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Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40

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Abstract

The chemical structure of an exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40 is studied, explaining earlier reported analytical discrepancies. The EPS was found to have a molecular mass of 6.8×10^5 g mol^{-1} and a molar ratio of rhamnose:galactose:glucose:phosphorus of 1:1.3:2:1.1. ^{31}P NMR indicated that a single phosphate group is present as a phospho*di*ester. EPS B40 was chemically modified using 0.6 M H_2SO_4, 28 M HF or 2 M NaOH. From these modifications it could be concluded that galactose 1-phosphate was linked at the 3-position of 1,2,3,4-linked galactose in the backbone of the EPS. Furthermore, it appeared that during the hydrolysis step of the sugar composition analysis the galactose 3-phosphate linkages were only partially split and that, as a result, the amount of galactose was underestimated in presence of phosphate. Finally, it was demonstrated that a crude cellulase preparation was able to degrade dephosphorylated and partially de-rhamnosylated EPS.

Keywords: Exopolysaccharide; Lactococcus; Chemical structure; Modification; Phosphorus

1. Introduction

Lactococci are generally regarded as safe (GRAS) in food production and are widely used as components of starter cultures for fermented dairy products (Teuber et al., 1992). Many lactic acid bacteria, among which lactococci, produce exopolysaccharides (EPS) which play an important role in the rheological behaviour and texture of fermented milks (Cerning, 1990). In order to gain insight in the physical properties of EPS from lactic acid bacteria, detailed structural studies have been performed on EPS from lactobacilli (Gruter et al., 1993; Yamamoto et al., 1994; Robijn et al., 1995a; 1995b; 1996a; 1996b; Staaf et al., 1996; Stingele et al., 1997), streptococci (Lemoine et al., 1997) and lactococci (Gruter et al., 1992; Nakajima et al., 1992).

Van Kranenburg et al. (1997) reported that EPS from *Lactococcus lactis* subsp. *cremoris* B40 consists of rhamnose, galactose and glucose in the ratio of 0.9:1.2:2.0 and that the molar ratio of carbohydrate and phosphorus is 4.7:1. From these data and from the ^{1}H and ^{13}C NMR spectra they suggest that EPS B40 is probably identical to EPS SBT 0495 (Nakajima et al., 1992) and consists of the repeating unit as presented in Fig. 1.

The present paper describes a more detailed study of the chemical structure of EPS B40 to clarify the discrepancy between the ratio of galactose reported by van Kranenburg et al. (1997) and Nakajima et al. (1992) and to gain a better understanding of the relationship between the chemical structure and the physical properties. For this purpose, effort is directed towards the modification of this chemical structure in order to be able to vary the physical properties. Since enzymes act very specifically, they can be used to bring about modifications in the EPS changing its functional properties. Therefore, in this study commercial enzyme preparations were screened for activity towards (chemically modified) EPS.

2. Experimental

2.1. Production and isolation of the exopolysaccharide

EPS was produced by *Lactococcus lactis* subsp. *cremoris* B40 in a medium based on whey permeate. The fermentation broth was centrifuged, ultrafiltered and lyophilised as

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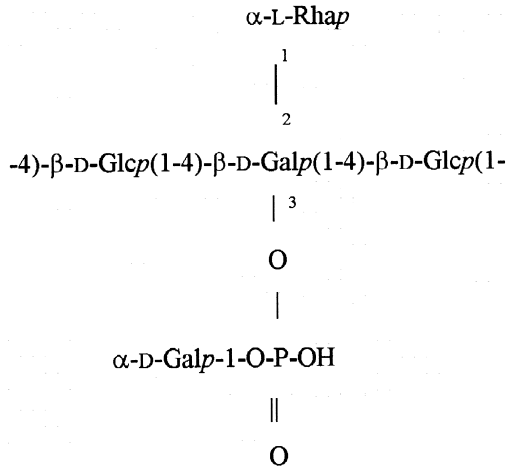

Fig. 1. Structure of the repeating unit from EPS SBT 0495 (Nakajima et al., 1992).

described by Tuinier et al. (1998) and kindly supplied as crude EPS by the Netherlands Institute for Dairy Research (NIZO).

To remove proteins, crude EPS was stirred in 4% (*w*/*v*) trichloroacetic acid (TCA) for 2 h at 4°C (1.7 g L^{-1}) and centrifuged (28 100 *g*, 30 min, 4°C). The supernatant was collected, neutralised with 2 M NaOH and two volumes of 96% ethanol (4°C) were added in order to precipitate EPS (2 h, 4°C). After centrifugation (28 100 *g*, 30 min, 4°C), the pellet was dissolved in distilled water, dialysed and lyophilised.

Further purification was performed by use of ion-exchange chromatography on a column (52 × 5 cm) of DEAE Sepharose fast flow (Pharmacia), using a Biopilot System (Pharmacia). Therefore, TCA/ethanol purified EPS (0.76 g) was dissolved in 5 mM NaOAc pH 6 (375 mL) and applied to the column equilibrated with the same solution. The flow rate varied from 10 mL min^{-1} during application to max. 50 mL min^{-1} during the gradient of 5 mM NaOAc pH 6 → 2 M NaOAc pH 6. The absorption of the eluent at 280 nm was measured on-line and the protein concentration was estimated by using the extinction coefficient of β-casein (0.46 cm^2 mg^{-1}; Swaisgood, 1992). Material still bound to the column was eluted with 0.5 M NaOH (20 mL min^{-1}). The fractions (c. 100 mL) were analysed for their total sugar content using the orcinol method (Tollier and Robin, 1979) with glucose as standard. The sugar composition and phosphorus content of the pooled fractions were analysed as described below.

2.2. Chemical modifications of EPS

2.2.1. H_2SO_4 treatment

To a solution of 25 mg EPS in 6.25 mL distilled water, 6.25 mL 0.6 M H_2SO_4 was added. The solution was kept at 37°C for 2 h, cooled on ice and neutralised (2 M NaOH).

2.2.2. HF treatment

50 mg EPS was stirred (48 h, 0°C) in 2.5 mL 28 M (=48%) HF and neutralised on ice with 6 M NaOH.

2.2.3. NaOH treatment

12.5 mL 4 M NaOH containing 1 mg mL^{-1} $NaBH_4$ was added to a solution of 25 mg EPS in 12.5 mL distilled water. After 3 min at room temperature, the solution was heated for 4 h at 80°C, cooled on ice and neutralised with 2 M HOAc.

After the chemical modifications, samples were taken for analysis by HPAEC. The remaining samples were dialysed and split: one part was concentrated and analysed by HPSEC and light-scattering analysis; the other part was lyophilised for sugar composition analysis and phosphorus analysis.

2.3. Analytical methods

2.3.1. Sugar composition

EPS was pre-treated in 12 M H_2SO_4 (1 h at 30°C) and hydrolysed in 1 M H_2SO_4 (3 h at 100°C), using inositol as internal standard. The released sugars were converted into their alditol acetates (Englyst and Cummings, 1984) and analysed on a J&W DB-225 column (15 m × 0.53 mm) in a Carlo Erba 4200 GC. The temperature program was: 1 min isothermal at 180°C, 180 → 220°C at 2.5° min^{-1} and 3 min isothermal at 220°C. The FID detector temperature was 275°C and He was used as carrier gas.

2.3.2. Sugar linkage composition

Purified EPS and HF-treated EPS were methylated according to Hakomori (1964) and subsequently dialysed against water and lyophilised. The methylated polysaccharide was hydrolysed in 2 M trifluoroacetic acid (1 h, 121°C). After evaporation (air stream, < 20°C), the partially methylated sugars were converted into alditol acetates (Englyst and Cummings, 1984) and analysed by GC–FID in a Carlo Erba HRGC 5160 gas chromatograph as described by Vincken et al. (1994). Partially methylated alditol acetates were quantified according to their effective carbon response (Sweet et al., 1975). Identification of the compounds was confirmed by GC–MS using a CP Sil 19 CB capillary column (25 m × 0.25 mm, 0.2 μm film thickness, Chrompack) in a HP 6890 gas chromatograph coupled to a HP 5973 mass-selective detector and using an HP Chem Station (Hewlett–Packard). The temperature program was 160 → 185°C at 0.5° min^{-1}, 185 → 230°C at 10° min^{-1} and 230°C for 5.5 min. Besides the samples mentioned above, methylated EPS was also treated with HF prior to derivatisation into alditol acetates to obtain information on the position of phosphate within the EPS.

2.3.3. Phosphorus determination

The amount of phosphorus in the samples was analysed with and without treatment in 72% perchloric acid (20 min 180°C) to correct for the free phosphorus. After dilution, the samples were analysed as described by Chen et al. (1956).

To find out whether all sugar phosphate linkages were cleaved during the hydrolysis in the sugar composition analysis, hydrolysed samples were analysed for their free phosphorus content as well.

2.3.4. ^{31}P NMR analyses

121.500 MHz ^{31}P NMR spectra were recorded in H_2O with 5–10% D_2O on a Bruker AMX-300 spectrometer, equipped with a 10 mm probe, at a probe temperature of 27°C. Chemical shifts were referenced to added L-α-glycerophosphorylcholine (GPC; Sigma) (δ 0.49). Chemical shifts caused by pH changes were measured to establish the presence of phosphomonoesters and/or phosphodiesters. The pH of the sample was adjusted by adding 25 mM HCl or 25 mM NaOH.

2.3.5. ^{1}H and ^{13}C NMR

1D 400.13 MHz ^{1}H NMR spectra and proton-decoupled 100.63 MHz ^{13}C NMR spectra of HF-treated EPS B40 were recorded in D_2O (8 mg mL^{-1}) at 60°C on a Bruker DPX-400 spectrometer equipped with a 5 mm probe.

2.3.6. Monosaccharide release

High-performance anion-exchange chromatography (HPAEC) was performed using a Dionex system, which included a gradient pump, an eluent degas (He) module, a CarboPac PA1 column (4 × 250 mm) with CarboPac PA100 guard column and a pulsed electrochemical detector (PED-2) in the pulsed amperometric detection (PAD) mode. A Spectra–Physics AS3000 autosampler was used and chromatograms were recorded using PC1000 software. The effluent was monitored using the PED-2 detector (reference electrode Ag/AgCl) containing a gold electrode. Potentials of *E1* 0.1, *E2* 0.7 and *E3* −0.1 were applied for duration times of *T1* 0.4 s, *T2* 0.2 s and *T3* 0.4 s. The gradient (1 mL min^{-1}) was obtained by mixing Millipore water, 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH. After equilibration with 16 mM NaOH, 20 μL of the sample was injected. The elution program was 0 → 20 min, 16 mM NaOH isocratic, followed by a washing step: 20 → 25 min, 0 → 1 M NaOAc; 25 → 30 min, 1 M NaOAc; 30 → 35 min, 0.1 M NaOH; 35 → 40 min, 0.1 M → 16 mM NaOH; 40 → 55 min, re-equilibration using 16 mM NaOH isocratic.

2.3.7. Galactose 1-phosphate release

Galactose 1-phosphate was analysed by HPAEC using the system described above. The gradient (1 mL min^{-1}) was obtained by mixing 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH. The elution program was 0 → 5 min, 0.1 M NaOH isocratic; 5 → 72 min, linear gradient of 0 → 0.6 M NaOAc; 72 → 77 min, 0.6 M NaOAc isocratic, followed by a washing step: 77 → 82 min, 0.6 → 1 M NaOAc; 82 → 87 min, 1 M NaOAc; 87 → 102 min, re-equilibration using 0.1 M NaOH. α-D-Galactose 1-phosphate (Sigma) was used as a standard.

2.3.8. Molecular mass determination

The molecular mass and radius of gyration of (modified) EPS B40 were determined by high-performance size-exclusion chromatography (HPSEC) with on-line refractive index and static light-scattering (SLS) analysis as described by Tuinier et al. (1998).

2.3.9. Enzymatic degradation

Enzyme-treated samples were analysed for degradation by HPAEC (see above) and HPSEC. HPSEC was performed on a SP8700 HPLC (Spectra–Physics) equipped with three Bio-Gel TSK columns in series (60XL, 40XL and 30XL; each 300 × 7.8 mm) preceded by a TSK XL guard column (40 × 6 mm). The system was eluted (30°C) with 0.4 M NaOAc pH 3.0 at 0.8 mL min^{-1} and the eluate was monitored on-line using a refractometer (Viscotek). Dextrans (Mw 4000–500 000 g mol^{-1}) were used for calibration.

2.4. Enzymatic degradation of EPS

(Chemically modified) EPS B40 was incubated with over 15 commercial polysaccharide-degrading enzyme preparations (e.g. pectinases, cellulases and hemicellulases). The incubation conditions depended on the optimal conditions for the enzymes and since the cellulase Maxazyme CL2000 from *Trichoderma viride* (Gist brocades, Delft, The Netherlands) was the only preparation which showed activity towards (modified) EPS B40, only this incubation method is described below.

Purified EPS, H_2SO_4-treated EPS, NaOH-treated EPS and HF-treated EPS were dissolved (2 mg mL^{-1}) in 50 mM NaOAc buffer pH 5.0 + 0.01% (*w*/*v*) NaN_3. Maxazyme CL2000 was dialysed against the same buffer and the final protein concentration was determined (Bradford, 1976) using bovine serum albumin (BSA, Sigma A 4503) as a standard. The substrates were incubated with dialysed Maxazyme CL2000 (protein content: 0.02%) for 24 h at 30°C. After incubation, the samples were heated (15 min, 100°C) to inactivate the enzymes, centrifuged and analysed by HPSEC and HPAEC.

3. Results and discussion

3.1. Purification and characterisation of EPS

EPS produced by *Lactococcus lactis* subsp. *cremoris* B40 was extracted from the crude EPS with TCA, followed by precipitation with ethanol. The yield, sugar content and the sugar composition of the crude EPS and the fractions obtained are shown in Table 1. The total sugar yield of the fractions was 94%. Some lactose (c. 5%) which was still present in the crude EPS was not recovered, since the fractions were analysed after dialysis. The ethanol-precipitated material contained the bulk of the sugars, as

Table 1
Sugar yield, sugar content and sugar composition of crude EPS and of the different fractions obtained after purification of EPS B40 with TCA and ethanol

	Yield[a] (*w*/*w* %)	Sugar content (*w*/*w* %)	Rha	Xyl	Man	Gal	Glc
			(mol%)				
Crude EPS	100	51	23	1	5	29	42
TCA precipitate	8	17	22	2	10	24	42
Ethanol precipitate	84	63	23	1	4	29	44
Ethanol supernatant	2	9	41	8	9	17	26

[a]Expressed as mg sugar per 100 mg sugar in crude EPS.

was expected. The total sugar content increased from 51% in the crude EPS to 63% in the ethanol-precipitated material due to removal of protein-like material. Although the sugar composition of the ethanol-precipitated material was similar to the sugar composition of crude EPS small amounts of rhamnose, xylose and mannose were removed during purification with TCA/ethanol.

The ethanol-precipitated material was purified further on an anion-exchange chromatography column. The elution patterns of sugar and protein are shown in Fig. 2. The retention of EPS B40 on the DEAE column illustrates the negative charge of the polysaccharide. After elution with 2 M NaOAc a sugar recovery of only 72% was obtained, while another 25% was recovered after elution with 0.5 M NaOH (NaOH concentration is not shown in Fig. 2). From preliminary results, the first part of the sugar peak was known to contain a relatively high amount of mannose and was therefore pooled separately. In Table 2, the sugar yield, the total sugar content, the sugar composition and the phosphorus content of the different pools are given. Pool I indeed contained a relatively high amount of mannose in addition to rhamnose, galactose, glucose and phosphorus. It appears that in this pool mannan-rich material, for which we have strong indications that it originates from the yeast extract in the medium (unpublished results), co-eluted with EPS. Pool II appeared to be similar to pool III in sugar composition, but the analysed total sugar content in pool II was higher than in pool III, which agrees with the

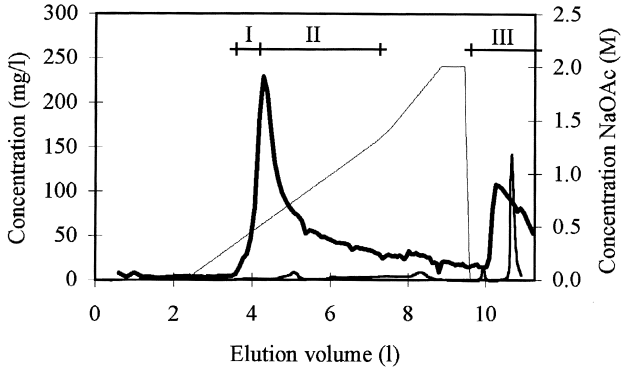

Fig. 2. Fractionation of EPS B40 on DEAE Sepharose Fast Flow, eluted with a gradient of NaOAc (-), followed by 0.5 M NaOH. The total sugar content (-) is expressed as glucose equivalents. The protein concentration (-) was estimated by dividing the measured A280 by the extinction coefficient of β-casein.

higher protein concentration in pool III (Fig. 1). The molar ratio of Rha:Gal:Glc in both pools was 1:1.3:2 and the phosphorus content was 0.05 g g^{-1} sugars, which corresponds to 1.1 mol phosphorus per 4.3 mol sugar. However, since not all galactose was detected during the sugar analysis (see below), it is more accurate to express the phosphorus content relative to the amount of rhamnose or glucose instead of relative to the total amount of sugar analysed.

Purified EPS SBT 0495 contained Rha:Gal:Glc:P in a ratio of 1.5:2.4:2:1.1 (Nakajima et al., 1990). The amount of phosphorus, relative to the amount of glucose in EPS B40 was the same as in SBT 0495. Although the relative amount of rhamnose analysed in EPS B40 was lower than in EPS SBT 0495, it approaches the proposed structure for EPS SBT 0495 (Nakajima et al., 1992) better. Most striking is the relative amount of galactose, which is much lower in EPS B40 than reported for EPS SBT 0495. This was analysed further in pool II and will be discussed below.

3.2. Chemical modifications of EPS

EPS B40 was chemically modified for three reasons. Firstly, because it might be useful in the structure elucidation of EPS B40. Secondly, because screening of chemically modified EPS besides native EPS increases the chance of detecting EPS-degrading enzymes. Finally, chemically modified EPS are of interest for physical studies. Therefore, EPS B40 was treated with H_2SO_4, HF and NaOH and the sugar composition and phosphorus content of the resulting polymers were analysed (Table 3).

It appears (Table 3) that after H_2SO_4 treatment the relative amount of galactose had decreased by a factor of 0.2, whereas the relative amounts of rhamnose, glucose and phosphorus did not change. Directly after H_2SO_4 treatment, the sample was analysed for the presence of sugar monomers using HPAEC. Only galactose monomer was detected and the amount was consistent with the loss of galactose found for the polymer. Since it is known that sugar 1-phosphate linkages are sensitive to dilute acid (Hancock and Poxton, 1988), galactose is probably terminally linked to phosphate, which agrees well with the proposed structure (van Kranenburg et al., 1997; Nakajima et al., 1992). The amount of galactose liberated was, however, less than expected from this proposed structure.

Table 2
Sugar yield, sugar content, sugar composition and phosphorus content of the different pools obtained after fractionation of EPS B40 on a DEAE Sepharose column

Pool	Yield (*w*/*w* %)	Total sugar content (*w*/*w* %)	Rha	Xyl	Man	Gal	Glc	Phosphorus (g/g sugars)
			(mol%)					
I	9	57	18	1	35	19	28	0.03
II	50	57	23	1	2	30	45	0.05
III	25	45	23	1	1	29	46	0.05

Possible explanations are that not every terminal galactose is released by H_2SO_4 treatment, or that not every repeating unit contains a terminally linked galactose. This was further analysed with ^{31}P NMR and will be discussed later.

HF treatment removed all phosphorus from the EPS and resulted in a polysaccharide with lower amounts of galactose and rhamnose relative to glucose (Table 3). Under these conditions, HF selectively cleaves sugar–phosphate linkages without hydrolysis of sugar–sugar bonds (Hancock and Poxton, 1988; Nakajima et al., 1992). However, some cleavage of rhamnose linkages during treatment with HF has been reported (Lugowski and Jennings, 1984; Jansson et al., 1988; Robijn et al., 1995a). As discussed below (Fig. 3), the removal of phosphate and rhamnose did not cause a large decline in hydrodynamic volume and, consequently, they must have been present as side groups of the EPS and not in the backbone. Moreover, the removal of galactose indicates that galactose is linked to the backbone of the polysaccharide via phosphate. The amount of rhamnose monomer released during HF treatment and detected by HPAEC was consistent with the rhamnose removal in the polymer. However, the amount of galactose detected by HPAEC was larger than the loss of galactose in the polymer (Table 3). Converted into a relative amount, 0.7 mol galactose per 2 mol glucose in the starting EPS was recovered by HPAEC. It can therefore be concluded that the removal of galactose from the polymer during HF treatment was much larger than determined as alditol acetates.

During HF treatment all phosphorus and therefore all terminally linked galactose was removed, while in H_2SO_4-treated EPS most of the terminally linked galactose but almost no phosphorus was removed. Remarkably, the relative amount of galactose in the polymer found after H_2SO_4 treatment was lower than the relative amount of galactose found after HF treatment (Table 3). These results strongly suggest that galactose in the backbone (see also the results of the sugar linkage composition) of the EPS is only *partially* analysed in presence of phosphorus. In order to investigate whether the low amount of galactose found as alditol acetates is caused by incomplete hydrolysis of galactose–phosphate linkages, hydrolysates were also analysed (prior to further derivatisation) for the amount of phosphorus. The results showed that almost no free phosphorus was present in native EPS. After hydrolysis, 30–40% of the total amount of phosphorus in the EPS was detected as free phosphorus. This means that only part of the phosphate linkages to the galactose in the backbone of the EPS were hydrolysed. As a result, the galactose in the backbone was determined for only 30–40% whereas the terminal galactose was determined completely after hydrolysis. This explains why a Gal:Glc ratio of 1.3:2 (Table 3) was found in the native EPS while a ratio of 2:2 was expected according to the proposed structure. It also explains the relatively small decrease in the galactose content of the polymer after HF treatment; the real removal of (terminally linked) galactose was diminished by the fact that all galactose in the backbone was analysed in our assay due to the removal of phosphorus.

NaOH treatment of the EPS revealed a polysaccharide with a decreased content of phosphorus and galactose (Table 3). Unlike HF treatment, NaOH treatment did not remove all phosphorus from the EPS and all rhamnose linkages were kept intact. Despite the fact that the galactose content had decreased after NaOH treatment, no galactose was found by HPAEC. There was, however, a peak that eluted at the retention time of galactose 1-phosphate followed by a second peak of similar size which has not yet been characterised.

From the chemical modifications it can be concluded that

Table 3
Total sugar content, sugar composition and phosphorus content of purified EPS B40 before and after chemical modification. (Traces of mannose and xylose were still present)

	Total sugar content (*w*/*w* %)	Rha	Gal	Glc	Phosphorus
		(molar ratio)			
Purified native EPS	57	1.0	1.3	2.0	1.1
0.6 M H_2SO_4-treated EPS	42	1.0	0.6	2.0	1.1
28 M HF-treated EPS	85	0.8	1.0	2.0	0.0
2 M NaOH-treated EPS	60	1.0	1.0	2.0	0.1

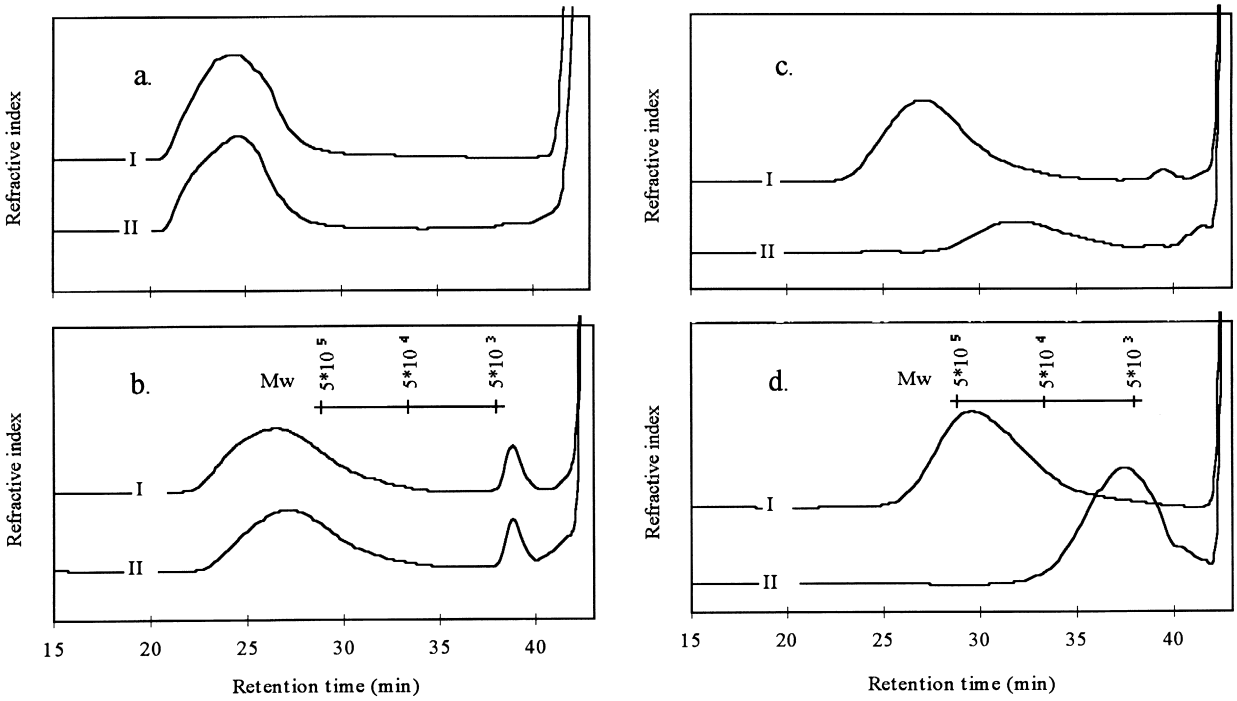

Fig. 3. HPSEC patterns of (modified) EPS B40 after incubation with a crude cellulase: (a) purified EPS (b) H_2SO_4-treated EPS (c) NaOH-treated EPS (d) HF-treated EPS; (I) substrate (II) substrate with enzyme. Calibration was performed by using dextrans and the molecular mass is given in g mol^{-1}.

the galactose in the backbone of purified EPS B40 was underestimated because of incomplete hydrolysis of the phosphate ester with the galactose in the backbone. This probably explains the relatively low amount of galactose found after hydrolysis in 4 N HCl, as reported by van Kranenburg et al. (1997). Nakajima et al. (1990) treated their EPS with 48% HF followed by hydrolysis with 2 N trifluoroacetic acid. Since their EPS was not dialysed after HF treatment, no galactose was lost and a ratio of Rha:Gal:Glc of 1.5:2.4:2 was found. In conclusion, the discrepancy between the relative amount of galactose in EPS B40 (van Kranenburg et al., 1997) and EPS SBT 0495 (Nakajima et al., 1990) was caused by differences in methods rather than differences in EPS structures.

3.3. Sugar linkage composition of EPS

Methylation analyses were performed on native EPS and HF-treated EPS, while methylated samples of native EPS were also HF-treated prior to derivatisation into alditol acetates. The data (Table 4) show that in native EPS terminally linked rhamnose, 1,4-linked glucose and traces of 1,2,4-linked and 1,2,3,4-linked galactose were found. A poor stoichiometry in the methylation analyses of phosphorylated polysaccharides, due to partial dephosphorylation during methylation and incomplete hydrolysis of phosphate esters, has been reported previously (Jansson et al., 1988; Nakajima et al., 1992; Robijn et al., 1995a; Robijn et al., 1996a). HF treatment after methylation caused a higher recovery of 1,2,3,4-linked galactose compared to native EPS. This can probably be explained by the fact that since all sugar–phosphate linkages are split during HF treatment, phosphorus can no longer interfere with the hydrolysis and, accordingly, all galactose in the backbone of the EPS is analysed. HF treatment prior to methylation analysis instead of HF treatment after methylation caused a change of 1,2,3,4-linked galactose into 1,2,4-linked galactose. This demonstrates that 1,2,3,4-linked

Table 4
Sugar linkage composition data of native EPS (**1**), methylated and HF-treated EPS (**2**) and HF-treated and methylated EPS (**3**)

Derivative	Linkage type	Molar ratio[a]		
		1[b]	**2**[b]	**3**
2,3,4-Rha[c]	1-Rha	1	1	1
2,3,6-Glc	1,4-Glc	2	2	2
3,6-Gal	1,2,4-Gal	Trace	Trace	1
6-Gal	1,2,3,4-Gal	Trace	1	Trace

[a]2,3,6-glc was taken as 2.
[b]Unmethylated material was left out of consideration (see text).
[c]1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, etc.

galactose in native EPS is substituted with phosphorus at *O*-3, which again agrees with the proposal of van Kranenburg et al. (1997) that the structure of EPS B40 resembles the structure of EPS SBT 0495 (Nakajima et al., 1992).

It should be remarked that the methylation analyses of native EPS B40 resulted in undermethylation, probably due to the low solubility of EPS in Me_2SO. However, the sugar composition of the unmethylated material (not shown) resembled that of the EPS. Moreover, the results (Table 4) were quite reproducible and no di-*O*-methyl sugars and mono-*O*-methyl sugars were observed for terminally linked rhamnose and 1,4-linked glucose. Therefore, it was assumed that the methylated part of the EPS was representative for the total EPS.

The results of the methylation analysis of EPS B40 (Table 4) correspond to the results of EPS SBT 0495 reported by Nakajima et al. (1992), except for the fact that we could not detect any terminally linked galactose. For HF-treated EPS the latter was expected since galactose was split off during HF treatment and was lost during dialysis. For native EPS, however, the loss of terminally linked galactose was not expected. Since the methylation analysis takes place under strongly alkaline conditions, it seems possible that the galactose 3-phosphate linkage is split during methylation and that galactose 1-phosphate is lost during dialysis. However, Table 4 shows that HF treatment after methylation results in 1,2,**3**,4-linked galactose, which means that during methylation the phosphate group was still present. Nakajima et al. (1992) found some terminally linked galactose after purifying the partially methylated, HF-treated and again methylated polysaccharide by extraction, but less than they had expected. Since they removed the excess of HF under vacuum over KOH in a desiccator, they retained the terminally linked galactose that was released during HF treatment. If, however, terminally linked galactose was split off during methylation, they would have retained the galactose also. Therefore, the difference in recovery of terminally linked galactose reported here and reported by Nakajima et al. (1992) might be explained by differences in the purification procedure.

3.4. NMR analyses of EPS

The ^{31}P NMR spectrum of purified EPS showed one resonance (δ − 0.8) at pH 7.0, indicating the presence of a single phosphate group in a repeating unit (Moreau et al., 1988). The chemical shift of this resonance did not change significantly during titration experiments (pH 6.1–10.0), corroborating the presence of a phosphodiester (Ilg et al., 1996). The fact that after purification no phosphate monoester was found shows that during purification with TCA the acid labile galactose 1-phosphate was kept intact and that all phosphate groups are present in diester form. However, it should be noticed that *prolonged* exposure of EPS to TCA can result in degradation of the phosphodiester. In that case we observed a phosphomonoester besides a phosphodiester resonance in the ^{31}P NMR spectrum.

H_2SO_4-treated EPS gave a resonance (δ 0.1 at pH 4.0) which shifted during titration experiments (δ 3.1 at pH 8.4), which is indicative for a titratable phosphomonoester. Since only a very small phosphodiester signal was found in this sample, almost all terminally linked galactose must have been removed during H_2SO_4 treatment.

HF-treated EPS was also measured and no resonances could be detected in ^{31}P NMR experiments, which corresponds with complete dephosphorylation of EPS.

^{1}H and ^{13}C NMR spectra (not shown) of dephosphorylated EPS B40 were similar to the spectra of dephosphorylated EPS SBT 0495 (Nakajima et al., 1992). These results agree with the suggestion of van Kranenburg et al. (1997), that the structure of the repeating unit of EPS B40 is identical to the structure published for EPS SBT 0495 (Nakajima et al., 1992).

3.5. Molecular mass and radius of gyration of (modified) EPS

The change in hydrodynamic volume caused by the different chemical treatments of EPS B40 are illustrated in Fig. 3 (lines I). All treatments caused a small decrease in hydrodynamic volume. To find out whether this decrease can be totally explained by the removal of substituents, native and chemically modified EPS were analysed by HPSEC/SLS to determine the molecular mass (M_n), the polydispersity (M_w/M_n) and radius of gyration (R_n). The results for purified EPS B40 were: $M_n = 6.8 \times 10^5$ g mol^{-1}, $M_w/M_n = 1.7$ and $R_n = 76.7$ nm and the results after H_2SO_4 treatment were: $M_n = 5.2 \times 10^5$ g mol^{-1}, $M_w/M_n = 1.4$ and $R_n = 67.2$ nm. The reduction in molecular mass during H_2SO_4 treatment approached the loss of all terminally linked galactose. It can therefore be concluded that this chemical modification was rather specific and that the resulting polymer might be interesting for further physical characterisation. The results of NaOH-treated EPS and HF-treated were not reliable due to problems with the solubility of modified EPS and a bad fit of the Debye plot, respectively.

3.6. Enzymatic degradation of EPS

Since enzymes can be used to modify polysaccharides very specifically, (chemically modified) EPS was incubated with various commercial enzyme preparations and analysed for degradation. In case of *endo*-activity, a shift in hydrodynamic volume can be observed (HPSEC), while *exo*-activity causes a release of sugar monomers or galactose 1-phosphate (HPAEC).

Most of the tested commercial enzyme preparations showed no activity towards EPS B40. There was however a crude cellulase preparation from *Trichoderma viride* (Maxazyme CL2000) which showed *endo* activity towards

HF-treated EPS and, to a much lesser extent, towards NaOH-treated EPS (Fig. 3). Since (H_2SO_4-treated) EPS B40 was not degraded by this crude cellulase, it can be concluded that the removal of phosphate and/or rhamnose is essential for the enzyme to be active on this EPS. Preliminary research on the degradation products on HPAEC showed a regular series of oligomers and it seems that although a crude enzyme preparation was used only one enzyme acted very specifically. In the future, more effort will be directed toward further identification of the oligosaccharides and revealing the mode of action of this enzyme since a well-characterised enzyme can be used as a tool for characterising other polysaccharides (Voragen et al., 1993).

4. Conclusions

As a first step in the study of the relationship between the chemical structure and the physical characteristics of EPS B40, the structure was characterised. The results indicate that EPS B40 is probably identical to the structure that was reported for EPS SBT 0495 by Nakajima et al. (1992). This has been proposed before by van Kranenburg et al. (1997) based mainly on similarities in sugar composition, phosphorus content and ^{1}H and ^{13}C NMR spectra.

Chemical modification of the EPS B40 with H_2SO_4, HF and NaOH appeared to be helpful not only to get information about the structure, but also to find an enzyme able to degrade modified EPS B40. This enzyme is subject of further investigation.

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