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Structural studies of the extracellular polysaccharide produced by *Butyrivibrio fibrisolvens* strain H10b

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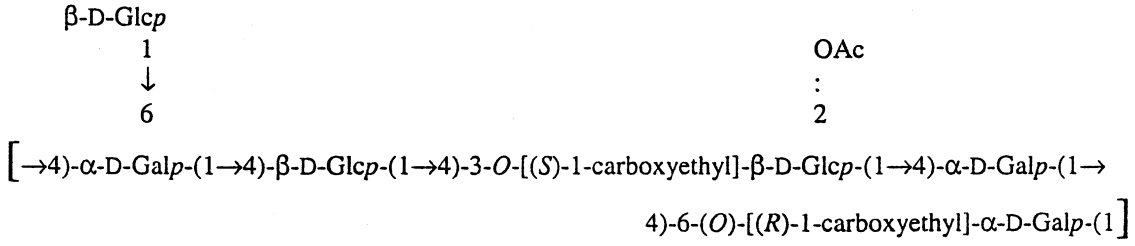
Abstract

The extracellular polysaccharide produced by *Butyrivibrio fibrisolvens* strain H10b, when grown under strictly anaerobic conditions with glucose as carbohydrate source, has been studied by chemical and spectroscopic techniques. The results demonstrate that the polysaccharide consists of hexasaccharide repeating units with the following structure:



The isolated polysaccharide was found to be ~65% acetylated at O-2 of the 3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p* residue. The absolute configuration of the 1-carboxyethyl groups was determined by circular dichroism.

Keywords: *Butyrivibrio fibrisolvens*; Bacterial polysaccharide; (1-Carboxyethyl)-galactose; (1-Carboxyethyl)-glucose; CD; Structures

1. Introduction

The anaerobic bacterium *Butyrivibrio fibrisolvens* is commonly found in the ruminal and cecal portions of the gastrointestinal tracts of ruminant animals.[1] It is involved in the degradation of cellulose and other plant polysaccharides into readily accessible carbohydrates. There are large variations within the genus *B. fibrisolvens* with respect to morphology, nutritional requirements, substrates fermented and fermentation products. When grown in vitro on a defined medium[1] most strains of the bacterium produce extracellular polysaccharides which contain an assortment of unusual monosaccharide constituents such as L-iduronic acid,[2] L-altrose[3] and several (1-carboxyethyl)-sugars.[1]

The detailed structures of the repeating units for the extracellular polysaccharides produced by *B. fibrisolvens* strains X6C61,[4] 49[5] and CF3[6] have been reported. We continue these studies with other selected strains to define the structures of the major classes of extracellular polysaccharides produced by *B. fibrisolvens*. A previous study with strain H10b, a bacterial isolate from the ovine ceccum, has shown the presence of glucose, galactose and two (1-carboxyethyl)-hexoses.[1] We now report further structural studies of this polysaccharide.

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2. Results and discussion

The crude polysaccharide material, prepared as described,[1] contained large amounts of high-molecular impurities as shown by the ^{1}H NMR spectrum with signals in the δ 7–8 region together with other broad non-carbohydrate signals. Treatment with a mixture of DNAse, RNAse and Proteinase K gave a material after dialysis, which had increased water solubility and reduced viscosity. A ^{1}H NMR spectrum showed that signals from contaminants were drastically reduced. Further purification on DEAE-Sephadex A-50, using an aqueous NaCl gradient, resulted in the isolation of a polysaccharide, which was eluted by 0.2–0.3 M NaCl. The polysaccharide was partly acetylated as determined from a signal at δ 2.19 in the ^{1}H NMR spectrum (Fig. 1(a)). Removal of the acetyl groups (0.1 M NaOH) followed by dialysis and lyophilization gave a homogenous material (Fig. 1(b)).

Monosaccharide analysis of the polysaccharide showed only glucose and galactose when the products after acidic hydrolysis were analyzed as the alditol acetates by GC–MS. The traces of rhamnose, which were reported in a preliminary study of a less pure sample of the same polysaccharide,[1] could not be detected. However, when the products were analyzed by GC–MS as the pertrimethylsilylated alditols glucitol, galactitol, 3- and 6-*O*-(1-carboxyethyl)hexitol were found in a 0.9:1.0:0.7:0.6 ratio. The absolute configuration of D-glucose and D-galactose was determined by the method of Gerwig and co-workers[7] where the retention times for the pertrimethylsilylated 2-(*S*)-butyl glycosides were compared with authentic pertrimethylsilylated 2-(*S*)- and (*R*)-butyl glycosides on GC–MS. The monosaccharides released after acidic hydrolysis of the acidic polysaccharide were also studied by ^{1}H NMR spectroscopy. Comparison of the signal intensities of the CH_3 groups of the 1-carboxyethyl substituted components with the rest of the observed signals showed that there was a 2:1 ratio of neutral and 1-carboxyethyl substituted components. The acidic components were separated from the neutral components by anion exchange chromatography and the ^{1}H NMR spectrum obtained for the mixture of the acidic components was compared to spectra obtained for synthetic 3-*O*-[(*R*)- and (*S*)-1-carboxyethyl]-substituted D-galactose, D-glucose and D-mannose and 6-*O*-[(*R*)- and (*S*)-1-carboxyethyl]-D-galactose. The components had chemical shifts and coupling patterns identical with those of 3-*O*-[(*S*)-1-carboxyethyl]-D-glucose and 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose identifying them as these monosaccharides or their enantiomers.

To determine the absolute configuration of 6-*O*-(1-carboxyethyl)-galactose and 3-*O*-(1-carboxyethyl)-glucose a combination of ^{1}H NMR and CD data was used. The polysaccharide was hydrolysed and the monosaccharides were separated by chromatography on Dowex 50 WS-2 (Ca^{2+}) resin. The absolute configuration of the 1-carboxyethyl group of the respective monosaccharide was determined from the sign and shape of the ellipticity maximum at 210 nm observed in the CD spectrum[8] (Fig. 2) recorded for samples in H_2O at pH 2. The spectra were compared with those of (*R*)- and (*S*)-lactic acid and the 1-carboxyethyl substituted monosaccharides were identified as 6-*O*-[(*R*)-1-carboxyethyl]-galactose and 3-*O*-[(*S*)-1-carboxyethyl]-glucose. Since the ^{1}H NMR spectra of these compounds were identical to the spectra obtained for synthetic 6-*O*-[(*R*)-1-carboxyethyl]-D-galactose and 3-*O*-[(*S*)-1-carboxyethyl]-D-glucose and significantly different from the spectra obtained for 6-*O*-[(*S*)-1-carboxyethyl]-D-galactose and 3-*O*-[(*R*)-1-carboxyethyl]-D-glucose, the absolute configuration of both sugars must be D. Further evidence for the D configuration was obtained upon treatment of

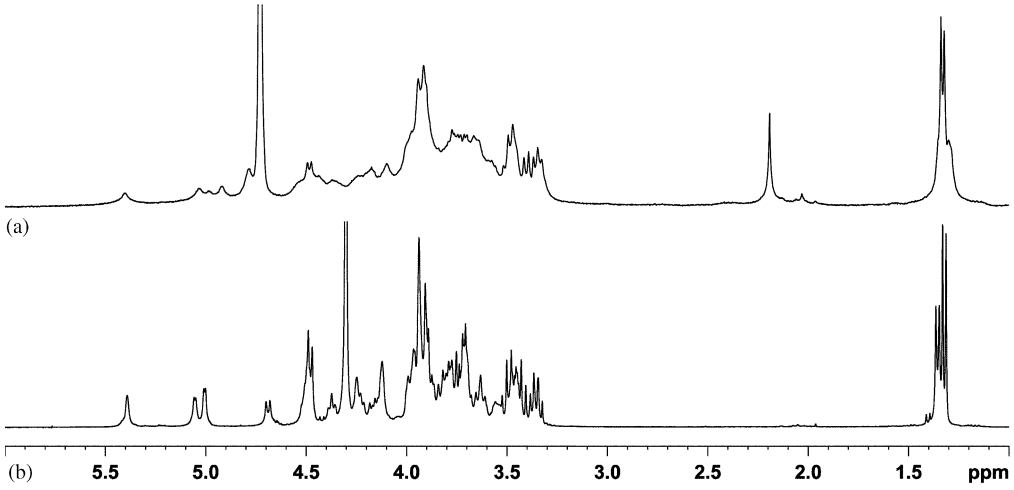

Fig. 1. ^{1}H NMR spectra of: (a) the extracellular polysaccharide produced by *B. fibrisolvens* strain H10b; and (b) the deacetylated polysaccharide.

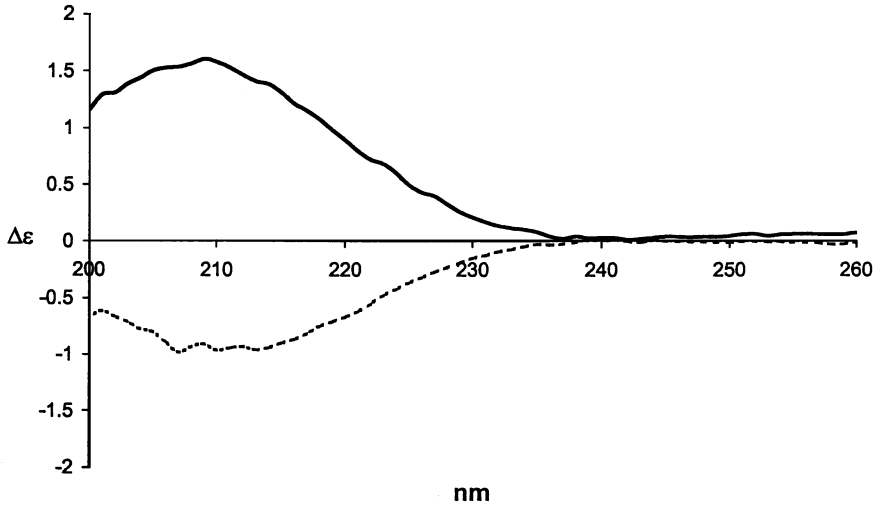

Fig. 2. CD spectra of 6-*O*-[(*R*)-1-carboxyethyl]-D-Gal (dashed line) and 3-*O*-[(*S*)-1-carboxyethyl]-D-Glc (solid line).

the 3-*O*-[(*S*)-1-carboxyethyl]-D-glucose with acetic acid-d_4 at 60 °C, which resulted in lactone formation almost exclusively to the position 4 as determined from a ^{1}H NMR spectrum.[9] In a 150 ms NOESY experiment, a strong cross peak was observed between H-2′ and H-3, while no cross-peak could be detected from H-3′. These findings are in agreement with a lactone formation to the position 4 of methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-D-glucopyranoside provided that the lactone ring is in a pseudo chair conformation.[9]

To identify the linkage positions, the deacetylated polysaccharide was carboxyl reduced[10] using $NaBH_4$ as the reducing agent and then subjected to methylation analysis.[11] The analysis by GC–MS revealed the presence of six partially methylated alditol acetates derived from 2,3,4,6-tetra-*O*-methyl-glucose, 2,3,6-tri-*O*-methyl-glucose, 2,3,6-tri-*O*-methyl-galactose, 2,3-di-*O*-methyl-galactose, 3-*O*-(2-methoxy-1-methylethyl)-2,6-di-*O*-methyl-glucose (Fig. 3) and 6-*O*-(2-methoxy-1-methylethyl)-2,3-di-*O*-methyl-galactose.

The NMR samples used for structural studies (5–15 mg/mL) were viscous and gave relatively broad signals in the acquired spectra. ^{1}H NMR and HSQC spectra of the deacetylated polysaccharide displayed signals from six anomeric protons and carbons indicating a hexasaccharide repeating unit. Signals at δ_H/δ_C 1.35/18.7 and 1.32/19.1 supported the presence of two different (1-carboxyethyl)-hexoses.[1] Because of overlapping signals in ^{1}H and ^{13}C NMR spectra, assignments of signals and identification of the sugar residues had to be done by combinations of different two-dimensional techniques and comparison of the chemical shifts with published data on similarly substituted sugar residues. The identities of the monosaccharides were shown by comparison of the observed chemical shifts with NMR data reported for respective monosaccharide[5,6,12,13] and from estimated $^3J_{H,H}$-values extracted from the cross peaks in the 2D spectra. The anomeric configurations were obtained from the chemical shifts and the $^1J_{H,C}$- and $^3J_{H,H}$-values for signals of the anomeric atoms. Six

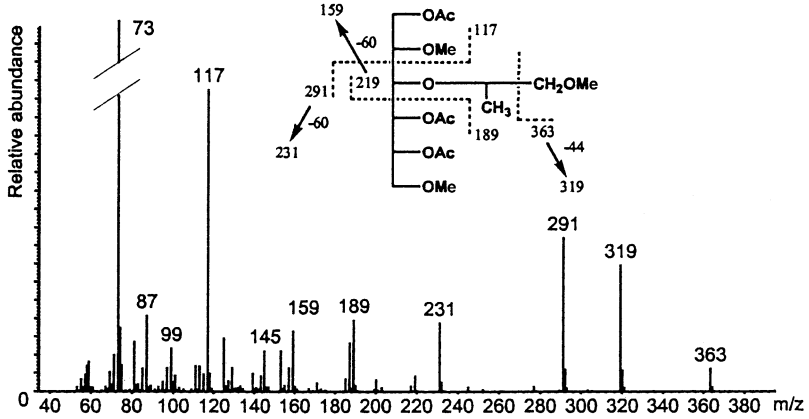

Fig. 3. EI mass spectrum and the primary fragments of 1,4,5-tri-*O*-acetyl-3-*O*-[(*S*)-2-methoxy-1-methylethyl]-2,6-di-*O*-methyl-D-glucitol.

signal pairs (δ_H 5.39/δ_C 101.0, 5.05/100.7, 5.00/101.1, 4.69/104.4, 4.48/102.2 and 4.48/103.4 ppm) were identified in the HSQC spectrum and the chemical shifts in combination with measured $^1J_{H,C}$-values suggested three α- and three β-hexopyranosides.

Determination of the sugar residues started with assignment of the spin systems containing the anomeric protons (H-1). Then H-2 could be assigned from the cross peaks in the COSY spectra but, due to severe overlap of ring proton signals, the rest of the spin systems had to be assigned by combinations of different COSY, TOCSY and 1D-TOCSY with several mixing times, and HSQC experiments. Using the identified ^{1}H spin systems and $^1J_{C,H}$-connectivities, the ^{13}C signals were assigned from HSQC, HSQC–DEPT and HSQC–TOCSY experiments. The linkage positions were determined from the downfield shifted signals from the substituted carbons. The ^{1}H and ^{13}C signals for all residues could be assigned (Table 1) from the procedures described and the assignments were verified by cross peaks found in NOESY and HMBC spectra (Tables 2 and 3, respectively).

Residue **I** with the H-1/C-1 signals at δ 5.39/101.0 and a small $^3J_{H\text{-}1,H\text{-}2}$-value was assigned as a 4,6-disubstituted α-D-Gal residue due to the similarities between the ^{1}H chemical shifts with those of α-D-Gal, the small coupling constants between H-1 and H-2 and H-3, H-4 and H-5 and the high chemical shift values for the C-4 and C-6 signals. The spin system from H-1 to H-5 was detected in a TOCSY experiment and 1D TOCSY and HSQC experiments indicated that the H-2 and H-3 and C-2 and C-3 signals overlapped at δ 3.94 and 69.8, respectively. Residue **II** with the H-1/C-1 signals at δ 5.05/100.7, $^3J_{H\text{-}1,H\text{-}2}$ 3.9 Hz, was assigned as a 4-substituted 6-*O*-(1-carboxyethyl)-α-D-Gal residue due to the similarities between the ^{1}H chemical shifts with those of 6-*O*-(1-carboxyethyl)-α-D-Gal,[5] the small coupling constants between H-1 and H-2 and H-3, H-4 and H-5 and the high chemical shift value for the C-4 signal. A cross peak in the HMBC spectrum identified a three-bond connectivity between C-6 and H-2′ of the (1-carboxyethyl)-substituent (δ 3.89) which corroborated the assignment. Residue **III** with the H-1/C-1 signals at δ 5.00/101.1, $J_{H\text{-}1,H\text{-}2}$ 3.8 Hz, was identified as 4-substituted α-D-Gal from the similar ^{1}H chemical shifts and coupling constants as those of residues **I** and **II** and the high chemical shift of the C-4 signal (δ 79.3). Residue **IV** with the H-1/C-1 signals δ 4.69/104.4, $J_{H\text{-}1,H\text{-}2}$ 7.8 Hz, was identified as 4-substituted 3-*O*-(1-carboxyethyl)-β-D-Glc from the ^{1}H chemical shifts, the large vicinal coupling constants between all ring protons and the high chemical shifts of the C-3 (δ 80.1) and C-4 (δ 76.8) signals. The position of the 1-carboxyethyl substituent was also corroborated by a cross peak in the HMBC spectrum between the signals from C-3 and H-2′ of the 1-carboxyethyl group. Residue **V** with the signals for H-1/C-1 at δ 4.48/102.2, $J_{H\text{-}1,H\text{-}2}$ 7.8 Hz, was identified as 4-substituted β-D-Glc from the ^{1}H chemical shifts, the large vicinal coupling constants between all ring protons and the high chemical shifts of the C-4 (δ 78.8) signal. Residue **VI** with the signals for H-1/C-1 at δ 4.48/103.4, $J_{H\text{-}1,H\text{-}2}$ 7.8 Hz, was identified as terminal β-D-Glc from the large vicinal coupling constants between all ring protons and the similar ^{1}H and ^{13}C chemical shifts with those of β-D-Glc. The weak HMBC cross peaks observed between the H-5 (δ 3.46) and C-6 (δ 62.0) signals in combination with HSQC results differentiated the signals of H-6a/b in this residue from

Table 1
^{1}H and ^{13}C NMR data obtained at 70 °C (pH 5.3) for deacetylated extracellular polysaccharide produced by *B. fibrisolvens* strain H10b

Residues	H-1 C-1	($J_{H\text{-}1,H\text{-}2}$, Hz) ($J_{C\text{-}1,H\text{-}1}$, Hz)	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a/H-6b C-6	 C-1′	H-2′ C-2′	H-3′ C-3′
→4,6)-α-D-Gal*p*-(1→	5.39	(n.r.) [a]	3.94	3.94	4.11	4.23	3.90/4.16			
I	101.0	(173)	69.8	69.8	78.4	71.4	68.6			
→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→	5.05	(3.9)	3.88	3.99	4.12	4.51	3.70/3.70		3.89	1.32
II	100.7	(170)	69.9	69.8	78.8	70.7	67.8	181.6	78.8	19.1
→4)-α-D-Gal*p*-(1→	5.00	(3.8)	3.93	3.97	4.25	4.37	3.70/3.79			
III	101.1	(172)	70.2	70.7	79.3	71.2	61.3			
→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→	4.69	(7.8)	3.45	3.63	3.84	3.55	3.80/3.98		4.48	1.35
IV	104.4	(158)	73.7	80.1	76.8	76.2	61.1	182.0	77.6	18.7
→4)-β-D-Glc*p*-(1→	4.48	(7.8)	3.37	3.75	3.63	3.49	3.77/3.92			
V	102.2	(161)	74.2	76.9	78.8	75.8	61.9			
β-D-Glc*p*-(1→	4.48	(7.8)	3.35	3.50	3.40	3.46	3.72/3.91			
VI	103.4	(156)	74.1	76.8	70.8	76.8	62.0			

Table 2
Significant NOEs observed for the anomeric protons of the deacetylated polysaccharide of *B. fibrisolvens* strain H10b

Residues		Anomeric proton		NOE to	
		δ_H	δ_H	Proton	Residues
I	→4,6)-α-D-Gal*p*-(1→	5.39	3.63	H-4	**V**
			3.94	H-2	**I**
II	→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→	5.05	4.11	H-4	**I**
			3.88	H-2	**II**
III	→4)-α-D-Gal*p*-(1→	5.00	4.12	H-4	**II**
			3.93	H-2	**III**
			3.70	H-6	**II**
IV	→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→	4.69	4.25	H-4	**III**
			3.63	H-3	**IV**
			3.55	H-5	**IV**
V	→4)-β-D-Glc*p*-(1→	4.48	3.84	H-4	**IV**
			3.75	H-3	**V**
			3.49	H-5	**V**
VI	β-D-Glc*p*-(1→	4.48	4.16	H-6b	**I**
			3.90	H-6a	**I**
			~3.49	H-3/5	**VI**

those of H-6a/b in residue **V**. The presence of two 1-carboxyethyl groups was confirmed from the COSY spectrum in which the two doublets at δ 1.32 and 1.35 coupled to quartets at δ 3.89 and 4.48, respectively. The ^{13}C NMR spectrum contained two signals at δ ~182 consistent with two 1-carboxyethyl substituents.

The sequence of the sugars in the repeating unit was obtained by the observed inter-residue connectivities generated by NOESY and HMBC experiments. NOE connectivities (Table 2) were observed between **I** H-1 and **V** H-4, **V** H-1 and **IV** H-4, **IV** H-1 and **III** H-4, and **VI** H-1 and **I** H-6a/b. The assignment of disaccharide elements from NOE connectivities between H-1 of residues **II** and **III** and H-4 of residues **I** and **II** was less obvious as the similar chemical shifts of the signals for H-4 of residues **I** and **II** overlap. However, residue **II** cannot be linked to itself and thus the observed connectivities must derive from NOE between **III** H-1 and **II** H-4 and **II** H-1 and **I** H-4. The assignment of the disaccharide elements was further supported by the inter-residue $J_{C,H}$-connectivities (Table 3), observed in the HMBC spectrum.

Table 3
Significant interresidue $^3J_{H,C}$-connectivities from the anomeric atoms of the deacetylated extracellular polysaccharide of *B. fibrisolvens* strain H10b, observed in a 100 ms HMBC spectrum

Residues	H-1/C-1	Connectivities to		
	δ_H/δ_C	δ_C/δ_H	Atom	Residues
→4,6)-α-D-Gal*p*-(1→	5.39			
I	101.0	3.63	H-4	**V**
→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→	5.05	78.4	C-4	**I**
II	100.7	4.11	H-4	**I**
→4)-α-D-Gal*p*-(1→	5.00	78.8	C-4	**II**
III	101.1	4.12	H-4	**II**
→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→	4.69	79.3	C-4	**III**
IV	104.4	4.25 [a]	H-4	**III**
→4)-β-D-Glc*p*-(1→	4.48	76.8	C-4	**IV**
V	102.2	3.84	H-4	**IV**
β-D-Glc*p*-(1→	4.48	68.6	C-6	**I**
VI	103.4	4.16	H-6	**II**

[a] Observed in a 65 ms HMBC spectrum.

Part of the sequence of sugars in the repeating unit was confirmed with identification of di- and trisaccharide elements. Partial acid hydrolysis of carboxyl-reduced polysaccharide (0.5 M TFA, 4 h, 80 °C) and fractionation on Bio-Gel P-2 gave products from monosaccharides to larger oligosaccharides. These fractions were lyophilized and further fractionated on the same column. MALDI-TOF spectra recorded for the fractions obtained showed that di- and trisaccharides occurred in the same fractions as tri-, tetra- and pentasaccharides with and without 2-hydroxy-1-methylethyl substituents. Further HPLC fractionation of these fractions was performed using a graphitized carbon column. Small amounts ($\sim$100 μg) were isolated of a di- and a trisaccharide and these were characterized by NMR spectroscopy (Table 4), which identified the following two structures:

β-D-Glc*p*-(1→4)-3-*O*-[(*S*)-1-carboxyethyl]-D-Glc*p*

β-D-Glc*p*-(1→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→4)-D-Gal*p*

A MALDI-TOF mass spectrum was recorded for an oligosaccharide mixture obtained by partial acid hydrolysis of purified polysaccharide using the same conditions as described above for the carboxyl reduced material. Major ions below 1200 Da observed in the mass spectrum were *m/z* 1157, 995, 833, 671 and 599. The observed ions are in agreement with a hexasaccharide of mass 1134 Da (*m/z* 1157 $[M+Na]^+$) and with oligosaccharides derived from further hydrolysis of hexose and (1-carboxyethyl)hexose residues.

Results of the monosaccharide and methylation analyses together with NMR data (Tables 1–3) and the structures of the isolated di- and trisaccharides, demonstrated the structure of the repeating unit:

VI
β-D-Glc*p*
1
↓
6

I **V** **IV** **III**

$$\left[\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}3\text{-}O\text{-}[(S)\text{-1-carboxyethyl}]\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow\right.$$

$$\left.4)\text{-}6\text{-}(O)\text{-}[(R)\text{-1-carboxyethyl}]\text{-}\alpha\text{-D-Gal}p\text{-}(1\right]$$

II

The substitution position of the *O*-acetyl group was determined from a comparison of ^{1}H NMR (Fig. 1(a)), COSY, TOCSY and HSQC spectra of the native polysaccharide with those of the deacetylated polysaccharide. Only the chemical shifts for the H-1 to H-3 and C-1 to C-3 signals of the →4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→ residue differed significantly with the corresponding ^{1}H signals at δ 4.78, 4.78 and 3.80 and ^{13}C signals at δ 102.8, 74.3 and 78.3. The chemical shift differences are thus 0.09, 1.33 and 0.17 ppm for proton signals and −1.6, 0.6 and −1.8 ppm for carbon signals. These shifts are similar to those observed for methyl 2-*O*-acetyl-β-D-glucopyranoside[14] relative to the non-acetylated methyl β-D-glucopyranoside.

The extracellular polysaccharides made by *B. fibrisolvens* contain interesting structural features. Both strain H10b and CF3 contain two (1-carboxyethyl)hexoses, 6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p* common to both, and 3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p* found in strain H10b and 4-*O*-[(*R*)-1-carboxyethyl]-β-D-Glc*p* in strain CF3. Though there are differences between the investigated structures of the polysaccharides made by *B. fibrisolvens,* strains H10b, X6C61 and 49 do share a common trisaccharide:

→4)-6-*O*-[(*R*)-1-carboxyethyl]-α-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→

A slightly modified version of this trisaccharide is also found in strain CF3 indicating a structural relationship between these strains. However, the →4)-α-D-Gal*p*-(1→ residue of the trisaccharide is 3-O-acetylated in strain 49, whereas strain X6C61 has an α-L-Rha*p* group at C-2, strain H10b a β-D-Glc*p* group at C-6. The corresponding C-5 epimer L-altrose in strain CF3 has a β-D-Glc*p* group at C-2. The identified structure of the polysaccharide from strain H10b further supports our

Table 4
^{1}H and ^{13}C NMR data obtained at 30 °C for oligosaccharides obtained by partial hydrolysis of deacetylated carboxyl reduced polysaccharide from *B. fibrisolvens* strain H10b

Residue	H-1	($J_{H-1,H-2}$ Hz)	H-2	H-3	H-4	H-5	H-6a/b	H-1′a/b	H-2′	H-3′
	C-1		C-2	C-3	C-4	C-5	C-6	C-1′	C-2′	C-3′
Disaccharide										
→4)-3-*O*-[(*S*)-1-carboxyethyl]-α-D-Glc*p*	5.22	(3.7)	3.58	3.74	3.74	3.96	3.88/3.95	3.44/3.83	3.95	1.25
	93.1		73.1	79.6	76.0	72.0	60.6	64.9	80.5	17.3
→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*	4.65	(8.0)	3.27	3.59	3.74	3.59	3.83/3.96	3.44/3.83	3.95	1.25
	97.0		76.0	82.3	76.0	76.3	60.8	64.9	80.5	17.3
β-D-Glc*p*-(1→	4.53	(7.9)	3.28	3.51	3.29	3.50	3.67/3.95			
	103.0		74.5	76.5	70.9	77.7	62.2			
Trisaccharide										
→4)-α-D-Gal*p*	5.27	(3.7)	3.86	3.94	4.22	[a]	~3.7			
	93.3		[a]		79.5	[a]	~62			
→4)-β-D-Gal*p*	4.61	(7.9)	3.55	3.74	4.16	[a]	~3.7			
	97.3		73.3	75.3	78.6	[a]	~62			
→4)-3-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*-(1→	4.68	(7.9)	3.40	3.63	3.73	3.55	3.84/3.99	3.44/3.82	3.96	1.25
	104.5		75.4	82.3	75.8	76.2	60.6	64.8	80.4	17.3
β-D-Glc*p*-(1→	4.53	(7.8)	3.29	3.51	3.30	3.50	3.67/3.96			
	102.8		74.5	76.4	70.9	77.7	62.2			

[a] Signals were not detected.

proposal that the structural modifications on the (1→4)-linked backbone make the *B. fibrisolvens* polysaccharides resistant to hydrolysis by the multitude of hydrolytic enzymes found within the rumen.

3. Experimental

3.1. General methods

Gel filtration was performed using a column (80 × 2.6 cm) of Bio-Gel P-2 irrigated with water and the fractions were monitored with a Knauer Differential Refractometer. All ^{1}H detected NMR spectra were recorded at 600 MHz (^{1}H) with a Bruker DRX 600 instrument and ^{13}C NMR spectra at 100 MHz with a Bruker DRX 400 instrument. Chemical shifts are given in ppm using acetone (δ_H 2.225, δ_C 31.1) as internal reference for samples measured in D_2O solutions. Assignments of signals were made from 2D COSY, TOCSY, NOESY, HSQC and HMBC experiments with the aid of 1D analogues of TOCSY and NOESY experiments. In the 2D TOCSY experiments, spectra were recorded with mixing times of 60, 100 and 150 ms while in the 1D TOCSY analogues spectra were acquired with 4–5 different mixing times between 20 and 150 ms depending on the spin system studied. In NOESY experiments, a mixing time of 50 ms was used and HMBC experiments were run with delay times of 65, 100 and 150 ms. ^{1}H NMR chemical shifts of overlapping signals were obtained from the center of the cross peaks in the 2D spectra. The selective excitation in 1D TOCSY and NOESY was achieved by the application of shaped pulses calculated with the shape tool included in the Bruker software[15] applying Hermitean line shape. Spin systems were assigned from the order of appearance of the signals as the mixing time was increased between experiments. MALDI-TOF mass spectra of the partially degraded polysaccharide were recorded in positive mode on a Bruker Reflex III instrument equipped with a reflector using 2,5-dihydroxybenzoic acid as matrix. Dialysis was performed in pre-boiled dialysis bags (Spectrapore, Mw cut off 6–8 kD) against deionized water at 4–8 °C.

3.2. Organism, growth conditions and isolation of extracellular polysaccharides

Butyrivibrio fibrisolvens strain H10b was grown at 37 °C under anaerobic conditions on chemically defined media specified by Cotta and Hespell[16] containing 1% D-glucose and supplemented with 0.3% trypticase. Cells were removed from the stationary phase by centrifugation. The crude polysaccharide was further purified by phenol extraction and dialysis as previously described.[1]

3.3. Purification of the native crude polysaccharide

Lyophilized crude polysaccharide (1.3 g) was added to a pH 7.3 buffer (1.2 mM $MgCl_2$, 7.2 mM $CaCl_2$ and 1.2 mM sodium phosphate in 250 mL water) in a flask. To the greyish lumpy suspension, RNAse (6 mg), DNAse (3 mg) and two drops of toluene were added. The suspension was gently stirred at room temperature (rt) for a week. To the lump free suspension, protease K (5 mg, 33 U/mg dissolved in 400 μL water) and another five drops of toluene were added and the reaction was left for 2 days without stirring before the reaction was dialysed against water (3×5 L). Further purification was afforded by anion exchange chromatography on a column (2.6×2 cm) packed with DEAE-Sephadex 50 A in the chloride form. In a typical run, the polysaccharide (100 mg) was dissolved in 200 mL deionized water, centrifuged (3000 rpm, 15 min) and filtered (45 μm filter) before application on the column at a rate of ~10 mL/h. The column was washed with 200 mL water before elution of the polysaccharide with a linear gradient of NaCl (0–0.5 M) in 500 mL at 25 mL/min. The polysaccharide fractions were detected by polarimetry and they were eluted mainly between 0.2 and 0.3 M NaCl. The yield after lyophilization was 59 mg (59%) of an almost pure polysaccharide as judged by its ^{1}H NMR spectrum. The overall yield of purified polysaccharide was 43% from the crude material.

3.4. Deacetylation of the polysaccharide

In a typical deacetylation experiment, the polysaccharide (25 mg) was treated with 0.1 M NaOH (5 mL) overnight at rt. The resulting material was dialyzed and freeze–dried to yield deacetylated polysaccharide (19 mg).

3.5. Carboxyl reduction of deacetylated polysaccharide

Carboxyl reduction was performed by the method of Taylor and co-workers[10] using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and $NaBH_4$ as the reducing agents. Repeating the procedure twice gave ~80% carboxyl reduction of the material as judged by ^{1}H NMR spectroscopy.

3.6. Partial degradation of carboxyl reduced polysaccharide and separation of oligosaccharides by SEC and HPLC

Deacetylated polysaccharide (25 mg) was partially hydrolyzed in 0.5 M TFA (5 mL) at 80 °C for 4 h. The reaction was stopped by neutralization with concn aq NH_3 followed by lyophilization. Gel-filtration gave six almost separated fractions. ^{1}H NMR and MALDI-TOF MS analysis revealed that they were heterogeneous and further separation was necessary. The fractions were purified by HPLC on a HyperCarb® column (100×4.6 mm) eluated with mixtures of water–MeCN (RI-detection). Two disaccharides were eluted with 97:3 water-MeCN and two trisaccharides were separated with 9:1 water-MeCN as eluent. Purification of tetrasaccharides and higher molecular mass fragments was not successful.

3.7. Determination of monosaccharide content and linkage analysis

Alditols of the monosaccharides from purified native polysaccharide (1 mg) were produced by hydrolysis in 2 M TFA followed by $NaBH_4$ reduction according to a procedure described.[8] The alditols were trimethylsilylated in 200 μL Sigma sil-A®, evaporated in a stream of N_2 and the trimethylsilylated alditols were extracted with 200 μL *n*-hexane.

Methylation analysis was performed on carboxyl-reduced polysaccharide using standard methods. Methylation of the polysaccharide was performed according to the Hakomori method[17] using sodium methylsulfinyl anion and CH_3I. The polysaccharide was then hydrolyzed, reduced and acetylated before analysis on GC–MS.

3.8. Identification of 1-carboxyethyl substituted hexose by NMR spectroscopy and circular dichroism spectropolarimetry

The O-deacetylated polysaccharide (6 mg) was hydrolyzed (TFA, 120 °C, 4 h), the solvent evaporated (N_2-stream, ~35 °C), neutralized (aq NH_3) and the acidic sugars were separated from the neutral sugars by passage through an anion exchange resin [Dowex 1 (Cl^-), 2×1 cm]. The acidic sugars were eluted with aq NaCl and desalted on a column of Bio-Gel P-2 (70×1 cm, irrigated with water) before they were lyophilized. ^{1}H NMR spectra of the mixture of the 6-substituted D-galactose and the unknown (1-carboxyethyl)hexose were recorded and compared to the ^{1}H NMR spectra of synthesized 3-*O*-[(*R*)- and (*S*)-1-carboxyethyl]-α-D-Glc*p* which were available from previous work.[18]

3.9. Separation of acidic monosaccharides released after hydrolysis of O-deacetylated polysaccharide

The O-deacetylated polysaccharide (3 mg) was hydrolyzed in 2 M TFA (1.5 mL) for 4 h at 120 °C followed by evaporation by a stream of N_2 at ~35 °C. After another evaporation from 1.5 mL water, the residue was dissolved in water (1.5 mL) and pH adjusted to ~10. After 1 h, TLC (12:3:3:1 EtOAc–MeOH–HOAc–water) showed an elongated spot at R_f ~0.3, which in connection with the absence of a spot with R_f ~0.8

indicated that no lactones were present in the sample. The neutral components were separated from the acidic components by passage of the mixture through an anion exchange column [2×1 cm Dowex 1 (Cl^-)]. The mixture of acidic components was eluted with aq NaCl, desalted on a column of Bio-Gel P-2 (70×1 cm, irrigated with water) and lyophilised. A ^1H NMR spectrum of the mixture showed that the two 1-carboxyethyl substituted monosaccharides were pure with respect to other monosaccharides.

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