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Structural elucidation of a cell wall fungal polysaccharide isolated from *Ustilaginoidea virens*, a pathogenic fungus of *Oriza sativa* and *Zea mays*

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ABSTRACT

The alkali-extractable water-soluble polysaccharides (F1SS) isolated from the outer cell wall of two strains of *Ustilaginoidea virens* have been studied by chemical and methylation analyses, and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The structures of these polysaccharides are very similar, and can be described by the following idealized repeating unit:

$$[\beta\text{-Gal}f\text{-}(1\rightarrow6)\text{-}\beta\text{-Gal}f\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p]_n \quad (6\downarrow,\ 1\downarrow)$$

$$[\alpha\text{-Gal}p\text{-}(1\rightarrow6)\text{-}\beta\text{-Gal}f\text{-}(1\rightarrow2)\text{-}\alpha\text{-Man}p]_m \quad (6\downarrow,\ 1\downarrow)$$

where n and m are approximately 1 and 2, respectively.

1. Introduction

Ustilaginoidea virens is a pathogenic fungus for maize (*Zea mays*), where it produces false smut, and for rice (*Oryza sativa*), where it causes green smut, affecting quality. Indeed, it produces brown smut balls that contaminate rice grain. In addition, the recent discovery of ustiloxins (cyclopeptidic phyto and mycotoxins produced by the pathogen) may be of concern to livestock and humans,[1,2] which has increased the need to follow and control the disease.

The taxonomical position of this species is uncertain, and it has been described as the asexual state of *Claviceps oryzae-sativa*.[3] The fungal alkali-extractable water-soluble cell wall polysaccharides (F1SS), which are minor components of the fungal cell wall (around 2–8%), constitute the glycidic moiety of peptido-polysaccharides or glycoproteins.[4–6] These polysaccharides are in close contact with the external environment,[7,8] are antigenically relevant,[9] and may serve for different biological functions, as its participation in cell-cell and/or cell-host recognition phenomena.[10] Connection of the parasite to the host cell surface probably involves binding of these surface polysaccharides to complementary host molecules, as it had been previously shown in other cases.[11] In addition, polysaccharides F1SS are considered as useful characters for the grouping of fungi and for the proposal of evolutionary theories. Then, the aim of this study is the determination of the structure of these polysaccharides in *U. virens*, for increasing the understanding of the biochemical events occurring during the first steps of infection as well as for assessing the correct taxonomic placement of this species.

2. Results and discussion

The polysaccharide F1SS amounted to 1.5% and 3.8% of the dry cell-wall material in strains 353.64 and 422.52, respectively. The samples were subjected to SEC, and showed to be composed of a single polysaccharide, with an apparent molecular mass around 50 kDa. GC analysis of their hydrolysates revealed the presence of galactose (61.8 and 54.4%), mannose (35.1 and 40.8%), and small amounts of glucose in strains CBS 422.52 and CBS 353.64, respectively. Absolute configuration analysis showed D-configuration for both sugars. Methylation analysis (Table 1) allowed the identification of four main types of residues: nonreducing ends of galactopyranose and galactofuranose, 6-O-substituted galactofuranose, and 2,6-di-O-substituted mannopyranose, in addition to other minor components.

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Table 1
Linkage types and their percentages deduced from methylation analysis of the polysaccharides F1SS isolated from *U. virens*

RT[a] (min)	Linkage type	Characteristic fragments (*m*/*z*)	Relative abundance (%)	
			CBS 422.52	CBS 353.64
6.78	Man*p*-(1→	87, 88, 102, 118, 129, 161, 205	2.3	2.1
6.91	Gal*f*-(1→	89, 102, 118, 162, 205, 278	10.3	10.6
7.21	Gal*p*-(1→	87, 88, 102, 118, 129, 161, 205	16.1	17.8
8.78	→2)-Man*p*-(1→	87, 88, 101, 129, 130, 161, 190	3.3	6.2
8.86	→5)-Gal*f*-(1→	87, 102, 113, 118, 131, 162, 173, 233	2.9	1.5
9.52	→6)-Man*p*-(1→	87, 88, 99, 102, 118, 129, 162, 189	4.3	4.8
10.25	→6)-Gal*f*-(1→	88, 101, 102, 117, 118, 127, 159, 233	27.2	23.0
12.54	→2,6)-Man*p*-(1→	87, 118, 129, 130, 189, 190	33.4	33.8

[a] Retention time.

Table 2
^{1}H and ^{13}C NMR chemical shifts (δ) for the alkali-extractable water-soluble polysaccharide F1SS isolated from *U. virens*

Units		1	2	3	4	5	6a	6b
A	^{1}H	5.14	4.17	4.09[a]	4.11[a]	*ca.*3.98	3.87	3.66
	^{13}C	106.5	82.1	77.7[b]	84.2	70.4	**70.0**	
B	^{1}H	5.13	4.16	4.10[a]	4.11[a]	3.98	3.86	3.66
	^{13}C	106.8	82.0	77.5[b]	84.0	70.3	**69.6**	
C	^{1}H	5.06	4.14	4.08[a]	4.07	ca. 3.85	3.72	3.68
	^{13}C	108.7	81.9	77.8[b]	84.2	71.8	63.6	
D	^{1}H	5.05	4.09	3.87	3.82	3.82	4.03	3.76
	^{13}C	98.0	**75.9**	71.6	67.6	72.0	**66.4**	
E	^{1}H	4.99	3.83	3.90	4.01	3.99	3.76	3.76
	^{13}C	99.4	69.3	70.3	70.1	71.8	62.0	

Underlined bold numbers represent glycosylation sites.
[a,b] These values may have to be interchanged.

The ^{1}H NMR spectra contained, inter alia, five main anomeric signals, which were labeled **A**–**E** from low to high field (Fig. 1a and b). The ^{13}C NMR spectrum (Fig. 1c), showed five singlets in the anomeric zone, one of them slightly splitted. 2D-shift correlation spectroscopy, that is, DQF-COSY and TOCSY (Fig. 2a), allowed the assignment of most of the signals of the proton spectrum. An HMQC experiment exhibited five crosspeaks in the anomeric region, two of them with very similar chemical shifts at both ^{1}H and ^{13}C frequencies. The HMQC also permitted assignment of most of the carbon signals (Fig. 2b). An HSQC-TOCSY experiment led to complete assignment of almost all the proton and carbon signals of the five main monosaccharide units (Table 2). Comparison of these experimental values with those of model compounds[12,13] permitted to infer that units **A** and **B** were 6-O-substituted Gal*f* , **C**, terminal Gal*f*, **D**, 2,6-di-O-substituted Man*p*, and **E**, terminal Gal*p*.

Concerning the anomeric configuration, **A**, **B**, and **C** had proton–proton coupling constants $J \leqslant 2$ Hz, which indicated β-configuration for all of them (compared with coupling ≈4 Hz for α derivatives),[14] corroborated by the carbon chemical shifts >106 ppm.[12] Unit **E** showed a coupling constant, $J_{1,2} = 3.8$ Hz, demonstrative of the α-configuration for Gal*p*. A coupled HMQC experiment also revealed that $^{1}J_{H\text{-}1,C\text{-}1} = 171.5$ Hz for residue **D**, which demonstrated the α-configuration for this Man*p* unit.[15]

With respect to the sequence of the glycosidic linkages, a 2D-NOESY (Fig. 2c) experiment (mixing time = 300 ms) exhibited, in addition to intraresidue crosspeaks, signals H-1**A**/H-1**D**, H-1**B**/H-1**D**, H-1**A**/H-2**D**, H-1**B**/H-2**D**, H-1**C**/H-6a**A**, 1**C**/H-6b**A**, H-1**D**/H-2**A**(**B**), H-1**D**/H-6a**D′**, H-1**D**/H-6b**D′**, H-1**D**/H-5**D′**, H-1**E**/H-6a**B**, H-1**E**/H-6b**B**, being **D′** a second molecule of **D**. Furthermore, an HMBC experiment (Fig. 3) showed intraring crosspeaks H-1**A**/C-4**A**, H-1**B**/C-4**B**, H-1**C**/C-4**C**, H-1**D**/C-5**D**, and H-1**E**/C-5**E**, demonstrating the furanoid character of rings **A**, **B**, and **C**, and the pyranoid structure of rings **D** and **E**. Also interunit connections H-1**A**/C-2**D**, H-1**B**/C-2**D**, H-1**C**/C-6**A**, H-1**C**/C-6**D′**, and H-1**E**/C-6**B** unequivocally demonstrated the primary sequences **C**→6**A**→**2**D→**6**D′ and

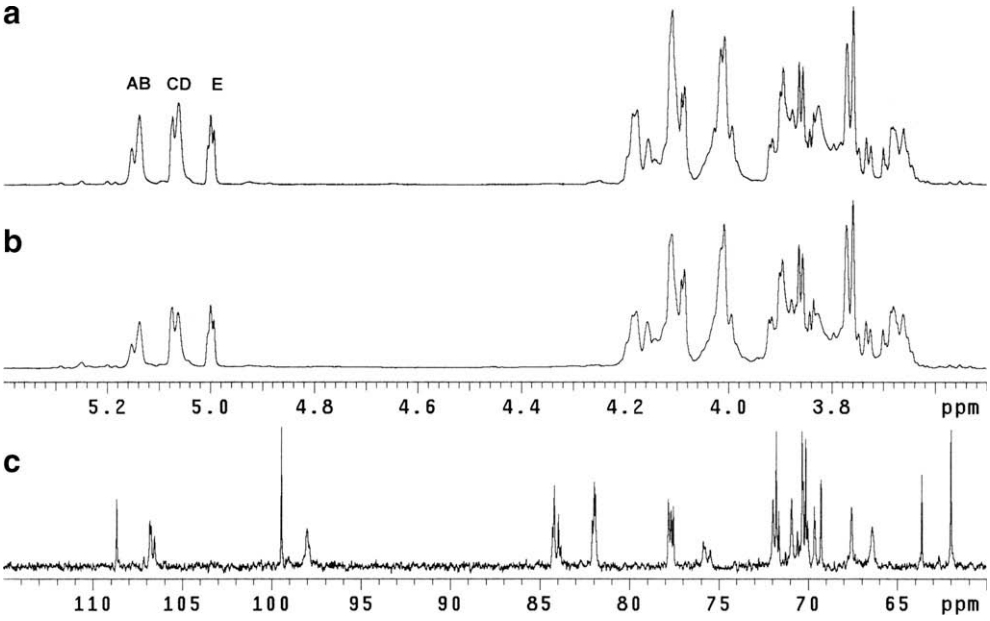


Figure 1. Spectra of the cell wall F1SS polysaccharide isolated from *U. virens*. (a, b) ^{1}H NMR spectra (500 MHz, D2O, 40 °C) of CBS 353.64 and 422.52, respectively. Anomeric peaks have been labeled. (c) ^{13}C NMR spectrum (125 MHz, D2O, 40 °C) of CBS 353.64.

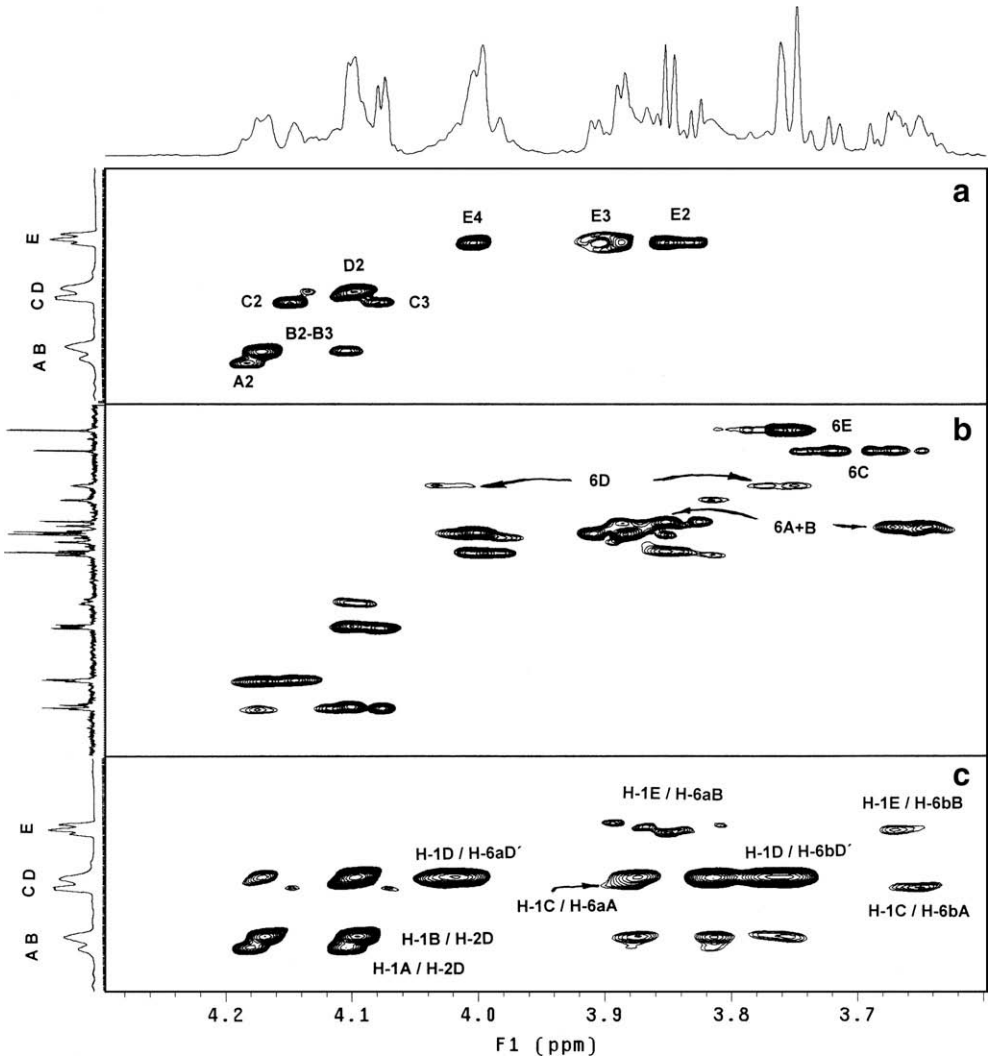
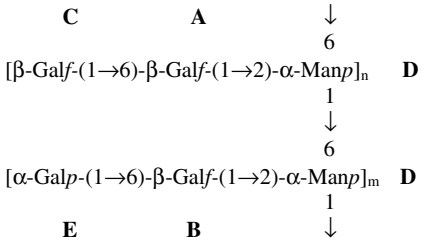


Figure 2. Selected regions of 2D NMR spectra of the F1SS polysaccharide from *U. virens*, showing relationships among: (a) TOCSY; (b) HMQC, and (c) NOESY. Significant crosspeaks have been labeled (being **D′** a second molecule of **D**).

E→6**B**→2**D**→6**D′**. The broadening of the anomeric protons **A**, **B**, **D**, and **E**, and also that of carbons C-1**D** and C-6**D**, in addition to the slight splitting of the peak at 106.8 ppm, indicated an irregular sequence of the chains linked to the mannan backbone although. In any case, from a global perspective, and from all the combined evidence, we may propose an idealized structure for the polysaccharide F1SS isolated from the cell walls of the two strains of *U. virens* such as:



where $n \approx 1$ and $m \approx 2$.

The small percentage of terminal, 2-O-substituted, and 6-O-substituted mannopyranoses found in the methylation analysis, very likely forms a part of a small mannan core, as had been previously observed in several fungal F1SS polysaccharides (see, for instance Prieto et al.[16] and Gómez-Miranda et al.[17]).

A galactomannan consisting of a main chain of α-(1→6)-mannose partially substituted at O-3 by single residues of β-D-galactofuranose or by the disaccharide β-D-Gal*f*-(1→5)-β-D-Gal*f*-(1→ has been reported from *Lineolata rhizophorae,*[18] and a galactomannan fully substituted at O-3 positions of the α-(1→6)-mannose chains was characterized from species of *Podospora*[19] and *Apodus*.[20] None of the F1SS polysaccharides from several species of the genus *Claviceps* and related genera have a linkage types composition compatible with the structure reported here (unpublished results). Then, it may be suggested that *U. virens* does not belong to the Clavicipitales as previously believed on the basis of morphological affinities.[3] Our results are in agreement with recent phylogenetic studies carried out using the sequences of the large subunit of ribosomal RNA gene.[21]

Fungal cell walls, and more specifically their polysaccharides, are implicated in the regulation of the primary events of contact between the parasite and the host.[22] Determinants on fungus-derived polysaccharides are recognized by conserved receptors in animals and plants and elicit a defence response.[23] It has been recently described that the fruitfly *Drosophila melanogaster* has specific Pattern Recognition Receptors for fungal cell wall polysaccharides, such as the polysaccharide-binding protein GNBP3.[24] Other studies show how polysaccharides isolated from fungal cell walls induce a specific response of enhanced resistance

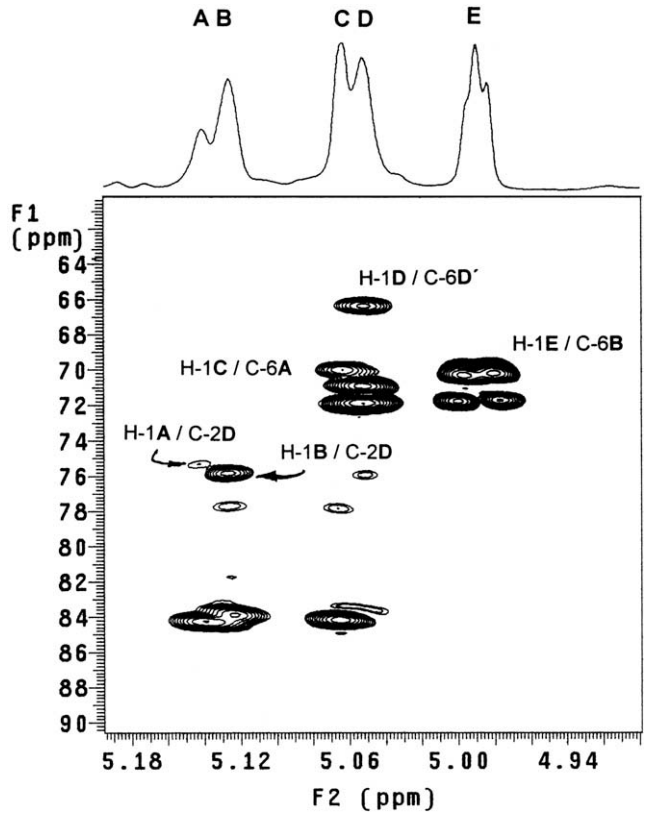


Figure 3. Partial 2D HMBC spectrum of the cell wall F1SS polysaccharide from *U. virens*, showing $^{1}H–^{13}C$ long range connectivities. The anomeric protons and significant crosspeaks have been labeled. **D**´ is a second molecule of **D**.

to pathogens in plants.[25] It has been also reported that during the recognition step previous to infection, a lectin of the fungus *Agaricus bisporus* recognizes and binds to a glucogalactomannan from the surface of the mycopathogen *Verticilllium fungicola.*[11]

3. Experimental

3.1. Microorganisms and culture media

The strains of *U. virens* used were CBS 422.52 and 353.64. The microorganisms were maintained on slants of Bacto potato dextrose agar supplemented with 1 g L^{-1} of Bacto yeast extract (Difco). The basal medium and growth conditions for mycelium production had been previously described.[26]

3.2. Wall material preparation and fractionation

The preparation of fungal cell-wall material[27] and the fractionation procedure[28] were performed as previously described. The crude polysaccharidic preparation extracted from the dry cell-wall material with 1 M NaOH at 20 °C contained water-soluble polysaccharides (Fraction F1S) and water-insoluble polysaccharides (F1I). Fraction F1S was re-fractionated by treatment with a small portion of water (about 50 mg mL^{-1}), followed by centrifugation (10,000*g*, 30 min), giving a solution which was freeze-dried (fraction F1SS), and a precipitate (fraction F1SI). F1I and F1SI were not further investigated. F1SS was treated with 50% ethanol in water. The precipitates were discarded, and the supernatants were dried to eliminate the ethanol, resuspended in water, and freeze-dried. The resulting material constituted polysaccharides F1SS. Purity of the polysaccharides was checked by Size Exclusion Chromatography (SEC). Polysaccharides F1SS (100 mg) were dissolved in 1 mL of 0.3 M NaOH centrifuged at 13,000*g* for 15 min to eliminate insoluble material. The supernatant was added to a column (65 × 2.6 cm) of Sepharose CL-6B, and eluted with 0.3 M NaOH at a flow of 22 mL h^{-1}. Fractions (3.5 mL) were collected and monitored for carbohydrate by the phenol sulphuric acid method.[29] The fractions that tested positive for carbohydrates were combined, dialyzed against water, concentrated to a small volume, and freeze-dried. The column was previously calibrated with a mixture of standards: Dextran Blue (2×10^6 Da), T70 (7×10^4 Da), and T10 (10^4 Da) dextrans (Pharmacia).

3.3. Chemical analyses

Neutral sugars were released by hydrolysis with 3 M trifluoroacetic acid (TFA) for 1 h at 121 °C. The products were reduced with sodium borohydride, and the corresponding alditols were acetylated with 500 μL of pyridine-Ac_2O (1:1) for 1 h at 100 °C. Identification and quantification were carried out by gas–liquid chromatography (GLC) in an Autosystem (Perkin Elmer) instrument equipped with a flame ionization detector. Helium was used as the carrier gas at a constant pressure of 30 psi. Injection was performed in the split mode (split ratio 50:1). Injector and detector were maintained at 250 °C, and samples were analyzed using a temperature program from 210 °C (1 min) to 240 °C, with a ramp rate of 15 °C min^{-1} and maintaining the final temperature for 7 min. Separation was made on a TR-CN100 capillary column (30 m × 0.25 mm, 0.2 μm film thickness). The absolute configuration of the sugars was determined as devised by Gerwig et al.[30]

3.4. Methylation analysis

The polysaccharides were methylated following the Ciucanu and Kerek's method.[31] and hydrolyzed sequentially with 0.15 M and 1.5 M TFA for 1 h at 121 °C. After each hydrolysis step, the products were reduced with sodium borodeuteride, and the mixture of partially methylated alditols was acetylated as above. The mixture of partially methylated alditol acetates obtained was examined by GC–MS in an Agilent 7980A-5975C instrument, using a HP-5 column (30 m × 0.25 mm, 0.2 μm film thickness) and a temperature program (160–210 °C, 1 min initial hold, 2 °C/min ramp rate). Quantifications were made according to peak area.

3.5. NMR analysis

The column-purified sample of polysaccharide F1SS (~20 mg) was dissolved in D_2O (0.8 mL) followed by centrifugation (10,000*g*, 20 min). The supernatant (ca. 0.7 mL) was used for recording the spectra. 1D- and 2D ^{1}H and ^{13}C NMR experiments were carried out at 35 °C on a Varian Unity 500 spectrometer with a reverse probe and a gradient unit. Proton chemical shifts refer to residual HDO at δ 4.66 and carbon chemical shifts to internal acetone at δ 31.07. The 2D NMR experiments (DQFCOSY, 2D-TOCSY, and HMQC) were performed using the standard VARIAN Software.

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