Enzymatic Synthesis of New Oligosaccharides Using Mannosyltransferases from Candida Species and Their NMR Assignments

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The outer layer of the cell wall of pathogenic fungi, Candida species, consists of mannan, which plays an important role in infection. In this study, we synthesized several oligosaccharides using mannosyltransferases obtained from Candida parapsilosis and Candida albicans. Namely, we synthesized mannotetrose [Manα1→2Manα1→2Man and Manα1→β(Manα1→6)Manα1→2Man] from mannotriose, Manα1→β(Manα1→2Man, and mannohexaoses [Manα1→2Manα1→3Manα1→3Manα1→2Manα1→2Man and Manα1→β(Manα1→6)Manα1→3Manα1→2Manα1→2Man] from mannopentaose. Manα1→3Manα1→2Manα1→2Man→2Man. The linkage sequence of these oligosaccharides was identified by a sequential 1H-NMR assignment method combined with rotating frame nuclear Overhauser enhancement spectroscopy and relayed coherence transfer spectroscopy. The steric effect by the α-1,6-linked branching mannose residue to the H-1 proton chemical shift of the neighboring 3-O-substituted mannose residue was different from that of the 2-O-substituted mannose residue. These oligosaccharides having novel structures seem to be useful as the substrate or ligand for glycocomics.

Key words Candida albicans; mannosyltransferase; NMR; oligosaccharide

Candida albicans is part of the normal human microbial flora that colonizes the mucocutaneous surfaces of the oral cavity, gastrointestinal tract, and vagina. In immunocompromised or intensive-care patients, increased mucosal proliferation secondary to the use of antibiotics, together with reduced host defenses and physical alteration of the mucosal barriers, may result in bloodstream invasion. The high levels of morbidity and mortality induced by C. albicans in hospitalized patients mean that this species is now one of the most prominent human pathogens.11

The outermost layers of the C. albicans cell wall are made of mannoprotein referred to as mannan. Mannan has been shown to play a role in host cell adhesion, immunomodulation, and antigenic variability.2–4 Furthermore, gene deletion experiments demonstrated that the mannan is important for the adherence to host cells and overall virulence.5–7 Mannan consists of a linear α-1,6-linked mannose polymer backbone with side chains composed of α-1,2-, α-1,3-, α-1,6-, and β-1,2-linked mannosides. The side chain mannooligosaccharide sequences are involved in the adhesion or attachment of C. albicans to the macrophage, dendritic cell, or epithelial cell through binding to the manno-receptor, dectin-2, the dendritic-cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin (DC-SIGN), and mannan-binding protein.9 The identification of pathogen-associated molecular patterns is controlled by pattern recognition receptors, such as the C-type lectin receptor, which are carbohydrate-recognizing proteins and mediate the pathogen recognition/clearance, immune cell interactions, and endothelium cell adhesion.

In the case of Saccharomyces cerevisiae, the side chains of the mannan consist only of α-1,2- and α-1,3-linkages. On the other hand, the side chains of the mannans of many Candida species have branched structures with α-1,6-linkages. Especially, the C. albicans,10 C. stellatoidea,11 and C. guilliermondii12 mannans contain many α-1,6-branched side chains. On the other hand, some Candida species, such as C. parapsilosis,10 do not contain a branched side chain, indicat

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Materials, Strains and Culture Conditions Substrate oligosaccharides, mannotriose (Manα1→3Manα1→2Man) and mannopentaose (Manα1→3Manα1→3Manα1→2Manα1→2Man) were prepared from the Saccharomyces cerevisiae serotype 1a mannan by acetylation.16 These oligosaccharides were isolated from the acetylate by HPLC and their structures were confirmed by 1H-NMR. The C. albicans NIH B-792 (serotype B) strain was the same specimen used in a previous study.10 The C. parapsilosis NBRC 0585 strain was obtained from the NITE Biological Resource Center (NBRC) of the National Institute of Technology and Evaluation (NITE). Yeast cells were grown at 28°C with shaking in

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yeast extract-peptone-dextrose (YPD) liquid medium (0.5% yeast extract, 1% peptone, 2% glucose) for 24 h.

**Enzyme Preparation** The preparation of the mannosyltransferase fractions of the *C. albicans* and *C. parapsilosis* cells was carried out by the following method as described in a preceding paper. The cells were grown in the YPD medium at 28 °C until the mid-exponential-growth phase. The cells were then harvested and washed with 5 mM Tris–HCl (pH 7.5) by centrifugation. The cells were resuspended in 15 ml of 5 mM Tris–HCl (pH 7.5) containing 3 mM MgCl2, 0.5% glycerol, 1.0% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) then homogenized with glass beads using a Bead Beater (Biospec Products, U.S.A.). The homogenate was centrifuged for 20 min at 15000×*g*, and the supernatant was further centrifuged for 1 h at 105000×*g*. The pellet was resuspended in 0.5 ml of 5 mM Tris–HCl (pH 7.5) containing 1% Triton X-100 and 1 mM PMSF and extracted for 2 h at 4 °C. The mixture was centrifuged for 60 min at 105000×*g*. The pellet was used as the enzyme fraction for the mannosyltransferase. This crude preparation seems to contain more than 30 mannosyltransferases. However, only two enzymes, an α-1,2-mannosyltransferase and an α-1,6-mannosyltransferase, require a non-reducing terminal α-1,3-linked mannose residue in the substrate oligosaccharides. Furthermore, it is conceivable that the activity of the two enzymes in the crude preparations from *C. albicans* serotype B and *C. parapsilosis* is significantly different. The crude preparation from *C. albicans* serotype B specifically contains the α-1,6-mannosyltransferase, while that from *C. parapsilosis* mainly contains the α-1,2-mannosyltransferase.

**Enzymatic Synthesis of Oligosaccharides Using Mannosyltransferase** The enzyme reaction was carried out in a total volume of 500 µl containing 5 mM mannotriose or mannopentaose, the enzyme fraction (6 mg of protein), 50 mM Tris–maleate buffer (pH 6.0), 10 mM MnCl2, 20 mM guanosine 5′-diphosphate (GDP)-mannose, 0.3% Triton X-100, and 1 mM PMSF. After incubation for 48 h at 30 °C, the reaction was stopped by boiling. After removal of the denatured protein by centrifugation, the reaction mixture was fractionated by HPLC. The enzyme reaction product was pooled, lyophilized, and used for the 1H-NMR analysis.

**Nuclear Magnetic Resonance Spectroscopy** Samples were exchanged twice in 2H2O with intermediate lyophilization, then dissolved at 1% (w/v) in 2H2O (99.97% atom 2H). The NMR spectra were recorded using JNM-LA400 spectrometers (JEOL, Tokyo, Japan) at 45 °C. The total correlation spectroscopy (TOCSY), rotating frame nuclear Overhauser enhancement spectroscopy (ROESY), two-dimensional 1H–1H correlated spectroscopy (COSY), and relayed coherence transfer spectroscopy (relayed COSY) were performed using the standard pulse sequence. The proton chemical shifts were referenced relative to the internal acetone at δ 2.225.

**RESULTS AND DISCUSSION**

The cell wall mannans of *C. albicans* serotype B and *C. parapsilosis* have a different structure. Although the former mannan contains α-1,6-branched side chains, the latter one does not. Furthermore, the α-1,3-linked mannose residues in the former mannan are present at the non-reducing terminal of the side chains, however, in the latter mannan, the α-1,3-linked mannose residues are fully substituted by the α-1,2-linked mannose residues. This difference in the structure indicates that *C. albicans* serotype B specifically contains an α-1,6-mannosyltransferase responsible for the synthesis of the α-1,6-branched side chains. On the other hand, *C. parapsilosis* contains a strong α-1,2-mannosyltransferase activity. Both mannosyltransferases require the non-reducing terminal α-1,3-linked mannose residue (Fig. 1). Taking advantage of the difference in the two mannosyltransferase activities in these species, we synthesized the mannoooligosaccharides with different structures. For the *C. albicans* mannan, the non-reducing terminal α-1,3-linked mannose residue is present in the pentaose side chains. On the other hand, for the *C. guilliermondii* and *S. cerevisiae* mannans, the α-1,3-linked mannose residue is present in the tetraose side chains. In these species, the α-1,6-mannosyltransferase reacts with these α-1,3-linkage-containing side chains to construct the corresponding α-1,6-branched side chains. Therefore, for the synthesis of the new oligosaccharides which have not been found in the cell wall mannans of the yeast, we used the α-1,3-linkage-containing triose (Manα1→3Manα1→2Man) and the consecutive α-1,3-linkage-containing pentaose (Manα1→3Manα1→3Manα1→2Manα1→2Man).

The mannotriose was first incubated with the enzyme fraction obtained from the *C. parapsilosis* or *C. albicans* for 48 h. The HPLC profile of each reaction product indicated that 30—40% of the substrate was transformed into mannotetraose (Fig. 2). The enzymatically synthesized mannotetraoses using the enzyme fraction from *C. parapsilosis* and *C. albicans* were designated as pManα, and aManα, respectively. The structure of the mannotetraoses was analyzed by a sequential 1H-NMR assignment method using the nuclear Overhauser effect (NOE). Figure 3 shows the partial ROESY spectra of the synthesized mannotetraoses. The boxed regions in Fig. 3 indicate intraresidue H-1–H-2 or H-1–H-3 connectivities, which were confirmed by COSY, relayed COSY, and TOCSY. On the other hand, the cross-peaks labeled with primed letters indicate interresidue H-1–H-2′ or H-1–H-3′ connectivities between the two adjacent mannose residues. The numbers on the labels indicate the corresponding ring protons. Figure 3B indicates the assignment of
pMan₄. Since the H-1–H-2-correlated cross-peak A₂ indicates the H-2 chemical shift of Man-A, the NOE cross-peak A₂' between the H-2 of Man-A and H-1 of Man-B was assigned. Similarly, the NOE cross-peak B₃' between the H-3 of Man-B, which was assigned from cross-peak B₃, and the H-1 of Man-C was assigned. Using this procedure, we could sequentially assign the H-1 signal from Man-A to Man-D as A₂–A₂'–B₃–B₃'–C₂–C₂'–D₂, and confirmed the following linkage sequence.

\[
\text{Man}_1 \rightarrow 2\text{Man}_1 \rightarrow 3\text{Man}_1 \rightarrow 2\text{Man}
\]

On the other hand, aMan₄ exhibits a signal corresponding to an α-1,6-linked mannose residue at 4.902 ppm (Fig. 3C). The attachment point of the mannose residue was determined to be Man-B based on the apparent downfield shift of the ring proton cross-peaks of Man-B in the TOCSY spectrum followed by the method of Hernandez et al.²⁷ Therefore, we determined the structure of aMan₄ as follows:

\[
\text{Man}_1 \rightarrow 3(\text{Man}_1 \rightarrow 6)\text{Man}_1 \rightarrow 2\text{Man}
\]

The reaction product from the enzyme prepared from \textit{C. albicans} serotype B predominantly contains the α-1,6-branched oligosaccharide. We did not detect any linear oligosaccharides in the reaction product. This result suggests that the activity of the α-1,2-mannosyltransferase in \textit{C. albicans} serotype B is fairly low compared to that in \textit{C. parapsilosis}. A substrate competition by the α-1,6-mannosyltransferase also seems to be involved in the predominance of the α-1,6-branched oligosaccharide in the reaction product.

Similarly, the incubation of mannopentaose with the enzyme fraction obtained from \textit{C. parapsilosis} or \textit{C. albicans} gave the mannohexaoses, pMan₆ and aMan₆, respectively. Figure 4B indicates the assignment of pMan₆. Using the same procedure, we could sequentially assign each proton from the H-1 of Man-A to Man-F. It has been shown that the H-1 signal of the α-1,3-linked mannose residue substituted by an α-1,2-linked residue causes a downfield shift (\( \Delta \delta = +0.24 \) ppm).²⁸ As shown in Fig. 4B, the Man-E of pMan₆ appeared at 5.359 ppm, which is 0.23 ppm more downfield than that of the substrate mannopentaose at 5.134 ppm. Moreover, the Man-F of pMan₆ exhibits a signal at 5.046 ppm, which

Fig. 2. Representative Elution Pattern of Oligosaccharides Obtained from the Enzyme Reaction

Mannotriose was incubated with the enzyme fraction prepared from \textit{C. parapsilosis} or \textit{C. albicans} serotype B at 30 °C for 48 h. HPLC was carried out using a column of amide-80. Elution was carried out with acetonitrile/water (52/48, v/v) and the eluate was monitored using a differential refractometer.

Fig. 3. Partial ROESY Spectra of pMan₄ (B) and aMan₄ (C) Obtained from Mannotriose (A) by the Action of the Enzymes Prepared from \textit{C. parapsilosis} and \textit{C. albicans} Serotype B, Respectively

The primed letters indicate the interresidue H-1–H-2' or H-1–H-3' NOE cross-peaks and the unprimed letters indicate the H-1–H-2 or H-1–H-3-correlated cross-peaks, which are boxed, due to J-coupling. The arrows indicate the direction of the sequential connectivity from the reducing terminal residue to the non-reducing terminal residue.
corresponds to a non-reducing terminal α-1,2-linked mannose residue. Based on these results, the Man-E of pMan₆ is substituted by the α-1,2-linked mannose residue, Man-F. Therefore, we determined the structure of pMan₆ as follows:

\[
\text{Man} \alpha_1 \rightarrow 2 \text{Man} \alpha_1 \rightarrow 3 \text{Man} \alpha_1 \rightarrow 2 \text{Man}
\]

The assignment results of these oligosaccharides are shown in Table 1. In a previous study, we found that the H-1 signal of the neighboring α-1,2-linked mannose residue of the 3,6-di-O-substituted one showed an upfield shift (Δδ = -0.08 ppm) due to the steric effect by the α-1,6-linked mannose residue. However, in the spectrum of aMan₄ and aMan₆, a small

Table 1. Proton Chemical Shifts of Mannooligosaccharides Obtained by Enzyme Reaction

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<th>Sugar residue</th>
<th>Proton</th>
<th>Chemical shift (ppm)</th>
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<td>aMan₄</td>
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The arrows indicate the direction of the sequential connectivity from the reducing terminal residue to the non-reducing terminal residue.
downfield shift (\(\Delta \delta = +0.01 \text{ ppm}\)) was found only for the 3,6-di-\(\text{O}\)-substituted mannose residue instead of the neighboring ones, indicating that the 3-\(\text{O}\)-substituted mannose residue is less affected by the presence of the 3,6-di-\(\text{O}\)-substituted one.

In this study, we synthesized four new oligosaccharides by the action of the enzyme fractions obtained from the *C. parapsilosis* and *C. albicans* strains. Since we found many characteristic side chain structures in the mannans of *Candida* species, we will be able to synthesize a number of novel oligosaccharides using various mannosyltransferases in these strains. These oligosaccharides seem to be useful for studying not only the substrate specificity of the mannosyltransferases, but also the binding specificity of animal and plant lectins. Man\(\alpha1\rightarrow3\)Man\(\alpha1\rightarrow6\)Man is present as a structural element in all of the \(N\)-linked oligosaccharides of glycoproteins, and the binding between the mannotriose and concanavalin A\(^{20,21}\) or DC-SIGN\(^{22,23}\) has been well studied. Animal lectin mediates the cellular recognition in the immune system, fertilization, cancer, infections, embryogenesis, and transport and targeting of glycoproteins. Therefore, the recognition process must be delineated to capitalize on the knowledge about the molecular recognition specificity for drug design.

*C. albicans* cells contain more than 30 mannosyltransferases and it is difficult to purify a specific mannosyltransferase from the cell homogenate. The function of some enzymes was studied using the deletion mutant of the genes. These studies indicated that some mannoooligosaccharide side chains are correlated with the pathogenicity of the *C. albicans* cells.\(^{7,24}\) However, there is no study for the substrate specificity of these enzymes especially for the *C. albicans* specific mannosyltransferases. To characterize these mannosyltransferases, we need to use the purified recombinant enzyme. This type of approach must be used for the characterization of the enzymes in the future.

REFERENCES