Supplementary Material (ESI) for Organic & Biomolecular Chemistry

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´GAGGAGAAGCCCGGTTAATAGACCTTGGGAACGTA3´;
5´GAGGAGAAGCCCGGTTAGTCCTGCCTGGCCCGCTG3´;
5´GAGGAGAAGCCCGGTTACGCATCCCAGATGTC3´;
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_{600} 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 (4500×g, 10 min, 4 °C) and lysed with 10 volumes of the non-ionic lytic detergent. After centrifugation of the lysate (20,000×g, 10 min, 4 °C), the debris was three times washed with 25 volumes of buffer (50mM Tris–HCl, pH 7.5).

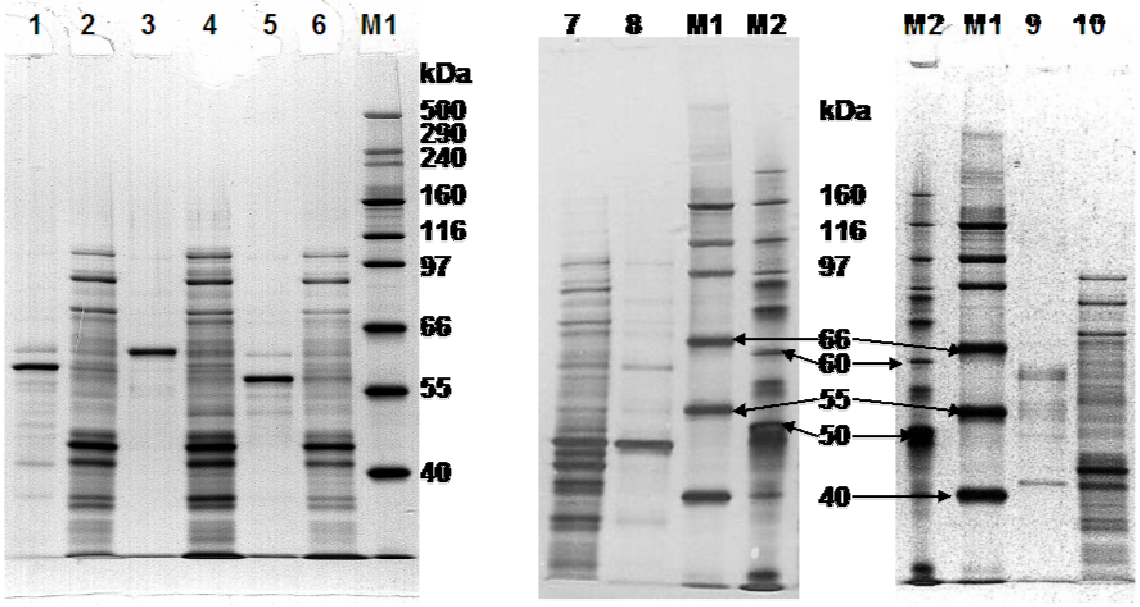

Fig.1 SDS-PAGE: *Lane 1&2* – insoluble & soluble fraction of CBDclos-SPO0224; *Lane 3&4* – insoluble & soluble fraction of CBDclos-SPO1256; *Lane 5&6* – insoluble & soluble fraction of CBDclos-SPO1727; *Lane 7&8* – soluble & insoluble fraction of CBDclos-b0910; *Lane 9&10* – insoluble & soluble fraction 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_2 column (150mm×3.3mm i.d.; 7 μm).

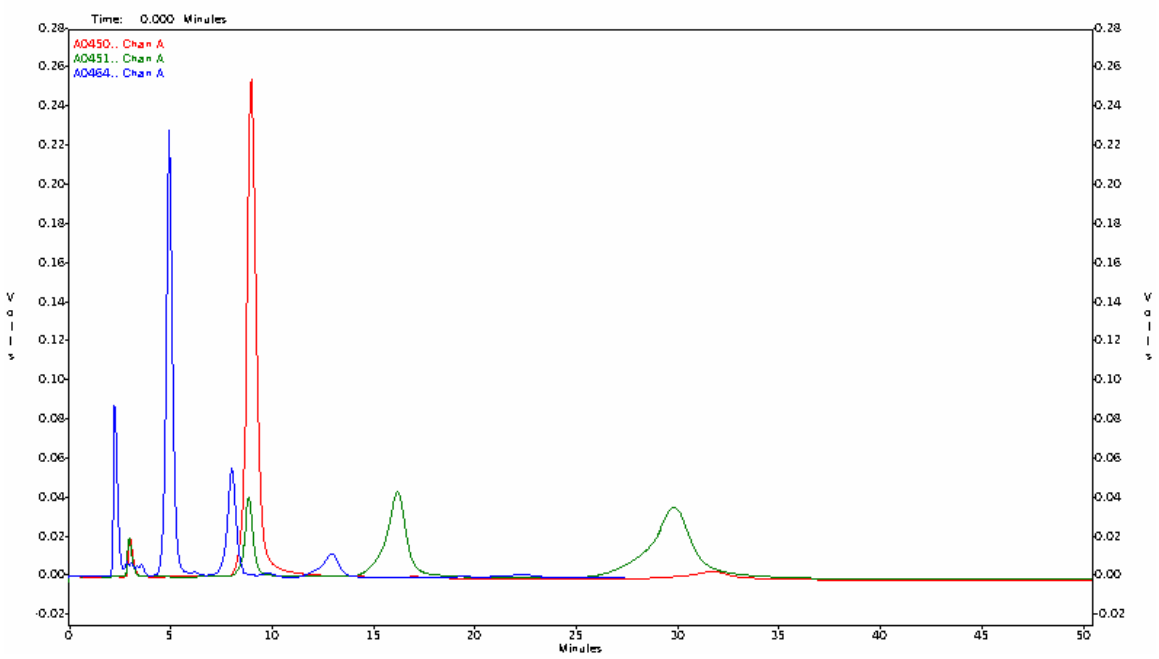

Fig. 2 HPLC of CMP-NeuAc synthesis. Time 0 – red line; time 1 h – green line; time 12 h - blue line, 254 nm. Isocratic elution with 50 mM H_3PO_4, 10 mM $MgCl_2$ (pH 6.4, triethylamine) at a flow rate of 0.5 ml/min.

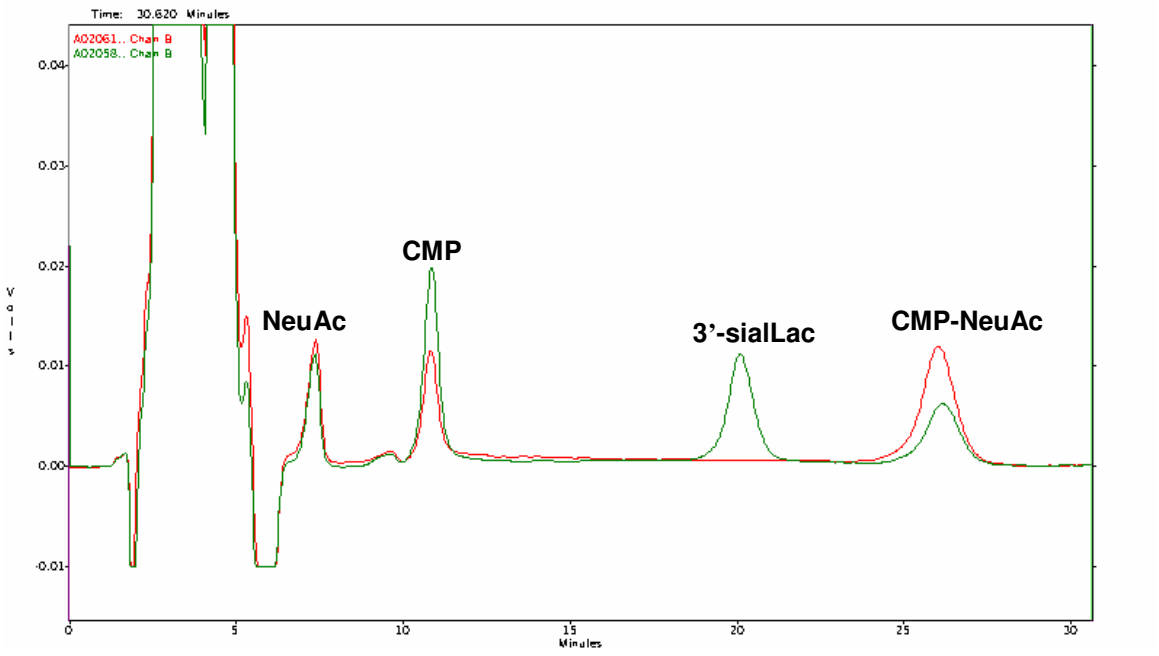

Fig. 3 HPLC of 3'-sialyllactose synthesis. Time 0 – red line, time 3 h – green line. Isocratic elution with mobile phase composed from 60% acetonitrile and 40% acid water solution (50 mM H_3PO_4, 10 mM $MgCl_2$), at a flow rate of 0.5 ml/min. RI detector.

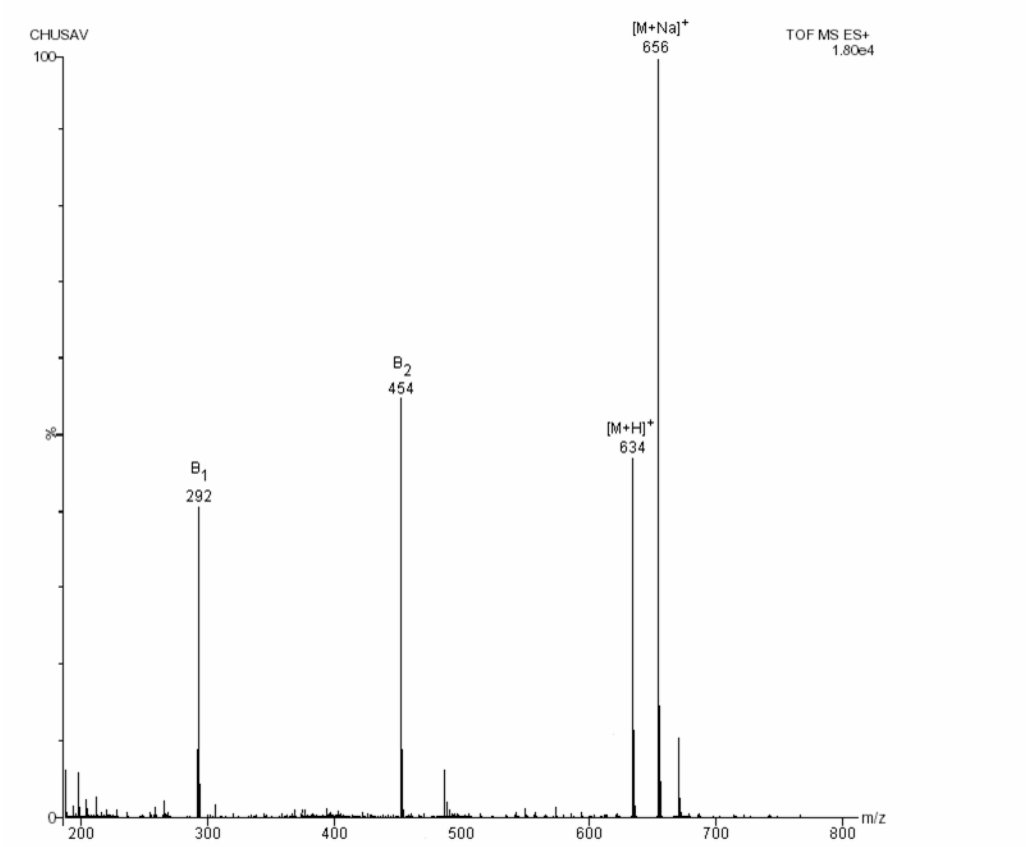

Fig. 4 HPLC–ESI-MS of 3'-sialyllactose synthesis. NH_2 Phenosphere column (150 mm × 2.0 mm I.D., 5 μm) from Phenomenex, Torrance, CA, USA. Acetonitrile and water (85:15, v/v), flow 1ml.min^{-1} was used as a mobile phase in isocratic HPLC coupled to electrospray ionization. Electrospray source in positive 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

1. G. G. Pan , L. D. Melton, Analysis of sialyl oligosaccharides by high-performance liquid chromatography-electrospray ionisation-mass spectrometry with differentiation of 2-3 and 2-6 sialyl linkages, *Journal of Chromatography* A, 2005, **1077**, 36–142.