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Metabolic engineering of *Escherichia coli* BL21 for biosynthesis of heparosan, a bioengineered heparin precursor

Chunyu Zhang [a], Long Liu [a,*], Liping Teng [b], Jinghua Chen [b], Jian Liu [c], Jianghua Li [a], Guocheng Du [a], Jian Chen [d,**]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[b] School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214122, China
[c] Division of Chemical Biology and Medical Chemistry, Eshelman School of Pharmacy, University of North Carolina, NC 27599, USA
[d] National Engineering Laboratory for Cereal Fermentation and Technology, Jiangnan University, Wuxi 214122, China

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ABSTRACT

As a precursor of bioengineered heparin, heparosan is currently produced from *Escherichia coli* K5, which is pathogenic bacteria potentially causing urinary tract infection. Thus, it would be advantageous to develop an alternative source of heparosan from a non-pathogeneic strain. In this work we reported the biosynthesis of heparosan via the metabolic engineering of non-pathogenic *E. coli* BL21 as a production host. Four genes, *KfiA*, *KfiB*, *KfiC* and *KfiD*, encoding enzymes for the biosynthesis of heparosan in *E. coli* K5, were cloned into inducible plasmids pETDuet-1 and pRSFDuet-1 and further transformed into *E. coli* BL21, yielding six recombinant strains as follows: sA, sC, sAC, sABC, sACD and sABCD. The single expression of *KfiA* (sA) or *KfiC* (sC) in *E. coli* BL21 did not produce heparosan, while the co-expression of *KfiA* and *KfiC* (sAC) could produce 63 mg/L heparosan in shake flask. The strain sABC and sACD could produce 100 and 120 mg/L heparosan, respectively, indicating that the expression of *KfiB* or *KfiD* was beneficial for heparosan production. The strain sABCD could produce 334 mg/L heparosan in shake flask and 652 mg/L heparosan in 3-L batch bioreactor. The heparosan yield was further increased to 1.88 g/L in a dissolved oxygen-stat fed-batch culture in 3-L bioreactor. As revealed by the nuclear magnetic resonance analysis, the chemical structure of heparosan from recombinant *E. coli* BL21 and *E. coli* K5 was identical. The weight average molecular weight of heparosan from *E. coli* K5, sAC, sABC, sACD, and sABCD was 51.67, 39.63, 91.47, 64.51, and 118.30 kDa, respectively. This work provides a viable process for the production of heparosan as a precursor of bioengineered heparin from a safer bacteria strain.

1. Introduction

Heparin and heparan sulfate (HS) are important glycosaminoglycans that involves a large number of biological processes such as blood coagulation, virus infection, cell differentiation, tumor metastasis and angiogenesis (Lidholt et al., 1988; Folkman et al., 1989; Lin et al., 2002; Marino et al., 2002; Sasisekharan et al., 2002; Tiwari et al., 2004; Tyrell et al., 1995). Heparin has been discovered as a drug to prevent blood coagulation since 1916 and has become the most popular anticoagulant (Baik et al., 2012; Bhaskar et al., 2012; Linhardt, 1991; Liu et al., 2009).

Heparin is currently extracted from animal tissues such as porcine intestine and bovine lung. The heparin supply chain was reportedly contaminated by over sulfated chondroitin sulfate, causing nearly 100 deaths alone in the USA in 2008 (Kishimoto et al., 2008; Laurencin and Nair, 2008). The accident raised the concerns over the vulnerability of animal sourced heparin. The US FDA then started inspection of foreign suppliers and upgraded the pharmacopeial monographs to reduce the likelihood of similar crisis (Linhardt and Liu, 2012). However, these efforts may lead to an insufficient supply of the critical drug as the animal sources for heparin preparation were very limited. With the increasing demand of the drug, the cost of heparin active pharmaceutical ingredient has increased 10-fold (Linhardt and Liu, 2012).

Many efforts have been made to synthesize heparin to overcome the side effects and insufficient supply of heparin. Among them, chemical synthesis and chemo-enzymatic synthesis are representative. Chemical synthesis is complicated and merely amenable to the synthesis of oligosaccharides less than hexasaccharide. An anticoagulant pentasaccharide, namely fondaparinux, was chemically synthesized by more than 60 steps, with yield as low as 0.5% (Liu and Liu, 2010). Although it was marketed and had good pharmacokinetic/pharmacodynamics properties, fondaparinux is unable to

* Corresponding author. Fax: +86 510 85918309.
** Corresponding author. Fax: +86 510 85910799.
E-mail addresses: longliu@jiangnan.edu.cn (L. Liu), jchen@jiangnan.edu.cn (J. Chen).

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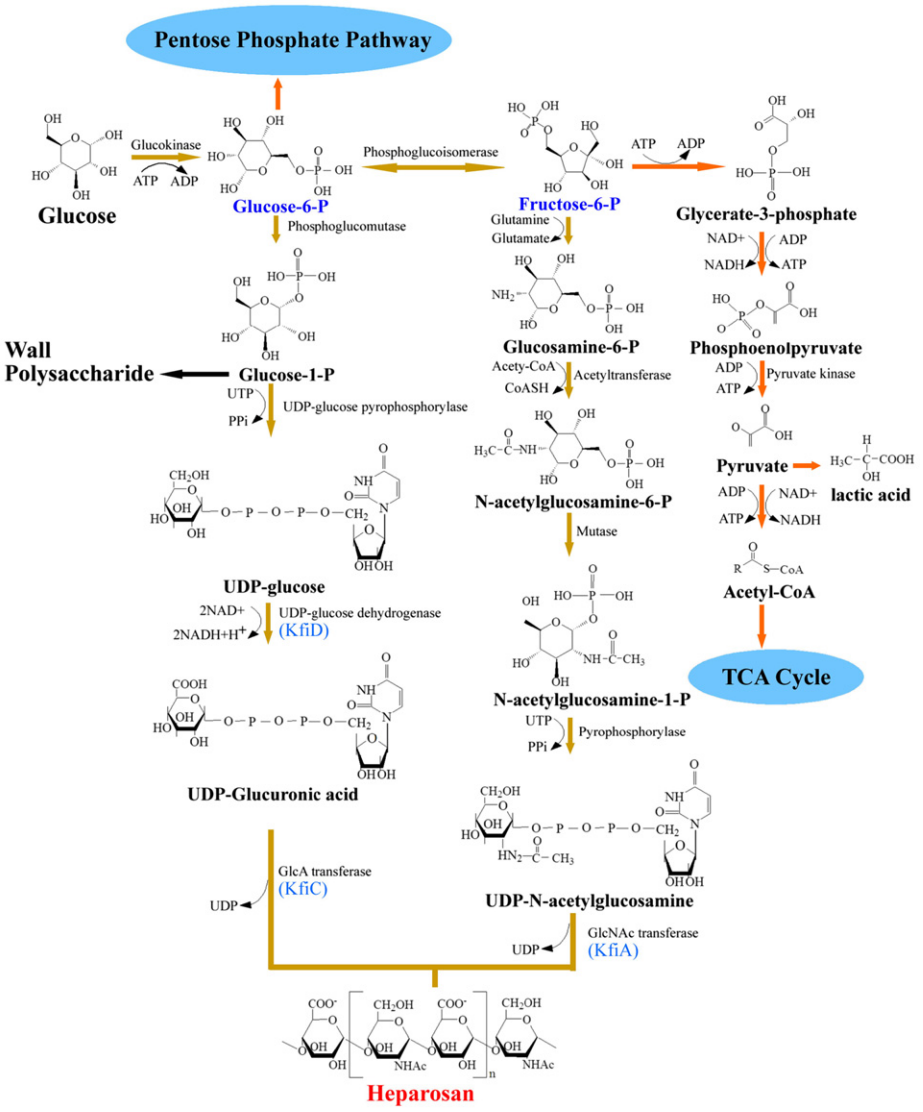

Fig. 1. Metabolic synthesis pathway of heparosan in *Escherichia coli* K5 strain.

replace heparin due to its high cost, difficulty to produce sufficient quantities and limited utility for kidney dialysis (Linhardt and Liu, 2012).

Chemoenzymatic synthesis was an alternative method to prepare bioactive heparin from heparosan (Laremore et al., 2009; Lindahl et al., 2005; Zhang et al., 2008). In this approach, heparosan is *N*-deacetylated using NaOH, and *N*-sulfonated with $(CH_3)_3N \cdot SO_3$, followed by three additional enzymatic modifications, namely, C_5-epimerization/2-O-sulfonation, 6-O-sulfonation and 3-O-sulfonation with C_5 epimerase/2-O-sulfotransferase, 6-O-sulfotransferase, and 3-O-sulfotransferase, and finally converted to anticoagulant heparan sulfate (Kuberan et al., 2003a). The yield (~1.1%) of chemoenzymatic approach is higher than that of the chemical synthesis (Kuberan et al., 2003b). Hence, bioengineered heparin from microbial heparosan has emerged as a new alternative for heparan sulfate or heparin production.

Heparosan is an acidic polysaccharide, comprised of a (-GlcA-1, 4-GlcNAc-1, 4-)$_n$ repeating disaccharide unit (Wang et al., 2011). *Escherichia coli* K5 and *Pasteurella multicida* are able to produce heparosan as bacteria capsule (DeAngelis et al., 2002). The open reading frames *KfiA-D* of the *E. coli* K5 located in region 2 that was essential for the biosynthesis of heparosan and contained four genes of *KfiA*, *KfiB*, *KfiC*, and *KfiD* (Wang et al., 2010). Fig. 1 shows the metabolic synthesis pathway of heparosan in *E. coli* K5. *KfiA* and *KfiC* catalyze the polymerization of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) to produce heparosan (Hodson et al., 2000). The *KfiB* encodes an enzyme stabilizing the multi-enzymatic complex during the elongation of polysaccharide chains but maybe it does not participate in catalysis directly (Hodson et al., 2000). The *KfiD* encoded protein is a uridine diphosphate-glucose dehydrogenase (UDP-glucose dehydrogenase) which could convert UDP-glucose to the precursor GlcA (Hodson et al., 2000). The heparosan is currently obtained from microbial culture of *E. coli* K5, which is pathogenic bacteria potentially causing urinary tract infection (Vann et al., 1981). Thus, it would be advantageous to develop an alternative source of heparosan that avoids the potential crisis.

E. coli BL21 is a widely used expression system and has the ability to overexpress multi non-native genes, and has been metabolically engineered for the production of many substances like hydrogen and fatty acids (Akhtar and Jones, 2009; Lu et al., 2008; Wells et al., 2011). In this work, we constructed a recombinant *E. coli* BL21 as a safer host for heparosan production. First, the heparosan synthesis genes of *KfiA*, *KfiB*, *KfiC* and *KfiD* from *E. coli* K5 were cloned and six plasmids pKfiA, pKfiC, pKfiAC, pKfiB, pKfiD and pKfiBD were constructed. The six plasmids were respectively transformed into *E. coli* BL21 and accordingly six