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NMR assignment of the galactomannan of *Candida lipolytica*

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Abstract The chemical structure of the cell wall galactomannan of *Candida lipolytica* was analyzed using two-dimensional NMR techniques without chemical fragmentation. The H-1-H-2-correlated cross-peaks of the galactomannan indicated that it consists of an α-1,6-linked mannan backbone moiety with side chains. A sequential NMR assignment of the side chains through nuclear Overhauser effect (NOE) cross-peaks indicated that the triose side chain contains an α-1,2-linked galactopyranose unit at the non-reducing terminal. The structure was significantly different from the galactomannan of *Trichophyton*. The molar ratio of the side chains calculated from the H-1 signal dimensions indicated that ca. 45% of the backbone α-1,6-linked mannose units are not substituted with side chains and are responsible for the reactivity of the galactomannan with factor 9 serum.

Key words: *Candida*; Galactomannan; NMR; Sequential assignment; Antigenic factor

1. Introduction

Candida lipolytica (teleomorph, *Saccharomycopsis lipolytica*, *Yarrowia lipolytica*, *Candida olea*), a *n*-alkane assimilating yeast, has been commercially used for the production of citric acid and single-cell protein. This species is a natural secretor of proteins and offers advantages over *Saccharomyces cerevisiae* as a eukaryotic host for the secretion of heterologous proteins [1] and is also known to contain a large amount of peroxisome. Because of the presence of the peroxisome assembly mutant, the mechanism of assembly of functional peroxisomes has been studied [2,3]. Furthermore, this species is known as one of the causes of new emerging yeast infections [4,5]. In common with many yeasts, the cell wall of *C. lipolytica* involves a mannan-containing polysaccharide. Although almost all of the yeast mannans do not contain other carbohydrate components except a trace of *N*-acetylglucosamine and glucose, *C. lipolytica*, *Schizosaccharomyces pombe*, *Trichosporon fermentans*, and *Trulopsis lactis-mondensi* are known to have galactomannan [6–8]. However, there is no report precisely describing the overall structure of the galactomannans.

Gorin and Spencer [8] tried to apply ^{1}H NMR spectra for the chemotaxonomy of yeasts. However, since there are so many different polysaccharide structures, the 1D-^{1}H NMR signals overlap with the different sugar units. On the other hand, 2D-NMR techniques, such as COSY, NOESY, 2D-HOHAHA, etc., give well-separated cross-peaks. The finding of an unidentified cross-peak near the backbone α-1,6-linked mannose unit in the 2D-HOHAHA spectrum of *Candida albicans* serotype B mannan [9] allowed us to demonstrate the existence of α-1,6-branched side chains in the mannan. In recent studies [10–12], we showed that we can determine the overall structures of cell wall mannans from the assignment results of the H-1-H-2-correlated cross-peaks and H-1 signal dimensions. Furthermore, we showed that we can sequentially assign the signals of fragment oligosaccharides [9,10,13–15] and some polysaccharides [16,17] using NOE or HMBC cross-peaks. Since the spectrum of the galactomannan of *C. lipolytica* was relatively simple, we tried to determine the structure of the polysaccharide using NMR techniques without chemical fragmentation. In this study, we used acetolysis only for the confirmation of the structure.

2. Materials and methods

2.1. Materials

The *C. lipolytica* IFO 1548 strain was obtained from the Institute for Fermentation (Osaka, Japan). 'Candida Check' (lot no. I751), developed by Tsuchiya et al. [18] for the identification of the clinically important *Candida* species, was purchased from Iatron (Tokyo, Japan).

2.2. Preparation and acetolysis of galactomannan

Yeast cells were grown at 28°C in a shaking liquid culture containing 0.5% yeast extract, 1% peptone, and 2% glucose. Galactomannan was extracted from the cells with water at 135°C for 3 h, and was separated by precipitation with Fehling's solution [9]. Acetolysis under mild conditions [19] was performed as described in preceding papers [9,15]. Fractionation of the resultant mannooligosaccharide mixture was achieved using a column (2.5×100 cm) of Bio-Gel P-2 (extra fine). Elution was carried out with water, and aliquots of the eluates were assayed for carbohydrate content using the phenol-sulfuric acid method [20].

2.3. Nuclear magnetic resonance spectroscopy

All ^{1}H NMR experiments were performed with a JEOL JNM-GSX 400 spectrometer at 400 MHz for ^{1}H and 100 Mz for ^{13}C. The spectra were recorded using a 1% (w/v) solution of each mannan or oligosaccharide in 0.7 ml D_2O at 45°C. Acetone (2.217 ppm) [21] and CD_3OD (49.00 ppm) were used as the internal standard for ^{1}H and ^{13}C NMR, respectively.

2.4. Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted as described in a preceding paper [22] using serial diluted factors 1, 4, 6, and 9 sera of 'Candida Check' from 8- to 2048-fold with phosphate-buffered saline.

2.5. Other methods

Total carbohydrate was determined by the phenol-sulfuric acid

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Abbreviations: DQF-COSY, double quantum filtered H-H-correlation spectroscopy; 2D-HOHAHA, two-dimentional homonuclear Hartman-Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; ELISA, enzyme-linked immunosorbent assay

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method of Dubois et al. [20] with D-mannose as the standard. Total phosphate was determined by the method of Ames and Dubin [23], using KH_2PO_4 as the standard.

3. Results and discussion

In the preceding studies, we assigned all of the H-1-H-2-correlated cross-peaks of the mannans of *C. albicans* [9], *C. guilliermondii* [10], *C. saitoana* [11], *C. famata* [11], *C. catenulata* [17], and *C. stellatoidea* [12], and the galactomannan of *T. mentagrophytes* [16]. Therefore, we tried to predict the structure of the galactomannan of *C. lipolytica*, designated as Fr. L, from the H-1-H-2-correlated cross-peaks in a COSY spectrum (Fig. 1A). We compared the cross-peaks with those of a galactomannan (Fig. 1B) and mannan (Fig. 1C) obtained from *T. mentagrophytes* [16]. The boxes in each panel indicate the regions of the H-1-H-2-correlated cross-peaks which were found in the spectra of the mannans of the *Candida* species [10–12,17]. On the other hand, circled cross-peaks had not be found in the *Candida* mannans. Cross-peak 1 is located near those of the intermediary α-1,2-linked mannose units of mannooligosaccharide side chains [10], suggesting that the unit which corresponds to this cross-peak is substituted with an α-1,2 linkage by other sugar unit. As shown in the preceding paper [12,24,25], cross-peaks 3 and 4, and 5 and 14 correspond to α-1,6-linked backbone mannose units substituted by α-1,2-linked oligosaccharides and mannose units, respectively. Cross-peak 6 indicates the non-reducing terminal α-1,2-linked mannose unit. Cross-peaks 7 and 8 indicate the presence of consecutive α-1,6-linked backbone mannose units substituted with no side chains [24]. The cross-peaks 6, 7, and 8 were also present in the spectrum of the mannan of *T. mentagrophytes* (Fig. 1C). Cross-peak 2 appeared in a significantly different region and $J_{1,2}$ and $J_{2,3}$ were 3.7 Hz and 10.3 Hz, respectively (Table 1). Furthermore, the chemical shifts of ring protons (H-1 = 5.161, H-2 = 3.820, H-3 = 3.893, H-4 = 3.984, H-5 = 4.073) and ^{13}C NMR signals (C-1 = 101.98, C-2 = 69.73, C-3 = 70.22, C-4 = 70.20, C-5 = 72.24, C-6 = 62.12)

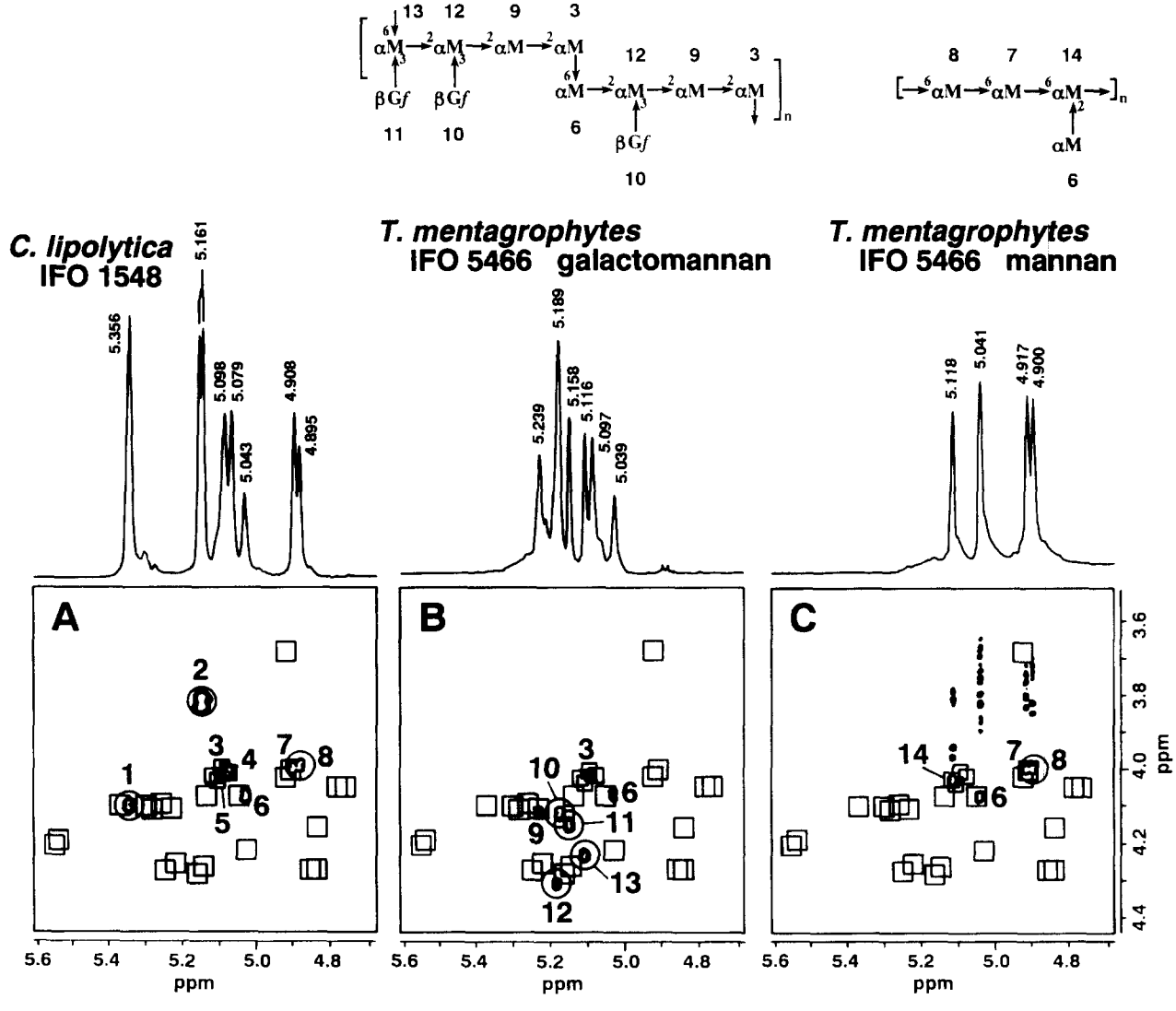

Fig. 1. Assignment of H-1-H-2-correlated cross-peaks of galactomannans. DQF-COSY spectrum of Fr. L (A) and 2D-HOHAHA spectra of the galactomannan (B) and mannan (C) of *T. mentagrophytes* are shown. The boxed regions in the spectra were the same as those in the 2D-HOHAHA spectra of the mannans obtained from *C. guilliermondii* and *C. stellatoidea* reported in the preceding papers [10,12]. The labeled cross-peaks in panels B and C have been assigned as shown in the above structures [16]. M and G*f* denote D-mannopyranose and D-galactofuranose units, respectively.

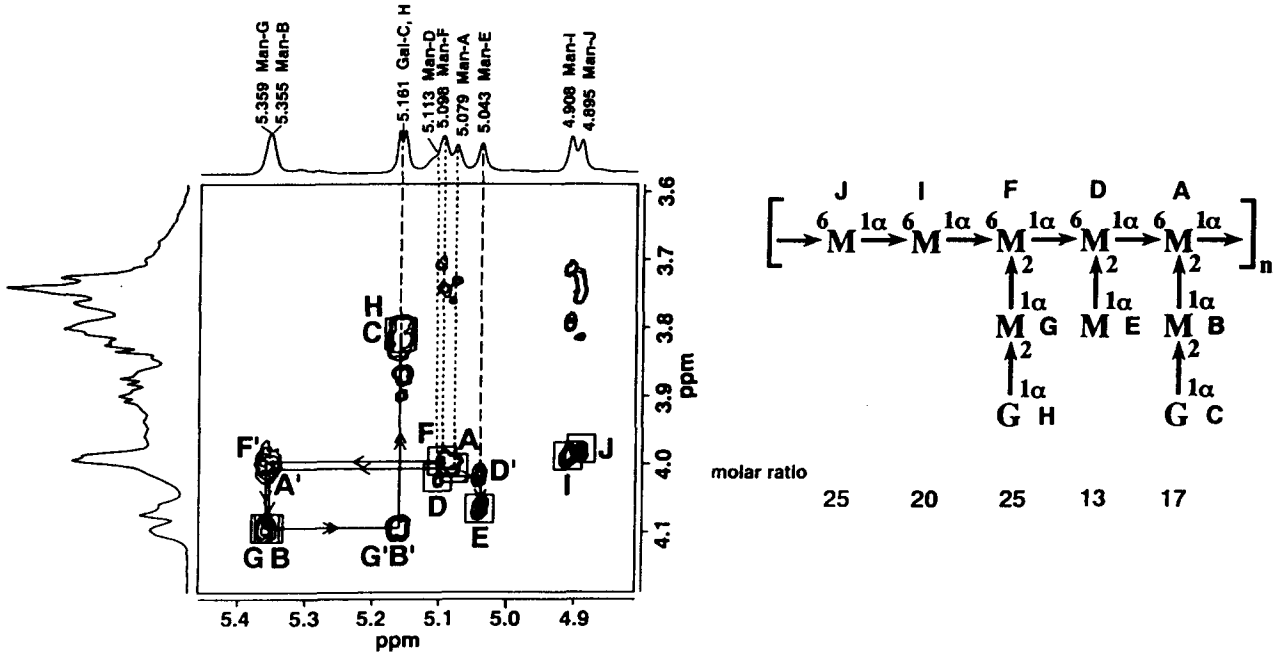

Fig. 2. Sequential connectivities of the side chains of Fr. L. In this NOESY spectrum, primed letters indicate interresidue H-1-H-2′ NOE cross-peaks and unprimed letters indicate the H-1-H-2-correlated cross-peaks, caused by *J-coupling*; e.g., A indicates the H-1-H-2-correlated cross-peak of a backbone α-1,6-linked mannose unit, Man-A, and A′ indicates the interresidue NOE cross-peak between the H-2 of Man-A and the H-1 of an adjacent mannose unit, Man-B. By this procedure, the H-1 and H-2 signals of a side chain were sequentially assigned from the H-1 of Man-A, A-A′-B-B′-C. Possible structure of Fr. L was also shown. M and G denote D-mannopyranose and D-galactopyranose units, respectively. The molar ratio of the side chains are calculated from the dimensions of the 1H-NMR signals in Fig. 1A.

indicate that the cross-peak 2 may correspond to an α-linked galactopyranose unit [26]. The backbone of the galactomannan of *T. mentagrophytes* has been identified as having a linear repeating structure of an α-1,2-linked mannotetraose connected by α-1,6 linkages and having galactofuranose units as the side chain [16]. In a preceding study [16], we identified that cross-peaks 10 and 11 correspond to β-1,3-linked galactofuranose units and cross-peaks 12 and 13 correspond to α-1,2-linked mannose units substituted by β-1,3-linked galactofuranose units. The cross-peaks of Fr. L were significantly different from those of the galactomannan of *T. mentagrophytes* (Fig. 1B), but similar to those of the mannan of *T. mentagrophytes* (Fig. 1C). These results suggest that the structure of the galactomannan of *C. lipolytica* is different than that of *T. mentagrophytes* and has a comb-like structure with α-1,6-linked backbone mannose units.

A sequential assignment study of the H-1 and H-2 signals of Fr. L was performed to determine the structure followed by previous descriptions [9,10,12,13,16]. Fig. 2 shows NOESY of Fr. L. In this figure, cross-peaks labeled with primed letters indicate through-space interresidue H-1-H-2′ connectivities between two adjacent mannose or galactose units. On the other hand, cross-peaks labeled with unprimed letters indicate intraresidue H-1-H-2-correlated cross-peaks. By using this procedure, the H-1 and H-2 signals of a side chain were sequentially assigned from cross-peak A of a backbone α-1,6-linked mannose unit, Man-A, A-A′-B-B′-C or from cross-peak F of Man-F, F-F′-G-G′-H. The assignment result indicates that these side chains are triose with the following structure.

Galα1→2Manα1→2Man

Furthermore, we could sequentially assign other signals from the H-1 of an α-1,6-linked mannose unit, Man-D, D-D′-E. This result indicates the presence of an α-1,2-linked

Table 1
^{1}H and ^{13}C chemical shifts of Fr. L

	Sugar residue									
	Man-A	Man-B	Gal-C	Man-D	Man-E	Man-F	Man-G	Gal-H	Man-I	Man-J
	Chemical shift, ppm									
H-1	5.079	5.355	5.161 ($J_{1,2}$ = 3.7)*	5.113	5.043	5.098	5.359	5.161 ($J_{1,2}$ = 3.7)*	4.908	4.895
H-2	4.011	4.100	3.820 ($J_{2,3}$ = 10.3)	4.031	4.074	4.003	4.009	3.820 ($J_{1,2}$ = 10.3)	3.997	3.989
H-3	3.911	3.910	3.893 ($J_{3,4}$ = 3.3)	3.931	3.807	3.934	3.910	3.893 ($J_{1,2}$ = 3.3)	3.814	3.832
	99.21	101.48	101.98	99.18	103.11	99.13	101.48	101.48	100.50	100.50
	79.73	80.24	69.73	–	70.96	79.64	80.24	69.73	70.96	70.96
	70.96	70.96	70.22	–	71.48	70.96	70.96	70.22	71.48	71.48

*Coupling constant, Hz.

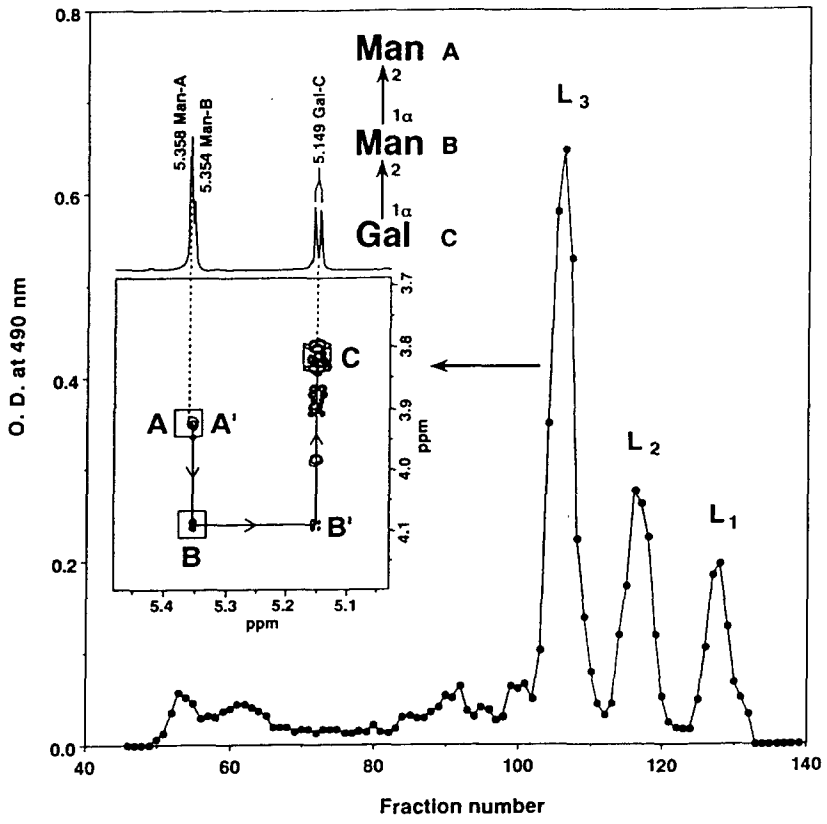

Fig. 3. Elution pattern of oligosaccharides obtained from Fr. L by acetolysis. Elution was performed with a column (2.5×100 cm) of Bio-Gel P-2. An inserted panel is a NOESY spectrum of triose, L3, obtained by acetolysis. We confirmed the structure of L_3 as shown in this figure using a sequential assignment method through NOE cross-peaks.

mannobiose side chain. The H-1 and H-2 chemical shifts of Man-D appeared little downfield than those of the backbone mannose unit substituted by long side chain. It seems that there is an additivity rule for the α-1,2-linked mannose unit [12]. The assignment result from H-1 to H-3 and from C-1 to C-3 were shown in Table 1. From these results we can depict the overall structure of the galactomannan as shown in Fig. 2. We think that there are branched and unbranched domains in the structure. In this structure, we also showed the molar ratio of the side chains calculated from the H-1 signal dimensions of the assigned cross-peaks. These results indicate that ca. 45% of the α-1,6-linked backbone mannose units are not substituted with side chains.

Fig. 3 shows the elution pattern of the acetolysate of Fr. L from a column of Bio-Gel P-2. As we expected, oligosaccharides were obtained up to triose. The oligosaccharides from the triose to monosaccharide were designated as L_3, L_2, and L_1. The ^1H NMR spectra of these oligosaccharides indicated that L_1 and L_2 were mannose and an α-1,2-linked mannobiose, respectively. On the other hand, the signals of L_3 were sequentially assigned using the NOE cross-peaks, and the structure was confirmed to be the one previously described.

Fr. L was tested for its reactivity to several factor sera by ELISA. As shown in Fig. 4, Fr. L showed a strong reactivity to factors 1 and 9 sera. These results support the finding that factor 9 serum contains an antibody against the α-1,6-linked backbone mannose units which have no side chains [10,11,27,28]. These results indicate that the use of antigenic factors for the classification of the structure of the cell wall polysaccharides is not perfect, because some of the factor sera have been known to contain antibodies against several antigenic structures [22,29]. Although the use of 1D-^1H NMR spectra for that purpose is also not perfect, the use of the H-1-H-2-correlated cross-peaks of the 2D-NMR spectra seems to be very effective and we will be able to distinguish

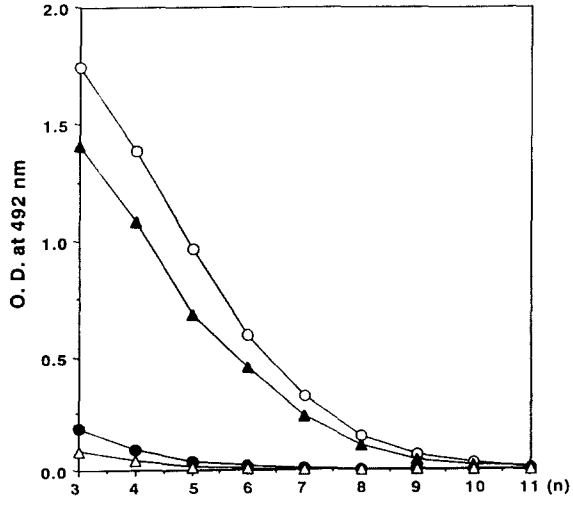

Fig. 4. Enzyme-linked immunosorbent assay of Fr. L. (○) factor 1 serum, (●) factor 4 serum, (△) factor 6 serum, (▲) factor 9 serum.

the difference in the structure of the polysaccharides which have the same antigenic factors or similar 1D-NMR signals.

There are many reports about the immunomodulating effect of cell wall mannans [30–35] and galactomannans [36,37]. Furthermore, there are studies about the contribution of the cell wall mannans to the adherence of *Candida* cells to host cells [38–40] and about the vaccination using monoclonal antibody against an epitope of the mannan [41]. For these studies, identification of the fine chemical structures of the cell wall mannans of pathogenic yeasts or fungi and their related ones seems to be essential.

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