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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

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In previous articles, we reported the presence of phosphate-bound β-1,2-linked oligomannosyl residues in the mannans of strains of *Candida albicans* serotypes A and B and *Candida stellatoidea*. To identify the antigenic factor corresponding to this type of oligomannosyl residue, a relationship between chemical structure and antigenic specificity in the mannans of *C. albicans* NIH B-792 (serotype B, B-strain) and *C. albicans* J-1012 (serotype A, J-strain) was investigated by using a combination of two-dimensional ^{1}H nuclear magnetic resonance spectroscopy of H-1, H-2, and H-5 regions in the mannans and an enzyme-linked immunosorbent assay that employed concanavalin A-coated microtiter plates. It was shown in the present ^{1}H nuclear magnetic resonance study that an examination of chemical shifts not only in the H-1 region but also in the H-5 region was useful for the quantitative determination of the phosphate-bound β-1,2-linked oligomannosyl residues. In the enzyme-linked immunosorbent assay using concanavalin A-coated plates, it was revealed that, of factor sera 1, 4, and 5, only factor serum 5 showed a reactivity proportional to the densities of the β-1,2-linked oligomannosyl residues of the mannan subfractions of different phosphate contents that had been prepared from the bulk B-strain mannan by DEAE-Sephadex chromatography. The above results indicate that the phosphate-bound β-1,2-linked oligomannosyl residues, Man*p*β1→(2Man*p*β1→)$_n$2Man (n = 0 ~5), correspond to antigenic factor 5.

The concept of using antigenic structure to correlate seven medically important *Candida* species with their serological specificities, as proposed by Tsuchiya and coworkers (50), has provided the basis for the development of an accurate serodiagnostic procedure for candidiasis, i.e., a kit of polyclonal factor sera, namely, Candida Check. Their study also revealed that the cells of a *Candida albicans* serotype A strain had four characteristic antigenic factors, 1, 4, 5, and 6, while those of a *C. albicans* serotype B strain involved antigenic factor 13b instead of factor 6 in addition to factors 1, 4, and 5. It is presumable that most of the epitopes corresponding to these antigenic factors are composed of mannose units and reside in the mannan, the major antigen of the parent cells (13).

At present, the general structure of the mannans of *C. albicans* species can be depicted as shown in Fig. 1, on the basis of the results of structural analyses by Shibata et al. (38–40, 42, 43) and Kobayashi et al. (20, 21). Namely, the mannans consist of acid-stable and acid-labile regions, which are connected by a phosphodiester group. The acid-stable region involves a backbone moiety consisting solely of α-1,6-linked mannopyranose units and many branches composed of α-1,2-, α-1,3-, and/or β-1,2-linked mannopyranose units that are connected to the C-2 positions of the large parts of α-1,6-linked mannopyranose units in the backbone moiety. The acid-labile region is composed of β-1,2-linked oligomannosyl residues, up to heptaose, that are linked to the phosphate group via the reducing terminal group of each oligosaccharide residue and is connected to the acid-stable region with another linkage of the phosphate group in a diesterified form. Because the linkage between each β-1,2-linked oligomannosyl residue and the phosphate group can readily be cleaved by a mild acid treatment, such as with 10 mM HCl at 100°C for 60 min, it is possible to investigate the antigenicities of the acid-stable and acid-labile regions by immunochemical procedures. In previous studies, it was revealed by Shibata et al. (38) and Kobayashi et al. (19, 20) that the side chains in the acid-stable region possessing one to three β-1,2-linked mannopyranose units corresponded to the serotype A-specific epitopes.

The phosphate-bound, acid-labile β-1,2-linked oligomannosyl residues are common structures in serotypes A and B and might be responsible for the majority of humoral antibody responsiveness of the parent whole cells (21, 39, 42, 43, 47) because of their strong antigenicity and low cross-reactivity with the constituents in mammals. Therefore, this acid-labile side chain would be a good target for detection of *Candida* antigen in sera of patients with candidiasis. The serotype A-specific side chains containing both α and β linkages, Man*p*β1→2Man*p*α1→2Man*p*α1→2Man*p*α1→2Man and Man*p*β1→2Man*p*β1→2Man*p*α1→2Man*p*α1→2Man*p*α1→2Man (side chains set in outline type in Fig. 1) (20, 38), were identified as corresponding to antigenic factor 6 (22). However, there is no report on the characterization of antigenic factors 1, 4, and 5. Therefore, another objective, the identification of phosphate-bound β-1,2-linked oligomannosyl residues with any antigenic factor, has yet to be achieved. Previously, Tojo et al. (47), who developed the monoclonal β-1,2-linked oligomannosyl antibodies, assumed that these residues might correspond to antigenic factor 4 or 5 on the

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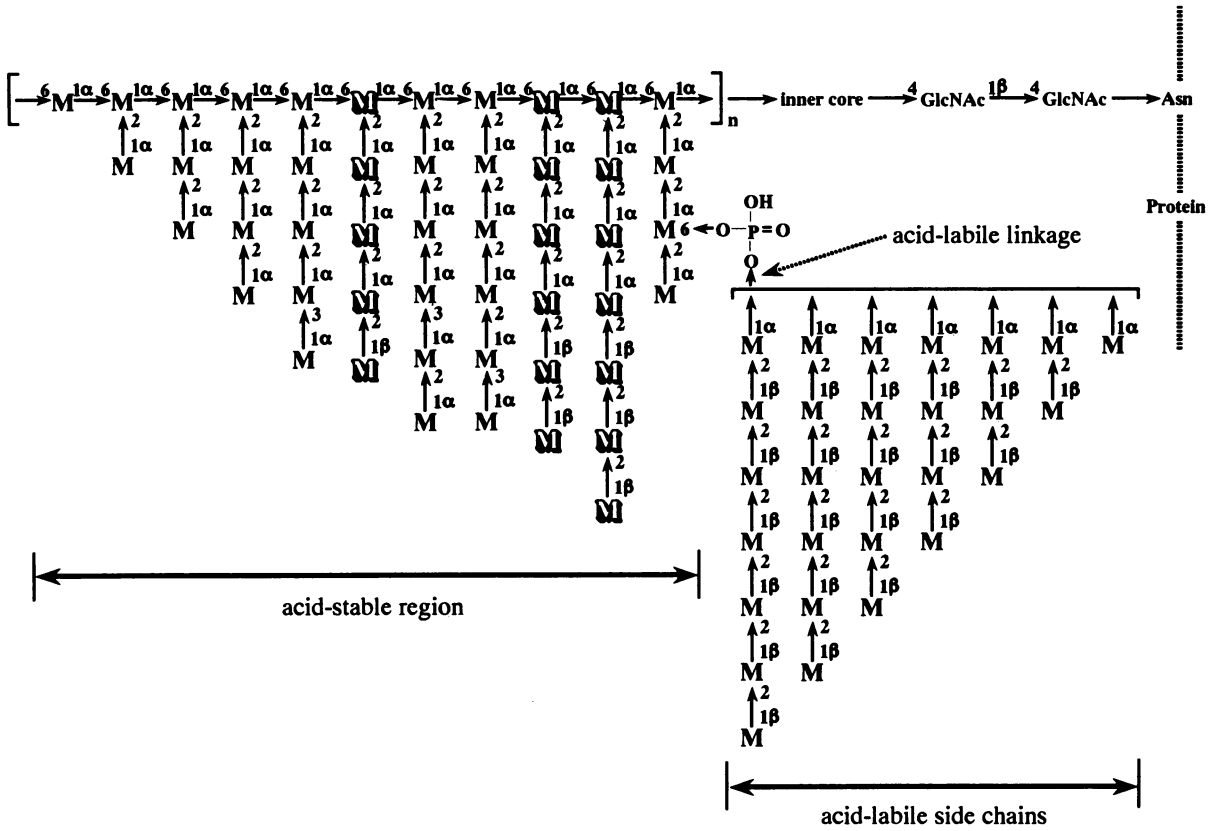

FIG. 1. Representative structure of *C. albicans* mannan. The phosphodiesterified β-1,2-linked oligomannosyl residues can be selectively removed by treatment with 10 mM HCl at 100°C for 60 min. Side chains depicted by the letter M set in outlined type indicate serotype A-specific structures containing β-1,2-linked mannopyranose units at the nonreducing terminal. Other side chains were common in mannans of serotype A and B strains.

basis of the results of an agglutination reaction between the whole cells of seven medically important *Candida* species and the monoclonal antibodies. To determine whether antigenic factor 4 or 5 corresponded to a β-1,2-linked oligomannosyl residue(s), we conducted a series of immunochemical studies of the mannans of *C. albicans* serotype A and B strains by using a combination of an enzyme-linked immunosorbent assay (ELISA) using a kit of polyclonal factor sera, Candida Check, and two-dimensional ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy.

MATERIALS AND METHODS

Materials. Mannans of *C. albicans* NIH B-792 (serotype B) and J-1012 (serotype A), designated Fr.B and Fr.J, respectively, were the same specimens used in previous studies (40, 46), which were shown to be the representative serotype B and A strains. Mannan of *Candida stellatoidea* IFO 1397, corresponding to serotype B of *C. albicans* species and designated Fr.S, has no phosphodiesterified β-1,2-linked oligomannosyl residues, is structurally and antigenically the same as acid-treated Fr.B, and is the same specimen used in a previous study (46). Bio-Gel P-2 (400 mesh) and DEAE-Sephadex A-50 were obtained from Bio-Rad. Concanavalin A (ConA, type IV) was purchased from Sigma. Polystyrene microtiter plates (Linbro/Titerteck, catalog no. 76-381-04) were purchased from Flow Laboratories, Rockville, Md. Factor sera 1, 4, 5, 6, and 13b of Candida Check (lot no. L157), rabbit polyclonal antibodies against *Candida* cells, were purchased from Iatron (Tokyo, Japan). Except for factor serum 1, which is an unabsorbed rabbit whole-cell serum against *C. albicans* cells, factor sera 4, 5, 6, and 13b are the sera of *C. albicans* absorbed with cells of *Candida parapsilosis*, *Candida guilliermondii*, *C. stellatoidea*, and *Candida tropicalis*, respectively, and are supposed to be monospecific (51).

DEAE-Sephadex chromatography of Fr.B. An aqueous solution of Fr.B (500 mg/4 ml) was applied to a column of DEAE-Sephadex A-50 (acetate, 2.5 by 35 cm). Elution was performed with water and subsequently with 0.025, 0.05, 0.1, and 0.25 M NaCl (30, 31, 44). Samples of eluates (20 μl) were assayed for their carbohydrate contents with phenol-sulfuric acid reagent (8). Eluates corresponding to each peak were combined, concentrated in vacuo to a small volume, dialyzed against water, and lyophilized. The results of chemical analyses indicated that the phosphate content was consistently proportional to the concentration of NaCl solutions used for elution. As shown in Fig. 2 and Table 1, the yield of mannan fraction B-1 was too low to use for further experiments.

Treatment of mannans with 10 mM HCl. The treatment of mannans with 10 mM HCl was conducted exactly as described previously by Shibata et al. (40). As shown by the cleavage point of mannan in Fig. 1, this mild acid treatment selectively eliminated the acid-labile β-1,2-linked oligomannosyl residues. The acid modification products of Fr.B and Fr.J were designated Fr.B-a and Fr.J-a, respectively.

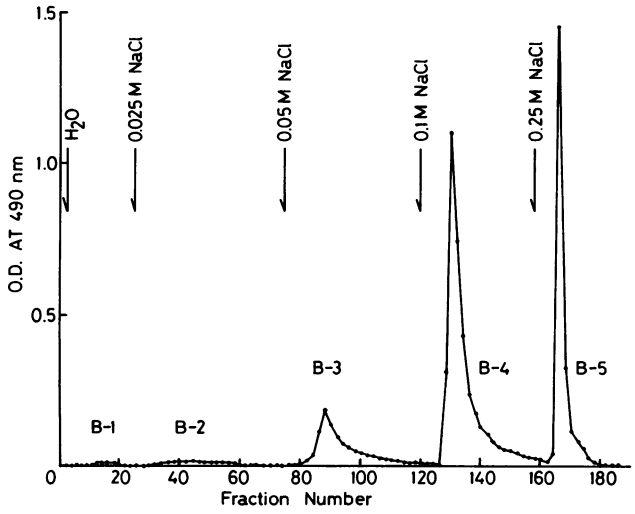

FIG. 2. Elution profile of the mannan of *C. albicans* NIH B-792 (serotype B) by DEAE-Sephadex chromatography (A-50, acetate, 2.5 by 45 cm) by stepwise elution with water and NaCl solutions.

ELISA of mannans. An ELISA of mannans was conducted as described previously (48) and as follows. Mannan solution (100 μg/ml) in 50 mM carbonate buffer (pH 9.6) was placed in the wells of a polystyrene microtiter plate, which was then kept at ambient temperature overnight. The plate was then washed three times with phosphate-buffered saline solution containing 0.1% (vol/vol) Tween 20 (PBST). PBST containing 1% bovine serum albumin (200 μl) was added to each well, and the plate was kept for 2 h at room temperature and then washed three times with PBST. Factor serum (100 μl), diluted from 10- to 20,000-fold with PBST, 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 immunoglobulin G (IgG)-horseradish peroxidase (HRPO) conjugate, diluted 1,000-fold with PBST (100 μl), was added to each well, and the mixture was left standing 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 left standing at room temperature for 30 min. After the addition of 2 M H_2SO_4 (50 μl) to each well, the color was measured at 492 nm in a Micro Plate Reader A4 (Tosoh, Tokyo, Japan).

A sandwich ELISA with ConA was performed as described previously (49) but modified as follows by means of goat anti-rabbit IgG alkaline phosphatase conjugate instead of goat anti-rabbit IgG HRPO conjugate as the antibody-linked enzyme. ConA solution (1 mg/ml) in PBS (100 μl) was placed in the wells of a plastic plate and kept at 4°C for 18 h. The plate was then washed three times with PBST. To each well, 1% bovine serum albumin solution in PBST (100 μl) was added. The plate was kept for 2 h at room temperature and then washed three times with PBST. Mannan solution (100 μg/ml) in PBS (100 μl) was added to each well. The plate was kept for 2 h at room temperature and then washed three times with PBST. Factor serum (100 μl), diluted 20-fold with PBST, was added to the wells, and the plate was kept for 2 h at room temperature and then washed three times with PBST. A 1,000-fold-diluted solution of goat anti-rabbit IgG alkaline phosphatase conjugate in PBST (100 μl) was added to the wells, and the plate was kept at room temperature for 2 h. After washing with PBST for three times, 100 μl of a substrate solution containing 1 mg of *p*-nitrophenylphosphate per ml in 100 mM glycine-HCl buffer (pH 10.4) was added to the wells, and the plate was kept at room temperature for 10 min. After the addition of 3 M NaOH (50 μl), the A_{405} was measured.

^{1}H NMR spectroscopy. ^{1}H NMR spectra of mannans and oligosaccharides were recorded on a JEOL JNM-GSX 400 spectrometer operating at 400 MHz at a probe temperature of 45°C. Each sample was dissolved in D_2O at a concentration of 1% (wt/vol), and acetone was used as the internal standard (2.217 ppm). Two-dimensional homonuclear Hartmann-Hahn (2D-HOHAHA) spectra were recorded by using the pulse sequence 90°-t_1-SL-acq, where SL stands for a multiple of the MLEV-17 sequence in accordance with the description of Bax and Davis (2). The spin-lock field strength corresponded to a 90° pulse width of 22 μs and a total spin-lock mixing time of 180 ms. The spectral width was 1,000 Hz in each dimension.

Other methods. Total carbohydrate was determined by the phenol-sulfuric acid method (8) with D-mannose as the standard. Total protein was determined by the Folin method of Lowry et al. (24) using bovine serum albumin as the standard. Total phosphate was determined by the method of Ames and Dubin (1) with KH_2PO_4 as the standard.

TABLE 1. Chemical composition and amount of β-1,2-linked mannopyranose unit in mannan fractions

Mannan fraction	Total protein (%)[a]	Total phosphate (%)[b]	Released oligosaccharide (%)[c]	β-1,2-linked mannopyranose (%)[d]	Yield (%)[e]
B	3.10	1.48	10.5	11.1	100
B-1					1.5
B-2	0.88	0.33	2.5	3.0	8.0
B-3	0.75	0.79	5.3	5.7	22.1
B-4	1.06	1.38	8.9	9.0	42.2
B-5	1.54	2.18	13.8	14.4	26.1

[a] Determined by the Folin method of Lowry et al. (24).
[b] Determined by the Ames-Dubin method to be $-PO_3H_2$ (1).
[c] Determined by quantitating the amounts of carbohydrate in the eluates of gel filtration chromatography of the acid degradation products. The values are expressed as percentages of weight basis of the parent mannans.
[d] Calculated from the dimension of the signals of regions I and III by formula 1 as described in the text.
[e] Weight basis of Fr.B.

RESULTS

^{1}H NMR of mannan subfractions. The four serotype B mannan subfractions, Fr.B-2, Fr.B-3, Fr.B-4, and Fr.B-5, prepared essentially as described by Okubo et al. (30), were measured for their ^{1}H NMR spectra at 45°C. A comparison of the patterns was made not only at the H-1 region, 4.75 to 5.60 ppm (designated region I), but also at the H-2 to H-6 region, 3.30 to 4.40 ppm (Fig. 3). In region I of the four mannan subfractions, signals corresponding to the 1-*O*-phosphorylated α-mannopyranose unit, 5.50 to 5.60 ppm (designated region I'), and β-1,2-linked mannopyranose unit, 4.75 to 5.00 ppm, a part of which is overlapped by that of the α-1,6-linked mannopyranose unit, 4.90 ppm (46), significantly increased in the order Fr.B-2→Fr.B-3→Fr.B-4→Fr.B-5. In addition to the above changes, the region from 4.15 to 4.40 ppm corresponding to the H-2 protons of β-1,2-linked mannopyranose units (designated region II) of

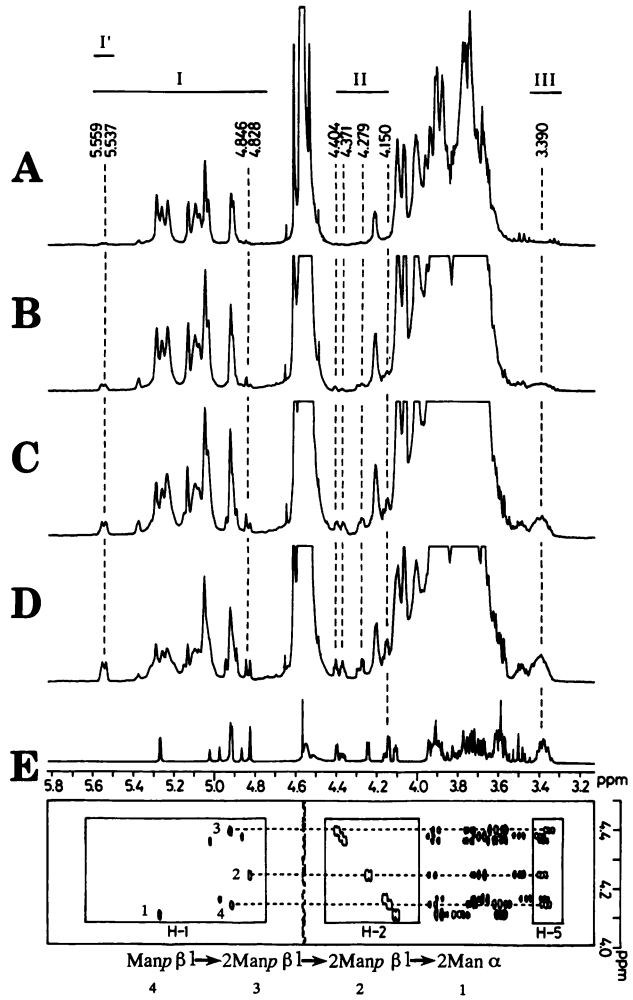

FIG. 3. ^{1}H NMR spectra of mannan subfractions and β-1,2-linked mannotetraose. 1D ^{1}H NMR of Fr.B-2 (A), Fr.B-3 (B), Fr.B-4 (C), and Fr.B-5 (D) and 2D-HOHAHA spectrum of β-1,2-linked mannotetraose (E), Manpβ1→2Manpβ1→2Manpβ1→2Man, obtained from Fr.B by mild acid treatment (41) were recorded with a JEOL JNM-GSX 400 spectrometer in D_2O at 45°C. In panel E, the cross-peaks of H-1 to H-6 of β-mannopyranose unit are connected with horizontal dotted lines.

these mannan subfractions also increased proportionally to the phosphate content. Furthermore, an increase in the signal at 3.30 to 3.45 ppm corresponding to H-5 protons of the β-1,2-linked mannopyranose unit (designated region III) was evident. It is therefore possible to state that the phosphate-bound β-1,2-linked oligomannosyl residues are able to manifest characteristic chemical shifts by participation not only with H-1 protons but also with H-2 and H-5 protons, and that this finding can be utilized in the qualitative and quantitative determination of β-1,2-linked mannopyranose units in these *C. albicans* mannans.

Figure 3E shows a 2D-HOHAHA spectrum of β-1,2-linked mannotetraose, corresponding to the major oligomannosyl residue of acid-labile side chains (19, 39, 40, 42) (Fig. 1). The existence of cross-peaks at 3.30 to 3.45 ppm (region III) on all mannopyranose units (i.e., Man-2, Man-3, and Man-4) except the α-anomeric reducing terminal mannose unit (Man-1) provides substantiating evidence for the assignment of the β-1,2-linked mannopyranose unit in the four mannan subfractions by ^{1}H NMR spectroscopy. Therefore, the percentage of β-1,2-linked mannopyranose unit in the parent mannan or each mannan subfraction can be calculated by the following formula:

$$\beta\text{-1,2-linked mannopyranose unit (\%)} = (III \times 100)/I \quad (1)$$

where *I* and *III* indicate the dimensions of the signals at 4.75 to 5.60 ppm and at 3.30 to 3.45 ppm, respectively.

As shown in Table 1, the percentages of β-1,2-linked mannopyranose unit calculated from the dimension of the ^{1}H NMR signal in the four mannan subfractions, Fr.B-2 to Fr.B-5, are consistent with those obtained from the elution pattern of the acid degradation product determined by the phenol-sulfuric acid method (8). Furthermore, the signals at 4.25 to 4.40 ppm, the other characteristic ones of β-1,2-linked oligomannosyl residues, were also shown to correspond to region H-2 of the intermediary β-1,2-linked mannopyranose unit as demonstrated by Shibata et al. (39).

ELISA of mannan subfractions with factor sera. It is well known that the serotype B cells undergo agglutination by factor sera 1, 4, 5, and 13b. Therefore, an investigation of the reactivities between these factor sera and the four mannan subfractions on a microtiter plate was performed as previously described by Tojo et al. (48). As shown in Fig. 4A, there are small differences among the four mannan subfractions in their reactivities against factor serum 1. On the other hand, the intensity of the reactivities of these mannan subfractions against factor sera 4 and 5 significantly increased in the order Fr.B-2→Fr.B-3→Fr.B-4→Fr.B-5. However, factor serum 13b did not show reactivity (data not shown). It was also shown by Tojo et al. (48) that the adhering behavior of mannans to a plastic surface was dominated by the hydrophobic property of the peptide moiety of the mannan. In other words, the amounts of mannan subfractions that adsorb to the plastic plate may vary depending on protein content of the subfraction. Therefore, we used polystyrene plates coated with ConA for a sandwich ELISA to test the reactivity of factor sera, because this plate adsorbed the same amount of mannans regardless of the protein content, as reported by Tojo et al. (49). It is noteworthy that the use of goat anti-rabbit IgG alkaline phosphatase conjugate instead of the corresponding HRPO conjugate as the antibody-linked enzyme gave a satisfactory result. As shown in Fig. 4B, the reactivities of factor serum 1 to the four mannan subfractions were identical. However, a significant increase in the reactivities of these mannan subfractions (from Fr.B-2 to Fr.B-5) with both factor sera 4 and 5 was observed in the order Fr.B-2→Fr.B-3→Fr.B-4→Fr.B-5. The increase in the reactivity is proportional to the augmentation of the peak dimension of the signal at region III in the ^{1}H NMR spectra of Fr.B-2 to Fr.B-5, indicating that both factor sera 4 and 5 are capable of recognizing the β-1,2-linked oligomannosyl residues.

2D-HOHAHA spectroscopy of Fr.B and its acid treatment product, Fr.B-a. To provide evidence for the structural requirement corresponding to factor sera 4 and 5, the serotype B mannan, Fr.B, and its acid treatment product, Fr.B-a, were examined by 2D-HOHAHA spectroscopy. As shown in Fig. 5A, 2D-HOHAHA spectroscopy of Fr.B gave approximately 10 H-1-to-H-2 correlated cross-peaks of β-1,2-linked mannopyranose units, in which H-5 protons give rise to distinct signals at region III (Fig. 5A, cross-peaks connected with dotted lines). Namely, the H-1 protons of β-1,2-linked mannopyranose units give signals not only at around 4.90 ppm, corresponding to an unsubstituted α-1,6-

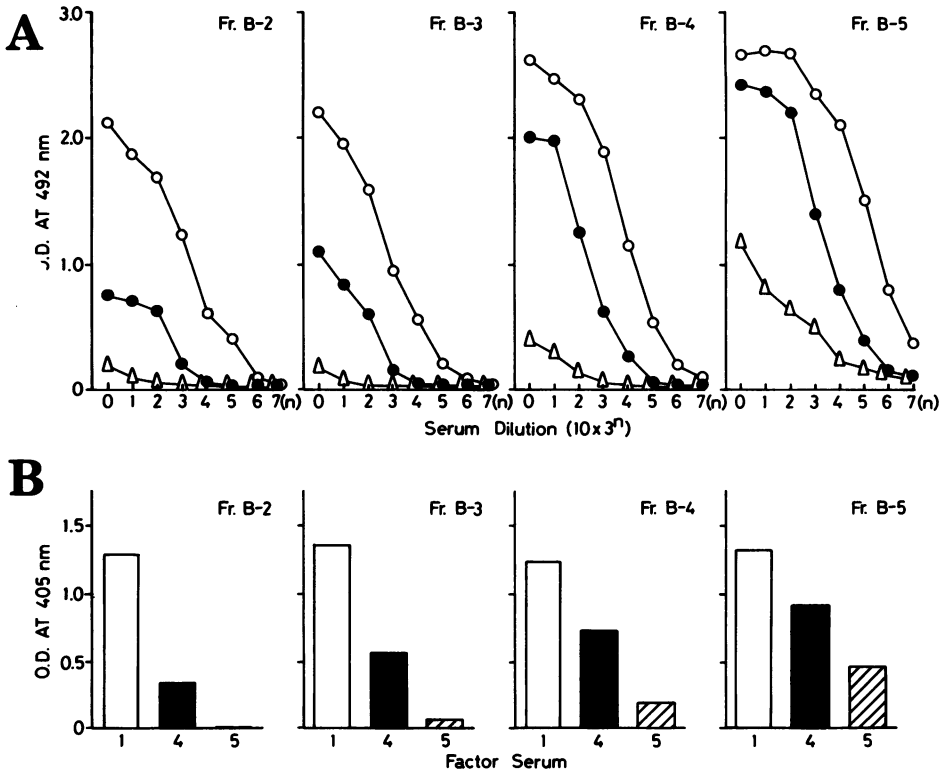

FIG. 4. ELISA of serotype B mannan subfractions with factor sera of Candida Check. (A) Conventional ELISA with anti-rabbit IgG HRPO conjugate antibody as the second antibody. (B) Sandwich ELISA with ConA and anti-rabbit IgG alkaline phosphatase antibody as the second antibody. Fr.B-2, Fr.B-3, Fr.B-4, and Fr.B-5 were the subfractions of *C. albicans* NIH B-792 (serotype B) strain mannan, Fr.B, prepared by DEAE-Sephadex chromatography as described in the legend to Fig. 2. Factor sera 1 (○), 4 (●), and 5 (△) were used separately as the first antibody.

linked mannopyranose unit, but also at 4.80 to 5.05 ppm. The 1-*O*-phosphorylated mannopyranose unit with an H-1-to-H-2 cross-peak at region I′ gave no cross-peak at region III. This result provides substantiating evidence that the anomeric linkage between phosphate and β-1,2-linked oligomannosyl residues must have an α configuration. On the other hand, Fr.B-a gave cross-peaks only of α-linked mannopyranose units, and the signals corresponding to region H-1 of β-1,2-linked mannopyranose units in region I and to region H-5 in region III completely disappeared after the mild acid treatment (Fig. 5B).

ELISA of Fr.B, Fr.B-a, and Fr.S with factor sera 1, 4, and 5. As shown in Fig. 6A, the mannan without β-1,2-linkage, Fr.B-a, did not react with factor serum 5 and slightly reacted with factor serum 4. The reactivities of Fr.S, the structure of which closely resembles that of Fr.B-a with respect to the lack of β-1,2-linked oligomannosyl residues (as demonstrated in a previous article [46]), were nearly identical to those of Fr.B-a with these factor sera (Fig. 6A). The sandwich ELISA with ConA gave similar results (Fig. 6B). This finding indicates that both factor sera 4 and 5 contain antibodies capable of recognizing the β-1,2-linked oligomannosyl residues and that the former serum involves an antibody (or antibodies) corresponding to an unidentified epitope(s) consisting of an α-linked mannopyranose unit(s) because of its positive reactivity in ELISA with Fr.B-a and Fr.S lacking β-1,2-linked oligomannosyl residues.

2D-HOHAHA spectroscopy of Fr.J and its acid modification product, Fr.J-a. As shown in Fig. 7, it is obvious that Fr.J, the serotype A mannan, has two kinds of β-1,2-linked oligomannosyl residues, i.e., one of which is being connected to the mannan via an acid-labile phosphodiester linkage as the common structure for the mannans of serotype A and B strains, and the other one of which occupies the nonreducing terminal sites of the side chains of the acid-stable region (28). The three H-1-to-H-2 cross-peaks connected with the boxed H-5 signals by dotted lines in Fig. 7B indicate the presence of serotype A-specific side chains containing β-1,2 linkage as reported previously (20, 22, 38). The total amount of β-1,2-linked mannopyranose units (acid-eliminable and acid-stable ones) in any serotype A mannan can also be determined from the corresponding ^{1}H NMR spectrum by formula 1. However, determination of the β-1,2-linked mannopyranose units in the phosphodiesterified oligomannosyl residue (acid-eliminable one) of serotype A mannan cannot be achieved by this procedure because of the inclusion of all chemical shifts of β-1,2-linked mannopyranose units; therefore, the following formula should be adopted for this purpose:

$$\text{Acid-eliminable } \beta\text{-1,2-linked mannopyranose unit (\%)} = (III - I'') \times 100/I \quad (2)$$

where I'' indicates the dimensions of the signals at 4.75 to 4.87 ppm corresponding to the amount of β-1,2-linked mannopyranose units in the serotype A-specific structures.

The peak dimensions of regions I, I′, I″, and III were 100, 2.9, 15, and 22, respectively. Therefore, the total amount of β-1,2-linked mannopyranose units can be estimated to be 22% by using formula 1. On the other hand, the amounts of the acid-eliminable and acid-stable β-1,2-linked mannopyra-

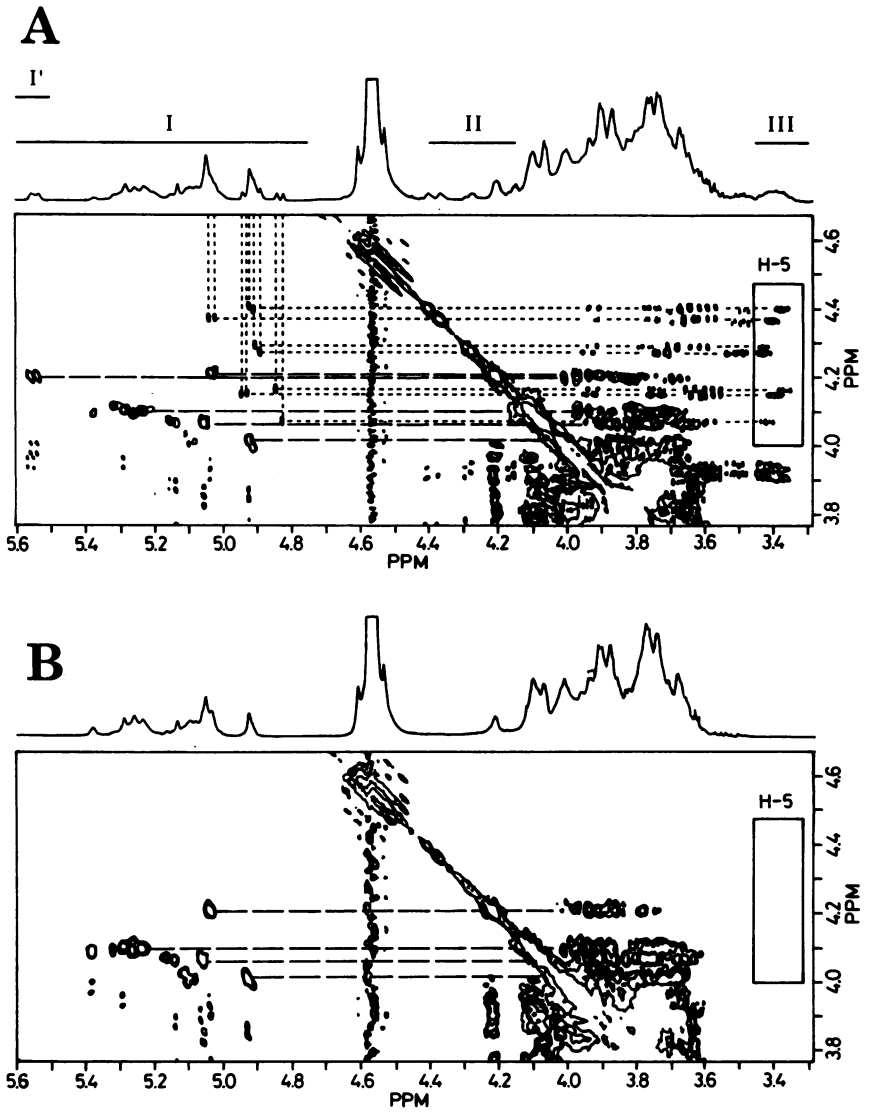

FIG. 5. 2D-HOHAHA spectra of *C. albicans* NIH B-792 (serotype B) mannan, Fr.B (A), and its acid modification (10 mM HCl at 100°C for 60 min) product, Fr.B-a (B). The dotted and dashed lines indicate the cross-peaks of β-1,2-linked and α-linked mannopyranose units, respectively.

nose units were calculated to be 7 and 15%, respectively, by formula 2. Although the amount of acid-eliminable β-1,2-linked mannopyranose units in Fr.J is identical to that of oligosaccharides released by mild acid treatment (20), the amount of acid-stable ones is ca. 20% larger than that estimated from the result of mild acetolysis (20), i.e., the mild acetolysis did not retain β-1,2 linkage quantitatively and cleaved ca. 20% of β-1,2 linkage, giving rise to mannose.

ELISA of Fr.J and Fr.J-a with factor sera 1, 4, 5, and 6. The reactivity of serotype A mannan Fr.J to these four factor sera determined by conventional ELISA was closely consistent with that determined by agglutination reaction of J-strain cells (Fig. 8A) (47). The acid-treated mannan, Fr.J-a, which lost phosphate-bound β-1,2-linked oligomannosyl residues, also strongly reacted with factor sera 1, 4, and 6, while the reactivity against factor serum 5 considerably decreased. It is therefore possible to state that factor serum 5 contains at least two kinds of antibodies, one of which recognizes relatively longer β-1,2-linked oligomannosyl residues and one of which reacts with relatively shorter ones. Because the latter oligomannosyl residues are also present at the nonreducing terminal sites in serotype A-specific epitopes (20, 38), it is presumable that the presence of antibodies against these oligomannosyl residues in factor serum 5 is responsible for the Fr.J-a reactivity remaining against this serum.

DISCUSSION

Several articles have been published by Fukazawa and coworkers on the structural identification of antigenic factors 8, 10, 18, and 18a as the side chains in the mannan of *Saccharomyces cerevisiae* (14, 29). On the other hand, these workers demonstrated that *C. albicans* cells possessed antigenic factors 1, 4, 5, and 6 for serotype A strains and antigenic factors 1, 4, and 5 for serotype B strains. Additionally, cells of both serotypes were shown to express antigenic

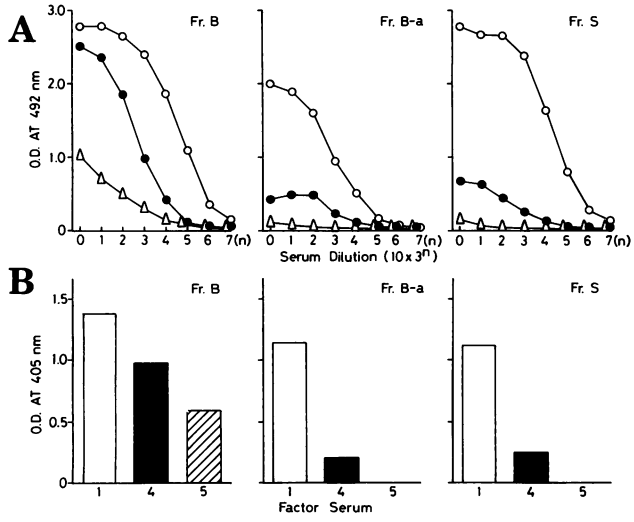

FIG. 6. ELISA of serotype B mannans with factor sera of Candida Check. (A) Conventional ELISA with anti-rabbit IgG HRPO conjugate antibody as the second antibody. (B) Sandwich ELISA with ConA and anti-rabbit IgG alkaline phosphatase antibody as the second antibody. Symbols of factor sera are the same as those described in the legend to Fig. 4. Fr.B and Fr.B-a were the intact mannan of *C. albicans* NIH B-792 (serotype B) and its acid modification product, respectively. Fraction S was a mannan of *C. stellatoidea* IFO 1397 (corresponding to serotype B of *C. albicans* species) and has no acid-labile β-1,2-linked oligomannosyl residues (49). Therefore, Fr.B-a and Fr.S have closely related chemical structures.

factor 13b (15). However, the chemical structures of these antigenic factors were reported to be composed only of α-linked mannopyranose units, and no mention was made of the presence of an antigenic factor containing β-1,2-linked mannopyranose units, although its presence in the mannans of *C. albicans* was suggested by Zhang and Ballou (53) in 1982. Thereafter, it has been reported by Shibata et al. (38) and Kobayashi et al. (20) that the chemical structures of serotype A-specific epitopes (antigenic factor 6) show a correspondence of mannopentaose and mannohexaose side chains with one and two β-1,2-linked mannopyranose units on their nonreducing terminal sites, respectively. However, the chemical structure of neither antigenic factor 1 nor antigenic factor 5 is yet known.

The first description of the presence of acid-labile phosphodiesterified β-1,2-linked oligomannosyl residues in the mannan of *Candida* species was made by Shibata et al. (40). Because H-1 signals of the manno-oligosaccharides released by mild acid treatment are complicated, the assignment was achieved by a combination of COSY and NOESY, providing the results that these oligosaccharides have a distorted conformation (39). It was demonstrated by H,C-COSY that the H-1 signals of β-1,2-linked mannopyranose units overlap those of unsubstituted α-1,6-linked mannopyranose units (46), since the quantitation of the amount of phosphodiesterified β-1,2-linked oligomannosyl residues was not possible by determining chemical shifts in the H-1 region by one-dimensional NMR (1D NMR). In the present study, we revealed the usefulness of H-5 signals of the β-1,2-linked mannopyranose unit as the key chemical shift for estimating the amount of β-1,2-linked mannopyranose unit in *Candida* mannans by 1D NMR. The signals at 4.77 and 4.83 ppm on region I of the ^{1}H NMR spectra of serotype A mannan directly indicate the presence of a β-1,2-linked mannopyranose unit (20, 38). For serotype B mannan that contains β-1,2-linked oligomannosyl residues in only phosphodiesterified form (21, 42), however, the determination by 1D NMR of H-5 signals of these units in region III is the only way to provide evidence for the existence of the β-1,2-linked mannopyranose unit, because H-1 protons of β-1,2- and α-1,6-linked mannopyranose units give intensively overlapping chemical shifts ranging from 4.80 to 5.05 ppm. Therefore, the method documented in the present work for determining the β-1,2-linked mannopyranose unit, either in acid-labile phosphodiesterified form (common structure in mannans of serotypes A and B) or in acid-stable form (serotype A-specific structure), by 1D NMR separately with formulae 1 and 2 seems to be useful for estimating the amounts and ratio of the major antigenic factors, 5 and 6.

The reactivities of factor sera 4 and 5 against the four mannan subfractions increased in the order Fr.B-2→Fr.B-3→Fr.B-4→Fr.B-5 in a manner proportional to the amount of phosphate-bound β-1,2-linked oligomannosyl residues. The reactivity of factor serum 1, which recognizes common branching moieties in the mannans of *S. cerevisiae* and *C. albicans* species, Manpα1→2Manpα1→2Man (41), also increased considerably in the order Fr.B-2→Fr.B-3→Fr.B-4→Fr.B-5. Because the protein moiety of a mannan-protein complex is the dominant factor in its ability to adhere to plastic plates (48), it is presumable that mannan subfractions with different protein contents are not adsorbed on plastic plates to the same degree, thus causing different reactivities of factor serum 1 to these mannan subfractions. To ensure that the plate was coated with the same amounts of mannan subfractions, the use of a plate precoated with ConA was preferred (49). HRPO, which has been widely used in ELISAs, is a glycoprotein with a high-mannose type of sugar moiety reactive with ConA. On the basis of this fact, the alkaline phosphatase-conjugated anti-rabbit antibody was used as a second antibody instead of HRPO to avoid nonspecific binding of the second antibody to ConA through its high-mannose type of sugar moiety. The result of the sandwich ELISA indicates that the reactivities of the factor serum 1 to four mannan subfractions were exactly the same, but those of factor sera 4 and 5 exhibited proportionally increasing reactivities to the amounts of the β-1,2-linked mannopyranose unit. Tojo et al. (47) revealed that the agglutination specificity of anti-β-1,2-linked oligomannosyl monoclonal antibodies to several *Candida* species is almost the same as that of factor serum 5. This observation is consistent with the results obtained in the present study. Furthermore, it is evident that the polyclonal factor serum 4 contains some anti-α-linked oligomannosyl antibodies in addition to anti-β-1,2-linked oligomannosyl antibodies, because factor serum 4 is reactive to Fr.B-a and Fr.S, which lack β-1,2-linked oligomannosyl residues. Kagaya et al. (17) reported the preparation of several monoclonal antibodies against factor 4 epitope and proposed a hypothetical model of the structures of factor 4 epitope consisting of α-1,6- and α1,2-linked mannopyranose units. Factor serum 5 was prepared by absorbing an anti-*C. albicans* whole-cell serum with the whole cells of *C. guilliermondii* (50, 51). Although the chemical structure of cell wall mannan of any *C. guilliermondii* species is not yet known, ^{1}H NMR data of the mannan of *C. guilliermondii* IFO 0566 clearly indicated the absence of a signal corresponding to the phosphodiesterified β-1,2-linked oligomannosyl residue in region I′ (41). Therefore, the use of whole cells of *C. guilliermondii* in absorption makes it possible to retain large amounts of anti-β-1,2-linked

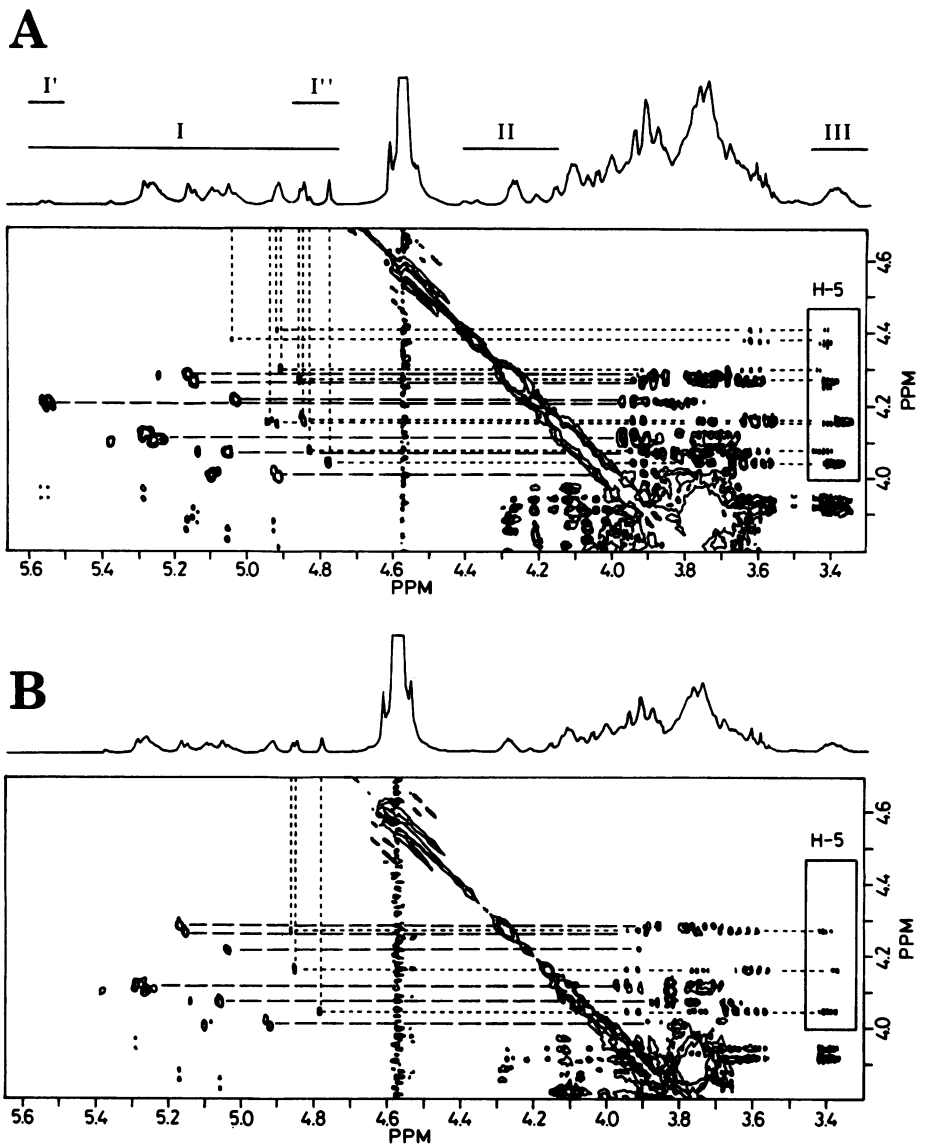

FIG. 7. 2D-HOHAHA spectra of *C. albicans* J-1012 (serotype A) mannans. Intact mannan, Fr.J (A), and its acid modification (10 mM HCl at 100°C for 60 min) product, Fr.J-a (B), were dissolved in D_2O, and 2D-HOHAHA spectra were recorded at 45°C. The dotted and dashed lines indicate the cross-peaks of β-1,2-linked and α-linked mannopyranose units, respectively.

oligomannosyl antibodies. The fact that Fr.J-a entirely lost the acid-labile β-1,2-linked oligomannosyl residue still reactive with factor serum 5 suggests that a part of the antibody in this serum can recognize the nonreducing terminal β-1,2-linked oligomannosyl residues on the acid-stable region. Namely, factor serum 5 is reactive with the nonreducing terminal side of factor 6 epitope, Man*p*β1→2Man*p*β1→ and/or Man*p*β1→2Man*p*β1→2Man*p*β1→, in addition to those of phosphodiesterified β-1,2-linked oligomannosyl residues, although factor serum 6 is able to recognize epitopes containing both α and β linkages, Man*p*β1→2Man*p*α1→ 2Man*p*α1→ and Man*p*β1→2Man*p*β1→2Man*p*α1→2Man*p*α1→ (22). The possible recognition sites of factor sera 5 and 6 are depicted in Fig. 9.

The agglutination reaction of yeast cells with a combination of factor sera is a well established method for the rapid identification of the pathogenic *Candida* species. Although there is a report on the application of factor serum to flow cytometric analysis of *Candida* cell wall antigen (5), the present article includes the first description of the use of factor sera in ELISA and sandwich ELISA using a plastic plate coated with ConA. Because both methods are very sensitive, giving rise to satisfactorily quantitative results, the analysis of the antigenic structures of the mannans of *C. albicans* is made possible by using a commercially available kit of factor sera. The fact that the β-1,2-linked oligomannosyl residues can be regarded as one of the specific epitopes, the presence of which has not been shown in mammals, is quite favorable for determining the mannan antigens in sera of patients for the diagnosis of candidiasis (9, 11, 23, 45).

Many reports have been published on the suppressive effects of the mannans of *C. albicans* and its catabolites (4, 6, 10, 12, 16, 27, 28, 32–35) on the immune system, and the mechanisms of several of them have been proposed as

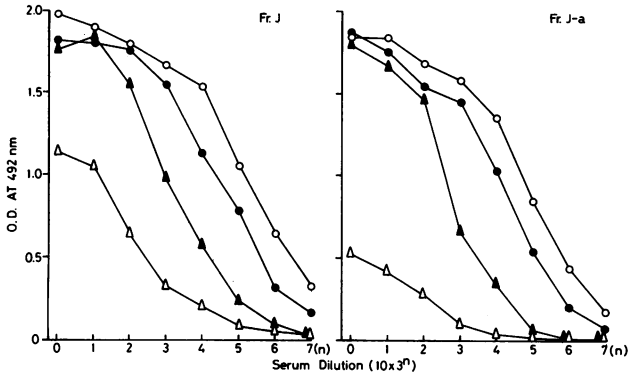

FIG. 8. ELISA of *C. albicans* J-1012 (serotype A) mannans with factor sera of Candida Check. Fr.J is an intact mannan, and Fr.J-a is a modified mannan, the acid-labile β-1,2-linked oligomannosyl residues of which were removed by the acid treatment. ▲, factor serum 6. Other symbols of factor sera are the same as those described in the legend to Fig. 4.

follows. (i) The homing receptor, an adhesion receptor on leukocytes to attach venular endothelial cells located adjacent to sites of infection, binds the phosphomannan monoester core of *Hansenula holstii* and mannose-6-phosphate (18, 52). Since phosphomannan of *C. albicans* also contains mannose-6-phosphate group (42), it is reasonable to expect that binding of *C. albicans* mannan to the homing receptor affects the function of these cells. (ii) A lectinlike property of tumor necrosis factor alpha (36), interleukin-1 (3), and interleukin-2 (37) was found to bind with uromodulin, an 85-kDa mannose-containing immunosuppressive glycoprotein (25, 26) obtained from pregnancy urine. Interleukin-2 especially is known to bind to ovalbumin, a high-mannose type of glycoprotein, and to *S. cerevisiae* mannan and has 27% homology with the carbohydrate-binding domain of human mannan-binding protein (37). It is, therefore, likely that the mechanism of the immunosuppressive effect of *C. albicans* mannan includes a binding to some cytokines with a lectinlike property which inhibits their immunomodulatory activities. (iii) *C. albicans* mannan and its catabolites were shown to induce suppressor T lymphocytes through the production of prostaglandin E_2 (35). This effect might be influenced by structural modification of the mannans (7), such as the removal of phosphodiesterified β-1,2-linked mannopyranose units, the amounts of which can be estimated by integration of the ^1H NMR signals of region III of mannan as described in this article. Furthermore, the results of the ELISA of mannan with factor sera 5 and 6 also show the amount of β-1,2 linkage-containing branching moieties. The development of a quantitative determination procedure of side chains participating in such biological effects might promote the analytical studies of immunomodulatory mechanisms of *C. albicans* mannans.

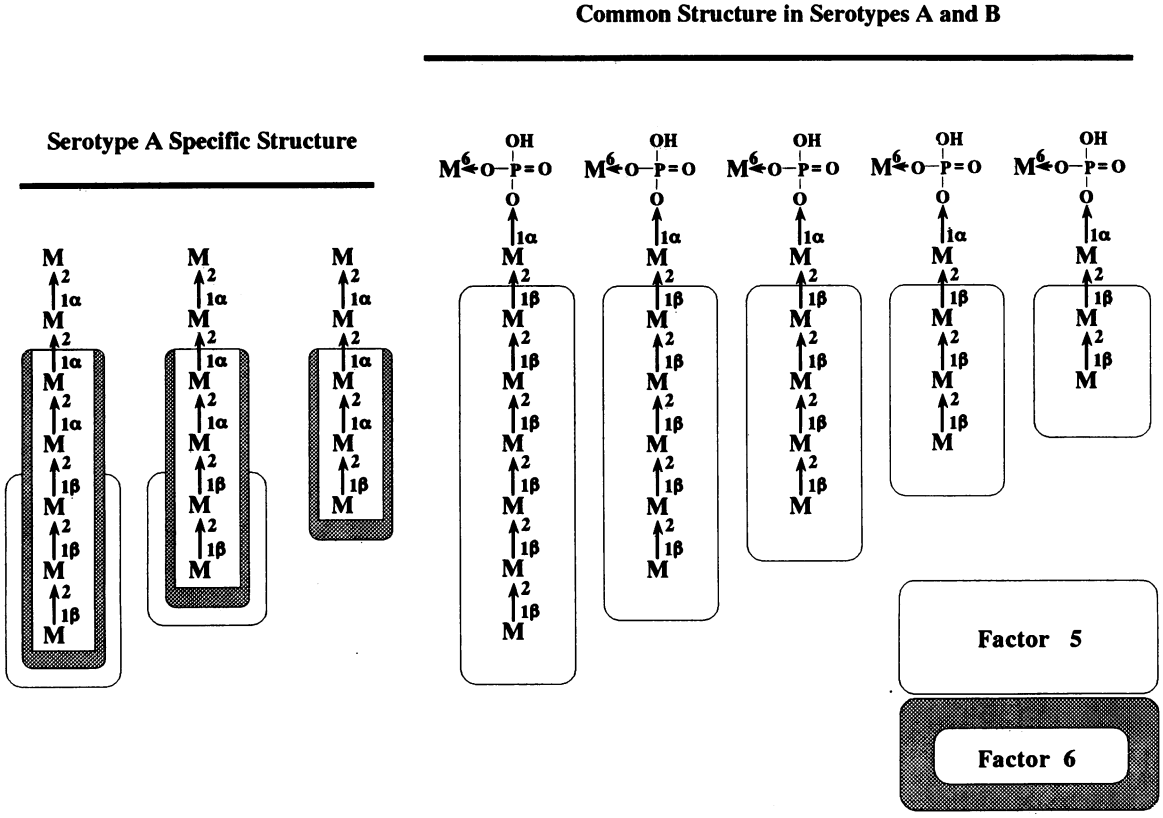

FIG. 9. Diagrammatic representation of the epitopes of antigenic factors 5 and 6 in *C. albicans* mannans. The antigenic factor 6 corresponds to a serotype A-specific side chain with one, two, and three β-1,2-linked mannopyranose units at the nonreducing terminal of α-1,2-linked mannotetraose side chains. On the other hand, the antigenic factor 5 corresponds to both the phosphodiesterified acid-labile β-1,2-linked oligomannosyl residues, the common structure for serotype A and B mannans, and the acid-stable β-1,2-linked oligomannosyl residues at the nonreducing terminal site of the serotype A-specific side chains and does not include any α-1,2-linked mannopyranose unit.

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