Identification of the antigenic determinants of factors 8, 9, and 34 of genus *Candida*

Hidemitsu Kobayashi*, Hiroko Oyamada, Akifumi Suzuki, Nobuyuki Shibata, Shigeo Suzuki[1], Yoshio Okawa

Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981, Japan

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Abstract **We investigated the antigenic determinants of factors 8, 9, and 34 of the genus *Candida* among pathogenic yeasts by enzyme-linked immunosorbent assay (ELISA) using mannans of *Saccharomyces cerevisiae* wild type and mutant types, *mnn* 1-*mnn* 4 and *mnn* 2. Results of ELISA including antisera against the antigenic factors of genus *Candida* (Candida Check, Iatron; FAbs) indicated that these three types of mannan distinctly react with FAbs 34, 8 and 9, respectively. To identify the recognition sites of these FAbs, we compared the ability of various oligosaccharides to inhibit the binding of the mannans to FAbs. The results indicated that FAb 34 preferentially recognizes linear side chains containing a non-reducing terminal α-1,3-linked mannose residue, Manα1→3Manα1→(2Manα1→)$_n$2Man ($n \geq 0$), and that one of the recognition sites of FAb 9 is linear α-1,6-linked oligomannosyl series, Manα1→(6Manα1→)$_n$6-Man ($n \geq 2$). On the other hand, the recognition site of FAb 8 apparently consisted of two α-1,2-linked oligomannosyl side chains and an α-1,6-linked mannose residue that originated from the mannan backbone, Manα1→2Manα1→2(Manα1→2-Manα1→6)Man.**

Key words: Pathogenic genus *Candida*; *Saccharomyces cerevisiae*; Cell wall mannan; Antigenic factor; Enzyme-linked immunosorbent assay

1. Introduction

Tsuchiya et al. [1] originally proposed that there is an immunochemical relationship among clinically significant species of the genus *Candida*, based on 10 cell surface antigenic factors. Thereafter, the Candida Check kit (Iatron, Japan) that included rabbit antisera to factors of the genus *Candida* was developed to identify clinical isolates from the candidiasis patients [2].

We showed that the antigenic determinants of factors 5, 6, and 9 correspond to three side chains containing β-1,2-linked mannose residue in cell wall mannan [3–5]. These side chains, which reside only in the genus *Candida* among all the pathogenic yeasts, have been investigated for use as the target antigen in diagnosing invasive candidiasis [6]. Although β-1,2-linked oligomannosyl units are involved in the induction of cytokines such as TNF [7,8], the α-linked mannooligosaccharides obtained from *Candida* mannan were potent inhibitors of lymphoproliferation induced by the parent mannan [9,10]. Furthermore, the α-linked units seem to participate in the adherence of *Candida* cells to mammalian tissues during the initial step of infection [11]. Therefore, the cell wall components containing mannose residues in the α configuration must be structurally and immunochemically characterized to help understand these host-parasite interactions. The antigenic determinants of factors 1 and 4 are linear α-1,2-linked oligomannosyl [12] and 3,6-branched oligomannosyl units [13], respectively. However, those of factors 8 and 34 corresponding to the specific antigens of *Candida kefyr* and *Candida glabrata*, which seemed to be α-linked oligomannosyl units, have not been identified. Here, we show that *Saccharomyces cerevisiae* wild and mutant-type mannans are recognized by several antisera to factors of the genus *Candida* and that the antigenic determinants correspond to specific α-linked oligomannosyl units.

2. Materials and methods

2.1. Strains and culture

S. cerevisiae wild type (X2180-1A; referred to as strain 1A) [14] and *S. cerevisiae mnn* 1-*mnn* 4 and *mnn* 2 mutant types (4484-24-D-1 and X2180-1A-5; referred as strains D1 and A5, respectively) [15] were gifts from Dr. T. Nakajima, Tohoku University, Sendai, Japan. *C. kefyr* (IFO 0586; strain K) [12] was obtained from the Institute for Fermentation Osaka (IFO), Osaka, Japan. These strains were cultivated in yeast extract-Sabouraud's liquid medium (0.5% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) D-glucose) at 27°C for 72 h on a reciprocal shaker.

2.2. Preparation of mannans

Mannans were extracted using hot water and precipitated with Fehling solution [16]. The purified mannan fractions obtained from strains 1A, D1, A5, and K were designated Frs. 1A, D1, A5, and K, respectively. The yields of Frs. 1A, D1, A5, and K were 7.8%, 7.2%, 7.3% and 6.0% of the dry cell weight, respectively. The structures of these mannans confirmed by ^{1}H-nuclear magnetic resonance (^{1}H-NMR) [17,18] are shown in Fig. 1.

2.3. Enzyme-linked immunosorbent assay (ELISA) of mannans

Antisera against factors 1, 6, 8, 9, and 34 of the genus *Candida* (FAbs 1, 6, 8, 9, and 34, respectively) were supplied with the Candida Check kits (lot nos. I-571 and R-572, Iatron, Tokyo, Japan). FAb 6, which recognizes β-1,2-linked mannose residue [4], was used as a negative control. Sera were diluted from 9- to 19 683-fold with phosphate-buffered saline containing 0.1% (w/v) Tween 20 (PBST). ELISA was performed according to a previously described procedure [3].

2.4. ELISA inhibition using mannooligosaccharides

A series of α-1,2-linked oligosaccharides, biose (M_2-2) to tetraose (M_4-2), was prepared from Frs. 1A, D1, K, and *C. albicans* mannans [16] by conventional acetolysis [19]. The α-1,6-linked series, triose (M_3-6) to pentaose (M_5-6), was prepared from Fr. A5 [17] by mild acetolysis [16]. Oligosaccharides containing a non-reducing terminal α-1,3-linkage, triose (M_3-3) to pentaose (M_5-3), were prepared from Fr. 1A and *Candida stellatoidea* type I [20] and *Candida krusei* mannans (Oyamada et al., unpublished) by mild acetolysis. A hexaose containing an internal α-1,3-linkage (M_6-2,3) was prepared from

*Corresponding author. Fax: (81) (22) 275-2013.

[1] *Present address:* Sendai Research Institute for Mycology, 1-14-34 Tohshogu, Aoba-ku, Sendai, Miyagi 981, Japan.

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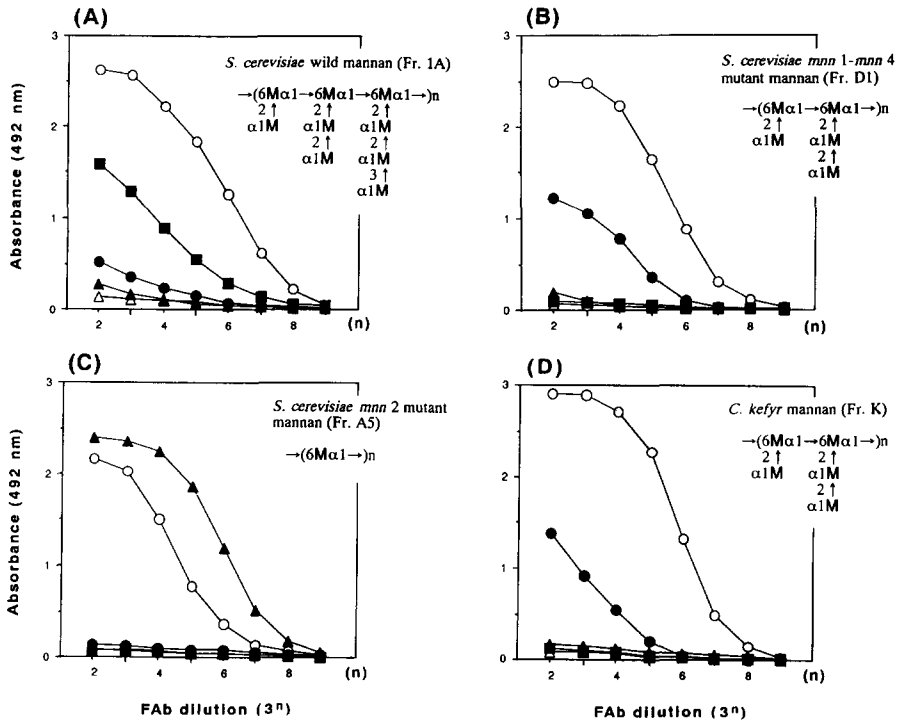

Fig. 1. ELISA of *S. cerevisiae* mannans, Fr. 1A (A), Fr. D1 (B), Fr. A5 (C), and Fr. K (D) with FAb 1 (○), FAb 6 (△), FAb 8 (●), FAb 9 (▲), and FAb 34 (■). The structure of each mannan is shown in the upper part of the corresponding graph. M denotes a D-mannose residue.

C. stellatoidea type I mannan by conventional acetolysis. A pentaose containing an α-1,6-linkage (M_5-2,6) was prepared from Frs. D1 and K by mild acetolysis. A 3,6-branched pentaose (M_5-3,6) was synthesized from M_4-3 by an enzymatic procedure using GDP-mannose and an α-1,6-mannosyltransferase of *C. albicans* serotype B [21]. The chemical structures of the oligosaccharides were confirmed by ^{1}H-NMR [13,18] and are shown in Table 1. The negative control, D-mannose (Sigma), and the inhibitors were diluted with PBST. The inhibition assay was performed according to Okawa et al. [22] with modification. FAbs 8, 9, and 34 were diluted 10-, 200-, and 20-fold with PBST, based on the ELISA results of Frs. D1, A5, and 1A, respectively. Diluted FAb, 50 μl, was incubated for 2 h at 30°C in the presence of 50 μl of inhibitor. This mixture was then added to the wells of microtiter plates coated with mannan, and the plates were processed as described by Shibata et al. [3]. The inhibition ratio (%) was calculated using the formula: Inhibition (%) = $(1-A/B)\times 100$, where A and B are the optical densities at 492 nm with and without inhibitor, respectively.

3. Results and discussion

The results of ELISA of Fr. 1A indicate that FAbs 1 and 34 are highly reactive, whereas FAbs 8 and 9 are only slightly reactive (Fig. 1A). This finding suggests that the recognition site of FAb 34 resides in the tetraosyl side chain containing the non-reducing terminal α-1,3-linked mannose residue, because short α-1,2-linked oligomannosyl side chains are the recognition sites of FAb 1 [12]. The results of ELISA-inhibition of Fr. 1A with diluted FAb 34 show that M_3-3, M_4-3, and M_5-3 inhibit the binding of this serum (Fig. 2A). These results indicate that FAb 34 recognizes a non-reducing terminal α-1,3-linked mannose residue but not the exact length of the inner α-1,2-linked oligomannosyl unit. M_5-3,6 did not inhibit under the same conditions, indicating that FAb 34 does not recognize the 3,6-branched side chain corresponding to the antigenic determinant of factor 4 [13]. The biosynthetic pathways of antigenic factors shown in Fig. 3 were inferred from both results and published mannan structures of *C. glabrata* [23] and *C. albicans* [13]. During biosynthesis of *C. glabrata* mannan (Fig. 3A), the side chain corresponding to the antigenic determinant of factor 34 appears to be preferentially synthesized compared to that of antigenic factor 6. On the other hand, during biosynthesis of *C. albicans* mannan (Fig. 3B), the intermediate side chain corresponding to factor 34 appears to be quickly presented as a substrate for the

Table 1
Chemical structures of mannooligosaccharides used for ELISA inhibition assay

Abbreviation	Structure of inhibitor[a]
M_2-2	Manα1→2Man
M_3-2	Manα1→2Manα1→2Man
M_4-2	Manα1→(2Manα1→)$_2$2Man
M_3-6	Manα1→6Manα1→6Man
M_4-6	Manα1→(6Manα1→)$_2$6Man
M_5-6	Manα1→(6Manα1→)$_3$6Man
M_3-3	Manα1→3Manα1→2Man
M_4-3	Manα1→3Manα1→2Manα1→2Man
M_5-3	Manα1→3Manα1→(2Manα1→)$_2$2Man
M_5-2,6	Manα1→2Manα1→2Man ↑6 α|1 Manα1→2Man
M_5-3,6	Manα1→3Manα1→2Manα1→2Man ↑6 α|1 Man
M_6-2,3	Manα1→2Manα1→3Manα1→(2Manα1→)$_2$2Man

[a] Man denotes a D-mannose residue. Chemical structures of oligosaccharides were determined based on ^{1}H-NMR assignments [13,16,18].

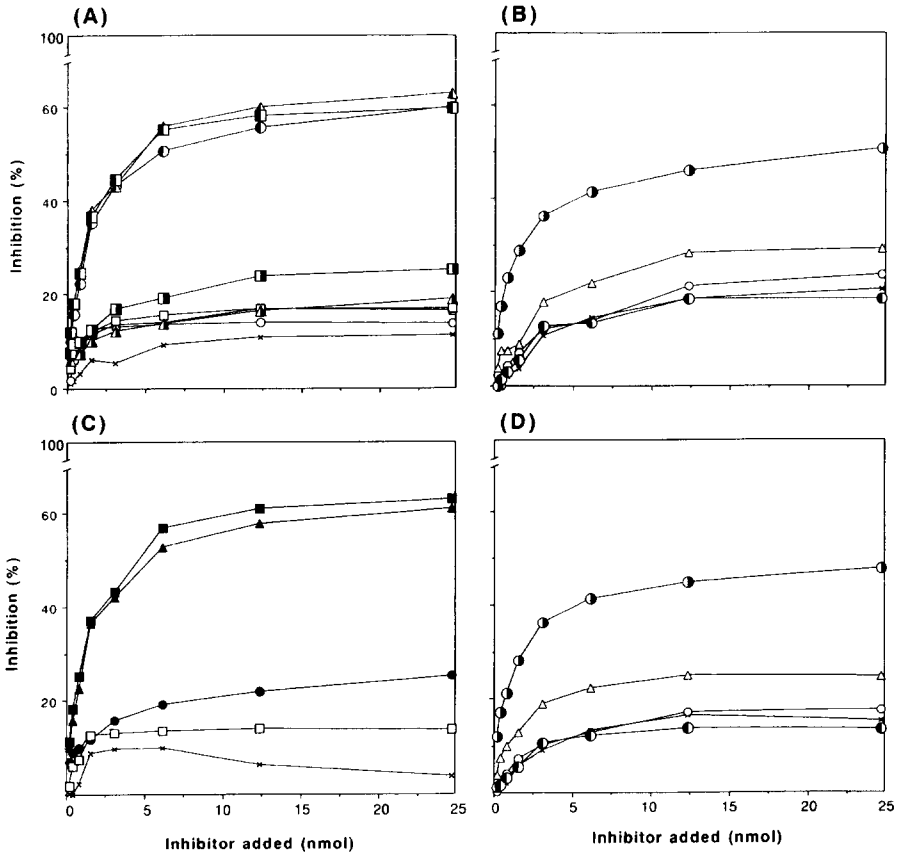

Fig. 2. ELISA inhibition assays of Fr. 1A with FAb 34 (A), Fr. D1 with FAb 8 (B), Fr. A5 with FAb 9 (C), and Fr. K with FAb 8 (D) using oligosaccharides, M_2-2 (○), M_3-2 (△), M_4-2 (□), M_3-6 (●), M_4-6 (▲), M_5-6 (■), M_3-3 (◐), M_4-3 (◭), M_5-3 (◧), M_5-2,6 (◑), M_5-3,6 (◨), and M_6-2,3 (◭), and D-mannose (negative control) (×).

downstream enzyme, α-1,6-mannosyltransferase, which participates in the synthesis of antigenic factor 4 [13] and requires a side chain possessing a non-reducing terminal α-1,3-linked mannose residue in *C. albicans* mannan [21]. As a result of this biosynthetic process in *C. albicans* mannan, antigenic factor 34 was not detected using FAb 34 [2].

We postulated that the immunodominant of FAb 8 resides in *C. kefyr* cell surface molecules other than the mannan [12]. On the other hand, Fukazawa et al. [24] reported that antigenic factor 8 corresponds to the α-1,2-linked mannobiosyl side chain of *C. kefyr* mannan. However, these conclusions should be revised according to the findings presented here. Fig. 1B,D shows that FAbs 1 and 8 recognize Frs. D1 and K, whereas FAbs 9 and 34 does not. In addition, M_5-2,6 similarly inhibited the binding of FAb 8 to these mannans (Fig. 2B,D), indicating that one of the determinants of FAb 8 is a unit consisting of two short α-1,2-linked oligomannosyl side chains and an α-1,6-linked mannose residue that originated from the mannan backbone. However, the possibility remains that FAb 8 requires other component(s), such as a larger molecule(s) composed of side chains joined by more than one α-1,6-linkage, which could not be prepared in this and prior studies. Despite the presence of an α-1,2-linked oligomannosyl side chain in Fr. 1A, the low reactivity of this mannan towards FAb 8 shown in Fig. 1A may be because a proper arrangement of two α-1,2-linked oligomannosyl side chains for the antigenic factor 8 is not widely distributed in this mannan.

Fig. 1C shows the results of ELISA of Fr. A5. These results support the findings of Ataoglu et al. [25] who defined the determinant of FAb 9 as a linear α-1,6-linked mannose polymer. In addition to their findings, the results of ELISA-inhibition assay of Fr. A5 with FAb 9 (Fig. 2C) indicate that the recognition size of FAb 9 is a linear α-1,6-linked oligomannosyl unit consisting of over three residues. However, it should be stressed that the true antigenic determinants of factor 9 are the specific side chains in *Candida guilliermondii* mannan corresponding to Manβ1→2Manβ1→2Manα1→3-Manα1→2Manα1→3Manα1→2Manα1→2Man, Manβ1→2Manβ1→2Manα1→3Manα1→2Manα1→2Man, and their branched forms substituted with an α-1,6-linked mannose residue [5]. We have previously demonstrated that the density of side chains substituting to the α-1,6-linked mannan backbone of *C. albicans* IFO 1060 is remarkably higher than that of *C. guilliermondii* IFO 10278 [5,26]. Therefore, FAb 9 is not monospecific since it was prepared simply by absorbing anti-IFO 10278 whole-cell serum with cells of the IFO 1060 [1].

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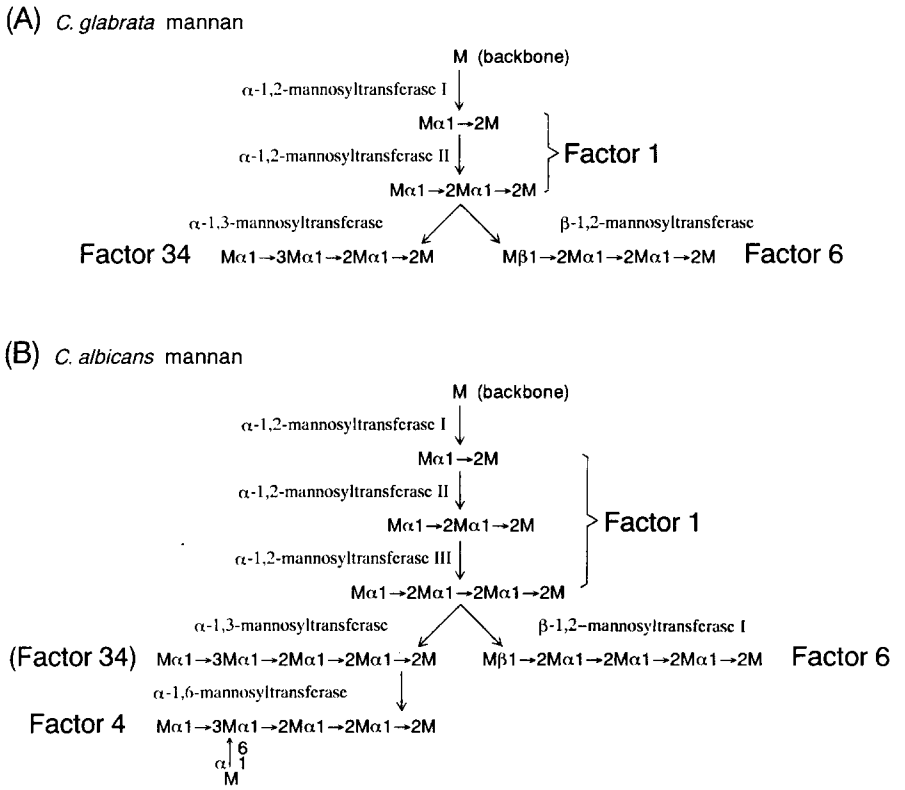

Fig. 3. Postulated pathway for biosynthesis of side chains corresponding to antigenic factors in cell wall mannans of *C. glabrata* (A) and *C. albicans* serotype A (B). M denotes a D-mannose residue.

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