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PII: S0144-8617(96)00056-2

Carbohydrate Polymers 31 (1996) 35–40

0144-8617/96/$15.00 + .00

Presence of acetate and succinate in the exopolysaccharide produced by *Zoogloea ramigera* 115SLR

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(Received 30 April 1995; revised version received 11 April 1996; accepted 11 April 1996)

The extracellular acidic polysaccharide produced by *Zoogloea ramigera* 115SLR contains *O*-acetyl and *O*-succinyl groups, in addition to glucose, galactose and pyruvate already reported. Methylation analysis of both the native and the chemically modified zooglan showed that these *O*-acyl groups are attached to position 6 of a 4-substituted glucosyl residue and of a terminal glucose. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Zoogloea ramigera 115, first isolated by Friedman and Dugan (1968a), is a Gram-negative, floc-forming bacterium that produces the exopolysaccharide, zooglan, which has strong affinity for metal ions (Friedman & Dugan, 1968b; Stauffer *et al.*, 1980) and unique rheological behavior (Easson *et al.*, 1987). Structural investigations (Friedman *et al.*, 1968; Parsons & Dugan, 1971; Ikeda *et al.*, 1982; Franzen & Norberg, 1984) of this biopolymer have established that zooglan is a highly branched exopolysaccharide composed of glucose, galactose and pyruvate.

A mutant derived from *Z. ramigera* 115 by nitrosoguanidine (NTG) mutagenesis had been previously isolated in our laboratory (Easson, 1987). This mutant, designated 115SL, is non-capsule forming, non-floc forming and produces an exopolysaccharide that does not remain bound to the cell wall and is consequently released into the culture broth. To facilitate further manipulations, a spontaneous rifampicin resistant strain derived from 115SL (designated 115SLR) had also been isolated (Easson, 1987).

Here, we present recent studies on the composition and structure of the exopolysaccharide from *Z. ramigera* 115SLR.

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EXPERIMENTAL

Bacterial strain

Z. ramigera 115SLR was grown at 30°C in trypticase soy broth (TSB) containing rifampicin (50 μg/ml). Defined medium for exopolysaccharide production consisted of 2.5 g glucose, 0.02 g $MgSO_4 \cdot 7H_2O$, 5 μg yeast extract, 0.2 g K_2HPO_4, 0.1 g KH_2PO_4 and 0.1 g NH_4Cl in 100 ml water (Norberg & Enfors, 1982). For exopolysaccharide isolation, 1 ml of an overnight culture of *Z. ramigera* 115SLR in TSB was added to a 500 ml buffled flask containing 200 ml of defined medium. The cultures were incubated at 30°C in a gyratory shaker at 200 rpm for 3 days.

Carbohydrate and protein determination

Total carbohydrate concentration in culture broths and polymer solutions was determined by the phenol reaction (Hodge & Hofreiter, 1962) using xanthan gum (Sigma Chemical Co.) as standard. Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories) and bovine albumin (Sigma Chemical Co.) for the calibration curve.

Preparation of the exopolysaccharide samples

To isolate the exopolysaccharide (EPS), the cultures were diluted with 2 volumes of hot water (50°C). Cell

pellets were removed by centrifugation at 25°C for 25 min (11 000 rpm, GSA rotor, Sorval RC5B centrifuge). The EPS was precipitated with 2 volumes of isopropanol at −20°C for 6 h and then recovered by centrifugation (3000 rpm for 10 min). EPS pellets were washed with water, recovered by isopropanol precipitation, redissolved in water and lyophilized. Prior to analytical procedures, EPS samples were dissolved in distilled water and dialyzed against distilled water for 24 h. After this purification procedure, the purity of the polysaccharide was found to be 97% as determined by the phenol reaction.

Compositional analysis by HPLC

For sugar and organic acid analysis, the dried exopolysaccharide (10 mg) was dissolved in 5 ml of 0.1 M trifluoracetic acid (TFA) and hydrolysed at 120 °C for 6 h. The hydrolysate was filtered using Millipore 0.22 mm Millex-GV filters and analyzed by high-performance liquid chromatography (HPLC). Sample analysis was performed at 40°C on a 1050 Hewlett Packard HPLC system equipped with both a refractive index detector (model 1047A) for sugar analysis and a u.v. detector (model 35900) at 220 nm for organic acid analysis. HPLC separation was performed with a Bio-Rad Aminex HPX-87H column using 0.008 N sulfuric acid at a flow rate of 0.6 ml/min as the mobile phase.

Calibration curves were obtained by injecting increasing quantities of glucose, galactose, pyruvic, acetic and succinic acids, within the range expected for these compounds. Since pyruvic acid and galactose coeluted, the pyruvic acid content was determined by HPLC using u.v. detection at 220 nm. The amount of galactose was calculated from the refractive index peak after subtracting the area due to pyruvic acid.

Methylation analysis

The native exopolysaccharide (20 mg) was methylated by three cycles of Hakomori's procedure (Hakomori, 1964). The location of the pyruvate groups in the exopolysaccharide was studied by treatment of the native exopolysaccharide with 0.25 mM H_2SO_4 at 100 °C for 1 h, neutralization with 1 M NaOH and dialysis against water. The absence of pyruvate in the resulting exopolysaccharide was confirmed by acid-hydrolysis and HPLC analysis. The depyruvylated exopolysaccharide was submitted to methylation by Hakomori's method (Hakomori, 1964).

To study the location of the O-acyl groups in the native exopolysaccharide, the Prehm methylation procedure was performed (Prehm, 1980). However, it has been reported that the Prehm methylation procedure could result in undermethylated products (Reinhold *et al.*, 1994). To account for this possibility, the exopolysaccharide was also treated with 10 mM potassium hydroxide for 5 h at room temperature prior to the Prehm methylation procedure so that these results could be compared with those obtained after Hakomori's methylation.

After methylation, the reaction mixture was poured into water (10 ml), extracted with chloroform (5 ml) and dried under vacuum at 40°C. Methylated exopolysaccharides were hydrolysed in 90% formic acid (3 ml) at 100 °C for 2 h, dried under vacuum at 40°C and the residue was dissolved in 0.25 M sulfuric acid (1 ml) and heated to 100°C for 10 h. After cooling, the solution was neutralized with barium carbonate, filtered and freeze-dried.

The resulting partially methylated aldoses were converted to partially methylated alditol acetates as described by Lindberg (1972). Gas chromatography/mass spectrometry (GC/MS) analyses were carried out on a Hewlett-Packard GC/MS equipped with a (0.22 mm × 50 m) HP-1 column. The temperature programming consisted of an initial temperature of 130°C for 5 min and a final temperature of 250°C, with a heating rate of 5°C/min. Quantitation of the partially methylated alditol acetates was carried out on a Hewlett-Packard gas chromatograph equipped with a (0.22 mm × 25 m) OV-1 column at 190 °C.

Periodate oxidation

Sodium periodate (0.1 M, 500 ml) was added to a solution of the exopolysaccharide (500 mg, in the sodium salt form) in water (500 ml). The solution was kept in the dark at 4°C. After 5 days, 10 ml of ethylene glycol were added and the solution was stirred for 1 h before dialysis for 3 days and freeze-drying. To ensure complete oxidation, the product from the first periodate oxidation (420 mg) was dissolved in water (420 ml), an equal volume of a solution of 0.1 M sodium periodate was added, and the mixture was kept in the dark at 4°C for 3 days. Addition of ethylene glycol (10 ml), dialysis and freeze-drying gave 394 mg of polyaldehyde. Reduction with sodium borohydride (0.4 g), dialysis and freeze-drying yielded 360 mg of polyol. A fraction of this polyol (10 mg) was subjected to methylation analysis. Another fraction of the product (50 mg) was hydrolysed in 1 M HCl at 80°C for 1 h. After dialysis and freeze-drying, the product was subjected to methylation analysis.

RESULTS AND DISCUSSION

Composition analysis

The composition of the exopolysaccharide was determined by acid-hydrolysis and HLPC analysis. This

Table 1. Linear regression parameters for calibration of glucose, galactose, pyruvate, acetate and succinate (R.I.: refractive index detector; U.V.: U.V. detector at $\lambda = 220$ nm)

Compound	Range of concentration (μmol/μl)	Slope	Intercept	Regression coefficient	Detector
Glucose	0.75–22.5	91.036	93.194	0.999	R.I.
Galactose	0.75–22.5	114.29	17.381	0.999	R.I.
Pyruvate	0.07–1.35	38.545	2.925	0.998	R.I.
Pyruvate	0.07–1.35	259.93	12.435	0.999	U.V.
Acetate	0.025–0.75	24.434	4.796	0.999	U.V.
Succinate	0.025–0.75	26.937	10.152	0.999	U.V.

Table 2. Reproducibility in the determination of glucose, galactose, pyruvate, acetate and succinate in EPS samples. Mean values ± standard deviation ($n = 4$)

Compound	μmol/mg of EPS	
	115SLR[a]	115SLR[b]
Glucose	2.45±0.08	2.18±0.08
Galactose	1.06±0.02	0.90±0.07
Pyruvate	0.86±0.01	0.85±0.10
Acetate	0.98±0.02	1.00±0.13
Succinate	0.12±0.05	0.14±0.05
Ratios:		
Glc:Gal	2.3±0.05	2.4±0.18
Glc:Acetate	2.4±0.02	2.2±0.28
Pyr:Acetate	0.87±0.02	0.85±0.02

[a]EPS from the same batch.
[b]EPS from different batches.

analysis confirmed the presence of glucose, galactose and pyruvate and led to the identification of succinic and acetic acids as side groups. The parameters of the resulting regression lines are shown in Table 1. The five tested compounds presented high correlation coefficients, showing a linear response within the range studied.

The reproducibility of the method was tested by analysis of the products of four separate acid-hydrolysis reactions of the same exopolysaccharide. Results are shown in Table 2. The low standard deviations for all the compounds indicate the reliability of the method for the analysis of the EPS composition.

When samples of exopolysaccharides obtained in different fermentations were analyzed, the standard deviation values were higher than those obtained when the same exopolysaccharide sample was analyzed multiple times (Table 2) even though all fermentations were carried out under the same conditions. Different batches showed small variations in the exopolysaccharide composition, with the highest degree of variability observed in the pyruvate and acetate content. The glucose:galactose ratio in the different samples varied between 2.2 and 2.7 with an average value of 2.4. Since the pyruvate and acetate contents are affected more by slight changes in the fermentation conditions, higher standard deviation values for the glucose:pyruvate and glucose:acetate ratios were observed among the samples. In contrast, the pyruvate:acetate ratio was very similar among all samples.

Structure analysis

Methylation analyses were carried out in order to further study the exopolysaccharide from *Z. ramigera* 115SLR. Methylation of the exopolysaccharides of two different batches via Hakomori's procedure demonstrated the presence of the sugar residues shown in Table 3. The same sugar residues were found by Franzen & Norberg (1984) after Hakomori's methylation of the capsular exopolysaccharide of *Z. ramigera* 115. However, since Hakomori's methylation method takes place under alkaline conditions in which *O*-acyl groups are removed, these sugar residues

Table 3. Partially methylated alditol acetates from the native and peroxidated EPS from *Z. ramigera* 115SLR. Methylation carried out by Hakomori's procedure (Hakomori, 1964)

Compound	t_R[a]	A[b](%)	B[b](%)	C[c](%)
2,3,4,6-tetra-*O*-methylglucose	1.00	23	22	—
2,3,6-tri-*O*-methylglucose	1.33	31	37	6
2,4,6-tri-*O*-methylgalactose	1.36	15	10	33
2,6-di-*O*-methylgalactose	1.58	17	15	28
2,3-di-*O*-methylglucose	1.84	3	3	8
2,4-di-*O*-methylgalactose	2.02	3	7	14
2-*O*-methylglucose	2.25	8	6	11

[a]Retention time relative to 2,3,4,6-tetra-*O*-methylglucose on OV-1 column at 190°C.
[b]A and B are two diffrerent samples of EPS.
[c]Periodate-oxidized exopolysaccharide.

Table 4. Partially methylated alditol acetates from the native and alkali-treated EPS from *Z. ramigera* 115SLR. Methylation carried out by Prehm's method (Prehm, 1980)

Peak No.	Compound	t_R[a]	Alkali-treated (%)	Native (%)
I	2,3,4,6-tetra-*O*-methyl-glucose	1.00	29	20
II	2,3,6-tri-*O*-methyl-glucose	1.33	18	8
III	2,4,6-tri-*O*-methyl-galactose	1.36	14	11
IV	2,3,4-tri-*O*-methyl-glucose	1.41	—	5
V	2,6-di-*O*-methyl-galactose	1.58	10	13
VI	2,3-di-*O*-methylglucose	1.84	8	24
VII	2,4-di-*O*-methyl-galactose	2.02	9	9
VIII	2-*O*-methylglucose	2.25	12	10

[a]Retention time relative to 2,3,4,6-tetra-*O*-methylglucose on OV-1 column at 190°C.

correspond only to the main backbone of the exopolysaccharide containing glucose, galactose and pyruvate.

In order to determine the location of the pyruvate groups, the exopolysaccharide was subjected to mild acid hydrolysis prior to methylation. The analysis of this sample showed the absence of the 2,3-di-*O*-methylglucose and 2-*O*-methylglucose residues found after Hakomori's methylation of the native exopolysaccharide and the presence of a new compound, 2,4,6-tri-*O*-methylglucose. These results are in agreement with those reported by Franzen and Norberg (1984) and indicate that the attachment of the pyruvate in the exopolysaccharide takes place in positions 4 and 6 of a terminal glucose and of a 1,3-substituted glucose.

In order to establish the position of the acetate and succinate groups in the exopolysaccharide, the methylation reactions were carried out by using the neutral procedure described by Prehm (1980). However, since it has been reported that the Prehm method could result in undermethylated products, the exopolysaccharide from 115SLR was also treated with 10 mM KOH and methylated by the Prehm methylation procedure. The results were compared to those found after Prehm's methylation of the native exopolysaccharide. Methylating native exopolysaccharide following the Prehm method gave rise to a more complex chromatogram than was obtained from the alkali-treated sample (Table 4). The main differences were a pronounced increase in 2,3-di-*O*-methylglucose, a decrease in 2,3,6-tri-*O*-methylglucose, the presence of a new compound, 2,3,4-tri-*O*-methylglucose, and a decrease in 2,3,4,6-tetra-*O*-methylglucose. These differences indicate that the side-chain substitutions of *O*-acyl groups in the exopolysaccharide occur mainly on position 6 of a 1,4-substituted glucose and of a terminal glucose, giving rise to an increase of 2,3-di-*O*-methyl- and the appearance of 2,3,4-tri-*O*-methyl-glucose.

Sugar analysis of the periodate oxidized exopolysaccharide showed a glucose:galactose ratio of ~1:3. The sugar residues found after methylation of the periodate-oxidized exopolysaccharide indicated that the periodate oxidation of the vicinal diols in the polymer was complete (Table 3). Treatment with 1 M HCl at 80°C for 1 h and methylation analysis showed that the only new residues after mild-hydrolysis were originated from the pyruvylated glucosyl residues.

CONCLUSIONS

The use of *Z. ramigera* strain 115SLR allowed the isolation of the exopolysaccharide directly from the culture broth by precipitation with isopropyl alcohol. This procedure avoids the use of the alkaline treatment required for the isolation of the EPS from *Z. ramigera* 115 wild-type which can cause the removal of *O*-acyl groups from the exopolysaccharide. The compositional analysis of the EPS from *Z. ramigera* 115SLR showed the presence of glucose, galactose, pyruvate, acetate and succinate. The glucose:galactose ratio was approximately 2:1 in agreement with that reported by Franzen and Norberg (1984), but lower than that of 11:3 reported by Ikeda *et al.* (1982). However, the analysis of the EPS from different batches showed some variations in the sugar and organic acid content.

The structural studies have revealed the presence of the residues shown in Fig. 1. The results obtained after methylation of the native and depyruvylated exopolysaccharide following Hakomori's procedure demonstrate that the attachment of the pyruvate groups takes place in positions 4 and 6 of a terminal glucose and of a 3-substituted glucose. These results, in agreement with those of Franzen and Norberg (1984), also imply the presence of 3-substituted galactosyl residues in the exopolysaccharide but, in disagreement with the results of Ikeda *et al.* (1982), no 3-substituted glucosyl residues others than those where the pyruvate groups are attached were observed.

After methylation of the exopolysaccharide under neutral conditions, the presence of a 2,3,4-tri-*O*-methylglucose and the marked increase of 2,3-di-*O*-methylglucose indicate that *O*-acyl groups are attached to position 6 of a 4-substituted glucosyl residue and of a terminal glucose. Since the acetate content in the exopolysaccharide is similar to that of the pyruvate and markedly higher than the succinate content, the acetate groups are probably attached to both the 4-substituted glucosyl residue and to the terminal glucose, whereas the succinate groups occupy only one position in the exopolysaccharide.

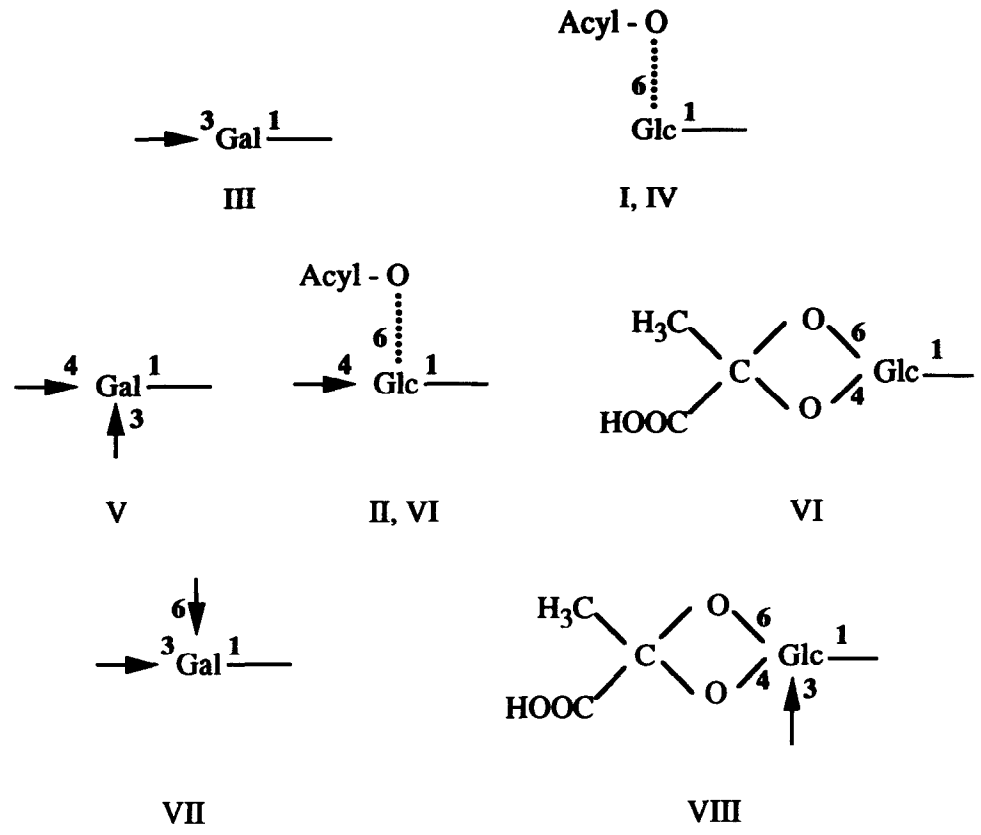

Fig. 1. Structural features demonstrated for the *Z. ramigera* 115SLR exopolysaccharide. The dotted line indicates that only some of the residues carry this substituent. The acetate and succinate substitutions are noted as Acyl-*O*. Roman numerals indicate the peak number of the methyl derivative shown in Table 4.

Periodate oxidation showed that the galactose residues are linked to each other or to a pyruvylated glycosyl residues and cannot be interspaced anywhere by 4-substituted glucoses.

The *Z. ramigera* 115SLR acidic exopolysaccharide has the same sugar and organic acid components as those of succinoglycan from *R. meliloti* (Leigh & Walker). However, the exopolysaccharide from *Z. ramigera* 115SLR has a different glucose:galactose ratio, a higher content of pyruvate and acetate per subunit and two branching residues which indicate a more irregular structure.

Further studies on the structure of this exopolysaccharide are necessary in order to propose the repeating unit of this polymer. The powerful thickening properties of this exopolysaccharide unable the preparation of concentrated solutions suitable for NMR analysis. We are now in the process of screening different microorganisms for the isolation of a depolymerase for this exopolysaccharide.

Since it is likely that changes in the rheological properties of this exopolysaccharide could he accomplished by modifying, either increasing or decreasing, the number of acetyl, succinyl and pyruvyl groups, we are investigating the roles of the acetyl, succinyl and pyruvyl transferases in the synthesis of zooglan.

ACKNOWLEDGEMENTS

This work was supported in part by Nalco Chemical Company. The authors thank Dr Ronald J. Allain and Dr Robert Wetegrove of Nalco Chemical Company for their valuable comments and support. E. Troyano was supported by a Ministerio de Educacion y Ciencia (Spain) post-doctoral fellowship.

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