Determination of the Substrate Specificities of *N*-Acetyl-D-glucosaminyltransferase†

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ABSTRACT: Heparan sulfate plays a wide range of physiological and pathological roles. Heparan sulfate consists of glucosamine and glucuronic/iduronic acid repeating disaccharides with various sulfations. Synthesis of structurally defined heparan sulfate oligosaccharides remains a challenge. Access to nonsulfated and unepimerized heparan sulfate backbone structures represents an essential step toward de novo enzymatic synthesis of heparan sulfate. The nonsulfated, unepimerized backbone heparan sulfate is similar to the capsular polysaccharide from *Escherichia coli* strain K5. The biosynthesis of this capsular polysaccharide involves in *N*-acetylglucosaminyltransferase (KfiA) and D-glucuronyltransferase (KfiC). In this study, we report the characterization of purified KfiA. KfiA was expressed in a C-terminal six-His fusion protein in BL21 star cells coexpressing chaperone proteins GroEL and GroES. The recombinant KfiA was purified to homogeneity with a Ni−agarose column. The binding affinities of various UDP-sugars for KfiA were determined using isothermal calorimetry titration, indicating that both the *N*-acetyl group and sugar type may be essential for donor substrates to bind KfiA. Kinetic analysis of KfiA toward different sizes of oligosaccharide revealed that KfiA is less sensitive to the size of the acceptor substrates. The results from this study open a new approach for the synthesis of the heparan sulfate backbone.

Heparan sulfate (HS)[1] is a linear polymer consisting of alternating hexuronic acid [D-glucuronic acid (GlcA) or L-iduronic acid (IdoUA)] and D-glucosamine (GlcN) units carrying sulfo groups. HS is widely distributed on the surface of mammalian cells and in the extracellular matrix. By interacting with various complementary proteins, HS can mediate and regulate many cellular events (*1*). Genetic knockout of the genes involved in the biosynthesis of HS has identified HS as an important regulator of cell growth and differentiation (*2*). The role of HS in regulating blood coagulation has a wide range of clinical applications which is evident in the fact that heparin, an analogue of HS, is the most commonly used anticoagulant drug (*3*). HS also plays essential roles in pathological conditions such as mediating tumor metastasis (*4*) and viral infection (*5*).

The biosynthesis of HS occurs in the Golgi apparatus, and the biosynthesis includes the elongation of a polysaccharide backbone as well as the modifications of the backbone (*6*). HS is initially synthesized as a copolymer of GlcA and N-acetylated glucosamine (GlcNAc) by D-glucuronyl and *N*-acetyl-D-glucosaminyltransferase, which is carried out by HS copolymerase, EXT1 and EXT2 (*7*). The backbone is then modified by several reactions, including N-deacetylation and N-sulfation of GlcN, C_5-epimerization of GlcA to form IdoUA residues, 2-O-sulfation of IdoUA and GlcA, and 6-O-sulfation and 3-O-sulfation of GlcN. The enzymes that catalyze those modifications have been cloned and characterized. The unique sulfation patterns of HS determine the specific functions of HS (*8*). However, the mechanism for the biosynthesis of the HS polysaccharide with specific sulfated saccharide sequences is poorly understood.

Our research efforts have been focused on understanding the structural and functional relationship of HS. One of the approaches is to synthesize structurally defined HS, which can be used to probe its function in a given biological system. Although chemical synthesis of structurally defined HS oligosaccharides has been shown to be the most efficient way to prepare polysaccharides smaller than hexasaccharides (*9*), it has become clear that chemical synthesis alone is currently incapable of generating a majority of larger oligosaccharide structures. Our laboratory and others began to explore a collection of HS biosynthetic enzymes for synthesizing HS with desired biological activities (*10*−*13*). However, it is unclear that the enzymatic approach offers the HS polysaccharide products with defined sequences in part due to the structural heterogeneity of starting backbone structures. Furthermore, the modification of the backbone structure could determine the preference for the subsequent enzymatic modifications and, consequently, regulate the synthesis of the polysaccharide with specific saccharide sequences (*8*). The availability of backbone structures with defined size and N-sulfation serves as an essential step toward the de novo synthesis of HS as well as a critical reagent for delineating the biosynthetic mechanism of HS.

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[1] Abbreviations: HS, heparan sulfate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GalNAc, UDP-*N*-acetylgalactosamine; UDP-GlcA, UDP-glucuronic acid; GlcN-1-P, glucosamine 1-phosphate; CoASH, coenzyme A; DTT, dithiothreitol; GlmU, glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase.

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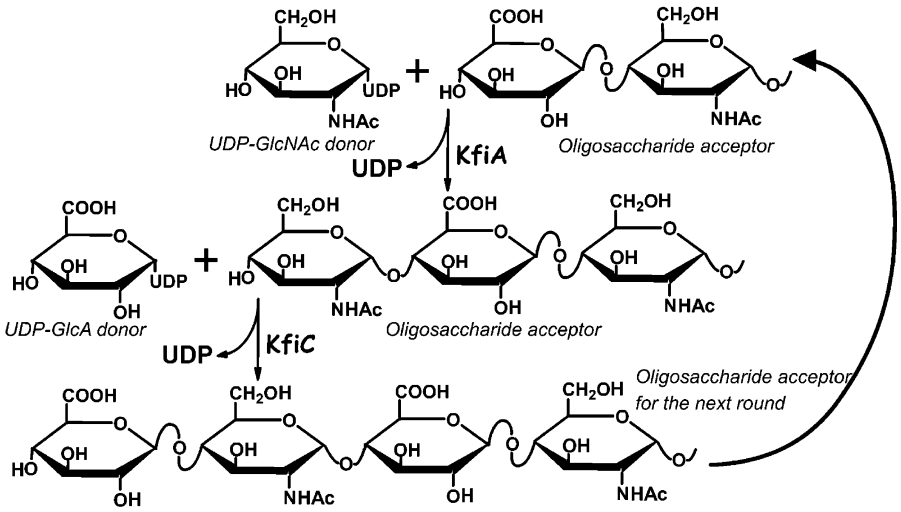


FIGURE 1: Reactions catalyzed by KfiA and KfiC. In the biosynthesis of K5 polysaccharide, KfiA and KfiC extend the polysaccharide chain via alternating additions of GlcA and GlcNAc from the corresponding UDP-sugars (donor) to the nonreducing end of the growing chain (acceptor).

Escherichia coli K5 capsular polysaccharide consists of GlcNAc and GlcA repeating disaccharide, an analogue of the unsulfated and unepimerized HS backbone (*14*). Using the enzymes involved in the biosynthesis of K5 polysaccharide offers an alternative approach to preparing the HS backbone structure (Figure 1). Previous studies (*15*) have indicated that the biosynthesis of K5 polysaccharide requires an *N*-acetyl-D-glucosaminyltransferase (KfiA). This enzyme, together with D-glucuronyltransferase, KfiC (*16*), alternatively incorporates GlcNAc and GlcA residues, from UDP-GlcNAc and UDP-GlcA to the nonreducing ends of the oligo- or polysaccharides (Figure 1). Although KfiA is of interest in the synthesis of HS backbones, the substrate specificity of this enzyme is still poorly understood. The lack of information about its biochemical features is primarily due to the unavailability of the purified enzyme. In this work, an efficient way to prepare purified KfiA in multimilligram quantities is described. Substrate specificities of KfiA were also characterized. The results from this study demonstrated the possibility of synthesis of HS backbone structure from a disaccharide structure using KfiA. The availability of KfiA and HS biosynthetic enzymes in large quantities could be employed in the de novo synthesis of HS with defined saccharide sequences.

MATERIALS AND METHODS

Materials. Unlabeled K5 polysaccharide was purified from *E. coli* strain K5 (from American Type Culture Collection) following a previous report (*14*). ^{3}H-labeled K5 polysaccharide was prepared from the bacterial media of an *E. coli* K5 overnight culture containing 0.1 mCi/mL [6-^{3}H]glucose (MP Biomedical). CoASH, coenzyme A synthetase, inorganic pyrophosphatase, ATP, UTP, UDP, UDP-glucose, UDP-GlcA, UDP-GlcNAc, and UDP-GalNAc were purchased from Sigma.

Expression and Purification of KfiA and KfiC. The *KfiA* and *KfiC* gene was amplified from *E. coli* K5 genomic DNA using two primers with the corresponding oligonucleotide sequences (KfiA, 5′-ATATATAAGGATCCGATGATTGTTGCAAATATGTC and 5′-ATAATATACTCGAGCCCTTCCACATTATACAC, where the cleavage sites of *Bam*HI or *Xho*I are underlined; KfiC, 5′-ATATATAAGCGGCCGCAATGAACGCAGAATATATA and 5′-AATTATAACTCGAGTTGTTCAATTATTCCTGA, where the cleavage sites of *Not*I or *Xho*I are underlined). The genes were cloned into the pET21b+ vector (Novagen) to yield the C-terminal six-histidine-tagged protein.

Expression of KfiA and KfiC was achieved in BL21 star (DE3) cells carrying pGro7 plasmid (Takara) which expresses chaperone proteins GroEL and GroES. Briefly, cells containing the plasmids expressing KfiA or KfiC, GroEL, and GroES were grown in LB medium supplemented with 50 μg/mL carbenicillin and 35 μg/mL chloramphenicol at 37 °C. When the A_{600} reached 0.6, the temperature was decreased to 22 °C, and arabinose (1 mg/mL) or isopropyl β-D-thiogalactopyranoside (IPTG, 1 mM) was added to induce the expression of chaperone and KfiA or KfiC, respectively. The culture was shaken overnight at 22 °C. The bacteria were harvested and lysed by sonication in a buffer containing 25 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 30 mM imidazole. The proteins were purified with Ni-Sepharose 6 Fast Flow (0.75 cm × 10 cm, Amersham), which was eluted with a linear gradient from 30 to 250 mM imidazole in 500 mM NaCl and 25 mM Tris (pH 7.5) in 60 mL. The purity of the resultant proteins was analyzed via precasted 12% SDS−PAGE (Bio-Rad).

Preparation of K5-Derived Oligosaccharides. Even-numbered oligosaccharides were prepared from a purified capsular polysaccharide of *E. coli* K5, with the structure $(GlcA\text{-}GlcNAc)_n$. The K5 polysaccharide (5.5 mg) mixed with ^{3}H-labeled K5 polysaccharide (80 000 cpm) was partially N-deacetylated by being treated with 2 M NaOH at 68 °C for 60 min followed by deamination with nitrous acid at pH 4.5 (*17*). The resultant oligosaccharides were reduced with sodium borohydride to yield a reducing terminal residue of 2,5-anhydromannitol ($aMan_R$). The oligosaccharides $(GlcA\text{-}[GlcNAc\text{-}GlcA]_n\text{-}aMan_R)$ were size-fractionated with a BioGel P-10 (Bio-Rad) column, which was eluted with 20 mM Tris-HCl (pH 7.4) and 1 M NaCl. ^{3}H radioactivity was determined. Those fractions corresponding to appropriate sizes of oligosaccharides ranging from disaccharides to octasaccharides were pooled separately, dia-

lyzed against double-deionized water for 12 h at 4 °C using a 3500 Da MWCO membrane (Fisher Scientific), and lyophilized. The amount of purified oligosaccharides was determined on the basis of the specific ^{3}H radioactivity of the polysaccharide.

Preparation of ^{3}H-Labeled UDP-GlcN[^{3}H]Ac. UDP-GlcN-[^{3}H]Ac was prepared from sodium [^{3}H]acetate (500 mCi/mmol, MP Biomedicals) using glucosamine-1-phosphate acetyltransferase/*N*-acetylglucosamine-1-phosphate uridyltransferase (GlmU) following a method described previously (*18*). Briefly, the GlmU gene was amplified from *E. coli* K12 (ATCC) genomic DNA with the 5′-ATATATAA-CATATGTTGAATAATGCTATG and 5′-AATTATAAG-GATCCTCACTTTTTCTTTACCGGAC primers and cloned into the pET21b+ vector using *Nde*I and *Bam*HI sites to prepare the C-terminal six-histidine-tagged GlmU protein. The expression was carried out in BL21 star (DE3) cells. The fusion protein was purified with Ni-Sepharose 6 Fast Flow resins as described above. The reaction mixture contained Tris (50 mM, pH 7.4), sodium [^{3}H]acetate (0.2 mM, 0.1 μCi/μL, 500 mCi/mmol), $MgCl_2$ (5 mM), dithiothreitol (DTT) (0.2 mM), UTP (2.5 mM), glucosamine 1-phosphate (GlcN-1-P) (2.5 mM), ATP (10 mM), CoASH (0.02 mM), acetyl coenzyme A synthetase (0.1 mg/mL), recombinant GlmU (0.4 mg/mL), and inorganic pyrophosphatase (0.01 unit/μL). The reaction mixture was incubated at 30 °C overnight with mild agitation. The yield of UDP-GlcN[^{3}H]Ac was monitored with RPIP-HPLC as demonstrated by a ^{3}H-labeled peak coeluted with unlabeled UDP-GlcNAc standard (Sigma) that has absorbance at 256 nm. The specific ^{3}H radioactivity of UDP-GlcN[^{3}H]Ac was estimated to be 172 mCi/mmol.

N-Acetyl-D-glucosaminyltransferase Assays. Experiments were carried out with purified KfiA and size-defined oligosaccharide acceptors. The assay was designed to assess the incorporation of the GlcN[^{3}H]Ac residue into defined oligosaccharide substrates. The reaction mixture contained 15 000 cpm of UDP-GlcN[^{3}H]Ac, 100 μg/mL KfiA, 1% Triton X-100, 25 mM Tris-HCl (pH 7.2), and 10 mM $MnCl_2$ (*15*). Reverse-phase ion-pairing HPLC (RPIP-HPLC) was employed to monitor the products.

Isothermal Titration Calorimetry (ITC). ITC was performed on a MicroCal VP-ITC device (*19*). Solutions were degassed under vacuum before being used. Purified KfiA was dialyzed against a buffer containing 25 mM Tris, 250 mM NaCl, and 10 mM $MnCl_2$ (pH 7.5) (Tris buffer) at 4 °C overnight. Experiments were conducted in Tris buffer using 50 μM KfiA (1.35 mg/mL). Titrations were performed by injecting 5 μL of 1.5 mM UDP and UDP-sugars (UDP-GlcNAc, UDP-GlcA, UDP-glucose, and UDP-GalNAc) in 25 mM Tris buffer [250 mM NaCl and 10 mM $MnCl_2$ (pH 7.5)]. Data analysis was completed using Origin version 7.

Inhibition Effect of UDP on KfiA Activity. Reactions were conducted at fixed concentrations of disaccharide (82 μM) and UDP-GlcN[^{3}H]Ac (8.1 μM, 1.4 μCi/mL) and UDP concentrations varying from 0 to 500 μM. Other conditions were identical to those of previous assays. The reaction mixtures were incubated at 37 °C for 2 min and the reactions quenched by heating to 100 °C. The products were quantified by RPIP-HPLC as described above.

Mass Spectrometry. The oligosaccharides purified with BioGel P-10 were further desalted by dialysis. The samples were then dried on a speed-Vac device, reconstituted in doubled-deionized water (50 μM, 200 μL), and introduced by direct infusion (10 μL/min) into the electrospray ionization mass spectrometer (Agilent 1100 MSD-Trap at the Mass Spectroscopy Core in the University of North Carolina School of Pharmacy) (*20*). Experiments were performed at negative ionization mode (350 °C, dry gas at 15 psi, nebulizing gas at 5 L/min).

Determination of the Concentration of K5 Polysaccharide. The concentration of K5 polysaccharide was determined by estimating the amount of the resultant disaccharide from a complete digestion of K5 polysaccharide with heparin lyase III (*21*). Briefly, K5 polysaccharide (10 μL) was mixed with heparin lyase III (10 μg) and incubated at 37 °C for 3 h. The resultant disaccharide (ΔUA-GlcNAc) was resolved via RPIP-HPLC and quantified by comparing the area of a UV_{232} peak with the same disaccharide standard (Seikagaku) with a known amount.

Kinetic Analysis of KfiA. Enzymatic activity of the purified KfiA was evaluated by monitoring the incorporation of GlcN-[^{3}H]Ac. Reactions were carried out at oligosaccharide acceptor substrate concentrations varying from 3 to 800 μM in a buffer of the enzymatic assay described above. To measure the K_m and K_{cat}/K_m values of UDP-GlcNAc, we conducted the reactions with the disaccharide (700 μM) as an acceptor substrate. To study the influence of KfiC on KfiA kinetics, enzymatic reactions toward disaccharide substrates were also prepared with KfiA at 2.7 μM and KfiC at 1.5 μM. Because the reaction rate remained constant up to 6 min, the average reaction rate of the first 2 min represented the initial velocity. The reaction mixtures were incubated at 37 °C for 2 min and reactions quenched by heating to 100 °C. The products were resolved with a C_{18} column (0.46 cm $\times$ 25 cm) (Vydac) under the reverse-phase ion-pairing HPLC (RPIP-HPLC) elution condition (*22*). Briefly, the column was isocratically eluted with different concentrations of acetonitrile depending on the size of oligosaccharide product in a solution containing 38 mM ammonium phosphate monobasic, 2 mM phosphoric acid, and 1 mM tetrabutylammonium phosphate monobasic (Fluka) at a flow rate of 0.5 mL/min. For the trisaccharide product, no acetonitrile was present in the elution buffer; for the penta- and heptasaccharide, 7.5% acetonitrile was present in the elution buffer, and for the nonasaccharide, 15% acetonitrile was present in the mobile phase. The eluent was monitored by an on-line radioactive detector (β-ram, INUS) for measuring ^{3}H radioactivity.

RESULTS

Expression and Purification of KfiA. KfiA was previously identified to carry *N*-acetylglucosaminyltransferase activity responsible for the synthesis of capsular polysaccharide in *E. coli* K5 using a site-directed mutagenesis approach in a cell-based assay (*15*). However, the biochemical character of this enzyme has not been fully elucidated because of the unavailability of purified enzyme. The potential application of KfiA in synthesizing defined-size HS backbone structures prompted us to investigate its donor and acceptor substrate specificities. The full-length KfiA gene was cloned from *E. coli* K5 genomic DNA and expressed in a format of C-terminal six-His fusion protein as described in Materials and Methods. Recombinant KfiA was purified by affinity

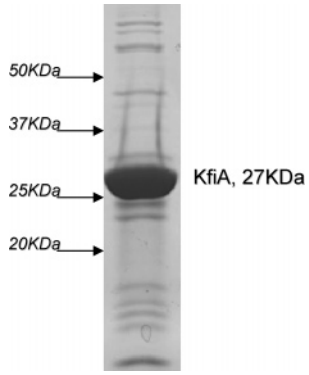


FIGURE 2: SDS−PAGE analysis of purified KfiA. Affinity chromatography-purified KfiA protein was resolved on a precasted 10% SDS−PAGE gel. The gel was stained with Coomassie blue. Migration positions of molecular markers (from Bio-Rad) are indicated. The apparent mass was approximately 27 kDa. The predicted molecular mass of KfiA is 27.3 kDa.

chromatography using a nickel column. The purified KfiA predominantly migrated as one major band at ~27 kDa on a 10% SDS−PAGE gel (Figure 2), which is nearly identical to the calculated molecular mass of KfiA (27.3 kDa). We estimated that the purity of KfiA was greater than 90%. Approximately 10 mg of purified KfiA was obtained from 1 L of bacterial culture.

Preparation of Even-Numbered Oligosaccharide Acceptors. To study the size specificity of the acceptor for KfiA, even-numbered oligosaccharides were prepared from K5 polysaccharide by partial N-deacetylation followed by deaminative cleavage. To monitor the polysaccharide during the purification, unlabeled K5 polysaccharide was mixed with metabolically ^{3}H-labeled K5 polysaccharide. The polysaccharide was deacetylated under a basic condition, and the resultant polysaccharide was subject to nitrous acid (pH 4.5) degradation. The oligosaccharide products with GlcA-[GlcNAc-GlcA]$_n$-aMan$_R$ structures were resolved on a Bio-Gel P-10 column based on the size of the oligosaccharides, such as di-, tetra-, hexasaccharide, etc. (Figure 3). Due to the resolution of the BioGel P-10 column, we were limited to obtaining up to octasaccharide with sufficient purity. The purified oligosaccharides were also subjected to the analysis of electrospray ionization mass spectrometry (ESI-MS) to confirm the molecular mass. The MS spectra of those analyzed oligosaccharides are shown in Figure 4. Furthermore, we did not observe the oligosaccharide with different sizes in a given sample, suggesting that the purified oligosaccharides are sufficiently pure for the kinetic studies as described below. The molecular masses of purified oligosaccharides were consistent with the anticipated size of the oligosaccharide (Table 1).

Determination of the Enzymatic Activity of KfiA. The activity of KfiA was determined using a purified tetrasaccharide as an acceptor, and the product was resolved via RPIP-HPLC. A ^{3}H-labeled peak that eluted at 18 min was observed [Figure 5A (—)], while this ^{3}H-labeled peak was absent in the control [without KfiA enzyme, Figure 5A (••••)], suggesting that a ^{3}H-labeled pentasaccharide was generated due to the action of KfiA. It should be noted that more than 90% of the UDP-GlcN[^{3}H]Ac had been converted to the ^{3}H-labeled pentasaccharide within 30 min under the standard assay condition, demonstrating that recombinant KfiA was highly active. This pentasaccharide was purified

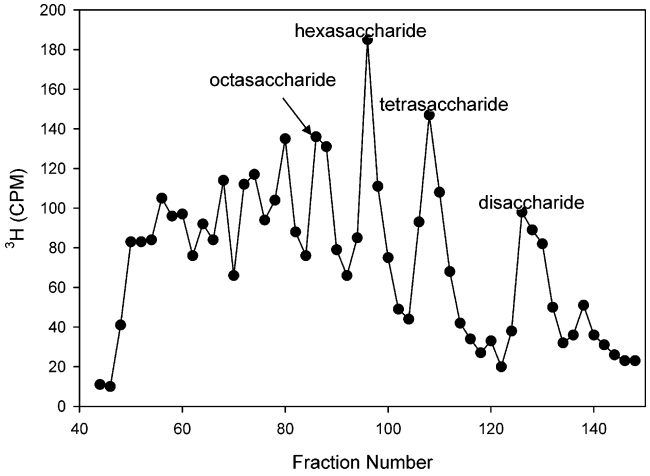


FIGURE 3: Fractionation of partially depolymerized K5 polysaccharide on BioGel P-10. K5 polysaccharide was partially digested as described in Materials and Methods. The products were fractionated by gel chromatography on BioGel P-10 eluted with 20 mM Tris-HCl (pH 7.4) and 1 M NaCl. The resultant oligosaccharides (from disaccharide to octasaccharide) were desalted and reconstituted in double-deionized water for mass spectrometry analysis and enzymatic assays.

by RPIP-HPLC, and its molecular mass was determined to be 922.6 Da ([M − 2H]$^{2-}$ = 460.3, M_r = 922.6 Da) by ESI-MS (Figure 5B), which is almost identical to the calculated molecular mass of the anticipated pentasaccharide (922.8 Da).

KfiA Transfers N-Acetylglucosamine to the Acceptors with Different Sizes. The requirement for the size of the substrate for KfiA was investigated by incubating the enzyme and the acceptor substrates with various sizes in the presence of UDP-GlcN[^{3}H]Ac, and the ^{3}H-labeled products were analyzed by RPIP-HPLC (Figure 6). It appears that KfiA is capable of transferring the GlcNAc residue to an acceptor as small as a disaccharide. To further quantify the susceptibility of the acceptors to KfiA modification, we determined the K_m and K_{cat}/K_m values of KfiA toward various acceptors. The amount of ^{3}H-labeled oligosaccharide product was determined by integrating the corresponding ^{3}H peak from RPIP-HPLC. As shown in Table 2, the binding affinities (as estimated by K_m) of various acceptors for KfiA are similar (between 256 and 674 μM), while the catalytic efficiency of KfiA (K_{cat}/K_m) moderately increases as the acceptor becomes longer (from 1.2 to 4.1). This suggested that elevated GlcNAc transfer efficiency could occur as the saccharide chain becomes longer. We attempted to determine the K_m and K_{cat}/K_m values of KfiA for K5 polysaccharide but failed to obtain consistent values partly because the impurities in the polysaccharide substrate interfered with the enzymatic reaction. We also examined the potential effect of purified recombinant KfiC on the K_m and K_{cat}/K_m values of KfiA toward a disaccharide substrate. We found that the K_m and K_{cat}/K_m values for KfiA (in the presence of 1.5 μM KfiC) are 315 μM and 1.4 M^{-1} s^{-1}, respectively, while the K_m and K_{cat}/K_m values for KfiA (in the absence of KfiC) are 256 μM and 1.2 M^{-1} s^{-1}, respectively. This observation suggests that purified KfiC has no significant effect on the kinetic character of KfiA in this system.

Donor Specificities of KfiA. It is believed that KfiA utilizes UDP-GlcNAc as a donor substrate in vivo. We further studied the donor specificity of KfiA. Isothermal titration

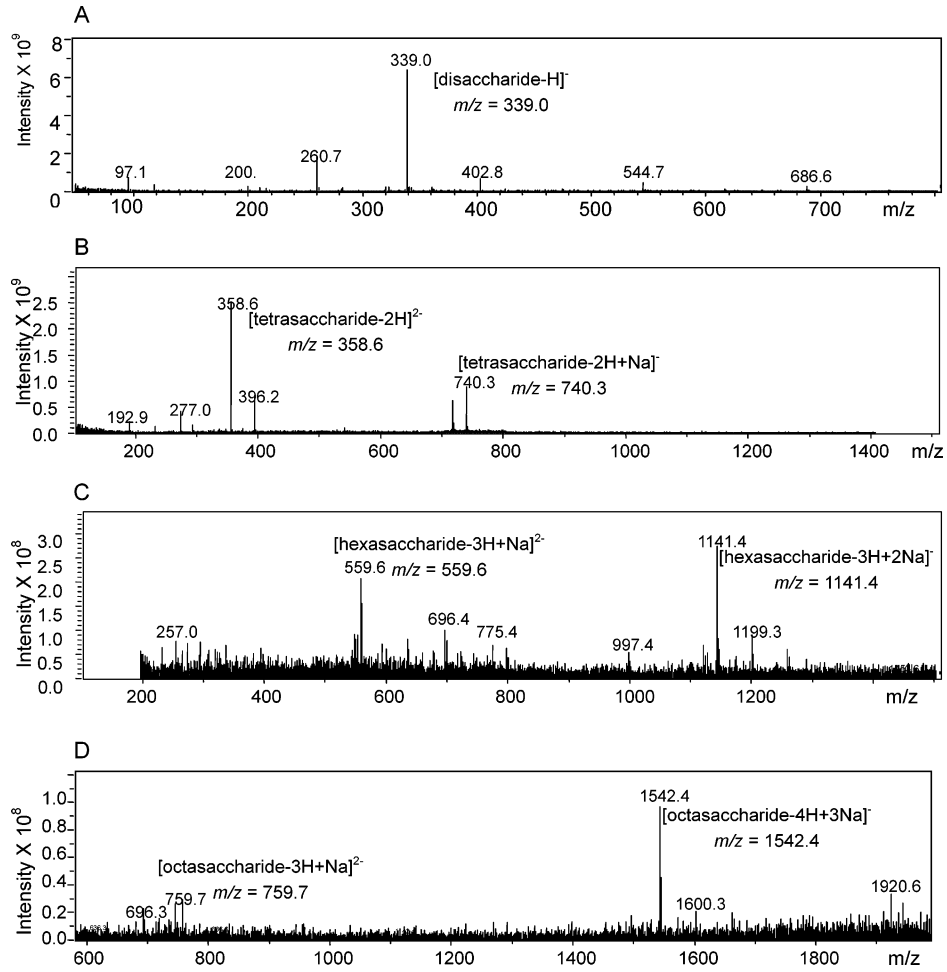


FIGURE 4: Electrospray ionization mass spectra of purified oligosaccharides. Panels A−D show the ESI-MS spectra of the disaccharide, tetrasaccharide, hexasaccharide, and octasaccharide, respectively. The samples were introduced by direct infusion (10 μL/min) into the mass spectrometer. The expected anions are indicated.

Table 1: Molecular Masses (M_r) of the Purified Oligosaccharides Determined by ESI-MS

sample	formula[a]	calcd M_r (Da)	detected M_r (Da)	detected molecular ion (*m*/*z*)
disaccharide	$C_{12}H_{20}O_{11}$	340.28	340.01	$[M - H]^- = 339.0$
tetrasaccharide	$C_{26}H_{41}O_{22}N$	719.60	719.25 ± 0.05[b]	$[M - 2H]^{2-} = 358.6$
				$[M - 2H + Na]^- = 740.3$
hexasaccharide	$C_{40}H_{62}O_{33}N_2$	1098.91	1098.84 ± 0.40[b]	$[M - 3H + Na]^{2-} = 559.6$
				$[M - 3H + 2Na]^- = 1141.4$
octasaccharide	$C_{54}H_{83}O_{44}N_3$	1478.23	1477.46 ± 0.01[b]	$[M - 4H + 2Na]^{2-} = 759.7$
				$[M - 4H + 3Na]^- = 1542.4$

[a] Molecular masses were calculated with a Molecular Weight Calculator (http://ncrr.pnl.gov/software/). [b] The value is the average ± the range of the detected molecular ions.

calorimetry (ITC) was employed to measure the binding affinities between KfiA and different UDP-sugars (Table 3). As the natural donor of KfiA, the dissociation constant (K_d) for UDP-GlcNAc was determined to be 62 μM, while we could not detect bindings between KfiA and UDP-GlcA, UDP-glucose, or UDP-*N*-acetylgalactosamine (GalNAc). This suggested that KfiA recognizes specific functional groups of the GlcNAc residue, including the *N*-acetyl group (the difference between glucose and GlcNAc is at the C2 position) and the orientation of the hydroxyl group (the difference between galactosamine and glucosamine is at the C4 position). UDP demonstrated a binding affinity (27 μM) comparable to that of UDP-GlcNAc, which implies that UDP might exhibit product inhibition of KfiA. Indeed, the IC_{50} of UDP on the *N*-acetyl-D-glucsoaminyltransferase activity of KfiA was measured to be 150 μM. The observation of the binding of UDP to KfiA, although somewhat surprising, is consistent with the previous studies on UDP-*N*-acetylhexosaminyltransferase, EXTL2 (*23*). The K_m of KfiA toward UDP-GlcNAc was determined to be 94 μM, which is very similar to the dissociation constant (K_d) (62 μM).

DISCUSSION

The bacterial capsular polysaccharide from *E. coli* strain K5 has a disaccharide repeating unit of -GlcA-β(1→4)-GlcNAc-, which is very similar to the unsulfated and unepimerized HS backbone structure. Our goal is to utilize K5 polysaccharide biosynthetic enzymes to synthesize the HS backbone structure with defined sizes and N-sulfation patterns. Previous reports demonstrated that at least four

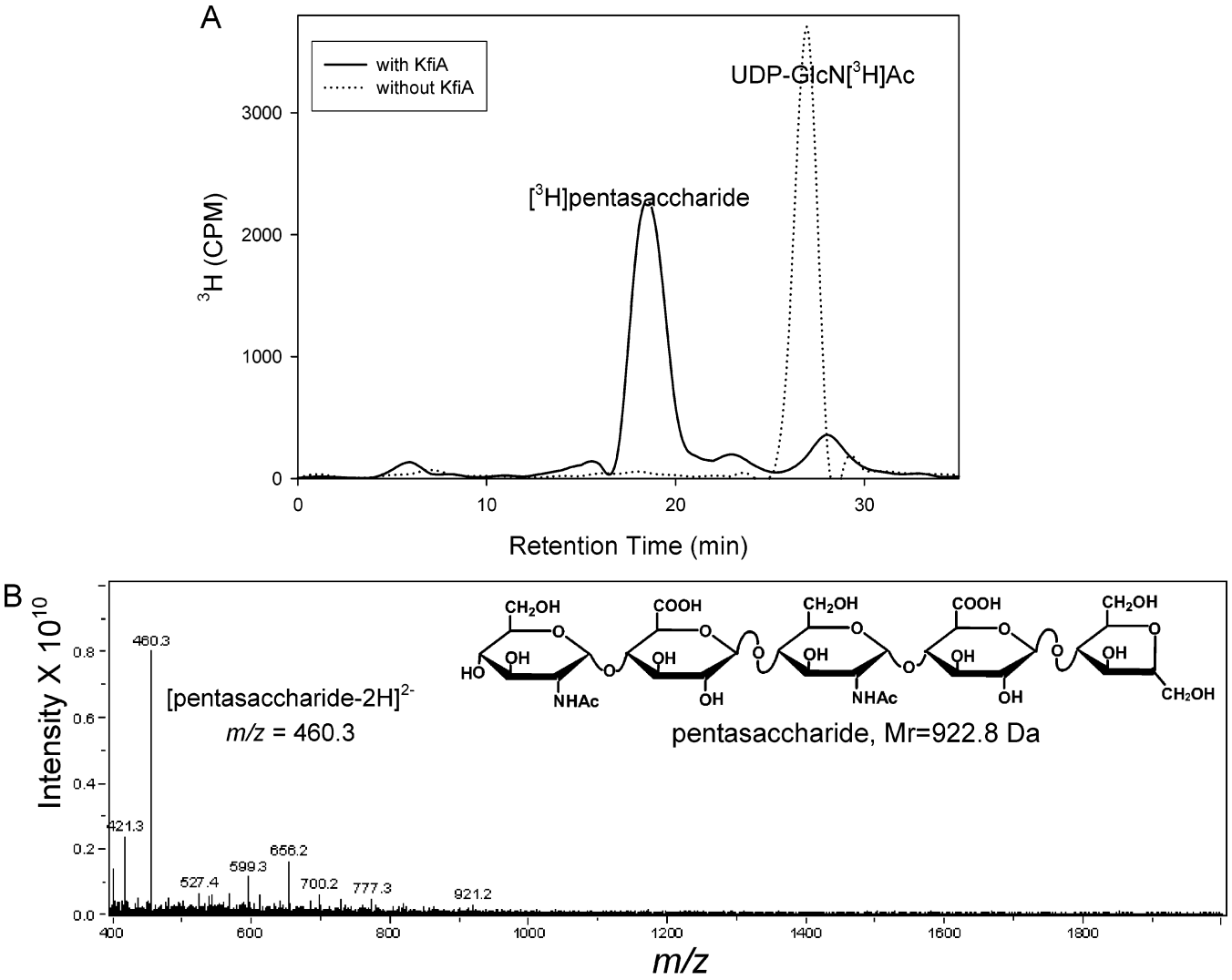


FIGURE 5: RPIP-HPLC chromatograms and ESI-MS spectrum of the KfiA-modified tetrasaccharide acceptor. (A) RPIP-HPLC chromatograms of KfiA-modified tetrasaccharide. UDP-GlcN[^{3}H]Ac was incubated with tetrasaccharide acceptors as described in Materials and Methods with (—) or without (···) KfiA. The reaction products were separated on RPIP-HPLC eluted with 50% acetonitrile in a solution containing 38 mM ammonium phosphate monobasic, 2 mM phosphoric acid, and 1 mM tetrabutylammonium phosphate monobasic at a flow rate of 0.5 mL/min. (B) ESI-MS spectrum of the KfiA-modified product. The pentasaccharide was prepared by incubating the tetrasaccharide, KfiA, and unlabeled UDP-GlcNAc. The product was purified by RPIP-HPLC, knowing the eluted position of [^{3}H]pentasaccharide. The resultant pentasaccharide was dialyzed and analyzed by ESI-MS. A doubly charged anion (*m*/*z* 460.3), corresponding to the pentasaccharide molecule, was detected. Thus, the determined M_r was 922.6 Da, which is consistent with the calculated M_r of the pentasaccharide (922.8 Da).

proteins are involved in the synthesis of K5 polysaccharide, including KfiA, KfiB, KfiC, and KfiD (*15*, *16*). Among them, KfiA and KfiC carry *N*-acetylglucosaminyl- and *N*-acetylglucuronyltransferase activity, respectively. Although KfiA has been overexpressed in *E. coIi* (*15*), the purified form has not been reported. Furthermore, the donor and acceptor substrate specificity of KfiA with purified protein have not been extensively studied. In this report, we report successful expression and purification of KfiA on a multimilligram scale in *E. coli* coexpressing chaperone proteins. The access of highly purified KfiA allowed us to determine substrate specificities using established approaches. Our results showed the purified KfiC has no significant effect on the kinetic parameters of KfiA toward a disaccharide acceptor. However, our results do not imply that KfiC does not affect the activity of KfiA in the synthesis of the capsular polysaccharide in vivo. In fact, null mutations in KfiC abolished the localization of KfiA on the cell membrane and vice versa (*15*), suggesting that KfiC may play not only a catalytic role in building the polysaccharide but also a structural role in maintaining the biosynthetic complex localized on the cytoplasmic face of the inner membrane.

It is interesting to note that KfiA activity is inhibited by UDP. Whether UDP competes with UDP-GlcNAc in the same binding pocket on KfiA and regulates the extent of the polymerization of capsular polysaccharide in vivo remains to be investigated. Ventura and colleagues discovered that the concentration of UDP-GlcUA affects the length of the capsular polysaccharide of *Streptococcus pneumoniae*, which is a copolymer of -Glc-GlcUA- catalyzed by the type 3 synthase (*24*). In a separate study, this group found that the efficiency of type 3 synthase for the polymer extension is controlled by the concentration of UDP-GlcUA as well as the temperature, providing a mechanism to explain the effect of the concentration of UDP-GlcUA on the length of the polysaccharide (*25*).

In addition to KfiA and KfiC, an enzyme with the capability of synthesizing K5 polysaccharide, known as

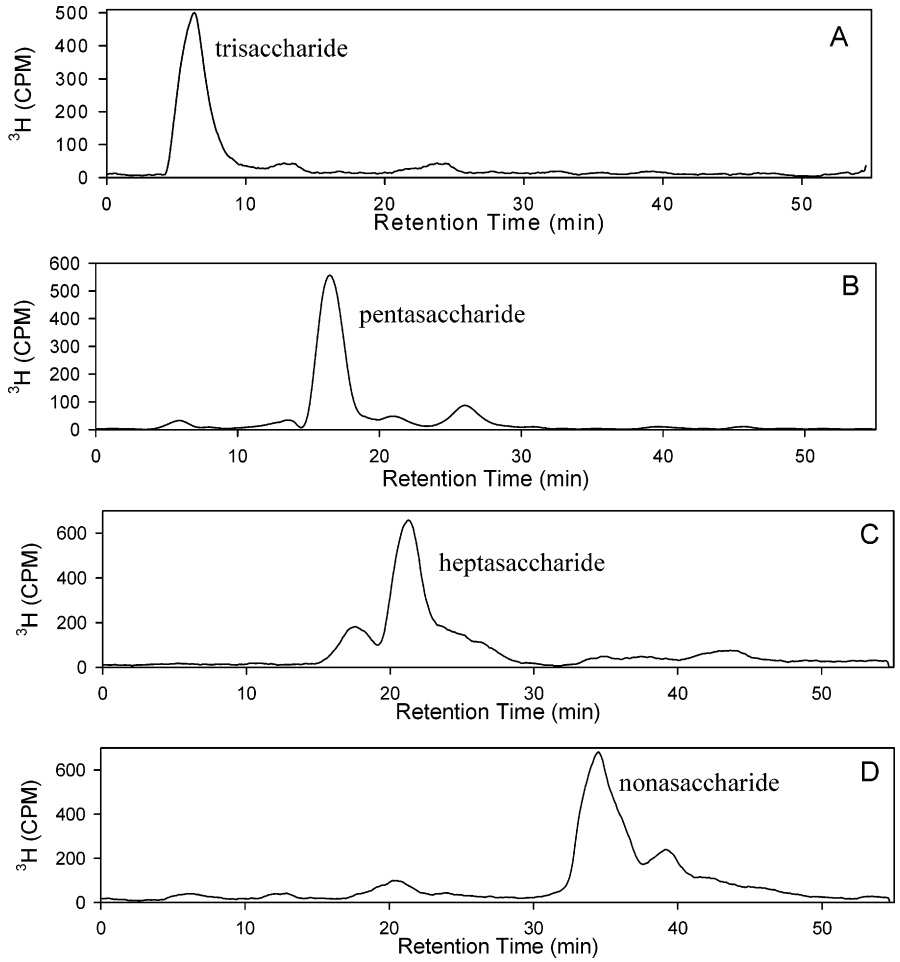


FIGURE 6: RPIP-HPLC chromatograms of KfiA-catalyzed *N*-acetylglucosaminyl reactions with different sizes of acceptor substrate. Various oligosaccharide substrates were incubated with purified KfiA and UDP-GlcN[^{3}H]Ac. The reaction was terminated by heating at 100 °C for 2 min. The resultant [^{3}H]oligosaccharides were resolved by RPIP-HPLC eluted with different concentrations of acetonitrile as described in Materials and Methods. Panels A−D show the chromatograms of disaccharide, tetrasaccharide, hexasaccharide, and octasaccharide substrates, respectively.

Table 2: K_m and K_{cat}/K_m Values of KfiA with Different Acceptors

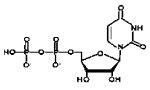
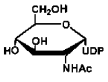
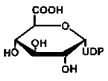
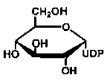
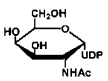
substrate	K_m (μM)	K_{cat}/K_m (M^{-1} s^{-1})	R^2 [a]
UDP-GlcNAc[b]	94	3.7	0.9935
disaccharide[c]	256	1.2	0.9968
tetrasaccharide	324	1.2	0.9976
hexasaccharide	674	2.7	0.9959
octasaccharide	254	4.1	0.9931

[a] R^2 represents the coefficient determination for the data in fitting to the Michaelis−Menten equation. [b] Disaccharide was used as an acceptor substrate as explained in Materials and Methods. [c] The concentration of UDP-GlcN[^{3}H]Ac used in the assays was 1270 μM.

heparosan synthase, has been identified in *Pasteurellas mutocida* type D (*26*). Unlike KfiA and KfiC, the heparosan synthase carries both *N*-acetylglucosaminyl- and *N*-acetylglucuronyltransferase activities. Mammalian HS polymerases, EXT1 and EXT2, were also identified (*27*). Both EXT1 and EXT2 carry both *N*-acetylglucosaminyl- and *N*-acetylglucuronyltransferase activities, and an EXT1−EXT2 complex was believed to be responsible for the synthesis of HS in vivo (*28*). There is no significant homology between KfiA and either heparosan synthase, EXT1, or EXT2, suggesting structural diversity among *N*-acetylglucosaminyltransferases.

The substrate specificities of KfiA are unique for the application in the synthesis of the HS backbone. Incubation of KfiA with oligosaccharide acceptors of various sizes all yielded extended oligosaccharide products. Our results suggested that KfiA can be employed in de novo HS backbone synthesis starting from a polysaccharide as short as disaccharide, provided that a disaccharide is readily

Table 3: Binding Affinities of Various UDP-Sugars

Donors	Structures of donor substrates	K_d (μM)	N[a]
UDP		27	1.25
UDP-GlcNAc		62	1.04
UDP-GlcA		ND[b]	ND[b]
UDP-Glucose		ND[b]	ND[b]
UDP-GalNAc		ND[b]	ND[b]

[a] Binding stoichiometry. [b] Not detected.

available from complete depolymerization of K5 polysaccharide with nitrous acid. We do not rule out the possibility that KfiA transfers a GlcNAc residue to other glycosaminoglycan substrates, including hyaluronic acid oligosaccharides, due to the unavailability. Clearly, an efficient expression of KfiA and detailed substrate specificity information will serve as an important tool in conducting in vitro enzymatic synthesis of HS as well as in probing the biosynthetic mechanism of HS.

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