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Note

Structure and conformational features of an alkali- and water-soluble galactofuranan from the cell walls of *Eupenicillium crustaceum*

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Fungal cell walls are mainly composed of polysaccharides. Some of these polymers are valuable chemotaxonomic markers and their characterisation can be of interest as a tool to rearrange some complex genera[1]. In addition, galactofuranose-containing polysaccharides are being thoroughly studied because these residues act as antigenic determinants[2,3]. A galactomannan, with side chains of (1 → 5)-linked-β-D-galactofuranosyl residues attached to the mannan, was extracted from hyphae of *Aspergillus niger*[4]. β-(1 → 5)-linked galactose polymers have also been found in *Penicillium citrinum* extracellular polysaccharide[5] and in the cell wall of some species of *Penicillium* and *Aspergillus*[6].

The chemical composition of cell-wall fractions has previously been studied for some species of the genus *Eupenicillium*[7]. Now we report on the structural characterisation of a polysaccharide purified from the alkali- and water-soluble cell-wall fraction of the type species, *E. crustaceum*.

Alkali-extractable fraction F1 (see Experimental) amounted to 13–18% of the wall material, and water-soluble fraction F1S represented 7–8% of the total F1. Gel filtration of F1S on Sepharose CL-6B gave one main polysaccharide (F1S-B; $M_W = 17$ k) and a small proportion of a high molecular weight polysaccharide (F1S-A). F1S and F1S-B contained galactofuranose as their major component and a small proportion of mannose (partial hydrolysis with 0.05 M H_2SO_4). Similar results were obtained with the Saeman hydrolysis.

Fraction F1S-B consumed 0.8 mol of periodate/hexose residue. Threitol was the main component detected by GLC after periodate oxidation, Smith degradation, and complete hydrolysis, revealing that the main type of linkage is either (1 → 4)- or (1 → 5)-linked galactosyl residues. The absorption bands at 870 and 810 cm^{-1} of the F1S-B fraction are characteristic of a β-galactofuranan[8]. Methylation

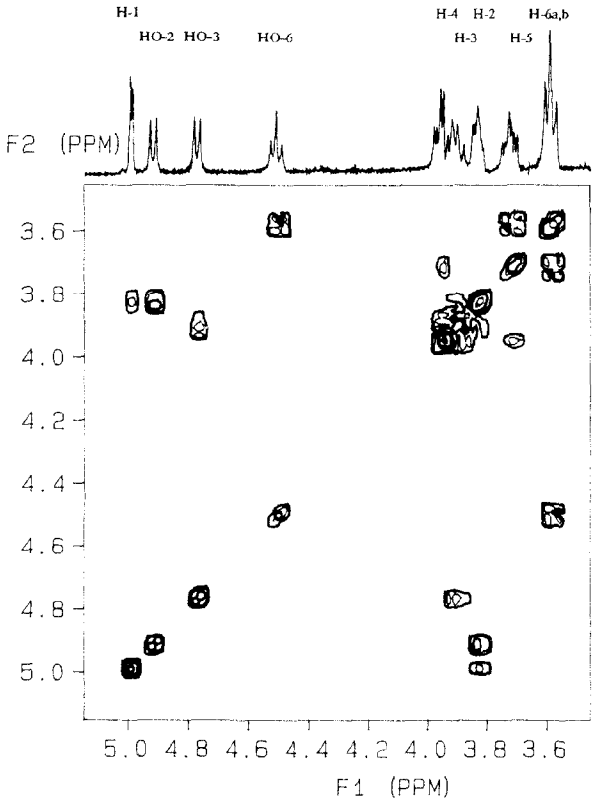

Fig. 1. COSY spectrum (Me_2SO-d_6, 80°C, 300 MHz) of the water-soluble F1S-B cell-wall polysaccharide of *E. crustaceum*. The hydroxyl groups and the ring protons have been labeled in the 1D spectrum.

analysis of F1S-B gave, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, consistent with (1 → 4)- or (1 → 5)-linked galactosyl residues.

The high-resolution ^1H NMR and proton-decoupled ^{13}C NMR spectra of F1S-B in Me_2SO-d_6 showed only one anomeric signal, indicating that the polysaccharide contains a monosaccharide repeating unit and that it is homogeneous. Three hydroxyl groups appeared in the Me_2SO-d_6 ^1H NMR spectrum. The peak at ca. 109 ppm for the anomeric carbon indicates a 1,2-*trans* furanoside[9]. The COSY spectrum (Fig. 1) allowed assignment of all the signals in a straightforward way, so that hydroxyl protons HO-2, HO-3, and HO-6 were unequivocally identified. On the other hand, the one-bond heteronuclear correlation experiment enabled assignment of the ^{13}C NMR spectrum, thus allowing comparison with previously reported chemical shifts for methyl furanosides.

All the data indicate that the polysaccharide consists of (1 → 5)-linked β-D-galactofuranosyl residues. The chemical shifts agree with those reported by Gorin and Mazurek[10] for a β-(1 → 5)-galactofuranose tetrasaccharide. The 2D NOE spectrum showed cross-peaks H-1/H-5 and H-1/H-6, thus supporting this conclusion.

TABLE I

Expected vicinal proton–proton coupling constants for the different conformations of the D-galactofuranose ring according to the equation proposed by Altona[12] and using $\Phi_m = 40$

P	Conformation	$\Phi_{1,2}$ (°)	$J_{1,2}$ (Hz)	$\Phi_{2,3}$ (°)	$J_{2,3}$ (Hz)	$\Phi_{3,4}$ (°)	$J_{3,4}$ (Hz)
18	${}_3E$	143	4.0	−162	6.2	167	7.3
36	${}^4T_3$	130	2.7	−155	5.3	169	7.5
54	${}^4E$	117	1.8	−146	3.9	167	7.3
72	${}^4T_0$	103	1.2	−134	2.9	160	7.0
90	${}_0E$	91	1.2	−120	1.6	151	6.3
108	${}^1T_0$	81	1.4	−107	1.0	138	5.1
126	${}^1E$	75	1.7	−95	1.0	125	3.6
144	${}^1T_2$	73	1.8	−85	1.4	111	2.2
162	${}_2E$	75	1.7	−79	1.8	99	1.5
180	${}^3T_2$	81	1.4	−77	2.0	89	1.2
198	${}^3E$	91	1.2	−79	1.8	83	1.1
216	${}^3T_4$	103	1.2	−85	1.4	81	1.1
234	${}_4E$	117	1.8	−95	1.0	83	1.1
252	${}^0T_4$	130	2.7	−107	1.0	89	1.2
270	${}^0E$	143	4.0	−120	1.6	99	1.5
288	${}^0T_1$	152	4.8	−134	2.9	111	2.2
306	${}_1E$	159	5.3	−146	3.9	125	3.6
324	${}^2T_1$	161	5.5	−155	5.3	138	5.1
342	${}^2E$	159	5.3	−162	6.2	151	6.3
360	${}^2T_3$	152	4.8	−164	6.4	160	7.0

The conformational analysis of the β-galactofuranose ring was carried out following a methodology similar to that recently described by Hoffmann et al.[11] for the α-L-arabinofuranosyl residues of several arabinoxylans. The protocol consists of a comparison of the observed proton–proton vicinal coupling constants with those expected for the different conformations involved in the pseudorotational itinerary of the D-galactofuranose ring. Molecular mechanics calculations can assist in distinguishing among the different solutions of the *J*-based analysis.

It is noteworthy that the pseudorotational itinerary of β-D-galactofuranoses is analogous to that of α-L-arabinofuranoses. For comparison purposes, we have adopted the definition of Hoffman et al.[11], N(north)-type conformers being those characterised by negative values of C-1–C-2–C-3–C-4 torsion angles; thus, the standard N-type conformation has the maximum negative C-1–C-2–C-3–C-4 torsion angle, i.e., the ${}^2T_3$ form.

Following the discussion by Hoffmann et al., a puckering amplitude Φ_m of $38 \pm 4°$ was used to calculate the possible proton–proton torsion angles of the D-galactofuranose ring. The vicinal proton–proton coupling constants were estimated from these torsion angles by using the extended Karplus equation proposed by Altona[12]. The couplings expected for the different conformations of the furanose ring [$\Phi_m(N) = \Phi_m(S) = 40°$] are given in Table I.

All *J*-couplings in both D_2O and Me_2SO-d_6 were sufficiently resolved to extract initial δ and *J* values for spectral simulation. The best values of ^1H chemical shifts

TABLE II

^{1}H NMR and ^{13}C NMR chemical shifts (δ, ppm) and vicinal coupling constants (J, Hz) for the water-soluble F1S-B galactofuranan of *E. crustaceum* in D_2O and Me_2SO-d_6

Atom	D_2O	Me_2SO-d_6	α-L-Arabinofuranoside residues in arabinoxylans[11]		
H-1	5.184	4.997			
H-2	4.128	3.814			
H-3	4.095	3.886			
H-4	4.146	3.945			
H-5	3.929	3.705			
H-6a	3.785	3.572			
H-6b	3.799	3.558			
$J_{1,2}$	2.1	2.1	0.8	1.0	1.6
$J_{2,3}$	4.2	4.4	2.3	2.5	3.2
$J_{3,4}$	6.8	6.9	5.0	5.4	5.8
$J_{4,5}$	3.4	3.1			
$J_{5,6a}$	6.2	6.3			
$J_{5,6b}$	5.0	5.1			
$J_{6a,6b}$	−12.1	−12.0			
C-1	107.8	108.1			
C-2	82.2	83.1			
C-3	77.4	78.1			
C-4	82.6	82.6			
C-5	76.5	76.2			
C-6	61.9	62.1			

and coupling constants are given in Table II along with the $^3J_{H,H}$ values observed by Hoffmann et al. for different α-L-arabinofuranosyl residues in arabinoxylans. The comparison of the values for F1S-B in both solvents indicates that the furanose ring has a similar conformational equilibrium in D_2O and Me_2SO-d_6.

The vicinal couplings for F1S-B are in all cases larger than those observed by Hoffmann et al. for arabinoxylans. According to Table I, the values of $J_{2,3}$ and mainly $J_{3,4}$ indicate a higher population of N-type conformers than those observed in arabinoxylans. In fact, the observed values are very close to those predicted by Altona's equation for the ^4E form. Nevertheless, a small contribution of S-type conformers in the $^1T_2$ region cannot be completely ruled out.

In order to have an idea of the steric interactions for the different forms of the five-membered ring, molecular mechanics calculations using the MM2 program were carried out for the model compound methyl 5-*O*-methyl-β-D-galactofuranoside. According to the calculations, the ^4E region is predicted to be favored, with the minimum having a geometry between the ^4E and the $^4T_3$ form. The $^1T_2$ form is destabilised by 2.0 kcal/mol, while the $^2T_1$ conformation, which corresponds to the crystal structure of methyl α-D-galactofuranoside, is destabilised by 2.4 kcal/mol; it is not a local minimum, and converges to the ^4E conformation.

Similarly to the α-L-arabinofuranosyl residues of arabinoxylans, the glycosidic oxygen adopts a pseudo-axial orientation, in agreement with the operation of the

TABLE III

Proton–proton torsion angles (°) and expected vicinal coupling constants (Hz) for the possible rotamers of the lateral chain of the galactofuranan from *E. crustaceum* [a]

Rotamer	$\Phi_{(H-4,H-5)}$	$J_{4,5}$	$\Phi_{(O-5,O-6)}$	$J_{5,6S}$	$J_{5,6R}$	Population (%)
g^-	−60	1.5	180	10.6	4.3	16.8
	−60	1.5	60	2.3	9.8	41.4
	−60	1.5	−60	2.1	2.3	0.2
g^+	60	4.5	180	10.6	4.3	0.1
	60	4.5	60	2.3	9.8	23.1
	60	4.5	−60	2.1	2.3	9.0
t	180	10.5	180	10.6	4.3	9.4
	180	10.5	60	2.3	9.8	0.1
	180	10.5	−60	2.1	2.3	0.2

[a] The calculated populations from MM2 calculations are also given.

anomeric effect, while the lateral chain has a pseudo-equatorial disposition to minimise steric interactions with the ring. No intramolecular hydrogen bonding seems to be present, at least in Me_2SO-d_6 solution, since the temperature coefficients for the three hydroxyl groups are very similar (ca. 7×10^{-2} ppm/°C) and high in magnitude. Also, the $^3J_{HO,H}$ values (ca. 6 Hz) indicate that there is not any preference for a particular orientation around the C–O bond of the hydroxyl groups.

Conformation of the lateral chain.—The $^3J_{4,5}$ value (ca. 3.3 Hz) indicates a different population for the three staggered rotamers around the C-4–C-5 bond. The expected values of J for the three possible conformations obtained by application of Altona's equations on the proton–proton torsion angles are given in Table III. Since several combinations could lead to the same J value, MM2 calculations were carried out for the possible rotations around C-4–C-5 and C-5–C-6. The results indicate a preference for the rotamer corresponding to $\Phi_{4,5} = -60$ (58%), followed by that with $\Phi_{4,5} = 60$ (32%) and by that with a *trans* proton–proton torsion angle (10%). The expected value for this combination agrees quantitatively with the experimental value.

Regarding the C-5–C-6 conformation, the calculations predict a conformational equilibrium 65:26:9 for the *gt* : *tg* : *gg* rotamers. However, the similar values of $J_{5,6a}$ and $J_{5,6b}$ are in agreement with a higher contribution of the *gg* rotamer. Thus, the observed J couplings can be explained by a 45 : 25 : 30 distribution of *gt* : *tg* : *gg* conformers.

Therefore, it can be concluded that the alkali- and water-soluble fraction of the cell-wall material of *Eupenicillium crustaceum* is a β-(1 → 5)-galactan. Our results also indicate that the five-membered ring of this polysaccaride has less conformational freedom than that in the arabinoxylans. Nevertheless, similarly to the arabinofuranosyl residues, O-1 has a pseudo-axial orientation, in agreement with the operation of the anomeric effect, while the lateral chain adopts a pseudo-equatorial disposition, to minimise steric interactions.

The conformational study of other galactofuranose rings from different fungal cell-wall polysaccharides is presently under way.

EXPERIMENTAL

Organism and growth conditions.—*Eupenicillium crustaceum* Ludwig, strain CBS 635.70 was obtained from the Centraalbureau voor Schimmelcultures (Baarn, Netherlands), and maintained on slants of Bacto potato dextrose agar (Difco) supplemented with 1 g/L of yeast extract (Difco). The basal medium and growth conditions have been described[13].

Cell-wall preparation and fractionation of wall material.—Cell walls were prepared from 5-day-old mycelium, and then dry wall material was extracted with 1 M NaOH at room temperature to give Fraction F1 as described elsewhere[14]. Fraction F1 (1 g) was further extracted with distilled water (2 × 150 mL), stirring at room temperature for 2 h each time. After centrifugation, a supernatant solution (F1S) and a precipitate (F1P) were obtained, and both of them were freeze-dried.

Gel filtration of the F1S fraction.—A solution of F1S (80 mg) in 0.3 M NaOH (1.5 mL) was centrifuged, and the supernatant solution was added to a column (40 × 2.6 cm) of Sepharose CL-6B and eluted with 0.3 M NaOH. Fractions (2 mL) were collected and monitored for carbohydrate by the phenol–H_2SO_4 method[15]. Appropriate fractions were pooled, dialysed against running tap water, concentrated to a small volume, and freeze-dried.

HPLC of main fraction.—A sample of the main fraction obtained by gel filtration was analysed in a Biogel TSK 40 column (300 × 7.5 mm) in order to determine its purity and molecular weight, eluting isocratically with 10 mM PBS in 0.308 M NaCl and detecting by the refractive index of the compounds. Various commercially available dextrans were analysed under identical conditions in order to obtain a calibration curve, from which the M_W of the sample was extrapolated.

Chemical analysis.—The polysaccharides F1S and F1S-B were hydrolysed with 0.05 M H_2SO_4 for 5 h at 100°C to release sugars in the furanose configuration, and by Saeman hydrolysis[16]. The neutral sugars, converted into their corresponding alditol acetates[17], were identified and quantified by GLC as described previously[18].

Periodate oxidation was carried out according to Aspinall and Ferrier[19]. The oxidised polysaccharide was subjected to Smith degradation and the products were analysed by GLC as the alditol acetates.

The IR spectrum was obtained by the KBr technique with a Perkin–Elmer 1420 ratio recording infrared spectrophotometer.

Methylation analysis.—Methylation of the sample was performed using a modification[20] of the Hakomori method[21]. The methylated fraction was hydrolysed, reduced with $NaBD_4$, acetylated, and analysed by GLC and GLC–MS as previously described[14].

NMR spectroscopy.—NMR spectra were recorded at 80°C with a Varian XL-300 spectrometer. Proton chemical shifts in D_2O were referenced to residual HDO at

δ 4.24 ppm. Proton chemical shifts in Me_2SO-d_6 were referenced to residual Me_2SO-d_5 at δ 2.49 ppm. Carbon chemical shifts in D_2O were referenced to internal dioxane at δ 67.4 ppm. Carbon chemical shifts in Me_2SO were referenced to residual Me_2SO-d_5 at 39.5 ppm.

2D Experiments were performed using the standard Varian software. The pure absorption 2D NOE spectrum was obtained using a mixing time of 300 ms. In both cases, data matrices of 256×512 points were used to resolve a spectral width of 1000 Hz. Shifted sine-bell functions were applied for processing in both dimensions. Zero-filling was used to expand the data to 512×1 K points. The relaxation delay was always set to 2 s.

The values of chemical shifts and coupling constants measured from the COSY and 1D spectra were refined by spectral simulation using the PANIC program (Bruker software).

The carbon–proton one-bond shift correlation spectrum was obtained in the ^{13}C-detection mode using F_1-decoupling. Proton decoupling during acquisition was achieved by the WALTZ scheme. 128 FIDs were collected, each consisting of 512 data points. The refocussing delay corresponded to J 145 Hz and the relaxation delay was set to 1.5 s.

Molecular mechanics calculations were carried out using the MM2 program.

The X-ray coordinates of methyl α-D-galactofuranoside were used as the starting point and modified as desired[22]. A dielectric constant of 1.5 D was used. All the possible arrangements of the O-4/O-5 and C-4/O-6 torsion angles were considered.

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