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ACIDIC POLYSACCHARIDES CONTAINING SUCCINIC ACID IN VARIOUS STRAINS OF *Agrobacterium**

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ABSTRACT

The water-soluble, exocellular polysaccharides from all 9 strains of *Agrobacterium* tested were shown to contain succinic acid as well as pyruvic and acetic acids as organic acid constituents. Succinic acid appeared to be linked to the polysaccharides through an ester linkage, and its content varied from 0.4 to 7.4%, whereas the contents of sugar and pyruvic acid were similar to each other. The structure of the polysaccharides was examined further by using a specific β-D-glucanase, succinoglucan depolymerase.

INTRODUCTION

Alcaligenes faecalis var. *myxogenes* 10C3, isolated in our laboratory, 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)[1–3]. When this strain was maintained on slants of nutrient agar, many mutant strains appeared that produced small amounts of succinoglucan and large amounts of curdlan[4] and colonies of these mutants were readily detected with Aniline Blue[5,6]. A similar appearance of mutants was observed with *Agrobacterium* species, and these mutants, forming blue colonies on Aniline-Blue plates, were also found to produce large amounts of a curdlan-type polysaccharide[7]. Zevenhuizen[8,9] studied the structure of the water-soluble polysaccharides of *Agrobacterium tumefaciens*, and showed that the sugar composition and the linkage pattern of the polysaccharides from the several strains are quite similar to those of succinoglucan[10,11]. Thus, *Alcaligenes* and *Agrobacterium* produce polysaccharides of similar structure, and are also similar in that the capacity of stock cultures to produce these polysaccharides is unstable.

This paper reports studies on the structure of water-soluble polysaccharides from 9 strains of *Agrobacterium*, showing that the polysaccharides contain succinic acid and that high-speed liquid chromatography is useful for the assay of organic acids in such polysaccharides.

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*Dedicated to Professor Dexter French on the occasion of his 60th birthday.
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EXPERIMENTAL

Organisms. — The following organisms were used: *Agrobacterium radiobacter* IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, IFO 13533, *Agrobacterium rhizogenes* IFO 13259, and *Agrobacterium tumefaciens* IFO 3058. These strains were obtained from the Institute of Fermentation, Osaka (IFO). Four of these strains (IFO 12665, IFO 13127, IFO 13256, and IFO 13259) produced mixtures of 2 forms of colonies, which could be distinguished on Aniline-Blue plates[7]. One form was more slimy and white than the other, and this form was used for preparation of the polysaccharide.

Flavobacterium sp. M64, which was used for growth tests on various polysaccharides, was isolated previously in our laboratory[12].

Cultivation of organisms. — The synthetic medium, containing 4% of D-glucose, described previously[13] was used for production of water-soluble polysaccharides by the organisms. Volumes of 95 ml of the medium in 500-ml conical flasks were inoculated with 5 ml of a seed culture that had been grown in the same medium. The cultures were shaken reciprocally at 120 strokes per min for 5 days at 30°.

Preparation of water-soluble polysaccharides. — The culture broth was centrifuged at 25,000 r.p.m. for 30 min, and the supernatant was decanted and mixed with sodium chloride (to 1% final concentration) and then with 2 volumes of ethanol. The polysaccharide was collected by filtration and washed with 1:2 (v/v) water–ethanol. It was then dissolved in distilled water, dialyzed against distilled water for 2 days, and passed through a column of Amberlite IR-120 (H^+ resin). The eluate was concentrated *in vacuo* at 30° and freeze-dried. The preparations contained 11.8–12.8% of water. Analytical values were calculated on a dry-weight basis. The preparations obtained by the foregoing procedure appeared homogeneous on ultracentrifugal analysis, and their protein content, determined by the method of Lowry *et al.*[14], was less than 0.4%.

Paper chromatography of organic acids. — Each polysaccharide sample (100 mg) was hydrolyzed in a sealed tube with 1 ml of M sulfuric acid for 60 min at 100° under nitrogen, and the resulting solution was extracted continuously with ether for 48 h. The extract was evaporated, applied to Toyo-Roshi No. 50 paper and developed with 75:25:1 (v/v) phenol–water–formic acid[15]. Hydroxamic acid derivatives were applied to Toyo-Roshi No. 50 paper and developed with water-saturated 2-methylpropanoic acid[15].

Quantitative analysis of organic acids. — Liquid chromatography was performed on a high-speed liquid chromatograph (HLC-802, Toyo Soda Ltd.) fitted with a refractive-index detector and a stainless-steel column (4 × 600 mm) packed with LS-222 (Toyo Soda Ltd.), a strong anion-exchange resin. A M solution of sodium formate was used as eluant[16]. Water-soluble polysaccharide (100 mg) was hydrolyzed in a sealed tube with 1 ml of M sulfuric acid for 60 min at 100° under nitrogen. The hydrolyzate was then neutralized with barium carbonate and centrifuged. The precipitate was washed and the supernatant and washings were combined and

evaporated to a syrup *in vacuo*. The syrup was dissolved in 1 ml of sodium formate solution, and injected into the analyzer. The amount of each organic acid was estimated by weighing the area of each peak on the chromatogram. Standard organic acids were also prepared and analyzed by the foregoing procedure.

Organic acids were also estimated colorimetrically; pyruvic acid and organic acids linked to esters were assayed by the methods of Koepsell and Sharpe[17] and McComb and McCready[18], respectively.

Quantitative analysis of sugars. — Water-soluble polysaccharide (20 mg) was hydrolyzed in a sealed tube with 1 ml of M sulfuric acid for 4 h at 100° under nitrogen. The hydrolyzate was neutralized with barium carbonate and evaporated. The syrupy residue was converted into a mixture of alditol acetates and analyzed[19] with a Hitachi K-53 gas chromatograph, fitted with a flame-ionization detector and a column (4 × 100 mm) of 3% of ECNSS-M on Gas-chrom Q at 170°. Xylose was used as the internal standard in these analyses.

Uronic acids were determined by the modified carbazole reaction described by Bitter and Muir[20].

Preparation of succinoglucan depolymerase. — Succinoglucan depolymerase was prepared as described previously[21].

Digestion of polysaccharides with succinoglucan depolymerase. — Polysaccharide (1 mg) was dissolved in 200 μl of 0.1M acetate buffer (pH 5.8) and incubated with 100 μl of succinoglucan depolymerase (2 units) for 5 h at 40°. The mixture was then boiled for 3 min, and passed through a column of Amberlite IR-120 (H^+ resin). The effluent was evaporated *in vacuo*, applied to Toyo-Roshi No. 50 paper, and developed with 6:4:3 (v/v) 1-butanol–pyridine–water. Sugars on the paper were detected with silver nitrate reagent.

Preparation of deacylated polysaccharides. — Deacylated polysaccharide was obtained by hydrolyzing a 0.1% solution of polysaccharide in 0.01M potassium hydroxide and 1% potassium chloride for 5 h at 20° under nitrogen, as described by Sloneker and Jeanes[22].

RESULTS AND DISCUSSION

Ether extracts of acid hydrolyzates and of their hydroxamic acid derivatives were subjected to paper chromatography. All of the polysaccharides gave spots having the same mobilities as succinic acid and pyruvic acid, although the polysaccharides from strains IFO 12665, IFO 13127, and IFO 13256 gave only weak spots corresponding to succinic acid. Furthermore, material from the spot corresponding to succinic acid was isolated crystalline from the hydrolyzates of the polysaccharides of strains IFO 12607 and IFO 13532, and then confirmed to be succinic acid by elementary analysis and by determining melting points of the free acids and their *p*-bromophenacyl esters. High-speed liquid chromatography by the method of Palmer[16] was used to determine the organic acids in the polysaccharide. As shown in Fig. 1, a standard mixture of acetic acid, succinic acid, and pyruvic acid was well

separated, although pyruvic acid gave two peaks. The patterns of the acidic components of the polysaccharides from strains IFO 13532 and IFO 13256 are shown in Figs. 2A and 2B. Peaks for acetic acid, succinic acid, and pyruvic acid were detected, but the minor peak before acetic acid was not identified. The proportions of organic acids in the polysaccharides were determined (Table I). The linkage pattern of the organic acids was examined by analyzing the deacylated polysaccharides. Liquid chromatography showed that acetic acid and succinic acid were removed, whereas pyruvic acid remained (Fig. 2C). Thus, the acetic acid and succinic acid are probably linked as esters, whereas pyruvic acid is linked in a somewhat alkali-stable form (possibly an acetal) to the polysaccharide. Organic acids were also determined colorimetrically (Table I). Data for pyruvic acid, obtained by liquid chromatography and by the colorimetric method based on hydrazone formation[17] were in good agreement. Acids, in the form of esters, were assayed on the basis of the reaction between carboxylic esters and hydroxylamine[18]. The total amounts of succinic and acetic acids obtained by liquid chromatography were similar to the values for the acids (as esters) obtained by colorimetry. This may be expected on the basis of the following assumptions: (*1*) only one carboxyl group of succinic acid is linked in the ester with a hydroxyl group of a sugar; (*2*) succinic esters give the same color intensity as acetic esters on reaction with hydroxylamine.

High-speed liquid chromatography is thus useful for quantitative and qualitative analyses of organic acids. There was a wide variation in the proportions of succinic acid in these polysaccharides. The polysaccharides of strains IFO 12607, IFO 12664, IFO 13532, IFO 13533, IFO 13259, and IFO 3058 contained 4.8–7.4% of succinic acid, but those of strains IFO 12665, IFO 13127, and IFO 13256 contained only about 0.5%. All of the polysaccharides contained 4.9–6.3% of pyruvic acid and 0.3–1.5% of acetic acid. The polysaccharides also contained 76–83% of D-glucose and 9.3–12% of D-galactose (Table I). No glucuronic acid or mannose was detected. The $[\alpha]_D^{20}$ values were $-9°$ to $-18°$ (*c* 1.0, water).

Pyruvic acid and acetic acid are known to be present in many exocellular polysaccharides produced by *Xanthomonas*[22], *Rhizobium*[8], *Azotobacter*[23], *Arthrobacter*[24], *Corynebacterium*[25], *Pseudomonas*[26], *Agrobacterium*[8], *Klebsiella*[27], and other genera of *Enterobacteriaceae*[28]. The occurrence of succinic acid in polysaccharides is rather rare, although the acid has been found in a polysaccharide of *Alcaligenes faecalis* var. *myxogenes* (succinoglucan)[2,3], the teichoic acid of *Actinomyces violaceus*[29], the lipopolysaccharide of *Mycobacterium phlei*[30], a glycerophosphate-oligosaccharide isolated from *Escherichia coli*[31], and the mannan in the membrane system of *Micrococcus lysodeikticus*[32]. Our preparations of polysaccharides from 9 strains of *Agrobacterium* seem similar to acidic β-D-glucans of *Agrobacterium tumefaciens*, composed of (1 → 3)-, (1 → 4)-, and (1 → 6)-β-linked D-glucose and a (1 → 3)-β-linked D-galactose, as described by Zevenhuizen[9]; they are also similar to the succinoglucan of *Alcaligenes faecalis* var. *myxogenes* 10C3, as shown in our previous papers[10,11]. Zevenhuizen did not report the presence of succinic acid as a constituent organic acid, apparently because he used a colorimetric assay-method. On the other hand,

TABLE I

COMPONENTS OF WATER-SOLUBLE POLYSACCHARIDES

Strain	$[\alpha]_D^{25}$ (*deg.*)[a]	D-*Glucose*[b] (%)	D-*Galactose*[b] (%)	*Pyruvic acid* (%)		*Succinic acid*[c] (%)	*Acetic acid*[c] (%)	*Acids as ester*[e] (%) *of succinic acid*
				I[c]	*II*[d]			
Agrobacterium radiobacter								
IFO 12607	−14	78.0	9.4	5.5	5.6	5.6	0.5	6.0
IFO 12664	−18	77.5	9.3	6.2	5.9	6.1	0.4	6.8
IFO 12665	− 9	83.0	10.5	5.0	5.3	0.4	0.3	0.7
IFO 13127	− 9	82.5	11.6	5.8	5.5	0.4	0.4	1.3
IFO 13256	−17	82.0	11.5	5.0	5.2	0.5	1.5	2.4
IFO 13532	−17	79.0	10.4	4.9	5.3	5.6	0.5	6.4
IFO 13533	−10	77.0	11.0	6.3	6.0	5.2	0.4	6.5
A. rhizogenes								
IFO 13259	−16	76.0	10.9	5.7	6.0	7.4	0.5	7.9
A. tumefaciens								
IFO 3058	−11	78.5	10.2	5.8	5.7	4.8	0.5	5.8

[a]In water, *c* 1.0. [b]Assayed by gas chromatography. [c]Assayed by high-speed liquid chromatography. [d]Assayed colorimetrically by the method of Koepsell and Sharpe[17]. [e]Assayed colorimetrically by the method of McComb and McCready[18] and expressed as succinic acid.

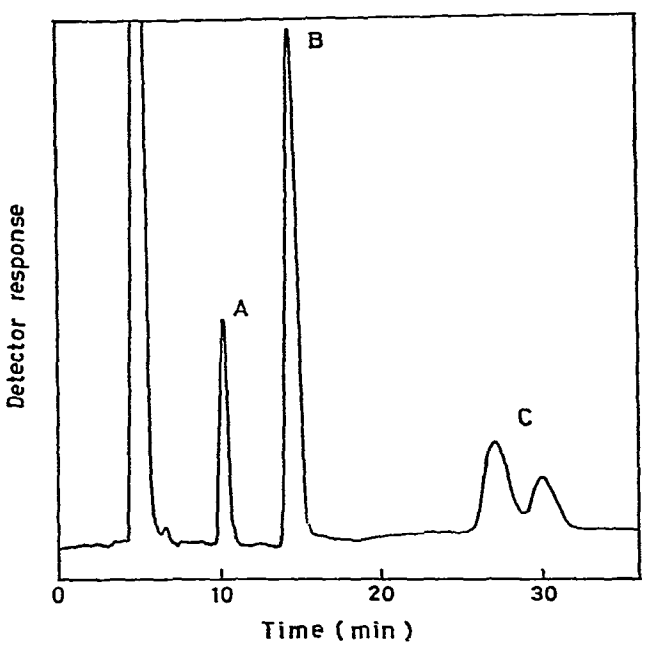

Fig. 1. High-speed liquid chromatogram of a calibration mixture containing 0.625% (w/v) of each of the indicated organic acids: A, acetic acid; B, succinic acid; C, pyruvic acid. Eluant, M sodium formate; separation column, 4 × 600 mm; flow rate, 1.0 ml/min. See text for details.

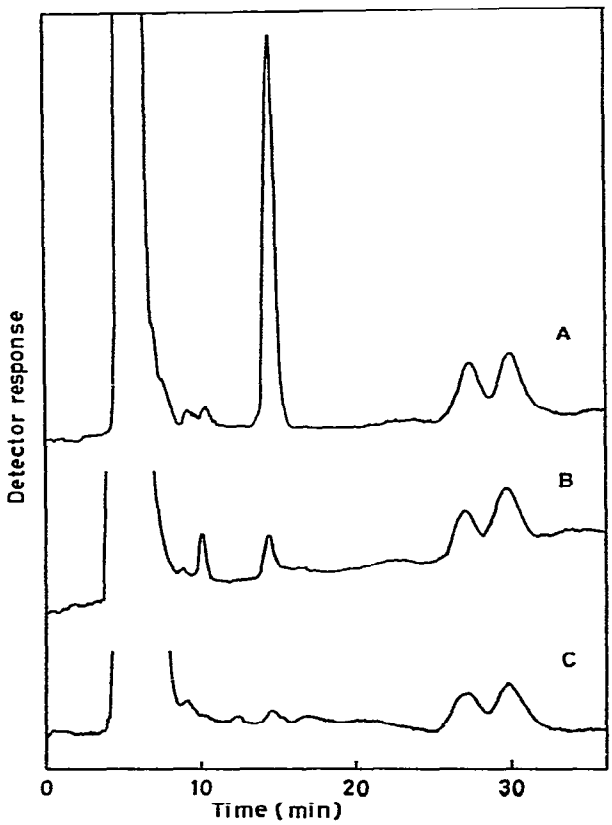

Fig. 2. Chromatograms of hydrolyzates of water-soluble polysaccharides and deacylated polysaccharide. A, polysaccharide of *A. radiobacter* IFO 13532; B, polysaccharide of *A. radiobacter* IFO 13256; C, deacylated polysaccharide of *A. radiobacter* IFO 13532. Chromatographic conditions are given in Fig. 1.

we did not previously report the presence of pyruvic acid in succinoglucan. We now report that it contains 5.8% of pyruvic acid in addition to 6.3% of succinic acid. This was shown by high-speed liquid chromatography.

Previously[12,21], *Flavobacterium* sp. M64, which can utilize succinoglucan as the sole source of carbon, was isolated from soil, and succinoglucan depolymerase was purified from it. This enzyme specifically hydrolyzed succinoglucan or deacylated succinoglucan to a polymer[21] of DP_n 12, and required a specific linkage-sequence in its substrate, because it did not hydrolyze such β-D-glucans as curdlan [β-D-(1 → 3)], cellulose [β-D-(1 → 4)], luteose [β-D-(1 → 6)], schizophyllan [β-D-(1 → 3), β-D-(1 → 6)], kefiran [β-D-(1 → 2), β-D-(1 → 6)], and laminaran [β-D-(1 → 3), β-D-(1 → 6)] or such oligosaccharides as 6-*O*-laminaratriosylglucose, laminaratetraose, laminaratriose, laminarabiose, gentiotriose, gentiobiose, cellobiose, and lactose[21]. To confirm the similarity between the polysaccharides from *Agrobacterium* and succinoglucan, we tested whether they could be hydrolyzed by succinoglucan

Fig. 3. Paper chromatogram of hydrolysis products of water-soluble polysaccharides produced by succinoglucan depolymerase: A–J: polysaccharides of *A. radiobacter* IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, IFO 13533, *A. rhizogenes* IFO 13259, *A. tumefaciens* IFO 3058, and *Alcaligenes faecalis* var. *myxogenes* 10C3; K, standard mixtures of glucose and gentiobiose.

depolymerase. We found by paper chromatography that all of the polysaccharides were hydrolyzed, giving one spot of reducing material near the origin, as shown in Fig. 3. Moreover, like succinoglucan, they could all serve as the sole source of carbon for *Flavobacterium* sp. M64. Thus, the polysaccharides from *Agrobacterium* are similar in structure to succinoglucan. Such highly specific enzymes as succinoglucan depolymerase are very useful for detecting and characterizing polysaccharides.

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