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

Jozef Nahálka* and Vladimír Pátoprstý

Received 15th December 2008, Accepted 13th March 2009
First published as an Advance Article on the web 23rd March 2009
DOI: 10.1039/b822549b

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.† 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.‡ 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.§ Thus, sialylation level (often called “capping”) is an important qualitative factor for biotherapeutic products based on glycoproteins.¶ Many attempts are now being made to enhance sialylation by:

(1) engineering animal, yeast or insect production cell lines,† and
(2) in vitro sialylation.¶

In principle, the mammalian, humanized yeast and insect cell culture systems used for production of most therapeutic glycoproteins 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 and the other uses trans-sialidase with 3′-sialyllactose or fetuin as a substrate.¶ 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.© 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.©

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.¶ Nousdays, inclusion bodies are not considered to be wasted cell material but can be used in reaction mixtures for efficient catalysis.¶ 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, while PPK1 used only 30%. The reaction mixture in these experiments contained 20 mM poly P, 30 mM MgCl₂, 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

Institute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, SK-84538, Bratislava, Slovak Republic. E-mail: nahalka@savba.sk; Fax: +421 2 59410222; Tel: +421 2 59410319
† Electronic supplementary information (ESI) available: Cloning and overexpression details; protein purification, SDS-PAGEs; HPLC and MS data. See DOI: 10.1039/b822549b
‡ See DOI: 10.1039/b822549b
Table 1  PNDK activities of PPK2 homologs from *Silicibacter pomeroyi*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Volumetric activities (U/L media)*</th>
<th>Specific activities (U/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PPK1</td>
<td>PPK2</td>
</tr>
<tr>
<td>ADP</td>
<td>18.5</td>
<td>115</td>
</tr>
<tr>
<td>GDP</td>
<td>16.5</td>
<td>140</td>
</tr>
<tr>
<td>CDP</td>
<td>5.9</td>
<td>14.9</td>
</tr>
<tr>
<td>UDP</td>
<td>5.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* The reaction mixture contained 75 mM poly(P) (as phosphate), 30 mM MgCl₂, 5 mM NDP, 50 mM Tris-HCl (pH 7.8) and was incubated at 30 °C.

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

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-His₆-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 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 synthases 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](image-url)  
**Scheme 1** Multiple-enzyme system for the synthesis of CMP-NeuAc.
not only for believe that PPK3 will provide the best future energy source proteins and that poly P can interfere with metal cofactors, we not all enzymes are generally active as CBDclos-tagged fusion Despite potential drawbacks of the proposed system, namely that significantly lower cost of the substrate (Sigma 2008; acetyl phosphate 456217-1G/51 EURO; poly P P8510-1KG/101 EURO). 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

Acknowledgements

This work was supported by the Slovak Research and Development Agency under contract no. APVV-51-040205 (http://www.biotecchem.sk/glytech).

Notes and references