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Structural characterization and rheological properties of an extracellular glucuronan produced by a *Rhizobium meliloti* M5N1 mutant strain

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ABSTRACT

The mutant strain M5N1 C.S. (NCIMB 40472) of *Rhizobium meliloti* M5N1 is able to produce during fermentation a partially acetylated extracellular (1 → 4)-β-D-glucuronan. At low concentration (1 $g \cdot l^{-1}$), in the presence of monovalent cations, this new glucuronate behaves as a thickening agent, whereas at higher concentration a thermoreversible gel is obtained. With such divalent cations as Ca^{2+}, a thermally stable gel can be formed.

INTRODUCTION

Since several years ago, the production of polysaccharides by fermentation has provided a valid alternative to traditional gums from plants and marine algae. Because of the wide range of polysaccharides thus synthesized having interesting and specific features, opportunities have been opened up for new processes and products. Even though significant progress has been made in the identification, characterization, and commercialization of microbial polysaccharides, only a few of them have been successfully developed, among them, the succinoglycan produced by different strains of *Rhizobium*[1–4]. From bacteria of the Rhizobiaceae family that are known to produce complex mixtures of extracellular polysaccharides (EPS) ranging from simple glycans to elaborate heteropolysaccharides[5,6], we aimed to obtain modified succinoglycans by mutation of a succinoglycan-producing strain[7]. The present paper describes the isolation, structural studies, and rheological properties of a glucuronan obtained by fermentation of a M5N1 mutant strain of *Rhizobium meliloti*.

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EXPERIMENTAL

Mutation by N-*methyl*-N′-*nitro*-N-*nitrosoguanidine (NTG) treatment.*—Exponentially growing cells in TY medium[8] were harvested by centrifugation (8000*g* for 5 min) and suspended in R medium[9] supplemented with D-glucose (1%) and NTG (0.1%). After 60 min of incubation at 30°C, the cells were harvested by centrifugation, washed twice with R medium, spread on R agar medium supplemented with glucose (1%), and incubated at 30°C.

Fermentation.—Among the mutants obtained by NTG treatment, the strain M5N1 C.S. (NCIMB 40472) that produced an extracellular material different from succinoglycan was selected. This strain was cultivated on RC medium[7] supplemented with sucrose (1%). The excretion was controlled according a method previously described[10].

EPS isolation.—After removal of the bacteria by centrifugation and microfiltration (pore size 0.2 μm), the EPS was precipitated from the culture medium with 1.5 vol of 2-propanol after addition of NaCl to bring the solution to 1 M. The precipitate (sodium salt of EPS) was washed several times with increasing concentrations of 2-propanol (75–99%) and dried for 48 h in vacuo at room temperature.

Reduction.—The uronic acids were reduced by action of a water-soluble carbodiimide {*N*-cyclohexyl-*N*′[β(*N*-methylmorpholino)ethyl]carbodiimide-*p*-toluenesulfonate]}, according to a method previously described [11].

Acid hydrolysis.—The reduced EPS (20 mg) was hydrolyzed in 2 mL of 70% H_2SO_4 for 30 min at room temperature. After addition of water (8 mL) the mixture was kept overnight at 100°C, and neutralized with $BaCO_3$ before concentration and filtration. The solution was then analyzed by HPLC.

Enzyme assay.—Reduced EPS (20 mg) was dispersed in water containing cellulase (Celluclast from NOVO) or amylase (alpha amylase from Boehringer). After 48 h of incubation at 50°C, the reaction was stopped (10 min at 100°C) and the solution filtered through Diaflomembrane (0.45 μm) before HPLC analyses.

High-performance liquid chromatography (HPLC).—Separation of neutral mono- and oligo-saccharides was achieved on Waters Associates equipment using a CHO-682 column from Interchim (France) eluted with water at 85°C. The absolute configurations of the sugars were assigned by using a Perkin–Elmer model 241 polarimeter as chromatographic detector[12].

Methylation.—Methylation of the reduced EPS was carried out by the Hakomori method[13]. A complete methylation was performed according to the Purdie method[14], and methylated polysaccharide was recovered by dialysis. After hydrolysis and conversion of the aldoses into partially *O*-methylated alditol acetates, the solution was analyzed by gas chromatography (GLC) and the identification confirmed by GLC–MS.

NMR experiments.—The ^{13}C (75 MHz) and ^{1}H (300 MHz) NMR spectra were obtained with a Bruker AC 300 spectrometer and a solution of sodium glycuronan

(10 mg) in D_2O (0.5 mL) at 85°C. The chemical shifts were referenced to internal acetone (^1H, 2.04 and ^{13}C, 29.8 ppm).

Weight-average molecular weight measurement ($\overline{M_w}$).—$\overline{M_w}$ values were determined by size-exclusion chromatography (SEC) on a modified Waters 150C apparatus equipped with multi-detection (differential refractometer, capillary viscometer, and low-angle laser light scattering: Chromatix CMX-100)[15]. Polysaccharides were chromatographed at 25°C on OHpak Shodex B-804 and B-805 columns (8×500 mm) in series with 0.1 M $NaNO_3$ as mobile phase at a flow rate of 1 $mL \cdot min^{-1}$.

Viscosity measurements.—The viscosities were determined as a function of the shear rate in a low-shear viscometer (Contraves 30) at controlled temperatures.

Elastic modulus measurements.—A 10 $g \cdot L^{-1}$ solution of sodium glucuronan was heated at 60°C and poured into dialysis tubing. After 5 h of immersion in 0.34 M $CaCl_2$ solution at 60°C, the solution was kept overnight at room temperature. The gel formed (diameter 14 mm) was introduced into a cylindrical mould, cut into a small cylinder (height 17 mm) and relaxed in 0.34 M $CaCl_2$ solution. Elastic modulus values were obtained by compression between parallel plates at room temperature with an Instron 4301 instrument.

RESULTS AND DISCUSSION

The *Rhizobium meliloti* M5N1 C.S. strain (NCIMB 40472) cultivated in a 20-L fermentor containing RC medium supplemented with sucrose (1%) excreted a viscous material. After 2 days of fermentation, the production was stopped. From the centrifuged and microfiltered broth, 30 g of white fibrous material was obtained by alcoholic precipitation. The composition, structure, and rheological properties of this product were investigated; it was free of protein (from microanalysis data).

Chemical characterization.—The polysaccharide secreted by the M5N1 C.S. strain (NCIMB 40472) was insoluble in 1 M H_2SO_4 and no hydrolysis occurred after heating for 12 h at 100°C. Only a slow degradation occurred, causing a brown coloration of the solution. Under more-drastic conditions (70% H_2SO_4 for 30 min at room temperature before dilution with water to bring the H_2SO_4 to 1 M) and heating overnight at 100°C, the amount of degradation increased; after neutralization and filtration, no neutral sugars were detected in the hydrolyzate. This behavior is as expected for uronic acid-rich polysaccharides.

Because of its high resistance towards acid hydrolysis, the EPS was reduced by the action of *N*-cyclohexyl-*N'*-[β-methylmorpholino)ethyl]carbodiimide-*p*-toluenesulfonate at pH 4.75 controlled with buffer, and reduction by $NaBH_4$ at pH 7 to prevent alkaline hydrolysis. This procedure was repeated twice to ensure complete reaction. The insoluble material obtained was dialyzed against water and freeze-dried. The reduced EPS was submitted to acid hydrolysis according to the

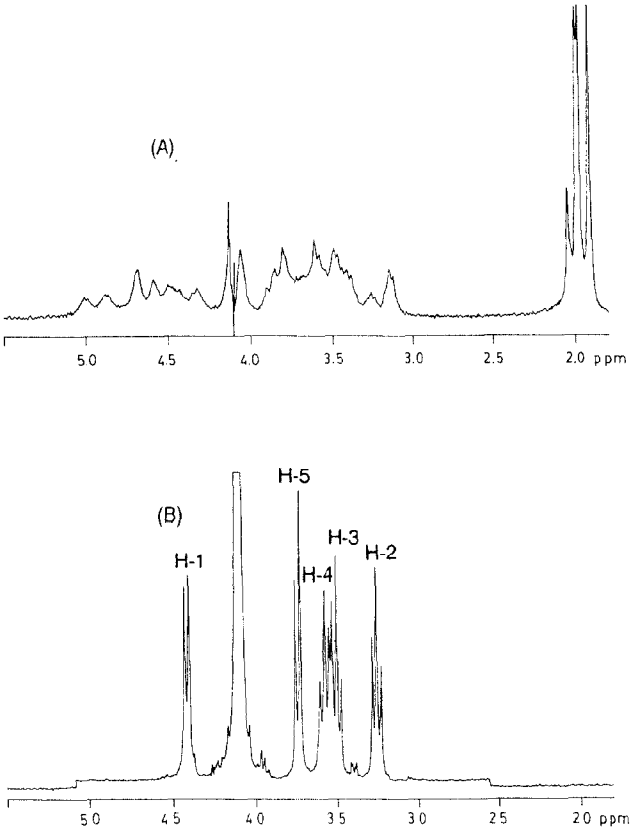

Fig. 1. ^{1}H NMR spectra (300 MHz) of the *Rhizobium meliloti* M5N1 C.S. mutant strain (NCIMB 40472) exopolysaccharide: (a) native, (b) after deacetylation.

procedure described previously. Only glucose was detected after analysis of the hydrolyzate by HPLC.

In order to carry out structural determinations, the lyophilized, reduced polymer was *O*-methylated and then hydrolyzed in CF_3CO_2H. The resulting sugars were reduced and acetylated. GLC analysis revealed the presence of a product having a retention time similar to that of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol; the result was confirmed by GLC–MS analysis.

At this point of the structure analysis, we were able to propose that the new polymer contained either 4-linked glucopyranosyluronic acid residues or 5-linked glucofuranosyluronic acid residues.

Enzymic hydrolysis.—Cellulase and alpha amylase were tested on the reduced EPS. Degradation occurred only with cellulose, to give monosaccharide, disaccharide, and trisaccharide, identified by HPLC with standards as glucose, cellobiose, and cellotriose respectively. These results were confirmed by ^{13}C NMR studies on the purified compounds. From its dextrorotation, the D chirality was concluded for the glucose unit.

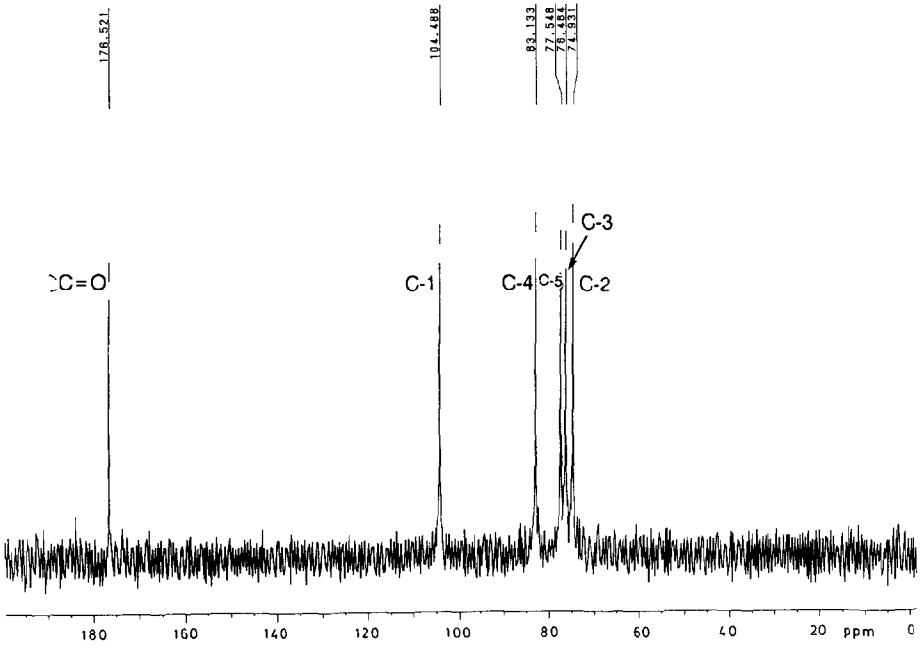

Fig. 2. ^{13}C NMR spectrum of deacetylated β-(1 → 4)-linked glucuronic acid polymer.

NMR experiments.—The ^{1}H NMR spectrum of the native polymer [Fig. 1(A)] shows a complex system in the ring-proton region and signals at 2 ppm characteristic of *O*-acetyl groups. To complete the determination of the structure, the deacetylated polymer was studied (deacetylation was performed by treatment with NaOH (pH > 8) at ambient temperature during at least 2 h. Only five protons of an uronic acid were detected [Fig. 1(B)]. The chemical shift of the anomeric proton (4.41 ppm) with a $J_{1,2}$ coupling constant of 7.7 Hz indicated a β linkage.

In the ^{13}C NMR spectrum obtained with the deacetylated polymer (Fig. 2), the six main peaks detected could be assigned to a β-(1 → 4)-linked uronic acid. Assignments were made by two-dimensional NMR techniques.

The extracellular polysaccharide produced by the M5N1 C.S. strain (NCIMB 40472) of *Rhizobium meliloti* is thus a (1 → 4)-β-D-glucuronan partially acetylated on 2 or/and 3 positions (Fig. 3).

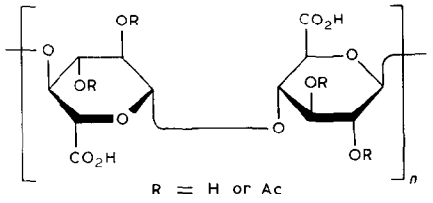

Fig. 3. Schematic representation of the glucuronan secreted by the *Rhizobium meliloti* M5N1 C.S. strain (NCIMB 40472).

This polysaccharide, known as mucoric acid, is present in the cell wall of Mucorales[16–19] and has been described recently as extracellular material of these mould species[20]. However, it is difficult to prepare large amounts of pure polysaccharide from this source and, because of its low molecular weight (30–60 glucuronic acid residues), no interesting rheological properties are to be expected.

Thus, to our knowledge, this is the first time that an extracellular glucuronan has been produced by bacteria belonging to the Rhizobiaceae family, although sequences of β-(1 → 4)-linked glucuronic acid residues have been found in EPS produced by *Rhizobium leguminosarum*[6].

Comparing this new polysaccharide with an "acid cellulose", the properties mentioned by Cesaro et al.[21] for cellulose oxidized at C-6 by $NaNO_2$ could be observed. However, the molecular weight of the oxidized cellulose was low (1.5×10^4 dalton) and it had a generally random distribution of oxidized groups. From their data, the C-6-oxidized cellulose behaves as a typical glycuronoglycan, being soluble as its Na^+ salt but forming a brittle gel at low pH values and insoluble salts with Ca^{2+}, Sr^{2+}, and heavy-metal cations.

Rheological behavior.—Preliminary studies have shown variations in molecular weight and in acetyl content depending on the cultivation conditions. In this study, the analyses refer to an EPS containing 16% of *O*-acetyl groups (w/w), the degree of acetylation being determined by ^1H NMR by comparison of integrals: both the ring and acetate protons.

The sodium salt of the glucuronan was very soluble at room temperature and a 1 $g \cdot L^{-1}$ solution was readily filtered through a 0.45-μm membrane. At pH values lower than 3, the EPS precipitated; this property may be used for extraction of the polymer from broth.

The molecular weight was measured by SEC with 0.1 M $NaNO_3$ as eluent, the results indicate a weight-average molecular weight, $\overline{M_w}$ in the range $6 \times 10^4 < \overline{M_w} < 4 \times 10^5$.

From the data, a viscosity relationship was obtained, $[\eta] = 2 \cdot 10^{-2} \times \overline{M_w}^{0.9}$ with $[\eta]$ the intrinsic viscosity in $mL \cdot g^{-1}$ and $\overline{M_w}$ the molecular weight. Comparable values have been obtained for alginates[22,23].

By increasing the EPS concentration (> 5 $g \cdot L^{-1}$) and/or the ionic strength (> 1 M) with monovalent salts, a thermally reversible gel was formed. The stability and strength of the gels depended on the counterion used and on the degree of acetylation of the polymer. The thermal stability at 100°C of 1 $g \cdot L^{-1}$ polysaccharide solutions was investigated: it was found that the viscosity decreases rapidly through chain degradation with $\overline{M_w}$ decrease and progressive deacetylation. After 2 h at 100°C, the molecular weight was one third that of the native polymer.

As with galacturonan and guluronan, a gel was formed with salts of divalent cations, except with Mg^{2+} ion, these gels were not thermoreversible. The strength of gels formed with a glucuronan containing 16% of *O*-acetyl groups (expressed by the Young modulus, E), was decreased by heating at 100°C in 0.34 M $CaCl_2$ only

TABLE I

Change of Young modulus [a], E, by heating at 100°C a Ca-gel obtained by dialysis of a 10 $g \cdot L^{-1}$ solution of the glucuronan [b] against water alone and 0.34 M $CaCl_2$

Solvent	Time				
	1	2	3	4	24
Water	1.2	1.1	1.1	0.9	[c]
0.34 M aq $CaCl_2$	1.3	1.3	1.3	1.25	1.2

[a] Values give $E \cdot 10^{-4}$ $N \cdot m^{-2}$. [b] $\overline{M_w}$ $2.2 \cdot 10^5$, 16% acetate. [c] Gel destroyed.

over a long period (Table I). In pure water, the gel swelled and was destroyed after 4 h, due to the osmotic pressure.

At the same concentration, the E values are smaller than those of alginates rich in guluronic acid, but an increase in modulus was observed when the degree of acetylation of the polymer decreased. When the degree of acetylation of the polymer is lower than 10%, the (1 → 4)-β-D-glucuronan is comparable to alginate (M/G = 0.28).

CONCLUSION

Mutation of a *R. meliloti* M5N1 strain producing a succinoglycan gave a strain excreting a polysaccharide different in structure and composition. Although a mutant of a succinoglycan-producing strain has been shown to produce a second exopolysaccharide containing a diglycosyl unit[24], this appears to be the first example of a homoglycuronan secreted by bacteria.

The solution properties of this polysaccharide suggest industrial uses where other glycuronans, pectins, alginates, and also gellan and other bacterial polysaccharide are currently employed.

When the biosynthetic pathway for *Rhizobium meliloti* polysaccharide becomes understood, it might be possible to obtain other polysaccharides by specific mutagenesis of strains belonging to the Rhizobiaceae family.

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