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Structure of Cell Wall Mannan of *Candida kefyr* IFO 0586

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We conducted a structural analysis of the antigenic cell wall mannoprotein (mannan) isolated from *Candida kefyr* (formerly *Candida pseudotropicalis*) IFO 0586. The result of two-dimensional homonuclear Hartmann-Hahn analysis of this mannan indicates that the molecule is constructed from α-1,2- and α-1,6-linked mannopyranose residues. Upon alkali treatment (β-elimination reaction), this mannan released two α-1,2-linked mannooligosaccharides, biose and triose. The structure of the alkali-stable mannan (outer chain) moiety was investigated by acetolysis. The structures of the resultant oligosaccharides, biose and triose, from the outer chain moiety were found to be the same as those of the alkali-released ones. Futher, the treatment of the parent mannan with an *Arthrobacter* GJM-1 exo-α-mannosidase gave a linear mannan consisting solely of α-1,6-linked mannopyranose residues. These results indicate that the mannan forms the long backbone of the α-1,6 linkage, with a large number of short α-1,2-linked oligomannosyl side chains forming a comblike structure. Moreover, we investigated the serological properties of this mannan by performing an inhibition assay of a slide agglutination reaction with mannooligosaccharides and polyclonal factor sera (Candida Check; Iatron). The result indicates that the factor 1 serum preferentially recognizes the α-1,2-linked oligomannosyl side chains in this mannan. On the other hand, the fact that the mannan does not contain an antigenic determinant(s) corresponding to factor 8 suggests that the epitope(s) of this factor resides in other molecules on the cell surface of this strain.

We have reported the structural determination of antigenic cell wall mannoprotein (mannan) obtained from several *Candida* species: *Candida albicans* (42), *C. stellatoidea* (22), *C. tropicalis* (12), and *C. glabrata* (13). We demonstrated that these mannans have two types of β-1,2-linkage-containing oligomannosyl side chains corresponding to *C. albicans* serotype A-specific epitopes (factor 6) (19) and the common epitopes throughout *C. albicans* serotypes A and B (factor 5) (36). Marquis et al. (28) revealed that factor 4 is located in a sugar-rich cell wall layer of *C. albicans*. Recently, Ataoglu et al. (2) reported that the antigenic determinants of *Candida* species corresponding to factors 1 and 9 are the O-linked sugar chains and the linear mannan consisting solely of the α-1,6-linked mannopyranose unit, respectively, on the basis of the results of an enzyme-linked immunosorbent assay (ELISA) with cell wall extracts obtained from *C. albicans* and *Saccharomyces cerevisiae mnn* mutants. In our previous studies (11, 17, 21), we have demonstrated that the mannans of *C. albicans* serotype A and *C. tropicalis* cells grown in yeast extract-Sabouraud liquid medium under acidic conditions possessed significantly modified structures, i.e., an increase in the number of α-linked mannopyranose residues with a concomitant decrease in the number of β-1,2-linked mannopyranose residues in the side chains. Additionally, Shibata et al. (37, 39) and Okawa et al. (34) reported that the mannans of *C. albicans* cells cultured at high temperature (37°C) showed a similar structural change. Thus, we expect that these modifications in linkage distribution would result in a significant alteration of the serological properties of the mannan.

The fact that β-1,2 linkage-containing oligomannosyl side chains can be regarded as one of the specific epitopes of the parent cells seems to favor the serologic determination of mannan antigens in the serum of patients with candidiasis (5, 6). The β-1,2 linkage-containing oligomannosyl side chains corresponding to factors 5 and 6 are now considered to be involved in the adherence of *C. albicans* cells to mouse spleen marginal zone macrophages (26) and human epithelial cells (25, 30), respectively. Nelson and his coworkers (32, 35) reported that the alkali-released oligosaccharides obtained from *C. albicans* mannan were potent inhibitors of lymphoproliferation induced by the parent mannan. This is of interest from the viewpoint of host-parasite interactions.

C. kefyr is one of the medically important pathogenic yeasts in humans, and its serological properties have been investigated by Fukazawa et al. (7). These workers stated that the presence of the cell surface epitopes corresponding to factors 1 and 8 was evident and that α-1,2-linked oligomannosyl side chains in the cell wall mannan were in large part responsible for specificity of factor 8. Although Fukazawa et al. (7) and Kogan et al. (24) have published the partial structure of the mannan of this species on the basis of methylation and nuclear magnetic resonance (NMR) analyses and/or acetolysis, its overall structure has not been reported. Therefore, we conducted the structural and immunochemical characterization of the mannan isolated from the yeast form cells of *C. kefyr* IFO 0586.

MATERIALS AND METHODS

General. *C. kefyr* IFO 0586 (abbreviated as K strain) was obtained from the Institute for Fermentation Osaka, Osaka, Japan. The yeast form cells of K strain were cultivated in yeast extract-Sabouraud liquid medium at 27°C for 72 h on a reciprocal shaker. Mannans of the *S. cerevisiae* 4484-24D-1

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TABLE 1. Chemical compositions and specific rotations of Fr K, Fr K-b, and Fr K-e

Mannan fraction	Total carbohydrate (%)	Total protein (%)	Total phosphate (%)	$[\alpha]_D^{25}$ (degrees)	Yield (%)[a]
K	94.6	2.2	0	+64.4	
K-b	93.9	2.8	0	+60.4	92.8
K-e	94.3	3.7	0	+68.8	41.2

[a] Fr K weight basis.

mnn1 mutant (lacking a nonreducing terminal α-1,3-linked mannopyranose residue of side chains in the outer chain moiety) and the *S. cerevisiae* X2180-1A-5 *mnn2* mutant (lacking all side chains composed of α-1,2- and α-1,3-linked mannopyranose residues in the same moiety) (3) were the same specimens as those used in a previous study (20). Polyclonal rabbit anti-*Candida* factor sera (PFAbs), (Candida Check; lot R156; Iatron, Tokyo, Japan) corresponding to antigenic factors 1, 4, 5, 6, 8, 9, 11, 13, 13b, and 34, as defined by Fukazawa et al. (8), were used. An *Arthrobacter* GJM-1 exo-α-mannosidase (9) was the same specimen as that used in previous studies (15). The column-packing material for gel filtration chromatography, Bio-Gel P-2 (400 mesh), with a fractionation range of 100 to 1,800 Da, was obtained from Bio-Rad, Richmond, Calif. The α-1,2-linked mannooligosaccharides biose and triose, obtained from *C. albicans* mannans by acetolysis (15, 16), were used as standards for determination of eluted positions in gel filtrations.

Preparation of mannan. Mannan was extracted with hot water and precipitated by short-term treatment with Fehling solution (15). The mannan fraction obtained from the cells of K strain was designated as Fr K. The yields of cell extract and purified Fr K were 23.5 and 5.8%, respectively, on an acetone-dried cell weight basis.

Treatment of Fr K with alkali (β-elimination). Fr K was treated with alkali as described by Shibata et al. (38). Briefly, 200 mg of Fr K was dissolved in 30 ml of 100 mM NaOH, and the solution was kept at 25°C for 18 h. Then the solution was neutralized with 1 M HCl, concentrated to a small volume, applied to a column (2.5 by 100 cm) of Bio-Gel P-2, and eluted with water (0.25 ml/min). The alkali-modified Fr K eluted in the void volume (Fr K-b). The yield of Fr K-b was 92.8% on an Fr K weight basis.

Treatment of Fr K with *Arthrobacter* GJM-1 exo-α-mannosidase. Fr K was treated with *Arthrobacter* GJM-1 exo-α-mannosidase as described previously (16). Briefly, 20 mg of Fr K was dissolved in 5 ml of 0.1 M phosphate buffer (pH 6.8) and 1 mg of exo-α-mannosidase was added. The solution was kept at 37°C for 18 h, concentrated, and applied to a Bio-Gel P-2 column under the same conditions as those for the treatment

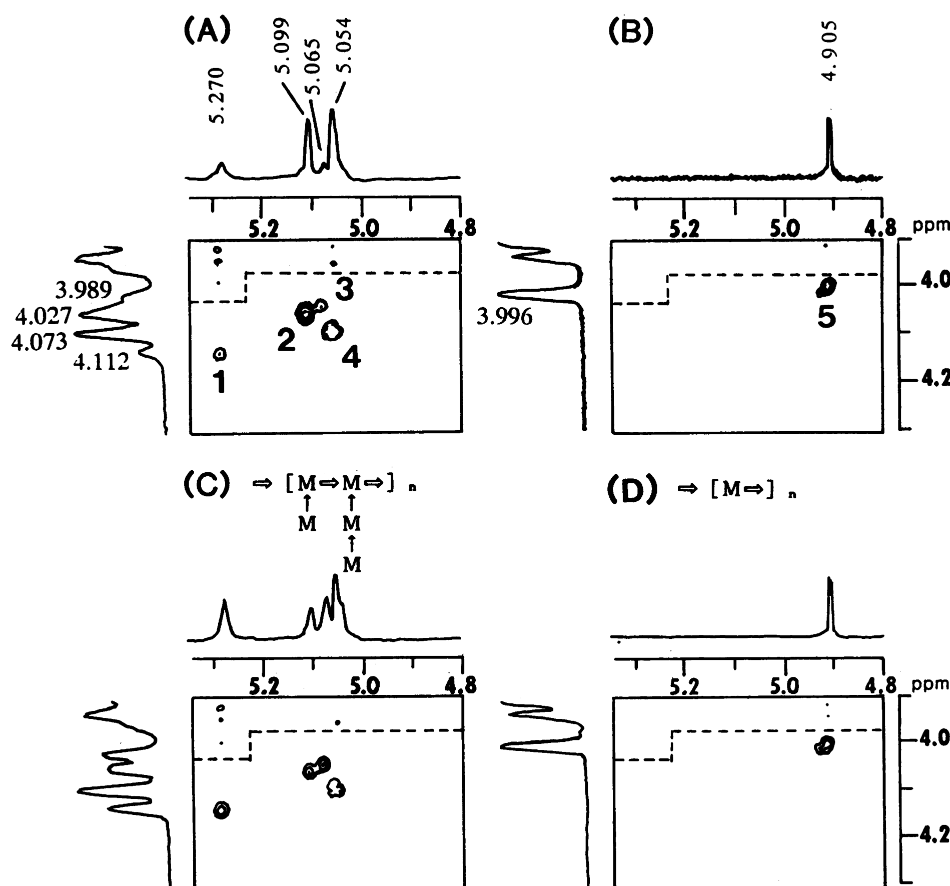

FIG. 1. 2D-HOHAHA (H1-H2 region) spectra of Fr K (A), Fr K-e (B), *S. cerevisiae* 4484-24D-1 (*mnn1* mutant type) mannan (C), and *S. cerevisiae* X2180-1A-5 (*mnn2* mutant type) mannan (D). The presence of intraresidue H1-H2 cross-peaks for mannopyranose residues is shown below the dashed line. M denotes a D-mannopyranose residue. Thin arrows (→) and open arrows (⇒) indicate α-1,2 and α-1,6 linkages, respectively.

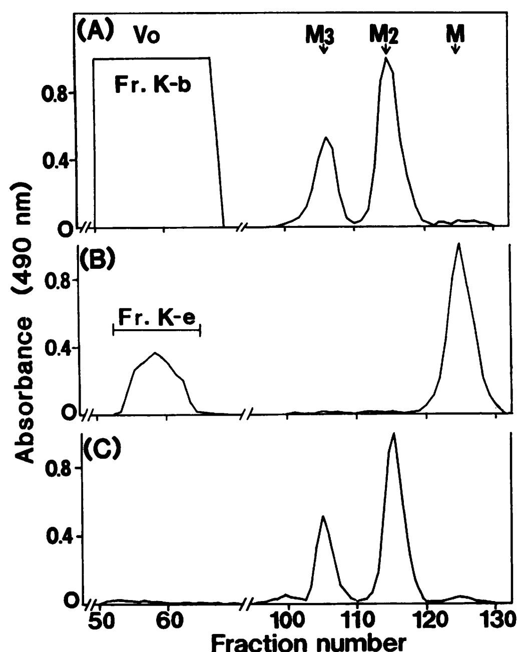

FIG. 2. Elution plofiles of degradation products obtained from Fr K by alkali treatment (β-elimination) (A) and *Arthrobacter* GJM-1 exo-α-mannosidase treatment (B) and obtained from Fr K-b by acetolysis (C). The carbohydrate content of each eluate was measured by the phenol-H_2SO_4 method (4). M_3, M_2, and M indicate mannotriose, mannobiose, and mannose, respectively. Vo refers to the void-volume region.

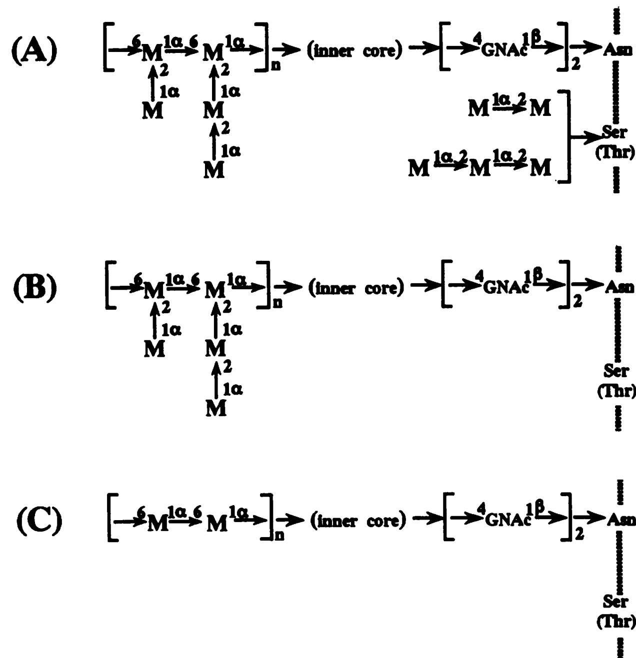

FIG. 3. Possible structure of the mannans Fr K (A), Fr K-b (B), and Fr K-e (C). M and GNAc indicate D-mannopyranose and 2-acetamido-2-deoxy-D-glucopyranose residues, respectively. The side chain sequence is not specified.

of Fr K with alkali. The enzyme-modified Fr K that eluted in the void volume was designated as Fr K-e. The yield of Fr K-e was 41.2% on an Fr K weight basis.

Acetolysis of Fr K-b. Fr K-b (100 mg) was acetolyzed by a modification (14) of the method of Kocourek and Ballou (23). A 10:10:1 (vol/vol/vol) mixture of $(CH_3CO)_2O$, CH_3COOH, and H_2SO_4 was used for the preferential cleavage of the α-1,6-linkage in Fr K-b. The resultant oligosaccharides were fractionated on a column of Bio-Gel P-2 under the same conditions as those for the alkali treatment of Fr K. The branching-frequency value of mannan was calculated by using the following formula in accordance with the previous descriptions (20): percent branching frequency = $(B + C) \times 100/(A + B + C)$, where A, B, and C represent the molar proportions of mannose (M), biose (M_2), and triose (M_3), respectively, in the gel filtration profile of the acetolysates of Fr K-b.

Slide agglutination of *C. kefyr* cells with PFAbs and its inhibition assay with mannooligosaccharides. Slide agglutination of K strain cells (10^8/100 μl of saline) with 100 μl of each PFAb (Candida Check) was done by a modification (19) of the method of Miyakawa et al. (29). The inhibition assay was conducted by a modification (19) of the method of Nishikawa et al. (33) as follows. Each PFAb was preincubated for 2 h at 37°C in the presence of inhibitor oligosaccharide solution (100 μl). This solution (200 μl) and heat-killed K strain cells (10^8/100 μl of saline) were mixed, incubated at 37°C for 2 h, and kept overnight at 4°C. The inhibitor oligosaccharides were obtained from Fr K-b and Fr K-e by acetolysis under conventional and mild conditions (18), respectively; each sample was purified by rechromatography on a column of Bio-Gel P-2.

ELISA of Fr K. ELISA of Fr K was conducted as described by Shibata et al. (36), with PFAbs 1, 8, and 6 (negative control). Mannan solution (0.1 mg/ml) in 50 mM carbonate buffer (pH 9.6) was placed in the wells of a polystyrene microtiter plate, which was kept at ambient temperature overnight, and then washed three times with phosphate-buffered saline solution containing 0.1% (by volume) 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) was diluted from 10- to 10,000-fold with PBST. Afterwards, a solution of goat anti-rabbit immunoglobulin G-horseradish peroxidase 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-rabbit immunoglobulin G 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 100 μl of 150 mM citrate buffer (pH 5.0) was added to each well, and the mixture was left at room temperature for 20 min. After the addition of 2 M H_2SO_4 (50 μl) to each well, the color in the plate was measured at 492 nm by a Micro Plate Reader type A4 (Tosoh, Tokyo, Japan).

Other methods. ^1H NMR spectra were recorded as previously described (16) on a JEOL JNM-GSX 400 spectrometer in D_2O at 70°C with acetone as an internal standard (2.217 ppm). Two-dimensional homonuclear Hartmann-Hahn (2D-HOHAHA) spectra were recorded on the same spectrometer in D_2O at 45°C with acetone as an internal standard (2.217 ppm) in accordance with the description by Shibata et al. (36). Total carbohydrate was determined by the phenol-H_2SO_4 method (4) with D-mannose as a standard. Total protein was determined by the Folin method of Lowry et al. (27) with bovine serum albumin (Sigma, St. Louis, Mo.) as a standard. Total phosphate was determined by the method of Ames and Dubin (1) with KH_2PO_4 as a standard. Specific rotations were

TABLE 2. Assignment of chemical shifts of ^{1}H NMR spectra of oligosaccharides obtained from *C. kefyr* mannan by alkali treatment (β-elimination) and acetolysis

Oligosaccharide	Sugar residue C B A	Chemical shift (ppm) C	B	A
Alkali released				
M_2-b	Man*p*α1-2Man(α)[a]		5.049	5.352
	Man*p*α1-2Man(β)		5.142	4.894
	Man*p*α1-2Glc(α)[b]		5.024	5.426
				5.435
	Man*p*α1-2Glc(β)[b]		5.250	4.711
				4.731
M_3-b	Man*p*α1-2Man*p*α1-2Man(α)	5.051	5.268	5.336
	Man*p*α1-2Man*p*α1-2Man(β)	5.051	5.268	4.890
	Man*p*α1-2Man*p*α1-2Glc(α)[b]	5.034	5.225	5.432
				5.437
	Man*p*α1-2Man*p*α1-2Glc(β)[b]	5.034	5.405	4.712
			5.412	4.733
Acetolysis released				
M_2-ac	Man*p*α1-2Man(α)		5.049	5.352
	Man*p*α1-2Man(β)		5.142	4.894
M_3-ac	Man*p*α1-2Man*p*α1-2Man(α)	5.051	5.268	5.336
	Man*p*α1-2Man*p*α1-2Man(β)	5.051	5.268	4.890

[a] Parenthetical letters denote anomers.
[b] These oligosaccharides were by-products of use of the diluted alkali solution (100 mM NaOH).

measured by means of a JAS DIP-360 digital polarimeter, after a 3-h dissolution of each sample (1%, wt/vol) in water.

RESULTS

Analysis of Fr K. The result of chemical analyses of Fr K is given in Table 1. Fr K has a large amount of carbohydrate (94.6%) and a small amounts of protein (2.1%). Figure 1A shows a partial 2D-HOHAHA spectrum (H1-H2 region) of Fr K. The chemical shifts of the H1-H2 cross-peaks, 1 to 4, correspond to the chemical shifts observed in *S. cerevisiae* 4484-24D-1 (*mnn1* mutant type) α-mannan except for the differences in peak intensities (Fig. 1A and C). The highly positive specific rotation of Fr K, +64.4^0, supports the following interpretation for the structure of Fr K: unlike other *Candida* mannans (12, 13, 22, 42), Fr K contains neither phosphate nor a β-1,2-linked mannopyranose residue as part of its structure (Table 1).

TABLE 3. Slide agglutination assay for whole cells of *C. kefyr* with PFAbs

Strain	Agglutination[a] with PFAb: 1	4	5	6	8	9	11	13	13b	34
C. kefyr IFO 0586	++	–	–	–	±	–	–	–	–	–
C. albicans NIH A-207[b] (serotype A)	++	++	+	+	–	–	–	–	–	–
C. albicans NIH B-792[b] (serotype B)	++	++	+	–	–	–	–	–	±	–

[a] Agglutination was scored from high (++) to low (±) and no agglutination (–).
[b] The agglutination reactions of *C. albicans* NIH A-207 (serotype A) and NIH B-792 (serotype B) are shown for comparative purposes.

Alkali treatment (β-elimination) of Fr K. Oligosaccharides corresponding to O-linked sugar chains were obtained by treating Fr K with alkali (β-elimination). As shown in Fig. 2A, the presence of alkali-labile oligosaccharides, biose (M_2-b) and triose (M_3-b), was evident. Yields of M_2-b and M_3b were 1.1 and 0.7%, respectively, on weight basis with respect to the parent mannan, Fr K. These oligosaccharides were identified as α-1,2-linked mannooligosaccharides, Man*p*α1-2Man and Man*p*α1-2Man*p*α1-2Man, by ^{1}H NMR analysis (Table 2) (these spectra are not shown). In this analysis, small amounts of epimerization products, Man*p*α1-2Glc and Man*p*α1-2Man*p*α1-2Glc, were detected as by-products in accordance with the previous description (14).

Exo-α-mannosidase treatment of Fr K. Fr K was digested with *Arthrobacter* GJM-1 exo-α-mannosidase (Fig. 2B). This treatment released D-mannose (57.7%) from Fr K. An enzyme-resistant mannan, Fr K-e, eluted in the void volume (41.2% on

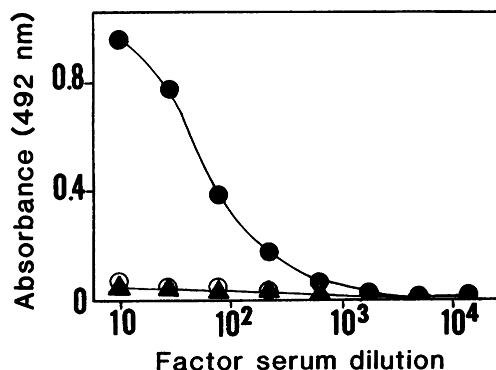

FIG. 4. ELISA of the *C. kefyr* mannan Fr K with PFAb 1 (●) and PFAb 8 (▲) and with PFAb 6 (○) as a negative control.

TABLE 4. Inhibition of agglutination of *C. kefyr* cells with PFAbs 1 and 8 by mannooligosaccharides obtained from Fr K-b and Fr K-e

Inhibitor[a]	Agglutination[b] with amt (μmol) of inhibitor:							
	2^1	2^0	2^{-1}	2^{-2}	2^{-3}	2^{-4}	2^{-5}	0
With PFAb 1								
Man	++	++	++	++	++	++	++	++
Man*p*α1-2Man	−	−	±	+	++	++	++	++
Man*p*α1-2Man*p*α1-2Man	−	−	−	±	+	++	++	++
Man*p*α1-6Man	++	++	++	++	++	++	++	++
Man*p*α1-6Man*p*α1-6Man	++	++	++	++	++	++	++	++
Man*p*α1-6Man*p*α1-6Man*p*α1-6Man	++	++	++	++	++	++	++	++
With PFAb 8								
Man	±	±	±	±	±	±	±	±
Man*p*α1-2Man	±	±	±	±	±	±	±	±
Man*p*α1-2Man*p*α1-2Man	±	±	±	±	±	±	±	±
Man*p*α1-6Man	±	±	±	±	±	±	±	±
Man*p*α1-6Man*p*α1-6Man	±	±	±	±	±	±	±	±
Man*p*α1-6Man*p*α1-6Man*p*α1-6Man	±	±	±	±	±	±	±	±

[a] α-1,2-Linked mannooligosaccharides were obtained from Fr K-b by acetolysis, and α-1,6-linked mannooligosaccharides were obtained from Fr K-e by mild acetolysis.

[b] Agglutination was scored from high (++) to low (±) and no agglutination (−).

an Fr K weight basis). The results of chemical analyses of Fr K-e indicate an increase in the protein content accompanying the digestion of the sugar moiety of Fr K (Table 1). Figure 1B shows the 2D-HOHAHA spectrum of Fr K-e. The presence of cross-peak 5 in this spectrum is identical to the spectrum observed in the *S. cerevisiae* X2180-1A-5 (*mnn2* mutant type) mannan (Fig. 1D). This result provides evidence that Fr K-e is a linear polymer consisting solely of α-1,6-linked mannopyranose residues corresponding to the backbone of the parent mannan, Fr K. Therefore, Fr K possesses a comblike structure comprising a long α-1,6-linked backbone substituted with many short side chains composed of α-1,2-linked mannopyranose residues.

Acetolysis of Fr K-b. Fr K-b was subjected to acetolysis to obtain the oligosaccharides corresponding to the side chains. The acetolysates were fractionated on a column of Bio-Gel P-2 (Fig. 2C). The small amount of mannose released indicates an extremely high density of side chains of Fr K-b, expressed as the high branching frequency, 98%, calculated from the peak areas of the elution profile of acetolysates. The yields of the resultant oligosaccharides, biose (M_2-ac) and triose (M_3-ac), were 53.9 and 24.2%, respectively, on an Fr K-b weight basis. M_2-ac and M_3-ac were identified as Man*p*α1-2Man and Man*p*α1-2Man*p*α1-2Man, respectively, possessing the same structures as those of oligosaccharides M_2-b and M_3-b, resulting from Fr K by β-elimination, as shown by the ^1H NMR analysis based on the previous finding (16) (Table 2, spectra not shown). Therefore, the overall structures of Fr K, Fr K-b, and Fr K-e are as shown in Fig. 3A, B, and C, respectively.

Immunochemical reactivities of *C. kefyr* cells and Fr K. Table 3 shows the result of the slide agglutination reaction of K strain cells with PFAbs (Candida Check). The cells agglutinated with PFAbs 1 and 8 in an identical manner to that for the same strain in the study by Fukazawa et al. (7). However, the agglutination of the cells with PFAb 8 was significantly weaker than with PFAb 1. Therefore, we conducted the ELISA of purified mannan Fr K with PFAbs 1, 8, and 6 (negative control) (Fig. 4). The result suggests that PFAb 1 can recognize Fr K but PFAb 8 cannot do so. Additionally, the ELISA of the *S. cerevisiae mnn1* mutant mannan gave a similar curve to that of Fr K (data not shown). As shown in Table 4, to identify the antigenic determinants in this mannan corresponding to factors 1 and 8, we also performed an inhibition assay of agglutination with oligosaccharides obtained from Fr K-b and Fr K-e. These results clearly indicate that PFAb 1 was unable to recognize the mannan backbone consisting solely of α-1,6-linked mannopyranose residues, whereas it recognized α-1,2-linked oligomannosyl side chains. The fact that the antigenic determinant(s) corresponding to factor 8 could not be found in these experiments indicates the factor 8 epitope may reside in other cell surface molecules of K strain, e.g., glycans other than the mannan, protein, and lipid.

DISCUSSION

The chemical structure proposed for the mannan of *C. kefyr* IFO 0586 is depicted in Fig. 3A. Fr K possesses a backbone consisting solely of α-1,6-linked mannopyranose residues and large numbers of short side chains consisting of α-1,2-linked mannopyranose residues. It lacks β-1,2- and α-1,3-linked mannopyranose residues and phosphate groups. Moreover, the O-linked sugar chains attached to the peptidic moiety corresponding to biose and triose were also composed of α-1,2-linked mannopyranose residues. Thus, the chemical structure of *C. kefyr* mannan is similar to that of *S. cerevisiae* 4484-24D-1 (*mnn1* mutant type) strain. This mannan, Fr K, consists of an α-1,6-linked polymannosyl backbone and α-1,2-linked mannobiosyl side chains commonly found in the mannans of *S. cerevisiae* (20, 31), *S. kluyveri* (40), and *C. glabrata* (13) and is essentially different from that of mannans of three other *Candida* species, *C. albicans* (42), *C. stellatoidea* (22), and *C. tropicalis* (12), which possess α-1,2-linked mannotriosyl side chains.

Ataoglu et al. (2) concluded from the result of ELISA with *C. albicans* cell wall extracts that one of the epitopes recognized by PFAb 1 was the O-glycosidically linked sugar chain of cell wall mannoprotein. In the present study, we have demonstrated that all side chains of *C. kefyr* mannan were composed of α-1,2-linked mannopyranose residues and that PFAb 1 preferentially recognized these side chains. In other words, the most common epitope among the medically important *Candida* species, antigenic factor 1, corresponding to α-1,2-linked oligomannosyl side chains, exists not only in the outer chain

but also in O-linked side chains in the peptide moiety of the parent cell wall mannoprotein.

Fukazawa et al. (7) reported that the *C. kefyr*-specific epitope, antigenic factor 8, corresponded to the α-1,2-linked oligomannosyl side chains of mannan on the basis of the result of a precipitation-inhibition assay on the homologous system of mannan-anti-*C. kefyr* whole-cell serum (not PFAb 8) with the mannooligosaccharides obtained from the parent mannan. However, our result of the agglutination inhibition assay with various mannooligosaccharides indicates that the mannan does not contain the epitope(s) corresponding to factor 8 and that PFAb 8 recognizes another cell surface molecule(s) than the sugar moiety of mannoprotein (e.g., glucan, protein, and lipid) on the slide agglutination assay of *C. kefyr* cells.

Recently, Kanbe and Cutler (10) reported that the acid-stable moiety of *C. albicans* serotype B mannan, which is composed of α-linked oligomannosyl side chains, is in large part responsible for yeast cell binding to the splenic marginal-zone macrophage. Stratford (41) stressed the importance of α-linked oligomannosyl side chains of cell wall mannan in the mechanism of several types of yeast flocculations. Therefore, it seems appropriate to conclude that the structural simplicity of *C. kefyr* mannan and the easy modifications of this mannan can be used to elucidate the cell-cell interaction mechanisms such as the above phenomena.

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