Bioresource Technology 247 (2018) 30–35

Contents lists available at ScienceDirect

Bioresource Technology

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

Dipotassium phosphate improves the molecular weight stability of polysialic acid in *Escherichia coli* K235 culture broth

Jianrong Wu, Xudong Fu, Yun Jiang, Xu Ma, Hongtao Zhang, Xiaobei Zhan*

Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Keywords:
Polysialic acid
Molecular weight stability
Dipotassium phosphate
Conformation

ABSTRACT

This work elucidated the intrinsic mechanism underlying the influence of K_2HPO_4 on PSA production and molecular weight (MW) stability. Among the different potassium salts mixed with K_2HPO_4 in the initial medium, those with buffering capacity were favorable for PSA production. In the bioreactor culture with pH control, adding an appropriate concentration of K_2HPO_4 could enhance PSA production. A dual-phase pH control strategy with ammonia water and KOH could also increase the yield and maintain the MW stability of PSA. Zeta potential test, UV/circular dichroism spectra, and transmission electric microscopy were utilized to explore the configuration of K_2HPO_4–PSA complex. The results from this study can serve a good basis for the industrial-scale production of PSA with stable MW.

1. Introduction

Polysialic acid (PSA) is a negatively charged, linear polysaccharide consisting of α-(2,8) and/or α-(2,9)-linked sialic acid (*N*-acetylneuraminic acid, Neu5Ac) with degree of polymerization (DP) range of 8–400; this polysaccharide is commonly found in the terminal end of the neural cell adhesion molecule of vertebrate cells or in the cell surface of some pathogenic bacteria, such as *Escherichia coli* K1 and *Neisseria meningitides* (Rodriguez-Aparicio et al., 1988; Inoue and Inoue, 2001; Nakata and Troy, 2005; Chau et al., 2011). In the vertebrate cells, PSA plays important roles in the plasticity of nervous system and the modulation of cell interactions, such as cell migration, cell recognition, and synapse formation. Given its good biocompatibility, high hydrophilicity, and non-immunogenicity, PSA is a potential biomaterial for tissue engineering, especially in nerve regeneration (Haile et al., 2008). PSA is also used as modification agent to improve the half-life and stability of protein drugs (Chen et al., 2012; Jain et al., 2003). Some groups also use PSA to formulate PSA-containing hydrogel or nanoparticle as drug delivery systems (Bader et al., 2011; Greco et al., 2013; Zhang and Bader, 2012; Wilson et al., 2014).

PSA is mainly obtained through bacterial fermentation with *E. coli* K1 or K235 (Rodriguez-Aparicio et al., 1988; Navasa et al., 2009). Sorbitol as a carbon source is generally favorable for PSA biosynthesis (Ferrero and Aparicio, 2010). PSA yield reaches approximately 5.6 g/L through pH-controlled and fed-batch fermentation (Liu et al., 2010). Using a dual-stage pH control strategy (pH is controlled at 6.4 before 16 h and is increased to pH 7.4 after 16 h), the molecular weight (MW) of PSA reaches 260 kDa (about 800 DP) (Zheng et al., 2013). Xylose and proline are also used as carbon and nitrogen sources for PSA production, respectively; however, the superiority of this combination declines when one of the components is replaced (Rode et al., 2008). High pyruvate content is observed in the culture with xylose used as a substrate. Although *E. coli* K1 grows well in an environment with glucose, glucose metabolism generates high titer of acetate in the broth, thereby affecting PSA synthesis and reducing PSA MW. Sialic acid monomer was previously detected in a culture with glucose as the carbon source. A low glucose concentration of 1.0 g/L controlled by glucose feeding is adopted to improve PSA production in a culture with glucose as the substrate; with pH controlled at 7.0 by ammonia water, the PSA yield reaches 16.1 g/L (MW of 113 kDa) (Chen et al., 2015).

The sialic acid residues in PSA contain a carboxyl group (–COOH), which contributes to the negative charge property of PSA. When PSA is dissolved in water, the α2,8-glycosidic linkages cleave preferably at the linkages between the two internal sialic acid residues under acidic conditions (Ferrero and Aparicio, 2010). Moreover, PSA can form lactones under mildly acidic conditions; such lactonized PSA is resistant to both enzyme- and acid-catalyzed glycosidic bond cleavages (Zhang and Lee, 1999). In general, PSA is unstable in acidic aqueous solutions, and its MW decreases after long period of storage.

In the submerged culture, PSA is released from the cell surface and encounters complicated conditions, such as organic acids (especially acetate), enzymes, various ions, and shear stress. In the flask culture for PSA production, K_2HPO_4 of high concentration (about 100 mM) is commonly used as a buffer. In the reactor culture with pH controlled at

* Corresponding author.
E-mail address: xbzhan@jiangnan.edu.cn (X. Zhan).

http://dx.doi.org/10.1016/j.biortech.2017.08.142
Received 18 July 2017; Received in revised form 19 August 2017; Accepted 22 August 2017
Available online 24 August 2017
0960-8524/

6.4–7.0 by ammonia water or NaOH, a low titer of K_2HPO_4 (2.5–10 g/L) is generally used. Thus, pH is an important factor for PSA biosynthesis because the protons (H^+) from organic acid can induce PSA degradation and reduce the MW of PSA.

In the present work, the mechanism underlying the interaction between PSA and K_2HPO_4 solution was investigated. During the fermentative production of PSA, the yield and MW of PSA were decreased at the final phase after reaching a peak. Various methods were applied to produce PSA with stable MW. K_2HPO_4 was found to stabilize the PSA MW in the final phase.

2. Materials and methods

2.1. Bacterial strain, materials, and media

E coli K235, which was previously developed by our laboratory and preserved as CCTCC M208088, was used to produce PSA (Liu et al., 2010). The seed medium was composed of NaCl (5.0 g/L), tryptone (10 g/L), beef extract (3.0 g/L), and yeast extract (2.0 g/L) (pH 7.2–74). The shake-flask fermentation medium consisted of sorbitol (40 g/L), $(NH_4)_2SO_4$ (5.0 g/L), $K_2HPO_4{\cdot}3H_2O$ (26.2 g/L), tryptone (1.5 g/L), and $MgSO_4$ (0.9 g/L) (pH 7.8). The fed-batch fermentation medium comprised sorbitol (40 g/L), $(NH_4)_2SO_4$ (5.0 g/L), $K_2HPO_4{\cdot}3H_2O$ (5.0 g/L), tryptone (1.5 g/L), and $MgSO_4$ (0.9 g/L) (pH 7.8). Additional 20 g/L sorbitol was added at 16 h of cultivation in the bioreactor.

2.2. Cultivation methods

The bacteria were grown in 50 mM shake flask with 10 mL of LB medium at 37 °C for 12 h. Subsequently, the culture was used to seed the 500 mL baffled flasks with 50 mL of seed medium at 5% inoculum (200 rpm, 37 °C for 12 h) to obtain the seed cultures. Shake-flask culture was performed in 500 mL baffled flasks with 40 mL of shake-flask fermentation medium at 200 rpm and 37 °C for 60 h.

Batch fermentation was conducted in a 7 L fermentor (BioFlo-415, New Brunswick Scientific, USA) at 1.0 vvm air flow and 400 rpm mixing speed with an initial working volume of 4 L of fed-batch fermentation medium and 8% (v/v) inoculum. The pH was controlled at 6.4 through the automatic feeding of 2 M HCl and base solution (ammonia water or 2 M KOH). Additional portion of sorbitol was fed at 16 h of cultivation to obtain a total of 60 g/L sorbitol.

2.3. Analytical methods

Biomass was determined by measuring the OD at 600 nm; 1.0 OD_{600} is equivalent to 0.4 g/L dry cell weight. The culture broth was centrifuged, and the supernatant was subjected to precipitation with three times the volume of ethanol. The precipitated PSA was dissolved in distilled water and was determined the resorcinol method described by Svennerholm (1957). Average DP was determined using the fluorescence method (Markely et al., 2010). Residual sorbitol and acetate in the fermentation broth were determined by HPLC (LC-2010, Shimadzu, Japan) equipped with an Aminex HPX-87H column (Biorad, USA) under the following conditions: mobile phase, 5 mM H_2SO_4; flow rate, of 0.6 mL/min; RI detector; 35 °C; and injection volume, 10 μL.

2.4. Zeta potential determination

Specific amounts of K_2SO_4, K_2HPO_3, $(NH_4)_2SO_4$, Na_2SO_4, and Na_2HPO_3 were added to the PSA solution (2 g/L) to obtain different salt concentrations (0, 25, 50, and 100 mM). The mixtures were incubated at 37 °C water bath for 1 h and subjected to zeta potential determination at 25 °C (ZetaPALS, Brookhaven, USA).

2.5. UV–visible spectroscopy

Aliquots of aqueous PSA (1.5 mL, 2 g/L) were introduced into a quartz cuvette. A specific concentration of K_2HPO_4 was added to obtain the different final concentrations of 0, 20, 50, and 100 mM. Absorption spectra were recorded using a 300 UV-vis spectrophotometer (Thermo Fisher Scientific, USA).

2.6. Circular dichroism (CD) spectroscopy

The CD spectrum of PSA was obtained using a spectropolarimeter (MOS-450, Bio-Logic, France). During CD measurements, an aliquot of 0.1 mL of aqueous PSA (2 g/L) was mixed in 1 cm optical cell (Hellma) with 0.1 mL of K_2HPO_4 solution of different concentrations (0, 20, 50, and 100 mM).

2.7. Transmission electron microscopy (TEM)

PSA–K_2HPO_4 complex was prepared by combining aqueous solutions of PSA (1 mL, 0.2 g/L) and K_2HPO_4 (1 mL, 100 mM) in a water bath at 37 °C and incubating this mixture for 3.5 h. After filtrating with a 0.45 μm filter, the PSA–K_2HPO_4 complex was observed with a TEM (S-4800, Hitachi, Japan). PSA solution in deionized water was used as a control.

3. Results and discussion

3.1. Effect of K_2HPO_4 and Na_2HPO_4 on PSA biosynthesis and MW

A high initial concentration of K_2HPO_4 (90–100 mM) is required for the high production of PSA in *E. coli* flask culture (Lin et al., 2015). If PSA fermentation is conducted in a bioreactor with controlled pH, then K_2HPO_4 concentration can be reduced to 2.5 g/L (Wu et al., 2010). In general, K_2HPO_4 functions as buffer to protect PSA due to its instability in acidic conditions. Two phosphates, namely, K_2HPO_4 and Na_2HPO_4, and their combination (for all cases, the total phosphate is 100 mM) were used for PSA fermentation to determine the intrinsic mechanism. As shown in Fig. 1, the biomass, PSA production, and MW of PSA were increased with the increasing ratio of K_2HPO_4. Thus, sodium was not favorable for PSA production as compared with K_2HPO_4. This

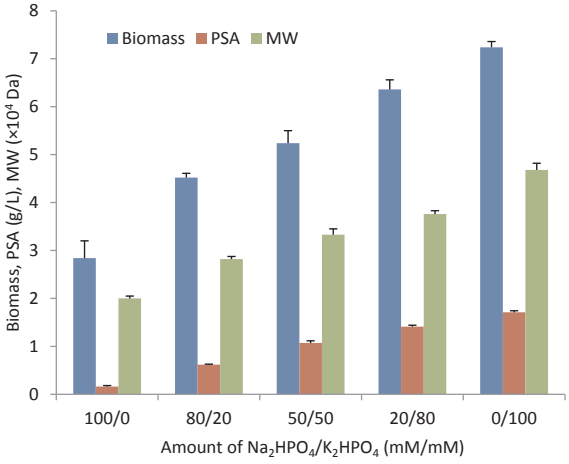

Fig. 1. Effect of different phosphates on the biosynthesis and molecular weight (MW) of polysialic acid (PSA). The fermentation parameters were from 48 h of cultivation in flasks.