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Human pathogen *Candida dubliniensis*: A cell wall mannan with a high content of β-1,2-linked mannose residues

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Abstract

A cell wall homopolymer – mannan has been isolated from the human pathogen yeast *Candida dubliniensis CCY 29-177-1*. It had $M_w = 35{,}000$ and optical rotation $[\alpha]^{20} = +48.1^0$. Results of compositional and methylation analyses show that the α-1,6-linked backbone of the *C. dubliniensis* mannan is to 83% substituted by side chains of d.p. 1–7, mostly in the form of tri-, di-, mono- and tetramers, at *O*-2 and some Man residues might be substituted at *O*-3. Substitution of the backbone with single βMan units was detected in an important quantity. With molecular weight increasing of side chains the quantity of oligosaccharides with terminal single β-, two consecutive ββ- and three consecutive βββ-1,2-linked Man residues was more significant, reaching the maximum in the highest fraction (75%).

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

1. Introduction

The research of the yeast *Candida dubliniensis* as a hazardous opportunistic human pathogen is very important from the immunological point of view. *C. dubliniensis* has been identified from HIV-infected and AIDS patients with recurrent oral candidiasis (Sullivan, Westerneng, Haynes, Bennet, & Coleman, 1995), as well from oral samples of HIV-seropositive pediatric patients (Brown, Jabra-Rizk, Falkler, Baqui, & Meiller, 2000). It 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). The cell surface hydrophobicity plays an important role in the pathogenicity of microorganisms. A correlation was demonstrated between the hydrophobicity status and changes in the acid-labile, phosphodiester-β-1,2-linked oligomannoside components of the *N*-linked cell wall mannoprotein of *C. albicans* (Masuoka & Hazen, 1997, 2004). The differences observed between *C. dubliniensis* and *C. albicans* in their cell surface hydrophobicity-related protein and mannosylation features could partially contribute to their differential virulence capabilities (Hazen, Wu, & Masuoka, 2001).

It has been found that the most of immunological effects observed with the intact yeast 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, Omaetxebarrıa, Elguezabal, Alvarez, & Moragues, 2001). The detailed structures of mannans from the cell wall of *C. albicans* (serotypes A and B) were proposed by Shibata et al. (1985, 1989, 1992a) and Kobayashi et al. (1990). Mannans consisted of acid-labile and acid-stable regions: the acid-labile region is composed of β-1,2-linked oligomannosyl residues, up to heptaose, that are linked to the phosphate group and connected to the acid-stable region with another phosphate group linkage in a deesterified form. The acid-stable region involves a backbone composed of α-1,6-linked mannopyranose units substituted at *O*-2 by many branches composed of α-1,2-, α-1,3- (serotype B), over serotype A α-1,2 -, α-1,3-, β-1,2-

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linked mannopyranose units. β-1,2-linked mannose units may be attached to the non reducing terminal of the α-l,2-linked mannotetraose side chains of the mannan *C. albicans* serotype A (Kobayashi, Shibata, Mitobe, Ohkubo, & Suzuki, 1989; Shibata et al., 1989), *C. tropicalis* (Kobayashi et al., 1994a) and *Candida glabrata* (Kobayashi et al., 1992a), or they may be connected to an α-l,3-linked mannose unit at the terminal end of an oligosaccharide chain in *Candida guilliermondii* (Shibata et al., 1996a, 1996b) and *Candida lusitaniae* (Shibata, Kobayashi, Okawa, & Suzuki, 2003). For fine structural features of mannan polymers, such as the number of 1,2-linked βMan residues attached to the non reducing terminal of αMan oligosaccharide chains, characteristic H1 and H2 chemical shifts data were used (Shibata et al., 1992a, 1992b, 1993, 1996b, 1997, 2003; Kobayashi et al., 1997; Kolarova, Matulová, & Capek, 1997). Oligosaccharides with one to three 1,2-linked βMan attached to the non reducing terminal of α-1,2-linked or α-1,2- and α-1,3-linked Man oligomers of different length have been reported, and NMR data some of them have been fully assigned (Shibata et al., 2003).

First results, based on the compositional and methylation analyses and NMR spectroscopy studies on the *C. dubliniensis* mannan and its oligomeric acetolysis products pointed to a highly branched structure of the polymer (Ližičárová, Matulová, Machová, & Capek, 2007). The backbone, composed of α-1,6-linked mannose residues, was found to be 83% branched at *O*-2 by single mannose residues as well as oligosaccharide side chains mostly in the form of tri-, di-, mono- and tetramers. Longer side chains, penta- to heptamers, were present in lesser amounts. NMR spectra of oligosaccharides showed that they were formed mostly by α-1,2- and α-1,3-linked mannose residues. 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 ^{1}H and HSQC NMR spectra of the polymer. In this study we describe the detailed analysis based on ^{1}H and COSY NMR spectra of the mannan and its oligosaccharide fractions obtained after a conventional acetolysis.

2. Methods

2.1. Cultivation of yeast

Candida dubliniensis strain (*CCY 29-177-1*) 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 according to the scheme (Fig. 1). 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 carbon, hydrogen, and nitrogen content using the EA 1108 device (FISONS Instruments, UK). The content of phosphorus was estimated using dithizone indicator.

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 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), a 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 Biogel P-2 (Bio-Rad) column (2 cm × 150 cm) by water elution. Their degree of polymerization (d.p.) was identified by comparison with elution volumes of mannooligosaccharides used as reference standards. Carbohydrates were monitored with a differential refractometer RIDK 32 (Laboratory Equipments, Prague, Czech Republic).

2.6. Methylation analysis

The dry sample of polysaccharide (~2 mg) was solubilized in dry Me_2SO (1 mL) and methylated by the Hakomori method (Hakomori, 1964). The methylated polysaccharide was purified using a Sep-Pak C_{18} cartridge (Waters Assoc.) to give fully methylated product. The permethylated polymer was hydrolyzed with 90% formic acid (1 h, 100 °C) and 2 M trifluoroacetic acid (1 h, 120 °C), reduced with $NaBD_4$, acetylated and analyzed by GLC-MS.

2.7. HPLC analysis

HPLC analysis was performed at ambient temperature with two columns (250 × 8 mm) packed with Biospher

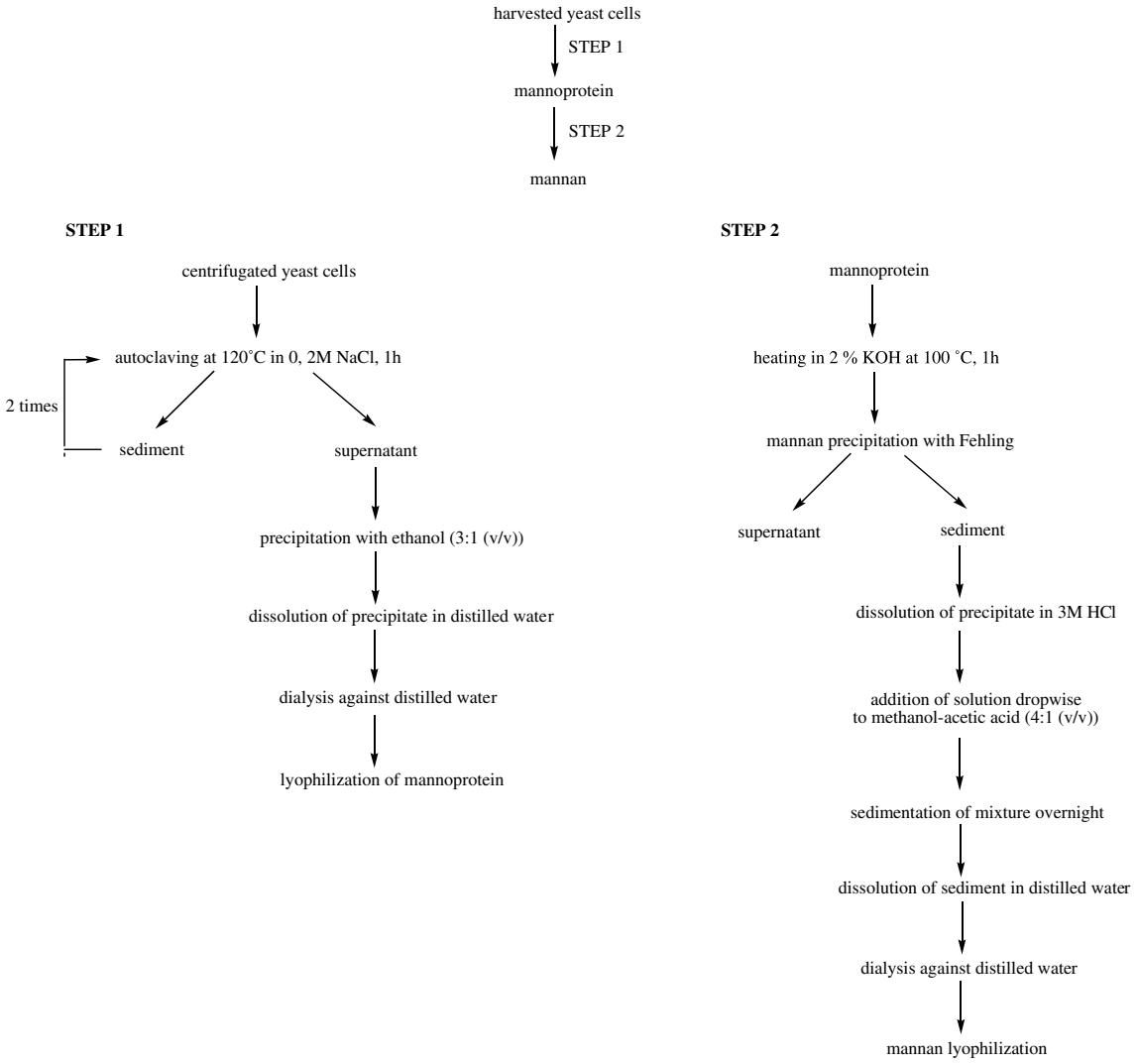

Fig. 1. Isolation scheme of *C. dubliniensis* mannan.

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{13C, 15N}PFG Triple Res IDTG600-5, respectively, (both with *z*-gradients). Carbon spectra were measured in 5 mm $^{1}H,^{13}C,^{15}N$, ^{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, ^{1}H and ^{13}C NMR spectra, chemical shifts are referenced to internal acetone (δ 2.217 and 31.07, respectively). ^{1}H-^{13}C Hetero-correlated HSQC spectra with optimisation on one bond coupling constant $^{1}J_{CH} = 165$ Hz and ^{1}H-^{1}H homocorrelated COSY spectra with gradient selection in absolute intensity mode were measured. Assignment of cross peaks was based on already published data (Shibata et al., 1992a, 1992b, 1993, 1996b, 1997, 2003; Kobayashi et al., 1997; Kolarova et al., 1997) and their quantification was made on the basis of cross peak intensities in COSY spectra.

3. Results

Candida dubliniensis mannan, prepared from the freeze-dried yeast biomass according to isolation scheme (Fig. 1) was composed of D-mannose and a trace amount of D-glucose residues. It had $M_w = 35{,}000$ and optical rotation was $+48.1^0$. The content of nitrogen was 0.05% (C, H, N analysis) and phosphorous 0.9% (dithizone indicator). No protein was found using modified Bradford method (Sedmak & Grossberg, 1977). Partial depolymerization of mannan by acetolysis followed by gel chromatography afforded

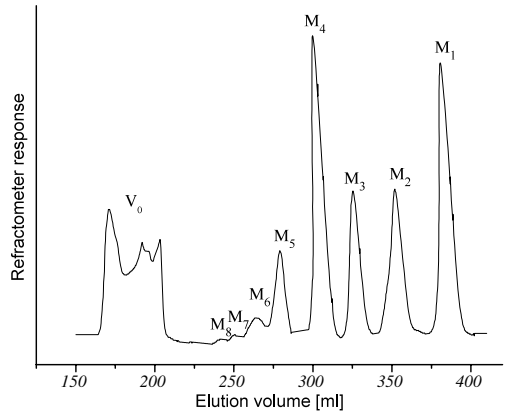

Fig. 2. BioGel P-2 profile of the mannan-derived oligosaccharides. (**M1**, mannose; **M2**–**M8**, mannobiose to mannooctaose; **V0**, void volume).

monomeric residues, a series of oligosaccharides fractions of d.p. **M2**–**M8** and a fraction eluted in the void volume (**V0**) of the column (Fig. 2). Monosaccharide analysis of **M2**–**M8** revealed the presence of D-mannose residues only and confirmed the homopolymer type of cell wall polysaccharide.

Linkage sugar analysis of *C. dubliniensis* mannan showed the presence of 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, and indicated the prevalence of terminal, 1,2- and 1,2,6-linked mannopyranose units, respectively, in the polymer. The low content of 2,3,4-tri-*O*-methylmannose derivatives demonstrated the presence of unbranched 1,6-linked mannopyranose residues in the polymer as well (Table 1). The results of the linkage analysis suggested a highly branched structure of the polymer with a backbone composed of 1,6-linked α-mannopyranose residues branched at the position *O*-2 by side oligosaccharide chains composed 1,2- and 1,3-linked mannose residues.

The conventional acetolysis of the *C. dubliniensis* mannan afforded after the separation 8 fractions. Fig. 3 shows ^{1}H NMR spectra of individual fractions (**M1**–**M8**), the void volume fraction (**V0**) and that of the polymer mannan (**P**). None of oligosaccharide spectra contained a H1 signal at δ 5.44 suggesting the presence of phosphodiesterified oligosaccharides (Shibata et al., 1985, 1992b). This signal was detected only in the polysaccharide spectrum (not shown) indicating their presence in a trace amount in accordance to the chemical analysis (0.9% w/w of phosphorous). H1 Signals characteristic of βMan residues appeared at δ 4.92–4.75 while those of αMan at δ 5.38–4.90. A comparison of both regions (Fig. 3) suggested higher quantity of βMan linked residues in hexa-, hepta- and octasaccharide fractions (**M6**–**M8**).

Two dimensional ^{1}H-^{1}H homocorrelated COSY NMR spectra (Fig. 4) were used to enhance the resolution e.g. to obtain H2 and H3 chemical shifts values. Chemical shifts of signals found in the COSY spectrum of the polymer were in accordance with the literature data (Kobayashi et al., 1997; Kolarova et al., 1997; Shibata et al., 1992a, 1993, 1996b, 1997, 2003) and they are shown in the Table 2. NMR analysis showed that the **M2**–**M8** fractions represent mixtures of oligosaccharides. Their composition and component quantifications were made on the basis of H1/H2 cross peak signals volumes characteristic of individual linkage types and fragments. Characteristic H1/H2 chemical shift values searched in oligosaccharide fractions, which were used for this structural analysis, were similar to that of the polymer. Data of the polymer are presented in the Table 2. In addition to H1 and H2 also H3 chemical shifts data are shown because they gave valuable information in this structural study. Table 3 shows the oligosaccharide composition in individual fractions.

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	56.7	→ 2)-Man*p*-(1→
2,3,4-Me$_3$-Man	3.2	→ 6)-Man*p*-(1→
2,4-Me$_2$-Man	2.7	→3,6)-Man*p*-(1→
3,4-Me$_2$-Man	16.9	→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.

3.1. Analysis of the *M1*–*M8* fractions

3.1.1. Fraction *M1*

NMR spectra of the fraction **M1** showed that it is composed of free mannose and traces of glucose residues (Fig. 3, **M1**) in agreement with results of sugar compositional analysis. In the COSY spectrum H1/H2 cross peaks due to free αMan and βMan were detected at δ 5.169/3.923 and δ 4.885/3.934, respectively, while those due to αGlc and βGlc at δ 5.218/3.519 and δ 4.633/3.229, respectively.

3.1.2. Fraction *M2*

Disaccharides with terminal α-1,2- and α-1,3-linked Man in Manα(1 → 2)Man (**2**$_1$) and Manα(1 → 3)Man (**2**$_2$) were found in the ratio 62% and 8%, respectively. In addition, 30% of the disaccharide **2**$_3$ with terminal β-1,2-linked Man was present. (Fig. 3, Table 3). The attribution of its signals was in agreement with literature data (Zhang & Ballou, 1981): Manβ(1 → 2)Manα (20%; H1/H2 (starting from the non reducing end) δ 4.753/4.040 and 5.276/4.12, respectively) and Manβ(1 → 2)Manβ (10%; H1/H2 δ 4.809/4.172 and 4.964/4.173, respectively). The configuration of mannose residues in all disaccharides was confirmed by one bond $^1J_{CH}$ coupling constants values: for βMan residues $^1J_{CH}$ 160.1–161.9 Hz and αMan $^1J_{CH}$ 169.4–170.2 Hz was found.

3.1.3. Fraction *M3*

A quantitative evaluation of individual components of the **M3** mixture was done on the basis of following characteristic H1/H2 cross peaks in the COSY spectrum (Table 2): the signal **2** at δ 5.29/4.105 due to internal α-1,2-linked Man and **9** at δ 5.050/4.064 due to terminal α-1,2-linked

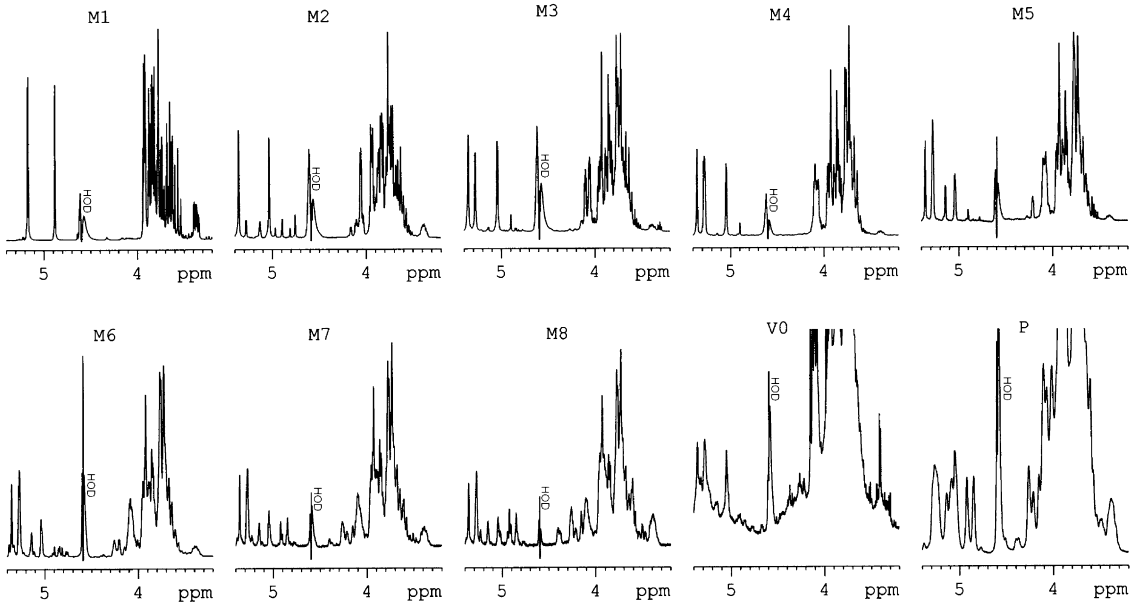

Fig. 3. ^{1}H NMR spectra of mannan polysaccharide P, void volume V0, and **M1**–**M8** oligosaccharide fractions obtained after conventional polysaccharide hydrolysis. HOD – residual signal of water.

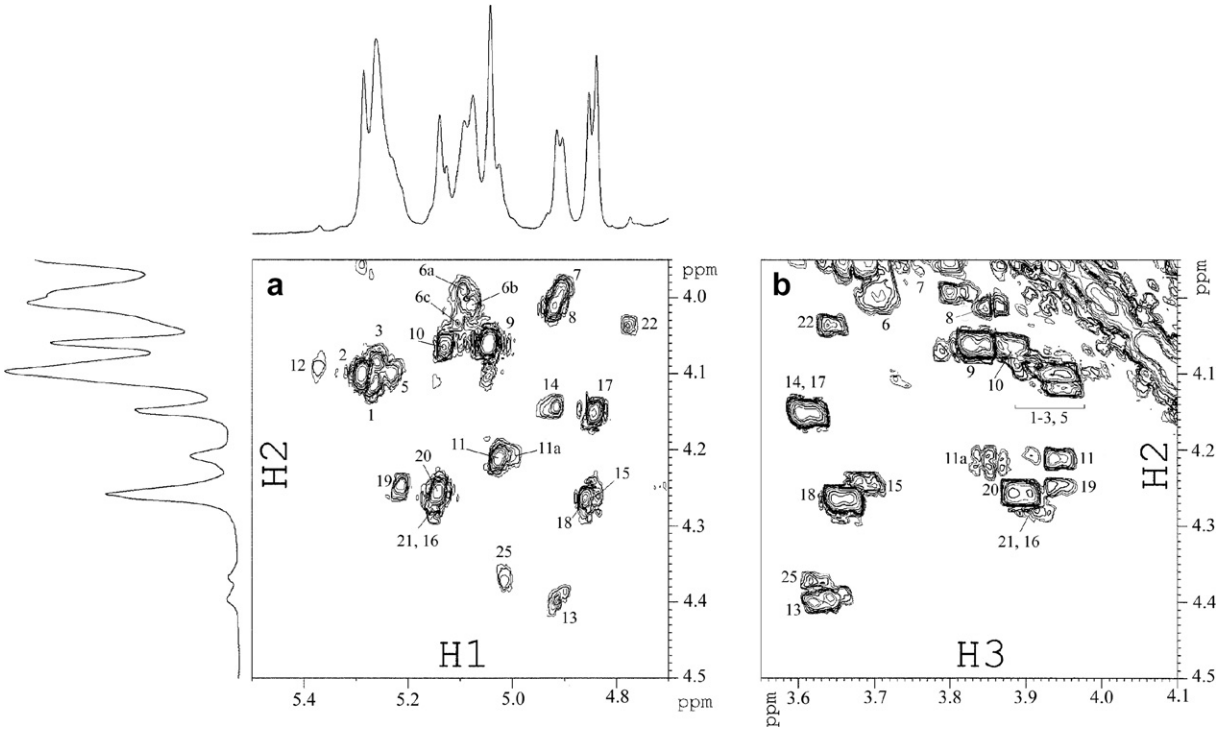

Fig. 4. ^{1}H-^{1}H homocorrelated 600 MHz COSY spectrum of the mannan polymer. a – region of H1/H2 cross peaks; b – the region of H2/H3 cross peaks. Attribution of signals was made according the Table 2.

Man showed that trisaccharide Manα(1 → 2)Manα(1 → 2)-Man (**3**$_1$) was a dominant component of the mixture (80%), while the intensity of the signal **10** at δ 5.14/4.06 due to the terminal α-1,3-linked Man unit in Manα(1 → 3)Manα(1 → 2)Man (**3**$_2$) indicated 10% of this oligosaccharide. H1 Signals due to two consecutive βMan residues at δ 4.839 (**17**) and 4.856 (**18**) revealed the presence of 7% of the Manβ(1 → 2)Manβ(1 → 2)Man (**3**$_4$) trisaccharide, while that one at δ 4.778/4.04 (**22**) due to the single terminal β-1,2-linked mannose unit suggested 3% of Manβ(1 → 2)Manα(1 → 2)Man (**3**$_3$) (Fig. 4, Table 3).

3.1.4. Fraction *M4*

Based on the signal intensities **2**, **9** and **10** the tetrasaccharide Manα(1 → 2)Manα(1 → 2)Manα(1 → 2)Man (**4**$_1$) was found to be dominant in the mixture (70%), while

Table 2
Assignment of H1, H2 and H3 chemical shifts of mannose unit in structure reporter fragments searched in the polymer mannan of *C. dubliniensis* and the quantity of those which were identified

Cross peak	Chemical shift/δ			Fragment structure	Quantity %
	H1	H2	H3		
1	5.271	4.110	3.87–3.98	$\alpha1 \rightarrow 2$**Man**$\alpha1 \rightarrow 2$	3.9
2	5.290	4.102	3.87–3.98	Manα 1$\rightarrow$ 2**Man**$\alpha1 \rightarrow 2$	14.8
3	5.260	4.084	3.87–3.98	$\alpha1 \rightarrow 2$**Man**$\alpha1 \rightarrow 2$	4.0
4	5.30	4.11	x	Man$\alpha1 \rightarrow$ 3Man$\alpha1 \rightarrow$ 2 **Man**$\alpha1 \rightarrow$ 3 Man$\alpha1\rightarrow$ 2 $\uparrow$6 $\uparrow$6 Manα1 Manα1	xx
5	5.240	4.100	3.87–3.98	Man$\alpha1 \rightarrow$ 3Man$\alpha1 \rightarrow$ 2 **Man**$\alpha1 \rightarrow$ 2 Man$\alpha1\rightarrow$ $\uparrow$6 Manα1	1.7
6a	5.095	3.990	3.700	$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6**Man**$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6 $\uparrow$2 $\alpha1 \rightarrow$ 2Manα1	2.3
6b	5.077	4.011	3.700	$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6**Man**$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6 $\uparrow$2 $\uparrow$2 $\uparrow$2 $\alpha1 \rightarrow$ 2Manα1	2.9
6c	5.103	4.029	3.700	$\alpha1 \rightarrow$ 6Man1$\alpha \rightarrow$ 6**Man**$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6 $\uparrow$2 $\uparrow$2 $\uparrow$2 Manα1	0.7
6d	5.120	4.01	x	$\alpha1 \rightarrow$ 6Man1$\alpha \rightarrow$ 6**Man**$\alpha1 \rightarrow$ 6Man$\alpha1 \rightarrow$ 6 $\uparrow$2 Manα1	0
7	4.904	3.991	3.807	$\alpha1 \rightarrow$ 6**Man**$\alpha1 \rightarrow$ 6	5.2
8	4.918	4.011	3.856	**Man**$\alpha1 \rightarrow$ 6	8.2
9	5.045	4.058	3.834	**Man**$\alpha1 \rightarrow$ 2	26.0
10	5.140	4.063	3.880	**Man**$\alpha1 \rightarrow$ 3	5.0
11	5.026	4.210	3.944	$\alpha1 \rightarrow$ **3Man**$\alpha1 \rightarrow$ 2	6.0
11a	5.000	4.207	3.854	$\alpha1\rightarrow$ **3Man**$\alpha1 \rightarrow$ 2 $\uparrow$6 Manα1	0.9
12	5.372	4.092	x	Man$\alpha1 \rightarrow$ 2**Man**$\alpha1 \rightarrow$ **3**Man$\alpha1 \rightarrow$ 2	0.3
13	4.913	4.395	3.634	Man$\beta1 \rightarrow$ 2**Man**$\boldsymbol{\beta}$**1** $\rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\alpha1 \rightarrow$ 2	0.6
14	4.916	4.142	3.612	**Man**$\boldsymbol{\beta}$1 $\rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\alpha1 \rightarrow$ 2	
15	4.858	4.264	3.657	Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2**Man**$\boldsymbol{\beta}$1 $\rightarrow$ 2Man$\alpha1 \rightarrow$ 2	
16	5.150	4.265	x	Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2**Man**$\alpha1 \rightarrow$ 2	
17	4.844	4.150	3.621	**Man**$\boldsymbol{\beta}$1 $\rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\alpha1 \rightarrow$ 2,3	
18	4.852	4.269	3.690	Man$\beta1 \rightarrow$ 2**Man**$\boldsymbol{\beta}$1 $\rightarrow$ 2Man$\alpha1 \rightarrow$ 3	
19	5.213	4.245	3.943	Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2**Man**$\alpha1 \rightarrow$ 3	1.7
20	5.142	4.255	3.893	Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2**Man**$\alpha1 \rightarrow$ 2	12.5
21	5.155	4.269	3.910	Man$\beta1 \rightarrow$ 2**Man**$\alpha1 \rightarrow$ 2, 3	
22	4.774	4.035	3.645	**Man**$\boldsymbol{\beta}$**1** $\rightarrow$ 2Man$\alpha1 \rightarrow$ 2	2.7
23	4.757	4.037	x	**Man**$\boldsymbol{\beta}$**1** $\rightarrow$ 2Man$\alpha1 \rightarrow$ 3	traces
24	5.246	4.276	x	Man$\beta1 \rightarrow$ 2**Man**$\boldsymbol{\alpha}$**1** $\rightarrow$ 3	0
25	5.014	4.370	3.626	Man$\beta1 \rightarrow$ 2**Man**$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\beta1 \rightarrow$ 2Man$\alpha1 \rightarrow$ 2	0.7

The quantity is shown only for identified fragment structures and it was based on the cross peaks intensities measured in 600 MHz COSY spectrum. x – not assigned, xx – not found due to signal overlaps. Searched chemical shifts for oligosaccharides had similar values to those of the polymer.

the quantity of the oligosaccharide with the terminal α-1,3-linked mannose Man$\alpha(1 \rightarrow 3)$Man$\alpha(1 \rightarrow 2)$Man$\alpha(1 \rightarrow 2)$-Man ($\mathbf{4}_2$) represented 17%. Further, the intensity of the cross peak **12** due to the substituted α-1,3-linked Man suggested the presence of Man$\alpha(1 \rightarrow 2)$Man$\alpha(1 \rightarrow 3)$Man$\alpha(1 \rightarrow 2)$Man ($\mathbf{4}_3$) tetrasaccharide (13%). On the

Table 3
M1–M8 fraction compositions obtained after a conventional acetolysis of the polysaccharide mannan found by NMR analysis and elution profile data

Fraction/ oligomer		Sugar residue H	G	F	E	D	C	B	A	Quantity % Elution	NMR tube
M1									$[Man^{\alpha1}]_n$ (↑6)	24.7	100
M2	2_1								$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)	18.1	62
	2_2								$Man^{\alpha1}\rightarrow{}^{3}Man$ (↑6)		8
	2_3								**$Man^{\beta1}\rightarrow{}^{2}Man$** (↑6)		30
M3	3_1						$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)			14.3	80
	3_2						$Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)				10
	3_3						**$Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)				3
	3_4						**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man$ (↑6)				7
M4	4_1					$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)				31.4	70
	4_2					$Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)					17
	4_3					$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)					13
	4_4					**$Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)					tr.
	4_5					**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)					tr.
	4_6					**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man$ (↑6)					tr.
M5	5_1				$Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)					8.2	73
	5_2				$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)						10
	5_3				$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)						10
	5_4				**$Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)						6
	5_5				**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)						tr.
M6	6_1			$Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)						2.7	22
	6_2			$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)							27
	6_3			**$Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}$**$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)							7
	6_4			**$Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}$**$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)							22
	6_5			**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}$**$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)							22
	6_6			**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}$**$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)							tr.
M7	7_1		$Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)							0.35	10
	7_2		$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								19
	7_3		$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								13
	7_4		**$Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								10
	7_5		**$Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								10
	7_6		**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								10
	7_7		**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)								14
	7_8		**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$** (↑6)								14
M8	8_1	$Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}$ (↑6)								0.25	25
	8_2	**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{3}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)									25
	8_3	**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}$****$Man^{\alpha1}$**$\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$ (↑6)									25
	8_4	**$Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\beta1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man^{\alpha1}\rightarrow{}^{2}Man$** (↑6)									25

Elution – relative quantity of individual fractions based on the surface of the signals in the elution profile; NMR tube – percentage of identified oligosaccharides in individual fractions calculated on the basis of integrals of structure reporter H1/H2 cross peaks in the COSY spectrum (Table 2). The total content of oligosaccharides in each fraction was equal 100%. Column A represents mannose units of the reducing end of the oligosaccharides previously 1,6-linked in the backbone, Columns B–H show mannose units of side chain.

basis of characteristic signals **14 - 22** trace amounts of a big variety of oligosaccharides ($\mathbf{4}_{4-6}$) with β-linkages could be deduced (Fig. 4, Table 3).

3.1.5. Fraction *M5*

Besides the signal due to the reducing end αMan (H1/H2) δ 5.356/3.92, following cross peaks could be resolved in the COSY spectrum of **M5**: δ 5.298/4.103 (signal **2**) due to the internal α-1,2-linked Man, δ 5.13/4.05 (**10**) due to the terminal α-1,3-linked Man, δ 5.045/4.037 (**9**) due to the terminal α-1,2-linked Man, δ 5.04/4.228 (**11**) due to the internal α-1,3-linked Man. On the basis of their intensities the quantity of two principal components of the mixture was estimated as follows: 73% of the pentasaccharide composed of Manα(1 → 3)Manα(1 → 2)Manα(1 → 2)-Manα(1 → 2)Man ($\mathbf{5}_1$), 10% of that one with α-1,2-linked Man ($\mathbf{5}_2$) units and 10% of that one with internal α-1,3-linked Man ($\mathbf{5}_3$). The signal **22** at δ 4.776/4.03 due to single terminal β-1,2-linked Man residue suggested 6% of Manβ(1 → 2)Manα(1 → 2)Manα(1 → 2)Manα(1 → 2)Man ($\mathbf{5}_4$) oligosaccharide (Fig. 4, Table 3).

3.1.6. Fraction *M6*

NMR spectra of **M6** pointed to the rich mixture of oligosaccharides. In the region characteristic of αMan units following H1/H2 signals were identified (Table 2): **1-3** due to the internal α-1,2-linked Man involved in different types of linkages; **9** due to the terminal α-1,2-linked Man; **10** due to the terminal α-1,3-linked Man; **11** indicating the presence of αMan next to the terminal or internal α-1,3-linked Man; **12** revealing the presence of the fragment Manα(1 → 2)Manα(1 → 3)Man(1 → ; and finally H1/H2 signal at δ 5.356/3.92 due to the reducing end αMan. In the region characteristic of α-1,3-linked Man signal besides **10** another signal at δ 5.15/4.02 was detected. The comparison with the literature data (Kolarova et al., 1997) suggested that it could be assigned to the reducing end α-mannose substituted at *O*-3. On the basis of this fact it might be deduced that some of α-1,6-linked Man unit of the backbone can be branched by hexasaccharide side chains at position 3.

M6 was the first higher molecular weight fraction with more significant cross peak intensities due to βMan units. Intensities of the signal **22** at δ 4.776/4.038 due to the fragment Man**β**(1 → 2)Manα(1 → **2**)Manα(1→ (fragment βα2α-) and **23** at δ 4.757/4.037 due to Man**β**(1 → 2)Manα(1 → **3**)Man**α**(1→ (fragment βα3α-) suggested that hexasaccharides with single βMan are more abundant than those with two consecutives βMan units in Man**β**(1 → 2)Man**β**(1 → 2)Manα(1 → **2**)Man**α**(1→ and Man**β**(1 → 2)Manβ(1 → 2)Manα(1 → **3**)Man**α**(1→ fragments, (ββα2α- and ββα3α-, respectively). Two consecutive βMan showed characteristic signals **17, 18** in the ^{1}H NMR spectra and could be easily recognized. However, signals **19** and **20** due to αMan units next to the βMan were used to distinguish the fragments ββα2α- and ββα3α-. Calculations showed that **M6** is composed of 49% of hexasaccharides (Table 3, $\mathbf{6}_{1-2}$) composed of αMan residues only and 51% of those (Table 3, $\mathbf{6}_{3-6}$) containing β-1,2-linked Man units (Table 3).

3.1.7. Fraction *M7*

The composition of **M7** was the most complex. The analysis of the COSY spectra was performed analogously as described above and the presence of heptasaccharides with identical fragments as in the case of **M6** containing βMan residues at non-reducing ends (βα2α-, βα3α-, ββα2α- and ββα3α-) was identified and quantified. However, in addition to previous fractions, in the COSY spectrum of **M7** characteristic signals of three consecutive β-1,2-linked Man units linked to α-1,2-linked Man residues were found as well (signals **13-15**, βββα-) (Shibata et al., 2003). For the quantitative evaluation the signals **13** and **14** were used. The results showed that the **M7** mixture was composed of 42% of heptasaccharides composed of α-1,2- and α-1,3-linked Man units only ($\mathbf{7}_{1-3}$), while 58% of oligosaccharides ($\mathbf{7}_{4-8}$) had βMan units at the non reducing end: 20% single terminal β-1,2-linked Man ($\mathbf{7}_4$,$\mathbf{7}_5$), 24% two consecutives β-1,2-linked Man units ($\mathbf{7}_6$,$\mathbf{7}_7$) and 14 % three consecutives βMan units ($\mathbf{7}_8$) e.g. terminal βββα- fragment (Table 3).

3.1.8. Fraction *M8*

In the **M8** mixture only 25% of the octasaccharide composed of α-1,2-linked Man residues ($\mathbf{8}_1$) was found. No terminal α-1,3-linked Man signal was detected (Table 3). No octasaccharide with single terminal β-1,2-linked Man was present. Equal amounts of oligosaccharides with two consecutive βMan residues in the fragment ββα2α- ($\mathbf{8}_2$, 25%) and ββα3α- ($\mathbf{8}_3$, 25%) and that of three consecutive βMan in the fragment βββα- ($\mathbf{8}_4$, 25%) were identified.

3.2. Polysaccharide analysis

Our preliminary study of the *C. dubliniensis* mannan structure was based on the analysis of ^{1}H NMR (Fig. 3) and ^{1}H -^{13}C heterocorrelated HSQC spectra (Fig. 5) (Ličičárová et al., 2007). In the ^{1}H NMR spectrum besides signals characteristic of α-1,2- and α-1,3-linked Man units (δ ~5.28, 5.13 and 5.04) those ones characteristic of two consecutives ββ-1,2-linked Man residues of high intensity were present (Fig. 3, P; Table 2): that one at δ 4.857 due to **15** or **18** and another one at δ 4.844 due to **17**. Intensities of signals due to substituted α-1,6-linked Man units **6a**, **6b**, **6c,** distinguished at δ 5.01–5.08, were much higher then those due to internal and terminal 1,6-linked residues (**7**, **8**). A rough calculation based on the comparison of intensities **6a**, **6b** and **7** suggested 75% of substituted and 25% of non substituted α-1,6-linked Man residues in the mannan backbone in agreement with results of methylation analysis (83%). H1/C1 Cross peaks in the HSQC spectrum (Fig. 5) were attributed on the basis of Kobayashi et al. (1992a); Tojo, Shibata, Ban, & Suzuki (1990) as follows: **12** at δ 5.375/100.75 due to the α-1,3-linked Man in a Man

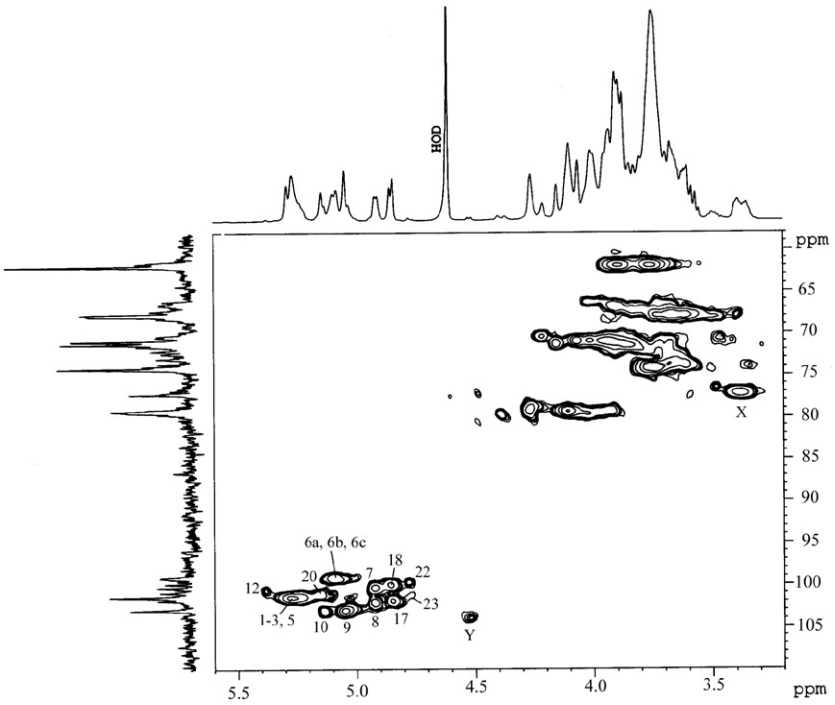

Fig. 5. ^{1}H-^{13}C HSQC 300 MHz spectrum of mannan polysaccharide. Signal attributions were made according the Table 2. X – H5/C5 cross peak due to βMan residues; Y – the cross peak due to glucose of glucan (impurity).

α(1→ 2) Manα(1→ 3) Manα (1→ fragment; the broad signal **1**–**3**, **5** at δ 5.29/101.44 due to internal α-1,2-linked Man units, **10** due to the terminal α-1,3-linked Man units at 5.13/103.1, **9** 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: **6a, 6b, 6c** at δ 5.10/99.12 to substituted α-1,2,6-linked internal Man units; **7** at δ 4.92/100.31 to non substituted α-1,6-linked Man and the signal **8** at δ 4.92/102.07 to terminal 1,6-linked ones. Cross peaks **17, 18, 22, 23** are characteristic of βMan residues involved in 1,2-linkage. Particularly, **17** at δ 4.844/101.902 is due to the terminal βMan unit of the Manβ(1 → 2)Manβ(1 → 2)-Manα(1→ fragment and the cross peak **18** at δ 4.856/99.97 due to its neighbouring βMan. Cross peaks **22** (δ 4.774/99.62) and **23** (4.757/101.15) indicated the presence of small amount of oligosaccharides with one terminal βMan unit in βα2α- and βα3α- fragments, respectively. The signal **21** (δ 5.15/101.10) was assigned to α-1,2- and α-1,3-linked Man units close to the terminal βMan. A signal with characteristic H5 and C5 chemical shifts due to β-1,2-linked Man residues appeared at δ 3.379/77.14 **(X)** (Kobayashi et al., 1990; Shibata et al., 2003). The cross peak **Y** (Fig. 5) was attributed to free glucose originated from glucan supposed to be a contaminant.

A 2D homocorrelated ^{1}H-^{1}H COSY spectrum, particularly, the H2/H3 signals enabled to reveal further fine structural features of the polysaccharide. Based on the H1/H2 signal assignment the attribution of their corresponding H3 signals was made. The H3 chemical shifts (Fig. 4b) due to βMan units were downfield shifted to the region at δ 3.6–3.7, while those due to αMan were observed between δ 3.82–3.94. The H3 chemical shifts due to substituted α-1,2,6-linked Man units of the backbone **6a**, **6b** and **6c** (Table 2) were downfield shifted to δ 3.70 (Fig. 4b, signal **6**). These observations allowed identify the H1/H2 signals configuration of mannose units in the case of overlapped H1/H2 cross peak signals (Fig. 4a).

H1/H2 Cross peak signals **17** and **18** were structure reporter signals of the fragments **Manβ**(1 → 2)Manβ (1 → 2)Manα(1 → 3)Manα(1→ (fragment **β**βα3α-) and Manβ(1 → 2)**Man**β(1 → 2)Manα(1 → 2)Manα(1→ (fragment ββ**β**α2α-), respectively, (Fig. 4a), while the presence of oligosaccharide chains with βββα- fragment was indicated by signals **13** (β**β**βα-) and **14** (**β**ββα-) (the position of the unit related to the given signal is in bold). However, the precise quantification of signals was not possible due to the signal overlaps. Particularly, the cross peak **18** (β**β**α2α-) was overlapped with **15** (β**β**βα-), while **20** (ββ2α-) was overlapped by with **21** (βαα-) and **16** (βββα-). Signals **21** and **16** were due to αMan neighboring βMan in βαα- and βββα- fragments, respectively. Chemical shifts due to H3 protons in Fig. 4b, were more disperse and thus they helped to the assignment and confirmed the cross peak attributions.

The H1/H2 cross peak at δ 5.014/4.370 (**25**) showed an unusual H2 chemical shift. The chemical shift of its corresponding H3 (δ 3.626) confirmed the β configuration of its corresponding Man residue (Fig. 4b). The comparison with the literature data (Shibata et al., 1992a) suggested that **25** might be due to βMan unit next the terminal βMan in the fragment of four consecutives βMan residues (ββββα-) oligosaccharide in the side chain (Table 2).

The intensity of the signal **8** was high, and it indicated the side chains branching by terminal α-1,6-linked Man residues. This hypothesis was confirmed by the presence of the cross peak **5** (δ 5.24/4.10) characteristic of the αMan unit next to the isolated α-1,3,6-linked Man residue (Shibata et al., 1995). The cross peak due to the fragment **4** (Table 2) due to αMan located between two 1,3,6-linked mannose residues could not be proved due to its chemical shifts very close to those ones due to **2**, but its presence is highly probable.

The signal **11** (Fig. 4a), characteristic of αMan substituted at *O*-3 by the terminal αMan, indicated an overlap with a non attributed signal **11a** (δ H1/H2 4.998/4.142). However, corresponding H2/H3 cross peaks of both, were well distinguished (Fig. 4b). The chemical shift of H3 signal at $\delta \sim 3.94$ was in agreement with a previous assignment of H3 due to 1,2-linked Man substituted at *O*-3 by terminal αMan found for a tetrasaccharide (Kolarova et al., 1997) and it was attributed to **11**. The chemical shift due to H3 of **11a** was in accordance with that one due to α-1,3,6-linked Man (Shibata et al., 1995, 1997). Consequently, it was deduced that **11a** might be due to α-1,3-linked Man units branched at *O*-6 by terminal αMan residues.

4. Discussion

The preparation of *C. dubliniensis* mannan was accomplished in two steps: (1) by obtaining of mannoprotein and (2) isolation of mannan in alkaline conditions at which mannooligosaccharides, linked to serine and threonine, were released. In the following step – Fehling's precipitation – phosphodiester linkages, through which β-1,2-oligomers are attached to side chains of the outer chain polymer were cleaved. However, the quantity of phosphorous estimated by elemental analysis was very low. Traces of glucose residues detected on hydrolysis of mannan were supposed to be due to the β-glucan which, together with chitin, constitutes the rigid structure of the cell wall.

Oligomers obtained after the conventional hydrolysis of the *C. dubliniensis* mannan polymer were identified and quantified on the basis of COSY spectra. In the COSY spectrum of the polymer partial structures of branching oligosaccharides were identified but quantification was not precise due to many overlaps caused by a high complexity of the polymer structure. Even 600 MHz COSY spectrum resolution was not enough in the case of such complex polymer and thus not all signals were identified. Obtained data show a great variety of partial structures (Table 2) and side chain oligosaccharide types (Table 3) caused mainly by single β-, two consecutive ββ- and three consecutive βββ-1,2-linked mannose residues at their terminal non reducing ends. The presence of side chains with ββββ-1,2-linked fragment is highly probable. The oligomers isolated from acid-stable region of *C. dubliniensis* mannan are similar to those ones obtained from *C. albicans* mannan (serotype A) (Kobayashi et al., 1992b; Shibata et al., 1992a) as well as from *C. lusitaniae* mannan (Shibata et al., 2003). Noteworthy, the presence of β-1,2-linked mannose residues linked at the non reducing ends in acid-stable part of *C. albicans* mannan (serotype A) depends on growth conditions: at pH 2.0 neither β-1,2-linked mannopyranosyl units were detected while in conventional medium at pH 5.9 the whole scale of α-1,2-, α-1,3- and β-1,2-linked mannose residues in oligomers was obtained (Kobayashi et al., 1994b).

4.1. Backbone branching

The high degree of substitution of the *C. dubliniensis* mannan backbone found by methylation analysis (83%) was in accordance with that found in ^{1}H NMR spectrum ($\sim$ 75%). The type and the frequency of the substitution of the α-1,6-linked Man due to backbone could be deduced from **6a, 6b** and **6c** signals (Fig. 4). The substitution of the backbone with longer oligosaccharide side chains was more frequent (**6a** and **6b)** then that one with single αMan units (**6c**). Further, the substitution by longer chains was observed in blocks (**6b**) but also on the isolated backbone units (**6a**), while the substitution with single α-1,2-linked Man was observed only in blocks (**6c**). The signal of α-1,6-linked Man substituted by single βMan was not attributed. This might be the reason a small difference between the backbone substitution degree found by the methylation analysis and NMR.

4.2. βMan containing side chain

A high degree of the backbone substitution by oligosaccharides terminated with two consecutive ββ-1,2-linked Man was evident from the NMR spectra of the polymer mannan, however, their quantity found in individual fractions was very low (Table 3). This fact suggests that β-linkages did not survive conventional acetolysis conditions. Further studies will be necessary to clarify the size and quantity of the β-linked oligosaccharides occurring in the *C. dubliniensis* mannan.

On the basis of obtained data following conclusions might be done:

(i) In the fraction **M2** 30% of single terminal β-1,2-linked Man remained after the hydrolysis
(ii) In **M3** and **M5** fractions less then 10% of βMan containing oligosaccharides were found, in **M3** 7% of them was with two consecutive ββ-1,2-linked Man
(iii) In **M4** only traces of βMan containing oligosaccharides were detected
(iv) The ratio of βMan containing oligosaccharides in individual fractions increased dramatically with increasing the molecular weight of oligosaccharides (Table 3). In **M6** it was 51%, **M7** 58% and **M8** 75% with important participation of ββ- and βββ-Man configuration of glycosidic linkages at the non reducing end.

5. Conclusion

Results obtained on the basis of compositional and methylation analyses show that the α-1,6-Man backbone of the *C. dubliniensis* polymer is to 83% substituted by side chains (d.p. 1–7) at *O*-2. NMR data indicate that some Man residues of the backbone might be substituted at *O*-3. Trimers with α-1,2-Man sequence were the most frequent side chains occurring in the polymer. Substitution of the backbone with single βMan was detected in an important quantity. Longer side chains (d.p. 5–7) were isolated in low quantity with higher content of β-1,2-linked Man units. Moreover, with increasing of their molecular weight the quantity of oligosaccharides with single β-, two consecutive ββ- and three consecutive βββ-1,2-linked Man fragments was increasing and reaching maximum in **M8** with 75%. Although oligosaccharides containing β-1,2-linked Man did not survive completely the condition of the conventional acetolysis they afforded a valuable information on side chain structures occurred in *C. dubliniensis* mannan.

Immunochemical properties of *Candida* cells depend on the chemical structure of the mannans. According to our study of *C. dubliniensis* cell wall mannan and identified oligosaccharides containing α-linked mannose units we could propose the presence of antigenic factors 1, 8, 13b, 34 (Suzuki, 1997). While, the most abundant oligosaccharides composed of both β-1,2 and α-1,2- linkages with common structure Manβ(1 → 2)Manα(1 → 2)Man correspond to an integral part of antigenic factor 6 (Kobayashi, Shibata, & Suzuki, 1992c; Shibata et al., 2003). Immunochemical properties will be the subject of further studies.

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References

Ahmad, S., Khan, Z., Mokaddas, E., & Khan, Z. U. (2004). Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection and biochemical methods for species identification. *Journal of Medical Microbiology, 53*(7), 633–637.

Brown, D. M., Jabra-Rizk, M. A., Falkler, W. A., Baqui, A. A. M. A., & Meiller, T. F. (2000). Identification of *Candida dubliniensis* in a study of HIV – seropositive pediatric patients. *Pediatric Dentistry, 22*, 234–238.

Gutiérrez, J., Morales, P., Gonzáles, M. A., & Quindós, G. (2002). *Candida dubliniensis*, a new fungal pathogen. *Journal of Basic Microbiology, 42*(3), 207–227.

Hakomori, S. (1964). A rapid permethylation of glycolipid and polysaccharide as catalyzed by methylsulfinylcarbanion in dimethyl sulfoxide. *Journal of Biochemistry (Tokyo), 55*, 205.

Hazen, K. C., Wu, J. G., & Masuoka, J. (2001). Comparison of the hydrophobic properties of *Candida albicans* and *Candida dubliniensis*. *Infection & Immunity, 69*(3), 779–786.

Jones, J. K. N., & Stoodley, R. J. (1965). Fractionation using copper complexes. *Methods in Carbohydrate Chemistry, 5*, 36–38.

Kobayashi, H., Matsuda, K., Ikeda, T., Suzuki, M., Takahashi, S., Suzuki, A., et al. (1994a). Structures of cell wall mannans of pathogenic *Candida tropicalis* IFO 0199 and IFO 1647 yeast strains. *Infection & Immunity, 62*, 615–622.

Kobayashi, H., Mitobe, H., Takahashi, K., Yamamoto, T., Shibata, N., & Suzuki, S. (1992a). Structural study of a cell wall mannan-protein complex of the pathogenic yeast *Candida glabrata* IFO 0622 strain. *Archives of Biochemistry and Biophysics, 294*, 662–669.

Kobayashi, H., Shibata, N., Mitobe, H., Ohkubo, Y., & Suzuki, S. (1989). Structural study of phosphomannan of yeast-form cells of *Candida albicans* J-1012 strain with special reference to application of mild acetolysis. *Archives of Biochemistry and Biophysics, 272*, 364–375.

Kobayashi, H., Shibata, N., Nakada, M., Chaki, S., Mizugami, K., Ohkubo, Y., et al. (1990). Structural study of cell wall phosphomannan of *Candida albicans* NIH B-792 (Serotype B) strain, with special reference to 1H and 13C NMR analyses of acid – labile oligomannosyl residues. *Archives of Biochemistry and Biophysics, 278*, 195–204.

Kobayashi, H., Shibata, N., Osaka, T., Miyagawa, Y., Ohkubo, Y., & Suzuki, S. (1992b). Structural study of cell wall mannan of a *Candida albicans* (serotype A) strain. *Phytochemistry, 31*, 1147–1153.

Kobayashi, H., Shibata, N., & Suzuki, S. (1992c). Evidence for oligomannosyl residues containing both β-1,2 and α-1,2 linkages as a serotype A-specific epitope(s) in mannans of *Candida albicans*. *Infection & Immunity, 60*, 2106–2109.

Kobayashi, H., Suzuki, J., Tanaka, S., Kiuchi, Y., Oyamada, H., Iwatade, N., et al. (1997). Structure of a cell wall mannan from the pathogenic yeast *Candida catenulata*: assignment of ^{1}H nuclear magnetic resonance chemical shifts of the inner α-1,6-linked mannose residues substituted by a side chain. *Archives of Biochemistry and Biophysics, 341*, 70–74.

Kobayashi, H., Takahashi, S., Shibata, N., Miyauchi, M., Ishida, M., Sato, J., et al. (1994b). Structural modification of cell wall mannan of *Candida albicans* serotype A strains grown in yeast extract-Sabouraud liquid medium under acidic conditions. *Infection & Immunity, 62*(3), 968–973.

Kocourek, J., & Ballou, C. E. (1969). Method for fingerprinting yeast cell wall mannans. *Journal of Bacteriology, 100*, 1175–1181.

Kolarova, N., Matulová, M., & Capek, P. (1997). Structure of glucomannan protein from the yeast *Cryptococcus laurentii*. *Journal of Carbohydrate Chemistry, 16*, 609–623.

Ližičárová, I., Matulová, M., Machová, E., & Capek, P. (2007). Cell wall mannan of human pathogen *Candida dubliniensis*. *Carbohydrate Polymers, 68*, 191–195.

Masuoka, J., & Hazen, K. C. (1997). Cell wall protein mannosylation determines *Candida albicans* cell surface hydrophobicity. *Microbiology, 143*, 3015–3021.

Masuoka, J., & Hazen, K. C. (2004). Cell wall mannan and cell surface hydrophobicity in *Candida albicans* serotype A and B strains. *Infection and Immunity, 72*, 6230–6236.

Pontón, J., Omaetxebarría, M. J., Elguezabal, N., Alvarez, M., & Moragues, M. D. (2001). Immunoreactivity of the fungal cell wall. *Medical Mycology, 39*, 101–110.

Sedmak, J. J., & Grossberg, S. E. (1977). A rapid sensitive and versatile assay for protein using Coomassie brilliant blue G-250. *Analytical Biochemistry, 79*, 552–554.

Shibata, N., Akagi, R., Hosoya, T., Kawahara, K., Suzuki, A., Ikuta, K., et al. (1996a). Existence of novel branched side chains containing β-1,2 and α-1,6 linkages corresponding to antigenic factor 9 in mannan of *Candida guilliermondii*. *Journal of Biological Chemistry, 271*, 9259–9266.

Shibata, N., Arai, M., Haga, E., Kikuchi, T., Najima, M., Satoh, T., et al. (1992a). Structural identification of an epitope of antigenic factor 5 in mannans of *Candida albicans* NIH B-792 (serotype B) and J-1012 (serotype A) as β-1,2-linked oligomannosyl residues. *Infection & Immunity, 60*, 4100–4110.

Shibata, N., Fukasawa, S., Kobayashi, H., Tojo, M., Yonezu, T., Ambo, A., et al. (1989). Structural analysis of phospho-D-mannan-protein complexes isolated from yeast and mold form cells of *Candida albicans* NIH A-207 serotype A strain. *Carbohydrate Research, 187*, 239–253.

Shibata, N., Hisamichi, K., Kikuchi, T., Kobayashi, H., Okawa, Y., & Suzuki, S. (1992b). Sequential nuclear magnetic resonance assignment of β-1,2-linked mannooligosaccharides isolated from phosphomannan of pathogenic yeast *Candida albicans* NIH B-792 strain. *Biochemistry, 31*, 5680–5686.

Shibata, N., Ichikawa, T., Tojo, M., Takahashi, M., Ito, N., Okubo, Y., et al. (1985). Immunochemical study on the mannans of *Candia albicans* NIH A-207, NIH B-792, and J-1012 strains prepared by fractional precipitation with cetyltrimethylammonium bromide. *Archives of Biochemistry and Biophysics, 243*, 338–348.

Shibata, N., Ikuta, K., Imai, T., Satoh, Y., Satoh, R., Suzuki, A., et al. (1995). Existence of branched side chains in the cell wall mannan of pathogenic yeast *Candida albicans* structure – antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. *Journal of Biological Chemistry, 270*, 1113–1122.

Shibata, N., Kobayashi, H., Okawa, Y., & Suzuki, S. (2003). Existence of novel β-1,2 linkage-containing side chain in the mannan of *Candida lusitaniae*, antigenically related to *Candida albicans* serotype A. *European Journal of Biochemistry, 270*, 2565–2575.

Shibata, N., Kojima, C., Satoh, T., Suzuki, A., Kobayashi, H., & Suzuki, S. (1993). Structural study of cell-wall mannan of *Saccharomyces kluyveri* IFO 1685 strain. Presence of a branched side chain and β-1,2-linkage. *European Journal of Biochemistry, 217*, 1–12.

Shibata, N., Onozawa, M., Tadano, N., Hinosawa, Y., Suzuki, A., Ikuta, K., et al. (1996b). Structure and antigenicity of the Mannans of *Candida famata* and *Candida saitoana*: comparative study with the Mannan of *Candida guilliermondii*. *Archives of Biochemistry and Biophysics, 336*, 49–58.

Shibata, N., Senbongi, N., Hosoya, T., Kawahara, K., Agaki, R., Suzuki, A., et al. (1997). Demonstration of the presence of α-1,6-branched side chains in the mannan of *Candida stellatoidea*. *European Journal of Biochemistry, 246*, 477–485.

Sullivan, D. J., Morgan, G. P., & Coleman, D. C. (2005). *Candida dubiniensis*: ten years on. *FEMS Microbiology Letters, 253*, 9–17.

Sullivan, D. J., Westerneng, T. J., Haynes, K. A., Bennet, D. E., & Coleman, D. C. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology, 141*, 1507–1521.

Suzuki, S. (1997). Structural investigation of mannans of medically relevant *Candida* species; determination of chemical structures of antigenic factors 1, 4, 5, 6, 9 and 13b. In S. Suzuki & M. Suzuki (Eds.), *Fungal cells in biodefense mechanism* (pp. 1–15). Tokyo, Japan: Saikon Publishing Co., Ltd..

Tojo, M., Shibata, N., Ban, Y., & Suzuki, S. (1990). Structure of the D-mannan of *Candida stellatoidea* IFO 1397 strain. Comparison with that of the phospho-D-mannan of *Candida albicans* NIH B-792 strain. *Carbohydrate Research, 199*, 215–226.

Zhang, W., & Ballou, C. E. (1981). *Saccharomyces kluyveri* cell wall mannoprotein, structure of the *O*- and *N*-linked carbohydrate components. *Journal of Biology and Chemistry, 256*, 10079.