Carbohydrate Research, 117 (1983) 229–239
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

IMMUNOCHEMICAL DETERMINANT OF *Candida parapsilosis*

MASAHIRO FUNAYAMA, AKEMI NISHIKAWA, TAKAKO SHINODA*,
Department of Microbiology, Meiji College of Pharmacy, Tanashi-shi, Tokyo 188 (Japan)

AND YOSHIMURA FUKAZAWA
Department of Microbiology, Medical University of Yamanashi, Nakakoma-gun, Yamanashi 409-38 (Japan)

(Received October 12th, 1982; accepted for publication, November 9th, 1982)

ABSTRACT

Acetolysis of *Candida parapsilosis* cell-wall D-mannan, obtained by alkali extraction and purified as a copper complex, gave six oligosaccharides by Bio-Gel P-2 filtration. Their chemical structure was examined by ^{1}H-n.m.r. spectroscopy, methylation analysis, and partial acid hydrolysis. The structures of the penta- and hexa-saccharide were α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man (M_5) and α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-D-Man (M_6), respectively. Inhibition of the oligosaccharides with anti-*C. parapsilosis* serum and homologous D-mannan indicated that although M_5 and M_6 exhibited strong inhibitory-activity, M_6 was the more effective inhibitor, and suggested that M_6 may be the immunodominant or may be responsible for the specificity of *C. parapsilosis* mannan in the precipitin-reaction system, or both.

INTRODUCTION

The past decade has witnessed increased interest in opportunistic infections by *Candida* species and other yeasts[1–3]. Tsuchiya *et al.* [4,5] established the relationship of the antigens of medically important yeasts and many other yeasts, and successfully used antigenic factors for identifying the members of the genus *Candida*. Recently, Shinoda *et al.* [6] reported that this relationship and the antifactor sera are useful for the rapid and accurate identification of the medically important species of *Candida*. Therefore, immunochemical analysis of the antigenic factors is of interest for both the basic and applied aspects of yeast serotaxonomy. We previously reported the immunochemical structures of several antigenic factors of *Saccharomyces* and *Candida* species[7–9], and we now report the results of further studies on the immunochemical structure of the specific determinant of *C. parapsilosis* cell-wall D-mannan, as related to the antigenic factors[5] 13 and 13b.

*To whom correspondence should be addressed.

0008-6215/83/$ 03.00

TABLE I

ANTIGENIC FACTORS OF *Candida parapsilosis* AND RELATED SPECIES

Species	*Strain*[a]	*Factors*
C. parapsilosis	M 1015	1,13,13b
C. albicans	M 1012 (serotype A)	1,4,5,6
C. albicans	M 1447 (NIH A-207, serotype A)	1,4,5,6
C. albicans	M 1445 (NIH B-792, serotype B)	1,4,5,13b
C. tropicalis	M 1017	1,4,5,6
C. stellatoidea	M 1016	1,4,5

[a]M strains from the Meiji College of Pharmacy, Tokyo, Japan. NIH strains from the National Institutes of Health, Bethesda, Maryland, U.S.A.

EXPERIMENTAL

Organism. — *C. parapsilosis* M 1015, *C. albicans* M 1012 (serotype A), and *C. albicans* M 1445 (serotype B), which are stock cultures in our laboratory, were used. The antigenic factors of *C. parapsilosis* and related species proposed by Tsuchiya *et al.*[4,5] are shown in Table I.

Extraction and purification of the polysaccharides. — Yeast cells grown on Sabouraud medium for 48 h at 27° were harvested, heated for 2 h at 100°, and washed three times with distilled water. After centrifugation, the D-mannan was extracted by alkali and purified as a copper complex[10].

Acetylation and acetolysis of D-*mannan*[11]. — A sample (100 mg) was acetylated in 1:1 anhydrous pyridine–acetic anhydride (10 mL) by heating in an oil bath for 8 h at 100°. The acetylated polysaccharide was dissolved in the acetolysis mixture (10.5 mL, 10:10:1, v/v acetic acid–acetic anhydride–conc. sulfuric acid). The solution was warmed in an oil bath at 40°. To cleave the α-D-(1→6) linkages completely, the acetolysis was carried out for 13 h. The reaction was stopped by adding anhydrous pyridine (2 vol.). The solvent was evaporated, the syrupy residue extracted with 1:1 (v/v) chloroform–water (50 mL), and the chloroform extract evaporated to dryness. The residue was dissolved in anhydrous methanol (2 mL), and then sodium methoxide in methanol was added dropwise until the solution became alkaline. After 20 min, it was centrifuged for 15 min at 3000 r.p.m., and the precipitate washed with dry methanol and dissolved in distilled water (2 mL). The solution was de-ionized with Amberlite IR-120 (H^+) cation-exchange resin. The deacetylated acetolysis products were separated by Bio-Gel P-2 filtration.

Column chromatography on Bio-Gel P-2. — The deacetylated acetolysis products were applied to a column (2.5 × 150 cm) of P-2 gel and eluted with distilled water at the rate of 20 mL/h at room temperature. The total carbohydrate content in the effluents was determined by the phenol–sulfuric acid method[12,13].

Methylation analysis. — The oligosaccharide was methylated by treatment with methylsulfinylcarbanion–methyl iodide in dimethyl sulfoxide according to the

method of Hakomori[14], as described by Hellerqvist *et al.* [15]. The partially methylated alditol acetates were analyzed by g.l.c.–m.s. at 190° with a JMS D-300 instrument (Japan Electron Optics, Tokyo) equipped with a 3% ECNSS-M column (2 mm × 2 m). The fragmentation patterns were compared to those reported by Björndal *et al.*[16].

^{1}H-N.m.r. spectra of oligosaccharides. — ^{1}H-N.m.r. spectra were recorded with a JEOL 100-MHz, nuclear magnetic resonance spectrometer (Japan Electron Optics) for solutions, in deuterium oxide, at 70°, of oligosaccharides obtained by acetolysis; sodium 4,4-dimethyl-4-silapentane-1-sulfonate was the internal standard. ^{1}H-N.m.r. spectra of oligosaccharides obtained by partial acid hydrolysis of the acetolysis compound M_6 were further recorded at 65° with a JEOL 270-MHz nuclear magnetic resonance spectrometer.

Partial acid hydrolysis. — Mannohexaose (M_6), obtained by acetolysis, was treated with 0.4M sulfuric acid for 1 h at 100°. The solution was made neutral with barium carbonate, applied to a 2.5 × 150-cm column of Bio-Gel P-2, and eluted with distilled water at room temperature.

Optical rotation of oligosaccharides. — Optical rotation was determined for aqueous solutions of the oligosaccharides, at 589 nm, with a DIP-4 polarimeter (JASCO, Tokyo).

Immunological methods. — *(a) Preparation of antisera.* Antisera were prepared by the method of Fukazawa *et al.*[17]. Yeast cells, grown on Sabouraud medium for 48 h at 27°, were harvested with a saline solution, heated for 2 h at 100°, washed three times with 0.5% formalinized saline solution, and adjusted to No. 9 on the McFarland scale. Rabbits were intravenously injected at 4-day intervals with 1.0-, 2.0-, 4.0-, 4.0-, and 4.0-mL cell suspensions.

(b) Quantitative precipitin- and inhibition-reactions. The quantitative precipitin-reactions and inhibitions of the precipitin-reaction system by acetolysis oligosaccharides were performed as described by Raschke and Ballou[18]. Quantitative precipitin-reactions between rabbit antisera (0.1 mL) and D-mannan antigens were performed in physiological saline solution (PSS); the final volume was 1.0 mL. The reaction mixture was incubated for 48 h at 4°, and the precipitate was washed twice with ice-cold PSS by centrifugation. Protein was measured by the method of Lowry *et al.*[19]. For inhibition of the precipitin reaction, the antiserum was incubated, for 2 h at 37°, in the presence of the acetolysis-oligosaccharide inhibitor. The antigen was added, and incubation (48 h, 4°) and subsequent steps were as described for the quantitative precipitin-reaction.

RESULTS AND DISCUSSION

The acetolysis of *C. parapsilosis* D-mannan, followed by gel filtration, gave 6 peaks (see Fig. 1). The molar ratios, calculated from the fraction areas, of the mono- and oligo-saccharides D-mannose (M_1) to D-mannobiose (M_2) to D-mannotriose (M_3) to D-mannotetraose (M_4) to D-mannopentaose (M_5) to D-man-

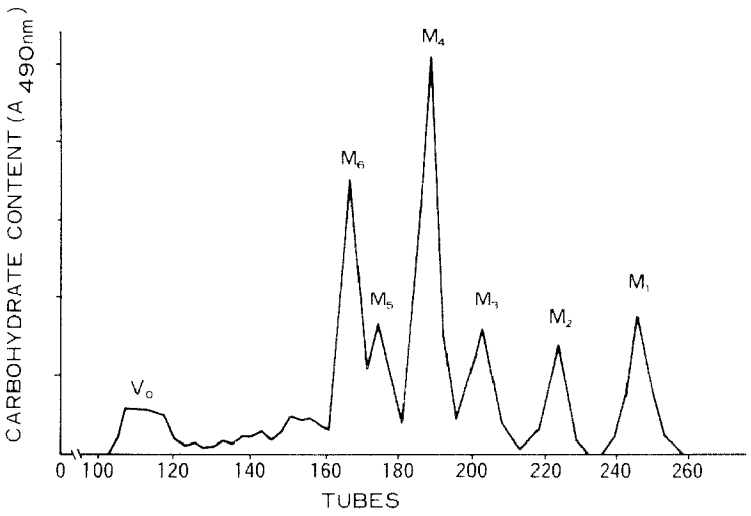


Fig. 1. Filtration profile of the oligosaccharides obtained by acetolysis of *C. parapsilosis* M 1015 D-mannan on a Bio-Gel P-2 column eluted with water. M_1, D-mannose; M_2, D-mannobiose, M_3, D-mannotriose, M_4, D-mannotetraose; M_5, D-mannopentaose, and M_6, D-mannohexaose. V_0, void volume. Each tube contained 2.5 mL.

TABLE II

METHYLATION ANALYSIS OF OLIGOSACCHARIDES OBTAINED BY ACETOLYSIS OF *C. parapsilosis* D-MANNAN

Partially acetylated mannitol derivative	*Relative retention time*	*Molar ratios*[a]				
		M_2	M_3	M_4	M_5[b]	M_6[b]
1,3,4,5,6-Penta-*O*-methyl	0.41				0.54	0.55
2,3,4,6-Tetra-*O*-methyl	1.00	1.00	1.00	1.00	1.00	1.00
3,4,6-Tri-*O*-methyl	1.78	0.96	1.77	2.43	1.94	2.73
2,4,6-Tri-*O*-methyl	1.93		0.16	trace	0.68	0.78
4,6-Di-*O*-methyl	2.94				trace	trace

[a]Relative to 2,3,4,6-tetra-*O*-methyl-D-mannitol. [b]Prior to methylation, M_5 and M_6 were reduced with sodium borohydride.

TABLE III

SPECIFIC ROTATIONS OF OLIGOSACCHARIDES DERIVED FROM *C. parapsilosis* D-MANNAN

Oligosaccharide	*Specific rotation*, $[\alpha]_D^{20}$ *(degrees)*[a]
D-Mannobiose	+37.50
D-Mannotriose	+47.72
D-Mannotetraose	+67.39
D-Mannopentaose	+55.17
D-Mannohexaose	+61.94

[a]In water, *c* 2.5

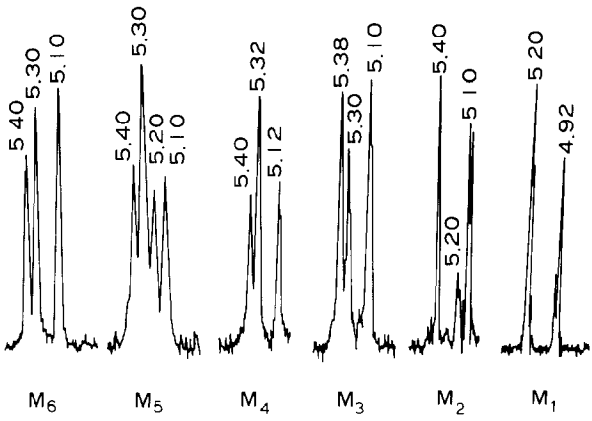


Fig. 2. ^{1}H-N.m.r spectra (δ, H-1 region) of oligosaccharides obtained by acetolysis of *C parapsilosis* D-mannan. For abbreviations, see the legend to Fig. 1.

nohexaose (M_6) were 19:7:6:14:3:6. M_4 and M_6 were the main oligosaccharides obtained. Methylation analysis (Table II) suggests that M_2, M_3, and M_4 contain mainly (1→2)-linkages, and M_5 and M_6 an additional (1→3) linkage. These results and those of ^{1}H-n.m.r. spectroscopy (see later) suggest that the (1→3) linkage in M_6 is not located at the nonreducing end. The specific rotations of the oligosaccharides (Table III) indicate α-D linkages.

The ^{1}H-n.m.r. spectra (H-1 region) of the acetolysis oligosaccharides are shown in Fig. 2. That of M_1 is identical to that of D-mannose. Those of M_2 and M_3 suggest oligosaccharides mainly α-D-(1→2)-linked, mixed with a small proportion of oligosaccharide having an α-D-(1→3) linkage, as shown by a small signal at δ 5.20. M_4 showed signals at δ 5.12, 5.32, and 5.40 with ratios of intensity 1:2:1, indicating the presence of only α-D-(1→2) linkages. M_5 showed a signal at δ 5.20 for an α-D-(1→3) linkage at the nonreducing end, and other signals indicating the presence of α-D-(1→2) linkages. M_6 showed signals at δ 5.10, 5.30, and 5.40 signals with the ratios of intensity ~1:1:1, suggesting the existence of α-D-(1→3) linkage within the chain, but not at the nonreducing end. The possible modes of linkage of oligosaccharides M_1–M_6, based on both the chemical shift assignment for manno-oligosaccharides reported by Cohen and Ballou[20] and on the results of the methylation analysis (Table II), are shown in Table IV.

To determine the location of the α-D-(1→3) linkage in M_6, reduced and unreduced oligosaccharides, and the fragments resulting from partial acid hydrolysis were examined by methylation analysis and ^{1}H-n.m.r. spectroscopy. Methylation analysis of reduced M_6 showed that the reducing end-group was linked at O-2 (Table II). Cohen and Ballou[20] reported that, in the reduced manno-oligosaccharides, a chemical shift of the H-1 signal of the adjacent D-mannose residue was modified because the H-1 signal of the reducing end-group may be moved into the ring-proton region of the spectrum. Our experiments on reduced and unreduced M_6 to determine the linkage of the residue adjacent to the reducing end-group

TABLE IV

POSSIBLE MODE OF LINKAGE AND H-1 CHEMICAL SHIFTS FOR MONO- AND OLIGO-SACCHARIDES OBTAINED BY ACETOLYSIS OF *C. parapsilosis* D-MANNAN

Structure[a]	*Chemical shift (δ)*[b]					
	H-1''''''	*H-1''''*	*H-1'''*	*H-1''*	*H-1'*	*H-1*
M						5.20 (5.165)
M→2M					5.10 (5.036)	5.40 (5.363)
M→3M					5.20 (5.133)	5.20 (5.143)
M→2M→2M				5.10 (5.039)	5.30 (5.280)	5.40 (5.352)
M→3M→2M				5.20 (5.138)	5.10 (5.033)	5.40 (5.363)
M→2M→2M→2M			5.12 (5.038)	5.32 (5.275)	5.32 (5.275)	5.40 (5.354)
M→3M→2M→2M→2M		5.20 (5.135)	5.10 (5.027)	5.30 (5.274)	5.30 (5.274)	5.40 (5.350)
M→2M→3M→2M→2M→2M	5.10	5.40	5.10	5.30	5.30	5.40
M→2M→2M→3M→2M→2M	5.10	5.30	5.40	5.10	5.30	5.40
M→2M→2M→2M→3M→2M	5.10	5.30	5.30	5.40	5.10	5.40

[a]Abbreviation: M, α-D-Man*p*. [b]H-1 chemical shifts in parentheses were reported by Cohen and Ballou[20].

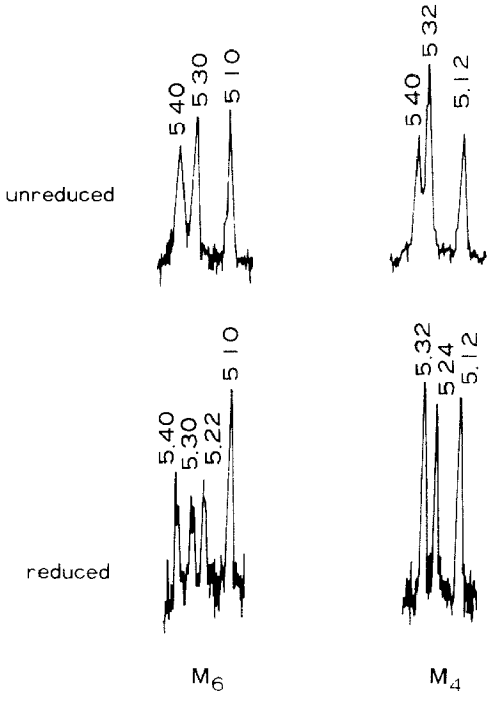


Fig. 3. ^{1}H-N.m.r. spectra (δ) of reduced and unreduced oligosaccharides M_6 and M_4

showed that reduction resulted in the disappearance of the signal at δ 5.40 corresponding to the reducing end-group, and in a chemical shift of the H-1 signal of the adjacent D-mannose residue from δ 5.30 to 5.22 (Fig. 3).

The comparable experiment with M_4, which has exclusively α-D-(1→2) linkages, showed that, in reduced M_4, the signal at δ 5.40 for the reducing end-group disappeared and a new signal at δ 5.24 appeared, and the chemical shift of the H-1 signal of the adjacent D-mannose residue changed from δ 5.32 to 5.24, although a definite assignment has not been established (Fig. 3). These results suggest that the linkage of the residue adjacent to the reducing end-group of M_6 is also an α-D-(1→2) linkage.

The nonreducing end-group of M_6 being α-D-(1→2)-linked, the presence of the H-1 signal at δ 5.10 and its absence at 5.20 (Fig. 2) suggest two possible structures (**1** or **2**). To ascertain the location of the α-D-(1→3) linkage, M_6 was partially acid hydrolyzed, and gel filtration gave six peaks (see Fig. 4). Two pentasaccharides (M_5) may be derived from M_6 one by loss of the nonreducing and the other of the reducing residue. Comparison of the ^{1}H-n.m.r. spectrum of M_5 derived from M_6 with the chemical shifts provided by Cohen and Ballou[20] (see Fig. 5-A) suggested that the signal at δ 5.21 corresponded to the nonreducing end-group α-D-(1→3)-linked. When the intensity of this signal was compared with those of the other signals, the ^{1}H-n.m.r. spectrum of M_5 was found to be complex, suggesting that it is a mixture of structures **3** and **4** in the ratio of ~5:1. These results suggest structure **1** for M_6. The possibility of a minor component (M_6) having structure **2** may be excluded, because the signal at δ 5.21 that indicates the presence of an α-D-(1→3) linkage of the nonreducing end-group was not detected in M_4 (Fig. 5-B).

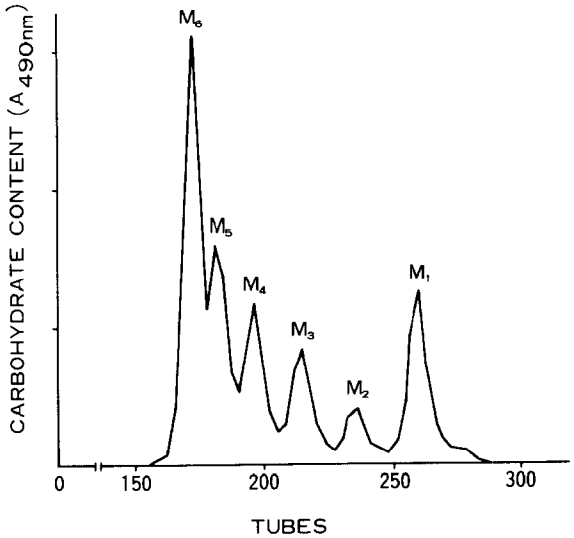
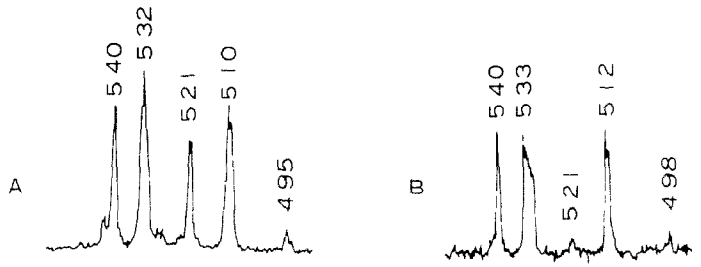


Fig. 4. Filtration profile, on a Bio-Gel P-2 column with water elution, of the oligosaccharides obtained by partial acid hydrolysis of *C. parapsilosis* M_6. Each tube contained 2.5 mL.



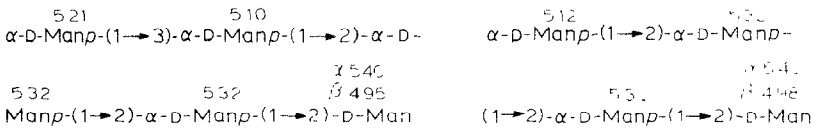
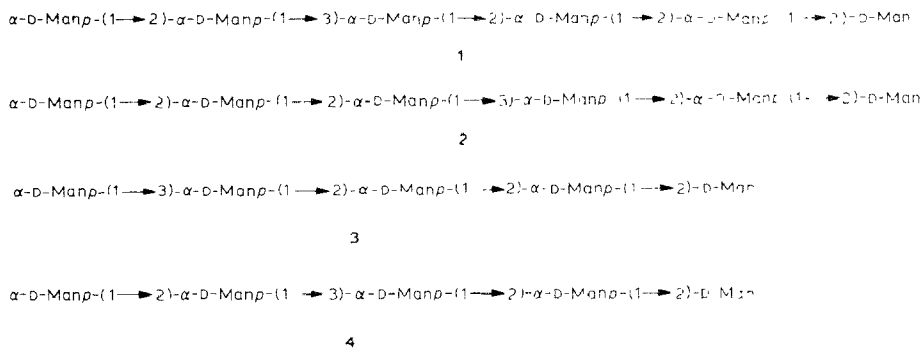


Fig. 5. ^{1}H-N.m.r spectra (δ, H-1 region) of M_5 (A) and M_4 (B) obtained by partial acid hydrolysis of *C. parapsilosis* M_6, and possible linkages deduced by assignment according to Cohen and Ballou[20]



To determine the sensitivity and specificity of the precipitin reaction between antibody and the D-mannan system, anti-*C. parapsilosis* serum was tested against homologous and *C. albicans*, serotype A and B D-mannans. In the quantitative precipitin tests, the antiserum to *C. parapsilosis* M 1015 cross-reacted with the *C. albicans*, serotype B D-mannan (M 1445), as well as with homologous D-mannan, but failed to cross-react with *C. albicans*, serotype A D-mannan (M 1012) (Fig. 6). These results suggest that the precipitin reaction has a specificity different from that of the agglutinin reaction, and that the precipitin pattern of this system may be responsible for the antigenic determinant(s) for the antigenic complex 13,13b, part of which is shared with *C. parapsilosis* and *C. albicans* serotype B. Therefore, to determine the specific determinant of the oligosaccharides for *C. parapsilosis*, the inhibition of the precipitin reaction between anti-*C. parapsilosis* M 1015 serum and homologous D-mannan by several inhibitors was examined. As shown in Fig. 7, D-

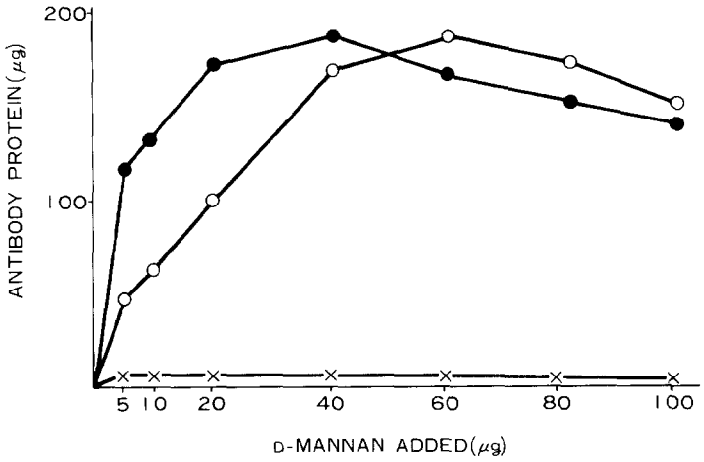


Fig. 6. Quantitative precipitin reaction of anti-*C. parapsilosis* serum with homologous and heterologous mannans: (●) homologous D-mannan; (○) *C. albicans* M 1445 (serotype B) D-mannan; and (x) *C. albicans* M 1012 (serotype A) D-mannan.

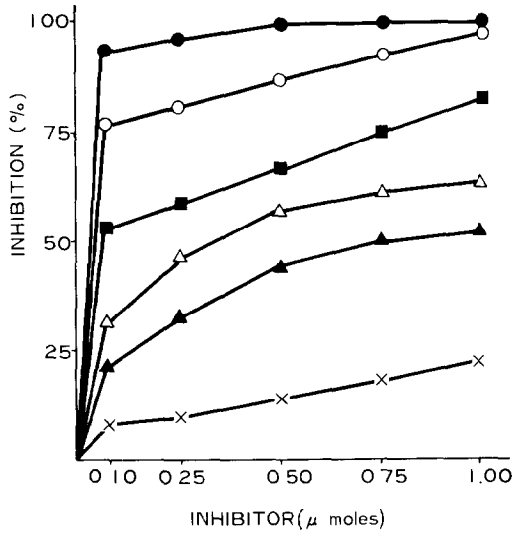
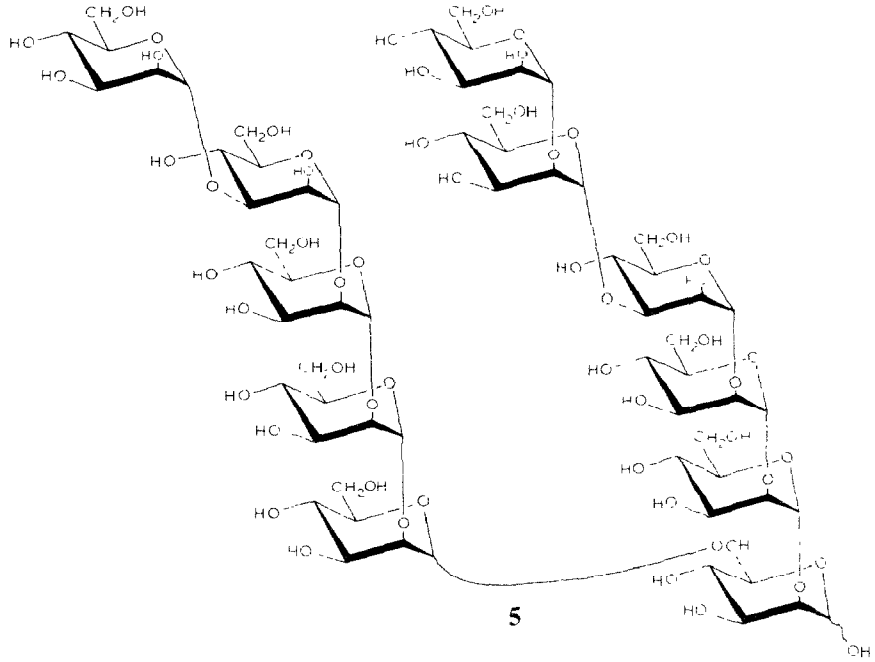


Fig. 7. Inhibition of the homologous precipitin-reaction of *C. parapsilosis* M 1015. Inhibitors: (●) D-mannohexaose, (○) D-mannopentaose, (■) D-mannotetraose, (△) D-mannotriose, (▲) D-mannobiose, and (x) D-mannose from *C. parapsilosis* M 1015 D-mannan.

manno-hexaose and -pentaose demonstrated effective inhibitory activity giving 100% inhibition even at a very low concentration (0.5 μmol), and 98% inhibition at 1 μmol. Although both D-manno-hexaose and -pentaose exhibited strong inhibitory activity, the observation that D-manno-hexaose was the most effective inhibitor suggests that this structure is the main determinant for the specificity of *C. parapsilosis* D-mannan. *C. parapsilosis* cell-wall D-mannan contains a large propor-

tion of side-chains composed of tetra- and hexa-saccharides. The acetolysis pattern of this D-mannan is characteristic when compared with the patterns of other *Candida* and *Saccharomyces* species[7–9,11].

M_2 and M_3 were also found to be mainly α-D-(1→2)-linked oligosaccharides, in admixture with a small proportion of oligosaccharide having an α-D-(1→3) linkage to the nonreducing end-group. M_4 was found to have only α-D-(1→2) linkages, M_5 an α-D-(1→3) linkage to the nonreducing end-group of M_4, and M_6 an α-D-(1→2) linkage to the nonreducing end-group of M_5.



5

A possible structure of the immunodominant or specific determinant of *C. parapsilosis* is that of **5**. Tsuchiya *et al.*[4,5] first detected the antigenic factor 13, specific for *C. parapsilosis*, by absorption experiments using a slide agglutination-test. Later, they found[21] that part of factor 13 is shared with *C. albicans* serotype B and named it factor 13b. We prepared, by absorption with heated *C. albicans* M 1012 (serotype A) cells, anti-factors 13,13b serum from anti-*C. parapsilosis* serum. The agglutinin titer of the anti-factors 13,13b serum was 1:256. However, this anti-factor serum did not precipitate *C. parapsilosis* D-mannan. Therefore, we used whole anti-*C. parapsilosis* serum for the quantitative precipitin reaction and its inhibition. In addition, as shown in Fig. 6, unabsorbed anti-*C. parapsilosis* serum failed to cross-react with *C. albicans* M 1012 (serotype A) D-mannan containing common antigenic factors with *C. parapsilosis*, which were determined by cell agglutination. The mechanism of the difference between precipitation and agglutination with respect to sensitivity and specificity is not known. However, at present,

we propose that the structure of M_6 may be the dominant determinant of factors 13,13b, shared with *C. parapsilosis* and *C. albicans* serotype B.

ACKNOWLEDGMENTS

The authors thank Dr. M. Suzuki for his kind comments, and appreciate the help of Mr. K. Sato in performing the ^{1}H-n.m.r.-spectral analyses.

REFERENCES

1 R. HURLEY, in F. A. SKINNER, S. M. PASSMORE, AND R. R. DAVENPORT (Eds.), *Biology and Activities of Yeasts*, Academic Press, New York, 1980, pp. 231–248.
2 F. C. ODDS, *Candida and Candidosis*, Leicester University Press, Leicester, 1979.
3 A. ITO, *Shinkin to Shinkinsho*, 21 (1980) 239–248.
4 T. TSUCHIYA, Y. FUKAZAWA, AND S. KAWAKITA, *Mycopathol. Mycol. Appl.*, 26 (1965) 1–15.
5 T. TSUCHIYA, Y. FUKAZAWA, M. TAGUCHI, T. NAKASE, AND T. SHINODA, *Mycopathol. Mycol. Appl.*, 53 (1974) 77–91.
6 T. SHINODA, L. KAUFMAN, AND A. A. PADHYE, *J. Clin. Microbiol.*, 13 (1981) 513–518.
7 Y. FUKAZAWA, A. NISHIKAWA, M. SUZUKI, AND T. SHINODA, in H. PREUSSER (Ed.), *Medical Mycology, Zentrabl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 1, Suppl.*, 8 (1980) 127–136.
8 A. NISHIKAWA, T. SHINODA, AND Y. FUKAZAWA, *Mol. Immunol.*, 19 (1982) 367–373.
9 M. SUZUKI AND Y. FUKAZAWA, *Microbiol. Immunol.*, 26 (1982) 387–402.
10 P. A. J. GORIN AND J. F. T. SPENCER, *Can. J. Chem.*, 46 (1968) 2299–2304.
11 J. KOCOUREK AND C. E. BALLOU, *J. Bacteriol.*, 100 (1969) 1175–1181.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
13 S. MOORE, in A. NOWOTNY (Ed.), *Basic Exercises in Immunochemistry*, Springer, Berlin, 1969, pp. 102–104.
14 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
15 C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Carbohydr. Res.*, 8 (1968) 43–55.
16 H. BJORNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem., Int. Ed. Engl.*, 9 (1970) 610–619.
17 Y. FUKAZAWA, T. SHINODA, A. NISHIKAWA, AND T. NAKASE, *Int. J Syst. Bacteriol.*, 30 (1980) 196–205.
18 W. C. RASCHKE AND C. E. BALLOU, *Biochemistry*, 10 (1971) 4130–4135.
19 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
20 R. E. COHEN AND C. E. BALLOU, *Biochemistry*, 19 (1980) 4345–4358.
21 Y. FUKAZAWA, T. SHINODA, A. NISHIKAWA, K. KAGAYA, AND Y. YONEZAWA, in K. IWATA (Ed.), *Yeasts and Yeast-like Microorganisms in Medical Science*, Univ. Tokyo Press, Tokyo, 1976, pp. 213–218.