효소반응을 통한 dTDP-4-keto-6-deoxy-D-glucose 합성

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One-pot enzymatic synthesis of dTDP-4-keto-6-deoxy-D-glucose

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서론

We have developed a synthetic method for dTDP-4-keto-6-deoxy-D-glucose with four enzyme system. We have used crude extracts from cultures of *Escherichia coli* BL21 strains harboring plasmids containing different sources. dTDP-4-keto-6-deoxy-D-glucose was synthesized by the combination of thymidine-monophosphate kinase, acetate kinase, dTDP-glucose synthase and dTDP-D-glucose 4,6-dehydratase in a batch system, starting the reaction with dTMP. The enzymatic synthesis strategy allowed a dTMP conversion with a 95%.

본론

Introduction

A large variety of deoxysugars is found as building blocks in the oligo-saccharide moieties of glycoproteins, glycolipids, and different classes of secondary metabolites, essentially determining their roles in intra- and intercellular communication. Activated ketoglucose derivatives play a key role in the biosynthesis of deoxygenated oligo- and polysacchrides. Because they represent the branching points in many biosynthetic pathways leading to 2,6- and 3,6-dideoxy- and aminodeoxysugars, and branched deoxy sugars. dTDP-4-keto-6-deoxy-D-glucose is the common intermediate in the biosyntheses of all dTDP-deoxysugars, forming the starting point for a broad-fanned branching(figure 1). The synthesis of dTDP-4-keto-6-deoxy
-D-glucose was reported enzymatically converting dTDP and sucrose with sucrose synthase and dTDP-D-glucose 4,6 dehydratase in fed batch system. However fed batch system is less convenient than batch system. Moreover, dTDP is more expensive than dTMP (about thirty five times) which is starting material in our synthetic system. [1-4]
In this present paper we have developed the method that dTDP-4-keto-6-deoxy-D-glucose was synthesized using four enzyme one-pot reaction from dTMP.

Materials and methods

Materials

All chemicals used in this study were purchased from Sigma (MO, USA) and enzymes used for DNA manipulations were from Boehringer Mannheim GmbH (Mannheim, Germany). Primers were synthesized from Bioneer Corporation (Chungwon, Korea). pGME-T easy vector easy vector system was from Promega

Corporation (WI, USA). pET15b and *E. coli* BL21(DE3) were from Novagen (WI, USA). and pET24ma were kindly donated from Dr. Hiroshi Sakamoto (Pasteur Institute, Paris, France).

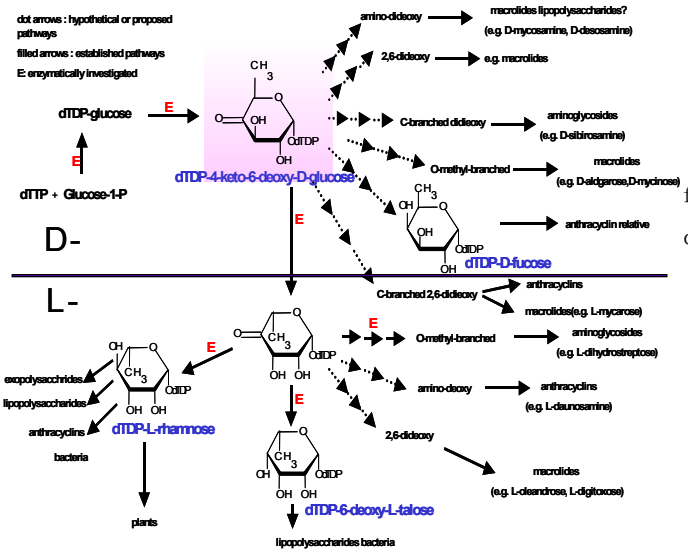

figure1. Biosynthesis of dTDP-activated deoxysugars.

Plasmid construction

The plasmid pET15b containing the pBR322 replication origin, ampicillin resistance gene, and T7 promoter was used for the construction of the plasimid expressing TMP kinases and plasmid pET24ma which contains the p15A origin, kanamycin resistance gene, and T7 promoter was used for the construction of the plasmid expressing acetate kinase and dTDP-glucose synthase. The TMP kinase, acetate kinase, and dTDP-glucose synthase genes were amplified by PCR using synthetic primers and the genomic DNA of *E. coli* K12 as the template. The PCR products of TMP kinase was cloned into pET15b, those of acetate kinase and dTDP-glucose synthase were cloned pET24ma, dTDP-D-glucose 4,6-dehydratase which is cloned from Salmonella enterica serovar typhimurium LT2 was donated Dr. Sohng(SnnMoon Univ, Chung Nam, Korea)

Expression and purification of enzymes

E. coli BL21 (DE3) carrying the expression plasmids containing TMP kinase and acetate kinase was grown at 37℃ in 50 ml LB medium containing ampicillin (100 mg/ml) or kanamycin (50 mg/ml) to OD600 of 0.5-0.8 , and then isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1mM. After further 6 hours. on the other hand *E. coli* BL21 (DE3) carrying the expression plasmids containing dTDP-glucose synthase and dTDP-D-glucose 4,6-dehydratase was grown at 37 C in 50 ml LB medium containing kanamycin (50 mg/ml) or ampicillin (100 mg/ml) to OD600 of 0.5-0.8, and then IPTG was added to a concentration of 0.4mM. After further 12 hours, cells were harvested and pelleted. on the other The extracts of the cells were obtained by sonication and dialysis.

Assay of enzymes and analytes

All enzyme activities were determined in Tris-HCl buffer (pH7.5) at 37C. TMP

kinase activity was assayed using 5 mM dTMP and 5 mM ATP. and Acetate kinase activity was assayed using 5mM dTDP and 20mM acetylphosphate. dTDP-glucose synthase activity was assayed 5mM dTTP and 20mM glucose-1-phosphate. additionally dTDP-D-glucose 4,6-dehydratase was assayed 5mM dTDP-glucose. One unit of the enzyme activity was defined as the amount catalyzing the formation of 1umol product per min. various nucleotides were analyzed with HPLC using a reverse phase column (3.9 x150 mm Symmetry C18, Waters) eluted at a flow rate of 1 ml/min by the following gradient program: 100% Eluent A (100 mM KH_2PO_4/K_2HPO_4 and 8 mM tetrabutylammonium hydrogen sulfate at pH 7.0) for 10 min; 0% to 10% Eluent B (methanol) over 10 min; 10% Eluent B for 10 min. Eluted nucleotides were monitored by absorbance at 270 nm.

Results and discussion

The enzymatic synthesis of dTDP-4-keto-6-deoxy-D-glucose was accomplished by a four-step synthesis strategy as depicted in figure2.

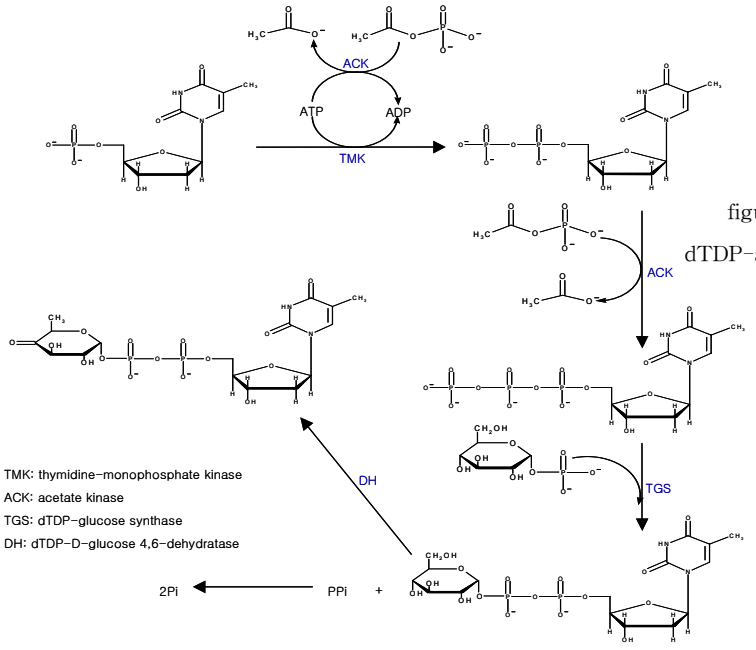

figure2. Biosynthetic pathway of dTDP-activated dexysugars.

The reaction was carried out on a 100ul scale at 37℃.
dTMP, ATP, acetyl-phosphate, and glucose-1-phosphate were added and sonicated crude extracts of cultures of *Escherichia coli* BL21 strains harboring plasmids containing different sources were also added to the reaction system.
Most of the enzymes involved in the process of figure2 requires metal ion, especially, Mg^{2+}, for their catalytic activities. We investigated that dTDP-4-keto-6-deoxy-D-glucose production rate could be maximized above 20mM $MgCl_2$, and the effect of acetyl phosphate to dTMP concentration on the conversion yield of dTDP-4-keto-6-deoxy-D-glucose of the process was investigated. Acetyl phosphate is used as the phosphate donor for the conversion of dTMP to dTTP , and theoretically approximately two equivalent mole of acetyl phosphate to dTMP are required for the complete conversion, if the catalytic amount of ATP is neglected. However at acetyl-phosphate/dTMP ratio of 3, above 95% conversion yield was

obtained. Perhaps, this would be caused by the instability of acetyl phosphate. additionally glucose-1 -phosphate/dTMP ratio of 4 was optimum reaction condition.

At our optimized reaction system, the profile of product formation is shown in fig3. The final yield of dTDP-4-keto-6-deoxy-D-glucose was 95% from dTMP.

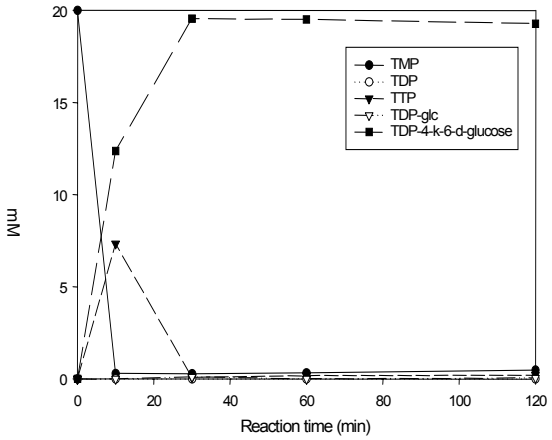

figure3. The profile of product formation

We analysed and identified the product formation with HPLC using a reverse phase column (3.9 x150 mm Symmetry C18, Waters). and are now going on product purification. In conclusion, we have developed the economical biosynthetic method for the production of dTDP-4-keto-6-deoxy-D-glucose from dTMP.

Reference

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결론

1. A new synthetic method for dTDP-4-keto-6-deoxy-D-glucose was developed.
2. dTDP-4-keto-6-deoxy-D-glucose was synthesized using four enzymes in one-pot reaction from dTMP and glucose-1-phosphate.
3. The enzymatic synthesis strategy allowed a dTMP conversion over 95%.