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A one-pot approach to bio-synthesize globotriose and its derivatives from simpler substrates

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ABSTRACT

Globotriose is involved in numerous pathogenic processes and drug development strategies. Recent studies have demonstrated that globotriosylceramide could be used in colon cancer therapy and as a crucial indicator for susceptibility to HIV-1 infection. Therefore, the cost-effective and facile approaches for large-scale production of globotiose as potential drugs are highly required. Here, a multi-enzyme one-pot system containing a galactokinase (SpGalK, E.C.2.7.1.6), a UDP-glucose pyrophosphorylase (SpGalU, E.C.2.7.7.9), a α-1,4-galactosyltransferase (LgtC, E.C. 2.4.1.44) and a commercial inorganic pyrophosphatase (PPase, EC 3.6.1.1) was designed to achieve globotriose on preparative scales. This method exploits a cheaper initial substrate, galactose, for donor UDP-galactose production. More importantly, the substrate specificity of SpGalK and SpGalU is highly promiscuous and various UDP-galactose derivatives obtained could be used as the donor substrates for LgtC. This pointcut of rapid preparation of globotriose derivatives is proposed for the first time. Finally, three globotriose analogs were achieved by this one-pot multi-enzyme system in our study.

1. Introduction

The globotriose is the carbohydrate portion of globotriosylceramide and it is involved in many physiologic events. For instance, the over-expression of globotriosylceramide often occurs in Burkitt's lymphoma [1], testicular carcinoma [2], colorectal adenoma [3] and breast cancer [4]. Besides, Desselle A. et al. recently found that globotriosylceramide is over expressed on proliferative endothelial cells, which could be utilized in anti-angiogenic tumor immunotherapy [5]. Globotriosylceramide could be also applied in colon cancer therapy as it induces selective apoptosis in the tumor cells [6]. Moreover, globotriosylceramide is engaged in the development of invariant natural killer T (iNKT) cells. The accumulation of globotriosylceramide is the major cause for iNKT cells decreasing and impeded interaction between iNKT cells and dendritic cells (DCs) [7]. The current research reports that globotriose status is a crucial indicator for susceptibility to HIV-1 infection. The increased resistance of HIV infection was observed when more globotriose expressed on the cell surface [8,9]. Thus, the production of globotriose and its derivatives may result in novel therapeutic approaches for the prevention of HIV/AIDS.

Many human pathogens initiate disease by binding their microbial adhesin proteins to sugar chains of glycoconjugates on host cell mucosal surfaces. The Shiga toxin (Stx) produced by pathogenic strain (O157:H7), often causes diarrhea and even life-threatening hemolytic uremic syndrome (HUS) in humans, by binding to an intestinal cell surface glycan, globotriose [Galα (1,4)Galβ (1,4)Glc] [10,11]. The interaction of Stx and globotriose provides a promising therapeutic mode against Stx infection [12–16].

Due to the importance of globotriose in preclinical and immunology research, preparative scale synthesis of globotriose *in vitro* has attracted big interest of biochemists in this field [17–20]. In general, chemical strategies of synthesizing oligosaccharides have been developed, but their application is always hampered by repetitive protection and deprotection steps. Ye group [21] has reported a one-pot sequential glycosylation approach which simplified the procedure of chemical synthesis. Whereas, their method is still laborious compared with bioenzymatic approaches for oligosaccharide synthesis [22]. The most promising approach of enzymatic synthesis of globotriose is directly using UDP-galactose

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(UDP-Gal) as a starting material catalyzed by galactotransferase. However, commercially available UDP-Gal is too expensive to be used in large scale oligosaccharide production. In our study, we showed a new enzymatic approach in which a cheaper substrate, galactose (Gal), was used as the starting material and finally large-scale high purity globotriose was obtained. This result demonstrates an efficient fashion of synthesis and purification of oligosaccharides. More interestingly, the enzymes used in this reaction system have a promiscuous substrate specificity, which provides a potential for the rapid preparation of globotriose derivatives.

2. Results

2.1. Expression and purification of recombinant enzymes

The expression and purification of recombinant SpGalK, SpGalU and LgtC were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The purified SpGalK, SpGalU and LgtC showed an apparent molecular weight about 47 kDa, 36 kDa and 32 kDa, which were consistent with the molecular mass calculated from their amino acid sequence. The expression levels of soluble SpGalK, SpGalU and LgtC were about 126.24 mg/L, 107.05 mg/L and 68.9 mg/L, respectively. The purity of the enzymes is more than 95%.

2.2. Enzymatic synthesis of globotriose

Preparative-scale production of globotriose was carried out in a 20 ml reaction system. In this multi-enzymatic system (Scheme 1), Gal is first phosphorylated at C1 position by SpGalK (galactokinase). The Gal-1-phosphate is subsequently pyrophosphorylated by SpGalU (UDP-glucose pyrophosphorylase). Commercially available PPase (inorganic pyrophosphatase) is used to hydrolyze the by-product PPi (pyrophosphate), thus driving the reaction forward. With the catalysis of LgtC (α-1,4-galactosyltransferase), the galactosyl is transferred from the resulting UDP-Gal (donor) to lactose (acceptor), generating the target product, globotriose. It is worth noting that all the reactions are carried out in a one-pot pattern.

The reaction mixture was analyzed by thin-layer chromatography (TLC) to examine whether globotriose can be produced by this approach. It can be observed from Fig. 2 that Gal was consumed rapidly for UDP-Gal production. After 20 min, a new spot was obviously emerged with the consumption of UDP-Gal and lactose (Lac). In the following experiments, we confirmed this new product was globotriose. The substrates were almost exhausted after 30 h and the yield of globotriose was more than 95% (Supplementary materials). Therefore, this one-pot multi-enzyme system is feasible and efficient for synthesizing globotriose, and the feature of little substrate residues is convenient for purification of target product. More importantly, a four-enzyme globotriose-generation strategy with Gal as the initial substrate is economical in large-scale production of globotriose.

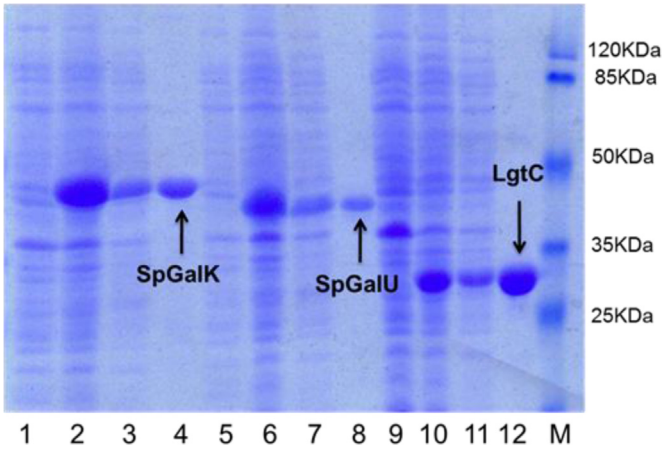

Fig. 1. SDS-PAGE analysis of the expression and purification of SpGalK, SpGalU and LgtC. Lane 1, 5, 9: SpGalK, SpGalU or LgtC whole cell before IPTG induction; Lane 2, 6, 10: SpGalK, SpGalU or LgtC whole cell after IPTG induction; Lane 3, 7, 11: SpGalK, SpGalU or LgtC cell lysate before purification; Lane 4, 8, 12: SpGalK, SpGalU or LgtC cell lysate after purification; M: protein molecular weight standards.

2.3. Enzymatic synthesis of globotriose derivatives

To illustrate the application of this one-pot four-enzyme system in biosynthesis of globotriose derivatives, we tried five monosaccharides as starting materials instead of Gal. These monosaccharides have a structural modification at C-2, C-4 or C-6 position (Scheme 1). The products were determined by TLC and mass spectrometry (MS) (Supplementary materials). As shown in Table 1, three of five monosaccharides were successfully added to Lac forming corresponding globotriose analogs. The MS results are given as follows. 2-deoxyGalα (1–4) Galβ (1–4) Glc, MS (ESI) *m/z*: calculated for $C_{18}H_{31}O_{15}Na$ (M + Na+) 511.1639, found 511.1590. 4-deoxyGalα (1–4) Galβ (1–4) Glc, MS (ESI) *m/z*: calculated for $C_{18}H_{31}O_{15}Na$ (M + Na+) 511.1639, found 511.1582. 6-deoxyGalα (1–4) Galβ (1–4) Glc, MS (ESI) *m/z*: calculated for $C_{18}H_{31}O_{15}Na$ (M + Na+) 511.1639, found 511.1583.

2.4. Globotriose separation and characterization

Globotriose was isolated from reaction mixture through two processes including activated charcoal absorption and gel filtration with Bio-Gel P-2. The purified globotriose was confirmed by MS and nuclear magnetic resonance (NMR) (Supplementary materials). Finally, 44.8 mg pure globotriose was obtained. The MS and NMR results are shown as follows. MS (ESI) *m/z*: calculated for $C_{18}H_{32}O_{16}Na$ (M + Na^+) 527.1588, found 527.1588. ^1H NMR (400 MHz, D_2O): δ 5.15 (d, $J = 3.76$ Hz), 4.88 (d, $J = 3.78$ Hz), 4.59 (d, $J = 8.07$ Hz), 4.44 (d, $J = 7.93$ Hz), 4.28 (d, $J = 6.38$ Hz, 1H), 3.97 (d, $J = 3.31$ Hz), 3.21 (t, $J = 8.27$ Hz).

3. Discussion

Biosynthesis of globotriose has been studied by several groups. Wang group [17] ever synthesized globotriose from sucrose and lactose fermented by the recombinant *Escherichia coli* NM522 containing designed plasmid pLDR20 (*susA- galE- lgtC-* pLDR20). Samain et al. [19] constructed the metabolically engineered *E. coli* K-12 for globotriose production from lactose with overexpressing α-1,4-Gal transferase gene. However, synthesizing oligosaccharides *in vivo* normally requires more steps for purification [19]. Advanced approach for oligosaccharide synthesis involves enzymatic glycosylation *in vitro*, which has been demonstrated as an effective glycosylated strategy. In general, globotriose was directly synthesized from UDP-Gal (donor) and D-Lac (acceptor) mediated by galactosyltransferase. But, expensive cost blocks its application in preparative scale production of oligosaccharide. So how to produce UDP-Gal economically becomes the key point to address this question [23]. Wang group reported an enzymatic method for synthesizing globotrihexose from a comparatively cheap substrate glucosyl-1-phosphate (Glc-1-P), which was used to obtain UDP-Gal by GalU (E.C.2.7.7.9), GalE (E.C.5.1.3.2) [20]. In the present study, we developed a multi-enzyme one-pot system to achieve globotriose substantially with a cost-effective approach. This simple strategy was achieved in the mild solution conditions and all the sequential