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NOVEL STRUCTURE OF THE CELLULAR MANNAN OF THE PATHOGENIC YEAST *Candida krusei*

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ABSTRACT

On the basis of methylation analysis, ^{13}C- and ^{1}H-n.m.r. spectroscopic data, and the results of immunological methods, a new structure for the cell-wall α-D-mannan of the pathogenic yeast *Candida krusei* is proposed. In contrast to the α-D-mannans of other pathogenic yeasts of the *Candida* species, which have (1→6)-linked main chains and many (1→2)- and/or (1→3)-linked side chains, the *C. krusei* mannan is lightly branched and contains (1→2) and (1→6) linkages in the ratio 3:1.

INTRODUCTION

The immunological properties and antigenic relationship of pathogenic yeasts have long been of interest in medicine and yeast taxonomy. Cell-wall α-D-mannan, which is the major antigen of the *Candida* species, has a (1→6)-linked backbone and side chains containing (1→2) and (1→3) linkages[1,2]. In the determination of the structure of the mannan, acetolysis played an important role, since it cleaves (1→6) linkages selectively, yielding a mixture of oligosaccharides that is characteristic for each yeast species[3]. Inhibition of the antigen–antibody reaction by the *O*-deacetylated acetolysis fragments showed that the side chains are the principal immunochemical determinants of mannans and yeast cell wall[4,5].

Mannans and mannose-containing glycoproteins occurring on the surface of the yeast cell wall may also play an important role in the adherence of microorganisms to host cells. There appears to be a relationship between the ability of the species to adhere to a cell surface and its propensity for causing infections. *Candida albicans* is the most virulent species, followed by *C. tropicalis* and *C. stellatoidea*, whereas *C. krusei, C. guilliermondii*, and *C. pseudotropicalis* show low virulence[6,7].

In spite of some similarities in the structures of the mannans of pathogenic *Candida*, there are marked differences in their biological activities. We have now analysed and compared the structures and immunological properties of the mannan

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of *C. krusei* on the one hand and those of *C. albicans* and closely related species on the other.

EXPERIMENTAL

Candida *strains*. — Cultures of *Candida krusei* CCY 29-9-5, *C. albicans* CCY 29-3-100 serotype A, *C. albicans* CCY 29-3-102 serotype B, *C. guilliermondii* CCY 29-4-16, *C. tropicalis* CCY 29-7-6, *C. pseudotropicalis* CCY 29-8-4, and *C. parapsilosis* CCY 29-20-6 were obtained from the Czechoslovak Collection of Yeast and Yeast-like Organisms[8] (CCY).

Extraction and purification of the polysaccharides. — The yeasts were grown for 3 days at room temperature on a gyrorotary shaker in a semi-synthetic liquid medium containing 2% of D-glucose[9]. The cells were harvested by centrifugation and the mannan was extracted[10] from the cellular paste by autoclaving thrice with 0.2M NaCl at 140°. The mannan was then purified using Fehling's reagent[11].

Acetylation and acetolysis of the mannan. — The method of Kocourek and Ballou[3] was used. For complete cleavage of the (1→6) linkages, acetolysis was carried out for 13 h; for partial cleavage, the reaction time was 4 h. The *O*-deacetylated products were eluted from a column (2 × 200 cm) of Bio-Gel P-2 with distilled water (containing 0.01% of sodium merthiolate for preservation) at 15 mL/h and room temperature, and detected with a differential refractometer RIDK 101 (Lab Equipments, Prague).

Methylation analysis. — The mannan was methylated by using methylsulphinylmethanide–methyl iodide in methyl sulphoxide[12] as described by Lindberg[13], the product was hydrolyzed, and the resulting sugar derivatives were reduced with borohydride and then acetylated. The resulting partially methylated alditol acetates were analyzed by using a JGC-20 K gas chromatograph equipped with a column (200 × 0.3 cm) of SP-2340 on Chromosorb WAW-DMCS (80–100 mesh), at 180° (4 min) and then →210° at 2°/min, with helium (inlet pressure, 101.3 kPa) as the carrier gas at 30 mL/min. The molar composition was calculated from the peak areas, using the response factors for individual methylated derivatives[14]. Mass spectra were obtained at 23 eV and an emission current of 300 μA, using a JEOL D 100 spectrometer. The inlet temperature was 220° and that of the ionising chamber 200°. The fragmentation patterns were compared with those reported by Jansson *et al.*[15].

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra (300 MHz) were recorded with a Bruker AM-300 FT-spectrometer at room temperature for solutions in D_2O (internal acetone, 2.2 p.p.m. relative to Me_4Si).

^{13}C-N.m.r. spectra (75.468 MHz) were recorded with the same instrument at room temperature on solutions in D_2O (internal MeOH, 50.15 p.p.m. relative to Me_4Si).

Preparation of antisera. — The antisera were produced in rabbits by intravenous injection of a suspension of heat-killed yeast cells (5 mg/mL). The animals were

injected with 1.5–2.0 mL of the suspension twice weekly for 4–8 weeks, and blood samples were taken 7 days after the last injection. The sera were combined and stored at −20°.

Immunodiffusion. — Diffusion of mannans and antibodies was carried out by the method of Ouchterlony[16], using 1.5% of agar in buffered saline. The concentration of antigen was 1 mg/mL and the antisera were used undiluted. Diffusion was allowed to take place at room temperature for 1–4 days.

Preparation of immunoglobulin. — Rabbit immunoglobulin was prepared by precipitation with ammonium sulphate and subsequent purification on DEAE-cellulose[17]. The concentration of immunoglobulin for checkerboard titration was adjusted to 1 mg/mL.

Indirect ELISA method[18]. — The cellular mannans were adsorbed from solutions (200 μL) in carbonate–hydrogencarbonate coating buffer (pH 9.6), on to the wells of plastic microtitre plates for 4 h at 37°. The coated wells were washed thrice for 3 min each with PBS-T [0.01M sodium phosphate buffer (pH 7.2), 0.14M sodium chloride, and 0.05% of Tween 20]. Working dilutions of unlabelled Ig were determined by checkerboard titration. Ig fraction from rabbit antisera was diluted 1:200 with PBS-T containing 2% of polyvinyl pyrrolidone, and 200 μL of this solution was added to mannan-coated wells and incubated for 18 h at 4°. The plates were then washed thrice for 3 min with PBS-T. Specific swine antiserum to rabbit Ig labelled with peroxidase (SEVAC, Prague) was diluted 1:1000 in PBS-T containing 2% of polyvinyl pyrrolidone and 0.02% of bovine serum albumin, and 200 μL was added to each well. The plates were incubated for 4 h at 37° and washed thrice for 3 min each with PBS-T, and 200 μL of fresh substrate buffer (pH 5, 29.4 g of sodium citrate, 0.1 g of sodium merthiolate, 1000 mL of H_2O, and 1 mL of aqueous 30% H_2O_2), containing 0.1 mg of 1,2-phenylene diamine were added. After incubation for ~30 min at room temperature, the reaction was stopped by adding 50 μL of 2M H_2SO_4 to each well. Absorbance was read at 492 nm with a Uniskan II (Flow Labs.).

RESULTS AND DISCUSSION

In order to determine the antigenic properties of the mannans from pathogenic *Candida*, antisera were prepared against *C. krusei*, *C. albicans* (serotypes A and B), and *C. pseudotropicalis*. After 4 weeks of immunization, all antisera against *Candida* sp. except *C. krusei* showed a titre of 1:640–1280, whereas *C. krusei* antiserum had a titre of only 1:80 (demonstrated by agglutination). After prolonged immunization up to 8 weeks, the titre of *C. krusei* antiserum was increased to 1:160.

In immunodiffusion, *C. albicans* antisera provided sharp and heavy precipitin lines with homologous and structurally closely related mannans. *C. krusei* mannan formed no precipitin line with *C. albicans* antisera (Fig. 1). On the other hand, antiserum of *C. krusei* gave a faint precipitin line with homologous *C. krusei* mannan.

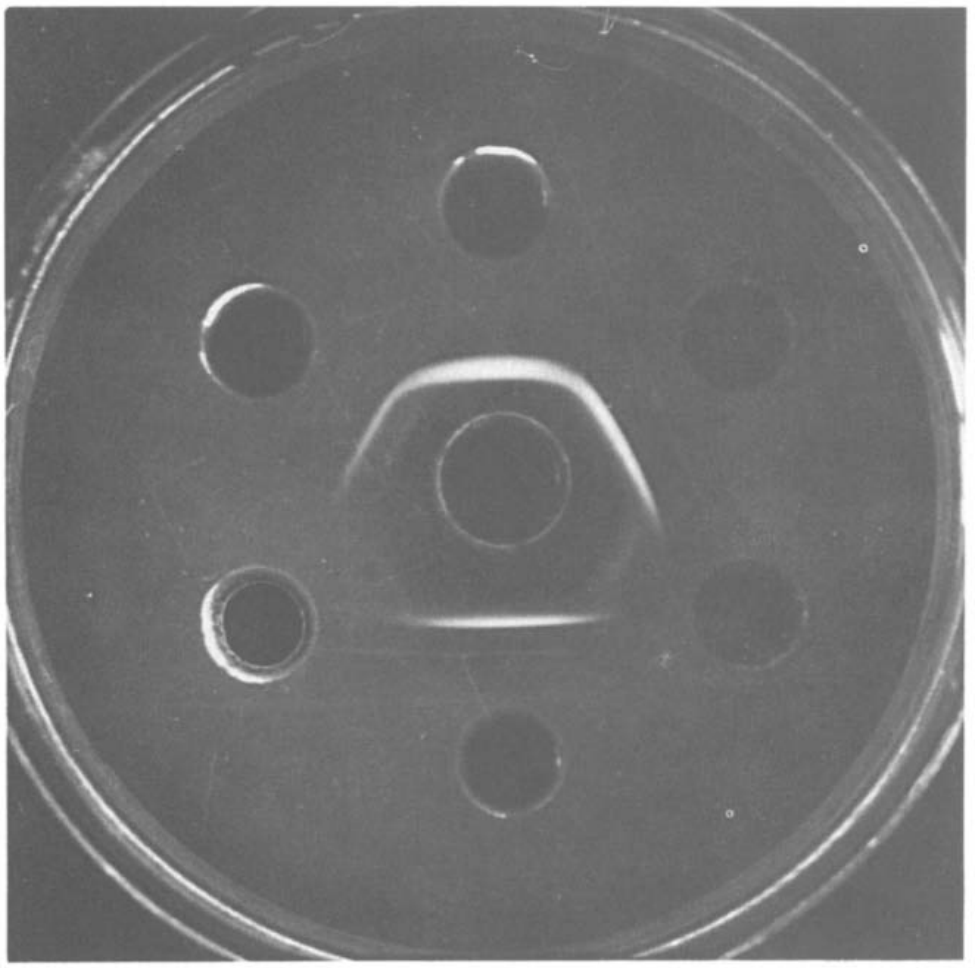


Fig. 1. Immunodiffusion of mannans and antiserum *C. albicans* serotype B: S, antiserum; mannans: 1, *C. albicans* serotype B; 2, *C. albicans* serotype A: 3, *C. guilliermondii*; 4, *C. tropicalis*; 5, *C. krusei*; 6, *C. parapsilosis*.

The cross-reactivity of *C. krusei* mannan with Ig's of *C. albicans* (serotypes A and B) and *C. pseudotropicalis* antisera were investigated by the indirect ELISA method. Mannans of these species represent different antigenic structures of pathogenic *Candida* species[4,5,18–20]. The results shown in Fig. 2 indicate very low cross-reactivity of *C. krusei* mannan with Ig of *C. albicans* (serotypes A and B) and *C. pseudotropicalis* antisera. Different properties of *C. krusei* mannan were found also by Shinoda[21], who examined mannans of eight *Candida* strains by ^{1}H-n.m.r. spectroscopy and by gel-diffusion precipitation against other *Candida* strains.

These facts indicate that the structure of the *C. krusei* mannan is different to those of the mannans of pathogenic *Candida* species.

Methylation analysis of *C. krusei* mannan (Table I) showed most of the mannopyranose residues to be 2- and 6-linked. The small amount of 2,3,4,6-tetra-*O*-methylmannitol diacetate (derived from terminal non-reducing groups) and 3,4-di-*O*-methylmannitol tetra-acetate (derived from 2,6-substituted units) indicates that the mannan is very lightly branched.

The results of methylation analysis are consistent with the results of ^{13}C-n.m.r. spectroscopy. The assignment of signals of ^{13}C-n.m.r. spectra (Fig. 3) of the *C. krusei* mannan was based on published data[22,23]. The signals at lowest field belong to C-1 of terminal non-reducing (103.39 p.p.m.), 2- (101.81 p.p.m.), 6- (100.65

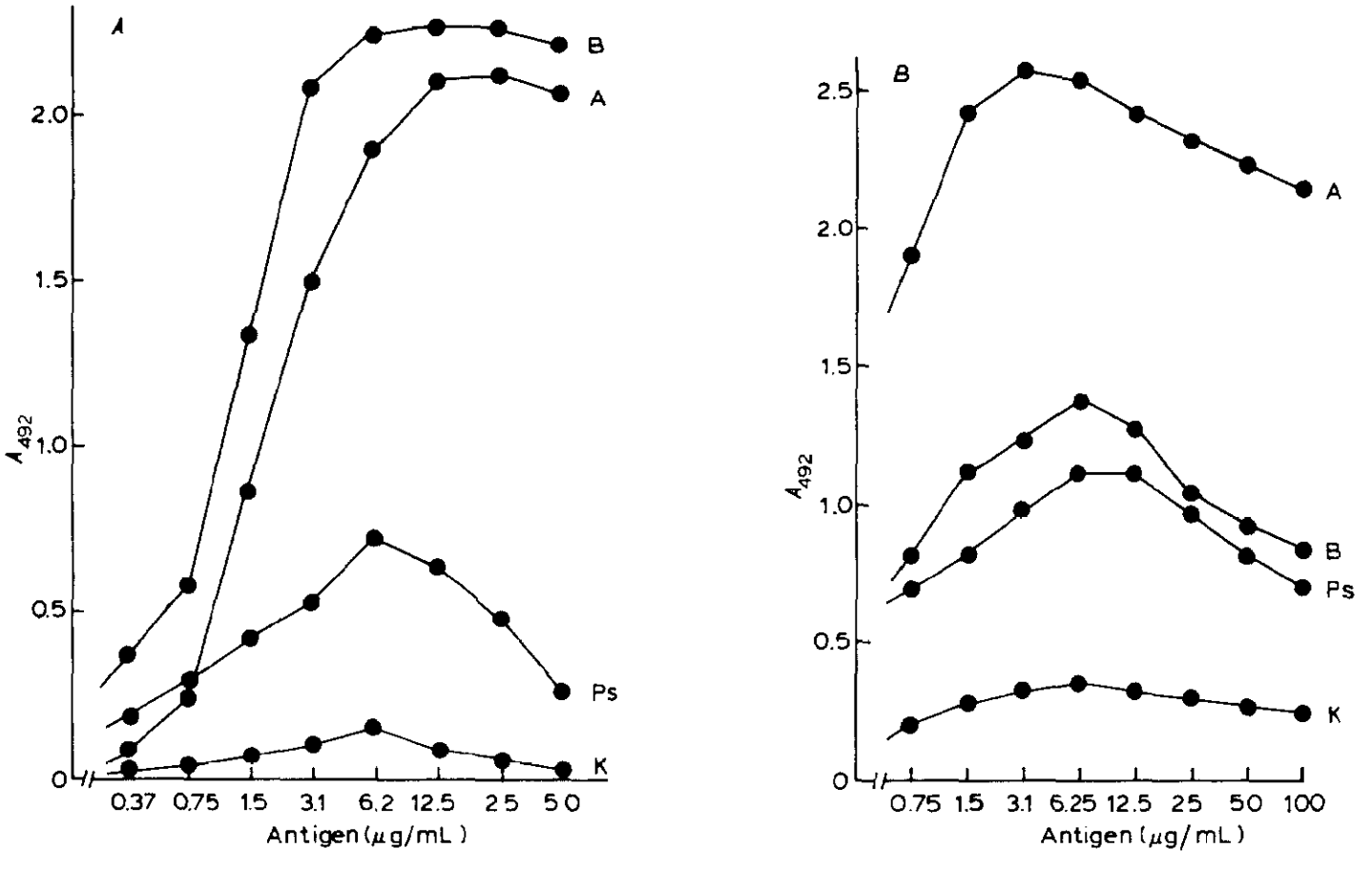


Fig. 2. Cross-reactivity of mannans with antisera determined by indirect ELISA. Antisera: *A*, *C. albicans* serotype B; *B*, *C. albicans* serotype A; *C*, *C. pseudotropicalis*. Mannans: A, *C. albicans* serotype A; B, *C. albicans* serotype B; Ps, *C. pseudotropicalis*; K, *C. krusei*.

TABLE I

METHYLATION ANALYSIS DATA FOR *C. krusei* MANNAN

Partially acetylated mannitol derivatives	*Molar percentage*
2,3,4,6-Tetra-*O*-methyl	4
3,4-Di-*O*-methyl	4
3,4,6-Tri-*O*-methyl	68
2,3,4-Tri-*O*-methyl	24

p.p.m.), and 2,6-substituted (99.39 p.p.m.) residues. The chemical shift data are presented in Table II. Apparent lack of agreement between the amount of 6-linked α-D-mannosyl residues of the main chain found by methylation analysis (24%) and the low intensity of the corresponding C-1 signal (at 100.65 p.p.m.), as well as the discrepancy between the low amount of non-reducing terminal units and branching points (4% each) and the high intensities of the corresponding C-1 signals, may be explained in terms of different n.O.e. enhancement of the signals of individual anomeric carbon atoms bound by different types of glycosidic linkages. Similar extremely low intensities of the C-1 signals of 6-linked main-chain α-D-mannosyl residues and enhancement of the other two signals have been observed in other investigations of yeast mannans by ^{13}C-n.m.r. spectroscopy[2,23–25].

Acetolysis of the *C. krusei* mannan, followed by gel filtration of the *O*-deacetylated products on Bio-Gel P-2, gave five peaks (Fig. 4). The molar ratios of the mannose (M_1) to manno-oligosaccharides (M_2–M_5) were 14:7:14:5:1. The ^{1}H-n.m.r. spectra of M_2–M_5 are shown in Fig. 5. The spectra of the *O*-deacetylated acetolysis fragments (M_3–M_5) contained signals at 5.04, 5.28, and 5.36 p.p.m. for H-1 of the non-reducing end, 2-linked, and reducing end-groups[1,26], respectively. The signal at 5.13 p.p.m., which could be ascribed to H-1 of 3-linked units[1,26], was observed only in the spectrum of M_2. Accordingly, it is suggested that this fragment contains mainly a 2-linkage with a small proportion of 3-linkage.

TABLE II

^{13}C-N.M.R. DATA FOR *C. krusei* MANNAN

Chemical shift data[a] *(p.p.m.)*				
Atom	*Terminal Man unit*	*2,6-Substituted Man*	*2-Substituted Man*	*6-Substituted Man*
C-1	103.39	99.39	101.81	100.65
C-2	71.87	79.82	79.29	71.32
C-3	71.87	71.32	70.80	71.87
C-4	68.19	67.44	68.19	68.19
C-5	74.45	72.74	74.45	72.74
C-6	62.23	66.56	62.23	65.03

[a]Solutions in D_2O (internal MeOH, 50.15 p.p.m. relative to Me_4Si).

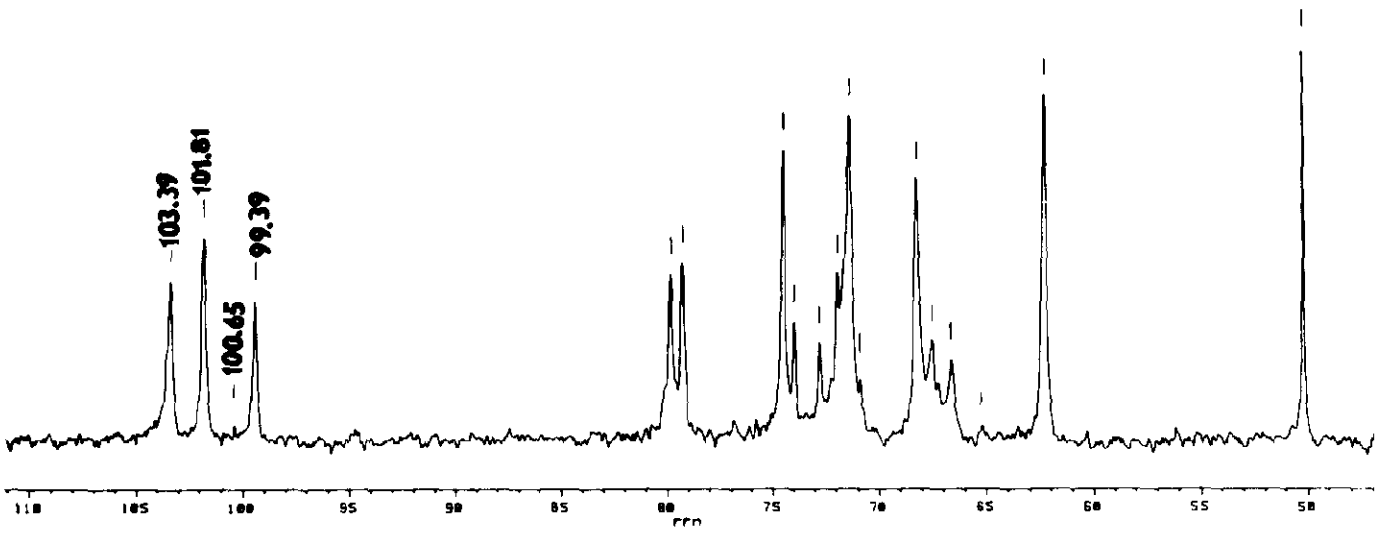


Fig. 3. ^{13}C-N.m.r. spectrum of *C. krusei* cell-wall mannan.

Many cell-wall mannans of yeasts possess a backbone of 6-linked α-D-mannosyl residues with 2- and 3-linked units as side chains[27–30]. The results of methylation analysis and acetolysis of the *C. krusei* mannan do not accord with this comb-like structure. Methylation analysis shows that the mannan is lightly branched and that the amount of 2-linked units is high. This finding could reflect the presence of long side-chains, but the acetolysis pattern involves fragments containing only 2–5 units. The hypothesis of a comb-like structure of the *C. krusei* mannan conflicts with the results of cross-reactivity with antisera of *C. albicans* (serotype A and B) and *C. pseudotropicalis*. Mannans of these strains contain the same 2-linked side-chains[20,31] that were present in fragments of acetolysis of the *C. krusei* mannan, but its cross-reactivity with their specific antisera was low.

Acetolysis cleaves (1→6) linkages quantitatively and selectively[3]. In order to investigate the fine structure of the *C. krusei* mannan, partial acetolysis (4 h) was performed. Gel filtration of the *O*-deacetylated products on Bio-Gel P-2 (Fig. 6) gave eight peaks (M_1–M_8) in the molar ratios 9:7:11:14:2:1:2:2. Mannotetraose (M_4) was the main oligosaccharide.

The ^{1}H-n.m.r. spectra of M_5–M_8 contained, in addition to signals at 5.04,

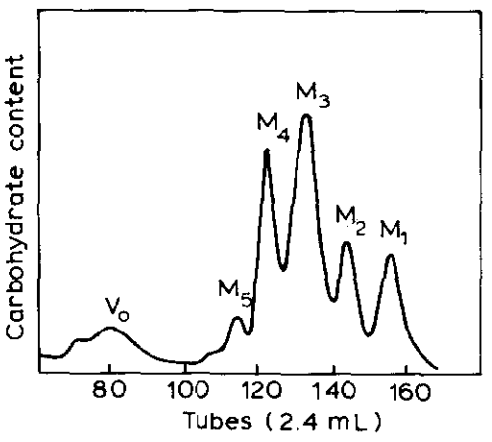


Fig. 4. Gel-filtration profile on Bio-Gel P-2 of the *O*-deacetylated oligosaccharides obtained on acetolysis of *C. krusei* mannan.

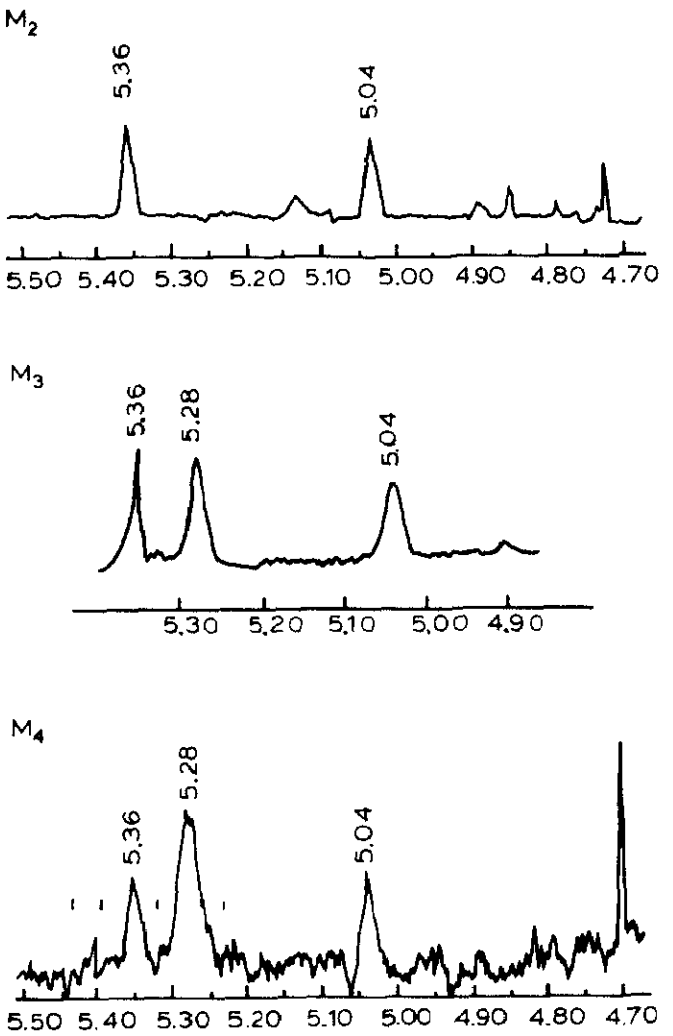


Fig. 5. ^{1}H-N.m.r. spectra of the *O*-deacetylated oligosaccharides obtained by acetolysis of *C. krusei* mannan.

5.28 and 5.36 p.p.m. for 2-linked units, a signal in the region 5.11–5.16 p.p.m. The signals for H-1 of 3- and 2,6-linked units overlap in this range[26]. Since methylation analysis and ^{1}H-n.m.r. spectra of the oligosaccharides obtained by complete acetolysis did not show the presence of 3-linked units, it is assumed that this signal belongs to 2,6-linked units.

The presence of these 2,6-linked units in M_5–M_8 was confirmed by the ^{13}C-n.m.r. data (Table III). The spectrum of M_2 contained, at lowest field, signals at 103.35 and 93.75 p.p.m. which correspond to C-1 of terminal non-reducing and reducing units, respectively. The spectra of M_3 and M_4 contained, in addition, a signal (101.71 p.p.m.) for C-1 of 2-linked units. The spectra of M_5–M_8 contained,

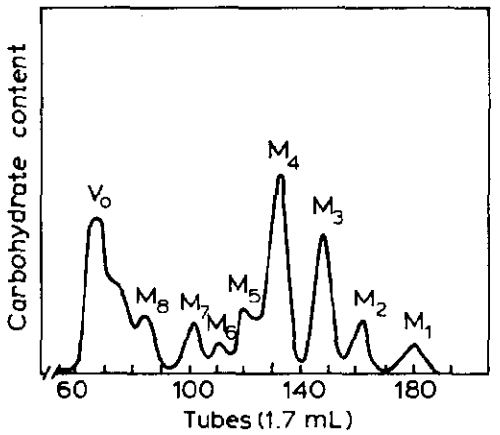


Fig. 6. Gel-filtration profile on Bio-Gel P-2 of the *O*-deacetylated oligosaccharides obtained after partial (4 h) acetolysis of *C. krusei* mannan.

TABLE III

^{13}C-N.M.R. DATA AND STRUCTURES OF *O*-DEACETYLATED OLIGOSACCHARIDES OBTAINED BY PARTIAL ACETOLYSIS OF *C. krusei* MANNAN

		Chemical shifts (p.p.m.)			
M_2	C-1	103.35	93.75	102.80	94.80
	C-2	71.22	80.29	71.58	79.55
	C-3	71.58	71.22	71.68	74.83
	C-4	68.10	68.28	68.06	68.15
	C-5	74.49	73.86	78.00	74.18
	C-6	62.29	62.29	62.29	62.29
		M1 →	2Mα	M1 →	2Mβ
M_3	C-1	103.35	101.72	93.69	
	C-2	71.59	79.86	80.52	
	C-3	71.21	71.59	71.20	
	C-4	68.06	68.26	68.26	
	C-5	73.62	74.38	74.38	
	C-6	62.26	62.26	62.26	
		M1 →	2M1 →	2M	
M_4	C-1	103.34	101.71	101.71	93.69
	C-2	71.59	79.63	79.86	80.52
	C-3	71.29	71.21	71.59	71.21
	C-4	68.06	68.26	68.26	68.26
	C-5	73.62	74.38	74.38	74.38
	C-6	62.26	62.26	62.26	62.26
		M1 →	2M1 →	2M1 →	2M
M_5	C-1	103.32	99.37	101.69	93.67
	C-2	71.59	79.85	79.85	80.54
	C-3	71.20	71.59	71.59	71.20
	C-4	68.06	68.25	68.25	68.25
	C-5	73.62	73.85	74.39	74.39
	C-6	62.25	62.25	62.25	62.25
		M1 →	2M1 →	2M1 →	2M
			6		
			↑		
			1		
			M		

Table III (continued)

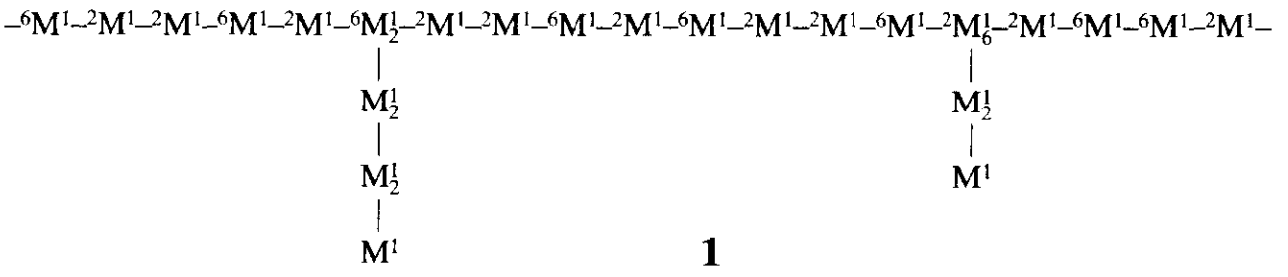
		Chemical shifts (p.p.m.)			
M_6	C-1	103.34	99.37	101.72	93.70
	C-2	71.59	79.65	79.86	80.54
	C-3	71.21	71.59	71.59	71.29
	C-4	68.06	68.27	68.27	68.27
	C-5	73.64	73.90	74.38	74.38
	C-6	62.21	62.21	62.21	62.21
		M1 →	2M1 →	$(2M1)_2$ →	2M
			6		
			↑		
			1		
			M		
M_7	C-1	103.35	99.37	101.72	93.69
	C-2	71.59	79.60	79.86	80.52
	C-3	71.21	71.59	71.59	71.21
	C-4	68.06	68.25	68.25	68.25
	C-5	73.60	73.89	74.39	74.39
	C-6	62.21	62.21	62.21	62.21
		M1 →	2M1 →	$(2M1)_3$ →	2M
			6		
			↑		
			1		
			M		
M_8	C-1	103.36	99.39	101.73	93.68
	C-2	71.60	79.80	79.36	80.48
	C-3	71.60	71.21	70.80	71.21
	C-4	67.47	68.24	68.24	68.24
	C-5	74.40	72.75	74.40	74.40
	C-6	62.22	62.22	62.22	62.22
		M1 →	2M1 →	$(2M1)_4$ →	2M
			6		
			↑		
			1		
			M		

in addition to the signals for C-1 of terminal non-reducing units (~103.35 p.p.m.), reducing (~93.69 p.p.m.), and 2-linked units (~101.72 p.p.m.), a signal at 99.37 p.p.m., which was assigned to C-1 of 2,6-linked units[22,23]. This signal was most intense in the spectra of M_7 and M_8. The signal of C-1 of 6-linked units was not detected in any fragments.

Thus, it is assumed that (1→6) linkages at otherwise unsubstituted units were cleaved more easily than those of 2,6-substituted units.

On the basis of the above results, the novel structure **1** for the *C. krusei* α-D-mannan is proposed. The main chain is lightly branched and consists of 2- and 6-linked units in the ratio 3:1. The side chains are short. This structure is more compatible with the low cross-reactivity of *C. krusei* with antisera of other

pathogenic *Candida* species, and its weak immunogenicity, than the classical highly branched 6-linked structures with longer side-chains.



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Apparently, our findings on *C. krusei* mannan disagree with those of Nishikawa *et al.*[4]. They found that M_8 obtained after partial acetolysis, which contained six 2-linkages and a 6-linkage in the middle, gave the highest inhibition of mannan–antimannan reaction. They assumed that this α-D-mannan had a branched 6-linked backbone and an octasaccharide side-chain as its antigenic determinant, although this seems to be too large to fit into the antibody recognition site[32]. It is surprising that a 6-linkage in the side chain survived the partial acetolysis when it should be more exposed to the treatment than the linkages in the main chain. Moreover, if the *C. krusei* and *C. albicans* mannans have similar structures with highly branched backbones and long side-chains, they would not be expected to show such different immunological properties.

Among the pathogenic *Candida* species, only *C. etchelsii* showed antigenic similarity to that of *C. krusei*[33]. It may be expected that structurally similar mannans will be found in some *Pichia* species, because of their serological cross-reactivity[34] and the resemblance of their ^{1}H-n.m.r. spectra to that of *C. krusei* mannan[35].

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