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Production of an extracellular polysaccharide by *Agrobacterium* sp DS3 NRRL B-14297 isolated from soil

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A bacterium isolated from soil and identified as *Agrobacterium* sp produced a water-soluble extracellular polysaccharide capable of producing highly viscous solutions. Gas chromatographic analysis revealed a sugar composition of glucose, galactose and mannose in the molar ratio of 7.5 : 2.4 : 1, together with 3.7% (w/w) pyruvic acid. Methylation analyses showed the presence of (1 → 3)-, (1 → 4)- and (1 → 6)-linked glucose, (1 → 3)- and (1 → 4, 1 → 6)-linked galactose and a small portion of (1 → 3)-linked mannose residues. Succinic acid was not present. The molecular weight of the polysaccharide was estimated by light scattering to be 2×10^6 Da. The viscosity of solutions containing the polysaccharide remained constant from pH 3 to 11, and decreased by 50% when heated from 5 to 55°C. Maximum yield of the polysaccharide, 20 g L^{-1}, was reached in 48 h at 30°C incubation.

Keywords: extracellular polysaccharide; *Agrobacterium*; viscous polysaccharide

Introduction

Microorganisms are known to accumulate various kinds of polysaccharide inside or outside of the cells. Many polysaccharides produced by bacteria have characteristic rheological and physiological properties that are different from those of natural gums and synthetic polymers. They are also susceptible to biodegradation in nature and are typically less harmful to the environment than many synthetic polymers. Therefore, some microbial polysaccharides are produced on industrial scales and used as raw materials for food processing and for medical and industrial purposes. Xanthan produced by *Xanthomonas campestris* [23,24] and alginate produced by *Pseudomonas aeruginosa* [2] are used as food stabilizers. Dextran produced by *Leuconostoc mesenteroides* [19] is used as a blood plasma extender, while Scleroglucan, produced by *Sclerotium glucanicum* [9], is used for industrial gum. Curdans from *Alcaligenes faecalis* var *myxogenes* [10,11] and *Agrobacterium radiobacter* [20] are used for either food or non-food materials as gelling agents.

We isolated a bacterium from soil which produces a water-soluble extracellular polysaccharide. The bacterium is a non-spore forming, Gram-negative rod and was identified as an *Agrobacterium* sp. Two types of polysaccharides produced by agrobacteria have been reported: a water-soluble acidic polysaccharide like succinoglycan [13,14]; and the water-insoluble β-(1 → 3)-glucans (curdlan-type) [21]. These polysaccharides were believed to promote nodule invasion by nitrogen-fixing microorganisms [8]. The polysaccharide produced by *Agrobacterium* sp DS3 described in this work was water-soluble and free of succinic acid; aqueous solutions of the polysaccharide were highly viscous. It contains glucose : galactose : mannose in the respective molar ratios of 7.5 : 2.4 : 1. The sugar components and their ratio found in this polysaccharide are different from other known microbial polysaccharides [4]. This paper describes the culture identification, purification of the polysaccharide, identification of sugar components and type of linkages, some physical properties and the optimum conditions for production of the polysaccharide.

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Materials and methods

Microorganisms

Microorganisms obtained from a variety of soil and water samples were screened for the ability to modify oleic or linoleic acids or to produce unusual products [15,16]. Each isolate from a single colony on TGY [5] agar plates was grown at 30°C aerobically in a 125-ml Erlenmeyer flask shaken at 200 rpm containing 50 ml of SMD medium with the following composition (per liter): dextrose, 20 g; K_2HPO_4, 5 g; yeast extract, 5 g; soybean meal, 5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4$, 0.014 g; $MnSO_4 \cdot H_2O$, 0.008 g; and nicotinic acid, 0.01 g. Prior to autoclaving it, the medium including dextrose was adjusted to pH 7.0 with dilute phosphoric acid. Cultures were maintained on agar (3%) slants of the same medium. Microbial isolates were identified by using Biolog GN Microplates and analyzed with the corresponding microstation (Biolog, Inc, Hayward, CA, USA).

Chemicals

Oleic acid [purity >99% by gas chromatography (GC)] was purchased from Nuchek Prep Inc, Elysian, MN, USA. Immobilized protease on 4% beaded agarose was obtained from Sigma (St Louis, MO, USA). Thin-layer precoated Kieselgel 60 F_{254} plates were obtained from EM Science (Cherry Hill, NJ, USA). All other chemicals were analytical grade reagents from commercial sources.

Quantitation of polysaccharide

Ten milliliters of culture broth were diluted with an equal volume of distilled water and centrifuged at 11 000 × *g* at 4°C for 20 min to remove bacterial cells. To the supernatant solution, two volumes of ethanol were added and the precipitate was collected by centrifugation. The precipitate was washed with 30% ethanol and centrifuged. The washed precipitate was taken up in water for quantitation of the polysaccharide by the phenol-sulfuric acid method [6] from a calibration curve using glucose as a standard.

Isolation and purification of polysaccharide

After the bacterial culture had grown for 48 h, an equal volume of 1 N NaOH was added to the viscous culture broth, and the mixture was centrifuged at 11 000 × *g* for 20 min to remove cells. The supernatant phase was neutralized with 3 N HCl and then centrifuged to separate water-soluble from insoluble curdlan-like polysaccharides. The majority of the polysaccharide remained in the supernatant phase. Two volumes of ethanol containing 2% KCl were added to the supernatant fraction, and the polysaccharide precipitate was collected by centrifugation. The precipitate was washed consecutively by suspension followed by centrifugation in 30% ethanol, acetone, and finally diethyl ether. The resulting precipitate was dried to a white powder with a stream of nitrogen. Fifty milligrams of the polysaccharide in 50 ml of water were incubated for 16 h with 100 mg of immobilized protease at 30°C with shaking. The mixture was then dialyzed against deionized distilled water for 24 h. The dialyzate was then precipitated with three volumes of ethanol and centrifuged. The resulting precipitate was again dried to a white powder.

Thin-layer chromatography of sugar components

Following hydrolysis of the polysaccharide in 2 M trifluoroacetic acid (TFA) for 1 h at 120°C in a sealed tube, the following solvents were used for separating component sugars on Kieselgel plates [18]: 2-propanol : acetone : 0.1 M lactic acid (4 : 4 : 2, v/v) and acetone : water (90 : 10, v/v). The sugars were made visible by means of the diphenylamine-aniline-phosphate spray reagent [1].

Gas chromatography of sugar components

To identify the native sugar components, the purified DS3 polysaccharide was hydrolyzed with 2 ml 2 M TFA for 1 h at 120°C in a sealed tube. After TFA was removed, the sugars were converted to the peracetylated aldononitrile (PAAN) derivatives [22] and analyzed by GC/MS incorporating a Hewlett-Packard 5970B mass selective detector operating at 70 eV and using a methylsilicone column (25 m × 0.022 i.d. × 0.1 μm thickness; Hewlett Packard, Wilmington, DE, USA). The column temperature was held for 3 min at 160°C and then raised at 5°C per min to 185°C. To identify the linkage sites in the polysaccharide, samples of purified DS3 were permethylated [22] with sodium methylsulfinyl methanide and methyl iodide in dimethyl sulfoxide. The methylated polysaccharide was then hydrolyzed with TFA and the sugars converted to PAAN derivatives. For the separation of these derivatives, the GC column temperature was held for 3 min at 130°C, raised at 5°C per min to 165°C, and then held for 10 min; helium was used as a carrier gas for both analyses.

Non-carbohydrate substituents

For analysis of substituent organic acids, 100 mg of DS3 polysaccharide was hydrolyzed with 10 ml 5% sulfuric acid (95°C, 10 h). The hydrolyzate was extracted twice with an equal volume of diethyl ether, and then the combined ether layer was washed with water and evaporated to dryness. A portion of this residue was methylated with diazomethane; the resulting fatty acid methyl esters were analyzed by GC isothermally at 200°C as described previously [15,16].

The aqueous layer of the hydrolyzate was neutralized with barium hydroxide and, after centrifugation, the precipitate was discarded. The supernatant fluid was lyophilized and the residue taken up in methanol, and methylated with diazomethane as previously described. The methyl ester derivatives were used for the analysis of smaller molecular weight dibasic organic acids, such as succinic acid, by GC on a crosslinked polyethylene glycol capillary column (30 m × 0.53 mm i.d. × 1.0 μm thickness; Hewlett Packard Innowax) and a temperature gradient program: 45°C for 1 min; 5°C per min to 240°C; and held 5 min at 240°C. Authentic dimethyl succinate has a retention time of 22 min.

Pyruvic acid was quantified by the enzymatic assay described by Duckworth and Yaphe [7].

Measurement of viscosity and surface tension

The viscosity of the crude culture broth was measured with a rotation viscometer (SynChro-Lectric with the #3 spindle; Brookfield, Stoughton, MA, USA). Measurement was at 25°C, and readings were taken after rotation for 2 min. The values obtained were averages of three measurements.

To determine the effects of temperature and pH on the viscosity of aqueous solutions of the polysaccharide, purified polysaccharide was dissolved in buffer, and the viscosity measured with a Brookfield Digital viscometer model DV-1 equipped with a temperature-regulated small sample adapter and the SC4-18/13R spindle. The temperature range evaluated was from 5 to 55°C; the system was allowed to equilibrate thermally between viscosity readings on the same sample. Solutions ranging from pH 3 to 11 were prepared from a stock polysaccharide solution and mixed with sodium acetate/sodium phosphate stocks (100 mM each) titrated to the appropriate pH. The pH of the buffered polysaccharide solution was determined prior to viscosity measurement (12 rpm, 30°C).

The surface tension of the polysaccharide solution was measured with a Kruss Tensiometer (Hamburg, Germany) using small ring (K-813) at 25°C.

Molecular weight

The molecular weight of the polysaccharide was determined by multiangle light scattering (Dawn DSP model B photometer; Wyatt Technology Corporation, Santa Barbara, CA, USA). Purified polysaccharide was dissolved in ultrapure HPLC grade water, filtered through a 0.45-μm pore size filter, and placed in a sample cuvette. Data were collected at several polysaccharide concentrations and analyzed by means of the Aurora software program (Wyatt Technology Corporation). The value determined was the average of molecular weights derived from the Zimm,

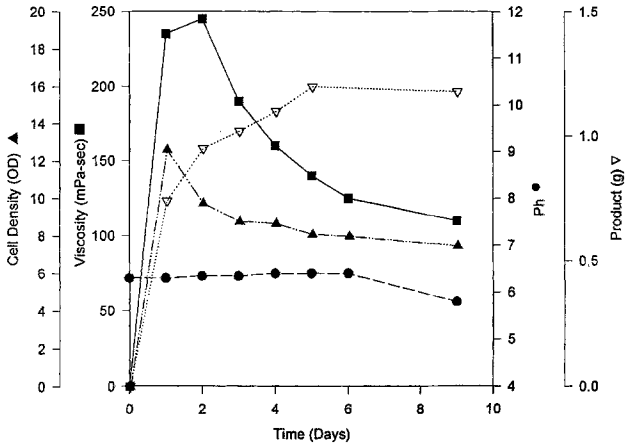

Figure 1 Production of water-soluble extracellular polysaccharide by *Agrobacterium* sp DS3, grown on SMD medium, 200 rpm, at 30°C. ▲—▲, Cell density ($OD_{660\,nm}$); ■—■, viscosity; ●——●, pH; ▽----▽, product polysaccharide (g).

Berry, and Debye plotting strategies that are part of the Aurora program.

Results and discussion

Identification of microorganism

Of many water and soil samples screened, only one culture, DS3, isolated from a dry soil sample collected at Peoria, IL, produced a significant amount of extracellular polysaccharide that was capable of producing viscous solutions. Strain DS3 is a Gram-negative, non-spore-forming rod (0.8 μm × 2 μm). Fifty-one of the ninety-six wells of a Biolog GN microplate yielded positive results: dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, *m*-inositol, D-lactose, lactulose, maltose, D-mannitol, D-mannose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, D-turanose, xylitol, acetic acid, formic acid, D,L-lactic acid, propionic acid, succinic acid, bromo succinic acid, alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline, L-serine, L-threonine, inosine, uridine, glycerol, glucose 1-phosphate and glucose 6-phosphate. By comparison to known strains, the Biolog GN microstation identified strain DS3 as belonging to the genus *Agrobacterium*, and having a 20% similarity to the closest species, *A. rhizogenes*. Therefore, strain DS3 is classified as *Agrobacterium* sp DS3 NRRL B-14297.

Production of polysaccharide

Production of extracellular polysaccharide by *Agrobacterium* sp DS3 was observed originally with our routine fatty acid bioconversion screen in which oleic or linoleic acid served as the substrate [15]. When the organism was grown at 30°C on a TGY agar plate for several days, translucent gelatinous colonies developed. It was also observed that the liquid culture medium became viscous during bacterial growth under aerobic conditions due to the formation of extracellular polysaccharide. Further investigation revealed

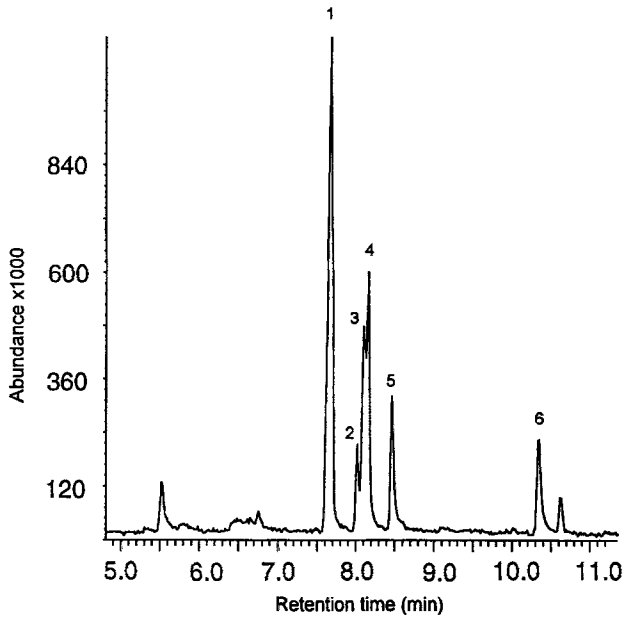

Figure 2 GC/MS of the peracetylated aldononitrile (PAAN) derivatives of sugars from strain DS3 polysaccharide. Column temperature was held for 3 min at 130°C, raised at 5°C per min to 165°C, and then held for 10 min. Peak identifications: (1) methyl-2,4,6-tri-*O*-methyl-D-glucose PAAN; (2) methyl-2,4,6-tri-*O*-methyl-D-mannose PAAN; (3) methyl-2,4,6-tri-*O*-methyl-D-galactose PAAN; (4) methyl-2,3,6-tri-*O*-methyl-D-glucose PAAN; (5) methyl-2,3,4-tri-*O*-methyl-D-glucose PAAN; (6) methyl-2,3-di-*O*-methyl-galactose PAAN.

that addition of fatty acid was not required for the production of polysaccharide. We therefore investigated the conditions for the optimum production of polysaccharide.

Strain DS3 was grown on SMD medium with 1, 2, 3 or 5% (w/v) of glucose. After five days of incubation, the polysaccharide in the culture medium was isolated and analyzed. Polysaccharide yields for 1, 2, 3, and 5% glucose were 0.3, 0.34, 1.03, and 1.2 g per 50 ml, respectively. Five percent glucose in SMD medium was used for subsequent studies.

The time course for the production of polysaccharide was also studied. Cell density, culture viscosity, pH and amount of polysaccharide produced were followed for 9 days. Figure 1 shows that the concentration of polysaccharide reached its maximum after 5 days of incubation, after which it remained relatively constant. There was not much change in pH of the culture broth during the course of investigation. The viscosity of the culture broth increased

Table 1 GC/MS analysis of PAAN derivatives of sugars from DS3

PAAN methyl ether[a]	Relative retention time	Molar ratio
2,4,6-tri D-glucose	1.00	8.7
2,4,6-tri D-mannose	1.05	1.0
2,4,6-tri D-galactose	1.06	3.7
2,3,6-tri D-glucose	1.07	5.2
2,3,4-tri D-glucose	1.11	2.9
2,3-di D-galactose	1.36	2.5

[a]Trivial names for the *O*-methyl sugars; eg 2,4,6-tri D-glucose is actually 2,4,6-tri-*O*-methyl-D-glucose PAAN.

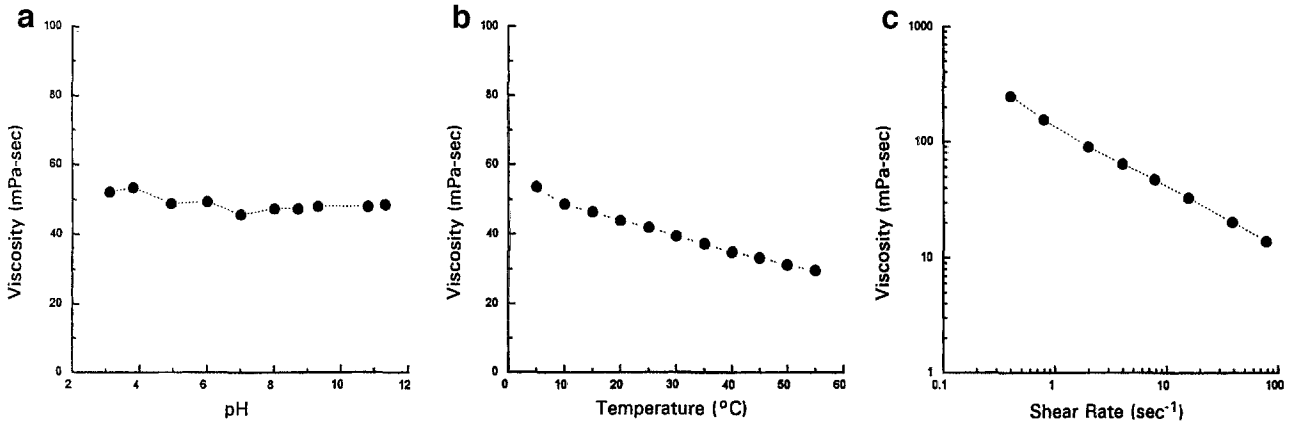

Figure 3 Rheological properties of the purified DS3 polysaccharide. (a) Effect of pH on viscosity at 30°C, 12 rpm; (b) effect of temperature on viscosity at pH 7, 12 rpm; (c) effect of shear rate on viscosity, 30°C, pH 7.

sharply (to 240 mPa-sec) during the first 2 days of incubation and then decreased significantly. Because the concentration of the polysaccharide remained constant, the decrease in solution viscosity suggests structural change(s) occurred in the polysaccharide.

Identification of sugar components of polysaccharide

When an acid hydrolyzate of the purified polysaccharide was analyzed by thin-layer chromatography, three sugars with R_f values identical to those of authentic D-glucose, D-galactose and D-mannose were detected with two different solvent systems. With 2-propanol : acetone : 0.1 M lactic acid as the solvent system, the R_f values for D-glucose, D-galactose, and D-mannose were 0.54, 0.41 and 0.57, respectively. With acetone : water as the solvent system, their respective R_f values were 0.55, 0.50 and 0.59. When peracetylated aldononitrile derivatives of these sugar components were analyzed by GC, three peaks with retention times of 7.39 min, 7.74 min, and 7.11 min were found which coincided with retention times of the derivatives from authentic D-glucose, D-galactose, and D-mannose, respectively. From these results, it was confirmed that the polysaccharide consisted of three sugars. Their mole ratios are glc : gal : man = 7.5 : 2.4 : 1.

The pyruvic acid content was 3.7% by weight. To determine whether other organic acid moieties existed in the polysaccharide, an acid hydrolyzate of the polysaccharide was extracted with diethyl ether. When methyl esters were prepared and analyzed by GC, no fatty acids were detected. This ruled out the existence of lipopolysaccharide. The possible existence of water-soluble organic acids such as succinic acid was also studied with the acid hydrolyzate. Results obtained from an HP-Innowax GC capillary column showed that there were no succinic or other water-soluble dibasic organic acids in the polysaccharide.

Structure analysis

Table 1 summarizes the methylation analysis of purified polysaccharide (Figure 2). The PAAN-hexose that most likely contained the pyruvate group (a 2-*O*-methyl PAAN) had a retention time of 12 min (result not shown). The results show that the most prominent sugar is (1 → 3)-linked glucose, followed in relative abundance by (1 → 4)-linked glucose; galactose is primarily (1 → 3)-linked, but some (1 → 4, 1 → 6)-linked galactose is also present. Mannose is the least abundant component of the polysaccharide, and is (1 → 3)-linked.

Zevenhuizen [25] identified an acidic polysaccharide similar to succinoglycan from *Agrobacterium tumefaciens*. Hisamatsu *et al* [13,14] found that water-soluble, exocellular polysaccharides from nine strains of *Agrobacterium* contained succinic acid as well as pyruvic and acetic acids. Their structures, which were similar to succinoglycan, were composed of (1 → 3), (1 → 4), and (1 → 6)-linked D-glucosyl and (1 → 3)-linked galactosyl residues. Zevenhuizen [25,26] also reported that *Rhizobium meliloti* YE-2SL secreted sucinnoglycans and a galactoglucan. *Alcaligenes faecalis* var *myxogenes* 10C3 produces large amounts of a water-soluble β-D-glucan containing succinic acid (succinoglucan) and small amounts of an insoluble (1 → 3)-β-D-glucan (curdlan) [10,11,14].

In contrast to several succinoglycans from *Agrobacterium* [13,14], *Alcaligenes* [10,11,14] and *Rhizobium* [3,26], strain DS3 polysaccharide contains mannose and pyruvic acid, and lacks succinic acid. The ratio of sugar constituents (glucose : galactose : mannose = 7.5 : 2.4 : 1) differs from any known microbial polysaccharide. Although there are some structural similarities between DS3 polysaccharide and succinoglycans, in that all have (1 → 3)-, (1 → 4)-, and (1 → 6)-linked glucose and (1 → 3)-linked galactose, the ratios of these linkages are quite different. In addition, the DS3 polysaccharide has (1 → 4, 1 → 6)-linked galactose.

Physical properties of the polysaccharide

The effects of temperature and pH on solution viscosity from the DS3 polysaccharide were investigated. Figure 3a shows that the solution viscosity remained relatively unchanged from pH 3 to 11. This stability of viscosity over a wide pH range is similar to that found for the succinoglycan from *Alcaligenes faecalis* var *myxogenes*, which was constant from pH 3 to 10 [12]. The solution viscosity of DS3 polysaccharide decreased with increasing temperature, and dropped by 40% as the temperature was increased from 5 to 55°C (Figure 3b). The viscosities of succinoglycan solutions were also reported to be temperature dependent, with the viscosity approaching that of water at 60°C [12].

The effect of shear rate on solution viscosity was also examined. As shown in Figure 3c, viscosity decreased with increasing shear rate. Many other microbial polysaccharides show pseudoplastic viscosity behavior [17].

Surface tensions of polysaccharide solutions (0.75% and 0.375% in water) were measured in order to determine whether the polysaccharide had surface-active properties. Both were 43.12 dynes cm^{-1}, indicating that the polysaccharide has moderate surface tension-lowering activity (water is 72.8 dynes cm^{-1} at 18°C).

Determination of molecular weight

A multiangle light scattering detector was used to measure the molecular weight of the polysaccharide in water. An average value of 2×10^6 Da was determined from analyzing data collected at several biopolymer concentrations, which is comparable to the values reported for other extracellular heteropolysaccharides [17]. The large size of the biopolymer is consistent with the decrease in solution viscosity as a result of shear thinning which is a typical phenomenon seen with high-molecular-weight polysaccharides.

Acknowledgements

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