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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

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ABSTRACT

The structure of the D-mannan of *Candida stellatoidea* IFO 1397 strain, which has properties identical to those of the phospho-D-mannan of *C. albicans* serotype B strain, does not contain phosphate groups, and its ^{1}H- and ^{13}C-n.m.r. spectra are quite similar to those of the phospho-D-mannan of *C. albicans* NIH B-792 strain. However, the ^{1}H-n.m.r. and ^{1}H–^{13}C-correlation n.m.r spectra of the products obtained by digestion with α-D-mannosidase of *C. stellatoidea* D-mannan considerably differed from those of the corresponding digestion products of the *C. albicans* phospho-D-mannan. Additionally, the enzyme-linked immunosorbent assay, by means of a monoclonal antibody corresponding to (1→2)-linked β-D-oligomannosyl residues, of the phospho-D-mannan of the same *C. albicans* strain indicated that the *C. stellatoidea* D-mannan does not contain any (1→2)-linked β-D-oligomannosyl residues. The absence of these residues may be used as one of the criteria of chemotaxonomical identification of *C. stellatoidea* spp.

INTRODUCTION

Candida albicans spp., an important pathogenic yeast for mammals, can be divided[1] into serotypes A and B. The serotype A strains of *C. albicans* spp. and *C. tropicalis* spp. are antigenically identical, and the serotype B strains of *C. albicans* spp. and *C. stellatoidea* spp. antigenically identical[2]. The D-mannans of the cell wall are mostly responsible for the serological specificities of the parent cells. Tojo *et al.*[3] reported earlier the preparation of monoclonal antibodies reactive with (1→2)-linked β-D-oligomannosyl residues of the phospho-D-mannan of *C. albicans* NIH B-792 strain. One of these monoclonal antibodies was found to agglutinate the heat-killed cells of three representative *C. albicans* strains, *i.e.*, NIH A-207, NIH B-792, and J-1012 (thereafter abbreviated as *A*-, *B*-, and *J*-strains, respectively), and of *C. tropicalis* IFO 0587, but not those of *C. stellatoidea* IFO 1397 strain (abbreviated as *S*-strain), suggesting that this *C. stellatoidea* strain lacks some common antigenic structure of the phospho-D-mannans of *C. albicans*[4–6]. Therefore, we investigated the structure of the D-mannan of *C. stellatoidea* IFO 1397 strain to clarify the structural differences between

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the D-mannans of *C. albicans* NIH B-792 and *C. stellatoidea* IFO 1397 and relate them to the serological properties of the parent yeast-form cells.

EXPERIMENTAL

Materials. — The *C. stellatoidea* IFO 1397 strain (*S*-strain) was obtained from the Institute for Fermentation Osaka, Japan. D-Mannans of *C. albicans* NIH A-207 and NIH B-792 strains, abbreviated as Frs. A and B, were the same specimens used in the previous study[4]. Bio-Gel P-2 (−400 mesh) was obtained from Bio-Rad. The *Arthrobacter* GJM-1 strain was kindly supplied by Dr. C. E. Ballou, University of California, Berkeley, through Dr. T. Nakajima, Tohoku University, Sendai, Japan. Monoclonal antibody (MAb) 18d7, specific for (1→2)-linked β-D-oligomannosyl residues, was prepared as described previously[3].

General methods.— Specific rotations were measured with an Applied Electric automatic polarimeter. ^{1}H-N.m.r. spectra of D-mannans and D-*manno*-oligosaccharides were recorded with a Jeol JNM-GSX 400 spectrometer for 0.7% solutions in D_2O at 70° and with acetone (δ 2.217) as the internal standard. ^{13}C-N.m.r. spectra were recorded with the same spectrometer for 7% solutions in D_2O at 70° and ($^2H_4$)methanol (δ 49.000) as the internal standard. ^{13}C–^{1}H-correlation spectra (C,H-COSY) were also recorded for solutions in D_2O at 70°. The total carbohydrate content was determined by the phenol–H_2SO_4 method[7] with D-mannose as the standard. The total phosphate content was determined by the method of Ames and Dubin[8] using KH_2PO_4 as the standard. The total protein content was determined by the modification of the Folin method by Lowry *et al.*[9] using bovine serum albumin as the standard.

Cultivation of S-strain. — The *S*-strain cells were grown, on a reciprocal shaker, at 28°, in 500-mL flasks containing Sabouraud liquid medium supplemented with 0.5% yeast extract. The cells in homogeneous yeast form were harvested after 48 h, washed three times with saline solution by centrifugation, and dehydrated with a large volume of acetone.

Preparation of the D-*mannan of* S-*strain.* — The D-mannan was prepared, according to the method of Kobayashi *et al.*[10] for the isolation of phospho-D-mannan of *C. albicans* J-1012 strain, by a combination of hot-water extraction, followed by fractional precipitation with Fehling solution within a short period.

Acetolysis of the D-*mannan of* S-*strain.* — Acetolysis of the D-mannan of *S*-strain was carried out as described previously[11]. Prior to acetolysis, the D-mannan was converted into its *O*-acetyl derivative according to Okubo and Suzuki[12]. D-Mannan (100 mg) was dissolved in anhydrous formamide (5 mL). To the solution was added 1:1 (v/v) acetic anhydride–anhydrous pyridine (20 mL), and the mixture was kept at 40° for 12 h. After evaporation *in vacuo* to dryness and removal of traces of volatile matter in high vacuum, the *O*-acetyl-D-mannan was dissolved in 10:10:1 (v/v) acetic anhydride–acetic acid–H_2SO_4 (10 mL) and the solution was kept for 13 h at 40° (ref. 13). The *O*-acetylated D-manno-oligosaccharide mixture was extracted with chloroform and *O*-deacetylated with sodium methoxide. The D-manno-oligosaccharides were then fractionated on a

column (2.5 x 100 cm) of Bio-Gel P-2. No peak of material was obtained at the void volume. The six peaks of material determined by neutral carbohydrate analysis in the elution profile of the acetolyzate were identified by t.l.c. with authentic specimens as D-mannohexaose to D-mannose (Man_6, Man_5, Man_4, Man_3, Man_2, and Man, respectively). Their amount was calculated from the peak dimensions and then converted into a molar ratio.

Enzyme-linked immunosorbent assay (ELISA) of D-*mannans.* — This assay was conducted as described previously[3,14,15] as follows. A 0.1% solution of each D-mannan in $NaHCO_3$ (60 mmol–L^{-1}, pH 9.6) was placed in the wells of a polystyrene microtiter plate (Falcon MicroTest III, Becton Dickinson and Co., USA), which was kept at ambient temperature overnight. Afterwards, the plate was washed three times with phosphate-buffered saline solution containing 1% (v/v) of Tween 20 (PBST). PBST-containing 1% bovine serum albumin (200 μL) was added to each well, the plate was kept for 2 h at room temperature, and then washed three times with PBST. Monoclonal antibody 18d7 (100 μL), diluted from 8- to 32768-fold with phosphate-buffered solution (PBS) was added to each well, the plate was kept for 2 h at room temperature, and then it was again washed three times with PBST. Afterwards, a solution of goat anti-mouse IgG-peroxidase conjugate, diluted 2000-fold with PBST (100 μL), was added to each well, and the mixture was kept at room temperature for 2 h. Excess peroxidase-labeled anti-mouse IgG antibody was then removed by washing three times with PBST. Finally, a substrate solution of 0.01% *O*-phenylenediamine and 0.006% H_2O_2 in 150 mM citrate buffer (100 μL, pH 5.0) was added to each well, and the mixture was kept at room temperature for 30 min. After addition of 2 M H_2SO_4 (100 μL) to each well, the color was measured at 492 nm in an Micro Plate Reader A4 (Tosoh, Japan). Each antigen was assayed in triplicate, and the average of each three values was used in plotting the reaction curve.

α-D-*Mannosidase treatment.* — This treatment was performed according to the method of Jones and Ballou[16] as follows. Fraction S or B (each 200 mg) was dissolved in 0.1M phosphate buffer (20 mL, pH 6.8) and crude *Arthrobacter α*-D-mannosidase (corresponding to 10 mg of protein) was added. The solution was kept for 24 h at 37°, dialyzed against water, and lyophilized to give Frs. S*E* and B*E*, respectively.

RESULTS

The yield of D-mannan of *S*-strain (Fr. S) obtained by hot-water extraction, followed by short-period treatment with Fehling solution was 4.8%. It contained ~95% of carbohydrate and 2.8% of protein, and no phosphate was detected (see Table I).

Examination of the H-1 region ^{1}H-n.m.r. spectra of Frs. S, A, and B (Fig. 1) showed that the spectrum of Fr. S resembled that of Fr. B rather than that of Fr. A, especially the lack of a signal at δ 4.839, which is characteristic for Fr. A[6]. In addition 1-phosphate groups (signals at δ 5.60–5.52) were absent in the spectrum of Fr. S, indicating the lack of phosphate-bound, (1→2)-linked β-D-oligomannosyl residues. A strong signal at δ 4.92 in the ^{1}H-n.m.r. patterns of Frs. S, A, and B could be assigned to

TABLE I

Chemical composition of the D-mannans of *S*-strain

Fraction	*Total carbohydrate*[a] *(%)*	*Total protein*[b] *(%)*	*Total phosphate*[c] *(%)*	$[\alpha]_D^{20}$ *(degrees)*[d]	*Yield (%)*[e]
S	95	2.8	trace	+47	4.8
S*E*	91	7.8	0.02	+24	1.3
B	96	1.3	1.45	+47	5.7
B*E*	93	2.2	2.84	+37	3.1

[a] Determined by the phenol–sulfuric acid method[7]. [b] Determined by the Folin method of Lowry *et al.*[9]. [c] Determined by the method of Ames and Dubin[8] as H_2PO_3. [d] In water, *c*, 1.0. [e] Weight basis of the acetone-dried whole cells.

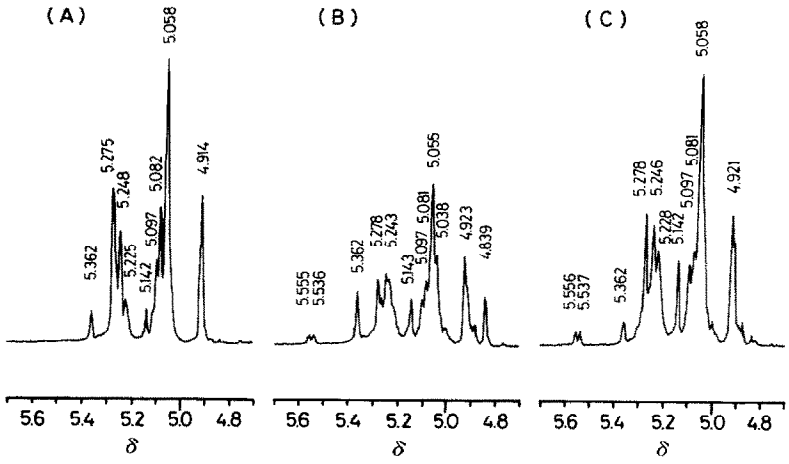


Fig. 1. H-1 Region of ^{1}H-n.m.r. spectrum of D-mannan and phospho-D-mannans isolated from: (A) *C. stellatoidea* IFO 1397, (B) *C. albicans* NIH A-207, and (C) *C. albicans* NIH B-792 (C) strain.

H-1 protons of α-(1→6)- and β-(1→2)-linked D-mannopyranose units, the latter existing as phosphate bound. A small peak at δ 5.362, which could be assigned to H-1 of the internal (1→3)-linked α-D-mannopyranose units[17,18], was observed in the three ^{1}H-n.m.r. spectra, in addition to a strong signal at δ 5.278–5.225 assigned to H-1 of the internal (1→2)-linked α-D-mannopyranose units. The elution profile of the acetolyzate of Fr. S showed six oligo- and mono-saccharides (Man_6, Man_5, Man_4, Man_3, Man_2, and Man) in the molar ratio of 1(sum of two isomers):2:6:6:15:32. These compounds were investigated by ^{1}H-n.m.r. spectroscopy, the signals being assigned on the previous reports of Cohen and Ballou[19], and Zhang and Ballou[17] (see Fig. 3 and Table II). The structures of the oligosaccharides were essentially identical to those of the branching components of the phospho-D-mannan of *B*-strain, namely, Man_2–Man_4 have structures **1**–**3**, Man_5 has structures **4** and **5** and Man_6 is a 10:3 mixture of structures **6** and **7**.

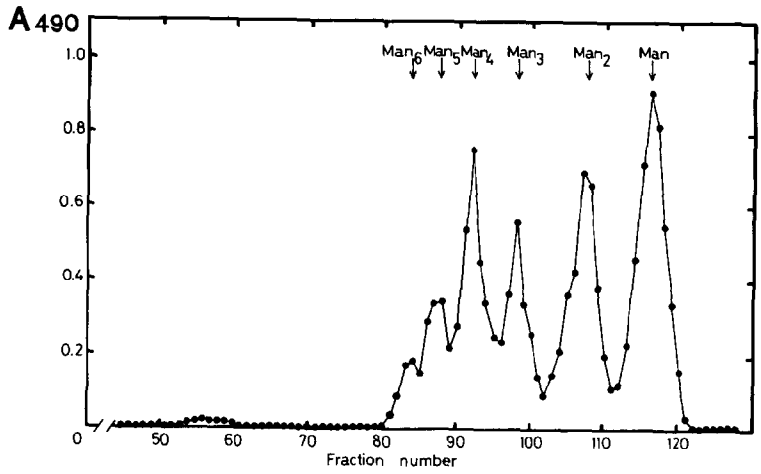


Fig. 2. Gel filtration of the acetolyzate of Fr. *S* on a column of Bio-Gel P-2, eluted with water (0.25 mL min^{-1}). The amount of carbohydrate was determined by the phenol–H_2SO_4 method[7] on aliquots of 100 μL. Man_6, Man_5, Man_4, Man_3, Man_2, and Man indicate D-mannohexaose, D-mannopentaose, D-mannotetraose, D-mannotriose, D-mannobiose, and D-mannose, respectively.

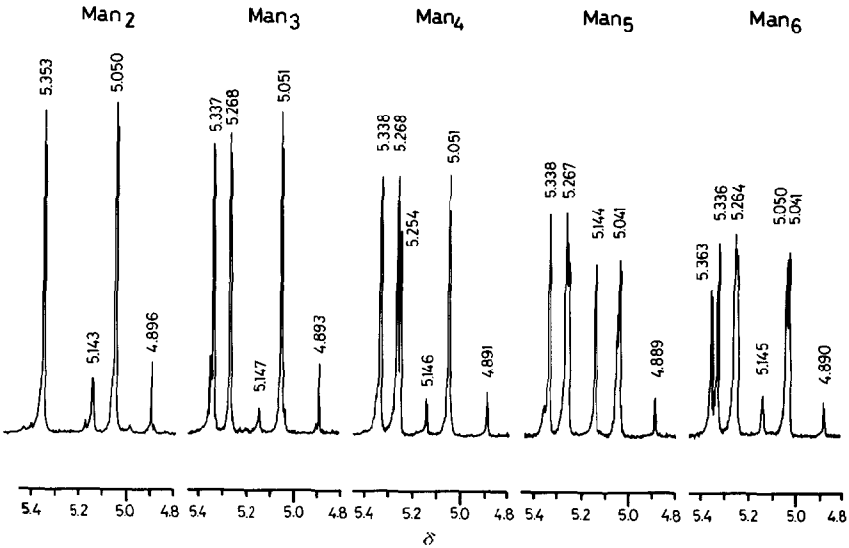


Fig. 3. ^{1}H-N.m.r. spectra of D-*manno*-oligosaccharides obtained by acetolysis of Fr. S.

α-D-Man$p$-(1→2)-D-Man

1

α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

2

α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

3

α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

4

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

5

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

6

α-D-Man$p$-(1→3)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

7

β-D-Man$p$-(1→2)-β-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

8

β-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-α-D-Man$p$-(1→2)-D-Man

9

TABLE II

Anomeric-proton chemical shifts for manno-oligosaccharides

Compound	*Chemical shift* (δ)						
	F	*E*	*D*	*C*	*B*	*A*(α)	*A*(β)
1					5.050	5.353	4.896
2				5.051	5.268	5.337	4.893
3			5.051	5.254	5.268	5.338	4.891
4		5.041	5.267	5.267	5.267	5.338	4.889
5		5.144	5.041	5.267	5.267	5.338	4.889
6	5.050	5.363	5.038	5.264	5.264	5.336	4.889
7	5.145	5.038	5.264	5.264	5.264	5.336	4.889

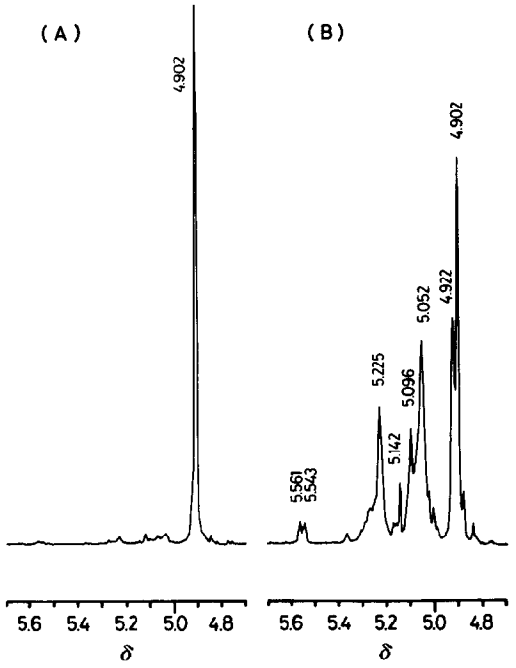


Fig. 4. ^{1}H-N.m.r. spectra of the D-mannan-protein and phospho-D-mannan-protein complex obtained by *Arthrobacter* GJM-1 α-D-mannosidase degradation of Frs. S and B: (A) Fr. S*E*, and (B) Fr. B*E*.

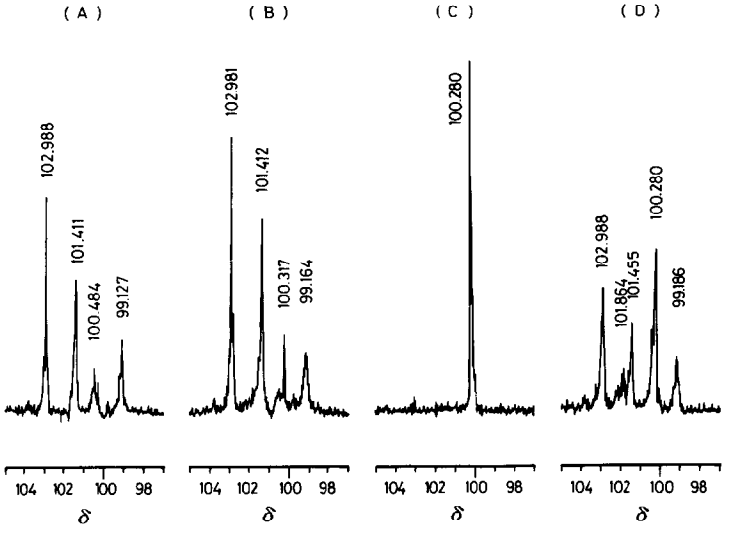


Fig. 5. ^{13}C-N.m.r. spectra of Fr. S (A), B (B), S*E* (C), and B*E* (D).

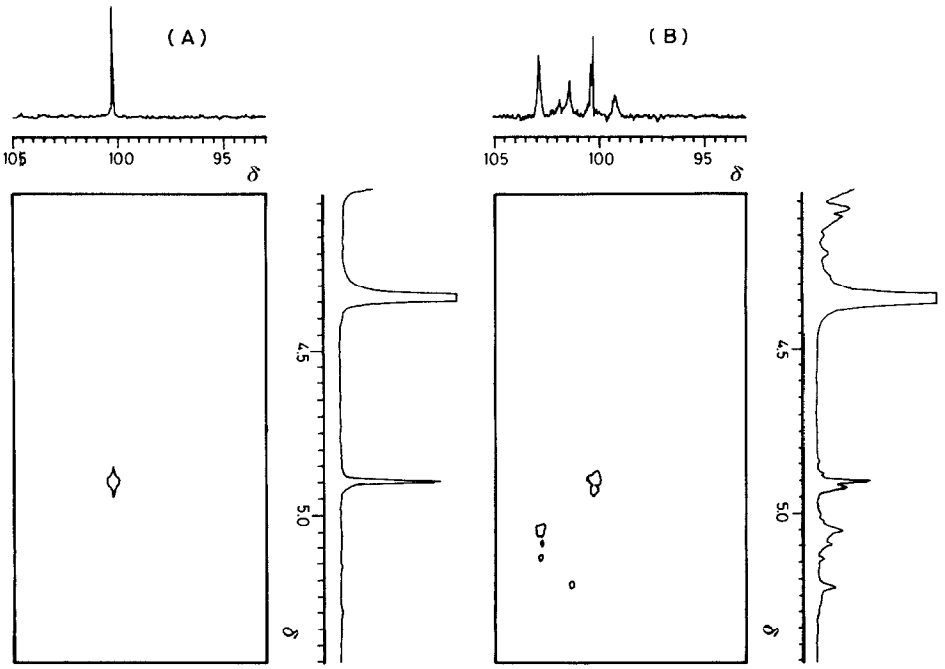


Fig. 6. ^{1}H–^{13}C-n.m.r. correlation spectra of: (A) Fr. S*E*, and (B) Fr. B*E*.

These latter two structures correspond to the epitopes dominating the serotypes B and A (refs. 18 and 20).

Upon treatment with *Arthrobacter* GJM-1 α-D-mannosidase, Frs. S and B released ~73 and 46% of D-mannose, respectively, to give Frs. S*E* and B*E*, respectively. Decrease of the values of specific rotation by this treatment (Table I) indicated the removal of a considerable proportion of α-linked D-mannopyranose units from both Frs. S and B. The increase of the signal at δ 4.902 in the ^{1}H-n.m.r. spectra of both Frs. S*E* and B*E* (Fig. 4) was attributed to the elimination of a part of the branch chains. This was substantiated by the increase of the reactivity of Frs. S*E* and B*E* against anti-*S. cerevisiae* X2180-1A-5 mutant strain serum (data not shown). The signal at δ 4.922 in the ^{1}H-n.m.r. spectrum of Fr. B*E*, was assigned to the proton(s) of the (1→2)-linked β-D-mannopyranose units.

In the ^{13}C-n.m.r. patterns of Frs. S, B, S*E*, and B*E* (Fig. 5), the difference between those of Frs. S and B is not significant, but the difference observed between those of Frs. S*E* and B*E* is significant as a large signal at δ 100.280 (Figs. 5C and 5D) could easily be assigned to be the core component consisting of consecutive (1→6)-linked α-D-mannopyranose units.

Confirmation of the difference between the signals attributed to the (1→2)-linked β-D-mannopyranose units of Frs. S*E* and B*E*, was obtained by two-dimensional, ^{1}H–^{13}C-n.m.r. correlation (Fig. 6). The signal at δ 4.902 in the ^{1}H-n.m.r. spectrum of Fr.

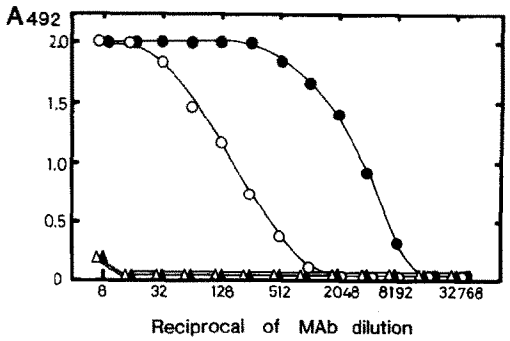


Fig. 7. Enzyme-linked immunosorbent assay of D-mannan-protein and phospho-D-mannan-protein complexes with mouse monoclonal antibody, MAb 18d7: Fr. S (▲), S*E* (△), B (●), and B*E* (○).

S*E* showed correlation with the signal at δ 100.28 of the ^{13}C-n.m.r. spectrum. However, the signal at δ 4.922–4.902 in the ^{1}H-n.m.r. spectrum of Fr. B*E* gave two correlation signals with the signal at δ 100.28 in the ^{13}C-n.m.r. spectrum. These results clearly suggested that the signal at δ 4.921 in the ^{1}H-n.m.r. spectrum of Fr. B*E* is an amalgam of two signals, corresponding to (1→6)-linked α-D-mannopyranose units of the core and to (1→2)-linked β-D-oligomannosyl residues of the internal part of branch chains.

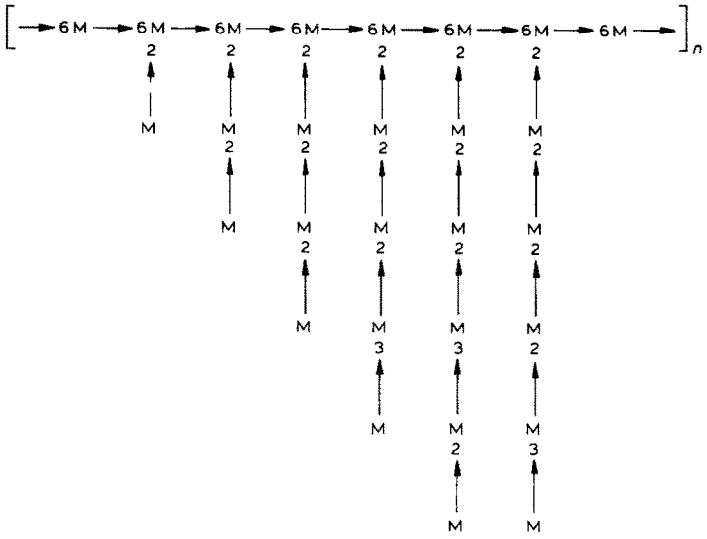
To explain the difference between the serological properties of *S*- and *B*-strain cells, we investigated the reactivity of Frs. S, S*E*, B, and B*E* against one of the monoclonal anti-*B*-strain-yeast-form-cell antibody (MAb 18d7); it had been shown to be reactive with (1→2)-linked β-D-oligomannosyl residues[3] by an ELISA procedure[15]. MAb 18d7 was able to distinguish clearly Frs. B from S, *i.e.*, Frs. B and B*E* showed high reactivities, whereas Frs. S and S*E* did not react (Fig. 7). These results clearly demonstrated the lack of (1→2)-linked β-D-oligomannosyl residues in the D-mannan of *S*-strain. That the reactivity of Fr. B*E* is lower than that of Fr. B might be attributed to the cleavage of part of the phosphodiester linkages by a phosphodiesterase that contaminated the α-D-mannosidase preparation. Supporting evidence for this explanation was obtained by comparing the ^{1}H-n.m.r. patterns of Frs. B and B*E*; the former pattern showed a distinct signal at δ 4.922–4.921, corresponding to an internal (1→2)-linked β-D-mannopyranose unit, whereas the latter pattern exhibited a weaker signal at the same δ value.

DISCUSSION

Shibata *et al.*[4] have reported that the phospho-D-mannans of both *A*- and *B*-strain contain (1→2)-linked β-D-oligomannosyl residues, and that these residues in the latter phospho-D-mannan exist only as an acid-labile form connected by 1-phosphate groups, whereas the former phospho-D-mannan contains two types of (1→2)-linked β-D-oligomannosyl residues, one located in the acid-stable and the other in the acid-labile regions. Shibata *et al.*[6] reported further the isolation of a mannohexaose (**8**) from the acetolyzate

of the partially acid-degraded phospho-D-mannan of *A*-strain. Because the acid-stable part of the phospho-D-mannan of *B*-strain did not contain any branch chain having a structure corresponding to a hexaose or a pentaose containing β-D-(1→2)-linkages, the presence of these hexaoses and pentaoses can be regarded as one of the characteristic features of *C. albicans* serotype A. Recently, Kobayashi *et al.*[10] reported the chemical structure of the phospho-D-mannan of *C. albicans* J-1012 strain which contains branch chains having a structure corresponding to the pentaose **9** in the acid-stable domain of the phospho-D-mannan.

The D-mannan of the *S*-strain and the phospho-D-mannan of the *B*-strain are difficult to distinguish by ^{1}H-n.m.r. analysis, because of the close identity of the spectra, excepting the presence of a quartet band at δ 5.60–5.52 (Fig. 1). Therefore, the structures of the D-mannan of *S*-strain and the phospho-D-mannan of *B*-strain were investigated by acetolysis, α-D-mannosidase treatment, and immunological methods, in addition to ^{1}H- and ^{13}C-n.m.r. spectroscopy. In the ^{1}H-n.m.r. pattern of Fr. A (Fig. 1B), the signal at δ 4.839 apparently corresponds to H-1 of the internal (1→2)-linked β-D-mannopyranose units of the hexaose **8**. However, we presume that the signal corresponding to the antigenic determinant that reacts with the monoclonal antibody is overlapping with the signal of consecutive (1→6)-linked α-D-mannopyranose units at δ 4.923–4.914. The ^{1}H-n.m.r. study of the D-*manno*-oligosaccharides obtained by acetolysis of Fr. S indicated that the structure of Fr. S is almost the same as that of the acid-stable domain of Fr. B, *i.e.*, Man_2, Man_3, and Man_4 (**1**–**3**) consist solely of (1→2)-linked α-D-mannopyranose units. However, Man_5 was shown to consist of α-D-(1→2)-linkage (**4**) and one α-D-(1→3)-linkage and, therefore, it was identified as **5**. Additionally, Man_6 was assumed to be a mixture of **6** and **7**. Degradation of Frs. S and B with *Arthrobacter* GJM-1 α-D-mannosidase substantiated the evidence that Fr. S does not contain phosphate groups[16] or β-linked D-mannopyranose units[21], because the increase of the signal at δ 4.902 in the spectrum of Fr. S*E* obviously corresponded to the formation of consecutive (1→6)-linked α-D-mannopyranose units by enzymic removal of the branches that are linked at O-2. On the other hand, splitting of the signal at δ 4.921 in the spectrum of Fr. B into signals at δ 4.922 and 4.902 by α-D-mannosidase treatment could be observed in the ^{1}H-n.m.r. spectrum of Fr. B*E* (Fig. 4B); the former signal could be assigned to be the consecutive (1→2)-linked β-D-mannopyranose units connected through phosphate groups, and the latter signal to the consecutive (1→6)-linked α-D-mannopyranose units. The patterns of ^{13}C-n.m.r. spectroscopy of Frs. S and B were nearly identical, whereas those of Frs. S*E* and B*E* were significantly different, *i.e.*, the signals corresponding to nonreducing, terminal, 2-*O*-substituted, and 2,6-di-*O*-substituted D-mannopyranosyl groups in Fr. S*E* at δ 102.988, 101.864, 101.455, and 99.186, respectively, were significantly smaller than the corresponding ones in the ^{13}C-n.m.r. pattern of Fr. B*E*. Furthermore, a signal at δ 100.317 was evident in the spectrum of Fr. B; it was not observed in the spectra of Frs. S and S*E*, and was split into a signal at δ 100.280 by treatment with α-D-mannosidase, COSY analysis of Frs. S*E* and B*E* apparently gave different correlation patterns that indicated the presence of consecutive (1→2)-linked β-D-oligomannosyl residues in Fr. B. Therefore, the most remarkable structural differ-



Scheme 1. Representative structure of the D-mannan component of *C. stellatoidea* IFO 1397 strain, Fr. S. M denotes an α-D-mannopyranose unit linked at O-1.

ence between Frs. S and B is the lack of (1→2)-linked β-D-oligomannosyl residues in Fr. S, resulting in its inability to react with MAb 18d7. A possible chemical structure of the D-mannan of the yeast-form cells of *C. stellatoidea* IFO 1397 strain (Fr. S) is depicted in Scheme 1.

In addition, the use of monoclonal antibody(ies) corresponding to (1→2)-linked β-D-oligomannosyl residues would be advantageous as serodiagnostic agent for the identification of the strains of *C. albicans* and *C. tropicalis* spp. as proposed by Tojo *et al.*[3], as a part of *C. stellatoidea* spp. can distinctly be distinguished from *C. albicans* spp. by means of a monoclonal antibody possessing the same specificity.

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