Enzymatic synthesis of sialylation substrates powered by a novel polyphosphate kinase (PPK3)

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Materials

HotStarTaq Master Mix Kit, QIAquick PCR Purification Kit, QIAprep Spin Miniprep Kit were from QIAGEN (Hilden, Germany). The linearized plasmid vector pET-34b(+) and T4 DNA Polymerase, that is suitable for ligation-independent cloning, were from Novagen (Madison, WI). S. pomeroyi and H. ducreyi genomic DNA were obtained from American Type Culture Collection (ATCC 700808D). Primers were from Invitrogen (Paisley, Scotland). CelLytic™ B Cell Lysis Reagent (Sigma, B7435-500ML) was used for active inclusion bodies preparation. All other reagents were of analytical grade from Sigma-Aldrich (St. Louis, MO).

Cloning, expression and isolation of active inclusion bodies

SPO0224; SPO1256; SPO1727; b0910 and Hd0053 genes were amplified from genomic DNA in 50 μL PCR reaction using forward

5´GACGACGACAAGTTGACCCATGAATCCGAC3´;
5´GACGACGACAAGTTGGAGACAGCAAAGCCC3´;
5´GACGACGACAAGTTGAACCGGAACGGCAGC3´;
5´GACGACGACAAGTTGACGGCAATTGCCCCG3´;
5´GACGACGACAAGTTGCTGATTCAACAAAATCTTG3´

and reverse

5´GAGGAGAAGCCCGGTTAATAGACCTTGGGGAACGTA3´;
5´GAGGAGAAGCCCGGTTAGTCTCTGCCTGGCCCGCTG3´;
5´GAGGAGAAGCCCGGTTACCGCATCCCAGATGTC3´;
5´GAGGAGAAGCCCGGTTATGCGAGAGCCAATTT3´;
5´GAGGAGAAGCCCGGTTAATTATGTATTGTACACAT3´

primers.
The target genes with LIC extensions was purified, treated with T4 DNA polymerase for preparation of overhangs and annealed with the linearized vector. Freshly transformed *E. coli* BL21(DE3) cells harboring the recombinant plasmid were grown overnight (30 °C, 225 rpm) in 30 ml of LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) supplemented with kanamycin (30 µg/ml). Then 10 ml of the culture was transferred into fresh LB (100 ml) medium containing kanamycin and grown at 37 °C. When A<sub>600</sub> value reached 0.9–1.0, temperature was decreased to 25 °C, the agitation rate was reduced to 100 rpm and after addition of 400 µM isopropyl-1-thio-β-D-galactopyranoside (IPTG) allowed to proceed for 20 h.

The cells were harvested by centrifugation (4500xg, 10 min, 4 °C) and lysed with 10 volumes of the non-ionic lytic detergent. After centrifugation of the lysate (20,000xg, 10 min, 4 °C), the debris was three times washed with 25 volumes of buffer (50mM Tris–HCl, pH 7.5).

**Fig.1** SDS-PAGE: *Lane 1&2* – insoluble & soluble fraction of CBDclos-SPO0224; *Lane 3&4* – insoluble & soluble fraction of CBDclos-SPO1256; *Lane 5&6* – insoluble & soluble fraction of CBDclos-SPO1727; *Lane 7&8* – soluble & insoluble fraction of CBDclos-b0910; *Lane 9&10* – insoluble & soluble fraction of CBDclos-Hd0053; *M1&M2* – marker proteins whose molecular masses are indicated in the picture; insoluble fractions were 20-times diluted in comparison with soluble fractions.

**Enzymatic activity and monitoring of the reactions**

10 µl aliquots were taken from the reaction mixture and frozen in 20 min intervals. The concentrations of NTPs were measured by HPLC.

The chromatography was performed on a Shimadzu system (LC-10AD, SPD-10AV, RID-10A) equipped with a TESSEK Separon SGX NH<sub>2</sub> column (150mm×3.3mm i.d.; 7 µm).
**Fig. 2** HPLC of CMP-NeuAc synthesis. Time 0 – red line; time 1 h – green line; time 12 h - blue line, 254 nm. Isocratic elution with 50 mM H₃PO₄, 10 mM MgCl₂ (pH 6.4, triethylamine) at a flow rate of 0.5 ml/min.

**Fig. 3** HPLC of 3'-sialyllactose synthesis. Time 0 – red line, time 3 h – green line. Isocratic elution with mobile phase composed from 60% acetonitrile and 40% acid water solution (50 mM H₃PO₄, 10 mM MgCl₂), at a flow rate of 0.5 ml/min. RI detector.
**Fig. 4** HPLC–ESI-MS of 3ꞌ-sialyllactose synthesis. NH₂ Phenosphere column (150 mm × 2.0 mm I.D., 5 µm) from Phenomenex, Torrance, CA, USA. Acetonitrile and water (85:15, v/v), flow 1ml.min⁻¹ was used as a mobile phase in isocratic HPLC coupled to electrospray ionization. Electrospray source in positive and negative mode was operated under experimental condition of splitting ratio 1:25, scan range 150 – 900, acceleration potential 3 500V.

In the positive mode ESI MS produced [M +H]⁺ at m/z 634, [M + Na]⁺ at m/z 656, and [M +K]⁺ at m/z 672 ions, and in the negative mode neuraminic acid moiety readily deprotonated to form [M-H]⁻ at m/z 632. Retention time of the peak and the fact that B2 fragment is absent in the mass spectrum of α2-6 sialyllactose confirm the identification of α2-3 linkage.¹

**References**