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Isolation of Mannooligosaccharides Corresponding to Antigenic Determinants of Pathogenic Yeast *Candida catenulata* Cell Wall Mannan

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ABSTRACT

To investigate the chemical structure of cell wall mannan of pathogenic yeast, *Candida catenulata* IFO 0745 strain, which possess the epitopes of antigenic factors 1, 9, and 34 to genus *Candida*, we previously performed the two-dimensional nuclear magnetic resonance (NMR) analysis of this mannan, Fr. C, without the need for harsh procedures. In this study, three oligosaccharides, biose, triose, and tetraose, and mannose were isolated from Fr. C by acetolysis. The results of NMR analysis indicate that the chemical structures of these oligosaccharides were identified to Manα1-2Man, Manα1-2Manα1-2Man, and Manα1-3Manα1-2Manα1-2Man. The most of resultant mannose seems to be originated from the α-1,6-linked mannan backbone which is recognized by antiserum to factor 9. The inhibition assay of slide agglutination reaction between Fr. C and antigenic antibodies using three oligosaccharides indicate that the Manα1-2Manα1-2Man and Manα1-3Manα1-2Manα1-2Man possess domains corresponding to immunodominants of antigenic factors 1 and 34, respectively.

Keywords: Cell Wall Mannan; Antigenic Factor; *Candida catenulata*; Acetolysis; Oligomannosidic Epitope

1. Introduction

Ten rabbit antibodies to antigenic factors of genus *Candida* (abbreviated as FAbs) were developed to identify clinical isolates from the patients with candidiasis by Fukazawa *et al*. [1] and Tsuchiya *et al*. [2,3]. We have reported the structure of cell wall mannans of genus *Candida*, for examples, *C. albicans* [4,5], *C. tropicalis* [6], *C. guilliermondii* [7], *C. glabrata* [8], and *C. lusitaniae* [9]. The determinants of antigenic factors 1, 9, and 34 to genus *Candida* were linear α-1,2-linked oligomannosyl side chains [10], linear backbone consisting of α-1,6 linkage [11,12], and linear oligomannosyl side chains containing a non-reducing terminal α-1,3 linkage [12], respectively. On the other hand, the antigenic determinants of factors 5 and 6 correspond to two kinds of β-1,2 linkage-containing side chains, a homologous series of β-1,2-linked oligomannosyl side chains [13], side chains composed of β-1,2 and α-1,2 linkages [14], respectively.

In carbohydrate chemistry, acetolysis is the one of the important procedures for the selective cleavage of glycosidic linkages, and was frequently used for the structural and immunochemical studies of various yeast mannans [15,16], and for the preparation of various substrates for enzymes in biosynthetic study of yeast mannans [17,18].

Candida catenulata is an opportunistic pathogen for responsible for candidiasis, and its cell wall mannan assumes the antigenicity of the cell surface. In the previous paper [19], the purified mannan obtained from NBRC 0745 (formerly IFO 0745) strain of this species, was utilized for the complete assignment of the nuclear magnetic resonance chemical shifts of all mannose residues in this molecule. In the present immunochemical study of *C. catenulata* mannan, we adopted acetolysis to obtain oligosaccharides corresponding to determinants of antigenic factors from the parent mannan.

2. Materials and Methods

2.1. Strains and Culture

Candida catenulata NBRC 0745 (formerly IFO 0745)

was obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan. This strain was cultivated in the yeast extract-Sabouraud's liquid medium [0.5% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) glucose] at 27˚C for 72 hr on a reciprocal shaker.

2.2. Preparation of Mannans

Mannan were extracted with hot-water and precipitated with Fehling solution [4]. The purified mannan obtained from the cells of the *C. catenulata* strain was designated Fr. C. The yields of Fr. C was 8.0% of the dry cell weight.

2.3. Acetolysis of Fr. C

Acetolysis under conventional conditions was carried out as described previously [20] by modifying the method of Kocourek and Ballou [15]. Namely, mannan, 150 mg, was dissolved in 3 ml of anhydrous formamide in 300-ml glass-stoppered round-bottomed flask, and the solution was added a 1:1 (v/v) mixture of $(CH_3CO)_2O$ and anhydrous pyridine, 100 ml. The clear solution was kept at 40˚C for 24 hr. The resultant solution was then evaporated *in vacuo* to dryness to an oil diffusion pump. The residue was dissolved in a 10:10:1 (v/v/v) ratio of mixture of $(CH_3CO)_2O$, CH_3COOH, and H_2SO_4, 150 ml, and the resultant solution was kept at 40˚C for 13 hr. This solution was evaporated *in vacuo* to a thick syrup after addition of pyridine, 15 ml. The residue was extracted by $CHCl_3$, 50 ml, and the extract was washed with water, 100 ml at three times. The $CHCl_3$ layer was separated and dried over anhydrous Na_2SO_4. After filtration, the solution was evaporated *in vacuo* to dryness, and the residue was dissolved in anhydrous CH_3OH, 2 ml. To the solution was added a few drops of 1 M methanolic CH_3ONa solution, and the mixture was left at room temperature until the precipitation of free sugars was accomplished. The mixture was then neutralized with 50% CH_3COOH and evaporated *in vacuo* to dryness. The residue was dissolved in 2 ml of water, applied to a column (2.5 × 100 cm) of Bio-Gel P-2 (-400 mesh), and eluted with water (0.25 ml/min). Aliquots (10 μl) of eluates were assayed for carbohydrate content by the phenol-H_2SO_4 method [21]. Eluate corresponding to each peak was combined and concentrated *in vacuo*. In order to remove small amounts of contaminated oligosaccharides of lower and higher molecular weight, the solution was rechromatographed on the same column of Bio-Gel P-2, and eluates containing a homogeneous oligosaccharide were combined and lyophilized after concentration *in vacuo*.

2.4. Calculation of Average Length of Side Chains and the Branching Frequency Value of Fr. C

The average length of side chains (X) and the branching frequency value (Y) of Fr. C were calculated by using the following formula in accordance with previous descriptions [6]:

$$X = \left[(A\times 1)+(B\times 2)+(C\times 3)+(D\times 4)\right]/(A+B+C+D),$$

and

$$Y = \left[(B+C+D)\times 100\right]/(A+B+C+D),$$

respectively, where A through D represent the molar proportions of mannose, biose, triose, and tetraose in the gel-filtration profile of the acetolysis products, and the numbers 1 through 4 indicate the degrees of polymerization of the mannose (M_1) and the three oligosaccharides, M_2 through M_4, respectively.

2.5. ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy

The NMR spectra conducted on a JEOL JNM-GSX 400 spectrometer at 400 MHz. It was recorded using a 0.5% (w/v) solution of each oligosaccharides in 0.7 ml of D_2O at 45˚C. Acetone (2.217 ppm) was used as an internal standard.

2.6. Inhibition Test of Slide Agglutination Assay Using Oligosaccharides

The inhibition assay of slide agglutination of *C. catenulata* cells with factor antibodies (FAbs) was conducted as previously described [13]. FAbs 1, 9, and 34 were prepared by Fukazawa *et al*. [1]. The inhibitor oligosaccharides, M_2, M_3 and M_4, were obtained from Fr. C by acetolysis.

3. Results and Discussion

The oligosaccharides mixture obtained from Fr. C by acetolysis were fractionated with water by gel-chromatography of Bio-Gel P-2 (**Figure 1**). The large amounts of oligosaccharides, tetraose (M_4) and triose (M_3), and the small amounts of oligosaccharides, biose (M_2) and mannose (M_1), were eluted. No product eluted at the position of void-volume (Vo) indicates that all α-1,6 linkages in Fr. C were completely cleaved by the acetolysis. The chemical structures of resultant oligosaccharides were analyzed by ^{1}H-NMR spectroscopy. The H-1 region signals of these oligosaccharides were shown in **Figure 2**. All spectra were identical to those of M_2, M_3, and M_4, which were previously isolated from the cell wall mannans of *Saccharomyces cerevisiae* wild-type [22] and *Candida glabrata* [8]. The structure of M_2 and M_3 were

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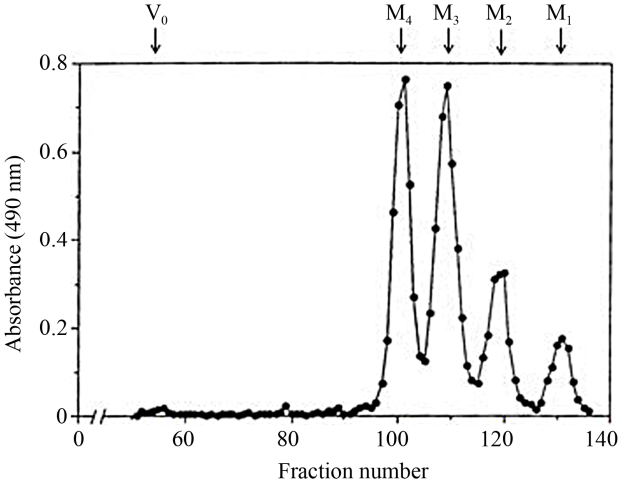

Figure 1. Elution profile of oligosaccharides obtained from *C. catenulata* mannan, Fr. C, by conventional acetolysis. Vo refers void-volume region. M_4, M_3, M_2 and M_1 indicate the eluted positions of standard monnooligosaccharides, tetraose, triose, and biose, and mannose, respectively.

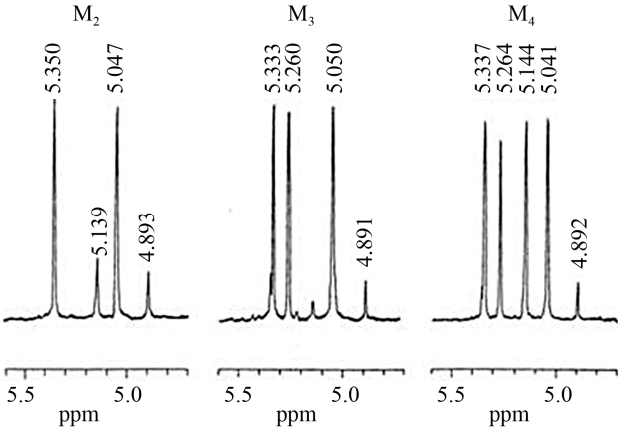

Figure 2. ^{1}H-NMR spectra of oligosaccharides obtained from *C. catenulata* mannan, Fr. C, by conventional acetolysis. Simbols are the same as in Figure 1.

identified to Manα1-2Man and Manα1-2Manα1-2Man, respectively. The H-1 signal at 5.144 ppm in the spectrum of M_4 indicates the presence of non-reducing terminal α-1,3-linked mannose residue linked to α-1,2-linked oligomannosyl unit. Therefore, the structure of M_4 was identified to Manα1-3Manα1-2Manα1-2Man. The chemical structure of all oligosaccharides and the assignment result of chemical shifts of all mannose residues based on the results of previous reports [8,23] were shown in **Table 1**.

As shown in **Table 2**, to identify the antigenic determinants in *C. catenulata* mannan corresponding to antigenic factor, we performed an inhibition assay of agglutination between *C. catenulata* cells and factor antibodies, FAbs 1, 9, and 34, with three inhibitor oligosaccharides, M_4, M_3, and M_2, and M_1 (mannose) obtained from Fr. C by acetolysis. The fact that the antigen determinant of factor 9 could not be found in this experiment indicates this epitope does not reside in the side chains of Fr. C. On the other hand, the result with FAb 1 clearly indicates that two α-1,2-linked oligomannosyl side chains corresponding to Manα1-2Man and Manα1-2Manα1-2Man possess antigenic determinant of factors 1. In contrast, FAb 34 unable to recognize α-1,2-linked oligomannnosyl side chain, whereas it recognized the side chain possessing terminal α-1,3-linked mannose, Manα1-3Manα1-2Manα1-2Man.

Table 1. Assignment of chemical shifts of H1 signals of oligosaccharides obtained from *C. catenulata* mannan, Fr. C, by acetolysis.

oligosaccharide	Sugar residue[a]				Chemical shft (ppm)[b]			
	D	C	B	A(α)[c]	D	C	B	A(α)
				A(β)				A(β)
M_2				Mα1-2M(α)		5.047	5.350	
				Mα1-2M(β)		5.139	4.893	
M_3			Mα1-2Mα1-2M(α)			5.050	5.260	5.333
			Mα1-2Mα1-2M(β)			5.050	5.260	4.891
M_4		Mα1-3Mα1-2Mα1-2M(α)			5.144	5.041	5.264	5.337
		Mα1-3Mα1-2Mα1-2M(β)			5.144	5.041	5.264	4.892

[a]M denotes a mannose residue; [b]This was measured at 45˚C using acetone (2.217 ppm) as a standard; [c]Configuration of reducing terminal residue.

Table 2. Inhibition of agglutination of *Candida catenulata* cells with FAbs 1, 9, and 34 by mannooligosaccharides obtained from *C. catenulata* mannan, Fr. C.

Agglutination[a] with inhibitor amt (μmol)						
Oligosaccharide						
	2^1	2^0	2^{-1}	2^{-2}	2^{-3}	0
With FAb 1						
M_1	+3	+3	+3	+3	+3	+3
M_2	+2	+2	+2	+2	+3	+3
M_3	+1	+1	+1	+2	+3	+3
M_4	+2	+2	+3	+3	+3	+3
With FAb 9						
M_1	+2	+2	+2	+2	+2	+2
M_2	+2	+2	+2	+2	+2	+2
M_3	+2	+2	+2	+2	+2	+2
M_4	+2	+2	+2	+2	+2	+2
With FAb 34						
M_1	+3	+3	+3	+3	+3	+3
M_2	+3	+3	+3	+3	+3	+3
M_3	+3	+3	+3	+3	+3	+3
M_4	+1	+1	+2	+2	+3	+3

[a]Agglutination was scored from high (+3) to low (+1).

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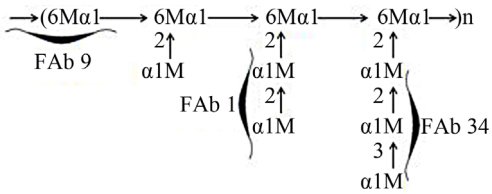
The chemical structure of the cell wall mannan obtained from *C. catenulara* IFO 0745 strain (Fr. C) and the recognition sites of factor antibodies 1, 9, and 34 were proposed as shown in **Figure 3**. The side chain distribution was calculated using the peak-dimensions in the gel-filtration profile of the acetolysis products (**Figure 3(a)**). The molar ratios of tetraosyl side chain were distinctly lower than that previously calculated from the dimension of H-1 signals in the ^{1}H-NMR spectrum of the same mannan (**Figure 3(b)**) [19]. The average length of side chains, 2.6, and the value of branching frequency, 77.1%, calculated from the peak-dimension of elution pattern of acetolysates (**Figure 1**) were lower than comparison with those calculated by the signal dimension of NMR spectrum (average length: 3.0, branching frequency: 91.3%). These findings showed that the acetolysis conditions make to cleave not only α-1,6 linkage of backbone but also non-reducing terminal part of the relatively longer α-linked side chains. In conclusion, although acetolysis is useful for the preparation of the oligosaccharides corresponding to side chains as haptens of immunochemical or biological function, the NMR analysis without using harsh procedure is useful for the detailed analysis for the distribution of side chains in the parent mannan.

In the previous study [10,12], we demonstrated that the α-1,2-linked mannooligosaccharides and the oligosaccharides containing a non-reducing terminal α-1,3-linked mannose residue corresponding to the epitopes of antigenic factors 1 and 34, respectively. In this study, we could prepare three oligosaccharides corresponding to the antigenic factors 1 and 34, Manα1-2Man, Manα1-2Manα1-2Man, and Manα1-3Manα1-2Manα1-2Man, which were isolated from α-1,6-linked polymannnosyl backbone of Fr. C by the selective cleavage method, acetolysis. Though Fr. C reacted weakly with FAb 9, we could not find the oligosaccharide which functions as an antigenic epitope of this antibody (**Table 2**). This phenomenon can explain that the site of factor 9 antibody is α-1,6-linked polymannnosyl backbone of yeast mannnan in accordance with previous finding [12]. Namely, it is concluded that the most of mannose (M_1) released by acetolysis arose from the backbone part that is not connected by the side chain.



Side chain distribution

(a)	22.2	:	22.4	:	33.3	: 22.1
(b)	8.7	:	17.1	:	38.8	: 35.8

Figure 3. Structure of *C. catenulata* mannan, Fr. C, and recognition sites of factor antibodies, FAbs 1, 9, and 34. (a) Side chain distribution was calculated based on the peak-dimensions in the gel-filtration profile of the acetolysis products; (b) Side chain distribution was calculated based on the dimensions of characteristic H1 signals of each side chain in the ^{1}H-NMR spectroscopy map [19]. Side chain sequence is not specified. M denotes a mannose residue.

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