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Structural studies of the acidic exopolysaccharide produced by a mucoid strain of *Burkholderia cepacia*, isolated from cystic fibrosis

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Abstract

The acidic exopolysaccharide produced by a mucoid strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient, was purified by cetyltrimethylammonium bromide precipitation and/or anion-exchange chromatography. Based on the sugar composition and permethylation analyses, supported by GLC–MS and NMR spectroscopy analyses, the repeating-unit of the polysaccharide was established as → 3)-β-D-Glc*p*-(1 → 3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1 → . © 1996 Elsevier Science Ltd.

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1. Introduction

Burkholderia cepacia, previously known as *Pseudomonas cepacia*, is the type-species of the new genus Burkholderia described by Yabuuchi et al. [1]. This bacterium was originally considered as a saprophyte causing soft rot of onions [2] and more generally, as a phytopathogen widely distributed in the environment (soil and water). However, in the last decade, *B. cepacia* emerged as an important opportunistic pathogen in nosocomial infection and notably as a real threat in patients with cystic fibrosis (CF) due to its association, in some cases, with a rapid and fatal decline in pulmonary functions [3–5].

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In contrast to *Pseudomonas aeruginosa*, another well-known CF pathogen, few information is available concerning the virulence factors and the molecular basis of the pathogenicity of *B. cepacia*. Indeed, several cellular and extracellular products have been listed as potential pathogenic determinants. These include iron-chelating siderophores, outer membrane proteins, haemolysin, lipases, proteases, lipopolysaccharides (LPS) and exopolysaccharides (EPS) [6,7]. However, the precise role for these compounds in the pathogenicity remains unclear and needs further studies.

In the case of *P. aeruginosa*, the EPS (an alginate-like polysaccharide) is postulated to play a role in the colonisation and the persistence of the microorganism in the respiratory tract of CF patients [8]. Although *B. cepacia* produces an EPS [9,10], the possible contribution of this molecule in the development of the pulmonary infection is still controversial. Furthermore, except for previous investigations on the general composition of the EPS indicating that it was mainly composed of glucose, mannose, galactose, rhamnose and glucuronic acid, with few exceptions [10,11] to date nothing is known about the chemical structure of this EPS. In the present study, we report the structural characterisation of an EPS produced by a mucoid strain of *B. cepacia* isolated from a CF patient. The structure of this EPS differs in composition from all the described EPS isolated from this bacterium species.

2. Results and discussion

Isolation and purification of the exopolysaccharide.—The crude EPS was obtained from agar-plate cultures by washing with 0.9% sodium chloride. The EPS was isolated by repeated ethanol precipitation followed by a fractionation procedure involving precipitation with cetyltrimethylammonium bromide (CTAB). This method led to the isolation of two EPS fractions: a major polysaccharide (**PS1**) exhibiting acidic behaviour, retained on anion-exchange chromatography on DEAE-Trisacryl and eluted from the gel as an homogenous large peak with 0.2–0.3 M NaCl, and a neutral minor component (**PS2**, 15% max of the total EPS), non-precipitable by CTAB, unretained on the DEAE-Trisacryl column. This latter fraction, which was composed mainly of rhamnose, galactose and a heptose, was not further considered.

The **PS1** polysaccharide was further purified on a cation-exchange resin column (Dowex H^+) and the resulting material was washed several times with chloroform in order to remove the possible peptidic and lipidic contaminants. Following this procedure, only trace amounts of proteins (or amino acids) and fatty acids (mainly palmitic, oleic and stearic acids) were found in the purified **PS1**. Using UV absorption at 260 nm, no detectable contamination by nucleic acids was observed. The purified **PS1** showed a broad distribution of molecular weight in gel permeation chromatography (GPC) when analysed on a Sephadex G-200 column with an apparent maximum at 180,000 Da.

Glycosyl composition of **PS1**.—GLC analysis of the derivatised acid hydrolysis products of **PS1** indicated that it was composed of D-galactose and D-glucose in the molar ratio 1:1, the absolute configuration being established as previously described [12]. It was found to be devoid of uronic acids both by colorimetric estimation [13] and GLC analysis. On solvolysis with 1.5 N methanolic hydrogen chloride followed by GLC

analysis of the resulting methyl glycosides, the composition indicated above was not found. On the GLC trace, however, four peaks were observed, two of them being identified as methyl glucoside derivatives. The two others unidentified peaks exhibited retention times different from those expected for the methyl galactoside derivatives (T_R relative to methyl glucoside 0.97 and 0.99 instead of 0.85 and 0.91). These unidentified peaks were not observed after treatment of the methanolysate with sodium borohydride followed by a second methanolysis. This treatment led to the identification of methyl glucoside and methyl galactoside derivatives in the molar ratio 1:1, as observed by acid hydrolysis of **PS1**. This results indicated that the galactosyl residue was linked to a substituent released by acid hydrolysis and by methanolysis only after a reduction step, suggesting the presence of a carboxyl function. The first evidence on the chemical nature of this substituent came from the analysis of the ^{13}C NMR spectrum (Fig. 3a) of the native **PS1**. A downfield resonance signal at 175.9 ppm was observed in the ^{13}C NMR spectrum and attributed to the resonance of a carboxyl carbon atom. In addition, an upfield resonance signal at 25.8 ppm was also seen in the spectrum and assigned to the resonance of a methyl carbon, whose protons resonate at 1.47 ppm in the ^{1}H NMR spectrum. These data suggested the presence of an *O*-(1-carboxyethylidene) group, which would be responsible for the acidic character of **PS1** [14,15].

*Characterisation of the 4,6-O-(1-carboxyethylidene)-*D*-galactose residue.*—Unambiguous characterisation and linkage positions of the pyruvate residue on the galactose unit were performed by GLC–MS analysis of the trimethylsilylated methyl glycoside derivatives, resulting from the methanolysis of **PS1**. The occurrence in the chemical ionisation (CI) mass spectrum of abundant ions at m/z 440 $[M + NH_4]^+$, 408 $[M - MeOH + NH_4]^+$ and less abundant ions at m/z 423 $[M + H]^+$ and 391 $[M - MeOH + H]^+$, was consistent with a molecular mass of 422 which corresponds to a trimethylsilyl derivative of a methyl *O*-(1-carboxyethylidenemethyl ester)-hexopyranoside. Confirmation of the structure and determination of the linkage positions of the pyruvate substituent were given by the analysis of the fragmentation pattern of the electron impact (EI) mass spectrum (Fig. 1). The occurrence of the characteristic ion at m/z 363 (M − COOMe, 44%), formed by an α-cleavage from the carboxyethylidene ring and that of the prominent ion at m/z 243 (97%), established a specific 4,6-*O*-(1-carboxyethylidene)-D-galactose structure [15]. The 4,6-linkage position of the carboxyethylidene group was also deduced by the occurrence in the EI mass spectrum of a prominent H-type ion at m/z 204 (47%), requiring adjacent trimethylsilyl groups at positions 2 and 3, and establishing a pyranose form for the galactose unit [16].

Glycosyl-linkage analysis.—GLC–MS analysis of the partially methylated alditol acetates of the depyruvated **PS1** (**PS1**$_{AA}$) led to the identification of 2,4,6-tri-*O*-methylglucitol and 2,4,6-tri-*O*-methylgalactitol derivatives, thus establishing the presence of C-3-linked glucosyl and C-3-linked galactosyl residues in the repeating-unit of the native **PS1**. In addition, a minor compound was observed in partially methylated alditol derivatives. It was identified as 2-*O*-methylgalactose, corresponding to a C-3,4,6-linked galactosyl residue which resulted from the incomplete removal of the pyruvate substituent and confirmed the C-4,6-linkage position of this group on the galactosyl residue.

NMR studies.—Data from both ^{1}H and ^{13}C NMR spectra (Figs. 2 and 3) were consistent with the composition and linkage analyses described above, and were also

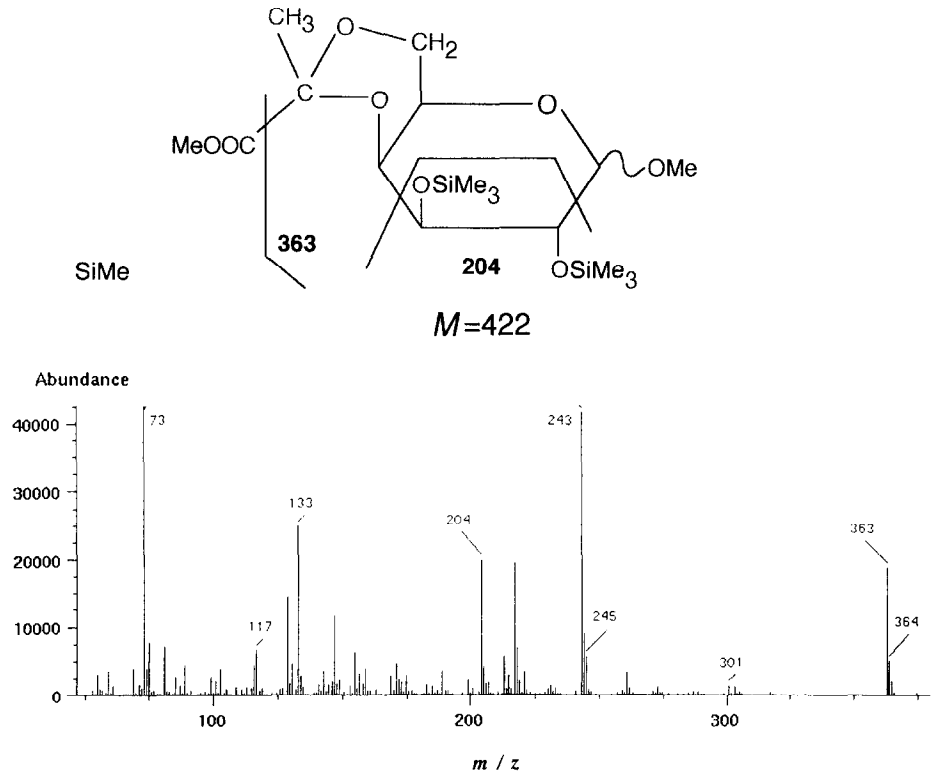


Fig. 1. Electron impact mass spectrum of methyl 2,3-di-*O*-trimethylsilyl-4,6-*O*-(1-methoxycarbonyl-ethylidene)-D-galactoside released by methanolysis of the acidic exopolysaccharide **(PS1)** of *B. cepacia*.

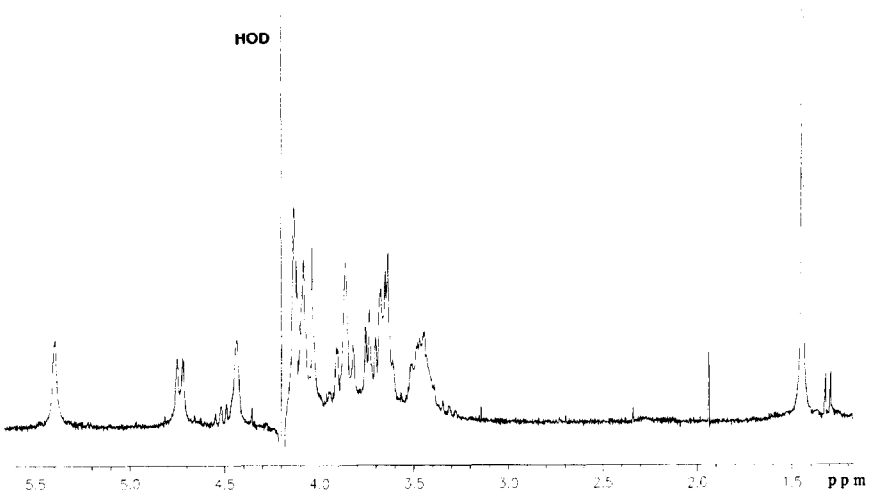


Fig. 2. ^{1}H NMR spectrum at 250 MHz (D_2O, 343 K) of the acidic exopolysaccharide of *B. cepacia* **(PS1)**.

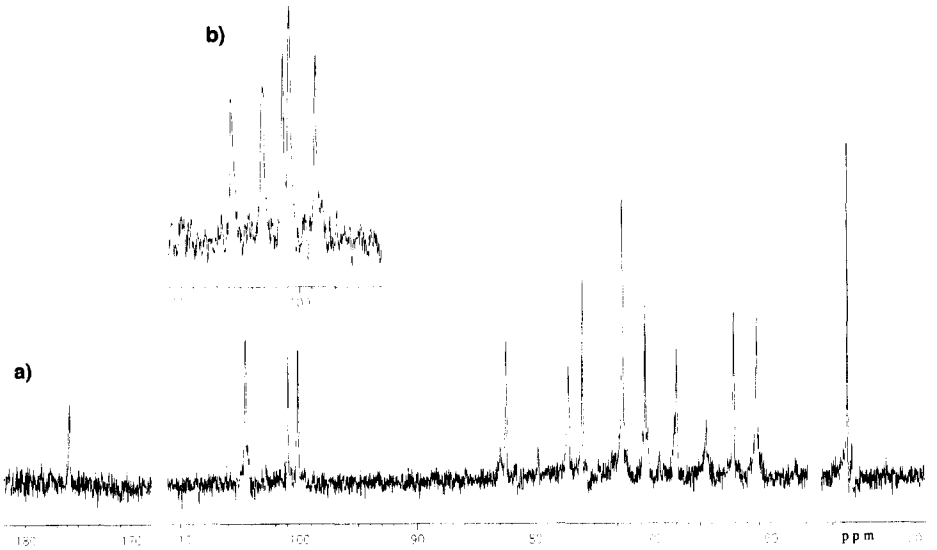


Fig. 3. (a) ^{13}C NMR spectrum at 62.5 MHz (D_2O, 333 K) of the acidic exopolysaccharide of *B. cepacia* (**PS1**). (b) Anomeric region of the coupled ^{13}C NMR spectrum of **PS1**.

indicative of a regular disaccharide repeating-unit bearing one pyruvate substituent with an *R* configuration for the acetal carbon, as indicated by the chemical shift observed at 25.8 ppm for the methyl carbon resonance [17]. The ^{1}H NMR spectrum (Fig. 2) of the native **PS1** (poorly resolved due to the high viscosity of the polysaccharide) contained, inter alia, three signals in the anomeric and other deshielded proton resonances region (Fig. 2) at 5.41 (unresolved, 1 H), 4.72 (doublet, $J_{1,2}$ 7.4 Hz, 1 H) and 4.44 ppm (unresolved, 1 H). Removal of the pyruvate substituent by treatment of **PS1** with 2% acetic acid (1.5 h, 100 °C), led to a significant decrease of the intensity of the signal at 1.47 ppm and of that at 4.44 ppm, demonstrating that the latter signal was not assignable to an anomeric proton. The decrease of the intensities of these signals were accompanied by the presence of a novel signal at 4.25 ppm. These data suggested that the resonance at 4.44 ppm belongs to H-4 of the galactosyl residue.

The ^{13}C NMR spectrum (Fig. 3a) showed three signals in the anomeric carbon region at 104.6, 101.1 and 100.2 ppm. Among these, two agreed with resonances of anomeric carbons as shown by the presence of two doublets in the coupled ^{13}C NMR spectrum in this region (Fig. 3b). Based on the chemical shift and coupling constant values, the signals at 104.6 ($J_{C\text{-}1,H\text{-}1}$ 163 Hz) and 100.2 ppm ($J_{C\text{-}1,H\text{-}1}$ 173 Hz) were assigned to β-pyranosyl and α-pyranosyl units, respectively. According to the literature data, the non-coupled resonance at 101.1 ppm was assigned to C-2 of the carboxyethylidene group [17]. In order to assign the anomeric configuration of the glycosyl and galactosyl residues, **PS1** was analysed by two-dimensional NMR experiments. From the homonuclear 2D shift-correlated spectroscopy (COSY) and the proton-detected heteronuclear multiple-quantum coherence (HMQC) spectra, it was easy to establish the complete spin system for the protons of the glucosyl and galactosyl residues (Table 1). Based on the

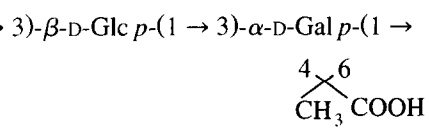
Table 1
^{1}H and ^{13}C NMR data [a] for **PS1**

Residue		^{1}H or ^{13}C chemical shifts (ppm)						
		1	2	3	4	5	6a	6b
→ 3)-β-D-Glc	H	4.72	3.48	3.66	3.66	3.44	3.72	3.86
	C	104.6	72.7	82.6	70.8	76.1	61.3	
→ 3,4,6)-α-D-Gal	H	5.41	4.12 [b]	4.13 [b]	4.44	4.10	4.03	3.85
	C	100.2	68.1	77.3	72.7	63.3	65.6	
Pyruvate	H			1.47				
	C	175.9	101.1	25.8				

[a] Chemical shifts in ppm relative to acetone, δ 2.22 and 31.07 ppm for ^{1}H and ^{13}C, respectively.
[b] Tentative assignment.

chemical shift and coupling constant values, the D-Gal*p* was shown to have the α configuration whereas the D-Glc*p* had the β configuration. In agreement with the methylation analysis, it was also shown that O-3 of the two hexoses were the sites of glycosylation in view of the significant deshielding observed for the chemical shifts of the resonances of the corresponding carbons [18,19]. Similarly, the downfield shifts observed at 65.6 ppm for C-6 [19] and at 72.7 ppm for C-4 [20] of the α-Gal residue, were indicative of a substitution on these positions, in agreement with the published results for the acidic EPSs of *Pseudomonas marginalis* [21] and *Arthrobacter* sp. [22]. The most striking feature of the ^{13}C NMR spectrum was the chemical shift observed at 63.3 ppm attributed to the C-5 resonance of the galactosyl residue. Such unusual chemical shift for a non-anomeric ring carbon resonance was, however, in accordance with previous studies on pyruvate acetal-containing saccharides [20] and was probably due to a strong β-effect of the aglycon moiety on this carbon [18].

Taken together, the data obtained from the chemical, mass and NMR spectroscopy analyses are consistent with the following structure for the repeating-unit of the EPS of *B. cepacia*:



The structure described above differs markedly in composition from those previously described for EPS produced from environmental and clinical strains of *B. cepacia* [10], and presents some similarity with EPS produced by *Pseudomonas putida* BR7 [23], *P. fluorescens* MC2 [23] and *P. marginalis* HT041B [21]. In the latter case, a structure similar to that described herein was found with, in addition, a succinyl substituent at either positions 2 or 4 of the glucosyl unit.

3. Experimental

General methods.—Evaporation and concentration was performed under diminished pressure or by flushing under a N_2 stream.

Depyruvated **PS1** (**PS1**$_{AA}$) was obtained by treatment of **PS1** with 2% AcOH for 1.5 h at 100 °C. The products were then passed through a mixed bed resin TMD 8 (Sigma), and lyophilised. Tests for the presence of uronic acids were performed according to the method described by Dische [13].

The EPS extracts were assessed for nucleic acids by ultraviolet absorption using a Perkin–Elmer (Lambda 5) spectrophotometer; protein and amino acid analyses were performed using UV detection and ninhydrin reaction after total hydrolysis of the **PS1** extract (HCl 6 N, 18 h, 110 °C), respectively. The lipid content of **PS1** was estimated by methanolysis and GLC–MS analysis of the methyl esters recovered in the ether phase of the methanolysate.

Analytical methods.—GLC–MS analyses were performed on a Hewlett–Packard 5890 Serie II gas chromatograph, fitted with an OV1 fused-silica capillary column (12 m HP-1, Hewlett–Packard, Avondale, PA, USA), connected to a Hewlett–Packard 5989X mass spectrometer. In the electron impact (EI) mode, an ionisation potential of 70 eV with an ion source at 200 °C was used. Ammonia was the reactant gas used in the chemical ionisation (CI) mode (ionisation potential 230 eV, ion source 200 °C). Samples were injected in the spitless mode and the temperature program used was: 80 °C (3 min, delay) and 8 °C/min to 290 °C.

Analytical GLC was performed on Me_3Si-derivatives of monosaccharides using a DELSI G30 chromatograph equipped with a OV1 WCOT fused-silica capillary column (25 m × 0.32 mm), and He as the carrier gas. The temperature gradient used was 100 to 290 °C at 3 °C/min.

Gel permeation and anion-exchange chromatography.—GPC was performed on a Sephadex G-200 column (95 × 1.7 cm) using 0.1 M NH_4OAc buffer (pH 7) as eluent (with 0.02% NaN_3 as preservative), equipped with a Iota refractive index detector. Anion-exchange chromatography was carried out on a DEAE-Trisacryl M column (Sepracor), equilibrated with a 10 mM NH_4Cl buffer (pH 8.3). The column (15 × 1.6 cm) was eluted with a 0–1 M NaCl gradient prepared in the same buffer. Fractions collected were assayed for carbohydrate using the anthrone–H_2SO_4 method [24]. Appropriate fractions were collected and lyophilised.

Methylation analysis.—Methylation of the depyruvated **PS1** (**PS1**$_{AA}$) was carried out according to the method described by Finne et al. [25]. Methylated **PS1**$_{AA}$ was dialysed in order to remove low-molecular-weight impurities. The products were then hydrolysed, with 2 M trifluoroacetic acid for 2 h at 110 °C, reduced by sodium borodeuteride (15 mg/mL, overnight) and acetylated with 1:1 Ac_2O–pyridine, 1 h at 100 °C. The resulting partially methylated alditol acetates were then analysed by GLC–EIMS.

Glycosyl composition.—Samples were subjected to hydrolysis with 2 M TFA for 2 h at 110 °C or methanolysis with anhydrous 1.5 N MeOH–HCl 16 h at 80 °C. Solvolysis was achieved on the native polysaccharide (**PS1**) or on the reduction products of previous first methanolysate. In the latter case, reduction was performed by sodium borohydride and the reduction products, recovered by cation-exchange resin (Dowex

H^+) followed by coevaporation of methyl borate with MeOH, was evaporated to dryness before a second methanolysis. The resulting products were trimethylsilylated [26] with 6:4:2 pyridine–hexamethyldisilazane–chlorotrimethylsilane, 15 min at room temperature, and analysed by GLC or GLC–MS. The percentage and identification of the different monosaccharides were determined by GLC using erythritol as the internal reference. The absolute configurations of the glucosyl and galactosyl residues were determined by GLC analysis of their corresponding trimethylsilylated (*R*)-2-butyl derivatives by comparison of their retention times with reference compounds [12].

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were recorded in D_2O on a Bruker AM 250 spectrometer at 70 and 60 °C, respectively. Chemical shifts were expressed in ppm relative to acetone as internal reference (δ_H 2.22, δ_C 31.07). Two dimensional homonuclear chemical-shift-correlated spectroscopy (COSY) and proton-detected heteronuclear multiple-quantum coherence (HMQC) experiments were recorded on a Bruker AM 500 using standard pulse sequences available in the Bruker software.

Growth conditions, purification and isolation of bacterial EPS.—A *Burkholderia cepacia* clinical strain was obtained from Dr T.L. Pitt (Central Public Health Laboratory, Colindale, London). A 18 h bacterial preculture was inoculated on a Pseudomonas Isolation Agar medium (PIA, Difco) containing 5% glycerol, recovered by a cellophane membrane and incubated for 72 h at 37 °C. The mucoid material was harvested by washing the agar-plate cultures with 0.9% NaCl using a bent glass rod. The suspension was stirred with glass beads, the cells were then removed by centrifugation at 10,000 *g* for 30 min at 4 °C. The resulting supernatant was filtered and precipitated overnight at 4 °C with 6 vol of 95% EtOH. Precipitated EPS was recovered by centrifugation and the EtOH precipitation step repeated once again. After centrifugation (12,000 *g* for 30 min at 4 °C), the pellet was dissolved in distilled water and extensively dialysed (8000–9000 mol wt cutoff tubing) against distilled water at 4 °C. The crude EPS extract obtained in this way, was subsequently purified and fractionated by precipitation with 2% cetyltrimethylammonium bromide (CTAB). The CTA-**PS1** complex was recovered by centrifugation at 12,000 *g* for 10 min and dissociated in 1 M sodium acetate. The solution was then washed several times with chloroform in order to remove cetyltrimethyl-ammonium acetate. The aqueous layer was dialysed, concentrated and lyophilised. The resulting material was referred to **PS1**. Possible peptidic contaminants were removed by cation-exchange chromatography through a Dowex 50 WX2 (H^+ form) column (Fluka). The polysaccharide **PS2**, not precipitable by CTAB, was obtained from the supernatant using the same protocol as for **PS1** except for the treatment with sodium acetate.

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