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Isolation and characterization of β(1-4)-D-glucuronans from extracellular polysaccharides of moulds belonging to Mucorales

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Glucuronic acid polymers were isolated after acid treatment (2M HCl, 100°C, 4 h) of immunochemically active extracellular polysaccharides from various moulds belonging to the order of Mucorales. NMR methods have been used to elucidate their structure. It was found that the polymers, which are mainly composed of β(1-4)-linked D-glucuronic acid, can be considered as characteristic fragments of extracellular polysaccharides of all mucoraceous moulds. However, it is unlikely that the glucuronic acid residues play an important role in the immunodominant part of these polysaccharides since no positive ELISA reaction with these polyuronides was found with antibodies raised against the intact native extracellular polysaccharides.

INTRODUCTION

Polyuronides occur fairly abundantly in nature as important components of carbohydrate polymers. Pectin, a plant cell wall polymer, contains α-D-(1-4)-galacturonic acid residues as a major constituent (Pilnik & Voragen, 1970). Alginate, a polysaccharide derived from brown marine algae, is composed of linear chains of β-D-(1-4)-mannuronic acid and α-L-(1-4)-guluronic acid (Sime, 1990). Fungal polyuronides are isolated from the fruiting bodies of fungi belonging to the taxon Phallales. These contain α-L-(1-4)-iduronic acid and β-D-(1-4)-glucuronic acid (Tsuchihashi *et al.*, 1983*b*). Mucoric acid, a homopolymer consisting of β-D-glucuronic acid, is present in a fraction isolated from the cell walls of moulds belonging to the order of Mucorales (Bartnicki-Garcia & Reyes, 1968; Datema *et al.*, 1977; Dow *et al.*, 1983; Tsuchihashi *et al.*, 1983*a*). Mucoric acid has been described as an acceptor for transferase enzymes in the biosynthesis of glucuronic acid containing cell wall compounds of *Mucor rouxii* (Dow *et al.*, 1981; Camacho-Aguero *et al.*, 1990). Sequences of three β-D-(1-4)-linked glucuronic acid residues are found in extracellular polysaccharides of the bacterium *Rhizobium* (McNeil *et al.*, 1986).

Recently, the authors studied the immunochemical and high-performance size-exclusion chromatographic properties of extracellular polysaccharides (EPS) of a number of mould species belonging to the order of Mucorales (De Ruiter *et al.*, 1991*b*). The immunochemical properties of these extracellular polysaccharides can be used for the detection of these moulds by immunoassay (Notermans & Heuvelman, 1985; De Ruiter *et al.*, 1991*a*). Mould species belonging to the order of Mucorales (class of the Zygomycetes) are important fungi which are distributed worldwide and are the causes of many cases of food spoilage (Pitt & Hocking, 1985). Certain *Rhizopus* and *Mucor* species are essential in the solid-state fermentation of soya beans to produce tempe (Nout & Rombouts, 1991). Medically, some members are significant because of their ability to cause mucormycosis in man (De Ruiter *et al.*, 1991*c*).

In previous studies of extracellular polysaccharides derived from various species of Mucorales the presence of fucose (11–25 %mol), mannose (9–30 %mol), galactose (4–13 %mol), glucose (0–39 %mol) and glucuronic acid (32–55 %mol) was established (Hough & Perry,

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Carbohydrate Polymers 0144-8617/92/$05.00

1955; Martin & Adams, 1956; Bartnicki-Garcia & Reyes, 1968; Miyazaki & Irino, 1972; De Ruiter *et al.*, 1991*c*). In this study, β-D-(1-4)-glcA polymers are described as a structural entity of these extracellular polysaccharides. These polyuronides, which are found to be characteristic of extracellular polysaccharides of Mucorales, are compared with the previously reported mucoric acids of fungal cell wall origin. Furthermore, the role of these polyglucuronides and their relation to the immunochemistry of the initial extracellular polysaccharides of these moulds are examined.

EXPERIMENTAL

Extracellular polysaccharides of moulds

Extracellular polysaccharides (EPS) of the strains of *Mucor hiemalis* (CBS 201.28), *Mucor circinelloides* (RIVM M 40), *Rhizopus stolonifer* (CBS 609.82), *Rhizopus oryzae* (LU 581), *Rhizomucor miehei* (CBS 371.71), *Absidia corymbifera* (LU 017) and *Syncephalastrum racemosum* (CBS 443.59) used in this study were isolated and purified as described (De Ruiter *et al.*, 1991*b*).

Preparation of polyglucuronic acid

Polyglucuronic acid was isolated from EPS by a slight modification of the method according to Bartnicki-Garcia and Reyes (1968) and Tsuchihashi *et al.* (1983*a*). The EPS preparation (200 mg) was dissolved completely in 10 ml of 2 M HCl and heated for 4 h at 100°C. The polyglucuronic acid containing precipitate was isolated by decanting the supernatant after centrifugation (15 min, 2000 *g*). The precipitate was extracted twice with 1 M NaOH (3 ml) at room temperature. A minor amount (3·5 mg) of alkali-insoluble residue remained. The combined extracts were dialysed against running tap water (24 h) and distilled water (48 h) followed by lyophilization.

Purification of β(1-4)-D-polyglucuronic acid

Polyglucuronic acid was isolated and purified from the lyophilized preparation using an anion-exchange column (15 cm × 1·7 cm) of DEAE-Sephacel (Pharmacia, Uppsala, Sweden) (Rombouts & Thibault, 1986; De Ruiter *et al.*, 1991*b*), which was equilibrated with 0·05 M sodium acetate buffer (pH 5·0). After loading of the sample (15 mg in 4 ml 0·05 M buffer, pH 5·0) the column was washed with 25 ml of buffer and then eluted at 30 ml/h with a linear gradient (100 ml) of 0·05–1 M sodium acetate buffer, followed by 25 ml of 1 M buffer. Fractions (1·7 ml) were assayed for glucuronic acid by the automated *m*-hydroxydiphenyl method (Thibault *et al.*, 1979) slightly modified by the addition of 0·0125 M sodium tetraborate to the sulphuric acid (Blumenkrantz & Asboe-Hansen, 1973). Glucuronic acid was used as the standard. Finally, the glucuronic acid containing fractions were combined, dialysed and lyophilized yielding purified β(1-4)-D-polyglucuronic acid as a white powder.

Chemical analysis

Neutral sugars were released by pre-treatment with 12 M H_2SO_4 for 1 h at 25°C followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100°C. Next, sugars were converted to their alditol acetates as described (Englyst & Cummings, 1984) and analysed by gas chromatography. Inositol was used as the internal standard. Methylation analysis was performed using dimethyl sulphinyl anion (Hakomori, 1964). Reduction of glucuronic acid was performed using the slightly modified (Shively & Conrad, 1976) method of Taylor and Conrad (1972). Protein was measured using coomassie brilliant blue (Sedmak & Grossberg, 1977) with bovine serum albumin as standard. The uronic acids in the supernatant after 2 M HCl hydrolysis were determined using a high-performance anion chromatography system of Dionex (Sunnyvale, CA) with electrochemical detection according to the slightly modified method of Martens and Frankenberger (1990). Circular dichroism was performed on a JASCO J-600 spectropolarimeter.

Immunochemical analysis

Immunochemical determination of the polyglucuronic acid preparations was performed using a sandwich enzyme-linked immunosorbent assay (ELISA) as described (Notermans & Heuvelman, 1985; De Ruiter *et al.*, 1991*b*) with polyclonal IgG antibodies raised against extracellular polysaccharides from *Mucor racemosus*.

Molecular weight distribution

The molecular weight distribution was studied using high-performance size-exclusion chromatography, which was performed on an SP8800 HPLC system (Spectra Physics, San Jose, CA) equipped with three Bio-Gel TSK columns in series (40XL, 30XL, 20XL; 300 mm × 7·5 mm; Bio-Rad Labs, Richmond, CA) in combination with a TSK-XL guard column (40 mm × 6 mm) at 30°C using 0·4 M acetic acid/sodium acetate (pH 3·0) as eluant with a flow rate of 0·8 ml/min (De Ruiter *et al.*, 1991*b*).

NMR experiments

The ^{13}C-(100·4 MHz) and ^{1}H-NMR spectra (399·65 MHz) were obtained with a JEOL GX-400 spectrometer on a solution of polyglucuronic acid derived from *M. hiemalis* in D_2O (10 mg/0·5 ml; pD 5) and for the

polyglucuronic acid preparations from the other moulds with a Bruker AC200E spectrometer (200 MHz) in 5 mm tubes at 25°C. The chemical shifts for the methyl group of internal acetone were taken to be 2·217 (^{1}H) and 31·07 ppm (^{13}C) with respect to the signals for Me_4Si.

RESULTS

Preparation and purification

Extracellular polysaccharides (EPS) of mould species belonging to the order of Mucorales are composed of glucuronic acid, mannose, fucose, galactose and glucose (De Ruiter *et al.*, 1991*b*). Treatment of these EPSs was performed with 2 M HCl, 100°C, 4 h. After alkali extraction of the precipitate a polyglucuronic acid fraction was isolated from the alkali-soluble material by anion-exchange chromatography from all species tested, belonging to five different genera within this order. As can be seen in Table 1, the yield varied from 5 to 16% relative to the initial amount of EPS. After hydrolysis, a considerable part of the initial amount of glucuronic acid was found in the polymeric acid-insoluble fraction, varying from 14–15% for species belonging to the genus *Rhizopus* and 25–28% for *Mucor* species to more than 50% for *Syncephalastrum racemosum*. Determination of the acid-soluble compounds in the supernatant revealed a non-binding DEAE-fraction, composed mainly of monomeric neutral sugars and a fraction binding to an anion-exchange column. The latter could be released using 0·1 M NaAc buffer (pH 5·0) as eluant, and was characterized as monomeric glucuronic acid by high-performance anion-chromatography. No oligomers or polymers of glucuronic acids could be determined in the supernatant. The minor fraction of insoluble residue after NaOH extraction did not contain neutral or acidic sugars but turned blue after the addition of Sedmak reagent (Sedmak & Grossberg, 1977).

Chromatographic homogeneity

The polyglucuronic acid fraction isolated from various preparations of extracellular polysaccharides showed a homogeneous molecular weight distribution in high-performance size-exclusion chromatography (Fig. 1). The molecular weight of the fractions varied from 5·5 to 6 kD for the species belonging to the genera *Mucor*, *Rhizopus* and *Absidia* whereas *Rhizomucor* and *Syncephalastrum* polyglucuronic acids had a higher molecular weight (9–10 kD) as determined by calibration with dextran standards.

Polyglucuronic acid was isolated in a yield of 60% from the acid-insoluble precipitate from EPS derived from *Mucor hiemalis*. Elution from a DEAE anion-exchange column revealed a single peak (Fig. 2) using 1 M NaAc buffer as eluant. Polyglucuronides from other mould species tested, were isolated from the acid-insoluble precipitates in yields varying from 51 to 82%, and eluted similarly from the anion-exchange column (patterns not shown).

Chemical characterization

The glucuronic acid content in the polyglucuronic acid fractions varied from 78 to 88% for the different preparations (Table 1). Neutral sugar analysis revealed traces of fucose (less than 2%) in most of the preparations. No protein could be detected.

Methylation analysis of the polyglucuronic acid preparations was not successful as degradation of the polymers took place after treatment with dimethyl sulphinyl anion. Complete reduction of the polymers by the method of Taylor and Conrad (Taylor & Conrad, 1972; Shively & Conrad, 1976) could not be achieved.

Table 1. Properties of glucuronic acid polymers isolated from extracellular polysaccharides of various mould species belonging to Mucorales

Species	Yield related to:		GlcA content[c]	Molecular weight[d]
	EPS[a]	GlcA[b]		
Mucor hiemalis	8	28	86	5·5
Mucor circinelloides	8	25	86	5·5
Rhizopus stolonifer	5	15	79	6·5
Rhizopus oryzae	5	14	80	5·5
Absidia corymbifera	5	20	86	6·0
Rhizomucor miehei	10	37	88	10·0
Syncephalastrum racemosum	16	52	78	9·0

[a] Expressed as percentage (w/w) of initial amount of EPS.
[b] Expressed as percentage (w/w) of initial amount of glcA in EPS.
[c] Determined by the automated *m*-hdp method (see Experimental), using glcA as standard.
[d] In kilo Dalton; calibration with dextran standards.

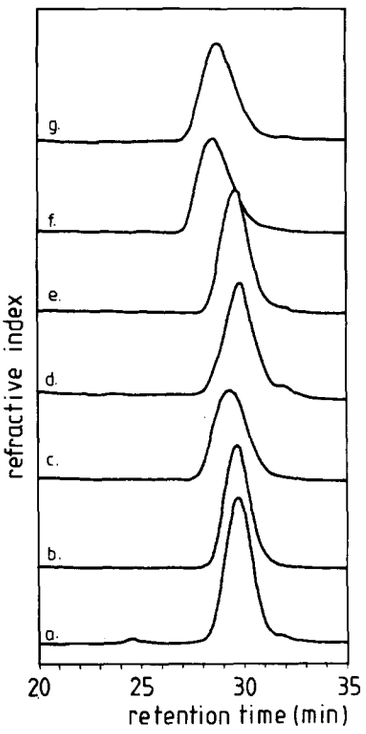

Fig. 1. High-performance size-exclusion chromatograms of polyglucuronic acid fractions derived by acid hydrolysis of extracellular polysaccharides from (a) *Mucor hiemalis*, (b) *Mucor circinelloides*, (c) *Rhizopus stolonifer*, (d) *Rhizopus oryzae*, (e) *Absidia corymbifera*, (f) *Rhizomucor miehei*, (g) *Syncephalastrum racemosum*.

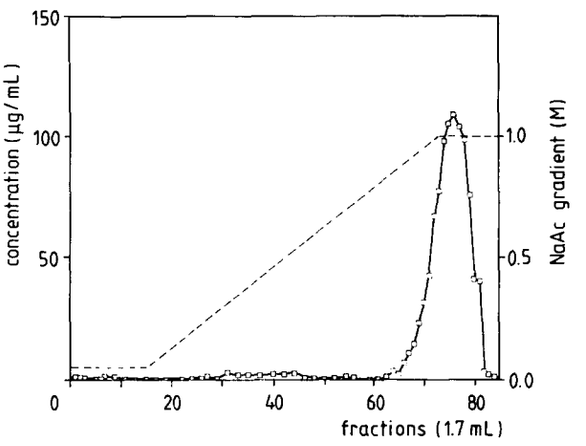

Fig. 2. Anion-exchange chromatogram of the polyglucuronic acid fraction derived from extracellular polysaccharides of *Mucor hiemalis*, using DEAE-Sephacel with a sodium acetate buffer (pH 5·0) gradient (see Experimental). Glucuronic acid, -□-□-: gradient, -------.

After three reduction steps the polymer still contained about 40% of the original *m*-hdp positive material.

NMR spectroscopy

The data of the ^{1}H-NMR spectrum from the polyuronic acid fraction derived from *M. hiemalis* are summarized in Table 2. A chemical shift of the anomeric proton of 4·52 ppm with a coupling constant ($J_{1,2}$) of 7·9 Hz indicates a β-linkage. Assignment of the remaining resonances was obtained by homonuclear decoupling experiments. Some minor peaks (data not given) could be assigned as differently linked β-glucuronic acid residues and as traces of L-fucose. The ^{13}C-NMR spectrum of polyglucuronic acid as given in Fig. 3 showed six main peaks, which could be assigned to a β(1-4)-D-glucuronic acid (Tsuchihashi *et al.*, 1983*a*). The ^{13}C-NMR data of polyglucuronic acid are listed in Table 3. Polyglucuronic acid preparations from other moulds belonging to the order of Mucorales, including the species *Mucor circinelloides*, *Rhizopus stolonifer*, *Rhizopus oryzae*, *Rhizomucor miehei*, *Absidia corymbifera* and *Syncephalastrum racemosum* gave similar NMR spectra (not shown).

Other properties

Immunochemical characterization of these polyglucuronic acid preparations by an ELISA showed no reactivity with antibodies raised against extracellular polysaccharides of *M. racemosus*. The water-soluble β(1-4)-glucuronic acid polymers were very stable towards acid hydrolysis as incomplete hydrolysis was obtained under the conditions of the Saeman hydrolysis (12 M H_2SO_4, 1 h, 25°C followed by 1 M, 3 h, 100°C. Circular dichroism measurement of the polyglucuronic acid preparations derived from *Mucor hiemalis* showed two bands, a main peak at around 203 nm and a small trough at around 215 nm (spectrum not shown).

Table 2. Chemical shifts[a] (ppm) and coupling constants (Hz) for ^{1}H-NMR resonances of the β(1-4)-linked glucuronic acid polymer derived from EPS of *M. hiemalis*

	Glucuronic acid atom				
	H-1	H-2	H-3	H-4	H-5
Chemical shift (ppm)	4·52	3·37	3·63	3·68	3·87
$J_{H,H+1}$ (Hz)	7·9	9·2	8·6	9·1	

[a] ±0·01 ppm.

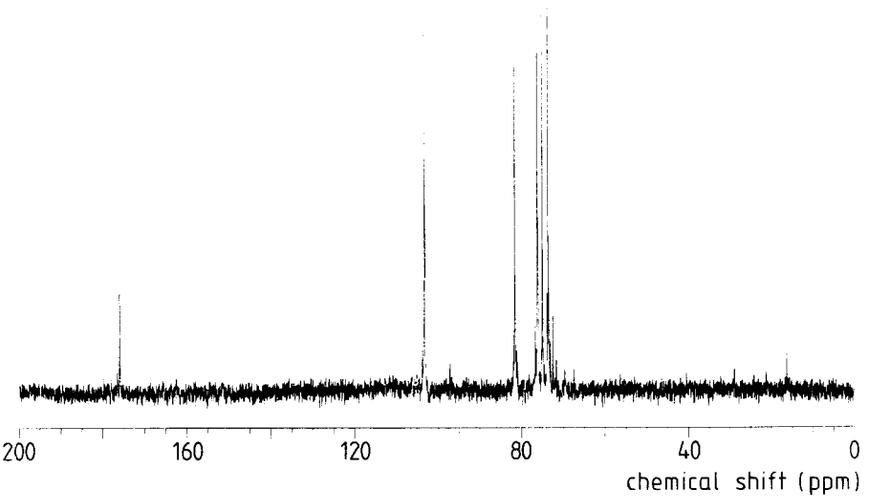

Fig. 3. ^{13}C-NMR spectrum (100·4 MHz) of β(1-4)-linked polyglucuronic acid derived from *Mucor hiemalis*.

Table 3. ^{13}C-NMR chemical shifts[a] (ppm) of the β(1-4)-linked glucuronic acid polymer derived from EPS of *M. hiemalis*

	Glucuronic acid atom					
	C-1	C-2	C-3	C-4	C-5	C-6
Chemical shift (ppm)	103·03	73·50	74·93	81·49	76·10	≈175·8

[a] ±0·01 ppm.

DISCUSSION

Acid-insoluble polyglucuronic acid preparations could be isolated by hydrolysis of immunochemically active extracellular polysaccharides of different mould species belonging to the order of Mucorales. With NMR methods it was shown that these acid-insoluble polymers were essentially homopolymers of β(1-4)-linked D-glucuronic acid containing a small amount of differently linked β-D-glcA residues and traces of fucose. However, in contrast with the initial polysaccharides (De Ruiter *et al.*, 1991*b*), no immunochemical activity was left with these glucuronic acid polymers in the ELISA assay with antibodies raised against native EPS of *Mucor racemosus*.

Characterization of the polyuronides by chemical methods was hindered by the very strong β(1-4)-glcA glycosidic linkages. The polymers were found to be very resistant towards acid hydrolysis, and also complete reduction could not be achieved. Incomplete reduction was previously mentioned by Dow *et al.* (1983) for glucuronic acid containing polymers.

The presence of fucose residues after the severe acid treatment can only be explained by assuming that single unit fucose residues served as aglycon in glycosidic linkages with glucuronic acid residues. This aldobiuronic type of linkage with the neutral sugar residue as reducing end is reported to be very acid resistant (BeMiller, 1967; Bartnicki-Garcia & Lindberg, 1972). The circular dichroism spectrum of polyglucuronic acid derived from *M. hiemalis* was very similar to the spectrum previously reported for β(1-4)-glcA in deacetylated xanthan (Morris *et al.*, 1977) and therefore consistent with the proposed structure.

As a result of this, the polymers of glucuronic acid can be considered as a genuine part of all extracellular polysaccharides of species belonging to the order of Mucorales tested. The molecular weight of these polymers varied from 5·5 to 10 kD, indicating an average of 30–60 glcA residues. The type of linkages of the residues liberated by acid hydrolysis remains unclear. It is unlikely that these residues originally were part of the β(1-4)-linked backbone as only monomeric glcA residues were found in the hydrolysates. However, they could be involved in glycosidic linkages with neutral sugars in sidechains of the native extracellular polysaccharides. Since all EPS preparations tested were hydrolysed identically, it is likely that the variations in length of the β(1-4)-glcA polymers, and the amount of glcA in these polymers relative to that in the initial EPS preparations, indicate structural differences between the various EPS preparations.

The β(1-4)-D-glucuronic acid polymers isolated from extracellular polysaccharides of mould species

belonging to Mucorales may be similar to mucoric acid, the polyuronide which was isolated from cell walls of these moulds (Bartnicki-Garcia & Reyes, 1968; Datema *et al.*, 1977; Tsuchihashi *et al.*, 1983*a*). However, in all preparations tested only one polyglucuronic acid fraction could be isolated from EPS as opposed to two distinct fractions varying in molecular weight and charge, isolated from cell walls of *Absidia cylindrospora, Mucor mucedo, Rhizopus stolonifer* (Tsuchihashi *et al.*, 1983*a*) and *Mucor rouxii* (Dow *et al.*, 1983). The β(1-4) polyglucuronides isolated from EPS preparations resembled mostly fraction II of the mucoric acid preparations isolated from mycelia by Tsuchihashi *et al.* (1983*a*), as they behave similarly on an anion-exchange column and in a size-exclusion chromatography system.

Biosynthesis of mucoran in the cell wall of *Mucor rouxii* is reported to be dependent on the action of fucosyl transferases using polyglucuronic acid as a precursor (Camacho-Aguero *et al.*, 1990). It is not clear whether the presence of fucose traces in the authors' isolated polyglucuronides indicate a role in the biosynthesis of extracellular polysaccharides as previously proposed (Dow *et al.*, 1981).

In conclusion, the polymers of β(1-4)-linked D-glucuronic acid can be considered as a common and characteristic structural element of extracellular polysaccharides of moulds belonging to Mucorales. However, the immunogenic properties of the native extracellular polysaccharides do not reside in these β(1-4)-glucuronic acid segments, but probably in the neutral sugar part.

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