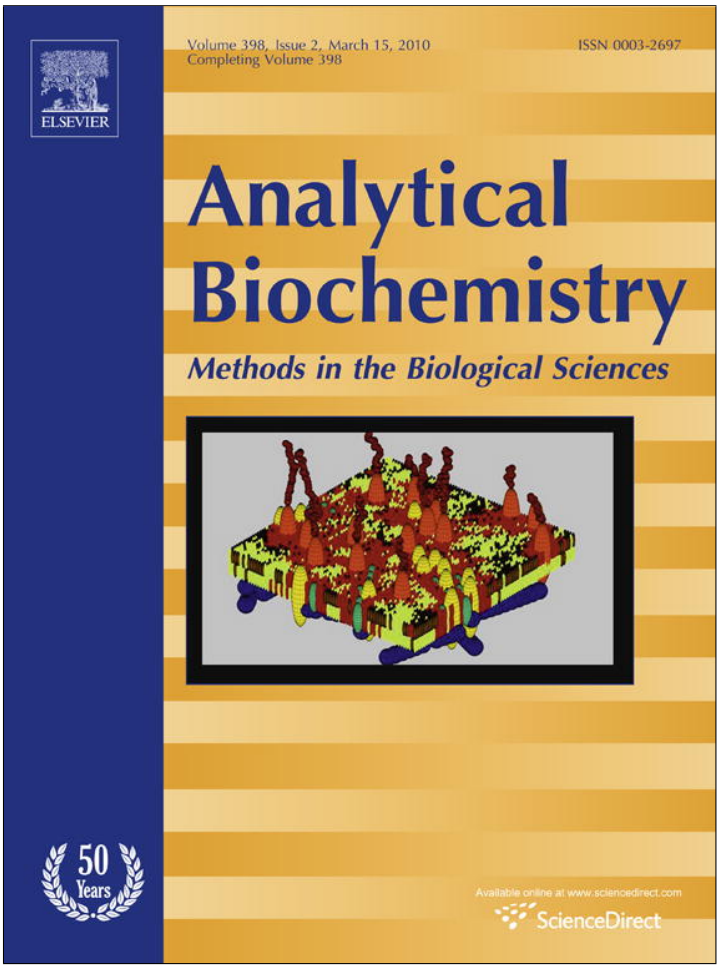

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Notes & Tips

Nuclear magnetic resonance quantification for monitoring heparosan K5 capsular polysaccharide production

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ABSTRACT

Traditional chromatographic quantification methods for heparosan produced from the *Escherichia coli* K5 strain rely on extensive purification requiring laborious sample preparation. These methods are time-consuming, often resulting in sample loss during purification, and thus might not accurately reflect the amount of heparosan in the original mixture. A simple, sensitive ^{1}H nuclear magnetic resonance (NMR) quantification method that directly quantifies heparosan K5 polysaccharide present in *E. coli* fermentation supernatant is described.

Heparosan is a polysaccharide with a β-1,4-D-glucuronic acid (GlcA)[3] and α-1,4-*N*-acetyl-D-glucosamine (GlcNAc), [→4)GlcA-β-(1–4) GlcNAc-α (1→]$_n$ repeating disaccharide unit. Heparosan is biosynthesized as a bacterial capsule, and this polysaccharide is identical to the precursor of the mammalian heparin and heparan sulfate in mammals [1]. Heparin and heparan sulfate participate in many important biological processes, including blood anticoagulation, viral and bacterial infection and entry, angiogenesis, inflammation, cancer, and development [2–4]. Heparin, extracted from porcine intestines, is one of the oldest drugs and is currently in widespread use for the prevention of blood clotting [2,5]. In 2008, a new, rapid-onset acute side effect resulting in hypotension was associated with certain lots of heparin contaminated with oversulfated chondroitin sulfate (OSCS) [6,7]. A bioengineered heparin prepared from heparosan offers a potential alternative for the preparation of a safer heparin [8,9]. Heparosan, of molecular weight greater than 10,000, is readily obtained from *Escherichia coli* K5 strain [10] and can be enzymatically modified to produce an anticoagulant polysaccharide similar to heparin [8,9]. Heparosan itself has also been explored as a biomaterial because of its stability and nonimmunogenic characteristics [11].

K5 heparosan is conveniently prepared by *E. coli* fermentation and recovered directly from the fermentation supernatant [12]. Thus, the heparosan concentration in the fermentation supernatant is a critical parameter for optimizing the fermentation process and calculating the purification efficiency. The carbazole assay has been used in the past to quantify polysaccharides that contain uronic acid [13]. Unfortunately, media components often interfere with this colorimetric assay. Capillary electrophoresis (CE) has been used to quantify purified heparosan, but media components may also interfere with CE analysis [14]. Disaccharide analysis using high-performance liquid chromatography (HPLC) and HPLC/mass spectrometry (MS) can also been used to quantify heparosan [15], but these methods require a time-consuming enzymatic digestion of the heparosan and the removal of proteins, enzymes, and buffer salts prior to sample analysis.

Nuclear magnetic resonance (NMR) is a powerful technique for elucidating the structure of molecules that can be used in quantitative analysis. ^{1}H NMR has been used for quantifying carrageenans in blends [16], for monitoring the wine and beer fermentation processes [17,18], for quantifying derivatized *Haemophilus influenzae* type b polysaccharide intermediate [19], and in many other quantitative applications [20,21]. The major advantages of NMR-based quantification are simple sample preparation and its nondestructive nature.

E. coli K5 (ATCC 23506) was cultured in batch on a medium consisting of 20 g/L glucose, 20 mg/L thiamine, 13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.4 g/L $MgSO_4{\cdot}7H_2O$, and 1.7 g/L citric acid, to which was added 10.0 ml of trace metal solution consisting of 10.0 g/L $FeSO_4{\cdot}7H_2O$, 2.0 g/L $CaCl_2$, 2.2 g/L $ZnSO_4{\cdot}7H_2O$, 0.5 g/L $MnSO_4{\cdot}4H_2O$, 1.0 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, and 0.02 g/L $Na_2B_4O_7{\cdot}10H_2O$ in 5 M hydrochloric acid. The feeding solution during the fed batch cultures consisted of: 250 to 1000 g/L glucose, 20 g/L $MgSO_4{\cdot}7H_2O$, and 0.15 or 0.25 g/L thiamine [22]. The batch growth phase began by inoculating 10 vol% seed culture

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[3] *Abbreviations used:* GlcA, β-1,4-D-glucuronic acid; GlcNAc, α-1,4-*N*-acetyl-D-glucosamine; OSCS, oversulfated chondroitin sulfate; CE, capillary electrophoresis; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple quantum correlation.

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prepared in a shake flask into 3 L of culture medium grown in an Applikon 7-L fermentor. The temperature was maintained at 37 °C. The pH was maintained between 6 and 8 by continuously adjusting with 29% ammonia solution. The culture was fed exponentially after the glucose in the medium was depleted. Samples were collected from the fermentor at various time points and centrifuged at 12,000*g* for 30 min to separate supernatant from cells, and 1-ml aliquots of supernatant were lyophilized.

Lyophilized supernatant was dissolved in 400 µl of D_2O and lyophilized, then redissolved in 400 µl of D_2O (99.96 atm%) containing 71 µg of sodium terephthalate before being transferred to a 5-mm NMR tube. (Water suppression can be used to eliminate the need for lyophilization and D_2O exchange steps but results in lower spectral quality.) Standards were prepared by dissolving purified K5 samples (0.2–1.6 mg) in 400 µl of D_2O containing 71 µg of sodium terephthalate.

^{1}H NMR (8 scans) and heteronuclear multiple quantum correlation (HMQC) NMR were performed on a Bruker 600-MHz NMR spectrometer, and acquisition of the spectra was carried out using TopSpin 2.0 software. All of the spectra were acquired at a temperature of 298 K. The relaxation delay time D1 was set to 20 s to ensure that the protons in the sodium terephthalate and *N*-acetyl group were adequately relaxed. ^{1}H NMR spectra were processed in MestRe-C software. The phase of the spectra was manually corrected, and the baseline of the spectra was adjusted with the "baseline correction–use polynomial" function. The integration of the peaks was performed using the "integration" function, with the peak area being selected manually. The two-dimensional NMR spectra were processed and analyzed using the program Sparky (version 3.114).

Sodium terephthalate was selected as a water-soluble, stable, and nonreactive internal standard for the K5 heparosan quantification because it shows a single peak at 7.91 ppm in the ^{1}H NMR in a region where there were no interfering peaks from the heparosan and fermentation components (Fig. 1). The *N*-acetyl peak for heparosan at 2.04 ppm was selected, and the peak area was normalized to the sodium terephthalate peak area. A standard curve, prepared from ^{1}H NMR spectra of triplicate samples at each heparosan concentration, showed good linearity (Fig. 2A).

E. coli K5 fermentation supernatant is a complicated mixture containing complex medium, proteins, metabolic products, and K5 polysaccharide (Fig. 1D). Most of the peaks in the ^{1}H NMR spectrum overlap with media components and cannot be used to quantify K5 polysaccharide. However, the peak at 2.04 ppm, corresponding to the methyl protons in *N*-acetyl groups of heparosan, was well resolved and diagnostic of heparosan in the fermentation supernatant. HMQC NMR confirmed the assignment of the peak at 2.04 ppm in the ^{1}H spectrum through its correlation with the ^{13}C signal 23.9 ppm (Fig. 1G) [9]. In a control experiment, *E. coli* BL21, a strain not producing K5 polysaccharide, was grown for 16 h in the same medium. The supernatant and cell pellet, after solubilization by sonication and centrifugation at 7000*g* for 30 min, were examined by ^{1}H NMR and showed no peaks at or around 2.04 ppm, eliminating the possibility that cell wall or cell lysis components from *E. coli* might interfere with the NMR quantification.

Integration of the *N*-acetyl peak at 2.04 ppm against the peak at 7.91 ppm for the internal standard afforded an accurate determination of heparosan concentration in the fermentation supernatant (Fig. 2B). The concentrations determined by ^{1}H NMR were in excellent agreement with concentrations determined by carbazole assay after heparosan recovery and purification. Heparosan concentration in the supernatant increased over the fermentation time, as expected, correlating with the increase in cell density.

In conclusion, ^{1}H NMR affords a simple and reliable method to quantify K5 heparosan from the fermentation. Other polysaccharides containing *N*-acetylhexosamine residues, such as chondroitin (from *E. coli* K4) and hyaluronan, should also be quantifiable using this method.

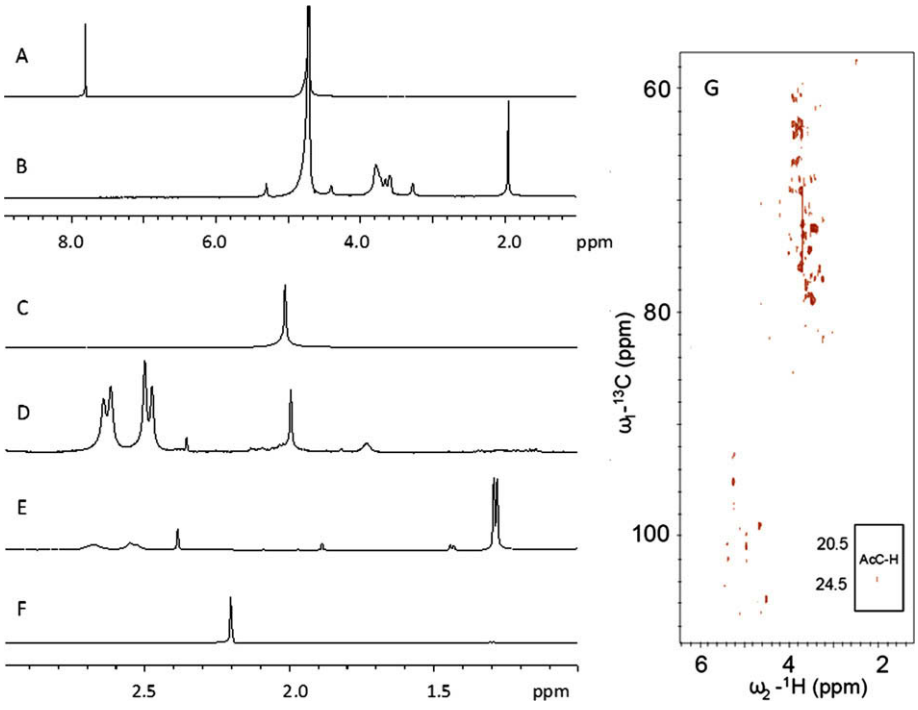

Fig. 1. (A,B) ^{1}H NMR spectra of sodium terephthalate (A) and K5 heparosan (B). (C–G) ^{1}H NMR spectra expanded between 1 and 3 ppm of K5 heparosan (C), *E. coli* K5 heparosan fermentation supernatant (D), *E. coli* BL21 culture supernatant (E), *E. coli* BL21 lysate (F), and HMQC NMR of purified K5 heparosan showing the correlation of ^{1}H and ^{13}C signals confirming assignments (G).

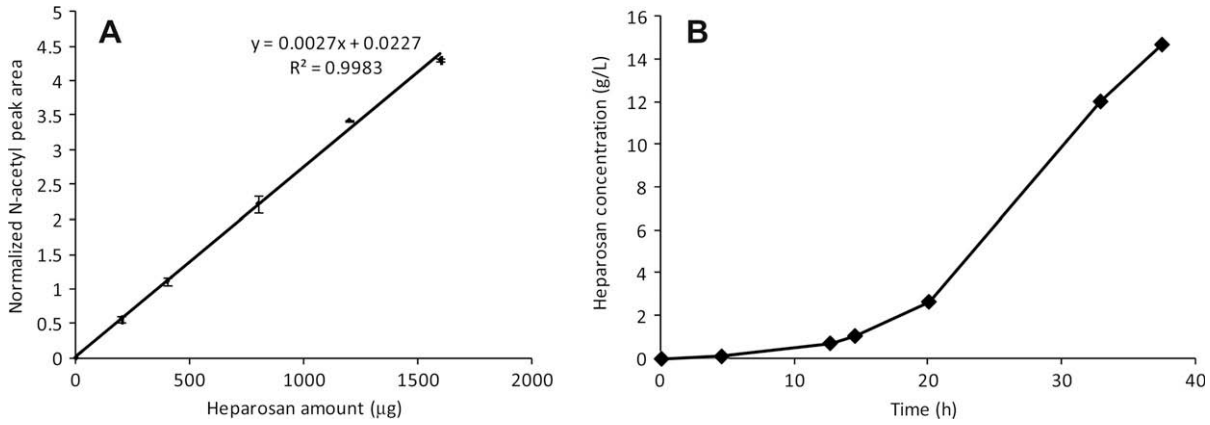

Fig. 2. (A) Standard curve for the quantification of K5 heparosan. *x* Axis: amounts of heparosan in the NMR samples. *y* Axis: normalized *N*-acetyl peak area from the ^{1}H NMR spectra. The equation and R^2 value are displayed. Data were acquired from triplicate experiments. (B) Time course of the K5 heparosan concentration during the fermentation process.

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