Carbohydrate Polymers 140 (2016) 424–432

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Efficient biosynthesis of polysaccharides chondroitin and heparosan by metabolically engineered *Bacillus subtilis*

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ARTICLE INFO

Article history:
Received 29 September 2015
Received in revised form 7 December 2015
Accepted 27 December 2015
Available online 29 December 2015

Keywords:
Polysaccharides
Chondroitin
Heparosan
Bacillus subtilis
Metabolic engineering

ABSTRACT

Chondroitin and heparosan, important polysaccharides and key precursors of chondroitin sulfate and heparin/heparan sulfate, have drawn much attention due to their wide applications in many aspects. In this study, we designed two independent synthetic pathways of chondroitin and heparosan in food-grade *Bacillus subtilis*, integrating critical synthases genes derived from *Escherichia coli* into *B. subtilis* genome. By RT-PCR analysis, we confirmed that synthases genes transcripted an integral mRNA chain, suggesting co-expression. In shaken flask, chondroitin and heparosan were produced at a level of 1.83 g L^{-1} and 1.71 g L^{-1}, respectively. Since *B. subtilis* endogenous *tuaD* gene encodes the limiting factor of biosynthesis, overexpressing *tuaD* resulted in enhanced chondroitin and heparosan titers, namely 2.54 g L^{-1} and 2.65 g L^{-1}. Moreover, production reached the highest peaks of 5.22 g L^{-1} and 5.82 g L^{-1} in 3-L fed-batch fermentation, respectively, allowed to double the production that in shaken flask. The weight-average molecular weight of chondroitin and heparosan from *B. subtilis* E168C/pP43-D and E168H/pP43-D were 114.07 and 67.70 kDa, respectively. This work provided alternative safer synthetic pathways for metabolic engineering of chondroitin and heparosan in *B. subtilis* and a useful approach for enhancing production, which can be optimized for further improvement.

1. Introduction

GAGs (namely Glycosaminoglycans) which locate at the mammalian extracellular matrix and bacteria capsular have attracted intensive research because of the wide biological and physiological functions (DeAngelis, 2002; Linhardt, 2003; Suflita, Fu, He, Koffas, & Linhardt, 2015; Yother, 2011). Among them, heparin (HP) and chondroitin sulfate (CS) have been deeply investigated and widely applied in clinic treatments (Ruffell et al., 2011; Wang et al., 2007). For instance, HP was mainly used as anticoagulant (Damus, Hicks, & Rosenberg, 1973) while CS was mainly used as anti-inflammatory drug for the treatment of osteoarthritis and Rheumatism (McAlindon, LaValley, Gulin, & Felson, 2000). In recent years, due to aging of the world population, the market demand of HP and CS has been dramatically increased.

Currently, HP and CS are extracted from animal tissues. However, the disadvantages such as potential risk of interspecies disease and over sulfation raised the concern of the animal sourced HP and CS (Guerrini et al., 2008; Laurencin & Nair, 2008). In view of these problems, development of safe and reliable alternatives to produce HP and CS is always a huge challenge (Laremore, Zhang, Dordick, Liu, & Linhardt, 2009). Accordingly, some de novo chemical synthesis routes for HP and CS with different length and sulfation have been developed (de Paz, Noti, & Seeberger, 2006; Xu et al., 2011). However, it will be unpractical to produce HP and CS in large-scale with these chemical methods because of the complex, time-consuming processes and the rare expensive substrates (Boltje, Buskas, & Boons, 2009). As an alternative approach, chemical synthesis of HP from the precursor heparosan with higher yield has also been reported (Laremore et al., 2009; Zhang et al., 2008). Consequently, semi-chemical synthesis and chemoenzymatic modification of the bioactive HP (which is composed of β-D-glucuronic acid (GlcUA) and *N*-acetyl-α-D-glucosamine (GlcNAc) repeating disaccharides) or CS (which consists a repeating

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http://dx.doi.org/10.1016/j.carbpol.2015.12.065
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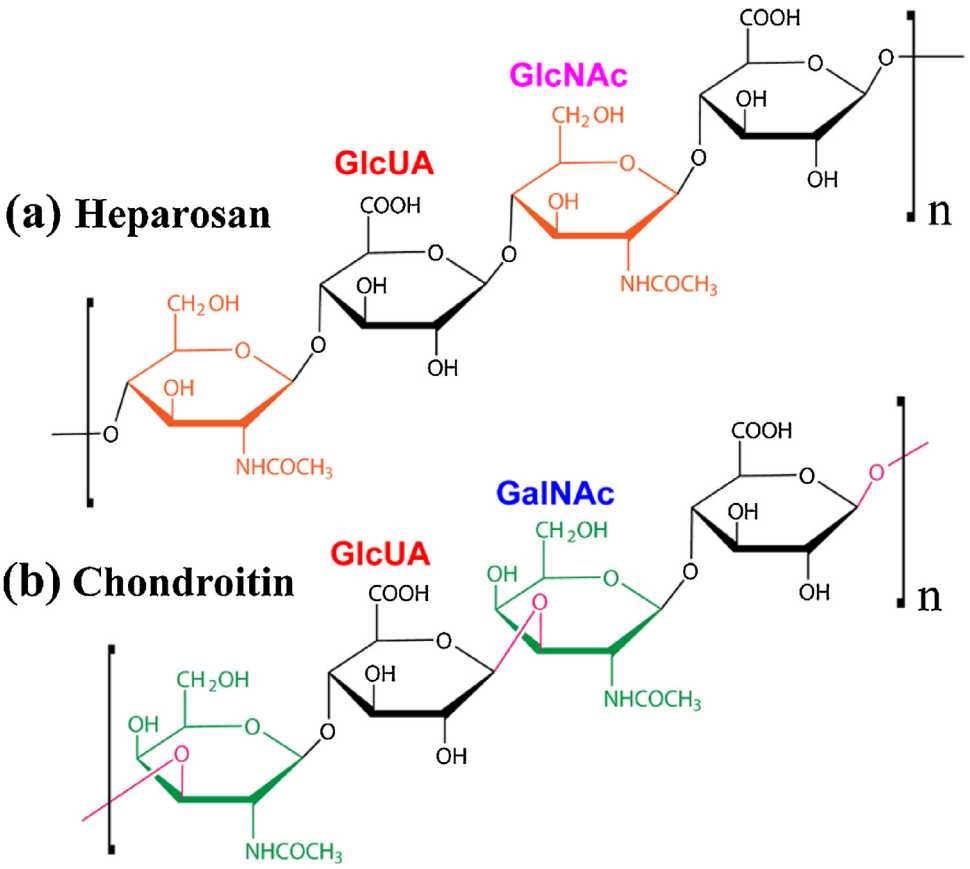

Fig. 1. The chemical structures of bacterial heparosan and chondroitin.

disaccharide unit of GlcUA and *N*-acetyl-D-galactosamine, GalNAc) from their precursors heparosan (Fig. 1a) and chondroitin (Fig. 1b) (Bhaskar et al., 2015; Li et al., 2014; Mikami & Kitagawa, 2013; Restaino et al., 2013) will be more attractive. Consequently, achievement of high yield production of the precursors heparosan and chondroitin is the key determinant.

In the past years, it has been found and demonstrated *Escherichia coli* K5 and K4 produce heparosan and chondroitin respectively (DeAngelis, 2012; Deangelis, Gunay, Toida, Mao, & Linhardt, 2002; Ninomiya et al., 2002; Zanfardino et al., 2010). Accordingly, many studies on optimization of cultivation process (Cimini, Restaino, Catapano, De Rosa, & Schiraldi, 2010; Wang et al., 2010; Wang, Dordick, & Linhardt, 2011) and engineering of the pathways (Cimini, De Rosa, Carlino, Ruggiero, & Schiraldi, 2013) have been carried out in these native strains. Nevertheless, the strains *E. coli* K5 and K4 are pathogenic bacteria and can cause urinary tract infection (Wiles, Kulesus, & Mulvey, 2008). In consideration of this disadvantage, the biosynthetic pathway for synthesis of chondroitin and heparosan have been individually constructed in *E. coli* BL21 (DE3) (He et al., 2015; Zhang et al., 2012) by introducing the corresponding synthases from *E. coli* K5 and K4 (Cress, Greene, Linhardt, & Koffas, 2013a, 2013b). Even though, due to the concern on food safety and the problem of phage contamination (Tanji, Hattori, Suzuki, & Miyanaga, 2008), the engineered *E. coli* strains will probably be confined in food industry. Consequently, construction of alternative robust engineered strains for producing GAGs at industrial scale should be more promising.

Bacillus subtilis, the best-characterized gram-positive bacterium, is regarded as GRAS (generally recognized as safe) strain (Kang, Yang, Du, & Chen, 2014; van Dijl & Hecker, 2013; Westers, Westers, & Quax, 2004) and has been widely used for the production enzymes and chemicals that used in food industries (Shi, Chen, Zhang, Chen, & Zhao, 2009; Song, Nikoloff, & Zhang, 2015; Wang, Fu, Zhang, & Chen, 2012; Yang et al., 2015). Compared with *E. coli*, *B. subtilis* has no significant codon bias and shows stronger tolerance to different environments. In addition, according to the genetic information (Kunst et al., 1997) and previous study on hyaluronan (Widner et al., 2005), *B. subtilis* hosts seem unlikely to encode enzymes degrading heparosan and chondroitin which will benefit the accumulation of heparosan and chondroitin.

In the present study, we firstly constructed and investigated the heparosan and chondroitin biosynthetic pathways in *B. subtilis*. By further optimization of the synthetic pathway, the production of heparosan and chondroitin were enhanced to 5.82 g L^{-1} and 5.22 g L^{-1}, respectively. The present work paved the way for large-scale production of heparosan and chondroitin and its derivatives in the GRAS *B. subtilis* strain.

2. Materials and methods

2.1. Strains and plasmids construction

The bacterial strains, plasmids, and primers used in this study were listed in Tables 1 and 2, respectively. Molecular cloning and manipulation of plasmids were done with *B. subtilis* 168. The polymerase chain reaction (PCR) was performed in 50-μL volumes using 1 μL DNA template, 10 pmol of each primer, 25 μL 2× Super *Pfu* PCR Master Mix (Hangzhou Biosci Co., Ltd, China) under the following conditions: 94 °C for 3 min; 32 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min; 72 °C for 5 min. The strong ribosome binding site (RBS) sequence (TAAAAAGGAGGCATTTACAT) was used as the translation initiation signal for *kfoA* and *kfiA* genes. To integrate the operons *kfoC–kfoA* and *kfiC–kfiA* on the genome of *B. subtilis*, the genes *kfoA* and *kfoC* were amplified from *E. coli* K4