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Determination of α-1,3-Linked Mannose Residue in the Cell Wall Mannan of *Candida tropicalis* NBRC 1400 Strain

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Abstract

To investigate the chemical structure of cell wall mannan obtained from pathogenic yeast, *Candida tropicalis* NBRC 1400 (former antigenic standard strain, IFO 1400). As a result of two-dimensional NMR analysis, it was shown that the mannan of this strain is composed of α-1,6-, α-1,3-, α-1,2- and β-1,2-linked mannose residues. In this research, the mannan was subjected to three degradation procedures, acid-treatment, α-mannosidase, and acetolysis under two conditions in order to determine the chemical structure of the antigenic oligomannosyl side chains in this molecule. The ^{1}H-nuclear magnetic resonance spectra of resultant oligosaccharides, pentaose and hexaose, demonstrated the existence of the oligomannosyl side chains corresponding to Manα1-3Manα1-2Manα1-2Manα1-2Man and Manα1-3Manα1-2Manα1-2Manα1-2Manα1-2Man, respectively, which have previously also been found in *Candida albicans* serotype A strain mannans. These findings indicate that *C. tropicalis* and *C. albicans* serotype A have no significant difference in the chemical structure of these cell wall mannans. Therefore, it can be interpreted that it is extremely difficult to distinguish both species by targeting the antigenic group in these mannans.

Keywords

Candida tropicalis, Pathogenic Yeast, Cell Wall Mannan, Antigenic Oligomannosyl Side Chain, Acetolysis, α-1,3-Linked Mannose Residue

1. Introduction

Mannan present in the yeast cell wall is mainly composed of mannose residues, which forms a complex with protein (mannoprotein) and/or phospholipid (phospholipomannan) [1] [2] [3] [4]. Since these molecules are located in the outermost layer of the cell wall, it is well-known to function as major antigenic determinants [1] [2]. For this reason, structural and immunochemical studies on the cell wall mannans of pathogenic *Candida* species have been actively conducted since ancient times [5] [6] [7].

In order to identify clinical isolates from patients with candidiasis, Tsuchiya and his coworkers developed ten rabbit antibodies to antigenic factors of the genus *Candida* (abbreviated as FAbs) [8] [9] [10]. These FAbs recognize the antigenic determinants in cell wall mannan [11]. We reported the structure of cell wall mannans of several *Candida* species, including *C. tropicalis* [12], *C. albicans* [13] [14], *C. glabrata* [15], *C. guilliermondii* [16], and *C. krusei* [17]. Moreover, we revealed that the determinants of antigenic factors 5, 6, and 9 correspond to the oligomannosyl side chains consisting of a homologous β-1,2-linked series [18], of β-1,2 and α-1,2-linked mannose residues [19], and of β-1,2 and α-1,3-linked mannose residues [20] [21], respectively. On the other hand, the determinants of *Candida* factors 1, 4, 13b, and 34 are α-linked oligomannosyl side chains, a linear homologous α-1,2-linked series [12], a 3,6-branched series [22], an internal α-1,3-linkage-containing linear series [22] [23], respectively.

Kobayashi *et al.* [12] showed that cells of *C. tropicalis* IFO 0199, IFO 0589, IFO 1400 and IFO 1647 are clearly aggregated with FAbs 1, 4, 5, and 6, and they reported that it the same as those of *C. albicans* serotype A (J-1012 strain) cells. Then, they selected two strains with the clearest immunochemical reactivity, IFO 0199 and IFO 1647, and performed a detailed structural analysis. As a result, it was revealed that mannans derived from these two strains were composed of α-1,2, α-1,6 and β-1,2-linked mannose residues. After a while, the results of two-dimensional NMR analysis of several *C. tropicalis* mannans indicated that the α-1,3-linked mannose residues could be found as the fourth bond form in the mannan of IFO 1400 strain [24].

We elucidated the location of α-1,3-linked mannose residues in *C. tropicalis* NBRC 1400 (formerly IFO 1400) strain and then mentioned the immunochemically relationship of *C. tropicalis* against *C. albicans* serotype A. Therefore, the results obtained will lead to a final judgment as to the possibility of constructing an immunochemical approach to distinguish *C. tropicalis* and *C. albicans* serotype A.

2. Materials and Methods

2.1. General

Candida tropicalis NBRC 1400 strain was obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan. This strain was maintained on Sabouraud agar slants. Jack bean α-mannosidase (EC 3.2.1.24)

was obtained from Sigma Chemical Co. (St. Louis, Mo.). Column packing for gel filtration chromatography (Bio-Gel P-2; 400 mesh), with a fractionation range of 100 to 1800 Da, was obtained from Bio-Rad (Richmond, Calif.).

2.2. Cultivation of *C. tropicalis* and Preparation of Mannan

Cultivation of *C. tropicalis* NBRC 1400 and preparation of mannan were performed as described for *C. albicans* J-1012 [25]. This strain was cultivated in Sabouraud liquid medium at 27˚C for 72 h on a reciprocal shaker. Preparation of mannan was conducted by a combination of hot-water extraction and Fehling solution method [13]. The purified mannan obtained from the cells of the *C. tropicalis* NBRC 1400 was designated Fr 1400.

2.3. Treatment of Fr 1400 with 10 mM HCl

Treatment of Fr 1400 was done as described by Shibata *et al.* [26]. Briefly, mannan was dissolved in 10 mM HCl, and the solution was heated in a boiling water bath for 1 h. The solution was neutralized with 100 mM NaOH concentrated *in vacuo*. The hydrolysate was applied to a column of Bio-Gel P-2 (2.5 by 100 cm) and eluted with water (0.25 ml/min). The acid-modified Fr 1400 was designated Fr 1400-a.

2.4. Conventional Acetolysis of Fr 1400-a

Conventional acetolysis of Fr 1400-a was done by a modification [27] of the method of Kocourek and Ballou [28]. A 10:10:1 (vol/vol/vol) mixture of $(CH_3CO)_2O$, CH_3COOH, and H_2SO_4 was used for the acetolysis. After de-*O*-acetylation, the resultant oligosaccharides were fractionated on a column (2.5 by 100 cm) of Bio-Gel P-2.

2.5. Mild Acetolysis of Fr 1400-a

Mild acetolysis of Fr 1400-a was done with a 100:100:1 (vol/vol/vol) mixture of $(CH_3CO)_2O$, CH_3COOH, and H_2SO_4 as described previously [27]. Separation of the region containing longer-chain oligosaccharides than hexaose was unsatisfactory in the case of mild acetolysis of Fr 1400-a. This was due to the presence of several isomers as judged by observation of the peak shape in the elution profile. This region was further treated with jack bean α-mannosidase to degrade the isomer(s) consisting of α linkages as described below.

2.6. α-Mannosidase Treatment of the Fraction Consisting of Oligosaccharide Isomers with Longer Chains Than Hexaose Obtained from Fr 1400-a by Mild Acetolysis

This treatment was conducted by the method of Shibata *et al.* [29]. Briefly, each longer-chain oligosaccharide fraction was dissolved in 50 mM sodium acetate buffer (pH 4.6), to a concentration of 5 mg/ml, and 10 U of α-mannosidase per ml was added to the solution. After incubation at 37˚C for 48 h, each reaction

mixture was applied to a column (2.5 by 100 cm) of Bio-Gel P-2 and eluted with water.

2.7. Other Methods

Total carbohydrate was determined by the phenol-sulfuric acid method [30] with D-mannose as the standard. Total protein was determined by the Folin method of Lowry *et al.* [31] with bovine serum albumin (Sigma) as the standard. Total phosphate was determined by the method of Ames and Dubin [32] with KH_2PO_4 as the standard. Four-hundred-megahertz ^{1}H-NMR spectrum analyses were conducted exactly as described previously [13] with acetone as the standard (2.217 ppm). Specific rotations were determined by means of a JAS DIP-360 digital polarimeter. The sample was dissolved in water, and measurement was done after 3 h of dissolution of each sample in water.

3. Results

3.1. Isolation of the Mannan Fr 1400 from Cell of NBRC 1400 Strain

As shown in **Table 1**, Fr 1400 was mostly composed of carbohydrates (applox. 90%) and contained a small amount of protein and phosphate groups. The low specific rotation value of mannan, +36.1 degree, indicates existence of β-linked mannose residues in Fr 1400.

3.2. Acid Treatment of Fr 1400

Fr 1400 was treated with 10 mM HCl at 100˚C for 1 h to isolate oligosaccharides linked through phosphate. Each hydrolysate was fractionated on a column of Bio-Gel P-2. As shown in **Figure 1**, acid treatment of Fr 1400 resulted in two oligosaccharides, triose (M3) and tetraose (M4), in amounts of 1.1%, on the basis of parent mannan. The ^{1}H-NMR spectra of two oligosaccharides were apparently identical to those of oligosaccharides isolated from mannans of *Candida* species, which was described previously [13] [14] [19] [32] [33] (H1 signals of oligosaccharides were not shown). Therefore, triose and tetraose were identified as Manβ1-2Manβ1-2Man and Manβ1-2Manβ1-2Manβ1-2Man, respectively, on the basis of the assignment of H1 signals [34] [35] (**Table 2**). The acid-stable fraction, Fr 1400-a, was obtained as the void-volume (Vo) regions in the gel filtration patterns (**Figure 1**).

Table 1. Chemical compositions and specific rotations of Fr 1400.

Fraction	Total Carbohydrate (%)[a]	Total protein (%)[b]	Total phosphate (%)[c]	$[\alpha]_D^{25}$ (degree)[d]	Yield (%)[e]
1400	89.55	4.7	0.39	+36.1	4.67

[a]Determined by the phenol-H_2SO_4 method [30]. [b]Determined by the Folin method of Lowry *et al.* [31]. [c]Determined by the Ames-Dubin method [32] as-KH_2PO_4. [d]1% (wt/vol) solution in water. [e]Weight basis of the acetone-dried whole cells.

Table 2. [1]H chemical shifts (anomeric region) of oligosaccharides (α anomer) obtained from Fr 1400 by acid-treatment (I) and acetolysis (II).

Oligosaccharide[a]	Sugar residu[b] G F E D C B A	Chemical shift (ppm)[c] G	F	E	D	C	B	A
I								
M3	Mβ1-2Mβ1-2M					4.825	4.823	5.264
M4	Mβ1-2Mβ1-2Mβ1-2M				4.910	4.917	4.816	5.272
II								
M2	Mα1-2M						5.049	5.352
M3	Mα1-2Mα1-2M					5.051	5.268	5.336
M4	Mα1-2Mα1-2Mα1-2M				5.051	5.255	5.269	5.338
M5	Mα1-2Mα1-2Mα1-2Mα1-2M			5.053	5.255	5.255	5.268	5.336
	Mα1-3Mα1-2Mα1-2Mα1-2M			5.144	5.041	5.255	5.268	5.336
M6	Mα1-2Mα1-2Mα1-2Mα1-2Mα1-2M		5.054	5.254	5.254	5.254	5.265	5.336
	Mα1-3Mα1-2Mα1-2Mα1-2Mα1-2M		5.144	5.055	5.254	5.254	5.265	5.336
	Mα1-2Mβ1-2Mα1-2Mα1-2Mα1-2M		4.837	4.837	5.143	5.251	5.251	5.333
M7	Mβ1-2Mβ1-2Mβ1-2Mα1-2Mα1-2Mα1-2M	4.838	4.906	4.838	5.143	5.251	5.251	5.335

[a]I, acid-labile oligosaccharide; II, acetolysis-labile oligosaccharide. [b]M denotes a D-mannose unit. [c]Chemical shift was indicated on the basis of a value of acetone (2.217 ppm) as an internal standard [13].

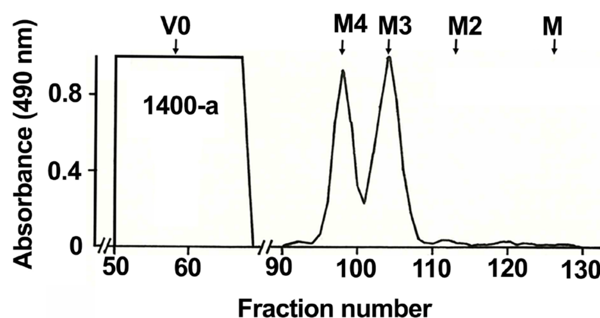

Figure 1. Gel filtration profile of the products obtained from Fr 1400 by treatment with 10 mM HCl at 100˚C for 1h on a column (2.5 by 100 cm) of Bio-Gel P-2 by elution with water at 0.25 ml/min. The carbohydrate in the eluate was determined by the phenol-sulfuric acid method [30]. M, M2, M3, and M4 indicate D-mannose, mannobiose, mannotriose, and mannotetraose, respectively. Vo refers to the void volume.

3.3. ^{1}H-NMR Analysis of Fr 1400 and Fr 1400-a

Figure 2(a) shows the ^{1}H-NMR spectra (H1 region) of Fr 1400, demonstrating that these signals closely resemble those of the mannan of *C. albicans* serotype Astrain [36]. The weak signals, 5.542 and 5.563 ppm, and a strong signal, 4.839 ppm, indicate the presence of phosphate-bound side chains corresponding to *Candida* antigenic factor 5 and the β-1,2-linkage-containing side chain corresponding to *Candida* antigenic factor 6, respectively (**Figure 2(a)**). On the

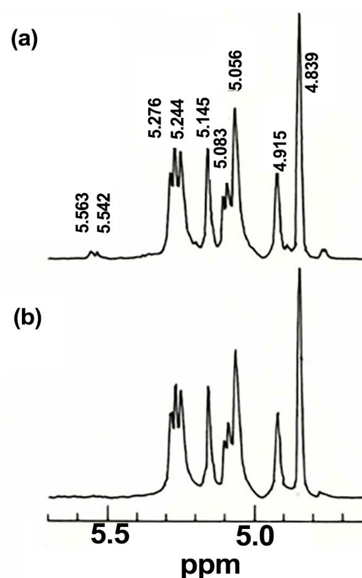

Figure 2. ^{1}H-NMR spectra in the anomeric region (H-1) resonances of parent (a), acid-modified (b) mannans isolated from *C. tropicalis* strain. This analysis was conducted with a JEOL JNM-GSX 400 spectrometer in D_2O at 70˚C with acetone as an internal standard (2.217 ppm).

^{1}H-NMR spectra of Fr 1400-a, loss of the weak signals mentioned above is evidence that the phosphate-bound side chains corresponding to *Candida* antigenic factor 5 were eliminated from each parent mannan by acid treatment (**Figure 2(b)**).

3.4. Acetolysis of Fr 1400-a

To obtain the *α*-linked oligosaccharides corresponding to side chains from the acid-stable domain of Fr 1400-a was at first subjected to conventional acetolysis, and the acetolysate was fractionated on a column of Bio-Gel P-2. The products isolated from this acetolysate were mannose (M), oligosaccharides, biose (M2) to hexaose (M6), and a phosphorylated oligosaccharide(s) eluted in the Vo region (**Figure 3(a)**). On the other hand, to isolate the *β*-1,2-linkage-containing oligosaccharide, Fr 1400-a was acetolysed under mild conditions. The elution pattern of the degradation products by this procedure indicates that a large amount of phosphorylated oligosaccharide(s) was eluted in the Vo region, and this oligosaccharide was followed by the fraction consisting of oligosaccharide isomers with longer chains than hexaose, the oligosaccharides with shorter chains than pentaose (M2 to M5), and mannose (M) (**Figure 3(b)**). The fraction consisting of longer-chain isomers than hexaose was then digested with the *α*-mannosidase, and the products were fractionated by gel filtration chromatography to remove the *α*-linked oligosaccharides from this fraction. Consequently, the *α*-mannosidase-resistant oligosaccharides, hexaose (M6) and heptaose (M7) remained (**Figure 3(c)**).

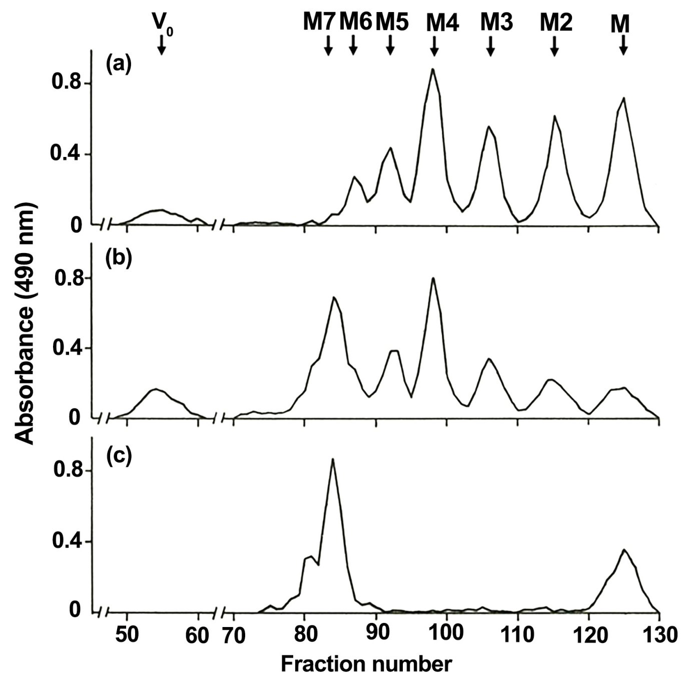

Figure 3. Gel filtration profiles of the products obtained from Fr 1400-a by acetolysis by using the same column and conditions as described in the legend to **Figure 1**. (a) Fr 1400-a acetolyzed under conventional conditions; (b) Fr 1400-a acetolyzed under mild conditions; (c) enzymolysis product with a jack bean exo-α-mannosidase obtained from the fraction illustrated panel B in corresponding to oligosaccharide isomers with longer chains than hexaose. M5, M6 and M7 indicate mannopentaose, mannohexaose, and mannoheptaose respectively. Other symbols are the same as those described in the legend to **Figure 1**.

3.5. ^{1}H-NMR Analysis of Oligosaccharides Obtained from Fr 1400-a by Acetolysis

All oligosaccharides were analyzed by ^{1}H-NMR (**Figure 4** and **Table 2**). The lower oligosaccharides, tetraose, triose, and biose were identified as Manα1-2Manα1-2Manα1-2Man, Manα1-2Manα1-2Man, and Manα1-2Man, respectively (signals are not shown), by correlation with data in the literature [13] [37] [38]. However, higher oligosaccharides, hexaose and pentaose, contain nonreducing terminal α-1,3-linked mannose units, because of the deposition of strong signals at 5.149 ppm; therefore, these oligosaccharides identified as Manα1-3Manα1-2Manα1-2Manα1-2Manα1-2Man, Manα1-3Manα1-2Manα1-2Manα1-2Man, respectively (**Figure 4(a)** and **Table 2**). The H1 signals of M6 and M7 obtained from Fr 1400-a by mild acetolysis followed by α-mannosidase revealed that these oligosaccharides were identified as Manβ1-2Manβ1-2Manα1-2Manα1-2Manα1-2Man and Manβ1-2Manβ1-2Manβ1-2Manα1-2Manα1-2Manα1-2Man, respectively, on the basis of assignment of the same oligosaccharides isolated from *C. albicans* serotype A and *C. stellatoidea* type II strains [13] [19] [36] (**Figure 4(b)** and **Table 2**). Additionally, the signals of the phosphorylated oligosaccharide(s) fraction

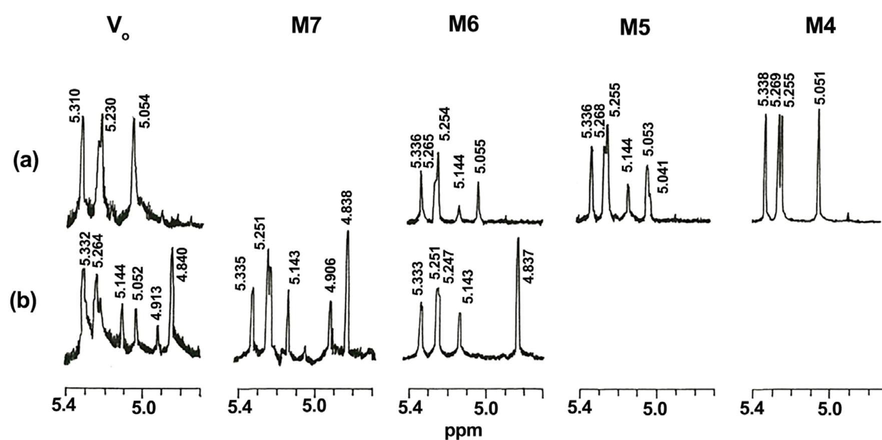

Figure 4. ^{1}H-NMR spectra in the anomeric region (H-1) resonances of oligosaccharides obtained from Fr 1400-a by conventional acetolysis (a) and by mild acetolysis followed by enzymolysis with exo-*α*-mannosidase (b). This analysis was conducted by using the same spectrometer and conditions described in the legend to **Figure 2**.

(Vo) obtained by acetolysis under two conditions indicated that the sugar moiety of this fraction was composed of both *α*-1,2- and *β*-1,2-linked mannose units (**Figure 4(a)** and **Figure 4(b)**).

4. Discussion

Previous report [12] has been shown that the mannans obtained from two typical *C. tropicalis* strains, IFO 0199 and IFO 1647, were constructed by the mannose residues with three forms, *α*-1,2-, *β*-1,2-, and *α*-1,6-linkages, and the phosphate group. However, later it has revealed the presence of a small amount of *α*-1,3 linked mannose residue in *C. tropicalis* NBRC 1400 (formerly IFO 1400) strain [24]. In this study, it is cleared that *α*-1,3 linked mannose residues exist in the oligomannnosyl side chains corresponding to pentaose and hexasaose, respectively, in this mannan molecule. Therefore, we propose that the overall structure of *C. tropicalis* NBRC 1400 strain mannan is as shown in **Figure 5**.

On the other hand, Okawa *et al.* [39] reported differences in lethal activity against mice and sucrose-utilization ability among *C. tropicalis* strains including IFO 1400 strain, and the IFO 0589 strain shows to be significantly weaker in both activities compared to the other strains. Additionally, in taxonomic gene analysis such as measurement of purine base content of DNA, the gene homology of *C. tropicalis* IFO 0589 is remarkably different from those of other strains [40]. In recent years, as a result of comprehensively judging these findings, the *C. tropicalis* IFO 0589 strain has been conserved in National Institute of Technology and Evaluation Biotechnology Centeras *Candida viswanathii* NBRC 0589 strain (as of November, 2019) [41]. Moreover, although the *α*-1,2-linked mannose residue is not detected in the mannan obtained from *C. tropicalis* IFO 1647 cells cultured at pH 5.9, this residue is clearly found in the mannan prepared from same cells grown at pH 3.0 [42]. Therefore, as in the case of *C. albicans* serotype A [25] [43] [44], the chemical structure of the cell wall mannan of *C.*

Molar ratio

26.1 : 15.0 : 12.8 : 20.2 : 5.9 : 3.1 : 4.3 : 0.9 : 8.4 : 2.2 : 0.4 : 0.7

Figure 5. Proposed structures for the cell wall mannan of *C. tropicalis* NBRC 1400 strain. Side chain distribution was calculated based on the peak-dimensions in the gel-filtration profile of the mild acetolysis products. M and GNAc denote D-mannopyranose and 2-acetamido-2-deoxy-D-glucopyranose units, respectively. The side-chain sequence is not specified.

tropicalis also seems to be changed according to growth conditions such as a hydrogen ion concentration and/or temperature.

5. Conclusion

Selective degradation procedures for several polysaccharides are useful for the preparation of the oligosaccharides corresponding to as haptens of immunochemical or biological function. The results of acetolysis against cell wall mannan purified from *C. tropicalis* NBRC 1400 strain showing typical antigenic activity demonstrated the presence of side chains containing non-reducing terminal α-1,3 linked mannose residues in this molecule. The presence or absence of an oligomannosyl side chain containing an α-1,3-linkage residue was thought to be an important point in distinguishing *C. albicans* serotype A from *C. tropicalis*. However, the finding in this study indicates that there is no significant difference in the chemical structure of cell wall mannans between *C. albicans* serotype A and *C. tropicalis*. Therefore, it can conclude that it is extremely difficult to develop a species identification method targeting antigenic groups in these mannans.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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