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SHORT COMMUNICATION

Structural analysis of the curdlan-like exopolysaccharide produced by *Cellulomonas flavigena* KU

WJ Kenyon and CS Buller

Department of Molecular Biosciences, The University of Kansas, Lawrence, KS 66045, USA

***Cellulomonas flavigena* KU produces large quantities of an insoluble exopolysaccharide (EPS) under certain growth conditions. The EPS has previously been shown to be a glucose polymer and to have solubility properties similar to curdlan, a β-1,3-D-glucan produced by *Alcaligenes faecalis* var. *myxogenes* 10C3K. Furthermore, EPS purified by alkaline extraction stains with aniline blue, a dye specific for curdlan-type polysaccharides. However, EPS-producing colonies of *C. flavigena* KU do not stain on aniline blue agar as do those of curdlan-producing bacteria. These facts prompted a more thorough structural analysis of the EPS. Here we report that purified EPS is indeed identical to curdlan in primary structure, but that the native form of the EPS may differ from curdlan in physical conformation.**
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Introduction

Cellulomonas flavigena KU was first isolated from a cellulose enrichment culture by Angelo *et al* [2]. The bacterial isolate produces large quantities of a water-insoluble exopolysaccharide (EPS) when grown aerobically in minimal media containing an excess of carbon/energy and a growth-limiting amount of nitrogen [4,23]. Such cultures form macroscopic aggregates of cells completely encapsulated by EPS. As described by Buller and Voepel [4], the EPS was first purified by extraction with sodium hydroxide and precipitation with acid. This alkali-extracted EPS was shown to be a glucose polysaccharide with solubility properties similar to curdlan, a β-1,3-D-glucan produced by *Alcaligenes faecalis* var. *myxogenes* 10C3K and some strains of *Agrobacterium* [4,7,8,10,15]. The alkali-extracted EPS also stained with the dye aniline blue, which specifically stains curdlan-type polysaccharides [4,15,16,23]. Therefore, the EPS was referred to as curdlan [4,23]. However, EPS-producing colonies of *C. flavigena* KU do not stain intensely blue on aniline blue agar as do colonies of other curdlan-producing bacteria [16]. This observation suggested a structural difference between the 'native' form of the EPS and the alkali-extracted form. One possible explanation for the aniline blue staining differences observed was that native EPS might contain ester-linked substituents (acyl groups) that are lost during the alkaline extraction procedure. The presence of such noncarbohydrate substituents, which are often hydrophobic or negatively charged [21], could conceivably interfere with the interaction between native EPS and the hydrophilic, positively charged aniline blue dye. To test this hypothesis, a milder purification method was developed involving extraction with the chemically inert solvent dimethyl sulfoxide (DMSO), and a more detailed structural analysis of both NaOH-EPS and DMSO-EPS preparations was undertaken.

Correspondence: Dr William J Kenyon, Department of Biomedical Sciences, University of South Alabama, Mobile, AL 36688, USA
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Materials and methods and results

Microorganism and culture conditions

C. flavigena strain KU was grown in M9 minimal medium [3] containing 0.05% yeast extract, 1.5% glucose, and 9.5 mM NH_4Cl (M9YE media) at 30°C with shaking for 72 h [4].

Preparation of NaOH-EPS and DMSO-EPS

Cultures were centrifuged at 10,800×*g* for 10 min at 4°C. Pelleted biomass containing bacterial cells and EPS was washed in saline and lyophilized. This biomass preparation was used for the preparation of both NaOH-EPS and DMSO-EPS and routinely contained greater than 90% carbohydrate by weight.

To prepare NaOH-EPS, dry biomass was suspended in 1 N NaOH at a ratio of 1:100 (wt/vol) and stirred for 30 min at room temperature. The suspension was centrifuged at 14,500×*g* for 20 min at 4°C. The extract was collected by decantation and filtered through a Whatman GF/A glass microfiber filter. EPS was precipitated by adding an equal volume of 1 N acetic acid while stirring.

To prepare DMSO-EPS, biomass was suspended in DMSO at a ratio of 1:100 (wt/vol) and stirred for 30 min at room temperature. The suspension was centrifuged at 25,300×*g* for 30 min at 25°C. The extract was collected by decantation and filtered through a Whatman GF/A glass microfiber filter. To precipitate the EPS, three volumes of distilled water were added to the filtrate while stirring.

The resulting hydrogels were collected by centrifugation at 10,800×*g* for 10 min at 4°C, washed four times with distilled water, lyophilized, ground to a fine powder, and stored in a desiccator. Authentic curdlan was obtained commercially (Wako Chemicals USA, Richmond, VA) and used for comparison.

The carbohydrate [5], protein [13], and phosphate [1] contents of EPS preparations from *C. flavigena* KU were compared to those of commercial curdlan from *A. faecalis* var. *myxogenes* (Wako Chemicals USA, Richmond, VA) and to that of dextran from *Leuconostoc mesenteroides* (Sigma Chemical Co., St. Louis, MO). Polysaccharides were dissolved in 25 ml of 0.25 N NaOH and then analyzed. All polysaccharide preparations contained approximately 90% carbohydrate and less than 1% protein by weight. Each polysaccharide preparation contained less than 0.2 nmol of total phosphate per milligram.

Monosaccharide composition

EPS preparations were prehydrolyzed in 90% formic acid under argon followed by evaporation of formic acid under a stream of nitrogen. Hydrolysis was then completed in trifluoroacetic acid (TFA) under argon. After evaporation of TFA, the hydrolysate was dissolved in distilled water and analyzed by thin-layer chromatography (TLC).

TLC of carbohydrates was performed using acetonitrile/water (85:15, vol/vol) [17]. Plates were sprayed with H_2SO_4/MeOH (1:3, vol/vol) and heated for 5 min at 110°C to give brown to black spots.

Hydrolysates of both EPS preparations gave a single spot with an R_f value identical to that of glucose, which agrees with what had previously been reported [4].

Polarimetry

Polysaccharides were dissolved in 1 N NaOH and in neat DMSO at a concentration of 10 mg/ml and specific optical rotations determined using a Perkin-Elmer polarimeter (Perkin-Elmer, Wellesley, MA). Readings were obtained at room temperature, using the sodium D-line and a path length of 1 dm.

The specific optical rotations of NaOH-EPS, DMSO-EPS, and commercial curdlan dissolved in 1 N NaOH were $+12.7^0$, $+12.7^0$, and $+16.0^0$, and those in DMSO were $+14.2^0$, $+14.2^0$, and $+14.6^0$, respectively. From these data, it can be concluded that the glycosidic bonds in both EPS preparations from *C. flavigena* KU are predominantly β-linked because of low, positive optical rotation values.

Methylation analysis

Methylation analysis was performed as described by Harris *et al* [10]. After acid hydrolysis of the methylated polysaccharides, methylated sugar residues were dissolved in methanol and identified by TLC using methylene chloride/methanol (9:1, vol/vol) [14].

After permethylation and acid hydrolysis of both EPS preparations from *C. flavigena* KU and commercial curdlan, the same two methylated products were detected by TLC. The major product was identified as 2,4,6-tri-*O*-methyl-D-glucopyranose, resulting from internal anhydro-glucose residues. The minor product was 2,3,4,6-tetra-*O*-methyl-D-glucopyranose, resulting from nonreducing end residues. Similar results were obtained for curdlan by Harada *et al* [9]. These results suggest that most, if not all, of the glycosidic linkages in the EPS are 1,3-linkages and that there are no detectable branch point residues.

Aniline blue staining

For each polysaccharide preparation, 5 ml of aniline blue solution, 0.001% (wt/vol) in 50 mM phosphate buffer (pH 7.0) was mixed with 50 mg of polysaccharide and allowed to stand at room temperature for 15 min. Fifteen milliliters of 95% ethanol was then added, and polysaccharide was pelleted by centrifugation (10,800×*g* for 10 min at 25°C). After four washes in ethanol, the extent of staining was compared.

The two types of EPS preparation from *C. flavigena* KU, commercial curdlan, and three other glucans were treated with aniline blue in this manner. As reported by Nakanishi *et al* [15], curdlan stained, but laminarin, microcrystalline cellulose, and soluble starch did not. Both NaOH-EPS and DMSO-EPS preparations from *C. flavigena* KU stained with aniline blue. These results do not support the hypothesis that groups present on native EPS are responsible for its staining resistance.

Ester linkages

The hydroxamic acid assay [7] was used to test for the presence of esters. Curdlan-type polysaccharides precipitate during the final steps of the assay. Therefore, precipitated polysaccharide was removed by centrifugation at 10,800×*g* for 10 min at 25°C before reading absorbance.

As much as 10 mg of NaOH-EPS, DMSO-EPS, and commercial curdlan gave no reaction in this test for esters. Considering the sensitivity of the assay, it can be concluded that there are no ester-linked substituents present in these polysaccharide preparations.

Infrared spectroscopy

Infrared absorption spectra were obtained from polysaccharide/KBr pellets using a Perkin-Elmer spectrometer (Perkin-Elmer, Wellesley, MA). Infrared spectroscopy of EPS preparations was used to determine the anomeric configuration of glycosidic bonds and to detect esters. The IR spectra of NaOH-EPS and of DMSO-EPS were essentially identical, and neither spectrum showed absorbance due to esters from 1720 to 1750 cm^{-1}. Both spectra showed absorption near 890 cm^{-1} indicative of β-linked

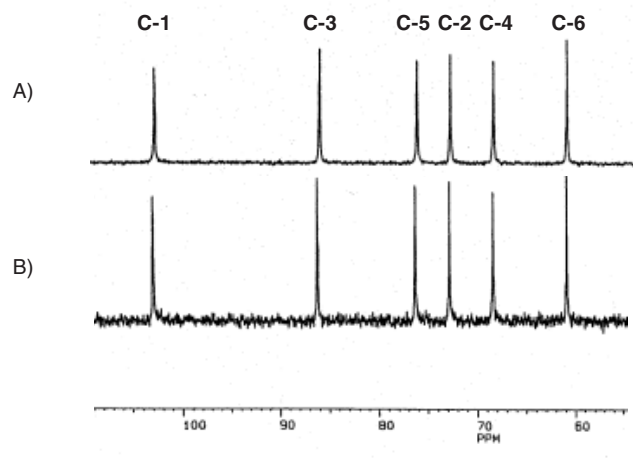

Figure 1 [^{13}C]NMR spectra of EPS preparations. (A) DMSO-EPS preparation and (B) NaOH-EPS preparation. EPS was dissolved in deuterated dimethylsulfoxide (d_6-DMSO) at a concentration of 5% (wt/vol). Spectra were obtained using a Bruker AM-500 NMR spectrometer operating at 125.77 MHZ. Carbon atoms of anhydro-glucose residues are labeled.

glycosidic bonds, and no absorption near 840 cm^{-1} from α-linked glycosidic bonds.

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were obtained for NaOH-EPS and DMSO-EPS dissolved in deuterated DMSO at a concentration of 5 mg/ml using a Bruker-AM-500 NMR spectrometer. The [^{13}C]NMR spectra are shown in Figure 1. Both are essentially identical to the spectrum for curdlan dissolved in 0.22 N NaOH [19], and do not indicate the presence of noncarbohydrate substituents.

Degree of polymerization (DP)

The average DP of the EPS preparations was determined by using the phenol–H_2SO_4 assay to determine the total number of monosaccharide residues and the BCA-reducing sugar assay to determine the number of reducing ends [6,24]. The DP of both NaOH-EPS and DMSO-EPS preparations was consistently around 500. Similar values have been reported previously for curdlan [8,18].

Discussion

All of the new structural data presented herein provide conclusive evidence that EPS produced by *C. flavigena* KU is identical to curdlan in primary structure:

$$[\rightarrow 3) - \beta - \text{D} - \text{Glc}p - (1]_{\sim 500}$$

The data also prove that noncarbohydrate side groups are not responsible for the poor aniline blue staining properties of native EPS. An alternative explanation may lie in the fact that, like paramylon, native EPS is highly resistant to both acid and enzymatic hydrolysis [23]. Paramylon is a β-1,3-D-glucan produced by *Euglena* species in the form of an intracellular storage granule. Paramylon granules are also resistant to aniline blue due to their highly crystalline conformation [12]. The differences in crystallinity between paramylon and curdlan from *A. faecalis* var. *myxogenes* 10C3K have been compared to the 'native-regenerated' forms of cellulose [12]. Furthermore, the curdlan produced by *A. faecalis* var. *myxogenes* 10C3K is not a closely associated capsule as it is in *C. flavigena* KU [23]. Thus, the native form of the EPS produced by *C. flavigena* KU may have a more crystalline structure than the hydrogel form, which results following solubilization and precipitation.

Curdlan has been studied extensively and has found a variety of industrial and medical applications [11,20,22]. *C. flavigena* KU is capable of producing up to 9 g of dry EPS per liter of aerated culture grown in minimal media with an excess of glucose and a growth-limiting amount of NH_4Cl [4,23]. This yield could probably be improved upon by optimizing culture conditions. Furthermore, EPS production from *C. flavigena* KU occurs during growth on cellulose particles in minimal media (unpublished data). However, the yield of curdlan is significantly less than that obtained from cultures grown on soluble sugars. One advantage of using *C. flavigena* KU over other bacterial strains for curdlan production is that it is Gram-positive and, therefore, it does not contain lipopolysaccharide (LPS), which could potentially contaminate curdlan preparations. In conclusion, *C. flavigena* KU represents an attractive alternative source of curdlan-type polysaccharide.

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