Cyclic (1→2)- β -D-Glucan and the Octasaccharide Repeating-unit of Succinoglycan Produced by *Agrobacterium*

By MAKOTO HISAMATSU,¹ AKINORI AMEMURA,^{1*}
TAKEKIYO MATSUO,² HISASHI MATSUDA² AND
TOKUYA HARADA¹

¹The Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka, 567, Japan

²The Institute of Physics, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan

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Nine strains of *Agrobacterium* produced extracellular cyclic (1→2)- β -D-glucan. Most of the strains produced an octasaccharide repeating-unit of succinoglycan. Mutant strains of *Agrobacterium* sp. and mutant strain 10C3K derived from *Alcaligenes faecalis* var. *myxogenes*, producing curdian without succinoglycan or with a slight amount of succinoglycan, produced only cyclic (1→2)- β -D-glucan. Cyclic (1→2)- β -D-glucan was shown by paper chromatography and methylation analysis to be composed of two components without other glucosidic linkages. These were confirmed to be heptadecaose and octadecaose by field desorption mass spectrometric analysis.

INTRODUCTION

A previous paper (Hisamatsu *et al.*, 1978a) reported that nine strains of *Agrobacterium* produced succinoglycan-type polysaccharides and five of the nine strains also produced curdian (Harada, 1977), a (1→3)- β -linked glucan capable of becoming a firm gel when its suspension is heated. Mutant strains of *Alcaligenes faecalis* var. *myxogenes* 10C3, which is taxonomically close to *Agrobacterium* sp., were found to produce the octasaccharide repeating-unit of succinoglycan (Hisamatsu *et al.*, 1978b). Many strains of *Agrobacterium* have been shown to produce (1→2)- β -D-glucan (McIntire *et al.*, 1942; Putman *et al.*, 1950; Gorin *et al.*, 1961; Barreto-Bergter *et al.*, 1980). The occurrence of cyclic (1→2)- β -D-glucan has been reported in cells of *Rhizobium* sp. (Zevenhuizen & Scholten-Koerselman, 1979). In the present work we have examined the production of the octasaccharide repeating-unit of succinoglycan and of the (1→2)- β -D-glucan by *Agrobacterium*. All the strains examined produced low molecular weight products composed of cyclic (1→2)- β -D-glucan with or without the octasaccharide repeating-unit of succinoglycan. We also examined some properties of the latter compound.

METHODS

General methods. Paper chromatography was conducted by the descending method on Toyo filter paper no. 50 (Toyo, Tokyo, Japan) with the following solvent systems: (A) propan-1-ol/ethyl acetate/water (7:1:3, by vol.); (B) butan-1-ol/pyridine/water (1:1:1, by vol.). Sugars on the paper were detected with sodium periodate–silver nitrate reagent (Yamada *et al.*, 1975).

GLC was performed with a GC7A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector. ¹H NMR spectra of 0.5% (w/v) solutions in deuterium oxide were recorded with a JEOL JNM-FX100 instrument (JEOL, Tokyo, Japan) at 85 °C with sodium 2,2-dimethyl-2-silapentane-5-sulphonate as an internal

standard. In order to determine the molecular weight of cyclic (1→2)- β -D-glucan, field desorption mass spectra were recorded by using a Matsuda-type double focusing mass spectrometer (Matsuo *et al.*, 1980).

Organisms. *Agrobacterium radiobacter* strains IFO 12607, IFO 12664, IFO 12665, IFO 13127, IFO 13256, IFO 13532, IFO 13533, *Agrobacterium rhizogenes* IFO 13259 and *Agrobacterium tumefaciens* IFO 3058 were obtained from the Institute of Fermentation, Osaka (IFO). *Agrobacterium radiobacter* IFO 12665b1, IFO 12665b2 and IFO 13127b were isolated as organisms forming blue colonies on aniline blue plates from stock cultures of strains IFO 12665 and IFO 13127, respectively. *Alcaligenes faecalis* var. *myxogenes* 10C3 and the mutant strains 10C3K, 22 and 22-33 were also used. Strain 10C3, isolated from soil in this laboratory, produces succinoglycan and curdian (Harada *et al.*, 1968). Strain 10C3K, obtained by spontaneous mutation from strain 10C3, is capable of producing only curdian in high yield (Harada *et al.*, 1966). Strain 22 was derived from strain 10C3, by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as an organism capable of producing only succinoglycan (Harada *et al.*, 1968). Strain 22-33 was derived from strain 22 as an organism capable of producing large amounts of the repeating-unit octasaccharide of succinoglycan (Hisamatsu *et al.*, 1978*b*).

Preparation of extracellular polysaccharides and a low molecular weight fraction. Synthetic medium containing glucose (4%, w/v) as the sole carbon source was supplemented with yeast extract (0.1%, w/v), as described previously (Hisamatsu *et al.*, 1978*b*). The medium (95 ml) in 500 ml conical flasks was inoculated with a culture (5 ml) which had grown in the same medium. Cultures (6 d) were centrifuged at 56 000 *g* for 30 min. Curdian was prepared from the precipitate as described previously (Harada *et al.*, 1968). The supernatant was mixed with two volumes of ethanol and centrifuged to remove succinoglycan-type polysaccharide. The supernatant was then concentrated to a small volume, again mixed with two volumes of ethanol and centrifuged. The supernatant was mixed with four volumes of ethanol, and the resulting precipitate was collected by centrifugation, dissolved in water and dialysed by vacuum filtration through a collodion membrane. The dialysable fraction was concentrated to a small volume and subjected to Sephadex G-10 chromatography to remove salt. Fractions in the void volume were collected and lyophilized (low molecular weight fraction). The amount of low molecular weight fraction produced reached a maximum in 6 d cultures.

DEAE-cellulose chromatography of the low molecular weight fraction. A sample of the low molecular weight fraction was applied to a column (3 × 12 cm) of DEAE-cellulose equilibrated with 1 mM-KCl. Material was eluted first with 150 ml of 1 mM-KCl and then with 700 ml of a linear gradient of 1 to 100 mM-KCl. Fractions of 10 ml were collected and their sugar content was measured by the phenol/H₂SO₄ method (Dubois *et al.*, 1956).

Quantitative analysis of sugars. Samples (2 mg) were hydrolysed in a sealed tube with 4 M-trifluoroacetic acid (TFA) for 6 h at 100 °C. The hydrolysate was converted to a mixture of alditol acetates and analysed in a column (3 mm × 2 m) of 3% ECNSS-M on GasChrom Q (Applied Science Laboratories) by GLC.

Quantitative analysis of organic acids. Organic acids were analysed quantitatively by HPLC as described previously (Hisamatsu *et al.*, 1978*a*).

Methylation analysis. Samples (2 mg) were methylated, as described by Hakomori (1964). The methylated sample was hydrolysed with 4 M-TFA for 6 h at 100 °C and analysed as alditol acetates in a column (3 mm × 3 m) of 0.3% OV275–0.4% GEXF 1150 on Shimalite W (Wako Pure Chem., Osaka, Japan) by GLC. The column temperature was held for 4 min at 140 °C and then raised to 180 °C at 0.5 °C min⁻¹.

Partial hydrolysis of (1→2)- β -D-glucan. A sample (5 mg) of (1→2)- β -D-glucan was hydrolysed in 2 ml of 0.1 M-TFA at 100 °C for 120 or 150 min. TFA was removed by passage through a column of Amberlite IR-400 (OH⁻ resin).

RESULTS AND DISCUSSION

The low molecular weight fractions produced by nine strains of *Agrobacterium* were methylated and the methylated sugars were analysed by GLC. Figure 1(*a*) shows the GLC pattern of the methylated sugars from the low molecular weight fraction of *A. radiobacter* IFO 12664. Peaks *a*, *c*, *d*, *e* and *f* were identified as 2,4,6-tri-*O*-methyl-D-glucose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose, respectively, by comparison with the methylated sugars derived from depyruvylated succinoglycan. Peak *b* was identified as 3,4,6-tri-*O*-methyl-D-glucose, as described later.

The low molecular weight fraction of strain IFO 12664 was subjected to DEAE-cellulose chromatography. As shown in Fig. 2, the sample was separated into four products, 1, 2, 3 and 4, with yields of 160, 25, 63 and 77 mg per 100 ml of culture medium, respectively. Results of component and methylation analyses of products 1, 2, 3 and 4 are summarized in Tables 1 and 2, respectively.

Fig. 1. GLC analysis of methylated sugars on a column of 0.3% OV275–0.4% GEXF 1150. Methylated sugars were derived from (a) the low molecular weight fraction of *A. radiobacter* IFO 12664 and (b) product 1 separated from the low molecular weight fraction by DEAE-cellulose chromatography as shown in Fig. 2.

Fig. 2. DEAE-cellulose chromatography of the low molecular weight fraction of *A. radiobacter* IFO 12664. A sample (330 mg) was applied to a column (3×12 cm) of DEAE-cellulose equilibrated with 1 mM-KCl and eluted with a linear gradient of 1 to 100 mM-KCl (---). Fractions were analysed for carbohydrate by the phenol/ H_2SO_4 method (●).

Table 1. Composition of products 1, 2, 3 and 4 obtained by DEAE-cellulose chromatography of the low molecular weight fraction of *A. radiobacter* IFO 12664

Product	Component (% w/w)			
	D-Glucose	D-Galactose	Pyruvic acid	Succinic acid
1	100	0	0	0
2	86.7	8.3	5.0	0
3	78.3	10.6	5.3	5.8
4	72.3	10.2	5.4	12.1

Product 1 was composed of D-glucose (Table 1) and did not show any reducing power by the Nelson–Somogyi method (Somogyi, 1952).

The GLC pattern of methylated sugars from product 1 showed only one peak (Fig. 1b) of a methylated sugar identified as 3,4,6-tri-*O*-methyl-D-glucose by mass spectrometry. The

Table 2. *Methylation analysis of products 1, 2, 3 and 4 obtained by DEAE-cellulose chromatography of the low molecular weight fraction of A. radiobacter IFO 12664*

Retention times (*T*) of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol are shown in parentheses. Chromatography conditions: column (3 mm × 3 m), 0.3% OV275–0.4% GEXF 1150 on Shimalite W.

Product	Methylated sugar (mol %)						
	2,3,4,6-Glc (<i>T</i> 1.00)	2,4,6-Glc (<i>T</i> 1.65)	3,4,6-Glc (<i>T</i> 1.73)	2,4,6-Gal (<i>T</i> 1.82)	2,3,4-Glc (<i>T</i> 1.99)	2,3,6-Glc (<i>T</i> 2.15)	2,3-Glc (<i>T</i> 3.22)
1	0	0	100	0	0	0	0
2	0	19.4	23.2	9.7	16.9	21.4	9.4
3	0	25.4	0	11.6	23.1	27.4	12.5
4	0	23.5	0	11.4	23.8	28.8	12.5

Fig. 3. Paper chromatography of partial hydrolysates of product **1** separated from the low molecular weight fraction (see Fig. 2). A sample (5 mg) was hydrolysed with 0.1 M-TFA at 100 °C for 120 min (a), or 150 min (b). (c) Native product **1** (0.5 mg) plus D-glucose. The paper was developed by the multiple descending method with solvent A and solvent B. Sugars on the paper were detected by spraying with sodium periodate–silver nitrate reagent. *G*₁ indicates the position of glucose and *G*₅–*G*₁₅ the position of the respective glucose oligomers.

Fig. 4. Field desorption mass spectrum of product 1. Molecular weight (M_n) of the sample is given by $162n$, where n is the degree of polymerization. Na-cationated ions ($M_n + 23$) and K-cationated ions ($M_n + 39$) corresponding to $n = 17$ and 18 are observed.

$[\alpha]_D^{20}$ of product 1 was -8.5° (c 1.0, water), suggesting a β -configuration of the sugar linkages. The anomeric-proton resonance at 4.88 p.p.m. with a coupling constant of 7 Hz in the ^1H NMR spectrum supported this configuration. These results led to the conclusion that product 1 is a cyclic and unbranched oligomer or polymer composed exclusively of (1 $\rightarrow$ 2)-linked- β -D-glucose residues.

To estimate the degree of polymerization, we partially hydrolysed product 1, and separated the products of hydrolysis by paper chromatography with solvents A and B. As shown in Fig. 3, the slowest-moving spot in the chromatogram of the hydrolysate seemed to be heptadecaose or octadecaose, and product 1 seemed to be a mixture of heptadecaose and octadecaose, or hexadecaose and heptadecaose. The field desorption mass spectrum of product 1 was recorded to confirm the molecular weight. As shown in Fig. 4, sodium and potassium cationated ions of heptadecaose and octadecaose were clearly observed. Thus, product 1 was a mixture of cyclic (1 $\rightarrow$ 2)- β -D-glucans with the degree of polymerization of 17 and 18.

Products 3 and 4 were composed of D-glucose, D-galactose, pyruvic acid and succinic acid in molar ratios of 7:1:1:1 and 7:1:1:2, respectively (Table 1). These compounds were identified as the octasaccharide structure previously reported (Hisamatsu *et al.*, 1980*a, b*) containing 1 or 2 mol succinic acid, respectively.

Product 2 seemed to be a mixture of product 1 and a deacylated form of product 3 or 4, judging by the results of component analysis and methylation analysis (Tables 1 and 2). When product 2 was treated with 0.05 M-KOH for 16 h at 25 $^\circ\text{C}$, it was separated into product 1 and deacylated product 3 or 4 by DEAE-cellulose chromatography, whereas when treated with 0.05 M-HCl for 16 h at 25 $^\circ\text{C}$ it did not separate. Thus, product 1 and deacylated product 3 or 4, might form a compound with an alkali-labile bond. The infrared absorption spectrum of product 2 showed no absorption about 1740 cm^{-1} due to an ester, but showed an absorption about 1580–1620 cm^{-1} due to carboxylic acid (K-form). Therefore, carboxyl groups of pyruvic acid in product 2 seem to exist in the free form, and ester bonds may be not concerned with the formation of the complex. This type of complex was also observed in low molecular weight fractions from other strains.

The average content of succinic acid per octasaccharide repeating-unit of the polysaccharide of strain IFO 12664 was 1 mol (Hisamatsu *et al.*, 1978*a*). However, repeating-units without succinic acid or with 1 or 2 mol succinic acid may exist in the polysaccharide as shown here.

These results indicate that *Agrobacterium* produces cyclic (1 $\rightarrow$ 2)- β -D-glucan and the octasaccharide repeating-unit of succinoglycan. Some workers (Putman *et al.*, 1950; Gorin *et al.*, 1961; Barreto-Bergter *et al.*, 1980) reported a (1 $\rightarrow$ 2)- β -D-glucan with some other linked D-glucose residues in the culture fluids of *Agrobacterium*. These minor linkages may be due to the presence of the octasaccharide repeating-unit of succinoglycan in their preparations. The existence of (1 $\rightarrow$ 2)- β -D-glucan with other minor glucosidic linkages was also reported by Dedonder & Hassid (1964) and York *et al.* (1980) in several strains of *Rhizobium*, which is taxonomically related to *Agrobacterium*. These minor linkages may also be due to

Table 3. Cyclic (1→2)- β -D-glucan, octasaccharide repeating-unit of succinoglycan, curdlan and succinoglycan produced by *Agrobacterium* strains and mutant strains of *Alcaligenes faecalis* var. *myxogenes* and *Agrobacterium radiobacter*

Strain	Cyclic (1→2)- β -D-glucan [mg (100 ml) ⁻¹]	Octasaccharide repeating-unit of succinoglycan [mg (100 ml) ⁻¹]	Curdlan [mg (100 ml) ⁻¹]	Succinoglycan [mg (100 ml) ⁻¹]	Cells [mg (100 ml) ⁻¹]
<i>A. radiobacter</i>					
IFO 12607	198	0	510	72	170
IFO 12664	176	155	0	277	155
IFO 12665	190	73	14	670	104
IFO 13127	229	98	80	400	119
IFO 13256	102	64	128	133	148
IFO 13532	6	11	0	315	101
IFO 13533	6	12	0	1200	83
<i>A. rhizogenes</i>					
IFO 13259	255	0	480	122	160
<i>A. tumefaciens</i>					
IFO 3058	58	11	0	178	62
<i>Alc. faecalis</i> var. <i>myxogenes</i>					
10C3	24	121	98	1190	174
22	0	84	0	1180	175
22-33	0	250	0	640	170
10C3K	35	0	1410	0	179
<i>A. radiobacter</i>					
IFO 12665b1	273	0	1100	9	113
IFO 12665b2	258	0	600	84	110
IFO 13127b	157	0	790	72	121

contamination with the repeating-unit oligosaccharide of extracellular acidic polysaccharide, because we have found that cyclic (1→2)- β -D-glucan without minor linkages and the repeating-unit of extracellular acidic polysaccharides, including succinoglycan, 13336 polysaccharide and other types of polysaccharides, are produced by many strains of *Rhizobium* (unpublished data).

Recently, Zevenhuizen & Scholten-Koerselman (1979) have reported that unbranched cyclic (1→2)- β -D-glucan was extracted from *Rhizobium* cells. The molecular weight of their glucan was about 3000, thus our glucan seems to have a molecular structure quite similar to it.

The amounts of cyclic (1→2)- β -D-glucan and the octasaccharide repeating-unit of succinoglycan with curdlan and succinoglycan per 100 ml culture medium produced by nine strains of *Agrobacterium* are shown in Table 3. The amounts of cyclic (1→2)- β -D-glucan and the octasaccharide repeating-unit were calculated from the results of GLC of methylated sugars from low molecular weight fractions by calculating the ratio of the peak of 3,4,6-tri-*O*-methyl-D-glucose derived from cyclic (1→2)- β -D-glucan and that of 2,3,6-tri-*O*-methyl-D-glucose derived from the octasaccharide. The former was produced by all the strains, although only in low yield by three strains incapable of forming curdlan. Seven strains produced the octasaccharide repeating-unit of succinoglycan, but two strains capable of producing quite large amounts of curdlan did not.

As shown in our previous papers (Hisamatsu *et al.*, 1978*b*, 1980*a*), *Alcaligenes faecalis* var. *myxogenes* strains 22 and 22-33, taxonomically close to *Agrobacterium* sp., produce the octasaccharide repeating-unit of succinoglycan. Therefore, the production of cyclic (1→2)- β -D-glucan and the octasaccharide repeating-unit of succinoglycan with curdlan and succinoglycan by these strains, the parent strain (10C3) and another mutant strain (10C3K) obtained from strain 10C3 were investigated. Strain 10C3, capable of producing succinoglycan and curdlan, produced the two products, whereas strains 22 and 22-33,

capable of producing only succinoglycan, produced only the octasaccharide and strain 10C3K, capable of producing only curdlan, produced only the cyclic (1 $\rightarrow$ 2)- β -D-glucan (Table 3). The octasaccharide is considered to be derived from an intermediate in the synthesis of succinoglycan (Hisamatsu *et al.*, 1978*b*). The use of strain 22-33 enabled us to elucidate the structure of the octasaccharide; strain 22-33 produces the octasaccharide in high yield but does not produce (1 $\rightarrow$ 2)- β -D-glucan.

The production of the two products by spontaneous mutant strains of *Agrobacterium* capable of producing curdlan in high yield with small amounts of succinoglycan were investigated (Table 3). Strains IFO 12665b1, 12665b2 and 13127b produced only cyclic (1 $\rightarrow$ 2)- β -D-glucan.

REFERENCES

- BARRETO-BERGTER, E., CAMARGO, C. R., HOGGE, L. R. & GORIN, P. A. J. (1980). Minor structures in β -D-(1 $\rightarrow$ 2)-linked D-glucopyranans from *Agrobacterium tumefaciens* and *Agrobacterium radiobacter*. *Carbohydrate Research* **82**, 366–371.
- DEDONDER, R. A. & HASSID, W. Z. (1964). The enzymatic synthesis of a (β -1,2)-linked glucan by an extract of *Rhizobium japonicum*. *Biochimica et biophysica acta* **90**, 239–248.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- GORIN, P. A. J., SPENCER, J. F. T. & WESTLAKE, D. W. S. (1961). The structure and resistance to methylation of 1,2- β -glucans from species of *Agrobacteria*. *Canadian Journal of Chemistry* **39**, 1067–1073.
- HAKOMORI, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalysed by methylsulfinyl carbanion in dimethyl sulfoxide. *Journal of Biochemistry* **55**, 205–208.
- HARADA, T. (1977). Production, properties and application of curdlan. In *ACS Symposium No. 45. Extracellular Microbial Polysaccharides*, p. 265. Edited by P. A. Sandford & A. Laskin.
- HARADA, T., MASADA, M., FUJIMORI, K. & MAEDA, I. (1966). Production of a firm, resilient gel-forming polysaccharide by a mutant of *Alcaligenes faecalis* var. *myxogenes* 10C3. *Agricultural and Biological Chemistry* **30**, 196–198.
- HARADA, T., AMEMURA, A., SAITO, H., KANAMARU, S. & MISAKI, A. (1968). Formation of succinoglycan and curdlan by parent and mutant strains of *Alcaligenes faecalis* var. *myxogenes* 10C3. *Journal of Fermentation Technology* **46**, 679–684.
- HISAMATSU, M., SANO, K., AMEMURA, A. & HARADA, T. (1978*a*). Acidic polysaccharides containing succinic acid in various strains of *Agrobacterium*. *Carbohydrate Research* **61**, 89–96.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T. (1978*b*). Formation of an oligosaccharide, the repeating unit of succinoglycan, by *Alcaligenes faecalis* var. *myxogenes*. *Carbohydrate Research* **66**, 289–294.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T. (1980*a*). Structure of the linear repeating unit of succinoglycan accumulated in cultures of *Alcaligenes faecalis* var. *myxogenes*. *Agricultural and Biological Chemistry* **44**, 461–462.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T. (1980*b*). Structural elucidation of succinoglycan and related polysaccharides from *Agrobacterium* and *Rhizobium* by fragmentation with two special β -D-glycanases and methylation analysis. *Agricultural and Biological Chemistry* **44**, 1049–1055.
- MATSUO, T., MATSUDA, H. & KATAKUSE, I. (1980). New Silicon emitter for field ionization and desorption mass spectrometry. In *Advances in Mass Spectrometry*, vol. 8A, pp. 990–996. Edited by A. Quayle. London: Heyden.
- MCINTIRE, F. C., PETERSON, W. H. & RIKER, A. J. (1942). A polysaccharide produced by the crown-gall organism. *Journal of Biological Chemistry* **143**, 491–496.
- PUTMAN, E. W., POTTER, A. L., HODGSON, R. & HASSID, W. Z. (1950). The structure of crown-gall polysaccharide. *Journal of the American Chemical Society* **72**, 5024–5026.
- SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.
- YAMADA, T., HISAMATSU, M. & TAKI, M. (1975). A method for detection of non-reducing saccharides. *Journal of Chromatography* **103**, 390–391.
- YORK, W. S., McNEIL, M., DARVILL, A. G. & ALBERSHEIM, P. (1980). Beta-2-linked glucans secreted by fast-growing species of *Rhizobium*. *Journal of Bacteriology* **142**, 243–248.
- ZEVENHUIZEN, L. P. T. M. & SCHOLTEN-KOERSELMAN, H. J. (1979). Surface carbohydrates of *Rhizobium*. I. β -1,2-Glucans. *Antonie van Leeuwenhoek* **45**, 165–175.