

Structural Modification of Cell Wall Mannans of *Candida albicans* Serotype A Strains Grown in Yeast Extract-Sabouraud Liquid Medium under Acidic Conditions

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The cell wall mannans of two *Candida albicans* serotype A strains, NIH A-207 and J-1012 (abbreviated as A and J strains, respectively), cultured in yeast extract-Sabouraud liquid medium at pH 2.0, contained neither a phosphate group nor a β -1,2-linked mannosyl unit (H. Kobayashi, P. Giummelly, S. Takahashi, M. Ishida, J. Sato, M. Takaku, Y. Nishidate, N. Shibata, Y. Okawa, and S. Suzuki, *Biochem. Biophys. Res. Commun.* 175:1003-1009, 1991). In this study, the mannans obtained from A and J strains grown in pH 2.0 medium (abbreviated as mannans A2 and J2, respectively) exhibited quite different reactivities against rabbit anti-*C. albicans* and anti-*Saccharomyces cerevisiae* sera compared with those of mannans from the corresponding strains cultured in conventional medium at pH 5.9 (abbreviated as mannans A and J, respectively). Namely, mannans A2 and J2 lost reactivity against the former serum but reacted with the latter serum to a higher extent than mannans A and J. In order to account for these difference in more detail, mannans A2 and J2 were subjected to acetolysis. Elution profiles of the acetolysates were completely different from those of acetolysates obtained from mannans A and J reported in our previous papers. The ^1H nuclear magnetic resonance spectra of the oligosaccharides from mannans A2 and J2 obtained by this procedure indicate that the side chains are composed of α -linked mannosyl units densely linked to the α -1,6-linked backbone. The long side chains containing one α -1,3-linked mannosyl unit are markedly increased.

We previously reported structural analyses of the antigenic mannan-protein complexes (mannans) of three *Candida albicans* strains, NIH A-207 (serotype A) (13, 28), J-1012 (serotype A, formerly serotype C) (11), and NIH B-792 (serotype B) (12, 30, 31) (abbreviated as A, J, and B strains, respectively, in this report), utilizing a sequential degradation procedure involving treatment with hot 10 mM HCl, acetolysis under conventional and mild conditions, and enzymolysis with an *Arthrobacter* strain GJM-1 exo- α -mannosidase. We also showed that both β -1,2 and α -1,2 linkages containing oligomannosyl side chains in cell wall mannan functioned as *C. albicans* serotype A-specific epitopes (15) and that the β -1,2-linked oligomannosyl side chains, which are attached to phosphate, serve as major common epitopes in *C. albicans* serotypes A and B (27). As can be seen in the chemical structures of these mannans (Fig. 1), the structural identity is evident: i.e., each possessed a fundamental structure that included a backbone consisting of consecutive α -1,6-linked mannosyl units and a large number of α -1,2-linked mannosyl side chains.

We found (7) that A and J strains could grow in Sabouraud liquid medium containing yeast extract (pH 2.0), whereas the B strain could not. The mannans of the two former *C. albicans* serotype A strains grown in this medium at pH 2.0 contained neither phosphate nor the β -1,2-linked mannosyl unit.

It was of interest to investigate in detail the structural features of the mannans of these serotype A strains grown at pH 2.0 to help elucidate the biosynthetic process of *C. albicans* mannans. In the present study, therefore, the chemical structures of these mannans were investigated by means of acetol-

ysis followed by ^1H nuclear magnetic resonance (NMR) analysis of the resultant oligosaccharides.

MATERIALS AND METHODS

Materials. *C. albicans* strains A and J were kindly supplied by T. Shinoda, Department of Microbiology, Meiji College of Pharmacy, Tokyo, Japan. Rabbit polyclonal antisera to B strain and *Saccharomyces cerevisiae* X2180-1A wild type (abbreviated as the 1A strain), called antisera B and 1A, respectively, were used as in our previous studies (17, 29). Mannans of B and 1A strains were also described in our previous studies (12, 16). For gel filtration chromatography, Bio-Gel P-2 (400 mesh; fractionation range, 100 to 1,800 Da), obtained from Bio-Rad Laboratories (Richmond, Calif.), was used.

Cultivation of *C. albicans* strains. Five batches of A strain (10^5 cells per 100 μl of saline) were cultured in yeast extract-Sabouraud medium at 27°C for 72 h at pH 5.9 \pm 0.1, 5.0 \pm 0.1, 4.0 \pm 0.1, 3.0 \pm 0.1, and 2.0 \pm 0.1 on reciprocal shakers. The pH 5.9 medium was prepared by autoclaving 200 ml of 0.5% (wt/vol) yeast extract, 1% (wt/vol) peptone, and 4% (wt/vol) D-glucose in 500-ml cultivation flasks at 121°C for 20 min. The pH was adjusted from 5.0 to 2.0 by adding 6 N HCl to 200 ml of the pH 5.9 medium. Two batches of J strain were cultured in the same medium as the A strain at pH 5.9 \pm 0.1 and 2.0 \pm 0.1.

Preparation of mannans. Methodologies for preparation of mannans have been described before (11). Briefly, a combination of hot-water extraction and short-term precipitation with Fehling solution was used. The mannan fractions obtained from A strain cells cultured at pH 5.9, 5.0, 4.0, 3.0, and 2.0 were designated mannans A, A5, A4, A3, and A2, respectively. Also, mannan extracted from the A strain cultured at pH 2.0 and

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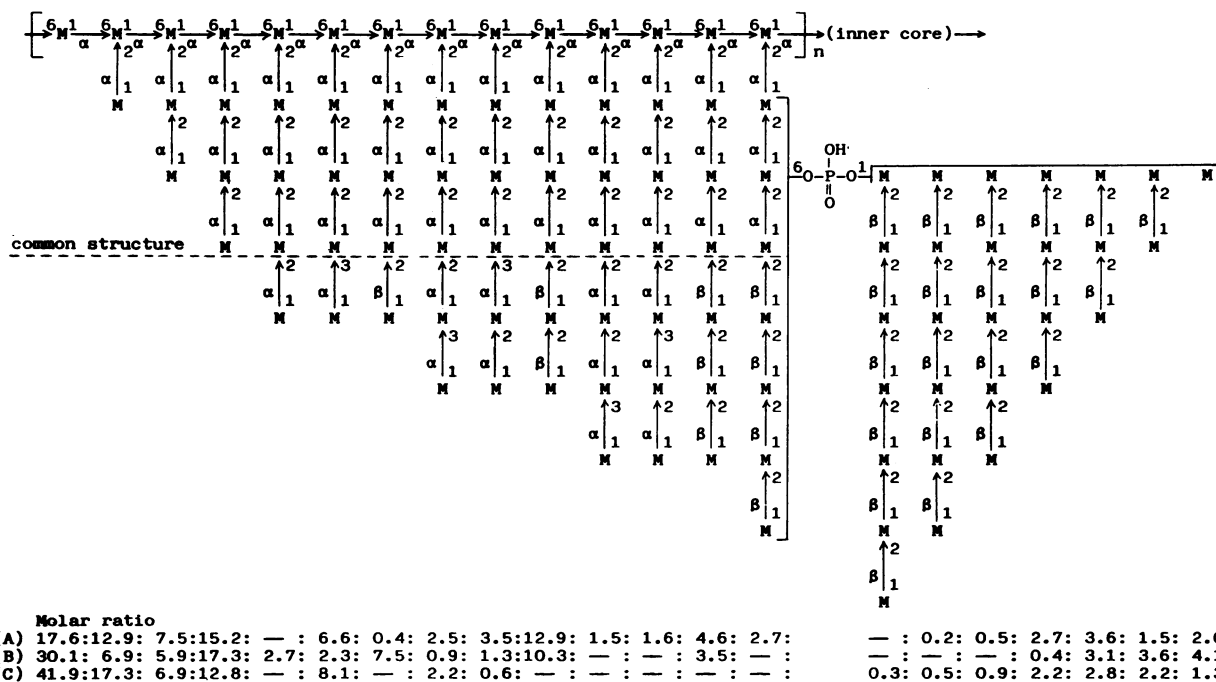


FIG. 1. Structures of cell wall phosphomannans (mannans) of *C. albicans* NIH A-207 (serotype A; A, bottom) (13), J-1012 (serotype A; B, bottom) (11), and NIH B-792 (serotype B; C, bottom) (12). M denotes a D-mannopyranose unit. The side chain sequence is not specified. The dashed line represents the borderline between common and variable parts of the side chains.

then at pH 5.9, each for 72 h, was designated mannan A'. The mannans obtained from the J strain cultivated at pH 5.9 and 2.0 were designated mannans J and J2, respectively.

Quantitative precipitin assay. The precipitin assay was performed as previously described (17, 25). Briefly, 500 μ l of saline containing serial quantities of mannan (1 to 50 μ g) was added to 100 μ l of antiserum in small test tubes (1.6 by 10.4 cm). The tubes were incubated at 37°C for 1 h, stored at 4°C for 16 h, and then centrifuged at 1,500 \times g for 10 min. Total protein in each precipitate was determined by the method of Lowry et al. (23).

Acetolysis of mannans A2 and J2. For acetolysis of mannans A2 and J2, we used the procedure described by Kobayashi et al. (14), which is a modification of the method of Kocourek and Ballou (20). Mannans A2 and J2, (150 mg of each) were dissolved in 3 ml of anhydrous formamide in a stoppered 50-ml round-bottomed flask by heating in a boiling water bath. The resultant solution was cooled to room temperature. Twenty milliliters of a 1:1 (vol/vol) mixture of $(\text{CH}_3\text{CO})_2\text{O}$ and pyridine was added, and the clear solution was maintained at 40°C for 12 h. The reaction mixture was concentrated in vacuo to a thick syrup, and the rest of the volatile materials was removed by means of an oil diffusion pump to leave O-acetylated mannan. The residue was then dissolved in 15 ml of a 10:10:1 (vol/vol/vol) mixture of $(\text{CH}_3\text{CO})_2\text{O}$, CH_3COOH , and H_2SO_4 , and the solution was maintained at 40°C for 13 h. After termination of the reaction by the addition of 2 ml of pyridine, the mixture was concentrated in vacuo to a thick syrup and dissolved in 15 ml of CHCl_3 . The solution was shaken with 15 ml of water three times and dried over anhydrous Na_2SO_4 . After filtration, the solvent was evaporated to dryness in vacuo and trace CHCl_3 was removed by codistillation with 5 ml of toluene. The residue was dissolved in anhydrous CH_3OH , 2.5 ml, and a 1 M CH_3ONa solution of

CH_3OH was added dropwise until de-O-acetyl precipitation began. After a 15-min interval, the mixture was neutralized with 50% CH_3COOH and evaporated to dryness in vacuo.

The residue was dissolved in 2 ml of water, applied to a column (2.5 by 100 cm) of Bio-Gel P-2, and eluted with water (0.25 ml/min). Aliquots (10 μ l) of the eluates were assayed for carbohydrate content by the phenol- H_2SO_4 method (3). Eluates corresponding to each peak were combined and concentrated in vacuo. In order to remove small amounts of contaminated oligosaccharides of lower or higher molecular weights, the solution was rechromatographed on the same column of Bio-Gel P-2, and eluates containing a homogeneous oligosaccharide were combined and lyophilized after concentration in vacuo.

Calculation of average chain lengths (X) and branching frequency values (Y) of mannans. The average chain lengths of the branching moieties and the branching frequency values of mannans A2 and J2 were calculated by using the following formulas in accordance with previous descriptions (16, 18): $X = [(A \times 1) + (B \times 2) + (C \times 3) + (D \times 4) + (E \times 5) + (F \times 6) + (G \times 7)] / (A + B + C + D + E + F + G)$; $Y (\%) = [(B + C + D + E + F + G) \times 100] / (A + B + C + D + E + F + G)$, where A through G represent the molar proportions of mannose (M) and the manno-oligosaccharides biose (M_2), triose (M_3), tetraose (M_4), pentaose (M_5), hexaose (M_6), and heptaose (M_7) in the gel filtration profiles of the acetolysis products of mannans A2 and J2, and the numbers 1 through 7 indicate the degrees of polymerization of the mannose and the six oligosaccharides M_2 through M_7 , respectively.

Other methods. Total carbohydrate was determined with phenol- H_2SO_4 (3), using D-mannose as the standard. Total protein was determined by the method of Lowry et al. (23), using bovine serum albumin (Sigma Chemical, St. Louis, Mo.) as the standard. Total phosphate was determined by the

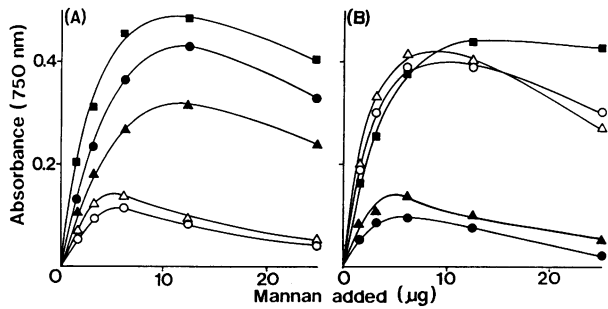


FIG. 2. Quantitative precipitin curves of mannans A (●), A2 (○), J (▲), and J2 (△) and of homologous mannans (■) against antisera B (A) and 1A (B). The homologous mannans were obtained from the corresponding strains grown under conventional conditions (pH 5.9).

method of Ames and Dubin (1), using KH_2PO_4 as the standard. The 400-MHz $^1\text{H-NMR}$ spectra were conducted as described by Kobayashi et al. (11) on a JEOL JNM-GSX 400 spectrophotometer in D_2O at 70°C , using acetone (2.217 ppm) as an internal standard. Specific rotation was determined with a JAS DIP-360 digital polarimeter after a 3-h equilibration of each sample in water.

RESULTS

Antibody precipitation assay of mannans against antisera of yeast form cells of *C. albicans* and *S. cerevisiae*. Figure 2 shows the comparative antibody precipitation assays of mannans A, A2, J, and J2 against the heterologous antisera B and 1A, which are specific for β -1,2- and α -1,3-linked mannopyranose units, respectively (17). Against antiserum B (Fig. 2A), mannans A2 and J2 were nonreactive, suggesting that the β -1,2-linked mannopyranose unit in these mannans was lost. Against antiserum 1A (Fig. 2B), mannans A2 and J2 were intensely reactive compared with mannans A and J. This finding suggests that the side chains containing the α -1,3-linked mannopyranose unit contribute greatly to the alteration of serological properties of mannans A2 and J2.

Analyses of mannans. In order to account for the change in immunochemical reactivities of mannans A2 and J2, these mannans were subjected to several chemical analyses. The chemical composition, specific rotation, and yield of the mannan fractions in the present study are shown in Table 1. A

TABLE 1. Chemical compositions of mannans

Mannan	Total carbohydrate (%) ^a	Total protein (%) ^b	Total phosphate (%) ^c	$[\alpha]_{\text{D}}^{25}$ (°) ^d	Yield (%) ^e
A	93.9	1.7	0.82	+43.0	12.2
A5	94.0	1.8	0.80	+44.2	12.3
A4	93.6	2.0	0.59	+49.0	10.4
A3	94.4	2.6	0.28	+54.6	10.8
A2	95.0	2.8	0	+60.2	11.3
A'	93.5	2.0	0.84	+44.0	10.9
J	94.5	2.3	0.62	+38.8	12.0
J2	96.1	2.7	0	+62.2	11.8

^a Determined by the phenol- H_2SO_4 method (3).

^b Determined by the method of Lowry et al. (23).

^c Determined by the Ames-Dubin method (1).

^d Determined with a JAS DIP-360 digital polarimeter.

^e Weight basis of acetone-dried whole cells.

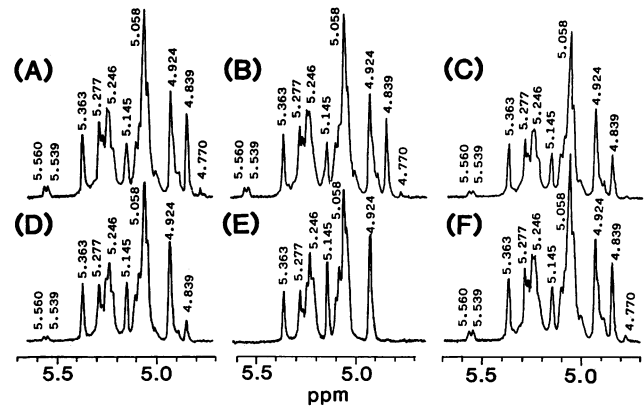


FIG. 3. $^1\text{H-NMR}$ spectra of mannans A (A), A5 (B), A4 (C), A3 (D), A2 (E), and A' (F). Spectra were recorded with a JEOL JNM-GSX 400 spectrometer in D_2O at 70°C , using acetone (2.217 ppm) as the standard.

decrease in the phosphate content to zero in A2 and J2 as well as an increase of the positive specific rotation values of these mannans were evident as the medium pH decreased from 5.0 to 2.0. Figure 3 shows the $^1\text{H-NMR}$ spectra of the six mannans of A strain: A, A5, A4, A3, A2, and A'. Mannans A5, A4, and A3 showed decreases in signal intensity at 4.770 and 4.839 ppm (corresponding to the β -1,2-linked mannopyranose unit) and 5.539 and 5.560 ppm (corresponding to the 1-O-phosphorylated mannopyranose unit) concomitantly with a decrease in pH from 5.0 to 3.0; these signals completely disappeared in mannan A2. On the other hand, mannan A' gave exactly the same signals as mannan A (Fig. 3A and F). Therefore, it is reasonable to state that the structural change in these mannans caused by differences in medium pH is entirely reversible and does not represent mutation of the parent cells. Similar results were obtained with mannans J and J2. The results of chemical analyses of the phosphate content and the measurement of specific rotation values also substantiate these findings (Table 1).

Acetolysis of mannans A2 and J2. In order to investigate the chemical structures of mannans A2 and J2 in detail, these fractions were acetolyzed with a 10:10:1 (vol/vol/vol) mixture of

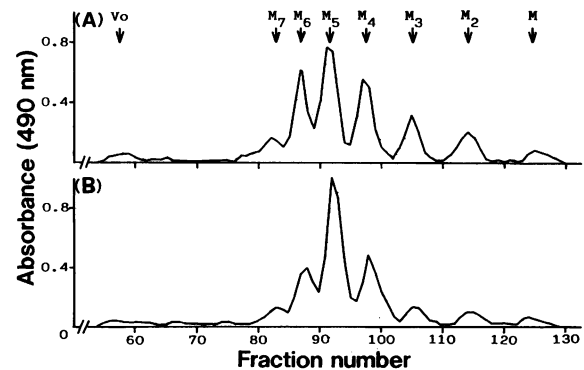


FIG. 4. Elution profiles of oligosaccharides obtained from mannans A2 (A) and J2 (B) by acetolysis on a column (2.5 by 100 cm) of Bio-Gel P-2. M_7 , M_6 , M_5 , M_4 , M_3 , M_2 , and M indicate mannohexaose, mannohexaose, mannopentaose, mannotetraose, mannotriose, mannobiose, and mannose, respectively. V_0 , void volume.

TABLE 2. Molar ratios of oligosaccharides and mannose produced by acetolysis from mannans A, J, A2, and J2^a

Mannan	Molar ratio							Avg chain length	Branching frequency (%)
	M ₇	M ₆	M ₅	M ₄	M ₃	M ₂	M		
A ^b	0.20	0.39	0.43	1.00	0.49	0.85	1.16	3.15	74.34
J ^c		0.13	0.29	1.00	0.34	0.40	1.74	2.51	55.40
A2	0.18	0.59	1.06	1.00	0.68	0.97	0.69	3.63	86.65
J2	0.17	0.59	1.51	1.00	0.45	0.66	0.30	4.11	93.59

^a Molar ratios are expressed with mannotetraose (M₄) as unity.

^b Based on a previous description by Kobayashi et al. (13).

^c Based on a previous description by Kobayashi et al. (11).

(CH₃CO)₂O, CH₃COOH, and H₂SO₄ (14, 20). Figure 4 shows the elution profiles of the acetolysates of mannans A2 and J2. The oligosaccharides isolated from these acetolysates were neutral, ranging from biose to heptaose (M₂ to M₇) and mannose (M). The amount of mannose was apparently less than that in acetolysates obtained from the mannans of both *C. albicans* strains grown in conventional medium, as previously reported (11, 13). Additionally, the absence of a void-volume region indicates that mannans A2 and J2 do not contain phosphate, because the phosphorylated oligosaccharides obtained from mannans A and J were eluted in this region (11, 13). In contrast, an increase in the amounts of the higher oligosaccharides (M₅ to M₇) was also evident. Thus, the results obtained by acetolysis of mannans A2 and J2 indicate that the branching frequency and average length of α -linked side chains, which are attached to the α -1,6-linked mannan backbone, remarkably increased in comparison with those of mannans A and J (Table 2).

All oligosaccharides (M₂ to M₇) were structurally identified by ¹H-NMR spectroscopy (Fig. 5). The anomeric proton chemical shifts of each oligosaccharide were assigned by adopting the findings of Cohen and Ballou (2), Zhang and Ballou (35), and Kobayashi et al. (11) (Table 3). The lower oligosaccharides (M₂ to M₄) were composed of α -1,2-linked mannosyl residues. On the other hand, each of the higher oligosaccharides (M₅ to M₇) contained one α -1,3-linked man-

nopyranose unit at either the nonreducing terminal or an intermediary position.

DISCUSSION

The serological classification studies of *C. albicans* species conducted by Hasenclever and Mitchell (4, 5) revealed that this species could be subclassified into two different groups, serotypes A and B, and that their properties resembled those of the closely related species *C. tropicalis* and *C. stellatoidea*, respectively.

In our recent serological study (15), we reported that the oligosaccharides containing both β -1,2 and α -1,2 linkages, Man β 1-2Man α 1-2Man α 1-2Man α 1-2Man and Man β 1-2Man β 1-2Man α 1-2Man α 1-2Man α 1-2Man, correspond to the specific epitopes of *C. albicans* serotype A (factor 6) and that the β -1,2-linked oligomannosyl residues, which are attached to the phosphate groups, served as a major common epitope, factor 5, in *C. albicans* serotype A and B strains (29), emphasizing the importance of the β -1,2-linked mannosyl unit as a component of epitopes of *C. albicans*. We also reported on the structures of the mannans of other *Candida* species, *C. stellatoidea* (8, 19), *C. tropicalis* (9), and *C. glabrata* (10), pointing out the presence of oligomannosyl side chains containing the β -1,2-linkages (factor 5 and/or 6) in these mannans. Recently, it was suggested that the β -1,2-linkage-

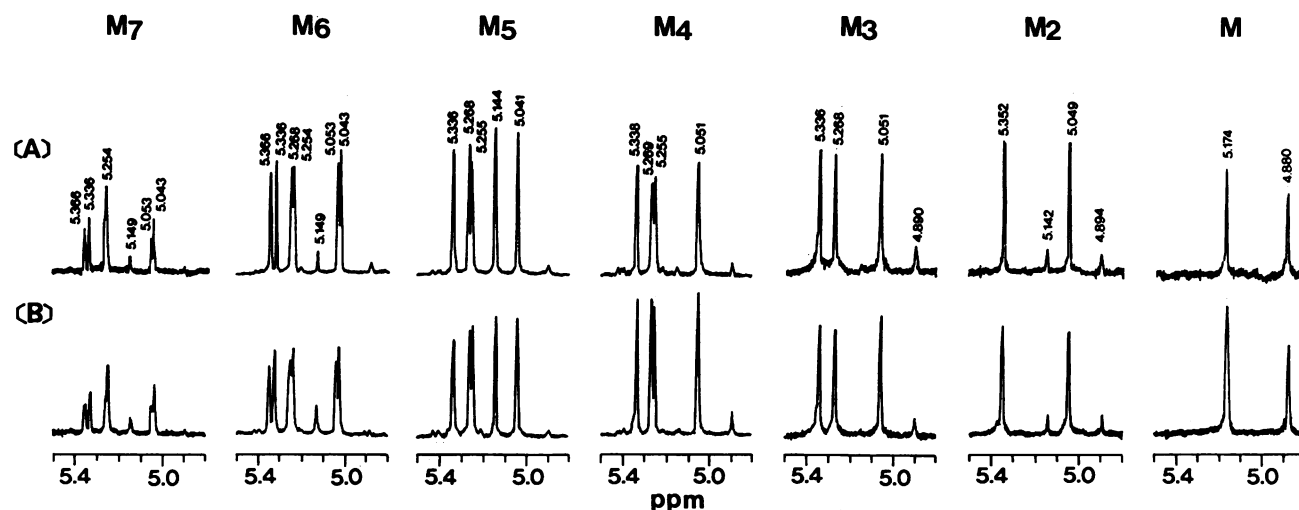


FIG. 5. ¹H-NMR spectra of oligosaccharides obtained by gel filtration of acetolysates of mannans A2 (A) and J2 (B). Each oligosaccharide, corresponding to one of the peaks in Fig. 4, was rechromatographed on the same Bio-Gel P-2 column to remove contaminated lower and/or higher oligosaccharides. These were recorded under the same conditions described in the legend to Fig. 3. For definitions, see legend to Fig. 4.

TABLE 3. Anomeric proton (H-1) chemical shifts for oligosaccharides and mannose produced by acetolysis from mannans A2 and J2^a

Oligosaccharide or mannose	Sugar residue							Chemical shift (ppm)						
	G	F	E	D	C	B	A	G	F	E	D	C	B	A
M							M(α)							5.174
							M(β)							4.880
M ₂							Mα1-2M(α)						5.049	5.352
							Mα1-2M(β)						5.142	4.894
M ₃							Mα1-2Mα1-2M(α)					5.051	5.268	5.336
							Mα1-2Mα1-2M(β)					5.051	5.268	4.890
M ₄							Mα1-2Mα1-2Mα1-2M					5.051	5.269	5.338
M ₅							Mα1-3Mα1-2Mα1-2Mα1-2M			5.144	5.041	5.268	5.255	5.336
M ₆							Mα1-2Mα1-3Mα1-2Mα1-2Mα1-2M		5.053	5.366	5.043	5.268	5.254	5.336
							Mα1-3Mα1-2Mα1-2Mα1-2Mα1-2M		5.149	5.043	5.268	5.254	5.254	5.336
M ₇							Mα1-2Mα1-3Mα1-2Mα1-2Mα1-2Mα1-2M	5.053	5.366	5.043	5.254	5.254	5.254	5.336
							Mα1-3Mα1-2Mα1-2Mα1-2Mα1-2Mα1-2M	5.149	5.043	5.254	5.254	5.254	5.254	5.336

^a M denotes a D-mannopyranose unit. Parentheses denote anomer.

containing oligomannosyl side chains corresponding to factors 5 and 6 function as an adhesin in the recognition of mammalian cells (22, 24).

Stratford (33) suggested that the receptors for Flo1 and Newflo phenotype yeast flocculations recognize side chains consisting of nonreducing terminal α-linked mannosyl units in cell wall mannan. In this study, we detected the α-1,3-linked mannosyl unit as a substitute of β-1,2-linked one in the mannans of *C. albicans* serotype A strains cultivated at a pH below 5.9. Figure 6 shows the structures of A and J mannans grown in medium at pH 2.0. Photomicroscopic observation of A and J strain cells grown in the low-pH medium and suspended in phosphate-buffered saline (pH 7.2) revealed that these cells were aggregated in grape-like clusters (7). In this case, the relationship between the flocculation phenomenon and the significant change in the structure of mannan side chains seems to support Stratford's hypothesis (33). Thus, our findings suggest that the structural changes in a portion of the α-linked side chains of cell wall mannans serve as agglutination sites for other cells by interacting with an

exposed peptidic moiety with a lectin-like activity located on the cell surface. The aggregation mechanism may be of relevance to the mechanism of adherence of *C. albicans* cells to other cells.

In yeasts, mannosyltransferases responsible for cell wall mannan (outer chain) biosynthesis are found in the internal membranes of the Golgi apparatus (21). In mammalian cells, it has become clear that several glycosyltransferases, which catalyze the transfer of a sugar unit to the nonreducing terminal site of glycoproteins, reside at the cell surface as well as within the Golgi apparatus and that the cell surface enzymes may have an adhesin function in cell-cell recognition (26, 32, 34).

In this study, the transfer of mannosylphosphate groups and β-1,2-linked mannosyl units during biosynthesis of the cell wall mannan of the *C. albicans* serotype A strain was suppressed under the low-pH environment of parent cells. In contrast, the transfer of the α-1,3-linked mannosyl unit was increased under the same conditions. Therefore, we presume that two membrane-bound enzymes, β-1,2- and α-1,3-mannosyltransferases, compete for the acquisition of GDP-mannose as a mannosyl donor and the α-1,2-linked oligomannosyl side chain as an acceptor. However, whether the low pH affects the secretory organelles, particularly the endoplasmic reticulum and/or the Golgi apparatus, or whether the altered mannan is an indirect consequence of some other defect, such as induction of additional factors in relation to expression and/or suppression of a gene, has not been determined.

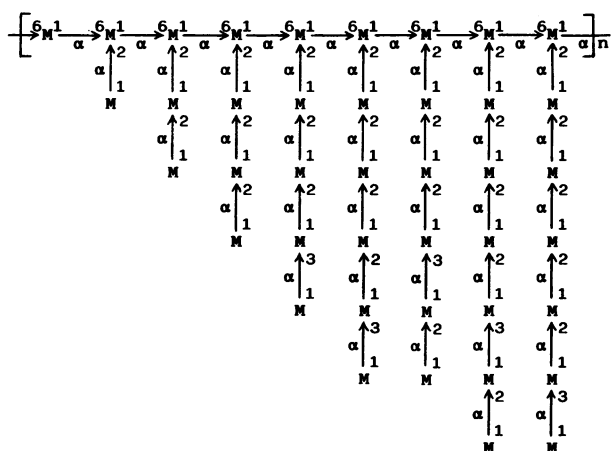
The findings obtained in this study, however, may be helpful for elucidation of the locations and characterizations of several enzymes in the biosynthesis of mannan, such as the β-1,2- and α-1,3-mannosyltransferases or mannosylphosphate transferase. In this regard, we have initiated experiments on mannan biosynthesis in *C. albicans*.

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Molar ratio

- (A) 13.35:18.76:13.16:19.34:20.50: 1.71: 9.70: 2.44: 1.04
 (B) 6.41:14.10: 9.62:21.37:32.26: 3.78: 8.83: 2.18: 1.45

FIG. 6. Structures of *C. albicans* serotype A mannans A2 (A) and J2 (B), obtained from cells growing in yeast extract-Sabouraud liquid medium at pH 2.0. Abbreviations are the same as those in the legend to Fig. 1. The side chain sequence is not specified.

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