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Novel succinylated and large-sized osmoregulated periplasmic glucans of *Pseudomonas syringae* pv. syringae

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ABSTRACT

Osmoregulated periplasmic glucans (OPGs) are intrinsic components of the Gram-negative bacterial envelope and are important for bacterial–host interactions. The OPGs of *Pseudomonas syringae* pv. syringae have been known to be highly branched linear glucans ranging from 6 to 13 glucose residues devoid of any substituents, while having backbone structure similar to those of *Escherichia coli* and *Erwinia chrysanthemi*. Here, we report for the first time succinylated and large-sized OPGs from *P. syringae* pv. syringae. The glucans were isolated with trichloroacetic acid treatment and various chromatographic techniques. These were further characterized by thin-layer chromatography, matrix-assisted laser desorption/ionization time of flight mass spectrometer, and 1D ^{1}H nuclear magnetic resonance spectroscopy. The results demonstrate that novel anionic glucans with one succinyl residue at the C-6 position of the glucose unit as well as neutral glucans including large-sized glucans with up to 28 degrees of polymerization are produced in *P. syringae* pv. syringae. Furthermore, the succinylated and large-sized OPGs of *P. syringae* pv. syringae are necessary for hypoosmotic adaptation.

1. Introduction

A plant pathogen, *Pseudomonas syringae* can infect a wide range of plant species, and exists as over 50 different pathovars.[1] Among these pathogens, *P. syringae* pv. syringae is the causal agent of brown spot disease of *Phaseolus vulgaris* L., the common bean. Controlling the pathogenicity of *P. syringae* pv. syringae appeared to be related to *opgGH* genes necessary for the synthesis of osmoregulated periplasmic glucans (OPGs).[2,3]

OPGs are found in the periplasmic space of Gram-negative bacteria and function as regulators to low osmolarity. Generally, OPGs are classified into four families on the basis of the polyglucose backbone.[4] In family I, OPGs are linear glucans containing 5–13 glucose residues. The backbone consisting of β-(1→2)-linked glucose units is branched by β-(1→6) linkages. In family II, OPGs are composed of a β-(1→2)-linked cyclic glucan backbone containing 17–25 glucose residues. Family III consists of β-(1→6) and β-(1→3) cyclic glucans containing 10–13 glucose units. In family IV, the backbone structure of OPGs consists of a β-(1→2) cyclic glucan containing one α-(1→6) linkage.

OPGs can be modified by non-glucose residues. However, the correlation between the structure of the glucan and the nature of the substituents remains unclear. Recently, various derivatized OPGs such as succinylated cyclic β-(1→2) glucans from *Brucella abortus*,[5] glycerophosphorylated α-cyclosophorohexadecaoses from *Xanthomonas campestris*,[6] and acetylated α-cyclosophorotridecaoses of *Ralstonia solanacearum* have been reported.[7] Prior to these reports,[5–7] most early studies reported that these three OPGs are only neutral molecules without any substituents.[1,8–10] In the present study, we report for the first time that *P. syringae* pv. syringae, in which neutral OPGs alone were previously thought to be synthesized, produces one succinylated anionic OPGs.

The length of OPGs is under strict control and recent studies on proteins related to the synthesis of OPGs have revealed the size controlling activity of those proteins.[11,12] As a family I OPGs member, *Escherichia coli* produces OPGs composed of 5–12 glucose units. However, the distribution of the various glucose backbones is strictly conserved by the *mdoD* gene.[12] Similarly, cyclic β-(1→2)-glucans, family II OPGs members, have 17–25 glucose residues under strict control of a β-(1→2)-glucan phosphorylase.[11] Herein, we first demonstrate that OPGs of *P. syringae* pv. syringae, a member of family I, have a much higher degree of polymerization (DP), ranging from 6 to 28, compared with the reported DP 6–13.[13] Furthermore, no strict size control was observed, despite that, like *E. coli*, they have a *mdoD* gene.

2. Results and discussion

2.1. Osmoregulation of neutral and anionic OPGs produced in *P. syringae* pv. syringae

Cell associated glucans in the periplasmic space were isolated from *P. syringae* pv. syringae. As the bacteria were grown in the

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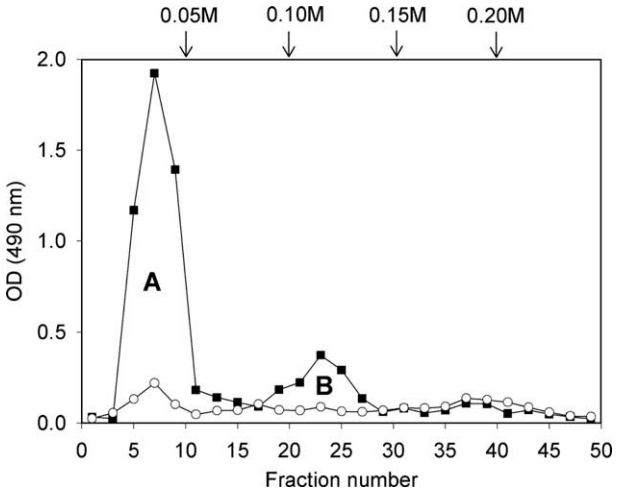

Figure 1. DEAE-Sephadex anion-exchange column chromatography profiles of OPGs from 1 L cultivation of *P. syringae* pv. syringae in LO medium (■) or LO medium with 0.3 M NaCl (○). Peaks A and B reflect the neutral OPGs and anionic OPGs, respectively. Ionic strength was increased by steps of 0.05 M NaCl at the fractions indicated by the arrows. The fractions (5 mL) were collected at a flow rate of 1 mL/min. Total carbohydrate concentrations were determined by a phenol/sulfuric acid procedure as described in Experimental.

low osmolarity (LO) medium supplemented with 0.1 M, 0.2 M, and 0.3 M NaCl, the amount of putative neutral glucan fraction eluted through the Bio-Gel P-4 column was strikingly reduced (data not shown) as the concentrations of NaCl were increased. This correlation was also confirmed through DEAE Sephadex anion-exchange column chromatographic analysis (Fig. 1). With no salt addition to the LO medium, fractionation allowed the separation of two main sugar-containing compounds. In Figure 1, the first peak (A) reflects neutral glucans and the second peak (B) represents anionic glucans. The ratio of peak A to peak B is approximately 5–1. With the addition of 0.3 M NaCl to the LO medium, peak A was sharply reduced and peak B disappeared. These results indicated that anionic glucans as well as neutral glucans produced by *P. syringae* pv. syringae were osmoregulated. The amount of neutral glucans in the LO medium was approximately nine times larger than that in the medium with 0.3 M NaCl added, while anionic glucans were synthesized only in the LO medium. These chromatographic analyses demonstrate that anionic and neutral periplasmic glucans are necessary for hypoosmotic adaptation in *P. syringae* pv. syringae. The exact molecular mechanism accounting for the difference in osmotic regulation between neutral and anionic OPGs is unknown. It has been reported that anionic substituents of OPGs further enhance their osmotic effect through the binding of counterions and contribute to the Donnan potential across the outer membrane.[14,15]

2.2. Structural analysis of succinylated and neutral OPGs by nuclear magnetic resonance (NMR) spectroscopy

The anionic and neutral OPGs of *P. syringae* pv. syringae were analyzed by a ^{1}H NMR spectroscopic analysis (Fig. 2). Glycosidic linkages, determined by methylation analysis, were previously reported for OPGs of *P. syringae* pv. syringae, where 3,4-di-*O*-methyl glucose, 3,4,6-tri-*O*-methyl glucose, and 2,3,4,6-tetra-*O*-methyl

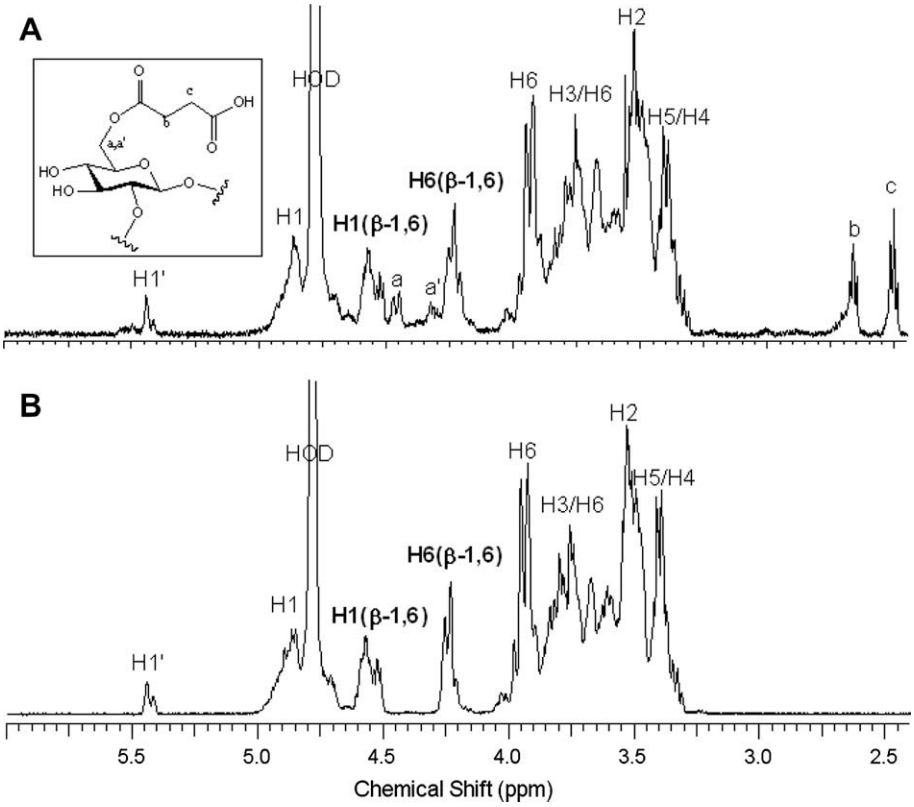

Figure 2. ^{1}H NMR spectra of succinylated OPGs (A) and neutral OPGs (B) isolated from *P. syringae* pv. syringae. In panel A, the inset shows a proposed structure of one succinylated OPGs, and a, a′, b, and c indicate the protons directly linked at carbons. Peaks ranging from 4.30 to 4.50 ppm indicated as a and a′, are attributed to H-6 and H-6′, respectively, of a glucose residue having a succinate linked at C-6. Two triplets between 2.45 and 2.75 ppm, denoted as b and c, correspond to the methylene protons of the succinate. Protons by the presence of β-(1→6)-linked and β-(1→2) linked glucoses, respectively, are also shown, and are designated in bold and ordinary type. HOD means partially deuterated water.

glucose were in a 1:1:1 molar ratio, indicating that the OPGs are highly branched at C-2 and C-6 of the glucose unit.[13] The OPGs have a β-(1→6) branched β-(1→2) linkage backbone structure, which was confirmed by the ^{1}H NMR analysis in this study. The ^{1}H NMR spectra show proton peaks indicating the presence of β-(1→6) linkages as well as β-(1→2) linkages for the glycosidic bond of the glucan. Figure 2A represents anionic OPGs, and two characteristic triplets identified as methylene protons of succinate are present between 2.45 and 2.75 ppm. The peaks ranging from 4.30 to 4.50 ppm are attributed to H-6 and H-6′ of glucose residues having a succinate linked at C-6 via an ester bond,[16] and the remaining signals are the same for the anionic and neutral glucans. The signals around 4.50–4.60 ppm and those between 4.20 and 4.30 ppm are assigned to H-1 and H-6 in the β-(1→6) branch linkages, respectively. The peak of H-1 in the β-(1→2) linkages is present near the HOD peak and the signals between 5.40 and 5.45 ppm, designated as H1′, are indicative of the H-1 of reducing glucose residues with a α-anomeric configuration. Other proton peaks from H-2 to H-6 and H-6′ in the β-(1→2) linked glucose units are designated as shown in Figure 2.

2.3. Structural analysis of succinylated and neutral OPGs by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)

The anionic and neutral OPGs of *P. syringae* pv. syringae were subjected to a MALDI-TOF MS analysis. Molecular ions at *m/z* 1292, 1454, 1616, 1778, 1940, 2102, 2264, 2426, 2588, 2750, and 2912 are present (Fig. 3A), indicating a [one succinylated OPGs+K^++H^+] containing DP 7–17. The mass spectrum shown in Figure 3B represents neutral OPGs and there are 18 potassium-cationized molecular ions [neutral OPGs+K^++H^+]. These molecular ions at 1030, 1192 1354, 1516, 1678, 1840, 2002, 2164, 2326, 2488, 2650, 2812, 2974, 3136, 3298, 3460, 3622, and 3784 are identical to those of linear glucans containing 6–23 glucose residues. The mass spectrum of anionic OPGs in Figure 3A shows a mass difference of 100, corresponding to the *O*-ester-linked succinyl residue, compared with that of neutral OPGs (Fig. 3B). Also, sodium-cationized molecular ions ([M+Na^++H^+]) were confirmed, with *m/z* increased by 16 below the masses of the corresponding potassium adduct ions ([M+K^++H^+]). These results indicate that *P. syringae* pv. syringae synthesizes anionic OPGs substituted by one succinyl residue and large-sized neutral OPGs containing more than DP 13, differently from known DP 6–13.[13]

2.4. DP distribution of neutral glucans by Thin-layer chromatography (TLC) and MALDI-TOF MS

The size distribution of neutral OPGs from *P. syringae* pv. syringae grown for one day could be confirmed through a TLC analysis (Fig. 4A). The lowest R_f value, close to 0.03 in fraction number 6, is attributed to the high DP OPGs (Fig. 4B) and the DP varied from 18 to 28. The R_f value of about 0.15 in fraction number 10 indicates DP ranging from 11 to 18 and the highest R_f value, approximately

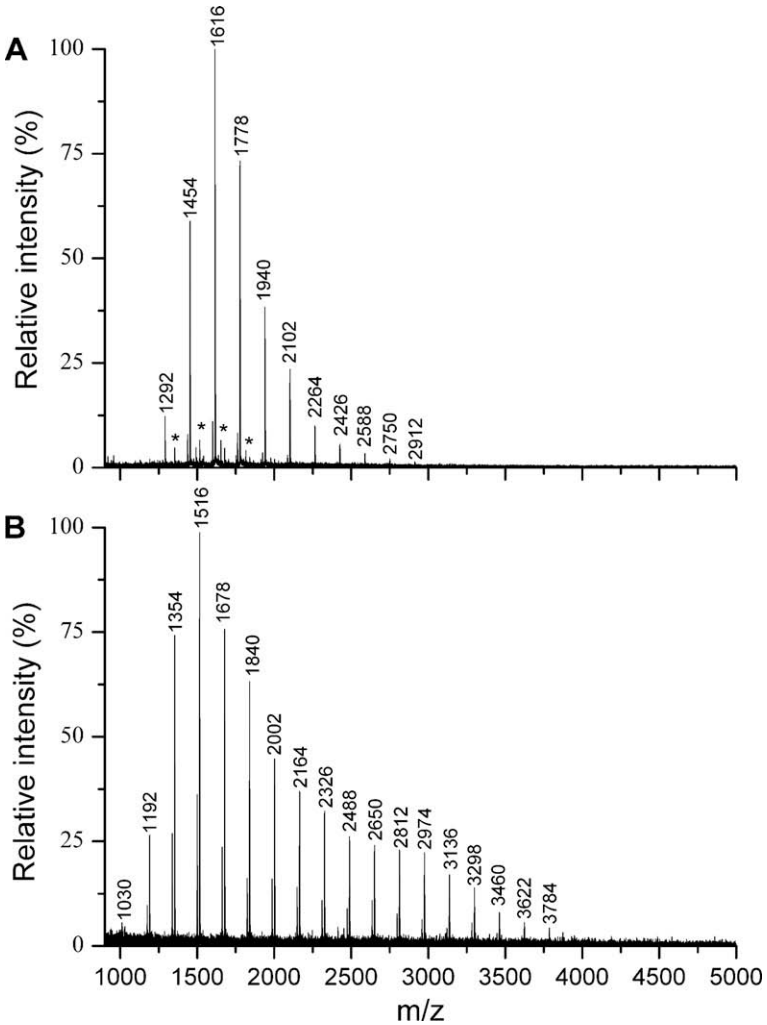

Figure 3. MALDI-TOF MS spectra of succinylated OPGs (A) and neutral OPGs (B) isolated from *P. syringae* pv. syringae. ∗: neutral OPGs, impurity.

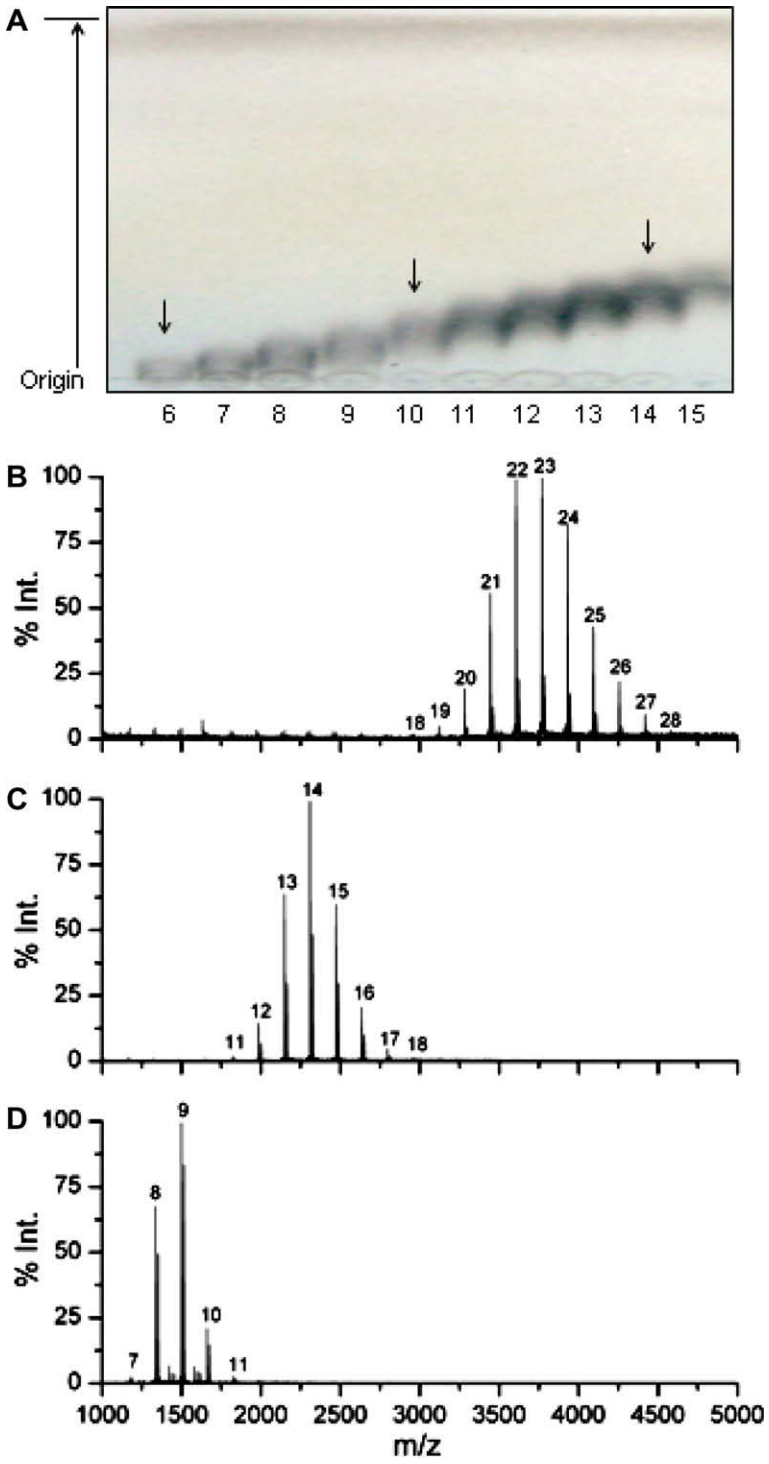

Figure 4. TLC analysis of purified neutral glucans fractionized by Bio-Gel P-4 column. (A) Three arrows indicate spots of fraction number 6, 10, and 14, respectively. MALDI-TOF MS spectra of fraction number 6 (B), 10 (C) and 14 (D) in TLC plate shown in (A). Numbers indicate DP of OPGs.

0.24 at fraction number 14 corresponds to the DP 7–11 of OPGs (Fig. 4C and D). The TLC and MALDI-TOF MS spectrum pattern of OPGs of *P. syringae* pv. syringae clearly indicates that the size is not controlled to DP 6–13.

As a family I OPGs member, the OPGs of *E. coli* are highly substituted with *sn*-1-phosphoglycerol, phosphoethanolamine, and succinyl ester residues,[17,18] while those of *E. chrisanthemi* are mildly substituted by *O*-acetyl and *O*-succinyl ester residues.[8] Hitherto, *P. syringae* pv. syringae OPGs in the same family are reported to have a neutral character with DP from 6 to 13. However, based on the results of the present study, this should be revised as a complicated character (anionic and neutral OPGs) with a much larger-sized DP. In particular, anionic OPGs substituted by one succinyl residue were induced at low osmolarity in *P. syringae* pv. syringae. Meanwhile, it was reported that *E. chrisanthemi* produced the succinylated OPGs at high osmolarity.[8] Recently, in *P. aeruginosa*, which

shares the same genus with *P. syringae* pv. syringae, linear OPGs containing a succinyl residue as a substituent were reported to be produced.[2]

Succinyl substituents in the anionic glucans are frequently found in *E. coli* and *E. chrisanthemi* as family I OPGs members,[8,18] *S. meliloti* and *B. abortus* as family II,[5,19] and *R. sphaeroides* as family IV.[9] The MdoC protein is known to be required to modify the glucans of *E. coli* with succinyl residues, while there have been no reports of proteins in *E. chrisanthemi* which add a succinyl residue to glucans. It is also known that MdoC and OpgC are two functional homologues that do not share significant amino acid sequence similarity. OpgC succinyltransferase,[20] which is responsible for succinylation of OPGs in *R. sphaeroides* and *B. abortus* have a gene, *cgm*,[5] which consists of an open reading frame coding for a predicted membrane protein of 393 amino acid residues 39% identical to *R. sphaeroides* succinyltransferase. As we identified succinylated OPGs of *P. syringae* pv. syringae, through a BLAST database search, a sequence 33% identical to *R. sphaeroides* glucan succinyltransferase (OpgC) was analyzed. This is predicted to be a membrane protein containing 376 amino acid residues (about 42 kDa). Also, as previously postulated,[4] the origin of succinyl residues might be succinyl-CoA through the membrane.

P. syringae pv. syringae has genes sharing homology with a locus of *E. coli* in synthesizing OPGs,[2,3] and thus it has *opgGH* (*mdoGH*) and *opgD* (*mdoD*) as well. In *E. coli*, *mdoD* controls the size of OPGs.[12] In addition, the distribution of DP is always strictly conserved, despite length heterogeneity (DP 5–12), and bacteria having *opgD* mutation synthesize higher DP OPGs. Unexpectedly, our results show that a conserved size distribution was not found in *P. syringae* pv. syringae. Through the present study, we found that novel succinylated and larger-sized osmoregulated periplasmic glucans are synthesized in *P. syringae* pv. syringae. Further investigations of other substituted or different-sized OPGs produced under different growth conditions of various bacterial species in nature are in progress.

3. Experimental

3.1. Bacterial cultures and conditions

P. syringae pv. syringae (ATCC 19310) was grown on a rotary shaker at 26 °C in LO medium (4 g of casein hydrolysate, 0.5 mg of $FeSO_4$, 2 mg of thiamin, 18 mg of $MgCl_2$, 200 mg of $(NH_4)_2SO_4$, and 175 mg of K_2HPO_4 per liter (pH 7.2)). To obtain a high osmolarity medium, NaCl was added to the LO medium at concentrations of up to 0.3 M.

3.2. Isolation and purification of neutral and anionic OPGs

Microorganisms were collected during the stationary phase (2 days) by centrifugation at 8000 rpm at 4 °C for 10 min. The cell pellets were extracted with 5% trichloroacetic acid,[8] and after centrifugation, the supernatant was neutralized with NH_4OH and desalted on a Sephadex G-25 column. The fractions containing the oligosaccharides were pooled by a colorimetric procedure using the phenol/sulfuric acid reagent and concentrated by rotary evaporation.[21] To separate the neutral and anionic glucans, the sample was applied to a DEAE-Sephadex column (2.5 × 17.5 cm), which was first eluted with 10 mM phosphate buffer (pH 8.0), and then with a linear gradient from 0 to 0.2 M KCl in the same buffer. Neutral and anionic fractions were, respectively collected and desalted on a Bio-Gel P-4 column (Bio-Rad). The column (2.5 × 52 cm) was eluted at room temperature with distilled water at a flow rate of 25 mL/h and then the desalted material was finally lyophilized.

3.3. TLC of OPGs

Silica Gel G-60 (E. Merck, 400–240 mesh) TLC plates were spotted with the analytes and developed under the solvent systems (5:5:4 butanol–ethanol–water) as described.[18] Glucans were detected by spraying dried plates with 5% sulfuric acid ethanol followed by heating at 120 °C for 10 min.

3.4. NMR spectroscopy

For NMR spectroscopic analysis,[7] a Bruker Avance 500 spectrometry was used to record ^1H NMR spectra. NMR spectroscopic analyses were done in D_2O at room temperature.

3.5. MALDI-TOF MS

The mass spectra of the oligosaccharides were obtained with a MALDI-TOF mass spectrometer (Voyager-DE™ STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix for carbohydrate analysis.

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