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Note

Structural analysis of the cell-wall D-glucuronans from the fungi *Absidia cylindrospora*, *Mucor mucedo*, and *Rhizopus nigricans**

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The cell walls of fungi belonging to the Zygomycotina consist of 20–30% of neutral sugar and uronic acid[2–6] which are thought to be present in two different types of acidic polysaccharide referred to as mucoran and mucoric acid[3,7,8]. Mucoran was extracted from the cell walls of *Mucor rouxii* by alkali[7], and from *Mucor mucedo* by alkali, nitrous acid treatment, and salt solution[9]. Mucoric acid resisted both acid and alkali treatments, but could be extracted by alkali after acid treatment[7]. This acid-insoluble polysaccharide was composed only of D-glucuronic acid[7] and was derived from mucoran by acid treatment[9]. The structures of mucoran and mucoric acid have not been elucidated in detail.

We now report on D-glucuronans Ac, Mm, and Rn from the fungi *Absidia cylindrosopra*, *Mucor mucedo*, and *Rhizopus nigricans*, respectively. These glucuronans were isolated from defatted cell-walls by extraction with alkali after treatment with acid. A typical elution profile for these glucuronans from DEAE-Sephadex A-25 (Cl^- form) is shown in Fig. 1*a*. Fraction Rn-I was eluted by a linear gradient of NaCl, and fraction Rn-II by M NaCl containing 0.02M NaOH. The yields of Rn-I and Rn-II were 30.8% and 41.7%, respectively. Similar results were obtained with Ac and Mm. The purified glycuronans were homogeneous in molecular size, as shown by their elution profile from Sephadex G-100 (illustrated for Mm in Fig. 2). The molecular sizes of Ac-I, Ac-II, Mm-I, Mm-II, Rn-I, and Rn-II were estimated as 3,400, 4,000, 3,300, 3,600, 3,300, and 3,600, respectively (Table I). Thus, the molecular weights of fractions I were slightly smaller than those of fractions II. However, when the latter fractions were re-chromatographed on DEAE-Sephadex A-25, their elution profiles were similar to that of the total alkali-solution material (illustrated for Rn-II in Fig. 1*b*). This behaviour was probably due to partial precipitation on the gel under the initial acidic conditions.

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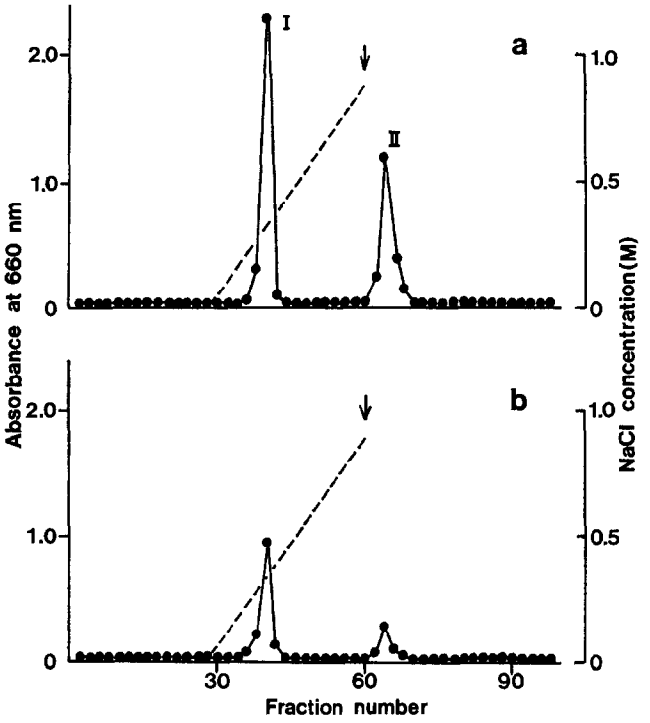

Fig. 1. Elution profiles of the glucuronans from DEAE-Sephadex A-25 (Cl^- form): (*a*) typical elution profiles of Ac, Mm, and Rn; (*b*) Rn-II.

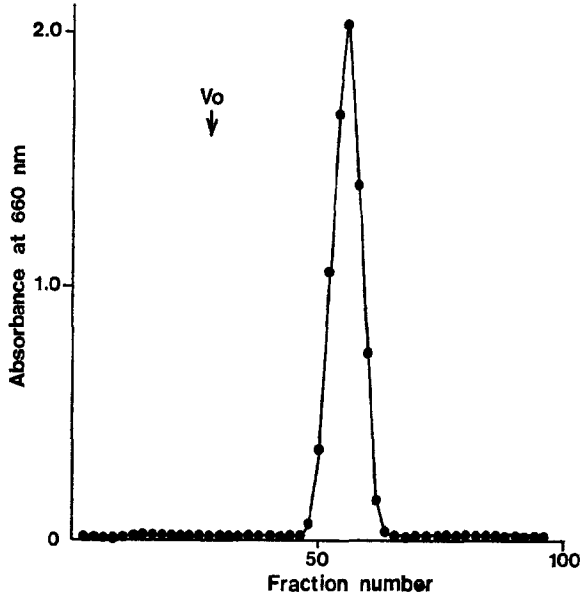

Fig. 2. Elution profile of Mm-I from Sephadex G-100.

TABLE I

ANALYTICAL DATA FOR THE GLYCURONANS

Glycuronan	$[\alpha]_D$ *(degrees)*	*Uronic acid content (%)*	*C/O ratio*	*Molecular size*[a]
Ac I	−93	96.7	0.99	3400
II	−92	95.0	1.07	4000
Mm I	−93	96.4	0.98	3300
II	−91	95.0	1.01	3600
Rn I	−92	95.4	1.03	3300
II	−92.5	96.4	1.02	3600

[a]Estimated by gel filtration on Sephadex G-100.

The properties of the purified glycuronans are shown in Table I. The uronic acid contents and the carbazole–orcinol ratios were consistent with glucuronans, and the $[\alpha]_D$ values were consistent with β-D-glucuronic acid residues. The glycuronans contained no nitrogen, phosphorus, or sulfate.

In the ^{1}H-n.m.r. spectra of these glycuronans (Table II), the signals for H-1 and the $J_{1,2}$ values were uniformly δ ~4.64 and ~8.0 Hz, respectively, consistent with β-D-glucuronic acid residues. The ^{13}C-n.m.r. (Fig. 3 and Table II) signals were similar to those[10] of (1→4)-linked β-D-glucuronic acid residues in protuberic acid. Thus, it is concluded that the glycuronans Ac, Mm, and Rn are linear, (1→4)-linked β-D-glucuronans and this was confirmed by methylation analysis of R-Rn, which gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol in the ratio 1:27.

Two fungal glycuronans have been described, namely, protuberic acid[10–13] and mucoric acid[7]. The former was found in the Phallales belonging to Basidio-

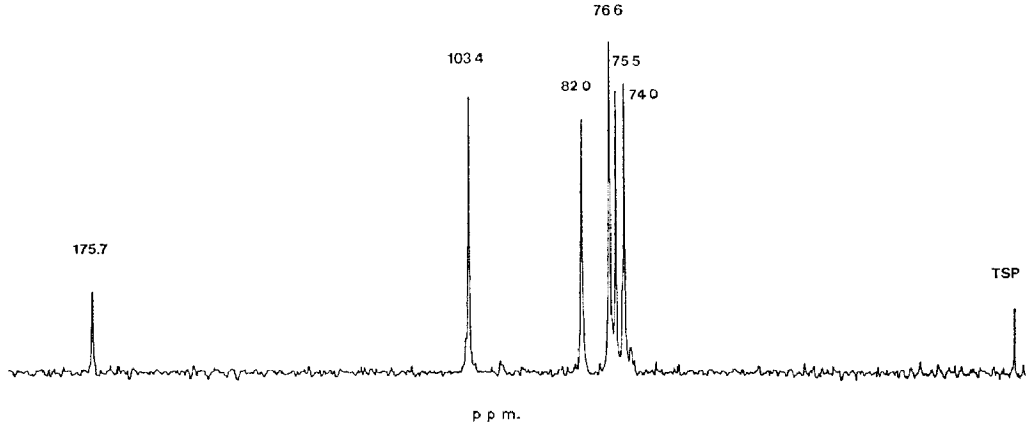

Fig. 3. ^{13}C-N.m.r. spectrum of Mm-I.

TABLE II

N.M.R. DATA FOR THE GLYCURONANS

Glycuronan	*Chemical shifts (p.p.m.)*							*($J_{1,2}$ in Hz)*
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*H-1*	
Ac I	103.5	74.0	75.5	82.0	76.7	175.7	4.64	(8.0)
II	103.5	74.0	75.6	82.1	76.7	175.5	4.63	(8.0)
Mm I	103.4	74.0	75.5	82.0	76.6	175.7	4.64	(8.0)
II	103.5	74.0	75.5	82.0	76.7	175.9	4.64	(8.0)
Rn I	103.5	74.0	75.5	82.2	76.6	175.5	4.63	(8.0)
II	103.5	74.0	75.6	82.3	76.7	175.5	4.64	(8.0)
PA[a]	103.5	74.2	75.4	82.2	76.6	176.1	4.55	(7.3)

[a]Signals for (1→4)-linked β-D-glucuronic acid residues in protuberic acid.

mycotina[14]. In the Mucoraceae belonging to Zygomycotina, several polysaccharides have been reported[5,15–19]. One of them, mucoran, is a single heteropolymer, which has an acid-resistant portion[7,9] that we have now shown to be a (1→4)-linked β-D-glucuronan. It is probable that D-glucuronans of this type are common to fungi belonging to the Mucoraceae.

EXPERIMENTAL

Fungi. — *Absidia cylindrospora* (IFO 4000), *Mucor mucedo* (IFO 6750), and *Rhizopus nigricans* (IAM 6070) were grown in YPG medium (6 L) containing Daigo yeast extract (3 g), Daigo polypeptone (10 g), D-glucose (20 g), and distilled water to make 1 L. The pH was adjusted to 4.0 with 0.5M H_2SO_4, and the medium was autoclaved at 120° for 20 min. Portions (150 mL) were inoculated with 1×10^5 spores from 7-day cultures on YPG agar (2% agar in YPG medium), and incubated at 27° on a reciprocating shaker (110 strokes/min). After 4 days for *A. cylindrospora* and *R. nigricans*, and 7 days for *M. mucedo*, the growth reached the late logarithmic phase. Each mycelium was collected by filtration and washed with distilled water.

Analytical procedures. — Uronic acid was determined as glucuronic acid by the orcinol[20] or carbazole[21] methods. Carbazole–orcinol (C/O) ratios were determined by the method of Hoffman *et al.*[22]. Phosphorus[23], nitrogen[24], and sulfate[25] were determined by literature procedures. Optical rotations were measured at 20° with a JASCO-DIP-Digital polarimeter.

Preparation of cell walls. — Cell walls were isolated by using a French press (1200 kg/cm^2), and were purified by repeated washing with cold saline and by fractional centrifugation. The cell walls were immersed in boiling ethanol–water (7:3) for 20 min. After two further such treatments, the cell walls were washed with distilled water and stored at −20°.

Preparation and purification of the glycuronans. — A modification of the method of Bartnicki-Garcia and Reyes[7] was used. The defatted cell-walls (3 g) were treated with 2M HCl (300 mL) for 4 h at 100°, isolated by centrifugation, and extracted with M NaOH (300 mL) at room temperature. The alkali extraction was repeated three times. The combined extracts were centrifuged, dialysed against running water for 48 h and then distilled water for 24 h, and lyophilised. The products are designated as Ac from *A. cylindrospora*, Mm from *M. mucedo*, and Rn from *R. nigricans*.

Aqueous solutions (50 mg/2 mL) of Ac, Mm, and Rn were severally fractionated on a column (1.6 × 20 cm) of DEAE-Sephadex A-25 (Cl^- form), equilibrated with 0.01M HCl, by gradient elution with NaCl (0→M) and then by M NaCl containing 0.02M NaOH. Fractions (4 mL) were collected at 20–25 mL/h. Two glycuronan-containing fractions (*e.g.*, Rn-I and Rn-II, Fig. 1) were obtained, each of which was dialysed against distilled water for 72 h, concentrated, and lyophilised. The molecular size and homogeneity of each purified glycuronan was assessed by using a column (1.2 × 120 cm) of Sephadex G-100, equilibrated and eluted with 0.2M NaCl, using dextrans T-70 (mol. wt. 70,000), T-40 (40,000), and T-10 (10,000) as standards.

N.m.r. spectroscopy. — N.m.r. spectra were recorded at 70° for solutions in D_2O (internal sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate) with JEOL-PS-100 (for ^1H at 100 MHz) and JEOL-FX-100 (for ^{13}C at 25 MHz) spectrometers; the latter was operated in the pulsed Fourier-transform mode with complete proton decoupling. Chemical shifts were expressed as p.p.m. downfield from that of Me_4Si. Proton-decoupled F.t. spectra were measured by using a repetition time of 2.0 s, a pulse width of 7 μs (45°), 8K real data points, a sweep width of 5,000 Hz, and, typically, 20,000–40,000 scans. Protuberic acid, isolated from *Kobayasia nipponica*[10], was used as a reference material for assigning the ^{13}C-chemical shifts.

Reduction and methylation analysis of Rn. — Reduced Rn was prepared by the method of Taylor and Conrad[26] from Rn (*i.e.*, Rn-I + Rn-II), methylated[11], and hydrolysed first with 90% formic acid at 100° for 5 h and then with M trifluoroacetic acid at 100° for 3 h. The hydrolysate was concentrated, and the syrup was reduced with borohydride and acetylated[11]. The methylated alditol acetates were then subjected to g.l.c. at 180° (injector 250°), using a glass column (0.3 × 200 cm) packed with 3% of OV-225 on Uniport KS (60/80 mesh) with nitrogen as carrier gas at 60 mL/min.

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