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Short communication

Cell wall mannan of human pathogen *Candida dubliniensis*

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Abstract

Cell wall mannan isolated and purified from the human pathogen yeast *Candida dubliniensis CCY 29-177-1* has been characterized. The polysaccharide of $M_w = 35{,}000$ was composed of D-mannose and trace amount of D-glucose residues. Compositional and methylation analyses of the polymer, acetolysis as well as ^1H and ^{13}C NMR measurements pointed to a highly branched structure of *C. dubliniensis* mannan. Its backbone was composed of α-1,6-linked mannose residues branched at *O*-2 by side mannose residues as single stubs, as well as side oligosaccharide chains of d.p. 2–7, mostly in the form of tri-, di- and tetramers. Longer side chains, penta, hexa- and heptamers, were found in lesser amounts. NMR spectra showed that oligosaccharide side chains were formed by α-1,2- and α-1,3-linked mannose residues. In addition, the presence of higher quantity of oligosaccharides with terminal Manβ(1 → 2)Manβ(1 → fragment was deduced on the basis of characteristic signals in the HSQC spectrum of polymer mannan.

Keywords: *Candida dubliniensis*; Cell wall mannan; Methylation analysis; Acetolysis; NMR

1. Introduction

Candida dubliniensis is a recently described human opportunistic pathogen that is closely related to *C. albicans* but differs from it with respect to epidemiology, certain virulence characteristics, and the developed fluconazole resistance (Ahmad, Khan, Mokaddas, & Khan, 2004; Gutiérrez, Morales, Gonzáles, & Quindós, 2002; Sullivan, Morgan, & Coleman, 2005). This strain was originally identified in oral specimens from Irish HIV-infected and AIDS patients with recurrent oral candidiasis (Sullivan, Westerneng, Haynes, Bennet, & Coleman, 1995). Recently, *C. dubliniensis* has been recovered from oral samples of HIV-seropositive pediatric patients (Brown, Jabra-Rizk, Falkler, Baqui, & Meiller, 2000), as well from insulin-dependent diabetic patients (Belazi et al., 2005). It has been found that the most of immunological effects observed with the intact fungal cells have been reproduced with cell-wall components, which were shown to be potent inducers of cellular and humoral immunity. Among them, mannans and mannoproteins were found to be the most significant (Pontón, Omaetxebarría, Elguezabal, Alvarez, & Moragues, 2001). Mannan is one of the major components of the yeast cell wall together with glucan, chitin and protein. It was found only as part of glycoconjugate – either glycoprotein or glycolipid – and not as an unconjugated oligosaccharide (Ballou, 1982). Cell wall mannoproteins containing mannose in either *O*- or *N*-glycosidic linkages are largely responsible for the adhesive properties (Kanbe, Han, Redgrave, Riesselman, & Cutler, 1993), immunomodulation ability (Domer, 1989), and antigenic variability (Barturen et al., 1995; Poulain, Hopwood, & Vernes, 1985). Generally, *Candida sp.* mannans are always composed of an α-1,6-linked backbone substituted mostly at *O*-2 by different number of linear or branched side oligomannosyl chains composed of α-1,2-, α-1,3- with or without terminal β-1,2-linkages (Shibata et al., 1992; Shibata et al., 1996; Shibata, Kobayashi, Okawa, & Suzuki, 2003; Kobayashi et al., 1994). Terminal β-1,2-linkages were found to function as the serotype epitops for individual species. Depending of their presence or absence, the serotype A and serotype B of *Candida albicans* mannans are distinguished (Kobayashi et al., 1990; Shibata et al., 1992).

The research of *C. dubliniensis* as a hazardous opportunistic human pathogen is very important from the

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immunological point of view. This communication gives the first preliminary results obtained during the study of *C. dubliniensis* mannan. Detailed investigation of structural features of this mannan is in progress and its results, which may significantly contribute to the understanding of pathogenicity of this yeast, will be reported in the following paper.

2. Material and methods

2.1. Cultivation of yeast

Candida dubliniensis strain (*CCY 29-177-1*, the gift from Dr. H. Bujdakova, Faculty of Natural Sciences, Bratislava) was grown on semi-synthetic liquid medium containing 2% D-glucose, 0.3% $(NH_4)_2SO_4$, 0.1% $MgSO_4$, 0.05% KH_2PO_4, 0.5% yeast autolysate and microelement solution for 4 days at 28 °C, then harvested by centrifugation, washed twice with distilled water and freeze-dried.

2.2. Isolation and purification of mannan

Mannoprotein was extracted from freeze-dried cells by autoclaving for 1 h at 120 °C three times with 0.2 M NaCl. The supernatant extracts were combined and a mannoprotein was precipitated with ethanol, dissolved in distilled water, dialyzed against distilled water for 24 h. The freeze dried mannoprotein was suspended in 2% KOH and heated for 1 h at 100 °C. Insoluble residue was separated by centrifugation, and mannan was precipitated from supernatant with Fehling reagent (Jones & Stoodley, 1965). Sedimented mannan–copper complex was dissolved in 3 M HCl, and added dropwise to methanol–acetic acid. The precipitate was centrifuged, dissolved in distilled water, dialyzed and freeze dried.

2.3. Elemental analysis

The mannan was analyzed for the carbon, hydrogen, and nitrogen content using the EA 1108 device (FISONS Instruments, UK). *Optical rotation* was measured with automatic polarimeter Perkin-Elmer Model 241.

2.4. Monosaccharide composition

Polysaccharide was hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C min^{-1})−165 °C (20 °C min^{-1}) and flow rate of hydrogen 20 cm^3 min^{-1}.

2.5. Acetolysis of mannan

Acetolysis of mannan was done as described by Kocourek & Ballou (1969). The deacetylated oligosaccharide products were separated on a column (2 cm × 150 cm) of Biogel P-2 (Bio-Rad) by water elution. Their degree of polymerization (d.p.) was identified by comparison with the elution volumes of mannooligosaccharides used as reference standard. Carbohydrates were monitored with a differential refractometer RIDK 32 (Laboratory Equipments, Prague, Czech Republic).

2.6. Methylation analysis

Methylation analysis was performed by the Hakomori procedure (Hakomori, 1964).

2.7. HPLC analysis

HPLC analysis was performed at ambient temperature with two columns (250 × 8 mm) packed with Biospher GM 300 and GM 1000 (Tessek, Prague, Czech Republic). 0.1 M aqueous $NaNO_3$ solution was used as a mobile phase. A set of pullulans (Gearing Scientific, Polymer Lab. Ltd, UK) was used for the calibration of the HPLC system.

2.8. NMR analysis

Spectra were measured in D_2O at 25 and 40 °C on Bruker 300 MHz Avance DPX and Varian 600 MHz UNITY INOVA 600 NB spectrometers, equipped with 5 mm multinuclear probe with inverse detection and 5 mm $^1H\{^{13}C,^{15}N\}$PFG Triple Res IDTG600-5, respectively, (both with *z*-gradients). Carbon spectra were measured in 5 mm ^1H, ^{13}C, ^{15}N, ^{31}P QNP probe on Bruker 300 MHz Avance DPX. Samples were freeze-dried from 95% D_2O and after they were dissolved in 99.98% D_2O. For both, ^1H and ^{13}C NMR spectra, chemical shifts are referenced to internal acetone (δ 2.217 and 31.07, respectively). ^1H–^{13}C Heterocorrelated HSQC spectrum was measured with optimisation on one bond $^1J_{CH}$ coupling constant on 165 Hz.

3. Results

Candida dubliniensis mannan was isolated from the freeze-dried yeast biomass according to isolation scheme (Fig. 1). The polymer was composed of D-mannose and a trace amount of D-glucose residues. It had $M_w = 35{,}000$ and optical rotation was +48.1°. No protein content was determined by elemental analysis. Partial depolymerization of mannan by acetolysis followed by gel chromatography afforded monomeric residues, a series of oligosaccharides of d.p. M_2–M_8 and a fraction eluted in the void of the column (Fig. 2). Monosaccharide composition of M_2–M_8 revealed on hydrolysis the presence of D-mannose residues only. It indicated that a low content of glucose residues detected on hydrolysis of mannan originated from cell wall glucan. The H1 signal of low intensity at δ 5.44 in the ^1H NMR spectrum (Shibata et al., 1985, 1992) reflected the presence of the 1-*O*-α-phosphorylated mannose in acid labile oligosaccharides in agreement with chemical analysis (2%).

Fig. 1. Isolation scheme of *C. dubliniensis* mannan.

In order to determine the linkage pattern of the monosaccharide components, the *C. dubliniensis* mannan was subjected to linkage sugar analysis. Three main sugar derivatives, i.e. 2,3,4,6-tetra-*O*-methyl-, 3,4,6-tri-*O*-methyl- and 3,4-di-*O*-methylmannose demonstrated the prevalence of terminal, 1,2- and 1,2,6-linked mannopyranose units in the polymer. The content of unbranched 1,6-linked mannopyranose residues, derived from 2,3,4-tri-*O*-methylmethylmannose derivatives was much lower (Table 1). The results of the linkage analysis suggested a highly branched structure of the polymer with a backbone composed of 1,6-linked mannopyranose residues branched at the position *O*-2 by side oligosaccharide chain composed mostly 1,2-linked mannose residues.

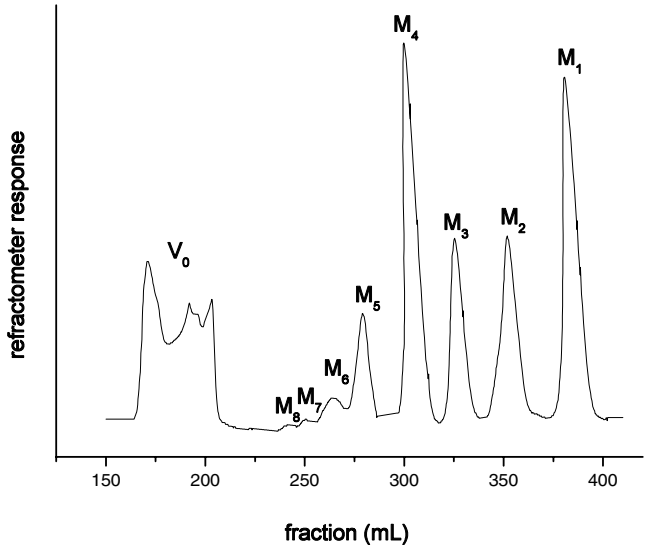

Fig. 2. BioGel P-2 profile of the mannan-derived oligosaccharides (M_1, mannose; M_2–M_8, mannobiose to mannooctaose; V_0, void volume).

Table 1
Methylation analysis data of *C. dubliniensis* cell wall mannan

Sugar derivative	Mole %	Mode of linkage
2,3,4,6-Me_4-Man[a]	20.5	Man*p*-(1→
3,4,6-Me_3-Man	54.7	→2)-Man*p*-(1→
2,3,4-Me_3-Man	3.5	→6)-Man*p*-(1→
4,6-Me_2-Man	tr.	→2,3)-Man*p*-(1→
2,4-Me_2-Man	tr.	→3,6)-Man*p*-(1→
3,4-Me_2-Man	20.3	→2,6)-Man*p*-(1→
Per-*O*-Ac-Man	tr.	→2,3,4,6)-Man*p*-(1→

2,3,4,6-Me_4-Man[a] = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, etc.

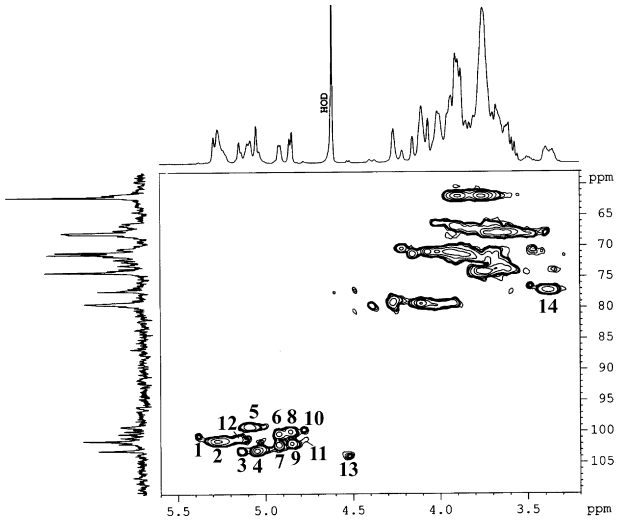

Fig. 3. ^{1}H–^{13}C heterocorrelated HSQC NMR spectrum of the *C. dubliniensis* mannan. HOD residual signal of water.

NMR spectra have shown the presence of both α and β mannose residues. In the ^{1}H–^{13}C heterocorrelated HSQC NMR spectrum of the polymer (Fig. 3) characteristic H1/C1 signals could be identified: cross peak number **1** at δ 5.375/100.75 due to the α-1,3-linked Man in a Manα(1 → 2)Manα(1 → 3) Manα(1 → fragment; the broad signal **2** at δ 5.29/101.44 due to internal α-1,2-linked Man units, **3** due to the terminal α-1,3-linked Man units at 5.13/103.1, **4** due to the terminal α-1,2-linked Man δ 5.04/103.01. For α-1,6-linked Man residues following signals could be attributed: **5** at δ 5.10/99.12 to substituted α-1,2,6-linked internal Man units; **6** at δ 4.92/100.31 to nonsubstituted α-1,6-linked Man and the signal **7** at δ 4.92/102.07 to the terminal 1,6-linked ones. Cross peaks **8**–**12** are characteristic of βMan residues involved in 1,2-linkage. Particularly, **8** at δ 4.856/99.97 is due to the terminal βMan unit of the Manβ(1 → 2) Manβ(1 → 2) Manα(1 → fragment and the cross peak **9** at δ 4.844/101.902 due to its neighbouring βMan. Cross peaks **10** (δ 4.777/99.62) and **11** (4.76/101.15) indicated the presence of small amount of oligosaccharides with one terminal βMan unit in Manβ(1 → 2)Manα(1 → 2) Manα(1 → and Man β(1 → 2)Manα(1 → 3) Manα(1→ fragments, respectively. The signal **12** (δ 5.15/101.10) is the complementary signal to **10** and **11** which is supposed to be due to α-1,2- and α-1,3-linked Man units close to the terminal βMan. Chemical shifts of these signals were in agreement with the literature data (Tojo, Shibata, Ban, & Suzuki, 1990; Kobayashi et al., 1992). Signals H5 and C5 due to β-1,2-linked Man residues have also very characteristic chemical shifts. They appeared at δ 3.379/77.14 (the cross peak **14**) (Kobayashi et al., 1990; Shibata et al., 2003). Sugar analysis revealed the presence of a low quantity of glucose originated from glucan, which is supposed to be a contaminant, and it was represented by the cross peak **13**.

From the results presented it can be concluded that cell wall mannan isolated from the human pathogen yeast *C. dubliniensis* displayed α-1,6-linked backbone pattern substituted at *O*-2 by side linear or branched chains, which is common for all *Candida sp.* mannans. However, some differences have been found in the length at the side chains, their frequence, and in the content of β-linked mannose residues.

Detailed investigation of structural features of mannooligosaccharides, which will clarify the complex structure of cell wall mannan from *C. dubliniensis*, is in progress and they will be published in the following paper.

Acknowledgements

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