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Note

Crystallographic data on bacterial (1 → 4)-β-D-glucuronan

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It was found recently [1] that the mutant strain M5N1 C.S. (NCIMB 40472) of *Rhizobium meliloti* was able to produce an extracellular (1 → 4)-β-D-glucuronan of relatively high molecular weight. Previously this glycuronan, known as mucoric acid, had never been isolated from any bacterial system. It had been described and characterized as a fragment of mucoran, a more complicated heteropolysaccharide, produced in the cell walls and extracellular media of some moulds belonging to the order *Mucorales* [2–5]. With mucoran, the extraction of pure poly[(1 → 4)-β-D-glucuronic acid] fractions required such a strong acid treatment that only a low molecular weight fraction could be obtained [2–5]. In contrast with the fungal mucoric acid, bacterial (1 → 4)-β-D-glucuronan was not only of perfect regularity but also had a weight average molecular weight $\overline{M}_w$, ranging from 6×10^4 to 5×10^5, and was easily extractable [1]. Depending on the fermentation conditions, it occurred as a partially acetylated product, acetylated on the C-2 and/or C-3 positions of the glucuronic acid residues. Removal of these acetyl groups produced the pure (1 → 4)-β-D-glucuronan.

An interesting property of bacterial (1 → 4)-β-D-glucuronan is its ability to form gels in the presence of divalent cations [1]. This behaviour is similar to that of other poly(glycuronic acids) such as pectins or alginates [6,7]. A classical application for such rapid gelation is in the industrial spinning of sodium alginate into a coagulation bath of aqueous calcium chloride [8]. The purpose of this study was to

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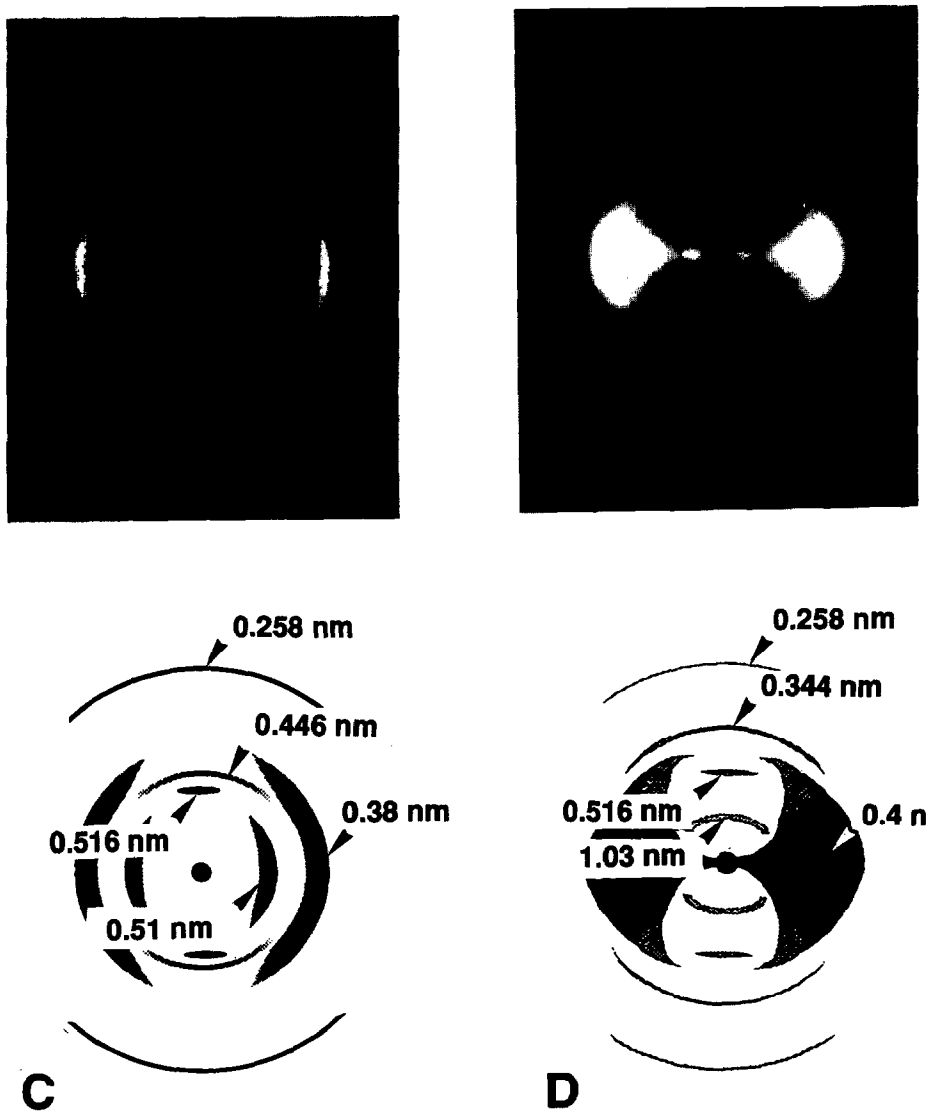

Fig. 1. (A) X-ray fibre diagram of deacetylated (1 → 4)-β-D-glucuronan. (B) X-ray fibre diagram of acetylated (1 → 4)-β-D-glucuronan. (C) Schematic diagram corresponding to the pattern in 1A. (D) Schematic diagram corresponding to the pattern in 1B.

see whether similar fibres could be obtained for bacterial (1 → 4)-β-D-glucuronan and, in that case, to measure their X-ray fibre diagrams.

Acetylated and deacetylated (1 → 4)-β-D-glucuronan fibres gave slightly different diffraction diagrams. These patterns, shown in Figs. 1A and B are schematically drawn in Figs. 1C and D. The pattern of the deacetylated sample (Fig. 1A)

displays a better resolution than that of the acetylated product (Fig. 1B). It consists of three rather sharp arcs (at 0.258 ± 0.003, 0.446 ± 0.003, and 0.516 ± 0.003 nm) along the meridian and two broad arcs centered at around 0.38 ± 0.01 and 0.51 ± 0.01 nm on the equator. Along the meridian, the arc at 0.446 nm has a much broader angular spread than those at 0.258 and 0.516, which obviously correspond to the same type of reflection but of different order. When the fibre was rotated by 14° about an axis perpendicular to the fibre direction, the meridional arc at 0.446 nm split whereas those at 0.258 and 0.516 nm did not. Thus, the periodicity along the fibre axis must correspond to a multiple of 0.516 nm.

Comparison of the pattern in Fig. 1A with known fibre diagrams of related β-(1 → 4)-D-linked polysaccharides, such as cellulose [9], chitin [10], mannan [11], or poly(β-D-mannuronic acid) [12], shows that a good correspondence exists between their fibre repeat at around 1.03 nm and that of the present sample. Thus, in the diagram shown in Fig. 1A, the arcs at 0.258 and 0.516 nm can be assigned to the 002 and 004 reflections. In such a scheme, the absence of reflections with indices 001 and 003 suggests a two-fold screw symmetry for this polysaccharide. The off meridional arc at 0.446 nm, which has a much broader angular spread, corresponds to diffraction planes giving reflections on the second layer line.

The pattern of the acetylated product in Fig. 1B is of poorer resolution. It consists of four rather sharp reflections along the meridian at 0.258 ± 0.004, 0.344 ± 0.006, 0.516 ± 0.003, and 1.03 ± 0.03 nm and a broad smear centered at around 0.4 nm on the equator. This diagram seems to correspond more to a Fourier transform of individual molecules than to a crystalline fibre pattern. The meridional reflections can be assigned to the first, second, third, and fourth order of 1.03 nm, that corresponds to the same fibre repeat as in Fig. 1A. The occurrence of the reflection at 1.03 and 0.344 nm, assigned to the first and third orders, indicates that a two-fold screw periodicity no longer exists in the acetylated fibres.

When the (1 → 4)-β-D-glucuronan was reduced, it gave a powder, the X-ray pattern of which corresponded to that of cellulose II. This is illustrated in Fig. 2 where the diagram of the reduced material 2A is compared with 2B, a powder pattern recorded on hydrolyzed fortisan. Despite a lower crystallinity, the reduced sample showed the three characteristic strong equatorial spacings of cellulose II at 0.718 ± 0.006, 0.440 ± 0.005, and 0.404 ± 0.005 nm [13].

The preliminary results presented in this study are, we believe, the first crystallographic data on (1 → 4)-β-D-glucuronan. Despite fibre diagrams of limited resolution, our results demonstrate that upon crystallization, this glucuronan adopts a ribbon-like two-fold conformation similar to that of many β-(1 → 4)-linked polysaccharides. In particular, the present sample adopts a two-fold screw conformation identical to that of its isomer: poly(β-D-mannuronic acid) [12]. In contrast to this constituent of alginic acid, bacterial (1 → 4)-β-D-glucuronan is structurally more regular and of higher molecular weight. It should therefore be superior both for fibre spinning and structural work. In comparing the present sample with poly[(1 → 4)-β-D-mannuronic acid], it remains to be seen whether it has a better ability to form strong gels in the presence of divalent cations.

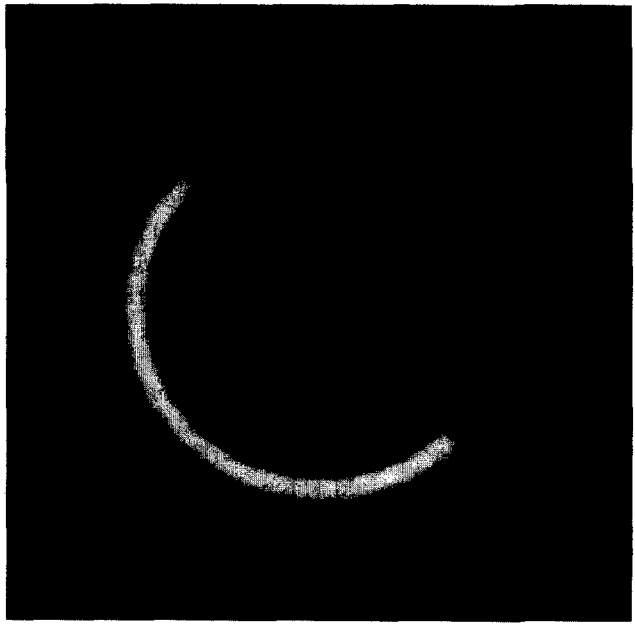

Fig. 2. (A) Powder diagram corresponding to the reduced (1 → 4)-β-D-glucuronan. (B) Powder diagram of hydrolyzed fortisan, taken as a cellulose II crystalline standard.

1. Experimental

Isolation of the polysaccharide.—(1 → 4)-β-D-Glucuronan was obtained by fermentation, using the mutant strain M5N1 C.S. (NCIMB 40472) of *Rhizobium meliloti* cultivated on a modified Laird R.C. medium [14]. Each liter of medium contained K_2HPO_4 (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), yeast extract (1.0 g), and sucrose (10 g). After fermentation, the culture medium was centrifuged and microfiltered in order to remove the bacteria.

Sodium chloride was then added to the culture medium to bring the solution to 1 M in salt. The polysaccharide was then precipitated by addition of 75% (v/v) 2-propanol. The precipitate was then washed several times with concentrations of 2-propanol increasing from 75 to 99%, before being dried for 48 h at room temperature in vacuo.

Some of the (1 → 4)-β-D-glucuronan was deacetylated for 4 h at 50°C after addition of sufficient aq NaOH to a dilute (1 g/L) polysaccharide solution in order to reach a pH of 10.

Reduction.—A fraction of (1 → 4)-β-D-glucuronan was reduced to cellulose by the action of a water soluble carbodiimide following a method described earlier [15].

Preparation of fibres.—The method was adapted from that used for the preparation of fibres from poly[(1 → 4)-β-D-mannuronic acid] [12]. Drops of an aq 1%

solution of (1 → 4)-β-D-glucuronan, neutralized as the sodium salt, were coagulated in a bath consisting of a satd aq $CaCl_2$ solution. The resulting gelatinous drops were then quickly pulled out from the coagulation bath to yield gelatinous fibres which were hung to dry under tension. The dried fibres were then immersed for 30 min in 7:3 EtOH–0.5 N HCl, before being washed several times with 50% aq EtOH and allowed to dry. The fibres were annealed overnight at 55°C at a relative humidity of 95% in an attempt to improve crystallinity.

X-ray diffraction analysis.—X-ray diffractograms were recorded with a Siemens Kristalloflex 2 X-ray generator equipped with a Wahrus flat film vacuum camera and operated with Ni-filtered Cu$K\alpha$ radiation. The diffractograms were calibrated by dusting the specimen with calcite. The *d* spacing values, together with their errors, were calculated from a number of diffractograms. The fibre diagrams were either recorded in vacuo or with the fibres sealed into thin-walled X-ray capillaries where a relative humidity of 95% was maintained.

Cellulose standard.—A standard of cellulose II was prepared from fortisan fibres by refluxing for 4 h a sample of fortisan fibres in 2.5 N HCl. This produced a microcrystalline powder of cellulose II which was neutralized, washed, and dried.

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