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Quantitation of heparosan with heparin lyase III and spectrophotometry

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ABSTRACT

Heparosan is *Escherichia coli* K5 capsule polysaccharide, which is the key precursor for preparing bioengineered heparin. A rapid and effective quantitative method for detecting heparosan is important in the large-scale production of heparosan. Heparin lyase III (Hep III) effectively catalyzes the heparosan depolymerization, forming unsaturated disaccharides that are measurable using a spectrophotometer at 232 nm. We report a new method for the quantitative detection of heparosan with heparin lyase III and spectrophotometry that is safer and more specific than the traditional carbazole assay. In an optimized detection system, heparosan at a minimum concentration of 0.60 g/L in fermentation broth can be detected.

Heparosan is the capsular polysaccharide of the *Escherichia coli* K5 strain. It serves as an important precursor in heparin biosynthesis and as an intermediate in the chemoenzymatic synthesis of bioengineered heparin [1]. Heparin has been used widely as an anticoagulant and antithrombotic drug, and it has attracted increasing interest in anticancer and anti-inflammation therapies. Approximately 100 tons of pharmaceutical-grade heparin products is produced and used annually worldwide [2]. The biological safety of heparin produced from animal source has received scrutiny since the crisis of heparin contamination in 2008 [3–5]. The preparation of a bioengineered heparin from a microbially produced heparosan offers a potentially safer alternative for animal-sourced heparin [6–8]. Microbial heparosan is composed of alternating *N*-acetyl-α-D-glucosamine (GlcNAc)[1] and β-D-glucuronic acid (GlcA) (Fig. 1A), identical to animal-derived heparosan and similar to heparan sulfate (HS) and heparin in the backbone structure.

A rapid and effective quantitative method for detecting heparosan is important in the large-scale production of heparosan. Currently, the most common method for quantifying heparosan uses the carbazole assay for uronic acids [9], which is not applicable directly for determining heparosan in fermentation broth because many substances, including glucose, proteins, and salts, can interfere with the accuracy of this colorimetric assay. Capillary electrophoresis (CE) has been used for quantifying purified heparosan [10]. In addition, nuclear magnetic resonance (NMR) spectroscopy is useful for the quantification of heparosan K5 capsular polysaccharides recovered from fermentation broth. In this NMR assay, heparosan concentration is determined from the area of the *N*-acetyl peak in the ^{1}H NMR spectrum [11]. Both CE and NMR determinations require multiple recovery, pretreatment, and purification steps and use special instrumentation. In this study, we developed a simple and reliable method for the quantitative detection of heparosan using heparin lyase III (Hep III, EC 4.2.2.8) and relying on a readily available and inexpensive spectrophotometer.

Heparosan fermentation was carried out in a 5-L fermentor (BIOSTAT B Plus, Sartorius Stedim Biotech, Germany) based on the previously reported culturing method for *E. coli* K5 [12]. The glucose-defined medium contains 20 g/L glucose, 4.5 mg/L thiamine–HCl, 13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.4 g/L $MgSO_4 \cdot 7H_2O$, 1.7 g/L citric acid, and 10.0 ml of trace metal solution (see online supplementary material for details). The supernatant of fermentation broth was obtained by centrifugation at 12,000*g* for 15 min (Sorvall Biofuge Stratos, Thermo Scientific, USA). Crude heparosan was precipitated by adding 3 volumes of ethanol left at 4 °C overnight and recovered by centrifugation (12,000*g*) for 30 min. The crude heparosan was recovered using DEAE Sepharose resin column (GE Healthcare, USA) and ethanol precipitation. Hep

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[1] *Abbreviations used:* GlcNAc, *N*-acetyl-α-D-glucosamine; GlcA, β-D-glucuronic acid; HS, heparan sulfate; CE, capillary electrophoresis; NMR, nuclear magnetic resonance; Hep III, heparin lyase III; PAGE, polyacrylamide gel electrophoresis.

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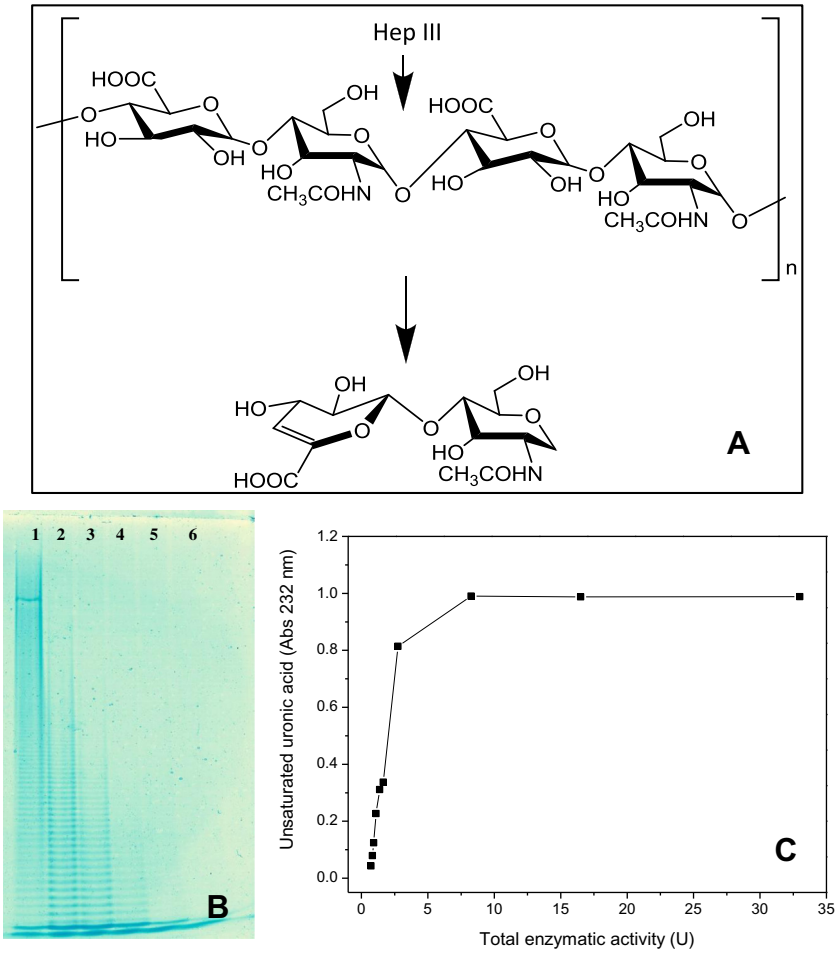

Fig.1. (A) Scheme of heparosan digestion with Heparin lyase III. (B) PAGE analysis of the enzymatic depolymerization of heparosan by Heparin lyase III at different time points, Lanes 1 to 6: Heparin lyase III digested heparosan at 0, 15, 30, 60, 90, and 120 min, respectively. (C) Effect of Heparin lyase III amount on heparosan digestion.

III was expressed in *E. coli* BL21(DE3) harboring a pET-15b–HepC vector (kindly supplied by Jian Liu, University of North Carolina at Chapel Hill) and purified using Ni-NTA agarose column. The enzyme activity was measured and calculated based on the increase in absorbance at 232 nm when HS was depolymerized to unsaturated uronic acid by Hep III.

Hep III catalyzes the eliminative scission of the bonds between GlcNAc and GlcA, depolymerizing heparosan into disaccharide product (Fig. 1A). Polyacrylamide gel electrophoresis (PAGE) analysis was performed, based on previously described methods [8,13], on depolymerized heparosan samples taken at various time points throughout the digestion (see supplementary materials for details). The PAGE results (Fig. 1B) indicate that the high-molecular-weight heparosan was gradually converted after a prolonged enzymatic reaction time to unsaturated disaccharide product. Heparosan (20 mg) was completely depolymerized after 90 min with the introduction of 41 U of Hep III (1 unit was defined as the amount of enzyme needed to form 0.1 μmol of unsaturated uronic acid residue per hour at pH 7.5 and 25 °C). The depolymerization product is an unsaturated disaccharide that can be measured with a spectrophotometer at 232 nm and used for quantifying heparosan.

It is necessary to use excess Hep III in the reaction to ensure complete depolymerization of heparosan to unsaturated disaccharide product. The effect of the Hep III amount on the depolymerization of heparosan (10 g/L) was evaluated. The result (Fig. 1C) shows that the absorbance stopped increasing (A_{232} kept at 0.981) when a greater Hep III amount than 8.25 U was added into a 300-μl enzymatic reaction, suggesting that 8.25 U Hep III is excess.

This enzymatic reaction was first tested with low concentrations of purified heparosan. The reagents were reagent A (20 mM Tris–HCl with 50 mM sodium chloride, 4 mM calcium chloride, and 0.01% [w/v] bovine serum albumin, pH 7.5), reagent B (heparosan solution in water), reagent C (Hep III in cold reagent A), and reagent D (50 mM HCl solution). To start the assay, reagent A (0.23 ml) and reagent B (0.05 ml) were mixed at 25 °C. Reagent C (Hep III, 0.02 ml) was added and incubated at 25 °C for 30 min, and the enzyme reaction was terminated by adding reagent D (2.7 ml). The absorbance at 232 nm of the final depolymerization product was measured with a spectrophotometer. The blank sample was prepared with the same procedure except that no Hep III was added. Fig. 2A shows an excellent correlation between heparosan concentration and the A_{232}, as can be seen in the linear fitting equation: $y = 0.1495x - 0.2024$ ($R^2 = 0.990$). Next, different amounts of heparosan were added to the supernatant of fermentation broth (the supernatant was collected when heparosan was in the low-yield period of K5 growth) to apply this method to the detection of crude heparosan in fermentation broth. The samples with these higher concentrations of heparosan in the fermentation broth (0.12 ml) were subject to Hep III enzymatic reaction (in 0.48 ml of reagent A) and spectrophotometry measurement. Fig. 2B shows an excellent linear calibration line: $y = 0.3239x - 0.0982$ ($R^2 = 1$). When we established the calibration line as in Fig. 2B, we accounted for the heparosan already present

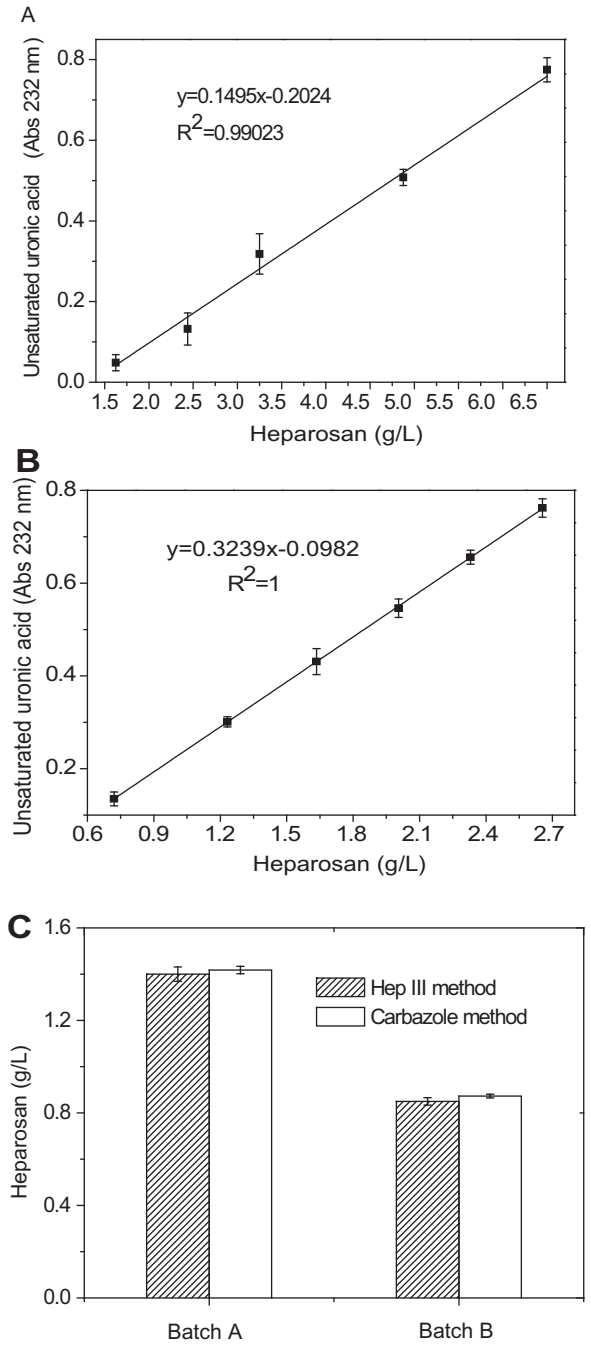

Fig.2. (A) Calibration line of heparosan detection in buffer enzymatic reaction system. (B) Calibration line of heparosan detection in enzymatic reaction system with fermentation broth. (C) Comparison of heparosan detection of two batches of broth using enzymatic method and carbazole assay. Error bars are standard deviations obtained from the triplicate measurements.

in the supernatant by using the supernatant when heparosan was in the low-yield period of K5 growth. The concentration of heparosan in the supernatant was confirmed to be approximately 0.02 g/L by carbazole assay, suggesting that the low heparosan content in the supernatant has a negligible effect on the assay. Differences between the slopes in Fig. 2A and B are the result of the different conditions used in these assays required to cover a large range of heparosan concentrations. Heparosan in two batches of fermentation broth was directly measured using this method. The results were compared with those of carbazole assay on the heparosan that was isolated by precipitation from the broth of the same batches. The results show that the values determined using the enzymatic method match well with those determined by carbazole assay (Fig. 2C).

In summary, we developed a simple and quick method for the quantitation of heparosan using Hep-III-catalyzed depolymerization detected with a spectrophotometer. This method can detect heparosan at a minimum concentration of 0.12 g/L in Tris–HCl buffer and 0.60 g/L in fermentation broth. It provides a safer and more specific procedure than the traditional carbazole assay. In addition, it also can be used for directly (i.e., without purification) quantitating heparosan in the broth of *E. coli* K5 fermentation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2013.10.029.

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