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COMPOSITION AND PROPERTIES OF THE EXTRACELLULAR POLYSACCHARIDE PRODUCED BY *Arthrobacter stabilis* NRRL B-3225

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ABSTRACT

The extracellular polysaccharide produced by *Arthrobacter stabilis* NRRL B-3225 contains D-glucose, D-galactose, pyruvic acid, *O*-succinyl, and *O*-acetyl in the approximate molar ratio of 6:3:1:1:1.5. Succinyl is linked as its half-ester, making it a readily removable, acidic function that imparts versatility to the polysaccharide both for fundamental research and for commercial use. The viscosity of aqueous, salt-free solutions of both native and deacylated polymer is relatively low, but atypical of anionic polysaccharides, increases rapidly in the presence of salts, acids, or alkali.

INTRODUCTION

The extracellular polysaccharide of *Arthrobacter stabilis* NRRL B-3225 is one of several polysaccharides having unusual solution properties to be studied at this laboratory. In addition to the practical significance of these polymers as thickening and suspending agents, they are of considerable fundamental interest for investigating the relationship between composition, structure, and solution behavior of various types of polysaccharide.

Polysaccharides of the genus *Arthrobacter* have been of further interest because few have been reported, and the availability of several strains in our culture collection makes possible the comparative, compositional studies of species of this genus.

Two polysaccharides produced by different strains of *A. viscosus* have previously been reported by this laboratory. The polysaccharide from *A. viscosus* NRRL B-1973 has been fully characterized as regards production[1], composition and properties[2], and structure[3]. The production[4] and composition[5] of polysaccharide from *A. viscosus* NRRL B-1797 have also been reported.

Isolation of the *A. stabilis* organism from garden soil, and procedures for production of the extracellular polysaccharides have been reported by Gill[6], who later deposited the culture in the NRRL collection, where it was assigned strain number

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

NRRL B-3225. In that report, the polysaccharide was characterized only to the extent of containing 6 mol of glucose, 2 mol of galactose, 4 mol of acetate, and 1 mol of uronic acid, as determined by "conventional chromatographic analysis". No methods were cited, nor was identification of constituents verified. We now report qualitative and quantitative compositional studies and pertinent rheological behavior of aqueous solutions of this polymer.

EXPERIMENTAL

Production of polysaccharide. — The production medium contained (g/100 ml): D-glucose, 5; tryptone, 0.2; peptone, 0.2; dipotassium hydrogenphosphate, 0.5. Also added was 0.5 ml of Speakman salts, solution B, which is an aqueous mixture containing 4% $MgSO_4 \cdot 7H_2O$, and 0.2% each of $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, and NaCl. The initial, unadjusted pH was 7.0. Portions (500 ml) were sterilized in Fernbach flasks and inoculated with 30 mL of vigorously growing culture. Flasks were shaken at 25° on a reciprocal shaker for 48 h, followed by 48 h on a rotary shaker. The smooth, mucoid culture had a viscosity of 3080 centipoises (cps). The culture was made 35% in ethanol and 1% in chloroform to kill the cells, diluted to a viscosity of 100 cps, and centrifuged in a Sharples continuous supercentrifuge. The bowl residue (killed cells) was discarded, the supernatant solution was made 1% in potassium chloride, and polymer was precipitated by addition of ethanol (1 vol). The fibrous precipitate was collected by centrifugation, redissolved, diluted to a viscosity of 50 cps, centrifuged twice more, and then twice reprecipitated. The purified precipitate was redissolved and dialyzed against distilled water until salt-free, as determined by conductivity measurement. The dialyzed sample had pH 6.1. The sample was filtered through sintered glass, concentrated, and lyophilized. Lyophilized product was bottled, and stored at 4°.

Preparation of deacylated polysaccharide. — A solution (0.1%) of polysaccharide was treated with 0.01M potassium hydroxide under nitrogen for 4 h at 20°. The solution was brought to pH 6.5 with hydrochloric acid, dialyzed against distilled water until salt free, adjusted to pH 6.5, lyophilized, and stored at 4°.

Analytical methods. — *A. Carbohydrate analysis.* Carbohydrate constituents were identified by paper chromatography; a 1% solution of polysaccharide was hydrolyzed for 4 h at 100° in M hydrochloric acid, neutralized with silver carbonate, treated with hydrogen sulfide to precipitate the excess of silver, evaporated to dryness, redissolved, and centrifuged to remove silver sulfide. The solution was spotted on Whatman No. 1 chromatography paper and developed in 2:5:5 pyridine–ethyl acetate–water, upper phase[7] and in 3:1:3 ethyl acetate–acetic acid–water, upper phase[7]. Sugars were detected on developed chromatograms by the silver nitrate dip-reagent[8].

Carbohydrate composition was quantitatively determined by radiochromatography[9]. A 1% solution of polysaccharide was hydrolyzed for 4 h in M hydrochloric acid at 100°, made neutral with 2M sodium carbonate, reduced with sodium [^{3}H]-borohydride, and chromatographed[10] in 9:1:1 butanone–acetic acid–saturated,

aqueous boric acid on 2-cm strips of Whatman No. 1 chromatography paper that had been previously dipped in saturated, aqueous boric acid and dried. Chromatograms were cut into 1-cm segments, which were counted in 0.4% PPO cocktail in a Beckman LS 250 liquid scintillation counter.

B. Determination of neutral equivalent. Polysaccharide solutions (0.1%) were decationized with BioRad AG-50 X-4 (H^+) resin, and then titrated with 10mM potassium hydroxide. End points were determined potentiometrically and conductometrically. To ascertain that decationization was complete, a replicate sample was passed through the ion-exchange column twice and titrated. The consumption of alkali remained constant.

C. Identification and analysis of pyruvate. The polysaccharide solution (1%) was hydrolyzed in M hydrochloric acid at 100°. Pyruvate was determined colorimetrically by the (2,4-dinitrophenyl)hydrazone method[11] and enzymically by the method of Duckworth and Yaphe[12]. Complete hydrolytic removal of pyruvate from the polysaccharide required 5 h. Positive identification of pyruvate was made by paper-chromatographic comparison of the (2,4-dinitrophenyl)hydrazone with that of an authentic sample[11].

D. Identification and analysis of acyl groups. *O*-Acyl content was measured by the hydroxamic acid method of McComb and McCready[13], and the following adaptation of that procedure was used as a means of identification of acyl constituents: a freshly prepared, 1:1 mixture of 9.5% sodium hydroxide and 3.25% hydroxylamine hydrochloride was added to a polysaccharide solution and allowed to react for 5 min at room temperature. The mixture was made neutral with methanolic hydrogen chloride and evaporated to dryness. Hydroxamates were extracted with ethanol, and the extract was evaporated to low volume to precipitate the excess of salt and decrease interference on chromatograms. The ethanolic solution was spotted on paper, and chromatographed in 4:1:5 butanol–acetic acid–water[14]. Spots were developed with ferric chloride spray[15].

Identification of acyl groups was verified by t.l.c. of the free acids: the polysaccharide (262 mg) was dissolved in M sodium hydroxide (100 mL), kept for 20 min at room temperature, and neutralized to pH 7.1. Polysaccharide was precipitated with ethanol; the supernatant solution and washings were evaporated to dryness, dissolved in water, acidified, and extracted with ether. The extract was made alkaline with 10% sodium carbonate, and evaporated to dryness. The residue was dissolved in water, acidified, and the solution distilled at 100°. Volatile and nonvolatile fractions were both extracted with ether, made alkaline with ammonium hydroxide, and re-extracted into aqueous solution. Solutions were spotted on Silica Gel G t.l.c. plates and developed with 100:12:16 95% ethanol–water–25% ammonium hydroxide[16] for 2 h, dried, sprayed with Bromocresol Green, and heated for 15–30 min at 115°. Spots were yellow on a deep-blue background.

Identification of succinic acid was verified by formation of the dihydronaphthazarine[17] derivative, which gives a red color having maximal absorption at 520 nm in benzene, and a blue color absorbing maximally at 610 nm when made alkaline and

extracted into aqueous solution. This test is specific for succinate. The method may also be used for quantitation, but was not successful in our work, because the relatively low sensitivity required a higher concentration of succinate than was feasible.

Total acyl content was verified by alkaline saponification, and determination of the amount of alkali consumed.

E. Optical rotation. Optical rotation was measured on 0.24% polysaccharide solutions in 0.1% potassium chloride with a Bendix automatic polarimeter operating at 546 nm and having a band width of ±8 nm in a 0.1967-dm cell. Approximate $[\alpha]_D$ values were obtained by multiplying by a factor of 0.8492, as calculated from National Bureau of Standards values for the specific rotation of sucrose. Measurement could not be made in salt-free solutions, because of high birefringence.

Rheological methods. — A Wells–Brookfield RVT cone–plate microviscometer was used for all viscosity measurements. Shear rates ranged from 1.92 to 384 sec^{-1}. For standard conditions, a shear rate of 3.84 sec^{-1} (speed of 1 r.p.m.) was used. Measurements were made of variation of viscosity with concentration, and the effect of salt and pH variation on viscosity, for both native and deacylated forms. The effect of salt was found by mixing aqueous potassium chloride solution with the polysaccharide solutions to obtain the desired concentration. To study the effect of pH variation. 0.1% solutions of polymer were decationized on AG-50 (X-4) columns and the sample titrated to determine the amount of potassium hydroxide required for neutralization to pH 7. Subsequent samples were treated with a volume of potassium hydroxide equivalent to the amount required for 25, 50, 75, 125, and 150% of neutralization. Samples were concentrated to 1%, and the pH and viscosity were measured. Measurements reported here were all on samples from the same batch of polymer. Tests on other batches showed the results to be reproducible.

RESULTS

Chemical properties and composition. — D-Glucose and D-galactose were the only carbohydrates detected by paper chromatography of acid hydrolyzates of the polymer. This observation was confirmed by quantitative radiochromatography. D-Glucose was found to comprise 64.5 and D-galactose 35.5% of the total carbohydrate composition. No uronic acid was detected by paper chromatography or radiochromatography. This observation was confirmed by applying carbazole tests used for specific identification of uronic acids[18], all of which were negative. However, when 4 mL of 0.2% Cetavlon (cetyltrimethylammonium bromide) solution was added to a 0.1% aqueous solution of the polysaccharide, the sample precipitated completely, indicative of an acidic polysaccharide.

Potentiometric titration of decationized, native polysaccharide established an equivalent weight of 929. The titration curve (Fig. 1) was typical of a dibasic acid, indicating that the polysaccharide contained two acidic groups, having different pK values. Titration of deacylated polysaccharide established a neutral equivalent of 1462; the titration curve was much sharper, indicating a single, acidic, functional

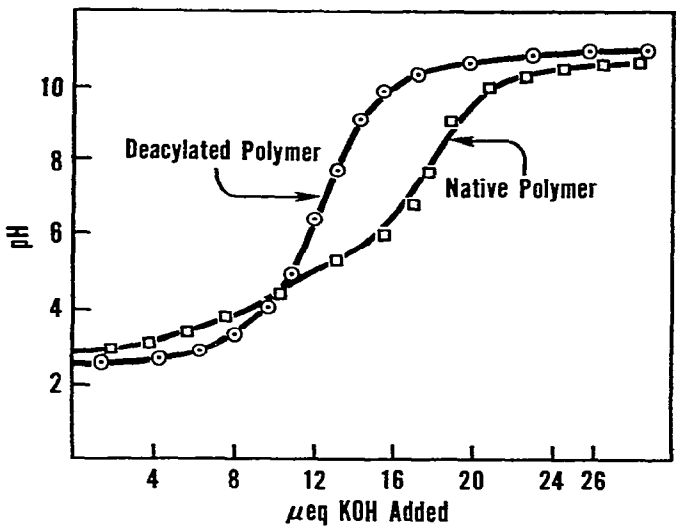

Fig. 1. Titration curves of native (18.5 mg) and *O*-deacylated (18.7 mg) B-3225 polysaccharide with 10 mM potassium hydroxide obtained with a Radiometer automatic titrator.

group. The difference between the titration behavior of native and deacylated samples indicated the presence of an alkali-labile group containing an acidic function, such as a dicarboxylic acid linked to the polymer as a half-ester.

The alkali consumption of neutral, native polysaccharide was 1.42 meq/g of polysaccharide, equivalent to 6.1% of *O*-acyl calculated as *O*-acetyl. *O*-Acyl determination by hydroxamic acid assay yielded 6.2%, calculated as acetyl. Identification of hydroxamate derivatives by paper chromatography showed one spot that migrated identically with the acetyl derivative, plus two other spots that migrated identically with the mono- and di-hydroxamates of succinic acid. The presence of dihydroxamate indicated some succinate to be in the diester form. However, because of the similarity in migration rates of these spots with those of other monocarboxylic acids, positive identification could not be made.

Positive identification of both acetyl and succinyl was made by t.l.c. of the ammonium salts of the free acids. The presence of succinyl was further confirmed by preparation of the dihydronaphthazarine derivative.

The alkali-stable, acidic substituent on the polysaccharide was identified and quantitated as pyruvic acid by its (2,4-dinitrophenyl)hydrazone and by enzymic analysis. Pyruvate (5.0%) was found in the native material, and 6.2% in the deacylated product. This is equivalent to one-half of the total acid-content of the native material. No identification of the mode of attachment to the polymer was made, but experience with other such polymers and the length of time required for complete hydrolysis, suggests that pyruvate is linked to galactose as the 4,6-acetal.

Succinyl content was quantitated by conductometric titration of the decationized polymer, whereby two end-points could be clearly defined. Total acid content was the same by potentiometric and by conductometric titration, namely,

TABLE I

COMPOSITION OF NRRL B-3225 POLYSACCHARIDE

Components	*Percent*	*Molar ratio*
Sugars		
D-Glucose	52.5	6.0
D-Galactose	28.9	3.2
Other		
Pyruvic acid	5.0	1.06
Succinic acid	5.9	1.08
O-Acetyl	3.7	1.60

neutral-equivalent values of 929 and 936, respectively. The pyruvate content equivalent to the first conductometric end-point was 5.0%, identical to the enzymic value. As no other acidic groups were found, the second conductometric end-point could be attributed to succinyl, giving a value of 5.9% of succinic acid.

The composition of the polysaccharide is summarized in Table I. Carbohydrate values were corrected for other constituents, and the *O*-acetyl value was corrected for succinyl. The composition of samples from different preparations was identical, indicating purity and reproducibility of the product, rather than a mixture of polymers. The nitrogen content (0.11%) and phosphorus value (0.04%) indicate that very little extraneous matter was present. The sulfated ash value was 8.0%, which is quite compatible with the content of carboxylic acids. The specific rotation of —23.9°, indicates mainly β linkages in the polymer.

Rheological characterization. — Fig. 2 shows viscosity–concentration relation-

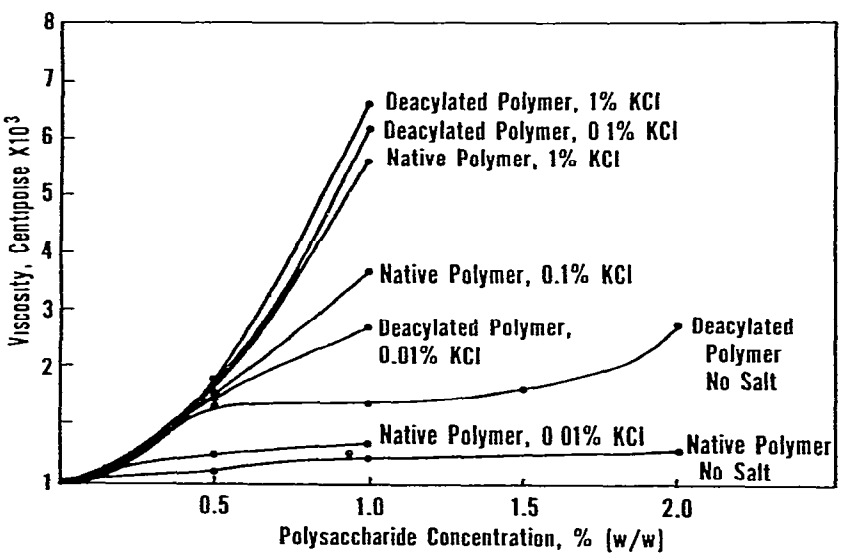

Fig. 2. Viscosity *vs.* concentration relationships of native and deacylated B-3225 polysaccharide in water and in various concentrations of salt.

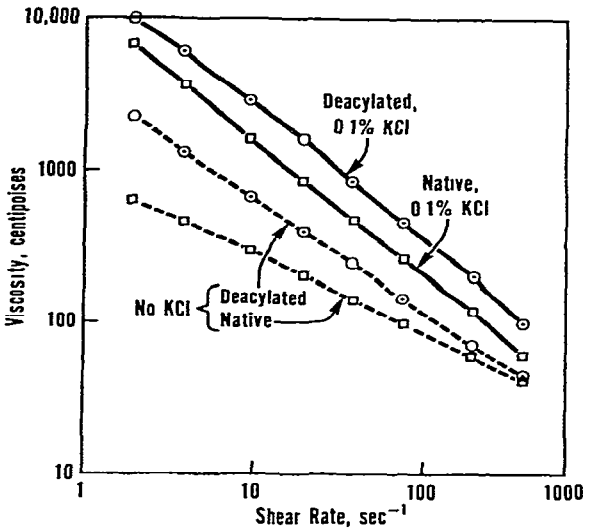

Fig. 3. Effect of shear rate on viscosity of 1% native and deacylated polysaccharide solutions

ships for native and deacylated forms in water and in presence of potassium chloride. The viscosity of the native polymer in water is extremely low, for example, 500 cps at a concentration of 1%. In contrast, the viscosities of 1% solutions of *A. viscosus* polysaccharides NRRL B-1797 and NRRL B-1973 are[5] ~10,000 cps.

The deacylated form of B-3225 polysaccharide has higher viscosity than the native, and is unusual in that there is a broad range of concentration in which viscosity changes very little. Both native and deacylated material show large viscosity increases when salt is added at polysaccharide concentrations of 0.5% or higher.

Effect of shearing on viscosity of 1% solutions is shown in Fig. 3. Both native and deacylated forms exhibit pseudoplastic or reversible shear-thinning behavior typical of macromulecules in the shear-rate range measured. The deacylated material decreases more rapidly with shear than does the native form, until viscosities at high shear-rates are quite comparable. Increased viscosity in the presence of salt is observed throughout the shear-rate range studied.

The effect if variation in degree of acidity on polysaccharide viscosity is shown in Fig. 4. Because the titration behavior of the native and deacylated forms differ so greatly, on account of the presence of two ionizable groups in the native and only one in the deacylated form, viscosity is plotted against degree of neutralization rather than pH, so that the two forms may be compared more readily. The pH values for each point are included. Both native and deacylated materials have much higher viscosities in the acid than in the neutral form, and both also show increase in the presence of an excess of alkali. The native form does, of course, undergo deacylation when kept in an alkaline medium and thus, with time develops a viscosity comparable to that of the deacylated sample. Addition of salt has little effect on the polymer viscosity in the acid state.

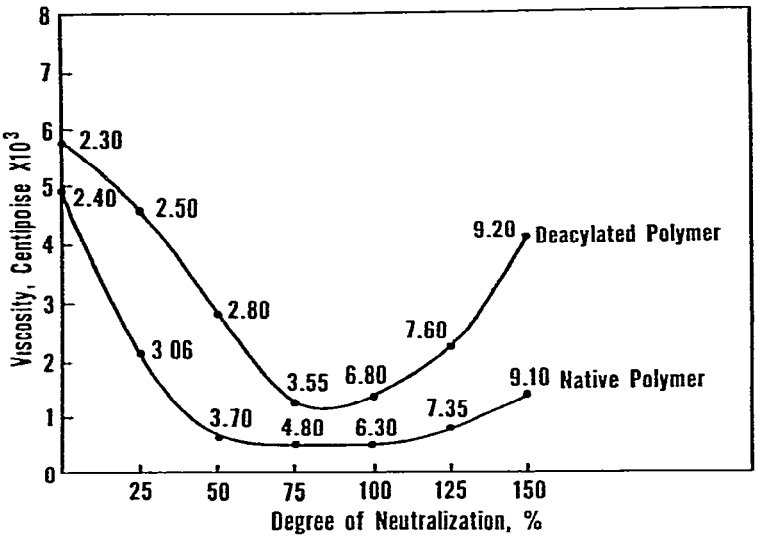

Fig. 4. Effect of acidic and alkaline environment on viscosity of 1% native and deacylated polysaccharide solutions. Number adjacent to each point is the pH at that degree of neutralization.

DISCUSSION

The composition of the B-3225 polysaccharide appears to be unique; only one other extracellular polysaccharide has been reported that contains succinic acid, namely the succinoglucan 10C3 of *Alcaligenes faecalis* var. *myxogenes*[19]. Pyruvic acid is common in polysaccharides from many species of microorganisms, but has only been found as the lone acidic function, or in combination with uronic acids. The presence of two acids of such widely differing pK values in the same molecule provides opportunity for investigating relationships between charge effects and solution properties. Ease of removal of succinic acid would facilitate such fundamental studies as well as increase versatility for practical application.

Comparison of the composition of the B-3225 polysaccharide with polysaccharides from *A. viscosus* NRRL B-1973 (ref. 2) and B-1797 (ref. 5) illustrates the variability found in this genus. The only common constituents are D-glucose, D-galactose, and *O*-acetyl, and relative amounts of these vary greatly. Acidic constituents are quite different; B-1973 contains only D-mannuronic acid, B-1797 contains D-glucuronic acid plus pyruvic acid, and B-3225 pyruvic acid plus succinic acid. This variation is in marked contrast to extracellular polysaccharides from *Xanthomonas*, in which polymer composition is quite consistent[20].

Because polyelectrolytes typically lose viscosity in the presence of salts or acids, the anomalous viscosity-increase described here is of both fundamental and practical interest.

Other polysaccharides have been reported that exhibit atypical solution behavior[21], but not to the extent of B-3225. Comparison of this polysaccharide with

other such polymers is helpful for gaining insight into possible explanations of these rheological phenomena.

An appropriate example of a polysaccharide exhibiting anomalous rheological behavior is xanthan, the extracellular polysaccharide from *Xanthomonas campestris* NRRL B-1459. Because of its widespread industrial application, relationships between its structure and rheology have been studied extensively. In addition, xanthan has other properties in common with B-3225 polysaccharide, such as strong birefringence in solution[22] and reversible thermal-gel melting[23], which indicate structural similarities and provide a basis for formulating some hypotheses for explaining the rheology of B-3225.

The primary structure of xanthan has been established[24, 25], and there is general agreement that the secondary structure consists of relatively stiff rods formed from helices having fivefold symmetry[26, 27]. Multiple helices (either double or triple) that are denatured upon heating and are re-formed upon cooling and addition of salt have been demonstrated by electron microscopy[28].

The ordered state of xanthan in salt-free aqueous solution appears to be analogous to that of B-3225. Both polymers exhibit strong solution-birefringence under crossed polarization which is dissipated by the addition of salt. The ordering is thus dependent upon the alignment caused by intermolecular, ionic repulsions. The fact that B-3225 is much less viscous than xanthan in this state indicates that the particle size of B-3225 must be considerably smaller than in xanthan. The stiff, expanded particles do not fill enough space in the solution to cause high viscosity.

Disruption of the order in solution by addition of salt causes moderate increases of viscosity in xanthan and large increases with B-3225. These increases apparently arise from intermolecular associations (such as hydrogen bonding), which effect an increase in particle size and permit formation of three-dimensional, gel-like networks in the solution.

The foregoing postulates regarding the effects of ionic charges on intra- and inter-molecular relationships is strengthened by examination of the data in Fig. 2. It may be seen that, when half of the anionic functional groups, namely, the succinyl, are removed from the molecule by deacylation, the resulting product requires only 0.1% salt to approach maximal viscosity, whereas, in the native material, 1% of salt is required. This gives strong evidence that ionic charges in the molecule prevent viscosity-building associations from occurring.

Any explanation of the rheological behavior in this polymer must remain tentative until the primary and secondary structures have been determined. However, the indirect evidence described here provides a basis for explanation and suggests directions for further study.

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