ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 327 (2000) 411–422

CARBOHYDRATE RESEARCH

Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B891

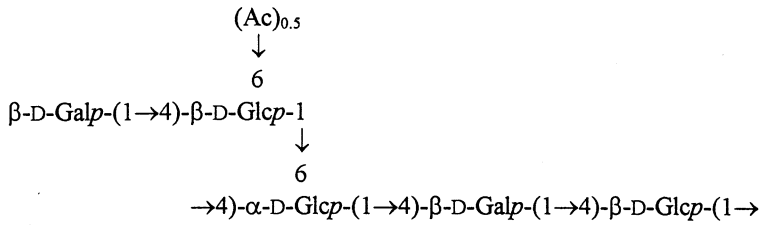
Willemiek H.M. van Casteren [a], Pieter de Waard [b], Cor Dijkema [c], Henk A. Schols [a], Alphons G.J. Voragen [a,*]

[a] *Department of Food Technology and Nutritional Sciences, Wageningen University, Laboratory of Food Chemistry, Bomenweg 2, NL-6703 HD Wageningen, The Netherlands*
[b] *ATO-DLO Agrotechnological Research Institute, Bornsesteeg 59, NL-6708 PD Wageningen, The Netherlands*
[c] *Department of Biomolecular Sciences, Wageningen University, Laboratory of Molecular Physics, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands*

Received 4 October 1999; accepted 16 February 2000

Abstract

Lactococcus lactis subsp. *cremoris* B891 grown on whey permeate produced an exopolysaccharide containing D-Gal and D-Glc in a molar ratio of 2:3. The polysaccharide was partially O-acetylated. By means of HF solvolysis, O-deacetylation, enzymic modification, sugar linkage analysis and 1D/2D NMR studies the exopolysaccharide was shown to be composed of repeating units with the following structure:



Keywords: Exopolysaccharide; *Lactococcus lactis* subsp. *cremoris*; Structural analysis; Enzymic modification

1. Introduction

Over the past 20 years, the food industry has developed an enormous range of convenience foods, but it is believed that the scientific understanding of the roles of hydrocolloids used has not kept pace adequately with innovations and changes in technology [1]. To be able to produce tailor-made polysaccharides for food applications, it is a prerequisite to know the influence of the chemical structure on the physical properties. Extracellular polysaccharides (EPSs) frequently consist of repeating units [2] and these regular structures make them very suitable for structure–function studies.

* Corresponding author. Tel.: +31-317-483209; fax: +31-317-484893.
E-mail address: fons.voragen@chem.fdsci.wau.nl (A.G.J. Voragen).

0008-6215/00/$ - see front matter © 2000 Elsevier Science Ltd. All rights reserved.
PII: S0008-6215(00)00065-3

In the last decade, the heteropolysaccharides of lactic acid bacteria have received considerable attention in view of their potential application as replacers of presently applied thickeners [3]. EPSs may also represent safe additives for novel food formulations because of their contribution to the peculiar rheology and texture of milk-derived products [2]. As a first step in structure–function studies, the chemical structures of several EPSs produced by lactic acid bacteria have been elucidated. For *Lactococcus lactis* subsp. *cremoris*, the investigated strains include H414 [4], SBT0495 [5], LC330 [6], B40 [7,8], and B39 [9].

Here, we report the structural analysis of the exopolysaccharide produced by *L. lactis* subsp. *cremoris* B891.

2. Experimental

Production, isolation and purification of EPS.—The production and isolation of EPS from *L. lactis* subsp. *cremoris* B891 was performed as described for EPS B40 [10] and the crude EPS was kindly supplied by NIZO food research (Ede, The Netherlands). The crude material was purified as described before [9].

O-deacetylation of the native polysaccharide.—The native EPS (1 mg/mL) was O-deacetylated by treatment with 5% NH_4OH at room temperature (rt) for 8 h [11]. The O-deacetylated EPS was recovered by freeze drying.

Enzyme preparation.—The commercial enzyme preparation Pectinex Ultra SP-L produced by *Aspergillus aculeatus* (Novo Nordisk Ferment AG, Dittingen, Switzerland) was used to modify O-deacetylated EPS B891.

Enzymic modification of the O-deacetylated EPS.—In the search for enzymes that are able to modify O-deacetylated EPS B891, partially purified EPS B891 was O-deacetylated. The resulting polymer (0.5 mL, 2 mg/mL in 50 mM NaOAc pH 5.0) was incubated (24 h, 30 °C) with 50 μL Ultra SP, which was dialysed against the same buffer. After incubation, the enzymes were inactivated (15 min, 100 °C) and the precipitate formed was removed by centrifugation. The supernatant was used to analyse the released monosaccharides and to determine the hydrodynamic volume of the remaining polymer. To obtain sufficient amounts of enzymically modified EPS B891 for sugar linkage analysis and NMR analysis, purified and O-deacetylated EPS B891 (10 mg) was dissolved in 50 mM NaOAc buffer pH 5.0 (5 mL). Ultra SP (1 mL) was dialysed against the same buffer and diluted to ca. 4 mL. The EPS solution was incubated (24 h, 30 °C) with 0.5 mL enzyme solution and subsequently heated (15 min, 100 °C). After centrifugation, the supernatant was dialysed against distilled water and freeze-dried for further analysis.

Partial HF solvolysis.—The solvolysis of EPS B891 with anhydrous liquid HF was performed according to Mort [12] at the Department of Plant Biochemistry and Biotechnology, Wesfälische Wilhelms-Universität Münster, Germany. The polysaccharide was partially solvolysed with HF in two batches. Therefore, freeze-dried polysaccharide (ca. 40 mg per batch) was treated with anhydrous, liquid HF (ca. 0.5 mL) for 30 min at −40 °C. To stop the reaction, 25 mL of ether, precooled with dry ice, was added. After the ether–HF mixture had warmed to rt, it was filtered through a Teflon filter using N_2 pressure. The precipitate was washed with more ether to remove any residual HF and then dried under vacuum. Finally, the precipitate was dissolved in distilled water and freeze-dried. The effect of the solvolysis was evaluated by high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis.

Partial purification of oligosaccharides obtained by HF solvolysis.—Freeze-dried oligosaccharides of EPS B891 (47 mg) were dissolved in distilled water (0.5 mL) and centrifuged. The supernatant was applied onto a Bio-Gel P-2 column (84 × 0.65 cm i.d., Bio-Rad Laboratories, Richmond, CA) and elution with distilled water (0.5 mL/min) was performed at 60 °C. The refractive index was monitored on-line (Shodex RI-72 detector) and relevant fractions (RI-signal) were analysed for their oligomeric content by HPAEC and MALDI-TOF MS.

Sugar (linkage) composition.—The sugar (linkage) composition and the absolute configurations of the monosaccharides were determined as described before for EPS B39 [9].

Monosaccharide release.—The release of monomers caused by enzymic hydrolysis of EPS B891 was verified by HPAEC, which was performed as described earlier [7].

Hydrodynamic volume of the polysaccharides.—High-performance size-exclusion chromatography (HPSEC) was performed as has been described earlier [7,8].

Oligosaccharide release.—The release of oligomers caused by solvolysis of EPS B891 using HF was examined by two methods: (I) the reaction product was analysed using the HPAEC equipment as described earlier [7]. After equilibration with 20 mM NaOAc in 100 mM NaOH, a 20 μL sample was injected and the elution program was started: 0 → 35 min, linear gradient of 20 → 50 mM NaOAc in 100 mM NaOH; 35 → 50 min, linear gradient of 50 mM → 1 M NaOAc in 0.1 M NaOH; 50 → 55 min, 1 M NaOAc in 0.1 M NaOH isocratic, followed by re-equilibration (55 → 70 min, 20 mM NaOAc in 100 mM NaOH isocratic). (II) The reaction product was analysed by MALDI-TOF MS as described earlier [8].

NMR spectroscopy.—Samples were analysed by NMR spectroscopy at probe temperatures of 80 °C (polysaccharides) or 27 °C (oligosaccharides) as described for EPS B39 [9].

Table 1
Sugar linkages of native EPS B891 (**1**), O-deacetylated EPS B891 (**2**) and enzymically modified O-deacetylated EPS B891 (**3**) [a]

Derivative	Linkage type [b]	Molar ratio [c]		
		1	**2**	**3**
2,3,4,6-Gal [d]	*t*-Gal	1.0	0.9	
2,3,6-Gal	1,4-Gal	0.5	0.5	0.5
2,3,4,6-Glc	*t*-Glc			1.2
2,3,6-Glc	1,4-Glc	2.1	2.1	1.2
2,3-Glc	1,4,6-Glc	1.0	1.0	1.0

[a] It should be kept in mind that the acetyl groups in **1** were removed during the procedure due to alkaline conditions.

[b] According to NMR experiments (vide infra) all glycosyl residues are in the pyranose ring form.

[c] 1,4,6-Glc was taken as 1.0.

[d] 1,5-di-*O*-Acetyl-2,3,4,6-tetra-*O*-methyl-galactitol-1-*d*, etc.

3. Results

Isolation, purification and chemical characterisation of EPS B891.—Crude EPS produced by *L. lactis* subsp. *cremoris* B891 was purified by CCl_3CO_2H extraction and EtOH precipitation, followed by size-exclusion chromatography on Sephacryl S-500. The purification step using CCl_3CO_2H and EtOH removed most of the proteins present in the crude EPS, while size-exclusion chromatography successfully removed a mannan population and remnants of protein. The mannan population is believed to originate from the yeast extract in the growth medium, as has been reported before [13]. The population was absent when EPS B891 was produced on a chemically defined medium [14] without yeast extract.

Sugar composition analysis of purified, native EPS B891 (**1**) and determination of absolute configurations revealed the presence of D-Gal and D-Glc in a molar ratio of 2.0:3.0. Sugar linkage analysis of **1** showed the presence of terminally-linked galactose, 4-substituted galactose, 4-substituted glucose, and 4,6-disubstituted glucose (Table 1). According to the results of NMR experiments (vide infra) and sugar linkage analysis before and after enzymic modification of EPS B891 (vide infra), all hexose residues are in the pyranose ring form.

O-deacetylation and enzymic modification of EPS B891.—Treatment of EPS B891 with 5% NH_4OH at rt resulted in complete O-deacetylation, as determined by NMR spectroscopy (vide infra). After incubation of O-deacetylated EPS B891 with Ultra SP, analysis by HPAEC showed that monomeric galactose had been released. The hydrodynamic volume of the enzymically modified EPS (**3**) had only been decreased slightly compared with O-deacetylated EPS (**2**) (HPSEC). After enzymic modification, sugar linkage analysis (Table 1) showed the disappearance of terminally-linked galactose and a decrease of 4-substituted glucose, while a new type of sugar linkage, terminally-linked glucose, appeared. Consequently, terminally-linked galactose is attached to a 4-substituted glucose residue and the branches, attached to the backbone of EPS B891, contain at least two sugar residues.

Table 2
^{1}H NMR chemical shifts [a] of O-deacetylated EPS B891 (**2**) recorded in D_2O at 80 °C

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A (1,4,6-α-D-Glc*p*)	4.948	3.63	3.87	3.75	4.364	4.18	3.98
B (1,4-β-D-Glc*p*)	4.608	3.39	3.69	3.67	3.63	3.99	3.84
C (1,4-β-D-Glc*p*)	4.531	3.40	3.68	3.65	3.60	3.98	3.82
D (1,4-β-D-Gal*p*)	4.515	3.60	3.75	4.053	3.79	3.92	3.85
E (*t*-β-D-Gal*p*)	4.463	3.57	3.67	3.95	3.73	3.81	3.77

[a] In ppm relative to the signal of acetone at δ 2.225.

1D NMR spectroscopy.—The ^{1}H NMR spectra of native EPS (**1**), O-deacetylated EPS (**2**) and enzymically modified O-deacetylated EPS (**3**) are shown in Fig. 1. In native EPS, the signal at δ 2.162 was assigned to the protons of an acetyl group. Based on the intensities of the relevant signals in the spectrum, the extent of O-acetylation of native EPS was determined to be 50%. Because of O-acetylation, the spectrum of native EPS (**1**) was more complex and showed less resolution than the spectra of (enzymically modified) O-deacetylated EPS (**2** and **3**). Therefore, the latter polysaccharides were used to solve the chemical structure of the repeating units, leaving the acetyl groups out of consideration for the moment. The ^{1}H NMR spectrum of **2** (Fig. 1) showed five signals in the anomeric region (δ 5.0–4.4), corresponding to a pentasaccharide repeating unit. The five monosaccharide units were labelled **A**–**E**. The coupling constant of the anomeric signal of residue **A** ($^3J_{1,2}$ 3.51) indicates the presence of an α-linked residue, whereas the coupling constants of the anomeric signals of residues **B**–**E** ($^3J_{1,2}$ 7.3–7.8) suggest four β-linked residues. In the ^{1}H NMR spectrum of enzymically modified O-deacetylated EPS (**3**) (Fig. 1), the anomeric signal of residue **E** (δ 4.463) disappeared almost completely, and based on the results of the sugar linkage analysis this residue was assigned to terminally-linked galactose. Since the chemical shift of **C*** H-1 in **3** shifted upfield relative to **C** H-1 in **2**, residue **C** was assigned to the 4-substituted glucose, which changed into terminally-linked glucose (residue **C***) upon enzymic modification.

The ^{13}C NMR spectrum (not shown) of native EPS B891 (**1**) did not contain signals typical for the carbon atoms of an acetyl group, probably because of the instability of acetyl groups at 80 °C (vide infra). The ^{13}C NMR spectrum (not shown) of O-deacetylated EPS B891 (**2**) is in agreement with the suggested pentasaccharide repeating unit, since five signals were observed in the anomeric region (δ 110–95). Based on their chemical shifts, the C-1 signal at δ 101.2 was assigned to the α-hexopyranosyl residue (**A**), while the other anomeric signals (δ 104.6, 104.4, 103.8, 103.6) were assigned to four β-hexopyranosyl residues. The resonances at δ 62.4, 61.9 and 61.7 were assigned to hydroxymethyl carbons (unsubstituted C-6) of hexopyranosyl residues, and one of these signals represents two carbons (vide infra). The signal at δ 68.9 was assigned to C-6 of the 4,6-disubstituted hexopyranosyl residue.

2D NMR spectroscopy of O-deacetylated EPS B891 (**2**).—By means of 2D COSY, TOCSY, and NOESY experiments, the ^{1}H chemical shifts of **2** (Table 2) were assigned. Taking the sugar (linkage) composition into account, the complete series of cross-peaks on the H-1 track of residues **A**, **B**, and **C** in the TOCSY spectrum (Fig. 2) together with the chemical shifts of **A** H-4, **B** H-4 and **C** H-4 indicate that these residues are glucosyl residues. For residues **D** and **E**, the H-1 tracks in the TOCSY spectrum showed cross-peaks to H-2, 3, 4 of the corresponding residue. Together with the typical chemical shifts of **D** H-4 and **E** H-4, this indicates that these residues are galactosyl residues. The H-5 chemical shifts of residues **D** and **E** were found via connectivities with H-4 in the COSY spectrum (not shown), and the H-5 assignment of residue **E** could be confirmed

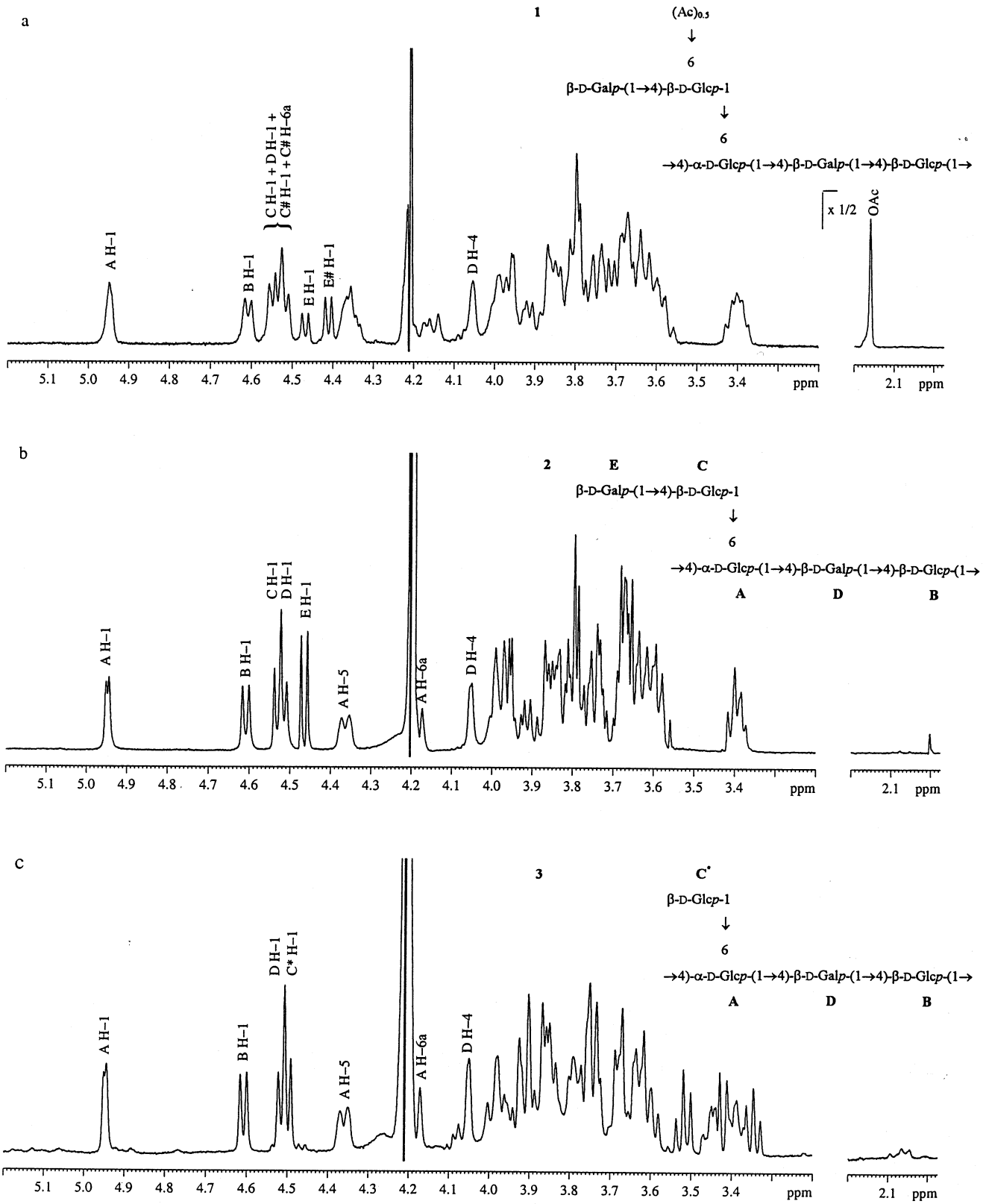


Fig. 1. 500-MHz ^{1}H NMR spectra of native EPS B891 (**1**) (a), O-deacetylated EPS B891 (**2**) (b), and enzymically modified O-deacetylated EPS B891 (**3**) (c), recorded in D_2O at 80 °C. The labelling of sugar residues in (a) is the same as in (b), and the signals of sugar residues, which were shifted because of the presence of an acetyl group, were labelled ($^{\#}$). For native EPS B891, the approximate relative amount of acetyl groups present (100% = 1) is given between brackets.

via the H-4 track in the TOCSY spectrum (not indicated in Fig. 2). The assignments of H-6a,6b from residues **D** and **E** were based on correlations with the corresponding H-5 in both COSY and TOCSY spectra, although for residue **E** no precise values could be given due to overlap. For this residue, the H-6a,6b chemical shifts were derived from HMQC measurements. The anomeric configuration was confirmed for all residues by intraresidual interactions in the NOESY spectrum (not shown).

Using the assignment of the ^{1}H signals, almost all ^{13}C resonances could be assigned as well by means of HMQC and HMBC experiments (Table 3). In conjunction with the sugar linkage composition, the relative downfield chemical shifts of **A** C-4 ($\Delta\delta$ 9.4), **A** C-6 ($\Delta\delta$ 7.3), **B** C-4 ($\Delta\delta$ 9.7), **C** C-4 ($\Delta\delta$ 9.8), and **D** C-4 ($\Delta\delta$ 9.3) compared with the ^{13}C chemical

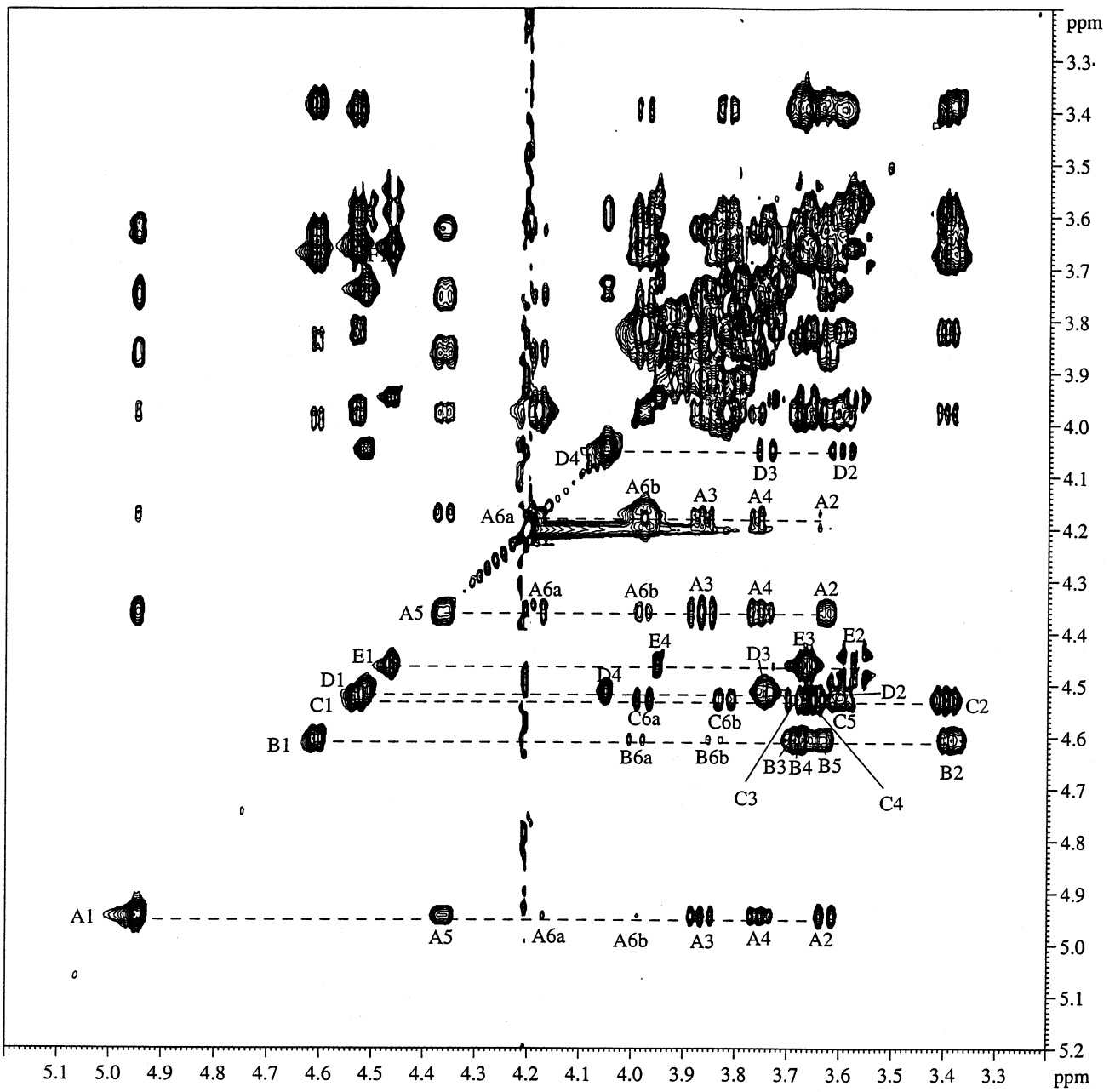


Fig. 2. 500-MHz 2D TOCSY spectrum, (mixing time = 140 ms) of O-deacetylated EPS B891 (**2**) recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons of H-5 and H-6a of residue **A**, and of H-4 of residue **D** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

Table 3
^{13}C NMR chemical shifts [a] of O-deacetylated EPS B891 (**2**) recorded in D_2O at 80 °C, as determined from 1D ^{13}C and 2D ^{1}H ^{13}C HMQC and HMBC experiments

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A (1,4,6-α-D-Glc*p*)	101.2	73.1	72.8	80.0	71.2	68.9
B (1,4-β-D-Glc*p*)	103.6	74.5	n.d. [b]	80.3	76.2	61.9
C (1,4-β-D-Glc*p*)	103.8	74.4	75.9	80.4	76.2	61.9
D (1,4-β-D-Gal*p*)	104.6	72.5	73.8	79.0	76.8	61.7
E (*t*-β-D-Gal*p*)	104.4	72.6	74.2	70.1	76.7	62.4

[a] In ppm relative to the signal of acetone at δ 31.55.
[b] Not determined.

shifts of the corresponding methyl aldosides [15] demonstrate that residue **A** is 4,6-disubstituted and residues **B**, **C** and **D** are 4-substituted. The $^{1}J_{C\text{-}1,\ H\text{-}1}$ values of 171 Hz for residue **A** and 162–164 Hz for residues **B**–**E** agree with the α-configuration for residue **A** and the β-configuration for all other residues.

The complete monosaccharide sequence of EPS B891 was determined via sugar linkage analysis after enzymic modification (vide supra), 2D HMBC spectra and 2D NOESY analysis. The results of the enzymic modification of EPS B891 already showed that residue **E** was linked to position 4 of residue **C**. This information appeared to be important for the correct sequence of the sugar residues within the repeating unit of EPS B891, since the chemical shifts of residues **B** and **C** are very alike. In the NOESY spectrum (not shown), no interresidual connectivities were found with **E** H-1, but the HMBC spectrum (Fig. 3) showed cross-peaks between **E** C-1 and **C** H-4 and between **C** C-4 and **E** H-1. The **C**(1→6)**A** sequence was suggested by correlations between **C** H-1 and **A** H-6a,6b in the NOESY spectrum and by the cross-peak between **A** C-6 and **C** H-1 in the HMBC spectrum. Interresidual connectivities in the NOESY spectrum between **A** H-1 and **D** H-4,5,6a,6b indicate the **A**→**D** sequence. This was confirmed by the **D** C-4, **A** H-1 cross-peak in the HMBC spectrum. Due to overlap in the NOESY spectrum, the **D** H-1, **B** H-4 cross-peak could only be assigned tentatively. However, the HMBC spectrum showed a cross-peak between **B** C-4 and **D** H-1, suggesting the **D**(1→4)**B** linkage. The **B**(1→4)**A** sequence was suggested by a strong interresidual cross-peak between **B** H-1 and **A** H-4 in the NOESY spectrum and this sequence was proven by **B** C-1, **A** H-4 and **A** C-4, **B** H-1 correlations in the HMBC spectrum.

The combined results from chemical, enzymic and NMR studies have demonstrated that O-deacetylated EPS B891 is composed of pentasaccharide repeating units with structure **2** as is shown in Fig. 1.

NMR spectroscopy of EPS B891 (**1**).—1D and 2D NMR experiments were performed on native EPS B891 (**1**) in order to locate the acetyl group within the repeating unit. Since **1** was only partially O-acetylated, the spectra were expected to contain signals of both O-acetylated and non-O-acetylated repeating units. Thus, the assignments of chemical shifts of **2** could be used to interpret the spectra of **1**. 1D ^{1}H NMR spectra of **1** and **2** (Fig. 1) showed changes in signal intensities in the anomeric region (δ 5.0–4.4): **2**: **A** H-1: **B** H-1: [**C** H-1 + **D** H-1]: **E** H-1 = 1:1:2:1, whereas **1**: **A** H-1: **B** H-1: [**C**$^{\#}$ H-6 + **C** H-1 + **C**$^{\#}$ H-1 + **D** H-1]: **E** H-1: **E**$^{\#}$ H-1 = 1:1:2.5:0.4:0.7. Signals of sugar residues, which were shifted because of the presence of an acetyl group, were labelled ($^{\#}$). In the ^{1}H spectrum of **1**, the signal at δ 4.410 was assigned to the anomeric proton of sugar residue **E**$^{\#}$. This assignment was based on the observation that the COSY and TOCSY (both not shown) correlation patterns of residue **E**$^{\#}$ in **1** were similar (but shifted) to the corresponding patterns of residue **E**. Furthermore, addition of the 1D signal intensities of **E** H-1 and **E**$^{\#}$ H-1 resulted in a ratio of 1 compared with the other

anomeric proton signals. The series of signals in the anomeric region of **1** with a total intensity of 2.5 was more difficult to unravel. However, strong indications were found in the COSY and TOCSY spectra of **1** (both not shown) that the chemical shifts of the protons of residue **C** shifted upon O-acetylation. Since the largest downfield shift was found for **C**$^{\#}$ H-6a,6b (signal **C**$^{\#}$ H-6a appearing in the anomeric region), EPS B891 is probably partially O-acetylated at C-6 of the glucosyl residue in the branches of the repeating unit (residue **C**$^{\#}$). In the NOESY spectrum of **1** (not shown), the CH_3 protons of the acetyl group had no cross-peaks with other protons. To prove the position of the acetyl group, a HMBC spectrum of **1** was recorded. However, in this spectrum (not shown) the expected multiple bond correlation between the C=O carbon of the acetyl group and the proton attached to the O-acetylated carbon could not be found. This was probably because **1** was only partially O-acetylated and because the acetyl groups appeared to be unstable at 80 °C; during the NMR measurements the degree of O-acetylation decreased from ca. 50% to ca. 20%. Performing measurements at higher concentrations or at lower temperature was no option because the sample viscosity would lead to considerable broadening of the signals. To overcome this problem, EPS B891 was partially solvolysed and the largest oligomers obtained were analysed by NMR.

HF solvolysis of EPS B891.—From work with other polysaccharides [16–18], the solvolysis of EPS B891 with liquid HF was expected to produce O-acetylated oligosaccharides useful to locate the acyl substituents. Especially the presence of only one α-linked sugar

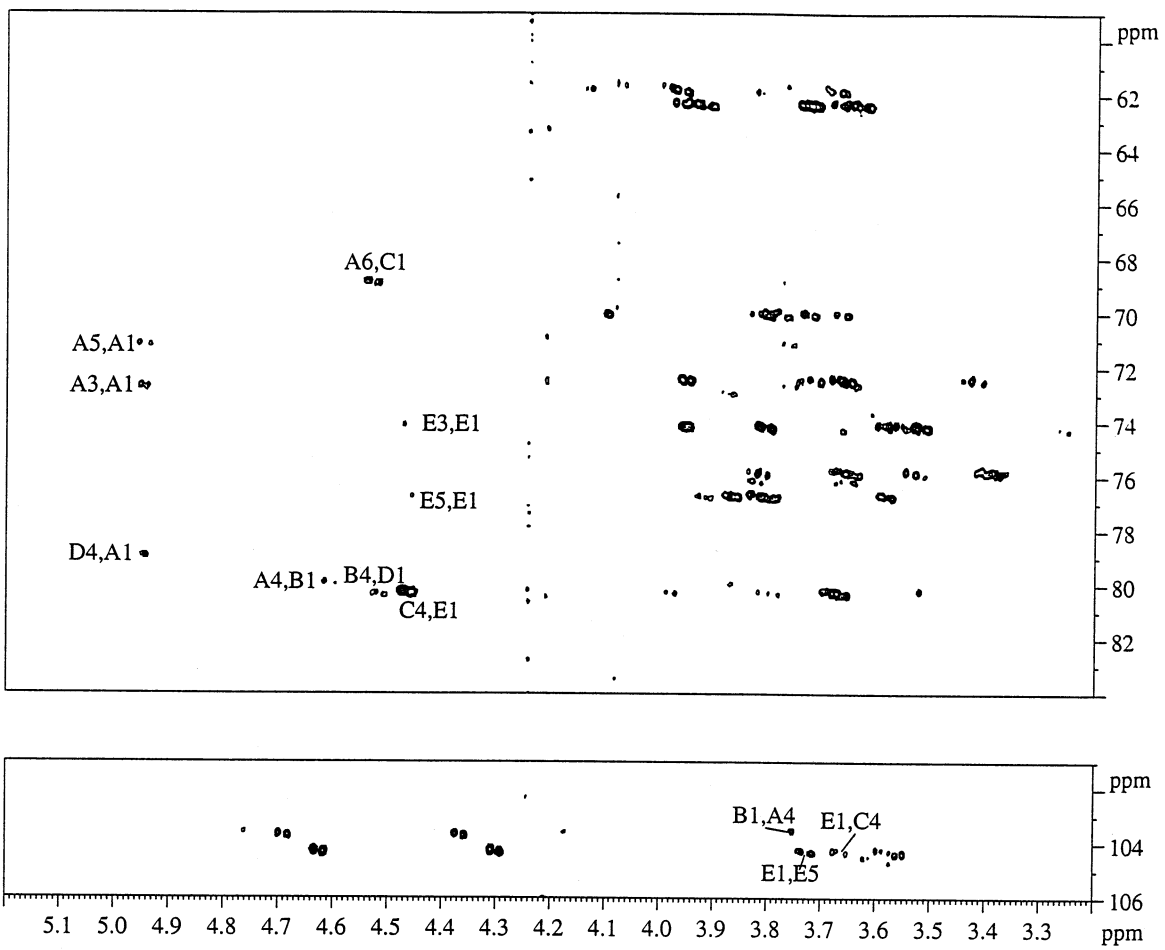


Fig. 3. 500-MHz 2D $^{1}H-^{13}C$ undecoupled HMBC spectrum (delay time between the first proton pulse and the first carbon pulse = 60 ms) of O-deacetylated EPS B891 (**2**) recorded in D_2O at 80 °C. The code B1,A4 corresponds to a long-range coupling between **B** C-1 and **A** H-4, etc.

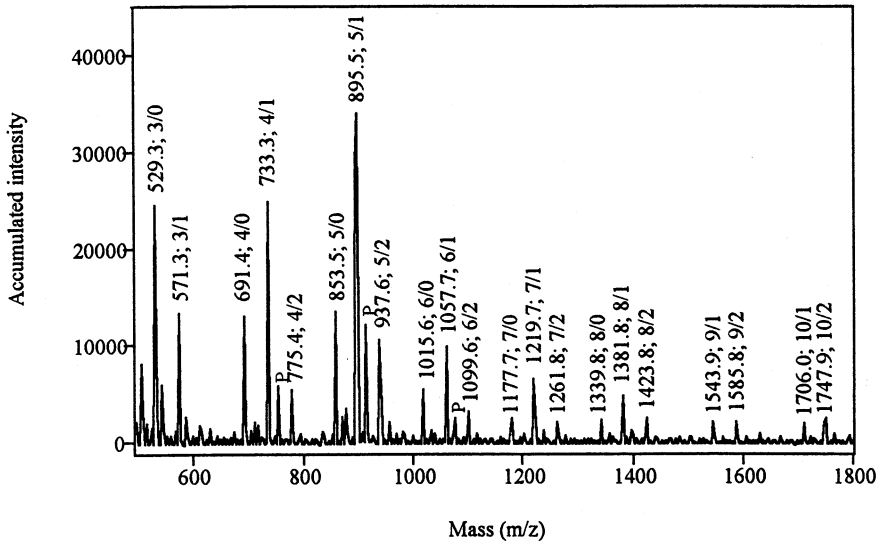


Fig. 4. MALDI-TOF mass spectrum of the reaction products obtained after treatment of EPS B891 in liquid HF (30 min at −40 °C). The masses of sodiated oligomers are indicated, followed by the corresponding number of hexoses and acetyl groups. P indicates the presence of potassium adducts. It should be kept in mind that the use of liquid HF gives glycosyl fluorides as products.

residue and its position in the backbone of the repeating unit of EPS B891 was expected to be in favour of the release of the repeating unit, since preferential cleavage of α- over β-linkages in HF has been noted previously [16,17]. Therefore, EPS B891 was treated with liquid HF and the obtained reaction product was analysed for the presence of oligomers. The HPAEC chromatogram (not shown) demonstrated the release of several oligosaccharides but since the retention time of oligosaccharides depends not only on the type and amount of sugar residues but also on the position of glycosidic linkages and on the overall conformational structure [19], no information was obtained about the identity of the oligomeric structures. Further analysis of the reaction product by MALDI-TOF MS confirmed the presence of oligomers. In fact, the series of signals in the spectrum (Fig. 4) indicated the presence of non-, mono- and di-O-acetylated oligosaccharides. The mono-O-acetylated pentahexosyl fluoride (sodiated, mass = 895.5) had the highest accumulated intensity and probably corresponds to the repeating unit of EPS B891. Attempted purification of the repeating unit from the HF reaction products on Bio-Gel P-2 resulted in the elution pattern shown in Fig. 5. Analysis of the fractions by HPAEC and MALDI-TOF MS (not shown) indicated that all oligomers were purified only partially. Since the presence of (non)reducing ends in a mixture of oligomers complicate NMR spectra, the fractions containing the largest oligomers (degree of polymerisation ≥ 6) were pooled for NMR analysis. To prove the multiple-bond correlation(s) between the C=O carbon of the acetyl group with the proton(s) attached to the O-acetylated carbon, a 2D HMBC spectrum of this pool was recorded. As expected, cross-2peaks were found (not shown) at ^{1}H δ 4.53; ^{13}C δ 175.3 and ^{1}H δ 4.35; ^{13}C δ 175.3, proving that the acetyl group is indeed located at C-6

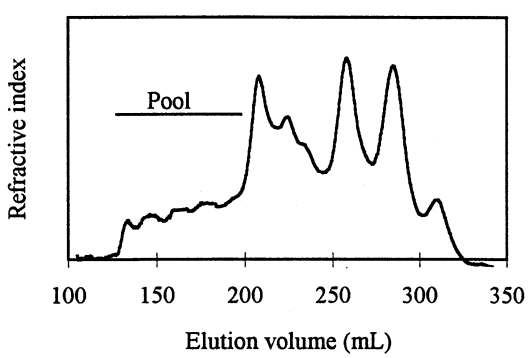


Fig. 5. Elution pattern on Bio-Gel P-2 of the oligosaccharides produced by treatment of EPS B891 in liquid HF for 30 min at −40 °C. The oligomers pooled for NMR analyses are indicated.

of sugar residue **C**$^{\#}$. Thus, the combined results from the analyses on O-(de)acetylated EPS B891 prove the chemical structure of the repeating unit of native EPS B891 (**1**) as is shown in Fig. 1.

4. Discussion

The chemical structure of EPS from *L. lactis* subsp. *cremoris* B891 was elucidated (Fig. 1(a)). As many other microbial polysaccharides, the EPS appeared to be O-acetylated. In native EPS B891, the acetyl groups were found in a non-stoichiometric ratio relative to the monosaccharides present. It has been mentioned before [11] that during the isolation and purification procedure of native EPS partial O-deacetylation may have occurred. Furthermore, it is possible that EPS contains acetyl substituents on alternate repeating units or more randomly distributed [20]. Since esterase activities capable of removing these substituents from exopolysaccharides have not been demonstrated [21] until very recently [22], EPS B891 was O-deacetylated using alkali and the chemical structure of the resulting polysaccharide was characterised.

Sugar linkage analysis of polysaccharides **1**, **2** and **3** did not provide a stoichiometric ratio of 4-substituted galactose to 4,6-disubstituted glucose (50% of theoretical, Table 1). These results were reproducible and there were no signals pointing towards undermethylation. Since base-catalysed O-acetylation has been shown to be not quantitative for all partially methylated alditols because of borate complexing [23], the partially methylated hydrolysate of EPS B891 was also O-acetylated under acid-catalysed conditions based on the method described by Harris et al. [23]. After acid-catalysed O-acetylation, the ratio of 4-substituted galactose to 4,6-disubstituted glucose appeared to be stoichiometric, although this time the amount of terminally-linked galactose was underestimated.

In order to prove the location of the acetyl group within the repeating unit, it was necessary to produce oligomers that could be analysed by NMR spectroscopy at low temperature and high concentration. Since the screening of enzymes on O-acetylated EPS B891 did not result in any endo-activity (unpublished results), oligomers were produced by solvolysis using HF. From the basic structure of EPS B891 and the behaviour of various polysaccharides in HF, a single cleavage was expected to occur at the α-linkage within the repeating unit. According to the analysis of the HF reaction product by MALDI-TOF MS, the solvolysis was not that selective, since not only single repeating units (and multimers of the repeating unit) were released. In the ^{1}H NMR spectrum of the pooled oligomers obtained after HF solvolysis (not shown), the signal of the anomeric proton of the α-linked sugar residue decreased drastically and a new doublet, probably α-glucosyl fluoride [18] based on $J_{1,F}$ 52.6 Hz, was centred at δ 5.69. Furthermore, the RI signal of the separation of oligomers on Bio-Gel P-2 (Fig. 5) combined with the MALDI-TOF MS spectra of the obtained fractions (not shown) proved that the reaction product of HF solvolysis contained a lot of oligosaccharides with degree of polymerisation ≤ 4. These results indicate that the HF solvolysis was rather selective, but that 30 min in liquid HF probably was too long and led to increased undesired cleavage of β-linkages. Nevertheless, the location of the acetyl group was proven by recording a 2D HMBC spectrum of the pooled oligomers. The approach for locating acetyl groups by measuring HMBC spectra has been described earlier for polysaccharides from fibre flax [24]. In EPS B891, the acetyl groups were present at the C-6 position of the 4-substituted glucosyl residues in the branches of the repeating units. The chemical shifts of the protons attached to the O-acetylated carbon were measured to be 4.53 and 4.35 ppm. These values are similar to the chemical shifts reported for the protons at the O-acetylated C-6 of a 4-substituted glucosyl residue in xyloglucan oligomers: 4.60 and 4.30 ppm [25]. Moreover, the effect of O-acetylation on the protons attached to the carbon, which bears the *O*-acetyl group in EPS B891 correspond to the observed shifts for the protons at the O-acetylated primary carbon (C-9) of sialic acid (0.5–0.6 ppm), which are lower than the observed shifts for the protons at the O-acetylated secondary carbons of sialic acid (1–1.5 ppm) [26].

Knowing the chemical structure of native EPS B891, most masses in the MALDI-TOF MS spectrum of the HF reaction product (Fig. 4) can be explained. However, the presence of masses corresponding to (four hexoses + two acetyl groups) and (five hexoses + two acetyl groups) indicates that the O-acetylation pattern has minor variants. Similar results have been reported before for gellan gum oligomers obtained by treatment in liquid HF [27]. Since the NMR spectra of native EPS B891 showed the presence of only one acetyl group, no further attention was paid to the unexpected masses in the MALDI-TOF MS spectrum of the HF reaction product.

The exact chemical structure of the repeating unit of EPS B891 can be represented in different ways. For the representation, data from the biosynthesis of the repeating unit by *L. lactis* subsp. *cremoris* B891 are taken into account. According to the results of van Kranenburg et al. [28], who demonstrated a glucosyltransferase linking glucose to the lipid carrier and a galactosyltransferase linking galactose to the lipid-linked glucose, the first two sugar residues synthesised for the repeating unit of B891 form lactose. These results led to the representation of the repeating unit of EPS B891 as is shown in Fig. 1(a).

As a first step in studying the structure–function relationship of exopolysaccharides by using EPS B891, the chemical structure was elucidated. Since the presence or absence of an acetyl group on each repeating unit can greatly alter the properties of a number of exopolysaccharides [29], comparison of the physical properties of O-acetylated and O-deacetylated EPS B891 would be very interesting. Likewise, additional physical analysis of enzymically modified O-deacetylated EPS B891 could give information about the influence of the presence or absence of the terminally-linked galactosyl residue in the branches of the repeating units.

To our knowledge, the chemical structure of EPS B891 has not been reported before for EPS produced by *L. lactis* subsp. *cremoris* or other lactic acid bacteria. Yet, the chemical structure of the branches of O-deacetylated EPS B891 are identical to the branches reported for EPS B39 [9]. This is reflected by the fact that the enzyme preparation Ultra SP is able to release galactose from both EPS B39 [9] and O-deacetylated EPS B891 (this research). Work on the purification and characterisation of the β-galactosidase responsible for the applied modification of both EPSs is in progress.

Acknowledgements

We gratefully acknowledge Professor Dr Moerschbacher (Department of Plant Biochemistry and Biotechnology, Westfälische Wilhelms-Universität Münster, Germany) for his kind cooperation with regard to HF solvolysis. This work was financially supported by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science and the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research programme of the Netherlands Association of Biotechnology Centres in the Netherlands (ABON).

References

[1] A.M. Stephen, S.C. Churms, in A.M. Stephen (Ed.), *Food Polysaccharides and Their Applications*, Marcel Dekker, New York, 1995, pp. 1–18.
[2] V. Crescenzi, *Biotechnol. Prog.*, 11 (1995) 251–259.
[3] J. Sikkema, T. Oba, *Snow Brand R&D Rep.*, 107 (1998) 1–31.
[4] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.*, 231 (1992) 273–291.
[5] H. Nakajima, T. Hirota, T. Toba, T. Itoh, S. Adachi, *Carbohydr. Res.*, 224 (1992) 245–253.
[6] V.M. Marshall, E.N. Cowie, R.S. Moreton, *J. Dairy Res.*, 62 (1995) 621–628.
[7] W.H.M. van Casteren, C. Dijkema, H.A. Schols, G. Beldman, A.G.J. Voragen, *Carbohydr. Polym.*, 37 (1998) 123–130.
[8] W.H.M. van Casteren, M.A. Kabel, C. Dijkema, H.A. Schols, G. Beldman, A.G.J. Voragen, *Carbohydr. Res.*, 317 (1999) 131–144.
[9] W.H.M. van Casteren, C. Dijkema, H.A. Schols, G. Beldman, A.G.J. Voragen, *Carbohydr. Res.*, 324 (2000) 170–181.
[10] R. Tuinier, P. Zoon, C. Olieman, M.A. Cohen Stuart, G.J. Fleer, C.G. de Kruif, *Biopolymers*, 49 (1999) 1–9.
[11] G.W. Robijn, D.J.C. van den Berg, H. Haas, J.P. Kamerling, J.F.G. Vliegenthart, *Carbohydr. Res.*, 276 (1995) 117–136.
[12] A.J. Mort, *Carbohydr. Res.*, 122 (1983) 315–321.
[13] G. Widmalm, K. Leontein, *Carbohydr. Res.*, 247 (1993) 255–262.

[14] P.J. Looijesteijn, J. Hugenholtz, *J. Biosci. Bioeng.*, 88 (1999) 178–182.
[15] K. Bock, H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
[16] A.J. Mort, *Abstr. Pap. Am. Chem. Soc. Meet.*, 181 (1981) CARB-49.
[17] A.J. Mort, J.-P. Utille, G. Torri, A.S. Perlin, *Carbohydr. Res.*, 121 (1983) 221–232.
[18] M.-S. Kuo, A.J. Mort, *Carbohydr. Res.*, 145 (1986) 247–265.
[19] Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151–162.
[20] I.W. Sutherland, *Biotechnology of Microbial Exopolysaccharides*, Cambridge University Press, Cambridge, 1990, pp. 1–11.
[21] I.W. Sutherland, *Carbohydr. Polym.*, 38 (1999) 319–328.
[22] W. Cui, W.T. Winter, S.W. Tanenbaum, J.P. Nakas, *Enzym. Microb. Technol.*, 24 (1999) 200–208.
[23] P.J. Harris, R.J. Henry, A.B. Blakeney, B.A. Stone, *Carbohydr. Res.*, 127 (1984) 59–73.
[24] J.M. van Hazendonk, E.J.M. Reinerink, P. de Waard, J.E.G. van Dam, *Carbohydr. Res.*, 291 (1996) 141–154.
[25] I.M. Sims, S.L.A. Munro, G. Currie, D. Craik, A. Bacic, *Carbohydr. Res.*, 293 (1996) 147–172.
[26] J. Haverkamp, H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, R. Pfeil, R. Schauer, *Eur. J. Biochem.*, 122 (1982) 305–311.
[27] M.-S. Kuo, A.J. Mort, A. Dell, *Carbohydr. Res.*, 156 (1986) 173–187.
[28] R. van Kranenburg, H.R. Vos, I.I. van Swam, M. Kleerebezem, W.M. de Vos, *J. Bacteriol.*, 181 (1999) 6347–6353.
[29] I.W. Sutherland, *Pure Appl. Chem.*, 69 (1997) 1911–1917.