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Identification of xanthans isolated from sugarcane juices obtained from scalded plants infected by *Xanthomonas albilineans*

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Abstract

The exudate gum produced by *Xanthomonas albilineans*, a specific sugarcane pathogen, has been isolated from juices of diseased sugarcane stalks, hydrolyzed with hydrochloric acid, and the hydrolysate analyzed by capillary electrophoresis. Sucrose, cellobiose, mannose, glucose, glucose-1-P and glucuronic acid were identified as the major components of the polysaccharide isolated from diseased stalks. Juices from healthy stalks contained maltose instead of cellobiose. The chemical nature of this polysaccharide is discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Xanthomonas albilineans*; Xanthans

1. Introduction

Xanthomonas albilineans, a specific sugarcane phytopathogen, produces a disease defined as leaf scald. The initial characteristic symptom is a white streak ('pencil-line') 1–2 mm wide on the leaf which follows the direction of the main veins. The streaks may later become more enlarged and the affected leaf becomes wilted and necrotic. The white pencil line may also be visible on the leaf sheaths. Symptoms of this phase are seen after ratooning or in young shoots growing from infected plant cane. Later, these symptoms may disappear, although plants remain infected [1]. Alternatively, plants may be infected, but grow without showing any symptoms. Mature stalks may suddenly wilt and die, sometimes without the previous appearance of other symptoms. *X. albilineans* induces closing of xylem vessels in both leaves and stalks by producing a gum which seems to be similar to xanthans [2]. As a consequence, a severe water stress appears as the main symptom of the disease in infected plants. During the first stages of the bacterial infection, sugarcane plants increase the production of glycoproteins containing a heterofructan as glycosidic moiety as a defense mechanism [3]. Thus, bacterial gum and plant heterofructans coexist during this period. The chemical nature of this gum has not yet been studied although another pathogenic species of *Xanthomonas* produces a gum known as xanthan.

Xanthan is an industrially important exopolysaccharide produced by the phytopathogenic, Gram-negative bacterium *Xanthomonas campestris* pv. *campestris*. It is composed of polymerized pentasaccharide repeating units which are assembled by the sequential addition of glucose-1-phosphate, glucose, mannose, glucuronic acid, and mannose on a polyprenol phosphate carrier [4]. The polymerization of

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lipid-linked repeat units occurs by the successive transfer of the growing chain to a new pentasaccharide-P-P-polyprenol. The reaction involves C_1 of glucose at the reducing end of the polyprenol-linked growing chain and C_4 of glucose at the nonreducing position of the newly formed polyprenol-linked pentasaccharide, generating a branched polymer with a trisaccharide side chain. Pyruvic acid acetal residues are transferred from phosphoenolpyruvate to the pentasaccharide-P-P-lipid [5].

In this paper, we attempt to analyze this exudate gum contained in stalks of diseased sugarcane by isolation and fractionation of juices, and acidic hydrolysis of the product according to Honda [6] and Morrison [7]. Hydrolysates were then analyzed by capillary electrophoresis, as selected between very different techniques for sugar separation in GC and HPLC, recently summarized by Legaz et al. [8].

2. Materials and methods

2.1. Biological material

Stalks from 14-month-old plants of *Saccharum officinarum* cv. Louissiana 55-5, healthy or spontaneously infected by *Xanthomonas albilineans*, field-grown, were used throughout this work.

2.2. Chemicals

All chemicals used for the preparation of buffers, sodium phosphate, sodium borate and sodium acetate, as well as other products such as hydrochloric acid, trichloroacetic acid (TCA), acetic acid, potassium chloride, ethanol and isopropyl alcohol were of analytical reagent grade (Merck, Darmstadt, Germany) and were used as received. Water was of Milli-Q grade (Millipore, Bedford, MA, USA). β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21.) was obtained from Sigma (St. Louis, MO, USA). Saccharide standards: D-sucrose, D-maltose, D-cellobiose, D-mannose, D-glucose, D-xylose, D-rhamnose, D-glucuronic acid and D-glucose-1-P were also from Sigma.

2.3. Instrumentation

Zone electrophoresis was performed using a Spectraphoresis 500 system from Spectra-Physics (Fremont, CA, USA). Microbore fused-silica tubing coated with polyimide (Scientific Glass Engineering, Milton Keynes, UK) of 75 μm I.D. and 190 μm O.D. with a total length of 70 cm and a separation length of 63 cm were used. The capillary was enclosed in a cassette for easy handling. On-line detection was performed with a variable-wavelength UV–Vis detector of band width 6 nm (Spectra-Physics). Detection of saccharides was monitored at 200 nm and electropherograms were recorded using a SP 4290 integrator (Spectra-Physics).

2.4. Preparation of sugarcane

Sugarcane stalks were mechanically crushed immediately after being cut and the crude juice was filtered through filter paper. Then, filtered juices were centrifuged at 2800 *g* for 15 min at 2 °C [9]. The pellet was discarded and 50 ml of the supernatant used for xanthan extraction after being lyophylized.

2.5. Extraction of xanthan

Extraction of xanthan was carried out according to the method described by Galindo et al. [10] introducing some modifications. Lyophilized juices was resuspended in 50 ml Milli-Q grade water at 60 °C, being shaken for 30 min and then, centrifuged at 10 000 *g* for 10 min at 2 °C. Supernatant (supernatant 1) was collected and pellet (pellet 1) were re-extracted with 25 ml Milli-Q grade water at 60 °C, shaking for 30 min and then centrifuged at 20 000 *g*. This process was twice repeated obtaining supernatants 2 and 3.

All the supernatants obtained (supernatants 1, 2 and 3) (100 ml) were mixed and precipitated with 100 ml of isopropyl alcohol containing 3% (w/v) of KCl in shaking and then the mixture was maintained at 4 °C for 2 h, being after this centrifuged at 14 000 *g* for 20 min at 2 °C. Supernatant was discarded and the pellet, containing precipitated xanthans was

dissolved in 10 ml of 10 m*M* sodium phosphate buffer, pH 6.8, and stored until used.

2.6. Preparation of xanthan-precipitated polysaccharides

Samples of 5.0 ml of xanthan preparation were filtered through a 15-cm×25-cm I.D column of Sephadex G-10 (Omnifit System) pre-equilibrated with 10 m*M* sodium phosphate buffer, pH 6.8.

The first 24×1.0-ml fractions of eluate were discarded. Fractions 25–41 were collected 8 ml of those being loaded onto a Sephadex G-50 column (30 cm×2.5 cm I.D), pre-equilibrated as above. Fractions 54–160 ml containing high-molecular-mass and mid-molecular-mass polysaccharides were monitored for the presence of carbohydrates by the method of Dubois et al. [11].

2.7. Acidic hydrolysis and sugar extraction

Xanthan precipitate and main fractions obtained from Sephadex G-50 filtration containing carbohydrates were hydrolyzed with 6 N HCl at 80 °C overnight and then were carried out to dryness under reduced pressure. Each residue was then ground with 3 ml of cold 80% (v/v) ethanol, stored for 2 h at 2 °C, and then, centrifuged at 19 000 *g* for 15 min at 2 °C. After evaporation to dryness, xanthan precipitates and main carbohydrate fractions were dissolved, respectively, in 700 and 400 μl 10 m*M* sodium borate buffer, pH 9.2, and used for CE analysis.

2.8. Enzymic hydrolysis of cellobiose

Cellobiose was enzymatically hydrolysed in reaction mixtures consisting of 200 μl of a substrate solution containing 0.2 mg of β-glucosidase in 0.5 *M* sodium acetate buffer, pH 4.5, and 200 μl of xanthan precipitate. The reaction mixture was incubated at 30 °C for 60 min and the reaction was stopped by adding 200 μl 7% (w/v) TCA. Protein was precipitated and the reaction mixtures centrifuged at 2000 *g* for 10 min at 2 °C. Supernatants were dried under reduced pressure, and the residues resuspended in 200 μl of 10 m*M* sodium borate buffer, pH 9.2, for CE analysis.

2.9. Capillary electrophoresis

A capillary just purchased was conditioned with 1 *M* NaOH for 10 min at 60 °C, 0.1 *M* NaOH for 10 min at 60 °C and Milli-Q grade water for 10 min at 60 °C. Equilibration of the capillary was then performed by washing with 25 m*M* sodium borate buffer, pH 9.2, for 30 min at 25 °C and finally with the same buffer for 30 min at 25 °C under applied voltage of 15 kV. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence: 0.1 *M* NaOH for 5 min, Milli-Q grade water for 5 min and 25 m*M* sodium borate buffer, pH 9.2, for 15 min. The buffer used as electrolyte was 25 m*M* sodium borate buffer, pH 9.2 [12].

Standard saccharides as well as sample solutions were prepared in 10 m*M* sodium borate buffer, pH 9.2. Voltage was applied in such manner that ions migrated from the anode to the cathode. Quantitation of monosaccharides in the hydrolysates was performed by interpolating area counts in the corresponding straight lines constructed with increasing concentrations of the corresponding standards.

3. Results and discussion

The product extracted with isopropanol–KCl treatment of lyophilized juices was analyzed by capillary electrophoresis. As is shown in Fig. 1, a mean peak at 9.20 min, that represented about 38% of the total peaks in terms of area counts, was obtained from juices of healthy plants. In addition, sucrose (9.67 min), maltose or cellobiose (10.25 min), rhamnose (11.90 min), mannose (12.96 min), glucose (16.99 min), xylose (17.31 min), traces of galactitol (17.78 min), and glucose-1-P (19.43 min), used as glucose donor during xanthan production, were revealed. The occurrence of sucrose could be explained as remaining after isopropanol extraction as part of the bulk of this disaccharide, the main component of sugarcane juices, but this is not probable, since sucrose would easily be hydrolyzed by the acid. More probably

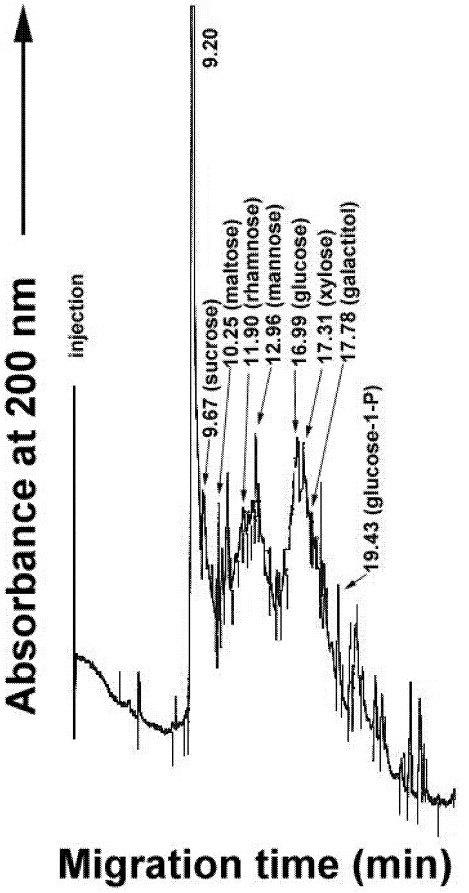

Fig. 1. Electropherogram of sugars released from total fraction extracted with isopropanol–KCl from sugarcane juices obtained from healthy stalks. Number near the peak shows the migration time value of the substance in HPCE.

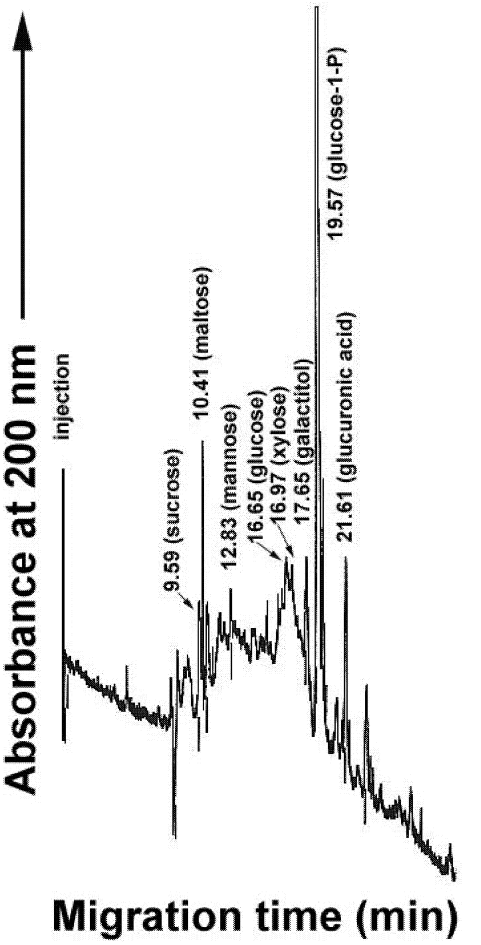

Fig. 2. Electropherogram of sugars released from total fraction extracted with isopropanol–KCl from sugarcane juices obtained from scalded stalks. Number near the peak shows the migration time value of the substance in HPCE.

sucrose could be removed from sugarcane heterofructans by incomplete acidic hydrolysis of the polysaccharide. The occurrence of galactitol in the hydrolysate could indicate that the mixture obtained after isopropanol extraction was contaminated with some of these heterofructans, composed of fructose and galactitol as previously described [13], produced by sugarcane stalks themselves, but the absence of free fructose discarded this possibility.

When the product obtained from diseased stalks was analyzed by CE, almost the same constituents were found (Fig. 2), e.g., sucrose (9.59 min), maltose or cellobiose (10.41 min), mannose (12.83 min), glucose (16.65 min), xylose (16.97 min), galactitol (17.65 min) and glucose-1-P (19.57 min), lacking rhamnose. However, the peak corresponding to an unknown substance, with migration time value of 9.20 min, only represented 5.3% of the total constituents of the mixture in terms of area counts. Moreover, the acidic hydrolysis of these samples revealed the presence of valuable amounts of glucuronic acid (21.61 min), which did not occur in samples obtained from healthy specimens. The mannose–glucuronic acid ratio, in area counts, was 3.63 ($>$1.0). Although the occurrence of glucuronic acid and mannose in the hydrolysate could indicate that one of the components of the original mixture could be a xanthan, or a xanthan-like polysaccharide [14],

Table 1
Quantitative composition of the total polysaccharide extracted with isopropanol from juices obtained from healthy and scalded sugarcane stalks

Monosaccharide (nmol in 36 nL of injected volume[a])	cv. Louissiana 55-5	
	Healthy	Scalded
Sucrose	61.0±5.8	9.8±1.1
Maltose (Cellobiose)	25.0±2.3	4.5±0.3
Rhamnose	3.6±0.3	–
Xylose	7.2±0.6	12.0±1.1
Mannose	–	2.0±0.3
Glucose	4.7±0.5	1.2±0.2
Glucose-1-P	–	167.0±15.4
Glucuronic acid	–	0.55±0.04
Galactitol	1.1±0.1	0.69±0.05

[a] Values are the mean of three replicates±standard error.

the ratio mannose to the uronic acid was very high for this purpose. Moreover, glucose–mannose and glucose–glucuronic acid ratios (Table 1) were 0.6 and 2.5, respectively, the first one very different to that expected for a true xanthan, in which the value of both ratios was always 3 [15]. This could be probably due to the occurrence of contaminating polysaccharides containing mannose extracted with the exudate gum from pathogenic bacteria.

To avoid the suspected contaminations by sugarcane polysaccharides from non-bacterial origin, both extracts were separated on a Sephadex G-50 column (30 cm×2.5 cm I.D.) and fractions eluted from 60 to 85 ml were collected (Fig. 3), hydrolyzed with 6 N HCl as above, and analyzed by CE. These fractions

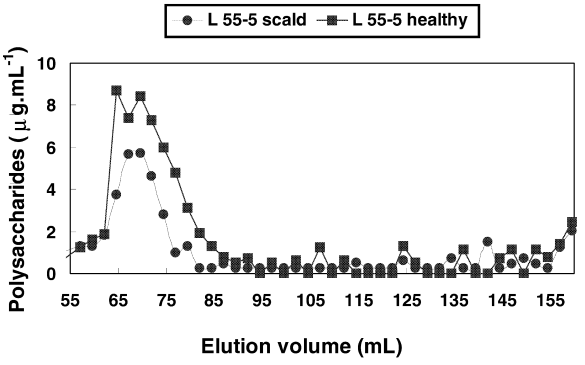

Fig. 3. Elution profile through a Sephadex G-50 column of total polysaccharides extracted with isopropanol–KCl from juices of healthy (■) or scalded (●) stalks of sugarcane. Sucrose, the main component of juices, eluted from 155 mL.

(60–85 ml) eluted from Sephadex G-50 at the same elution volume of high-molecular-mass heterofructans, previously described [16], isolated from sugarcane juices that have not been subjected to a previous extraction with isopropanol.

Polysaccharides isolated from healthy stalks and hydrolyzed with HCl were resolved in several peaks corresponding to sucrose (9.64 min), maltose or cellobiose (10.17 min), glucose (16.83 min) and glucose-1-P (19.80 min), as shown in Fig. 4. The main peak, with a migration time of 8.74 min, has not been identified, probably consisting of an oligosaccharide, produced by the incomplete hydrolysis of the main polysaccharide. Another possibility consisted on the spontaneous production of a cross-

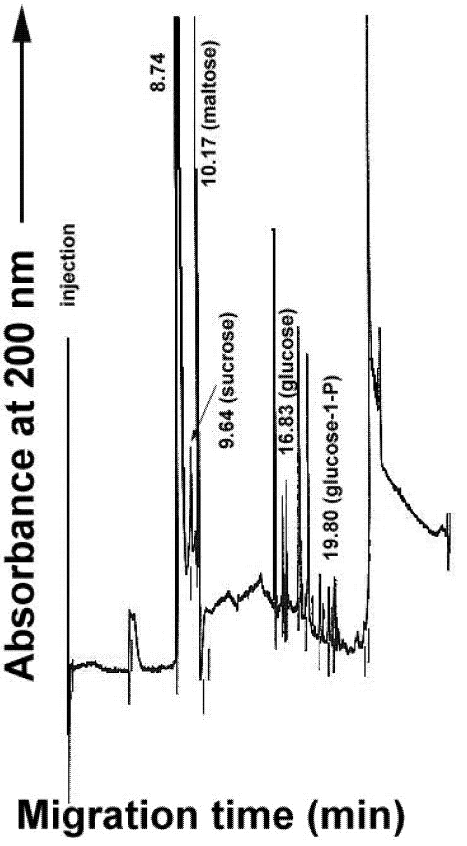

Fig. 4. Electropherogram of sugars released from polysaccharide fraction eluted from Sephadex G-50 after filtration of the extract obtained with isopropanol–KCl from juices of healthy sugarcane stalks. Number near the peak shows the migration time value of the substance in HPCE.

linked oligosaccharide from xanthan gum as a consequence of the borate treatment at pH values above 8.0 [17]. A peak with a migration time value of 10.17 min, could be maltose or cellobiose, since both disaccharides migrated at the same time in CE, in the described analytical conditions. Minor peaks of monosaccharides found in in complete fraction (Fig. 2), such as rhamnose, xylose, mannose and galactitol, completely disappeared after filtration of the sample through Sephadex G-50.

Analysis of the collected fractions obtained from diseased stalks revealed a main peak at 8.79, similar to that described for healthy juice, as well as those of sucrose (9.73 min), maltose or cellobiose (10.33 min), mannose (11.30 min), glucose-1-P (20.32 min) and glucuronic acid (21.94 min), as shown in Fig. 5. A Peak at 19.16 min has not yet been identified. The occurrence of both mannose and glucuronic acid could be considered as indicative of the existence of a xanthan-like polysaccharide in juices obtained from diseased sugarcane stalks but glucose was lacking from the hydrolysate to consider this hypothesis as true. However, the peak interpreted as maltose (4-*O*-α-D-glucopyranosyl-D-glucose) or cellobiose (β-D-glucosyl-[1→4]-D-glucose) in the electropherogram shown in Fig. 4, could be considered as the source of glucose needed to define this polysaccharide as a xanthan. As described in the literature, incomplete acidic hydrolysis of xanthan produced large amount of cellobiose [18], but unfortunately standards of maltose and cellobiose have identical values of migration time in CE.

To resolve this question, collected fractions from Sephadex G-50, corresponding to juices from healthy or diseased stalks, were incubated at pH 4.5 for 1 h at 30 °C with 0.1 mg β-glucosidase in a final volume of 200 μl. At the end of this period, protein was denatured by addition of 200 μl 7% (w/v) TCA and removed by centrifugation at 20 600 *g* for 15 min at 2 °C. The supernatant was dried in air flow, redissolved in 200 μl 10 m*M* borate buffer, pH 9.1, and analyzed by CE. Since monosaccharides form relatively stable, anionic complexes with borate in alkaline solution [19], which is decisively reflected by their electrophoretic mobilities [20], the alkalinization of dry residues after enzymatic hydrolysis was absolutely required. The peak with a migration time value of 10.50 min, obtained from extracts from

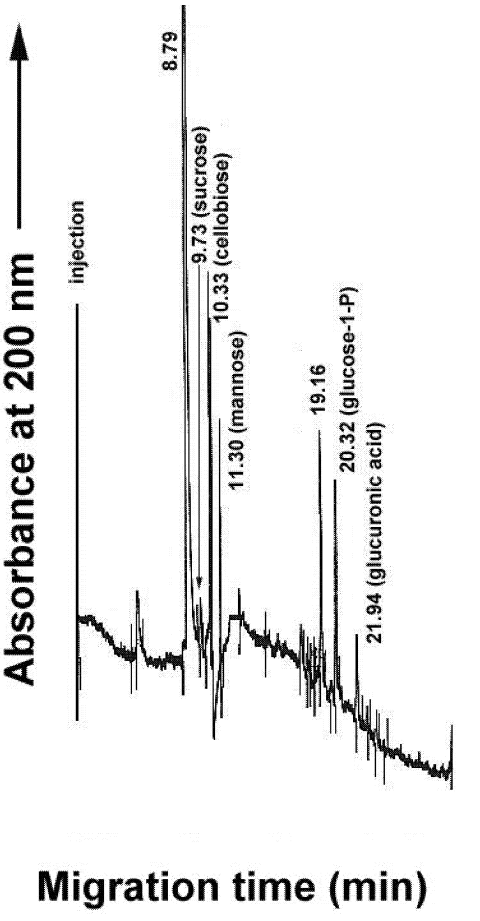

Fig. 5. Electropherogram of sugars released from polysaccharide fraction eluted from Sephadex G-50 after filtration of the extract obtained with isopropanol–KCl from juices of scalded sugarcane stalks. Number near the peak shows the migration time value of the substance in HPCE.

healthy stalks and filtered through Sephadex G-20 prior to acidic hydrolysis, remained practically unchanged after incubation with cellobiohydrolase, whereas it completely disappeared from extracts obtained from diseased stalks, being recovered as free glucose (Fig. 5). The ratio of free glucose and cellobiose to mannose or glucuronic acid (Table 2) was calculated as 2.1 and 2.04, respectively,the first of those more similar to 3, the value of these ratios for true xanthans. The hypothesis that the excess of mannose in the hydrolysate of the product of isopropanol extraction could be explained as a mannan contamination (Table 1) was then confirmed. As a

Table 2
Quantitative composition of the total polysaccharide obtained after filtering isopropanol-extracted fractions from juices prepared from healthy and scalded sugarcane stalks through a column of Sephadex G-50

	Healthy	Scalded
Sucrose	54.0±5.1	2.3±0.2
Maltose	37.0±4.1	–
Cellobiose	–	1.4±0.1
Mannose	–	1.8±0.2
Glucose	0.88±0.1	1.7±0.2
Glucose-1-P	–	14.0±1.6
Glucuronic acid	–	2.2±0.2

Values are the mean of three replicates±standard error.

conclusion, the gum produced and exudated by *X. albilineans* could be defined as a xanthan-like polysaccharide on the basis of its monosaccharide composition.

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