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Carbohydrate Research 337 (2002) 1547–1552

CARBOHYDRATE RESEARCH

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Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively

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Received 14 May 2002; accepted 18 July 2002

Abstract

Pasteurella multocida is a pathogenic Gram-negative bacterial species that infects a wide variety of animals and humans. A notable morphological feature of many isolates is the extracellular capsule. The ability to remove the capsule by treatment with certain glycosidases has been utilized to discern various capsular types called A, D and F. Based on this preliminary evidence, these microbes have capsules made of glycosaminoglycans, linear polysaccharides composed of repeating disaccharide units containing an amino sugar. Glycosaminoglycans are also abundant components of the vertebrate extracellular matrix. It has been shown previously that the major Type A capsular material was hyaluronan (hyaluronic acid). We report that the Type D polymer is an unmodified heparin (*N*-acetylheparosan) with a →4)-β-D-Glc*p*-UA-(1→4)-α-D-Glc*p*-NAc-(1→ repeating unit and the Type F polymer is an unmodified chondroitin with a →4)-β-D-Glc*p*-UA-(1→3)-β-D-Gal*p*-NAc-(1→ repeating unit. The monosaccharide compositions, disaccharide profiles, and ^{1}H NMR analyses are consistent with these identifications. The molecular size of the *Pasteurella* polymers is approximately 100–300 kDa as determined by gel electrophoresis and multi-angle laser light scattering; this size is much greater than the 10–30 kDa size of the analogous polymers isolated from animal tissues. The glycosaminoglycan capsular polymers are relatively non-immunogenic virulence factors that enhance microbial pathogenicity.

Keywords: Glycosaminoglycan; Capsular polysaccharide; Heparosan; Chondroitin; *Pasteurella multocida*

1. Introduction

Pathogenic bacteria often employ a variety of mechanisms to defeat host defenses. The extracellular capsule of bacteria was one of the earliest virulence factors to be recognized in the late 1800s. Extensive studies have been made on a variety of genera from both Gram-positive and Gram-negative organisms that plague animals and plants. Typically, the capsule is composed of an anionic polysaccharide, but uncharged polymers as well as proteinaceous molecules have also been described. Some of the potential roles of the capsular polymer during the bacterial life cycle include avoiding phagocytosis, defeating complement, modulating host responses or physiology, and resisting desiccation.[1] An astounding variety of polysaccharide structures have been elucidated. One potential 'flaw' of encapsulated pathogens, however, is that an immune response against the capsule is usually generated if the polymer structure is not normally found as a component of the vertebrate body. Typically, the host will successfully repel a second infection by a microbe with the same capsular polymer. In certain cases, however, the microbe employs molecu-

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00219-7

lar mimicry of host molecules. This clever strategy of utilizing relatively non-immunogenic polymers allows multiple infections to occur because an antibody response is not mounted. Indeed, if anti-self antibodies were generated, then potentially severe autoimmune complications could result.

The most vivid examples of mimicry are the production of glycosaminoglycan [GAG] or GAG-like capsules.[2] GAGs, linear polysaccharides with a repeating disaccharide backbone containing an amino sugar (either GlcNAc or GalNAc), are essential components of the extracellular matrices of animals. The vertebrate GAGs hyaluronan [HA], heparan sulfate/heparin, and chondroitin sulfate contain an uronic acid as the other sugar in the repeat while keratan sulfate has a galactose. These complex molecules play important structural, adhesion, and signaling roles in mammals. Group A and C *Streptococcus* and Type A *Pasteurella multocida* bacteria produce HA capsules; the microbial polymer is chemically identical to the vertebrate molecule, thus virtually no immune response is generated to the capsule. *Escherichia coli* K5 produces an unmodified heparin-like molecule called heparosan or *N*-acetylheparosan.[3] *E. coli* K4 produces a chondroitin-like molecule with fructose side branches on the 3-position of the glucuronic residues.[4] In all these cases, the wild-type bacteria are more virulent than acapsular mutants.

Carter originally recognized three *P. multocida* capsular types called A, B, and C.[5] Over time, some revision (Type C no longer recognized) and other types (D, E, and F) were described by Brogden, Heddleston, Rhoades, and Rimler.[6,7] As mentioned, the Type A microbe, the major fowl cholera pathogen and a causative agent of bovine shipping fever, produces a hyaluronan capsule.[8] Type B, the agent of hemorhagic fever in ungulates, produces a capsule of unknown structure composed of arabinose, mannose, and galactose. Type D isolates cause atrophic rhinitis in swine but are sometimes isolated from other organisms. Type E, an African serotype with a capsular polymer of unknown structure, infects cattle and buffalo. Type F is a minor causative agent of fowl cholera. Previous studies showed that the capsule of Type D and Type F bacteria were removed, as judged by light microscopy, by treatment with certain enzymes that selectively degrade GAGs. The Type D capsule was removed by treatment with heparinase III or chondroitin AC lyase.[6] The Type F capsule was removed by treatment with chondroitin AC lyase. Unlike many other non-GAG capsules, no typing antiserum could be generated for Types A, D, and F. The use of the basic dye, acriflavine, to flocculate these types was sometimes used, but the test is not robust and rather difficult to utilize by the non-expert. Therefore, the enzyme treatment was used as a vigorous proof to discriminate the Type D and F isolates. A newer polymerase chain reaction method based on the differences among genes in the various capsular loci has been described to distinguish the various strains,[9] but the exact nature of the Type D and F polymers was not known at the time.

In this report we utilize chemical, enzymatic, mass spectrometric, and nuclear magnetic resonance studies to identify definitively the Type D and Type F capsular polysaccharides.

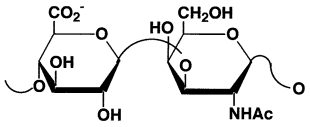
2. Results and discussion

The anionic capsular polymers were isolated from culture media of spent *P. multocida* by precipitation with cetylpyridinium chloride. Monosaccharide analysis of the Type D and F polymers both yielded only a hexosamine and a uronic acid. Type D polymer contained GlcN while Type F polymer contained GalN. Acid hydrolysis fragments the polymer, but also removes any *N*-acetyl groups as well as other labile modifications such as sulfates. Standards consisting of heparin and chondroitin sulfate derived from animal sources yielded very similar profiles as Type D and F, respectively.

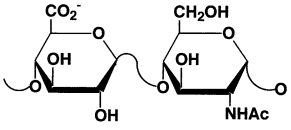
Size analysis of the Type D and F capsular polymers by both gel-filtration chromatography and electrophoresis indicate that the molecular size was in the 10^5 Da (~ 500 sugars) range. Polyacrylamide gel electrophoresis yielded apparent molecular weights of $\sim 50-100$ kDa for both polysaccharides using heparin or chondroitin sulfate standards. Gel-filtration chromatography coupled with multi-angle laser light scattering analysis yielded average molecular weights of ~ 330 kDa and ~ 270 kDa for Types D and F, respectively. The sizes of the capsular polymers are substantially larger that the chains observed in these GAGs derived from animal tissues (e.g., cartilage, trachea, and intestinal mucosa).

^{1}H NMR analysis of the Type F polymer at 298 and 318 K showed that it is an unsulfated chondroitin polymer. It consists of a $\rightarrow$4)-β-D-Glc*p*-UA-(1$\rightarrow$3)-β-D-Gal*p*-NAc-(1$\rightarrow$ repeating unit (Fig. 1). The region at 3.775–3.699 ppm comprises H-4, H-5 of Glc*p*-UA residue and H-6 of Gal*p*-NAc residue. The signals at 4.439, 3.595, 3.304 are H-1, H-3 and H-2 of Glc*p*-UA residue and signals at 4.068 and 1.969 are H-4 and *N*-acetyl methyl of Gal*p*-NAc residue, respectively (Fig. 2(A)). Enzymatic depolymerization[10] of sample with chondroitin AC lyase and chondroitin ABC lyase, followed by CE disaccharide analysis, showed a single peak that comigrates with ΔUA*p*-(1$\rightarrow$3)-α,β-D-GalNAc disaccharide standard. No other peaks corresponding to chondroitin sulfate- (or dermatan sulfate-) derived monosulfated, disulfated and trisulfated disaccharides were observed.

^{1}H NMR analysis of the intact Type D sample at 279, 298 and 318 K (Fig. 2(B)) suggested that it was an unsulfated heparan sulfate polymer, *N*-acetylheparosan, with a →4)-β-D-Glc*p*-UA-(1→4)-α-D-Glc*p*-NAc-(1→ repeating unit (Fig. 1). This sample showed an unusual peak in its ^{1}H NMR spectrum at 3.5 ppm, suggesting that it might be methylated as an ester or ether (but as described later, this peak corresponded to a contaminant).



Glycosaminoglycan F sample



Glycosaminoglycan D sample

Fig. 1. Disaccharide sequences of glycosaminoglycan F and D samples.

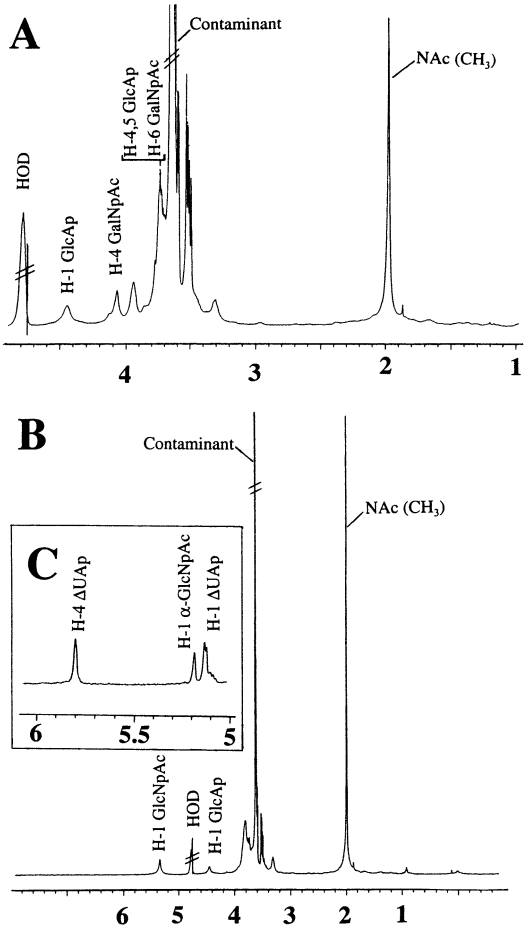


Fig. 2. 500 MHz ^{1}H NMR spectroscopy at 298 K. (A) Analysis of glycosaminoglycan F sample. (B) Analysis of glycosaminoglycan D sample. (C) Analysis of heparin lyase III digestion product of glycosaminoglycan D sample.

Exhaustive treatment of large amounts of the Type D sample with heparin lyase III yielded a product that showed absorbance at 232 nm. CE disaccharide analysis of the product mixture showed a single peak that comigrated with ΔUA*p*-(1→4)-α,β-D-GlcNAc disaccharide standard. No other heparin- (or heparan sulfate-) derived monosulfated, disulfated and trisulfated disaccharides were observed in this product. Analysis of this product mixture by ^{1}H NMR confirmed that it consisted primarily of ΔUA*p*-(1→4)-α,β-D-GlcNAc, the signals at 5.808, 5.120, and 4.199 corresponded to the H-4, H-1 and H-3 of ΔUA*p* residue and signals at 5.181, 4.688 and 2.014 corresponded to the α H-1, β H-1 and *N*-acetyl methyl of Glc*p*-NAc residue, respectively (Fig. 2(C)). Next, the major unsulfated disaccharide was isolated using analytical SAX-HPLC and assignment of the structure was done by ^{1}H NMR and ESIMS. In the ^{1}H NMR spectrum, the signal at 3.5 ppm was no longer present, proving that it is simply a contaminant. ESIMS analysis gave unsulfated disaccharide molecular ion mass $[M-H]^-$ 378.0 as a parent ion consistent with the structure. Therefore, the Type D polymer has the equivalent sugar structure as the capsular polymer of *E. coli* K5, *N*-acetylheparosan.[3] We found that the *Pasteurella* polymer, however, was larger than the *E. coli* polymer (~ 330 kDa versus ~ 90 kDa, respectively).

Recently, the genes encoding the enzymes responsible for polymerizing GAGs in *Pasteurella* have been molecularly cloned.[2] Each capsular polymer is produced by a distinct dual-action synthase that transfers both monosaccharides in an alternating fashion to the growing GAG chain. The Type A and F enzymes, pmHAS and pmCS, respectively, are about 90% identical at the DNA and protein level. This similarity appears logical because the only difference between the structure of HA and chondroitin polysaccharide backbones is the identity of the hexosamine. The amino acid sequence of the heparosan synthase of the Type D strain, pmHS, however, is not very similar to the Type A or Type F enzyme.[11] This finding is not surprising, because even though HA and heparin have the same monosaccharide composition, heparin contains alternating β and α glycosidic linkages, while HA is solely β-linked. Different reaction mechanisms are required to form the β linkage (inverting) or the α linkage (retaining) from the α-linked UDP-sugar precursors; therefore, the synthase polypeptide structures and sequences are expected to be different. The tests of the sugar transfer specificity of the recombinant pmCS and pmHS enzymes supplied

with various UDP-sugar nucleotides in vitro agree with our identifications of the GAG polymers; only the authentic substrates are utilized to synthesize high molecular weight polymers.[11,12]

In summary, the enzymatically released disaccharides from the Type D and F polymers migrated in an identical fashion to unsulfated disaccharide standards using capillary electrophoresis. The masses of these fragments were identical to the unsulfated standards. The Type D and F polymers gave NMR spectra consistent with unsulfated heparosan and chondroitin, respectively.

After various chemical and/or enzymatic treatments, the polymers from Type D and F strains may have utility in preparing defined GAGs for various medical treatments in the future. Currently, heparin derived from porcine intestinal mucosa is a widely used anticoagulant and antithrombotic agent. Chondroitin sulfate derived from shark or bovine sources is now used as viscoelastic aid, as well as a nutritional supplement with potential benefit for osteoarthritic disease. The various *Pasteurella* bacteria are defined, non-animal sources of GAGs that are free from viral or prion adventitious agents. After appropriate processing, the *Pasteurella* GAGs may serve as substitutes for these current applications.

3. Experimental

Strains, bacterial growth, and capsular polysaccharide isolation.—Type D (P-3881) and Type F (P-4679) *P. multocida* were obtained from the USDA collection (Ames, IA). For routine growth, the microbes were grown in brain heart infusion (Difco, Detroit, MI) or starch dextrose agar (BBL, Cockeysville, MD). For production of capsular polysaccharides, cultures were grown in a defined media that lacks crude extracts or complex polymer nutrients.[13] Growth at 37 °C with mild shaking for 24–48 h resulted in dense growth and luxurious capsule production. Cells were removed from the media by high-speed centrifugation (10,000 × *g*, 15 min), and the shed polymeric material in this supernatant was purified (the cell-associated material was very similar to the shed material, but more difficult to process).

The clarified spent media was subjected to repeated $CHCl_3$ extraction to deproteinize the mixture before quaternary amine detergent precipitation (1% cetylpyridinium chloride [CPC]). The resulting pellet was washed in water, redissolved in 1 M NaCl, the solution clarified by centrifugation, and the polymer was precipitated by the addition of 2.5 volumes of EtOH. The resuspension in salt solution, followed by EtOH precipitation, was repeated twice. The final pellet was then redissolved in water, treated with DNAase and RNAase (1 μg/mL final) for 1 h and then extracted with $CHCl_3$. The aqueous phase was harvested and passed through a reversed-phase SepPak cartridge (Waters, Milford, MA) to remove traces of CPC and protein. Uronic acid was quantified by the carbazole method with a glucuronic acid standard.[14]

Acid hydrolysis, composition analysis, and degradative enzymes.—Monosaccharide analysis by acid hydrolysis (2 M HC1, 100 °C, 4 h) and high-pH anion-exchange chromatography on a Dionex system with pulsed amperometric detection (Sunnyvale, CA) was performed as previously described.[12] Chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, chondroitin AC lyase (EC 4.2.2.5) from *Flavobacterium heparinum*, and heparin lyase III (EC 4.2.2.8) from *F. heparinum* were purchased from Sigma Chemical Co. (St. Louis, MO). After lyase-catalyzed digestion at 37 °C was completed, the enzymes were thermally inactivated by boiling for 2 min, and the products were analyzed by PAGE and/or CE.

Chondroitin lyase-catalyzed depolymerization of Type F polymer.—Samples (20 μg) were dissolved in 10 μL of 50 mM Tris–HCl and 60 mM sodium acetate buffer, pH 8.0, and digested overnight with either (a) 20 mU chondroitin AC lyase or (b) 20 mU chondroitin ABC lyase.

Lyase-catalyzed depolymerization of Type D polymer.—Samples (20 μg) were dissolved in (a) 10 μL of 50 mM Tris–HCl and 60 mM sodium acetate buffer, pH 8.0, for digestion overnight with 20 mU chondroitin ABC lyase or (b) 10 μL of 50 mM sodium phosphate buffer, pH 7.6, for digestion overnight with 20 mU heparin lyase III.

Preparative heparin lyase-catalyzed depolymerization of Type D polymer.—Sample (~800 μg) in 50 mM sodium phosphate buffer, pH 7.6 was treated exhaustively with heparin lyase III at 37 °C. At various time points, the absorbance at 232 nm was measured, and digestion was continued until the absorbance was constant (complete digestion). The digestion mixture was applied to Bio-Gel P4 (fine) column eluted with water (Bio-Rad; Richmond, CA). The major peak was pooled, lyophilized and analyzed by SAX-HPLC.

PAGE analysis.—Polyacrylamide gel electrophoresis (PAGE) was performed on a 32 cm vertical slab gel unit SE620, from Hoefer Scientific Instruments (San Francisco, CA), equipped with model 1420B power source from Bio-Rad (Richmond, CA). Polyacrylamide resolving gel (14 × 28 cm, 12% acrylamide) was prepared and run as described previously.[15] The molecular sizes of the carbohydrate were determined by comparing with chondroitin A and heparan sulfate standards.

Gel filtration/multi-angle laser light scattering analysis.—Polymers (50 μg) were separated on two tandem Toso Biosep TSK-GEL columns (6000PWXL, followed by 4000PWXL; each 7.8 mm × 30 cm; Japan) eluted in

50 mM sodium phosphate, 150 mM NaCl, pH 7, at 0.5 mL/min. The eluant flowed through an Optilab DSP interferometric refractometer and then a Dawn DSF laser photometer (632.8 nm; Wyatt Technology, Santa Barbara, CA) in the multi-angle mode. The manufacturer's software package was used to determine the absolute average molecular weight using a dn/dC coefficient of 0.153.

Disaccharide composition analysis by CE.—The experiments were performed with a capillary electrophoresis PACE 5500 system (Beckman Instruments, Fullerton, CA) at a constant capillary temperature of 18 °C with a potential of −22 kV by UV absorbance at 232 nm. The electropherograms were acquired using the manufacturer's System Gold software package. The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with saturated dibasic sodium phosphate as described previously.[16] Separation and analysis were carried out in a fused-silica capillary tube. This capillary was 50 μm inner diameter, 360 μm outer diameter, and 47 cm long, with a 40 cm effective length and was externally coated except where the tube passed through the detector. Prior to every run, the capillary was conditioned with 0.5 M NaOH (1 min, 20 psi or 138 kPa) and rinsed (1 min, 20 psi or 138 kPa) with running buffer. Samples were applied by pressure injection for 15 s at 0.5 psi.

SAX-HPLC analysis.—Strong anion-exchange high-performance liquid chromatography [SAX-HPLC] was performed on 5 μm Spherisorb column of dimension 0.46×25 cm from Waters. HPLC was performed on LC-10A*i* pumps from Shimadzu (Kyoto, Japan) with Shimadzu SPD-10A*i* variable-wavelength UV detector and data were processed using Shimadzu Class-Vp 4.03 chromatography data system running Windows-based acquisition and control software. The SAX-HPLC column was washed with 2.0 M sodium chloride, followed by a water wash, and equilibrated with water at pH 3.5. Fractions were analyzed using a 180 min linear gradient of 0–1.0 M sodium chloride at pH 3.5 at a flow rate of 1.0 mL/min, and the elution profile was monitored by absorbance at 232 nm at 0–0.2 absorbance units full scale (AUFS). The major peak (eluting at the same time with ΔUA*p* (1→4)-α,β-D-GlcNAc disaccharide standard prepared from heparan sulfate) was pooled, lyophilized and desalted on a Bio-Gel P4 column before analysis by ^{1}H NMR and ES-IMS.

NMR spectroscopy.—Samples were dissolved in D_2O (99.96% of atom), filtered through a 0.45-μm syringe filter, and freeze-dried to remove exchangeable protons. After exchanging the samples three times by freeze-drying from D_2O, samples were transferred to Shigemi tubes for analyses. One-dimensional (1D) ^{1}H NMR experiments were performed on a Varian 500 MHz VXR-500 spectrometer equipped with 5-mm triple resonance tunable probe with standard Varian software at 279, 298, 313 K.

ESIMS analysis.—Negative-ion spectrum was performed by using a Agilent Technologies 1100 MSD (Palo Alto, CA) equipped with an electrospray interface as described previously.[17] Nitrogen gas was used both as bath and nebulizer gas, at flow rates of 10 L/h and 60 psi (414 kPa), respectively. The electrospray ion source was held at 350 °C. Agilent's running mix in MeCN was used as the calibrant. The solid sample was dissolved in 1:1 1% NH_4OH–MeCN that was also used for the mobile phase. Multiple injections were performed. The spectra were obtained by 30–40 scans with the use of the manufacturer's HP LC–MSD Chem Station software.

Acknowledgements

We appreciate the aid of Bruce Baggenstoss and Long Nguyen for performing the light scattering size analyses and the late Dr. Richard Rimler for providing the bacterial strains and useful discussions. The authors acknowledge the support of a NSF grant MCB-9876193 (P.D.) and NIH grants GM38060 and HL52622 (R.J.L.).

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