^k) synthesis

Synthesis of TFAP-P^k was carried out according to the scheme in Fig. 1. 5-Trifluoroacetamidopentyl β-lactoside (TFAP-Lac) was prepared by a protocol described previously (Ogata et al., 2007). TFAP-Lac (40 mg, 0.076 mmol) and UDP-Gal (94 mg, 0.15 mmol) were first dissolved in a solution that contained 10.3 ml of 50 mM Tris–HCl (pH 6.8), $MnCl_2$ (34.6 mg), and BSA (15.3 mg), and 15.5 U (5 ml) of purified LgtC was then added. The mixture was then incubated for 4 h at 37 °C, and the reaction terminated by boiling for 5 min. The supernatant was isolated by centrifugation (8000 × *g*, 20 min), concentrated and dissolved in 5 ml of $CHCl_3/CH_3OH/H_2O$ (6:4:1), and loaded onto a Silica Gel 60 N column (4.5 × 30 cm). The same solvent at a flow rate of 10 ml/min was used as a running buffer and fraction sizes of 20 ml/tube. Aliquots from fractions 17–26 were then concentrated, dissolved in 2 ml of 20% methanol, and loaded onto an ODS column (2.5 × 30 cm) equilibrated with 20% methanol, at a flow rate of 2.0 ml/min. After washing the column with 280 ml of 20% methanol, the absorbed material was eluted with 40% methanol and a fraction size of 10 ml. The absorbance of the eluate was monitored at 210 nm. An aliquot from pooled fractions 3–4 was concentrated by evaporation and lyophilized. High resolution electrospray ionization mass spectrometry (HR-ESI–MS): *m/z* 708.23174 $[M+Na]^+$ (calcd for $C_{25}H_{42}F_3N_1NaO_{17}$, 708.23025); ^{1}H NMR (D_2O, 500 MHz): δ 4.84 (d, 1H, $J_{1'',2''}$ 4.0 Hz, H-1″), 4.40 (d, 1H, $J_{1'2'}$ 8.0 Hz, H-1′), 4.37 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.25 (1H, H-5″), 3.93 – 3.45 (18H), 3.23 (2H, H-ϵ), 3.19 (1H, H-2), 1.55 (2H, H-β), 1.51 (2H, H-δ), 1.30 (2H, H-γ); ^{13}C NMR (D_2O, 125 MHz): δ 158.9 (CF_3CONH-), 116.0 (CF_3CONH-), 103.3 (*C*-1′), 102.0 (C-1), 100.4 (*C*-1″), 78.8 (*C*-4), 77.4 (*C*-4′), 75.5 (*C*-5′), 74.9 (*C*-5), 74.6 (*C*-3), 73.0 (*C*-2), 72.2 (*C*-3′), 71.0 (*C*-5″), 70.9 (*C*-2′), 70.4 (*C*-α), 69.2 (*C*-3″), 69.0 (*C*-4″), 68.6 (*C*-2″), 60.6 (*C*-6″), 60.4 (C-6'), 60.1 (*C*-6), 39.7 (*C*-ϵ), 28.3 (*C*-β), 27.5 (*C*-δ), and 27.5 (*C*-γ).

2.5. Surface plasmon resonance (SPR)

SPR analyses were performed using Biacore 2000 (GE Healthcare Japan, Tokyo, Japan). TFAP-P^k was treated with NaOH to remove trifluoroacetic acid (TFA) from amino groups and neutralized with HCl. The P^k was then immobilized onto a CM5 sensor chip (GE Healthcare Japan) by amine coupling at pH 4.0 (1500–2000 RU). Stx1a-B or Stx2a-B were then injected into the sensor chip in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P-20 [GE Healthcare Japan], pH 7.4) at 30 μl/min. As a