Carbohydrate Polymers 88 (2012) 257–262

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Structural details of the Polyelectrolytic Exopolysaccharide (APE), the stabilizing component of the *Acinetobacter venetianus* RAG-1 emulsan complex

DeAnn Barnhart[a], David L. Kaplan[b,c], William T. Winter[a], Bruce J. Panilaitis[c], David J. Kiemle[d,*]

[a] *Cellulose Research Institute and Department of Chemistry, SUNY-Environmental Science and Forestry, One Forestry Drive, Syracuse, NY 13210, USA*
[b] *Department of Chemical and Biological Engineering, Tufts University, Medford, MA 02155, USA*
[c] *Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA*
[d] *Department of Analytical and Technical Services, SUNY-Environmental Science and Forestry, One Forestry Drive, Syracuse, NY 13210, USA*

ARTICLE INFO

Article history:
Received 21 May 2011
Received in revised form 1 November 2011
Accepted 1 December 2011
Available online 13 December 2011

Keywords:
Acinetobacter venetianus RAG-1
Acinetobacter Polyelectrolytic Exopolysaccharide
APE
Emulsan
NMR spectroscopy

ABSTRACT

Emulsan is a polysaccharide complex, secreted from *Acinetobacter venetianus* RAG-1, that has been studied extensively for its ability to effectively stabilize oil-in-water emulsions. In previous research, the active emulsion stabilizing component of the emulsan complex was isolated and termed *Acinetobacter* Polyelectrolytic Exopolysaccharide (APE). In the research presented here, both native and O-deacetylated APE were studied by ^{1}H and ^{13}C NMR spectroscopy, including 1D ^{1}H and ^{13}C and 2D ge-DQF COSY, TOCSY, NOESY, and ge-HSQC experiments. The native polysaccharide was found to contain 6-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranose, 2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid, and 2-acetamido-4-(3-hydroxybutyramido)-2,4,6-trideoxy-α-D-glucopyranose residues. The sequence of the linear trisaccharide repeating unit of the polysaccharide APE was determined to be →4)-α-D-Gal*p*NAc-6-OAc-(1 → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui*p*NAc4NHb-(1→.

1. Introduction

Polysaccharides have emerged as important biomaterials for industrial use as a result of their low toxicity and high biodegradability (Laplante, Turgeon, & Paquin, 2005; Ruijssenaars, Stingele, & Hartmans, 2000). Unfortunately, many of the polymers produced from plant or animal sources suffer from lack of specificity and high batch variability (Sutherland, 1998). However, these concerns can be mitigated through genomic and environmental controls (Dams-Kozlowska & Kaplan, 2007; van Kranenburg, Ingeborg, Kleerebezem, & de Vos, 1999). Thus, bacterially produced exopolysaccharides are of considerable interest as they are readily modified in ways that enhance their industrial utility (Dams-Kozlowska, Mercaldi, Panilaitis, & Kaplan, 2008; Kumar, Mody, & Jha, 2007).

Historically, the observation of certain bacteria accumulating at oil–water interfaces goes back, at least, to the work of Mudd and Mudd (1924). Beginning in 1981, studies by Rosenberg's laboratory focused on *Acinetobacter calcoaceticus* (Rosenberg & Rosenberg, 1981; Zosim, Gutnick, & Rosenberg, 1983) from which they isolated an emulsan, so named for its role in emulsifying hydrocarbon droplets to which the bacteria adhered. The reclassification of *Acinetobacter calcoaceticus* RAG-1 as *Acinetobacter venetianus* was made using restriction analysis of the amplified rDNA (Vaneechoutte et al., 1999). *A. venetianus* RAG-1, a Gram-negative seawater bacterium, was first isolated from surface water of the Venetian Lagoon (Di Cello, Pepi, Baldi, & Fani, 1997) and has been a target of investigation due to its ability to metabolize a variety of carbon sources, *e.g.*, long chain hydrocarbons, alcohols, fatty acids, and triglycerides (Di Cello et al., 1997; Mercaldi, Dams-Kozlowska, Panilaitis, Joyce, & Kaplan, 2008; Zhang, Gorkovenko, Gross, Allen, & Kaplan, 1997). For many years, it was thought that the emulsan complex acted as a potent biosurfactant consequential to its high degree of amphipathicity. This property was originally attributed to the presence of the hydrophobic fatty acids distributed along a hydrophilic polysaccharide backbone. These enabled the complex to stabilize diverse oil-in-water emulsions through associations across the oil–water interface (Gorkovenko, Zhang, Gross, & Kaplan, 1999).

Recently, Mercaldi et al. (2008) demonstrated that the emulsan complex was physically separable into a low molecular-weight lipopolysaccharide (~80%, w/w) ionically complexed to positively charged nitrogen atoms in an incompletely acylated high molecular-weight exopolysaccharide (~20%, w/w). Further, these authors reported that the emulsion stabilization properties of emulsan were derived solely from the exopolysaccharide fraction.

* Corresponding author. Tel.: +1 315 470 6883; fax: +1 315 470 6856.
E-mail address: dkiemle@esf.edu (D.J. Kiemle).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.12.006

This discovery surfaced when the hydrophilic properties of the exopolysaccharide component of the emulsan lead to their exploration of the emulsion stabilization potential of the separated, isolated lipopolysaccharide and exopolysaccharide fractions. After an allotted time, it was found that the exopolysaccharide fraction continued to stabilize the oil-in-water emulsion, while the lipopolysaccharide fraction displayed phase partitioning. Thus, it was theorized that the partial deacetylation of the N-acyl groups of the carbohydrate backbone was creating an amphiprotic polymer capable of emulsion stabilization (Dams-Kozlowska et al., 2008; Mercaldi et al., 2008). Such partial acylation in sea water is observed even with chitin (Kasaai, 2009).

Since studies with emulsan have focused on areas that exploit these emulsion stabilization features, such as the bioremediation of heavy metals and crude oil (Gutnick, 1987; Zosim et al., 1983), it is now appropriate to focus on the *Acinetobacter* Polyelectrolytic Exopolysaccharide (APE). This compound was isolated from the emulsan complex and purified to 98% (w/w) with protein as the major contaminant (Dams-Kozlowska et al., 2008; Mercaldi et al., 2008). Such a high degree of purity of APE, combined with the novel emulsification properties of this one component, suggests the need to consider the use of APE alone and in place of emulsan derivatives for biomedical applications such as vaccine adjuvants and drug delivery vehicles (Castro, Kamdar, Panilaitis, & Kaplan, 2005; Castro, Panilaitis, Bora, & Kaplan, 2006; Panilaitis, Johri, Blank, Kaplan, & Fuhrman, 2002). APE should be well suited for these tasks as its charge characteristics, as reported here, allow effective drug binding through mild ionic-complexation reactions. As a bacterial product, APE, like emulsan, has the potential for genetic modifications, which can alleviate, potentially, the need for post-synthetic modifications (Dams-Kozlowska et al., 2008). In order to utilize emulsan or its APE in future biomedical applications and to exploit the exopolysaccharide component for its emulsion stabilization properties, a better understanding of the molecular structure of the exopolysaccharide, APE, is needed. Prior to this study the molecular structure of emulsan's exopolysaccharide had been described as a linear polymer comprised of three repeating amino-sugars, *i.e.*, D-galactosamine, diamino-6-deoxy-D-glucose, and an unknown uronic acid, in an approximate 1:1:1 ratio, with O-acyl and N-acyl bound fatty acid side chains (Dams-Kozlowska et al., 2008; Mercaldi et al., 2008; Zhang et al., 1997; Zuckerberg, Diver, Peeri, Gutnick, & Rosenberg, 1979). Additionally, a 3-hydroxybutryic acid group is attached to the polysaccharide backbone via an amide bond (Mercaldi et al., 2008; Zhang et al., 1997). Thus, the goal of this research is to determine the primary structure, including sugars, ring shapes, configurations and linkages, of APE through the application of 1D and 2D NMR spectroscopic techniques.

2. Materials and methods

2.1. Materials

A. venetianus RAG-1 (ATCC 31012) was obtained from the American Type Culture Collection (Manassas, VA). APE production and purification methods followed those as described elsewhere (Mercaldi et al., 2008).

The sample was prepared for NMR analysis by dissolving 19.1 mg of native APE in 1.0 mL 99.97% D_2O (Cambridge Isotope Laboratory, Andover, MA) with 0.05% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) (Sigma, St. Louis, MO) as the internal standard (δ_H and δ_C at −0.017 ppm and −0.18 ppm, respectively) (van de Velde, Pereira, & Rollema, 2004). The O-deacetylated sample was prepared by treating native polysaccharide (21.2 mg) with 12.5% aqueous NH_3 solution (4 mL) overnight at 20 °C, followed by dilution with water and lyophilization. The sample was then twice redissolved in D_2O and lyophilized, and then brought up in 1 mL of D_2O (Dag et al., 2004).

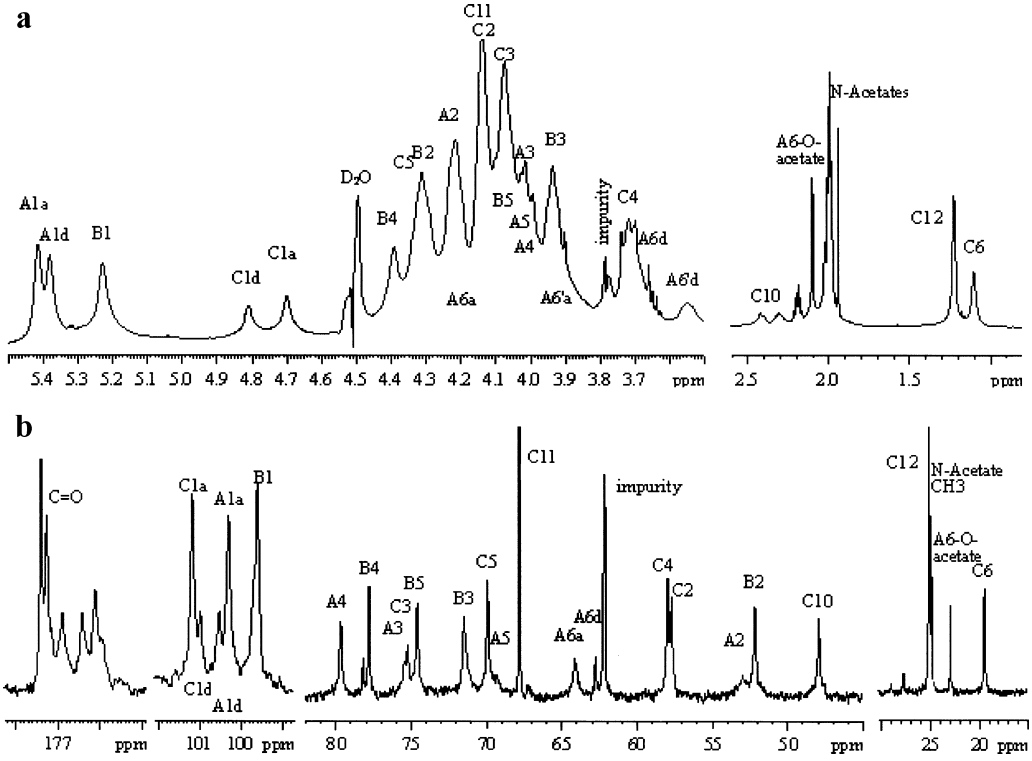

Fig. 1. (a) ^1H and (b) ^{13}C NMR spectra of the native form of APE.

2.2. ^{1}H, ^{13}C, and 2D NMR spectroscopy

All spectra were acquired at 50 °C with a Bruker AVANCE 600 spectrometer (600 MHz ^{1}H frequency) equipped with a 5 mm triple resonance *z*-gradient probe (Bruker, Billerica, MA). Data were acquired and processed in TOPSPIN v1.3 from Bruker BioSpin. Spectral widths of 4200 Hz and 36,000 Hz were used for ^{1}H and ^{13}C spectra, respectively. A series of 2D spectra, ge-DQF COSY, TOCSY, NOESY, and ge-HSQC, were recorded with standard pulse sequences using states-time proportion phase incrementation for quadrature detection in the indirect dimension. The isotropic mixing (MLEV17) time for the TOCSY was 120 ms; the mixing time for the NOESY was 80 ms. Spectral widths of 4200 Hz and 25,000 Hz were used for ^{1}H and ^{13}C spectra, respectively, for the ^{1}H–^{13}C ge-HSQC.

3. Results and discussion

3.1. 1D NMR analysis

For the purposes of discussing the ^{1}H and ^{13}C assignments, the three monosaccharide residues, D-galactosamine, D-galacturonic acid (in monovalent salt form), and diamino-6-deoxy-D-glucose are designated with a boldfaced letter **A**, **B**, or **C**, respectively. Additionally, **a** and **d** are used to denote native O-acetylated and O-deacetylated APE, respectively. Thus, the D-galactosamine in the native APE molecule is designated as **Aa**. After O-deacetylation, that same residue is labeled **Ad**. By extension, the specific positions in each sugar ring are designated **AIa**, where **I** = **1** for the anomeric carbon and hydrogen atoms and **2**–**6** for the other positions in the sugar. Upon O-deacetylation, these same positions are labeled **AId**.

The native APE ^{1}H spectrum (Fig. 1(a)) contained five anomeric proton resonances with chemical shifts in the range of 4.6–5.5 ppm. The peaks at 2.1 ppm suggested methyl groups of O-acetyl functional group origin, while the peaks at 2.0 ppm indicated methyl groups of N-acetyl functional group origin in acetamido sugars. The peak present at 1.12 ppm was indicative of the methyl hydrogen atoms of a 6-deoxy sugar. The peaks at 1.24, 2.32, and 2.42 ppm suggested the presence of an aliphatic side chain, subsequently identified as a 3-hydroxybutyramido group.

The ^{13}C NMR spectrum of native APE (Fig. 1(b)) also contained five anomeric peaks in the range of 99–102 ppm. Additionally present in the spectrum were six carbonyl signals in the range of 176–178 ppm. These peaks, with some possible overlapping of signals, confirmed the presence of at least three N-acetyl groups, one uronic acid, one O-acetyl group, and one additional group later identified as the 3-hydroxybutyramido side chain. Four peaks in the range of 52–58 ppm provided further evidence of four N-substituted ring carbon atoms, while two peaks in the 62–65 ppm range suggested the presence of two hydroxymethyl groups.

Although de-O-acetylation was incomplete, it did lead to a significant reduction in the intensity of peaks associated with the O-acetylated residue as well as the neighboring **C** residue. Thus, the de-O-acetylated form of APE gave anomeric ^{1}H and ^{13}C NMR spectra (Supplementary data, Fig. 7 and Fig. 8, respectively) recognizable as those of a regular linear polytrisaccharide with partial O-acetylation. The ^{1}H and ^{13}C spectra were each reduced to three anomeric signals with anomeric hydrogen chemical shifts of 4.82 (**C1d**), 5.24 (**B1**), and 5.39 (**A1d)** ppm, and anomeric carbon chemical shifts of 99.6 (**B1**), 100.5 (**A1d**), and 101.0 (**C1d**) ppm, respectively. For the ^{13}C NMR spectrum, five peaks were present in the 176–178 ppm range while one peak appeared in the 62–65 ppm window, suggesting O-deacetylation at the C6 (**A6a**) position of the galactosamine residue. Intensities of the anomeric signals in the native APE ^{1}H spectrum indicated approximately 50% O-acetyl substitution for both Residue **A** and Residue **C**.

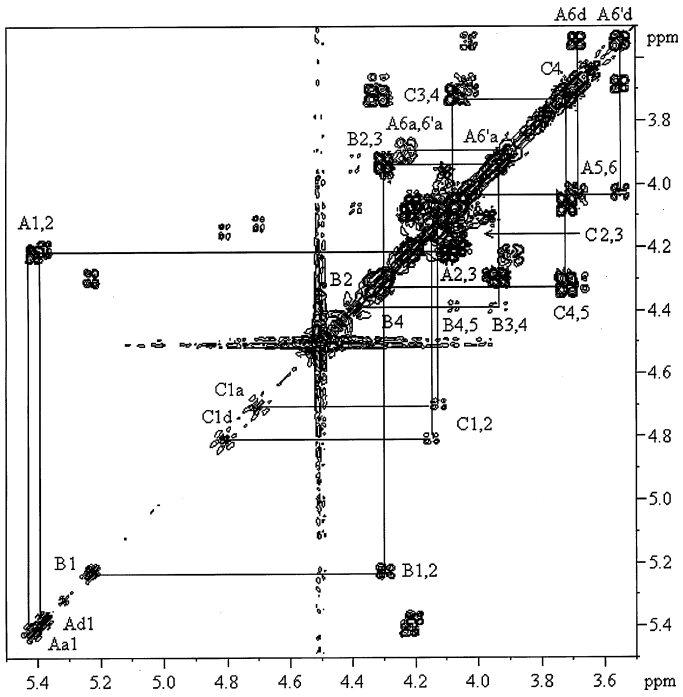

Fig. 2. ^{1}H–^{1}H DQF-COSY NMR spectrum of the native form of APE. Inter-residue cross signals are indicated.

3.2. 2D NMR analysis

Partial ^{1}H assignments of native APE were made from the DQF-COSY spectrum (Fig. 2). The anomeric resonances of Residue **Aa** and **Ad** at 5.42 and 5.39 ppm, respectively, gave cross-peaks to **A2**, and subsequent cross-peaks to **A3**. However, further spin connectivity could not be determined from **A3** to **A4** in the DQF-COSY. Cross-peaks from **A6a**, **A6a′** at 3.70, 3.56 ppm to **A5a** and **A6d**, **A6a′** at 4.21, 3.90 ppm to **A5d** were observed, but **A4**,**5** signals were likewise undetectable. The absence of further spin connectivity in the

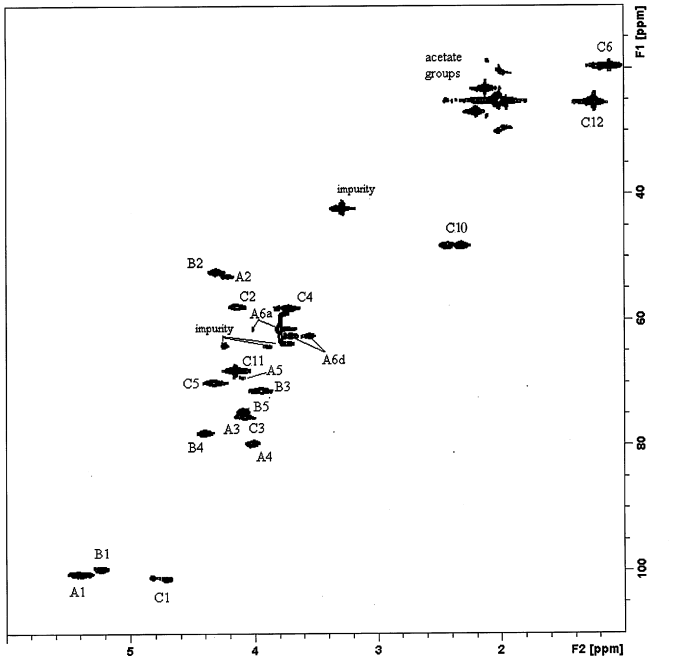

Fig. 3. ^{1}H–^{13}C HSQC NMR spectrum of native APE showing signals from all pyranosyl residues (**A**, **B**, and **C**) as well as the 3-hydroxybutyramido side chain of Residue **C**.

Table 1
^{1}H and ^{13}C chemical shift assignments in ppm.

Atom	Residue				
	Gal*p*NAc **Aa**	Gal*p*NAc **Ad**	Gal*p*NAcA **B**	Qui*p*N4NH$_b$ **Cd**	Qui*p*NAc4NH$_b$ **Ca**
H1	5.42	5.39	5.24	4.82	4.71
H2	4.23	4.21	4.30	4.16	4.14
H3	4.10	4.07	3.94	4.07	4.09
H4	4.01	3.99	4.40	3.71	3.73
H5	4.10	4.04	4.08	4.32	4.33
H6	4.21	3.70	–	1.12	1.12
H6′	3.90	3.56	–	–	–
H8	~2	~2	~2	~2	~2
H10	–	–	–	2.32	2.42
H11	–	–	–	4.14	4.14
H12	–	–		1.24	1.24
C1	100.3	100.5	99.6	101.0	101.2
$_1J_{C,H}$ (Hz)	177.8	177.4	172.6	168.6	173.0
C2	53.0	53.0	52.2	57.7	57.7
C3	75.5	75.5	71.5	75.4	75.4
C4	79.7	79.7	77.8	58.0	58.0
C5	69.4	69.4	74.6	69.9	69.9
C6	62.8	64.1	177.3	19.5	19.5
C7	~176–178	~176–178	~176–178	~176–178	~176–178
C8	~25	~25	~25	~25	~25
C9	–	–	–	~176–178	~176–178
C10	–	–	–	47.9	47.9
C11	–	–	–	67.8	67.8
C12	–	–	–	25.2	25.2

A, **B**, and **C** are residue names as assigned in Fig. 1.
a and **d** refer to the native acetylated and the O-deacetylated forms of the polymer.
^{1}H and ^{13}C NMR chemical shifts for the APE component of the emulsan complex of *Acinetobacter venetianus* RAG-1.

DQF-COSY spectrum was considered to arise from small (<3 Hz) $J_{3,4}$ and $J_{4,5}$ coupling constants, which prevented magnetization transfer past either **A3** or **A5**. This result was expected for a galactopyranosyl residue. In the case of Residue **B**, all protons, **B1** through **B5**, were identified, with **B3**,**4** and **B4**,**5** giving weak cross-peaks due to the aforementioned small coupling constants. The chemical shift of **B4** at 4.40 ppm was typical for a uronic acid. For Residue **C**, connectivity was established for all protons, **C1** through **C6**. Strong coupling throughout this spin system suggested a glucopyranosyl configuration with a 6-deoxy methyl group. Based upon the ^{1}H spin system beginning with the methyl resonance at 1.24 ppm (**C12**) coupled to a hydroxymethyl group at 4.14 ppm (**C11**) and finally to methylene groups at 2.32 and 2.42 ppm (**C10**), the side chain was

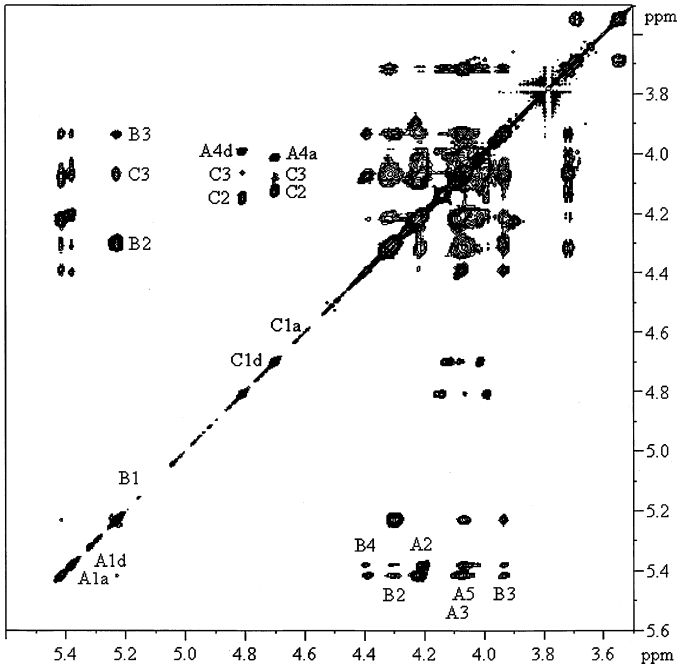

Fig. 4. ^{1}H–^{1}H NOESY NMR spectrum showing NOE connectivities related to the anomeric proton resonances of the three APE residues.

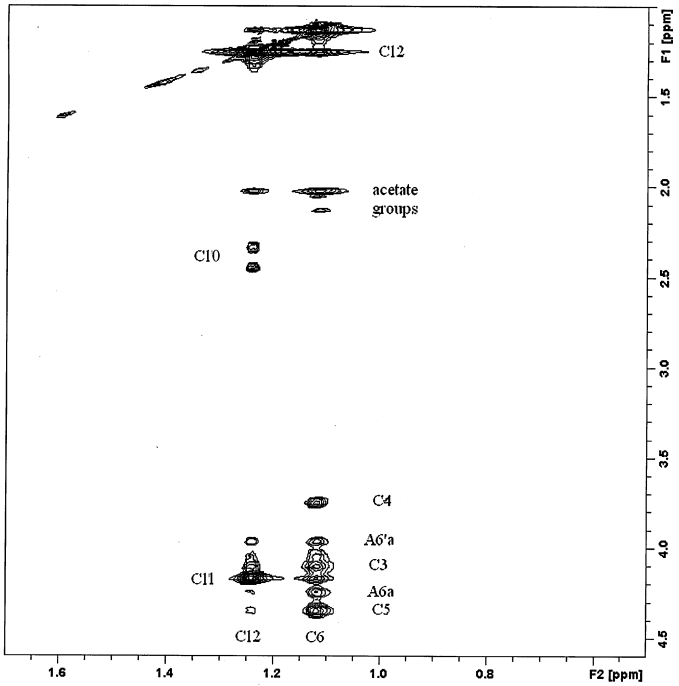

Fig. 5. ^{1}H–^{1}H NOESY NMR spectrum of native APE showing NOE connectivities related to the proton resonances of **C6**, the 6-deoxy methyl group of Residue **C**, and **C12**, H4 of the 3-hydroxybutyramido side chain. Interesting to note were the absences of **A6d** signals, further indicating that the acetyl group on Residue **A** may act to induce a conformational change to Residue **C**.

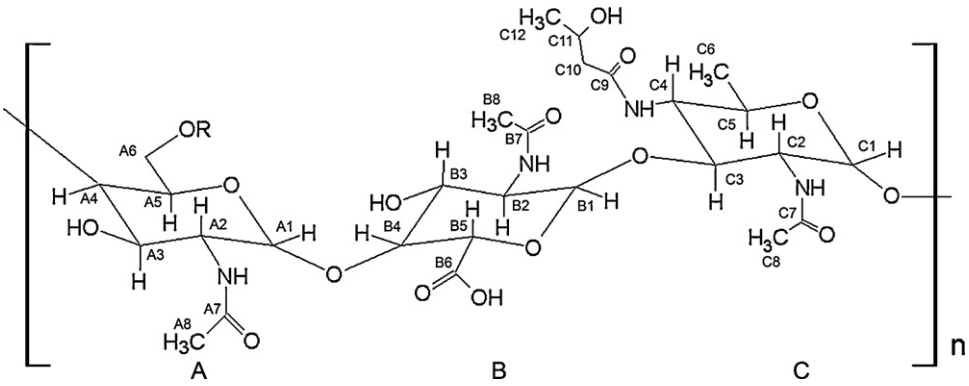

Fig. 6. Repeating unit for the APE component isolated from the emulsan complex of *Acinetobacter venetianus* RAG-1. R = acetyl or alcohol moiety.

shown to be a 3-hydroxybutyramido group (Supplementary data, Fig. 9).

Complete ^{1}H assignments (Table 1) for APE residues were achieved by means of a TOCSY experiment (Supplementary data, Fig. 10). Also confirmed from the TOCSY spectrum were ^{1}H assignments and connectivities for the 3-hydroxybutyramido side chain of Residue **C**. From those ^{1}H assignments, it was established that Residues **A**, **B**, and **C** were 6-O-acetyl-D-galactosamine, D-galacturonic acid, and 4-(3-hydroxybutyramido)-diamino-4,6-dideoxy-D-glucose, respectfully. Although a chemical shift was seen for the anomeric signal of Residue **C** upon O-deacetylation, suggesting a second O-acetylation site for APE, a more conventional interpretation was that O-acetyl groups can have long-range interactions affecting not only the repeating unit upon which they reside, but also the neighboring residue (Batley, Redmond, Packer, Liu, & Reeves, 1997; Dag et al., 2004). Previous research by Masoud, Ho, Schollaardt, and Perry (1997) had shown that upon O-deacetylation the anomeric proton signal of the acetylated residue underwent an upfield shift while concomitantly exhibiting a downfield shift in the anomeric proton signal of the neighboring residue. Additionally post de-O-acetylation, the anomeric carbon signal of the acetylated residue underwent a downfield shift while concurrently exhibiting an upfield shift in the anomeric carbon signal of the neighboring residue (Masoud et al., 1997). The similar, observed shifts for the anomeric signals of Residue **C** upon de-O-acetylation may be due to the acetyl group on Residue **A** inducing a conformational change to Residue **C**, perhaps due to hydrogen bonding between H1 (**C1**) of Residue **C** and O6 of Residue **A**. Alternatively, given the absence of sites for O-acetylation on Residue **C**, the possibility that the de-O-acetylation procedure that was adapted from Dag et al. (2004) also resulted in some N-deacetylation cannot be excluded. Following the analysis of changes in chemical shifts (Table 1) due to O-deacetylation, it was concluded that the most probable site for O-acetylation is on **A6** although **A3** and **B3** are also potential sites for such modifications.

Complete ^{13}C assignments (Table 1) of native APE were determined from ge-HSQC spectra (Fig. 3). In the O-deacetylated APE spectrum, the assigned anomeric proton resonances at 4.82, 5.24, and 5.39 ppm showed correlations to directly bonded carbon resonances 101.0, 99.6, and 100.5 ppm, respectfully. All $^{1}J_{C,H}$ coupling constants were in the range of 168–178 Hz (Supplementary data, Fig. 11), indicating α-anomeric configurations for all residues. The HSQC spectrum for native APE contained four resonances in the 52–58 ppm window, confirming the presence of three acetamido groups and one 3-hydroxybutyramido group. For each residue, the acetamido group was correlated to the C2 position. The 58.0 ppm signal for **C4** in Residue **C** verified it as the acylation site for the 3-hydroxybutyramido group. Thus the three residues of APE were identified as (**A**) 6-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranose (α-D-Gal*p*NAc-6-OAc), (**B**) 2-acetamido-2-deoxy-α-D-galactopyranosyluronic acid (α-D-Gal*p*NAcA), and (**C**) 2-acetamido-4-(3-hydroxybutyramido)-2,4,6-trideoxy-α-D-glucopyranose (α-D-Qui*p*NAc4NHb). The 6-deoxy sugar (Residue **C**) might alternately be described as a quinovose derivative.

3.3. Structural determination of APE

The sequence of the monosaccharide repeating units as well as the connectivity for the 3-hydroxybutyramido side chain were determined from the 2D NOESY spectrum (Figs. 4 and 5, respectively). Transglycosidic NOE correlations were observed for hydrogen atom pairs **C1** with **A4**; **A1** with **B2**, **B3**, and **B4**, as well as **B1** with **C3**. Due to the relative deshielding observed for the carbon resonance of **B4**, causing a significant downfield chemical shift as compared to the resonances of unsubstituted residues, it was concluded that **B4** was the glycosidic linkage site. From this, the sequence of the polysaccharide APE (Fig. 6) was determined to be →4)-α-D-Gal*p*NAc-6-OAc-(1 → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui*p*NAc4NHb-(1→ with approximately 50% O-acetyl substitution on the galactosamine residue. For all residues, the NOESY spectrum exhibited intra-residue NOE correlations that supported pyranosyl configuration. The NOE data showed some additional inter-residue correlations with respect to **A1** of Residue **A** and **B2** and **B3** of Residue **B** that were suggestive of an ordered solution conformation for APE. Further research in this area, possibly involving HR-MAS techniques, will be needed to elucidate this secondary structure.

4. Conclusions

The *Acinetobacter* Polyelectrolytic Exopolysaccharide (APE) structure was determined utilizing the ^{1}H and ^{13}C NMR spectroscopic methods of 1D ^{1}H and ^{13}C and 2D ge-DQF COSY, TOCSY, NOESY, and ge-HSQC experiments. Analysis was conducted through examination of first the ^{1}H and ^{13}C spectra, and then these results were compared to the more robust 2D techniques in order to render a structure. Furthermore, to accurately elucidate the structure, native APE was compared to the O-deacetylated polysaccharide. APE was found to contain 6-O-acetyl-2-acetamido-2-deoxy-α-D-galactopyranose, 2-acetamido-2-deoxy-α-D-galactopyranosyl-uronic acid, and 2-acetamido-4-(3-hydroxybutyramido)-2,4,6-trideoxy-α-D-glucopyranose residues. The sequence of the linear trisaccharide repeating unit of the polysaccharide APE was determined to be →4)-α-D-Gal*p*NAc-6-OAc-(1 → 4)-α-D-Gal*p*NAcA-(1 → 3)-α-D-Qui*p*NAc-4NHb-(1→.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.12.006.

References

Batley, M., Redmond, J. W., Packer, N. H., Liu, D., & Reeves, P. (1997). The relationship between the structures of the O polysaccharides from *Escherichia coli* O17 and O16. *Carbohydrate Research*, *303*, 313–318.

Castro, G. R., Kamdar, R. R., Panilaitis, B., & Kaplan, D. L. (2005). Triggered release of proteins from emulsan–alginate beads. *Journal of Controlled Release*, *109*, 149–157.

Castro, G. R., Panilaitis, B., Bora, E., & Kaplan, D. L. (2006). Controlled release biopolymers for enhancing the immune response. *Molecular Pharmaceutics*, *4*(1), 33–46.

Dag, S., Niedziela, T., Dzieciatkowska, M., Lukasiewicz, J., Jachymek, W., Lugowski, C., et al. (2004). The *O*-acetylation patterns in the *O*-antigens of *Hafnia alvei* strains PCM 1200 and 1203, serologically closely related to PCM 1205. *Carbohydrate Research*, *339*(15), 2521–2527.

Dams-Kozlowska, H., & Kaplan, D. L. (2007). Protein engineering of Wzc to generate new emulsan analogs. *Applied and Environmental Microbiology*, *73*, 4020–4028.

Dams-Kozlowska, H., Mercaldi, M. P., Panilaitis, B. J., & Kaplan, D. L. (2008). Modifications and applications of the *Acinetobacter venetianus* RAG-1 exopolysaccharide, the emulsan complex and its components. *Applied Microbiology and Biotechnology*, *81*, 201–210.

Di Cello, F. D., Pepi, M., Baldi, F., & Fani, R. (1997). Molecular characterization of an *n*-alkane-degrading bacterial community and identification of a new species *Acinetobacter venetianus*. *Research in Microbiology*, *148*(3), 237–249.

Gorkovenko, A., Zhang, J., Gross, R. A., & Kaplan, D. L. (1999). Control of unsaturated fatty acid substituents in emulsans. *Carbohydrate Polymers*, *39*, 79–84.

Gutnick, D. L. (1987). The emulsan polymer: Perspectives on a microbial capsule as an industrial product. *Biopolymers*, *26*(S0), S223–S240.

Kasaai, M. R. (2009). Various methods for determination of the degree of N-acetylation of chitin and chitosan: A review. *Journal of Agricultural and Food Chemistry*, *57*(5), 1667–1676.

Kumar, A. S., Mody, K., & Jha, B. (2007). Bacterial exopolysaccharides–a perception. *Journal of Basic Microbiology*, *47*, 103–117.

Laplante, S., Turgeon, S. L., & Paquin, P. (2005). Emulsion stabilizing properties of various chitosans in the presence of whey protein isolate. *Carbohydrate Polymers*, *59*, 425–434.

Masoud, H., Ho, M., Schollaardt, T., & Perry, M. B. (1997). Characterization of the capsular polysaccharide of *Burkholderia (Pseudomonas) pseudomallei* 304b. *Journal of Bacteriology*, *179*(18), 5663–5669.

Mercaldi, M. P., Dams-Kozlowska, H., Panilaitis, B., Joyce, A. P., & Kaplan, D. L. (2008). Discovery of the dual polysaccharide composition of emulsan and the isolation of the emulsion stabilizing component. *Biomacromolecules*, *9*(7), 1988–1996.

Mudd, S., & Mudd, E. B. H. (1924). The penetration of bacteria through capillary spaces IV. A kinetic mechanism in interfaces. *Journal of Experimental Medicine*, *40*, 633–645.

Panilaitis, B., Johri, A., Blank, W., Kaplan, D., & Fuhrman, J. (2002). Adjuvant activity of emulsan, a secreted lipopolysaccharide from *Acinetobacter calcoaceticus*. *Clinical and Diagnostic Laboratory Immunology*, *9*(6), 1240–1247.

Rosenberg, M., & Rosenberg, E. (1981). Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *Journal of Bacteriology*, *148*(1), 51–57.

Ruijssenaars, H. J., Stingele, F., & Hartmans, S. (2000). Biodegradability of food-associated extracellular polysaccharides. *Current Microbiology*, *40*, 194–199.

Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. *Trends in Biotechnology*, *16*, 41–46.

van de Velde, F., Pereira, L., & Rollema, H. S. (2004). The revised NMR chemical shift data of carrageenans. *Carbohydrate Research*, *339*(14), 2309–2313.

van Kranenburg, R., Ingeborg, C. B., Kleerebezem, M., & de Vos, W. M. (1999). Genetics and engineering of microbial exopolysaccharides for food: Approaches for the production of existing and novel polysaccharides. *Current Opinion in Biotechnology*, *10*, 498–504.

Vaneechoutte, M., Tjernberg, I., Baldi, F., Pepi, M., Fani, R., Sullivan, E. R., et al. (1999). Oil-degrading *Acinetobacter* strain RAG-1 and strains described as '*Acinetobacter venetianus* sp nov.' belong to the same genomic species. *Research in Microbiology*, *150*(1), 69–73.

Zhang, J., Gorkovenko, A., Gross, R. A., Allen, A. L., & Kaplan, D. (1997). Incorporation of 2-hydroxyl fatty acids by *Acinetobacter calcoaceticus* RAG-1 to tailor emulsan structure. *International Journal of Biological Macromolecules*, *20*, 9–21.

Zosim, Z., Gutnick, D., & Rosenberg, E. (1983). Uranium binding by emulsan and emulsanosols. *Biotechnology and Bioengineering*, *25*(7), 1725–1735.

Zuckerberg, A., Diver, A., Peeri, Z., Gutnick, D. L., & Rosenberg, E. (1979). Emulsifier of *Arthrobacter* RAG 1: Chemical and physical properties. *Applied and Environmental Microbiology*, *37*(3), 410–414.