Occurrence of Extracellular (1→2)- β -D-Glucans and (1→2)- β -D-Gluco-oligosaccharides in *Acetobacter*

By AKINORI AMEMURA,¹* TETSUYA HASHIMOTO,¹
KYOKO KOIZUMI² AND TOSHIKO UTAMURA²

¹*Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567, Japan*

²*Faculty of Pharmaceutical Sciences, Mukogawa Women's University, 4-16 Edagawa, Nishinomiya 663, Japan*

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Of 11 strains of *Acetobacter* tested, three strains of *A. xylinum* (IFO 3288, IFO 13693 and IFO 13772), two strains of *A. aceti* (IFO 3281 and IFO 3283), one strain of *A. pasteurianus* (IFO 3223) and one strain of *A. rancens* (IFO 3297) were found to produce 2–25 mg per 100 ml culture filtrate of a mixture of (1→2)- β -D-glucans and (1→2)- β -D-gluco-oligosaccharides as low-molecular-weight products. The glucans and oligosaccharides were linear without branching structures and their degrees of polymerization (DPs) ranged from 6 to about 42. The compounds with lower DPs did not seem to be produced by hydrolysis of those with higher DPs. *A. xylinum* IFO 3288 and *A. xylinum* IFO 13693 also produced extracellular acidic polysaccharides containing L-rhamnose, D-glucose, D-mannose and uronic acid.

INTRODUCTION

Strains of *Agrobacterium* and *Rhizobium* produce cyclic (1→2)- β -D-glucan both extracellularly (Hisamatsu *et al.*, 1982; Amemura *et al.*, 1983; Dell *et al.*, 1983; Zevenhuizen & van Neerven, 1983) and intracellularly (Zevenhuizen & Scholten-Koerselman, 1979; Abe *et al.*, 1982). This glucan is unique in molecular form (cyclic form) and molecular size (DPs 17–24 – Dell *et al.*, 1983; Hisamatsu *et al.*, 1983; Koizumi *et al.*, 1983; DPs 17–40 – Koizumi *et al.*, 1984) and has so far been found only in strains of these genera. Previously, Sandermann & Dekker (1979) reported that membrane preparations of *Acetobacter xylinum* catalyse the transfer of D-[¹⁴C]glucose from UDP-D-[¹⁴C]glucose to a β -(1→2)-linkage. This implies that this organism also produces (1→2)- β -D-glucan. In this work we prepared a low-molecular-weight (LMW) fraction from cultures of 11 strains of *Acetobacter* essentially by the procedures used to prepare cyclic (1→2)- β -D-glucan of *Agrobacterium* and *Rhizobium*, and examined whether this glucan is present in this fraction.

METHODS

Organisms. *Acetobacter xylinum* IFO 3288, IFO 13693 and IFO 13772, *A. acetigenum* IFO 3277, IFO 3278, IFO 3279 and IFO 3280, *A. aceti* IFO 3281 and IFO 3283, *A. pasteurianus* IFO 3223 and *A. rancens* IFO 3297 were used. These 11 strains were obtained from the Institute for Fermentation, Osaka, Japan (IFO).

Media. Strains were maintained on slopes of medium containing (per 100 ml distilled water): glucose, 0.5 g; peptone, 0.5 g; yeast extract, 0.5 g; MgSO₄·7H₂O, 0.2 g; agar, 1.5 g and CaCO₃, 1 g (pH 7.0).

The medium used to examine polysaccharide production contained (per 100 ml distilled water): glucose, 4 g; (NH₄)₂HPO₄, 0.15 g; KH₂PO₄, 0.1 g; MgSO₄·7H₂O, 0.05 g; NaCl, 1 mg; CaCl₂, 1 mg; MnCl₂·4H₂O, 1 mg; yeast extract, 100 mg; CaCO₃, 0.5 g (pH 7.0). The carbon source and CaCO₃ were sterilized separately and mixed with other components before use.

For pellicle formation by static cultures the glucose/yeast extract medium described by Steel & Walker (1957) was used.

Abbreviations: DP, degree of polymerization; LMW, low molecular weight; PC, paper chromatography; TFA, trifluoroacetic acid.

Culture conditions for polysaccharide production. The medium (95 ml) in 500 ml conical flasks with baffles was inoculated with a seed culture (5 ml) that had been grown in the same medium. The culture was shaken at 150 r.p.m. at 28 °C for 6 d on a rotary shaker.

Preparation of cells, acidic polysaccharide and LMW neutral fraction. The following procedures were essentially as described by Hisamatsu *et al.* (1982). The culture broth was centrifuged at 56 000 *g* for 30 min to yield the cells and insoluble CaCO₃. The CaCO₃ was removed by washing with 1 M-HCl and then the remaining insoluble material was washed with water and dehydrated with acetone. The cells obtained in this way included cell surface polysaccharide, when this was present.

The supernatant of the culture broth was mixed with 2 vols ethanol and centrifuged to remove a high-molecular-weight fraction including acidic polysaccharide. The supernatant was then concentrated to a small volume, again mixed with 2 vols ethanol and centrifuged. The supernatant was mixed with 4 vols ethanol, and the resulting precipitate was collected by centrifugation, dissolved in water and passed through columns of Amberlite IR-120B (H⁺-form) and Amberlite IR-45 (OH⁻-form). The effluent containing neutral materials was collected, concentrated to a small volume and lyophilized (LMW neutral fraction).

The high-molecular-weight fraction was dissolved in water to give a 0.5% solution and appropriate amounts of 10% (w/v) cetylpyridinium chloride solution were added to precipitate acidic polysaccharide. The precipitate was collected by centrifugation, washed with water and dissolved in 10% (w/v) NaCl solution. Two volumes of ethanol were added to the solution and the resultant precipitate was collected by centrifugation, washed with a mixture of ethanol and water (6:1, v/v) and with ethanol and dried *in vacuo* (acidic polysaccharide).

Paper chromatography (PC). Samples on Toyo no. 50 filter paper (Toyo, Tokyo, Japan) were developed at 25 °C for 64 h by the descending method with a solvent system of 1-butanol/pyridine/water (6:4:3, by vol.). Sugars on the chromatograms were detected with AgNO₃ reagent.

Thin-layer chromatography. Samples on an HPTLC plate Silica Gel 60 (Merck) were developed three times with a solvent system of 1-butanol/ethanol/water (5:5:4, by vol.) for 10 h each at 25 °C. Sugars on the chromatograms were detected by spraying with 5% (v/v) conc. H₂SO₄ in ethanol and heating at 100 °C.

Gas-liquid chromatography. A GC7A chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame-ionization detector was used.

High-performance liquid chromatography. Analyses were performed at room temperature with a Tri Rotar SR-1 pump, a VL-614 variable-loop injector (both from Jasco, Tokyo, Japan) and an SE-31 refractive index monitor (Showa Denko, Tokyo, Japan). An ERC-NH-1171 column (200 × 6 mm i.d.) was used (Erma Optical Works, Tokyo, Japan).

Acid hydrolysis. Samples of the LMW neutral fraction were hydrolysed with 0.2 M-trifluoroacetic acid (TFA) at 100 °C for 100 min or with 2 M-TFA at 100 °C for 6 h. The acid was removed by vacuum evaporation. The products were examined by TLC.

Enzyme hydrolysis. Samples of the LMW neutral fraction were digested with appropriate amounts of a mixture of endo-(1→2)-β-D-glucanase and β-D-glucosidase. These enzymes were partially purified from cells of *Cytophaga arvensicola* A21L as described previously (Mendoza & Amemura, 1983). The products were examined by PC.

Methylation. Materials were methylated with sodium methyl sulphoxide as described by Hakomori (1964). The methylated samples were hydrolysed with 2 M-TFA at 100 °C for 6 h and analysed as alditol acetates by GLC as described by Hisamatsu *et al.* (1980). Methylated sugars were identified by comparison of their retention times with those of standard sugars.

Analysis of sugars. Samples of acidic polysaccharides were hydrolysed with 90% (w/v) formic acid at 100 °C for 6 h and then with 2 M-TFA at 100 °C for 6 h and analysed as alditol acetates by GLC as described previously (Ghai *et al.*, 1981). Uronic acids were determined by the modified carbazole reaction described by Galambos (1967).

Analysis of organic acids. Pyruvic acid was analysed by the method of Koepsell & Sharpe (1952). Acyl groups were assayed by the method of McComb & McCready (1957).

Chemicals. The cyclic (1→2)-β-D-glucans used for reference purposes were prepared from culture filtrates of *Agrobacterium radiobacter* IFO 12665b1 and *Rhizobium meliloti* IFO 13336, as described previously (Hisamatsu *et al.*, 1982). A cyclic glucan component of DP 27 was isolated by HPLC from the glucan mixture of *R. meliloti* IFO 13336 as described previously (Koizumi *et al.*, 1984). A mixture of standard sophoro-oligosaccharides and polysaccharides was obtained by partial hydrolysis of cyclic (1→2)-β-D-glucan with 0.1 M-TFA at 100 °C for 2 h and then removal of the acid by vacuum evaporation. Sophoro-octaose was prepared by fractionation of partially hydrolysed (1→2)-β-D-glucan by PC.

Other chemicals used were commercial products of the highest purity available.

RESULTS

Characterization of the LMW neutral fraction of A. xylinum IFO 3288

The LMW neutral fraction obtained from the culture of *A. xylinum* IFO 3288 was readily soluble in water and its $[\alpha]_D^{20}$ was +2.4° (*c* 0.5, water). The material gave many spots on TLC,

Fig. 1. Thin-layer chromatograms of the LMW neutral fraction of *A. xylinum* IFO 3288 and its hydrolysis products. (a, e), Partial hydrolysate of standard cyclic (1→2)- β -D-glucan (DPs 17–24) of *Agrobacterium radiobacter* IFO 12665b1. (b) Native LMW neutral fraction. (c) Hydrolysate of the LMW neutral fraction with 0.2 M-TFA at 100 °C for 100 min. (d) Hydrolysate of the LMW neutral fraction with 2 M-TFA at 100 °C for 6 h. Samples of 100–140 μ g were applied to the plate. G1 indicates the position of glucose and G5–G20 indicate the positions of the respective sophoro-oligo- and polymers.

which were detected by spraying the chromatogram with H_2SO_4 . The R_F values of these spots coincided with those of (1→2)- β -D-glucan-oligosaccharides (sophoro-oligosaccharides) obtained by partial hydrolysis of standard cyclic (1→2)- β -D-glucan (Fig. 1 b). The spot with the highest R_F value was located in the same position as sophorohexaose. Two faint spots were also detectable between sophorose and sophoropentaose, which differed from sophoro-oligosaccharides in R_F values. Partial hydrolysis of the LMW neutral fraction with TFA resulted in formation of glucose and oligosaccharides with higher R_F values corresponding to those of sophorose, sophorotriose, etc. (Fig. 1 c). Complete acid hydrolysis of this fraction yielded only glucose (Fig. 1 d). Products with DPs of more than 25 could be detected on the chromatogram of the partial hydrolysate.

Next the LMW neutral fraction was hydrolysed with a mixture of endo-(1→2)- β -D-glucanase and β -D-glucosidase prepared from cells of *Cytophaga arvensicola* A21L. The former enzyme has

Fig. 2. Liquid chromatogram of the LMW neutral fraction of *A. xylinum* IFO 3288. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm i.d.); eluent, acetonitrile/water (55:45); flow rate, 1 ml min⁻¹; detector, Shodex RI SE-31 at 1 × 10⁻⁵ RI units full-scale; temperature, ambient.

high specificity for β -(1→2)-glucosidic linkages and readily hydrolyses the cyclic (1→2)- β -D-glucan of *Agrobacterium radiobacter* IFO 12665b1 and higher sophoro-oligosaccharides than sophoropentaose in a random manner. The latter enzyme is a specific β -D-glucosidase that hydrolyses only β -(1→2)- and β -(1→3)-glucosidic linkages. The products of enzymic hydrolysis of the LMW neutral fraction were found by PC to be glucose, oligosaccharides corresponding to sophoro-oligosaccharides and laminarabiose, formed by the transglucosylation activity of β -D-glucosidase, and the final product of hydrolysis, glucose. The faint spot seen between sophorotetraose and sophoropentaose on the thin-layer chromatogram (Fig. 1*b*) was observed as a more intense spot with AgNO₃ reagent on the paper chromatogram. This also shows that this material with strong reducing power is not sophoro-oligosaccharide, which gives a weak reaction with AgNO₃ reagent because of the linkage at carbon 2.

Methylation of the LMW neutral fraction and subsequent hydrolysis of the methylated product yielded mainly 3,4,6-tri-*O*-methyl-D-glucose with some 2,3,4,6-tetra-*O*-methyl-D-glucose. No appreciable amounts of di-methyl sugars or other tri-methyl sugars were found, indicating that the neutral fraction is composed predominantly of (1→2)-linked D-glucosyl residues without branching.

The LMW neutral fraction was subjected to HPLC on an NH₂-bonded silica column under various conditions. The elution profiles were superposable on those of a partial acid hydrolysate of cyclic (1→2)- β -D-glucan. One of the elution profiles is shown in Fig. 2. The DPs of the peaks shown were determined by comparison with the DPs of a partial acid hydrolysate of cyclic (1→2)- β -D-glucan. The last detectable peak corresponded to DP 42. Several peaks eluted earlier than sophorohexaose were not sophoro-oligosaccharide, judging from the retention times.

When the LMW neutral fraction was mixed with a partial hydrolysate of cyclic (1→2)- β -D-glucan (DP 27) and injected into the column, the peaks of the two samples were found to coincide exactly without any shoulders or broadened peaks (Fig. 3), while when mixed with cyclic (1→2)- β -D-glucan (a mixture of cyclic glucans of DPs 17–40) of *R. meliloti* IFO 13336, the peaks of the two samples did not coincide. Thus, the LMW neutral fraction was confirmed to consist of a mixture of linear (1→2)- β -D-glucans and (1→2)- β -D-glucan-oligosaccharides.

Although the lowest DP of the sophoro-oligosaccharides in the preparation was 6, it seemed possible that sophoro-oligosaccharides of DP lower than 6 might be present in the culture filtrate and might be removed from the filtrate during the alcohol fractionation used to prepare the LMW neutral fraction. To examine this possibility, the culture supernatant (6-d culture) was passed through columns of Amberlite IR-120B (H⁺-form) and Amberlite IR-45 (OH⁻-form) without alcohol fractionation, and subjected to TLC. Hexaose and higher oligosaccharides were found on the chromatogram, but lower sophoro-oligosaccharides were not.

Fig. 3. Liquid chromatogram of a mixture of the LMW neutral fraction and the partial hydrolysate (a mixture of glucose and linear (1→2)- β -D-gluco-oligo- and polymers with DPs of 2–27) of standard cyclic (1→2)- β -D-glucan (DP 27) of *R. meliloti* IFO 13336. Chromatographic conditions: eluent, acetonitrile/water (60:40); other conditions as for Fig. 2.

The next problem was whether the oligosaccharides and lower-molecular-weight glucans present in the preparation were produced by enzymic degradation of high-molecular-weight glucans during cultivation. We examined this problem by TLC of the LMW neutral fractions obtained from cultures on days 1, 2, 3, 4 and 6. The chromatographic patterns from all these samples were the same. Moreover, by TLC, we could not detect degrading activities against sophoro-octaose and the LMW neutral fraction in the cell-free extract and culture filtrate of strain IFO 3288, and this organism could not use the LMW neutral fraction, cyclic (1→2)- β -D-glucan of *Agrobacterium radiobacter* IFO 12665b1, or sophoro-octaose as the sole source of carbon. From these results we concluded that the oligosaccharides and lower-molecular-weight glucans are not produced from high-molecular-weight glucans by enzymic hydrolysis during cultivation.

Production of (1→2)- β -D-glucans and (1→2)- β -D-gluco-oligosaccharides by various strains of Acetobacter

LMW neutral fractions from 10 other strains of *Acetobacter* were prepared and examined by PC and TLC. *A. xylinum* IFO 13693 and IFO 13772, *A. aceti* IFO 3281 and IFO 3283, *A. pasteurianus* IFO 3223 and *A. rancens* IFO 3297 were also found to produce a mixture of (1→2)- β -D-glucans and (1→2)- β -D-gluco-oligosaccharides with various DPs. The chromatographic patterns of the (1→2)- β -D-glucans and oligosaccharides from each strain were similar to that of strain IFO 3288, and in all cases the smallest DP was 6. The preparations from strains IFO 13693, IFO 13772 and IFO 3223 were also analysed by GLC and confirmed to be composed of (1→2)-glucosidic linkages. Four strains of *A. acetigenum* (IFO 3277, IFO 3278, IFO 3279 and IFO 3280) did not produce these compounds although 1–3 mg of LMW neutral fraction was obtained. All 10 strains produced an unidentified reducing material located between sophorotetraose and sophoropentaose on paper and thin-layer chromatograms, as found in the preparation of strain IFO 3288.

The amounts of (1→2)- β -D-glucans and oligosaccharides of 11 strains produced per 100 ml of culture media are shown with the amounts of cells and extracellular acidic polysaccharide in Table 1. Two strains of *A. xylinum* (IFO 3288 and IFO 13693) produced an acidic polysaccharide composed of D-glucose, D-mannose, L-rhamnose and uronic acid in a molar ratio of 7–8:1:2:2 and some acyl groups. Pyruvic acid was not detected in the polysaccharide. Static

Table 1. *Formation of (1→2)-β-D-glucans and (1→2)-β-D-gluco-oligosaccharides, extracellular acidic polysaccharide and pellicle by strains of Acetobacter*

Strain	pH	Cells [mg (100 ml) ⁻¹]	(1→2)-β-D-Glucans and (1→2)-β-D-gluco- oligosaccharides [mg (100 ml) ⁻¹]	Extracellular acidic polysaccharide [mg (100 ml) ⁻¹]	Pellicle formation
<i>A. xylinum</i>					
IFO 3288	8.1	272	25.0	87.0	—
IFO 13693	7.0	388	5.4	27.8	+
IFO 13772	5.0	607	9.7	0	+
<i>A. acetigenum</i>					
IFO 3277	5.4	179	0	0	—
IFO 3278	6.7	504	0	0	—
IFO 3279	5.0	94	0	0	—
IFO 3280	6.2	127	0	0	—
<i>A. aceti</i>					
IFO 3281	5.2	139	2.0	0	—
IFO 3283	5.1	62	1.8	0	—
<i>A. pasteurianus</i>					
IFO 3223	4.9	239	15.0	0	—
<i>A. rancens</i>					
IFO 3297	5.1	68	2.3	0	—

cultures of strains IFO 13693 and IFO 13772 of *A. xylinum* produced a thick pellicle of cellulose on the surface of the liquid medium. Formation of this pellicle is a characteristic of *A. xylinum*, but was lost in the strain IFO 3288 we used.

DISCUSSION

Strains of *Acetobacter* were demonstrated to produce (1→2)-β-D-glucans and (1→2)-β-D-gluco-oligosaccharides with DPs of 6 to about 42 in their culture filtrates. These compounds have a linear structure, unlike the cyclic structure of (1→2)-β-D-glucans obtained from strains of *Agrobacterium* and *Rhizobium*. The lowest DP of the latter glucans so far reported is 17, whereas the glucans of *Acetobacter* contained components with lower DPs. (1→2)-β-D-Glucans were thought to be rarely occurring polysaccharides and were previously found only in strains of genera of the family *Rhizobiaceae*. But they may be widely distributed in other micro-organisms, as we found them in the genus *Acetobacter*.

The biological functions of (1→2)-β-D-glucans in bacteria are unknown, but there is evidence that cyclic (1→2)-β-D-glucan from *Rhizobium* influences symbiosis in leguminous plants. Abe *et al.* (1982) demonstrated that cyclic (1→2)-β-D-glucan from *R. trifolii* 4S increases infection thread formation and nodulation in clover, the host plant. *A. xylinum* is known to produce cellulose ((1→4)-β-D-glucan). Some workers (Walker & Wright, 1957; Weigel, 1961; Kjosbakken & Colvin, 1975; Colvin *et al.*, 1977) have suggested that a water- or alkali-soluble polymer may be a precursor of cellulose. The polymers they isolated are related to cellulose in chemical structure, although the polymer reported by Colvin *et al.* (1977) has branches of single glucosyl residues attached by β-(1→2)-linkages to the main chain of β-(1→4)-linkages. However, the water-soluble glucans examined in the present study are unlikely to be related to cellulose synthesis, since they were composed exclusively of β-(1→2)-linkages and their production was not related to ability of cellulose pellicle formation, as shown in Table 1.

The (1→2)-β-D-glucans isolated from the culture filtrates of *Acetobacter* are probably the same type as the glucan synthesized from UDP-D-[¹⁴C]glucose by membrane fractions of *A. xylinum* (Sandermann & Dekker, 1979). Their glucan has a much higher molecular weight, and is eluted in the void volume from Sephadex G-100, whereas the highest DP of the glucans detected here was only 42. We examined only LMW fractions obtained by ethanol fractionation of culture filtrates for the presence of (1→2)-β-D-glucan, but all the strains tested produced appreciable

amounts of high-molecular-weight fractions precipitated by addition of 2 vols ethanol. Therefore, (1→2)- β -D-glucans of high molecular weight, if present, may have been discarded. Acidic polysaccharides were isolated from the high-molecular-weight fractions of two strains (IFO 3288 and IFO 13693). The sugar constituents were the same as those of the polysaccharides of *A. xylinum* (Valla & Kjosbakken, 1981; Couso *et al.*, 1982).

REFERENCES

- ABE, M., AMEMURA, A. & HIGASHI, S. (1982). Studies on cyclic β -1,2-glucan obtained from periplasmic space of *Rhizobium trifolii* cells. *Plant and Soil* **64**, 315–324.
- AMEMURA, A., HISAMATSU, M., MITANI, H. & HARADA, T. (1983). Cyclic (1→2)- β -D-glucan and the octasaccharide repeating-units of extracellular acidic polysaccharides produced by *Rhizobium*. *Carbohydrate Research* **114**, 277–285.
- COLVIN, J. R., CHÉNÉ, L., SOWDEN, L. C. & TAKI, M. (1977). Purification and properties of a soluble polymer of glucose from cultures of *Acetobacter xylinum*. *Canadian Journal of Biochemistry* **55**, 1057–1063.
- COUSO, R. O., IELPI, L., GARCIA, R. C. & DANKERT, M. A. (1982). Biosynthesis of polysaccharides in *Acetobacter xylinum*. Sequential synthesis of heptasaccharide diphosphate prenil. *European Journal of Biochemistry* **123**, 617–627.
- DELL, A., YORK, K. W. S., MCNEIL, M., DARVILL, A. G. & ALBERSHEIM, P. (1983). The cyclic structure of β -D-(1→2)-linked D-glucans secreted by *Rhizobia* and *Agrobacteria*. *Carbohydrate Research* **117**, 185–200.
- GALAMBOS, J. T. (1967). The reaction of carbazole with carbohydrates. I. Effects of borate and sulfamide on the carbazole color of sugars. *Analytical Biochemistry* **19**, 119–132.
- GHAJ, S. K., HISAMATSU, M., AMEMURA, A. & HARADA, T. (1981). Production and chemical composition of extracellular polysaccharides of *Rhizobium*. *Journal of General Microbiology* **122**, 33–40.
- HAKOMORI, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalysed by methylsulfinyl carbanion in dimethyl sulfoxide. *Journal of Biochemistry* **55**, 205–208.
- HISAMATSU, M., ABE, J., AMEMURA, A. & HARADA, T. (1980). 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.
- HISAMATSU, M., AMEMURA, A., MATSUO, T., MATSUDA, H. & HARADA, T. (1982). Cyclic (1→2)- β -D-glucan and the octasaccharide repeating-unit of succinoglycan produced by *Agrobacterium*. *Journal of General Microbiology* **128**, 1873–1879.
- HISAMATSU, M., AMEMURA, A., KOIZUMI, K., UTAMURA, T. & OKADA, Y. (1983). Structural studies on cyclic (1→2)- β -D-glucans (cyclosophoraoses) produced by *Agrobacterium* and *Rhizobium*. *Carbohydrate Research* **121**, 31–40.
- KJOSBAKKEN, J. & COLVIN, J. R. (1975). New evidence for an intermediate polymer of glucose in cellulose biosynthesis by *Acetobacter xylinum*. *Canadian Journal of Microbiology* **21**, 111–121.
- KOEPSSELL, H. J. & SHARPE, E. S. (1952). Microdetermination of pyruvic acid and α -ketoglutaric acids. *Archives of Biochemistry and Biophysics* **38**, 443–449.
- KOIZUMI, K., OKADA, Y., HORIYAMA, S., UTAMURA, T., HISAMATSU, M. & AMEMURA, A. (1983). Separation of cyclic (1→2)- β -D-glucans (cyclosophoraoses) produced by *Agrobacterium* and *Rhizobium*, and determination of their degrees of polymerization by high-performance liquid chromatography. *Journal of Chromatography* **265**, 89–96.
- KOIZUMI, K., OKADA, Y., UTAMURA, T., HISAMATSU, M. & AMEMURA, A. (1984). Further studies on the separation of cyclic (1→2)- β -D-glucans (cyclosophoraoses) produced by *Rhizobium meliloti* IFO 13336, and determination of their degrees of polymerization by high-performance liquid chromatography. *Journal of Chromatography* **299**, 215–224.
- MCCOMB, E. A. & MCCREADY, R. M. (1957). Determination of acetyl in pectin and in acetylated carbohydrate polymers. *Analytical Chemistry* **29**, 819–821.
- MENDOZA, N. S. & AMEMURA, A. (1983). (1→2)- β -D-Glucan-hydrolyzing enzymes in *Cytophaga arvensicola*: Partial purification and some properties of endo-(1→2)- β -D-glucanase and β -D-glucosidase specific for (1→2)- and (1→3)-linkages. *Journal of Fermentation Technology* **61**, 473–481.
- SANDERMANN, H., JR & DEKKER, R. F. H. (1979). β -1,2-Glucosyl transfer by membrane preparations from *Acetobacter xylinum*. *FEBS Letters* **107**, 237–240.
- STEEL, R. & WALKER, T. K. (1957). Celluloseless mutants of certain *Acetobacter* species. *Journal of General Microbiology* **17**, 12–18.
- VALLA, S. & KJOSBAKKEN, J. (1981). Isolation and characterization of a new extracellular polysaccharide from a cellulose-negative strain of *Acetobacter xylinum*. *Canadian Journal of Microbiology* **27**, 599–603.
- WALKER, T. K. & WRIGHT, H. B. (1957). Oligosaccharide formation during synthesis of cellulose by *Acetobacter acetigenum*. *Archives of Biochemistry and Biophysics* **69**, 362–371.
- WEIGEL, J. (1961). Über den Stoffwechsel und die Cellulose-synthese von *Acetobacter xylinum* (Brown) Holland. *Archiv für Mikrobiologie* **38**, 350–360.
- ZEVENHUIZEN, L. P. T. M. & VAN NEERVEN, A. R. W. (1983). (1→2)- β -D-Glucan and acidic oligosaccharides produced by *Rhizobium meliloti*. *Carbohydrate Research* **118**, 127–134.
- ZEVENHUIZEN, L. P. T. M. & SCHOLTEN-KOERSELMAN, H. J. (1979). Surface carbohydrates of *Rhizobium*. I. β -1,2-Glucan. *Antonie van Leeuwenhoek* **45**, 165–175.