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

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Active inclusion bodies of polyphosphate kinase 3 and cytidine 5′-monophosphate kinase were combined with whole cells that co-express sialic acid aldolase and CMP-sialic acid synthetase. The biocatalytic mixture was used for the synthesis of CMP-sialic acid, which was then converted to 3′-sialyllactose by whole cells.

The sialic acids are a complex family of nine-carbon monosaccharide subunits that are typically found at the outermost end of glycan chains in all cell types.[1] These acidic subunits terminate cell surface glycans and most secreted proteins of vertebrates and higher invertebrates, mediating or modulating a variety of normal and pathological processes.[2] For example, they provide charge repulsion, preventing unwanted interactions of cells and proteins in the blood circulation; probably for these reasons, sialic acids are critical factors in determining the half-life of circulating red cells and glycoproteins. It was recently shown that the clearance of desialylated coagulation determinants (such as von Willebrand factor) serves to protect the organism from excessive intravascular coagulation during sepsis.[3] Thus, sialylation level (often called "capping") is an important qualitative factor for biotherapeutic products based on glycoproteins.[2] Many attempts are now being made to enhance sialylation by:

(1) engineering animal, yeast or insect production cell lines[4], and

(2) *in vitro* sialylation.[5]

In principle, the mammalian, humanized yeast and insect cell cultures[4] used for production of most therapeutic glycoproteins[6] have the capacity to produce fully sialylated glycoproteins. However, in practise, sialylation levels are often difficult to enhance or maintain because of the number of glyco-processing enzymes involved during the glycoprotein production and isolation. Therefore, a need exists for *in vitro* procedures to enzymatically increase glycan "capping". Two types of enzymatic procedures are currently known. The first uses sialyl transferases with cytidine monophosphate *N*-acetylneuraminic acid (CMP-NeuAc) as a substrate[5] and the other uses *trans*-sialidase with 3′-sialyllactose or fetuin as a substrate.[7] A major drawback of these processes is that the substrates are still quite expensive. We have developed an enzymatic approach that permits the synthesis of CMP-NeuAc and 3′-sialyllactose using cheap polyphosphates. The key to the improvement reported here is the discovery of a new polyphosphate kinase (PPK3), an enzyme that uses an inorganic polyphosphate as a donor to convert CDP to CTP. This enzyme's substrate distinguishes it from previously characterized polyphosphate kinases PPK1 and PPK2. PPK1, initially isolated from *Escherichia coli*, reversibly synthesises inorganic polyphosphate (poly P) from the terminal phosphate of ATP.[8] PPK2, initially isolated from the pathogenic bacteria *Pseudomonas aeruginosa*, performs a poly P-driven synthesis of GTP from GDP, and its nucleoside diphosphate kinase activity is 75-fold greater than the forward reaction, poly P synthesis from GTP.[9]

A BLAST search of nr-aa (GenBank, UniProt, RefSeq, PRF and PDBSTR) using the amino acid sequence of *Pseudomonas aeruginosa* PPK2 (GenBank accession no. NP_248831) identified over 500 homologs. Homologs were especially present in marine α-proteobacteria. A separate search in various species revealed that proteobacteria contained from one to six homologous genes. We probed a species, *Silicibacter pomeroyi* (marine α-proteobacterium in the roseobacter clade), with three putative homologous genes. The genes were cloned, expressed as CBDclos-tagged fusion proteins and purified as inclusion bodies.

Recently we developed a novel method of controlled precipitation *in vivo*, in which the target protein is *N*-terminally fused to the cellulose-binding domain of *Clostridium cellulovorans*, and expression in *E. coli* is performed under conditions that induce selective pull-down of the folded chimeric protein *via* intermolecular self-aggregation of the CBDclos.[10] Nowadays, inclusion bodies are not considered to be wasted cell material but can be used in reaction mixtures for efficient catalysis.[11] Additionally, their easy isolation by non-ionic detergent treatment and washing allows their simple separation from phosphatases, which are accumulated in the cells during overexpression of the recombinant kinases. Using this method, we were also able to clone and successfully express *E. coli* cytidilate kinase (CMK) as CBDclos-tagged fusion protein.

In the process of evaluation of polyphosphate kinases from *Silicibacter pomeroyi*, nucleoside diphosphate kinase (PNDK) activity was confirmed for all three gene products (Table 1). The homolog named as PPK3 showed the highest activity and selectivity for pyrimidine nucleoside diphosphates. PPK1 and PPK2 homologs showed selectivity for purine NDPs. In a second set of experiments, we measured the efficiency of polyphosphate utilization and found that PPK2 and PPK3 utilized 100% of poly P_{15} while PPK1 used only 30%. The reaction mixture in these experiments contained 20 mM poly P, 30 mM $MgCl_2$, 50 mM NDP, 50 mM Tris-HCl (pH 7.8) and was incubated at 30 °C. These results led us to hypothesize that *Silicibacter pomeroyi* uses PPK1 for poly P synthesis and energy storage and that PPK2 together

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Table 1 PNDK activities of PPK2 homologs from *Silicibacter pomeroyi*

Substrate (5 mM)	Volumetric activities (U/L media)[a]			Specific activities (U/mg protein)[a]		
	PPK1	PPK2	PPK3	PPK1	PPK2	PPK3
ADP	18.5	115	3550	0.032	0.31	3.8
GDP	16.5	140	4300	0.028	0.39	4.6
CDP	5.9	14.9	8800	0.010	0.04	9.5
UDP	5.0	4.2	8550	0.009	0.012	9.2

[a] The reaction mixture contained 75 mM poly(P) (as phosphate), 30 mM $MgCl_2$, 5 mM NDP, 50 mM Tris-HCl (pH 7.8) and was incubated at 30 °C.

with PPK3 are designated for consumption of saved energy. The bacterium uses PPK2 for purine and PPK3 for pyrimidine NTP synthesis. It should be emphasized that *S. pomeroyi* PPK1 has no homology with *E. coli* PPK1, and that the activities shown are only apparent. As mentioned above, the enzymes where expressed as CBDclos-tagged fusion proteins, which may affect the real activity values (native activity of non-tagged PPK2 can be higher).

To investigate how effective an energy source PPK3 could provide, CMK and PPK3 active inclusion bodies were combined with whole cells that co-express sialic acid aldolase (SAA) and CMP-sialic acid synthetase (CSAS). The bio-catalytic mixture was used for CMP-NeuAc synthesis (Scheme 1). Construction of these cells was described previously.[12] Fig. 1 depicts a time course of the synthesis. As Fig. 1 clearly shows, using PPK3 inclusion bodies confirmed our expectations that the efficiency of CTP-cofactor synthesis would be high enough to overcome whole cell degradation of the cofactor to cytidine, and 52 mM of CMP-NeuAc was accumulated in the reaction mixture.

Theoretically, up to 300 mg of CMP-NeuAc can be isolated from a 10 mL reaction mixture (Fig. 1).

Researchers from the laboratory of Prof. Xi Chen confirmed that the *Hd0053* gene of *Haemophilus ducreyi* encodes an α2,3-sialyltransferase.[13] They examined the *N*-His_6-tagged form in detail and showed that a divalent metal ion and disulfide formation are not required for α2,3-sialyltransferase activity (ST3) of the *Hd0053* protein. These qualities make it advantageous for our system, where the enzyme is physiologically aggregated in prokaryotic host and the chelating agent, polyphosphate, is used as energy

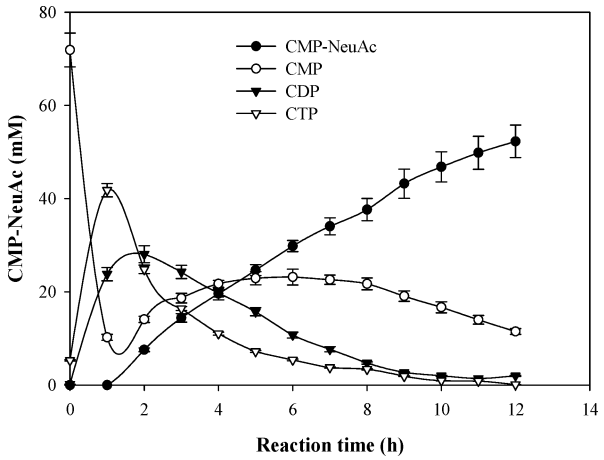
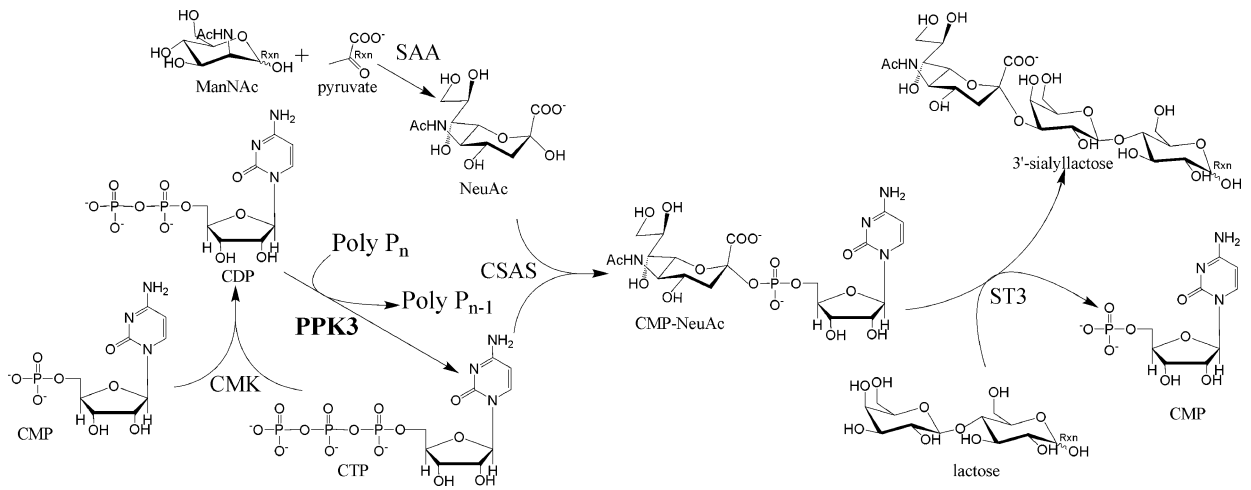


Fig. 1 The time course for CMP-NeuAc synthesis by bio-catalytic mixtures consisting of physiologically aggregated enzymes and whole *E. coli* cells. 10 mL of reaction mixture contained 4.8 mg CMK, 9.2 mg PPK3, 150 mM poly P, 150 mM pyruvate, 75mM ManNAc, 75 mM CMP, 50 mM $MgCl_2$, 5 mM CTP, 50 mM Tris (pH 7.8), incubated at 30 °C, and 50 mg dry *E. coli* (SAA/CSAS) was added after 1 hour.

source. We therefore cloned *Hd0053* and expressed it as CBDclos-tagged fusion protein. Unfortunately, after detergent treatment and isolation of inclusion bodies, the summation of ST3 activity in soluble and insoluble form represented only 5% of whole cell activity. For this reason, lyophilized cells that overexpress *H. ducreyi* α2,3-sialyltransferase were used for the conversion CMP-NeuAc into 3′-sialyllactose (Fig. 2). Despite the fact that quite low substrate and product degradation was observed, it is clear that the conversion is not complete. Inhibition of sialyl transferases with cytidine monophosphate is a serious limitation that has stimulated the development of cofactor recycling schemes. However, in our hands, separation between CMP-NeuAc and 3′-sialyllactose syntheses gave a better yield of 3′-sialyllactose. One would expect that using whole cells will shift up the plateau of product formation (Fig. 2) by constitutive phosphatases. It is not known now if CMP, poly P, or PPi/Pi has the main negative effect at the end of reaction. The process will need additional study for the scale up.



Scheme 1 Multiple-enzyme system for the synthesis of CMP-NeuAc.

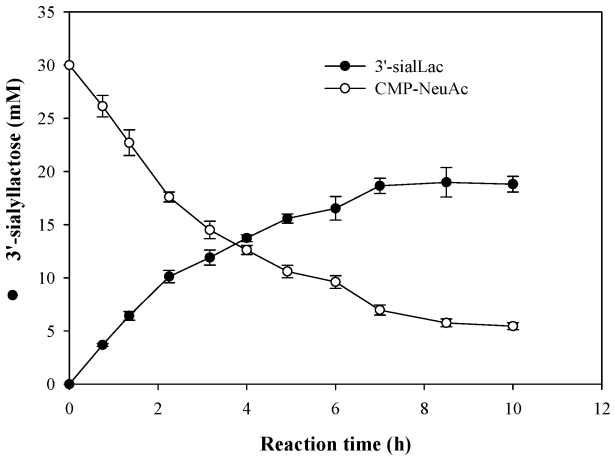


Fig. 2 The time course for synthesis of 3′-sialyllactose. 5.75 mL of the reaction mixture from CMP-NeuAc synthesis (Fig. 1) was mixed with 4.25 mL of a mixture containing 100 mM lactose and 50 mM Tris (pH 7.8, at 30 °C). Addition of 50 mg dry *E. coli* cells (ST3) started the reaction.

In summary, CMP-NeuAc and 3′-sialyllactose, important substrates for "capping" of the pharmaceutical glycoproteins, can be synthesized using a biocatalytic mixture composed of CMK and PPK3 active inclusion bodies and whole cells that express SAA/CSAS and ST3. PPK3 as a novel polyphosphate kinase utilizes 100% of poly P_{15} that would be more straightforward regarding the product purification in comparison with PPK1 from *E. coli* where a high load of poly P in reaction mixtures complicates the downstream processing.

With recent advances in genomics and genetic engineering, the use of biocatalysis for industrial synthetic chemistry is growing rapidly. However, cheap cofactor regeneration and enzyme immobilization are two factors that have been great hurdles in the industrialization of many promising chemo-enzymatic processes.[14] CMP-NeuAc synthesis is an example of a two-decade search for an effective CTP-(re)generation system. Pyruvate kinase/phosphoenolpyruvate, acetate kinase/acetylphosphate, creatine kinase/creatine phosphate, and poly P/PPK (PPK1 from *E. coli*), substrate/enzyme combinations have been proposed.[15] Comparing substrate price and kination efficiency, the acetate kinase/acetylphosphate system seems to be closest to achieving real mass production of CMP-NeuAc and 3′-sialyllactose.[16] However, the discovery of physiologically aggregated PPK3 offers an already immobilized enzyme with comparable activity but with significantly lower cost of the substrate (Sigma 2008; acetyl phosphate 456217-1G/51 EURO; poly P P8510-1KG/101 EURO). Despite potential drawbacks of the proposed system, namely that not all enzymes are generally active as CBDclos-tagged fusion proteins and that poly P can interfere with metal cofactors, we believe that PPK3 will provide the best future energy source not only for *in vitro* glyco-synthesis but also for *in vitro* protein synthesis and dNTPs synthesis for PCR-reactions, and that active inclusion bodies can be used as catalysts in chemical synthesis. Metal-chelating properties of poly P can be suppressed by 100% utilization of poly P by PPK3. In the event of unsuccessful pull-down or enzyme activity in inclusion bodies, whole cells can be used and various alternative enzyme immobilization strategies are known.[17]

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