Characterization of β-1,2-Mannosyltransferase in Candida guilliermondii and Its Utilization in the Synthesis of Novel Oligosaccharides*

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A particulate insoluble enzyme fraction containing mannosyltransferases from Candida guilliermondii IFO 10279 strain cells was obtained as the residue after extracting a 105,000 x g pellet of cell homogenate with 1% Triton X-100. Incubation of this fraction with a manno-Pentaose, Man-a-3-Man-a-6-Man-a-2-Man-a-2-Man, in the presence of GDP-mannose and Mn2+ ion at pH 6.0 gave a third type of β-1,2 linkage-containing mannooligosaccharide, Man-b-2-Man-b-1-3-Man-a-6-Man-a-2-Man-a-2-Man, which has been found to correspond to antigenic factor 9. Incubation of Candida albicans serotype B mannann with the enzyme fraction gave significantly transformed mannann, which contains the third type of β-1,2-linked mannann units.

We have studied the structures of the cell wall mannans of medically important Candida species for several years (1–3) and demonstrated that there are three types of β-1,2 linkage-containing side chains in its cell wall mannans. One is the β-1,2-linked manno-Pentaose, which is located in a phosphodiesterified oligosaccharide moiety, as the common epitope in the mannans of several Candida species (4–9). The second type is β-1,2-linked mannann units attached to the nonreducing terminal of the α-1,2-linked oligomannosyl side chains in the mannans of Candida albicans serotype A (10, 11), Candida tropicalis (12), and Candida glabrata (13). These two β-1,2 linkage-containing epitopes have been identified as corresponding to antigenic factors 5 (14) and 6 (15), respectively. The third type of β-1,2 linkage-containing side chains can be observed in the mnnan of Candida guilliermondii. This type of oligosaccharide contains β-1,2-linked mannann units attached to an α-1,3-linked mannann unit, the presence of which has been demon-

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FIG. 1. Biosynthetic process of mannan side chains. The biosynthetic pathway of the side chains of C. guilliermondii mannan was deduced from the findings of the structural analysis obtained in our laboratory.

Japan). GDP-mannose and jack bean α-mannosidase (EC 3.2.1.24) were obtained from Sigma. The TSK-Gel Amide-80 column (0.46 × 25 cm) and Toyopearl HW-40 (F) gel were obtained from Tosoh Co. (Tokyo, Japan). The cell wall mannan of C. albicans (serotype B) was prepared by the method described by a preceding paper (18). In this text, the oligosaccharides prepared from the mannan of C. albicans, S. cerevisiae, and Candida krusei were labeled with large letters, A, S, and K, respectively. Furthermore, the oligosaccharides synthesized by using enzymes of C. albicans and C. guilliermondii cells were labeled by the addition of small letters, a, g, and r, respectively. Substrate oligosaccharides, Manα1→2Manα1→2Manα1→2Man (AMan3), and Manα1→3Manα1→2Manα1→2Man (AMan4) were prepared from C. albicans mannan (24), Manα1→3Manα1→2Manα1→2Man (SMan4) and Manα1→3Manα1→2Manα1→2Man (SMan5) were from S. cerevisiae serotype 1a mannan (27). Manα1→3Manα1→2Man (KMan3) was from C. krusei mannan.1 Manα1→3Manα1→6Manα1→2Man (KMan3), Manα1→3Manα1→6Manα1→2Man (SMan3), 1 Manα1→3Manα1→6Manα1→2Manα1→2Man (SMan3), and Manα1→3Manα1→6Manα1→2Manα1→2Man (AMan3) were enzymatically prepared from corresponding linear oligosaccharides, KMan3, SMan4, AMan5, and SMan5, respectively, using the α-1,6-mannosyltransferase of C. albicans NIH B-792 cells followed by the method described in a previous paper (23).

Preparation of Pyridylaminol-oligosaccharides—The pyridylamination of the oligosaccharide was performed using the method of Yamamoto et al. (29) as follows: to an oligosaccharide (1 mg), 60 μl of a 2-aminoypyridine solution prepared by dissolving 1 g of 2-aminoypyridine in 0.65 ml of concentrated hydrochloric acid was added. After being sealed, the tube was heated at 90 °C for 10 min. The tube was then opened, and 60 μl of the supernatant of a mixture of 100 mg of sodium cyanoborohydride and 60 μl of water, as the reducing reagent, was added. The tube was resealed and heated at 90 °C for 1 h. The reaction mixture was diluted with 2 ml of water and applied to a column (1 × 50 cm) of Toyopearl HW-40 and separated from free 2-aminoypyridine by elution with 0.1 N ammonium acetate, pH 6.0.

Enzyme Preparation—The preparation of the mannoseyltransferase fraction of C. guilliermondii strain cells was carried out by the method described in a previous paper (22) as follows. The cells were grown in the YPD medium (0.5% yeast extract, 1% peptone, and 2% glucose) at 28 °C until the mid-logarithmic growth phase (A600 = ~6). The cells were then harvested and washed with 5 ml Tris/HCl, pH 7.5, by centrifugation. The cells (about 40 g of wet cells) were resuspended in 15 ml of 5 mM Tris/HCl, pH 7.5, containing 3 mM MgCl2, 0.5% glycerol, 1% 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and homogenized with a Bead Beater (Biospec Products) with 50 g glass beads. The homogenate was centrifuged for 20 min at 5,000 × g, and the supernatants were centrifuged for 20 min at 15,000 × g. Then the supernatants were recovered and were centrifuged for 1 h at 105,000 × g. The pellet was resuspended in 1 ml of 5 mM Tris/HCl, pH 7.5, containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride and extracted for 2 h at 4 °C. The mixture was centrifuged for 60 min at 105,000 × g. The pellet (fraction P) and supernatant (fraction S) were kept at −90 °C and were both assayed for protein contents and mannoseyltransferase activity.

Mannoseyltransferase Assay—The assay mixture containing fraction P (300 μg of protein), 5 mM pyridylaminol-oligosaccharide, 20 mM GDP-mannose donor, 50 mM Tris/maleate, pH 6.0, 20 mM MnCl2, and 0.3% Triton X-100 in a total volume of 25 μl was incubated for 1 h at 30 °C (standard assay). The reaction was initiated by the addition of GDP-mannose and terminated by heating the mixture for 10 min at 100 °C. After removal of the denatured protein by centrifugation, each reaction mixture was analyzed by HPLC as will be described below. The amount of product was estimated by its fluorescence intensity using pyridylaminomannose as a standard.

Analysis of Enzyme Reaction Products by HPLC—An Amide-80 column was used for the normal phase HPLC. The flow solvent was a 35:65 mixture of 3% acetic acid/triethylamine, pH 7.3, and acetonitrile, and the flow rate was 1.0 ml/min at 40 °C. Detection of the pyridylaminol-oligosaccharides was fluorospectrometrically conducted with excitation and emission wavelengths of 320 and 400 nm, respectively.

Large Scale Enzyme Reaction for NMR Analysis—For the NMR analysis, the enzyme reaction was carried out in a total volume of 500 μl containing 5–10 mg of oligosaccharide or mannan, fraction P (about 12 mg of protein), 20 mM GDP-mannose, 50 mM Tris/maleate, pH 6.0, 20 mM MnCl2, and 0.3% Triton X-100. After incubation for 24–48 h at 30 °C, the reaction was stopped by boiling, and the denatured protein was removed by centrifugation. The enzyme-modified oligosaccharide

The abbreviations used are: HPLC, high performance liquid chromatography; NOE, nuclear Overhauser effect; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; relayed COSY, relayed coherence transfer spectroscopy.

1 H. Oyama, H. Kobayashi, M. Suzuki, N. Shibata, S. Suzuki, and Y. Okawa, manuscript in preparation.
was fractionated by HPLC and lyophilized. The enzyme-modified mannan was dialyzed against water and lyophilized.

**NMR Spectroscopy**—All NMR experiments were performed using a JEOL JNM-GSX 400 spectrometer at 400 MHz for $^1$H in D$_2$O at a probe temperature of 45 °C. Acetone (2.217 ppm) was used as the internal standard for the $^1$H NMR.

**Protein Determination**—The protein was determined using the bicinchoninic acid protein assay kit (Pierce) (30) with bovine serum albumin as the standard.

**RESULTS**

**Enzyme Preparation**—The total amounts of protein in the membrane preparations, fractions P and S, prepared from the *C. guilliermondii* IFO 10279 strain cells were 48 and 38.5 mg, respectively. Transferase activities of the two fractions were assayed by incubation with GDP-mannose and pyridylamino-SaMan$_5$. Although the two reaction systems gave the same single product corresponding to pyridylaminomannohexaose by HPLC, the total activity observed in fraction P (2597 nmol h$^{-1}$) was about three times higher than that in fraction S (923 nmol h$^{-1}$). Therefore, we used fraction P as the enzyme preparation for further studies. To determine the structure of the enzyme reaction product by NMR, a large scale reaction mixture with free SaMan$_5$ was incubated for 36 h. The HPLC profile of the reaction products indicated that approximately 50% of the SaMan$_5$ was transformed into a hexaose.

**Sequential NMR Assignment of the Enzyme Reaction Product**—The linkage sequence of the mannohexaose obtained by the enzyme reaction, abbreviated as SagMan$_6$, was analyzed by a sequential NMR assignment method using rotating frame nuclear Overhauser effect spectroscopy (ROESY). This nonempirical assignment method has been demonstrated to give satisfactory results (8, 18, 24, 25, 31). The H-1 signal of the reducing terminal $\alpha$-mannose unit ($\delta = 5.35$ ppm) was able to be empirically assigned. Therefore, we started the sequential assignment of SagMan$_6$ from the reducing terminal mannose unit. The boxed regions in Fig. 2 indicate intraresidue H-1-H-2 or H-1-H-3 connectivities, which were confirmed by relayed coherence transfer spectroscopy (relayed COSY) and two-dimensional homonuclear Hartmann-Hahn spectroscopy (HOHAHA). On the other hand, cross-peaks labeled with primed letters indicate interresidue H-1-H-2 or H-1-H-3 connectivities between two adjacent mannose units. The numbers on the labels indicate the corresponding ring protons. Fig. 2, B and C, shows partial two-dimensional HOHAHA and ROESY spectra, respectively, of SagMan$_6$. 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 the H-1 of Man-B was easily assigned. Similarly, the NOE cross-peak B2 between the H-2 of Man-B, which was assigned from cross-peak B2, and the H-1 of Man-C was assigned. Since Man-C is substituted by an $\alpha$-1,3 linkage, the NOE cross-peak C3 was found through the H-1-H-3-correlated cross-peak C3. Usually, an $\alpha$-1,3 linkage gives weak H-1-H-2 NOE cross-peak in addition...
to strong H-1-H-3' NOE cross-peak. Therefore, we can also assign through the H-1-H-2-correlated cross-peak C2 and the NOE cross-peak C2' between the H-2 of Man-D and the H-1 of Man-C. Additionally, we could find the NOE cross-peak D2' between the H-2 of Man-D and the H-1 of Man-E, of which the signal, 4.764 ppm, corresponds to the β-1,2-linked mannose unit (10, 11, 17). Using this procedure, we could sequentially assign the H-1 signal from Man-A to Man-E as A2-A2'-B2-B2'-C3-C3'-D2-D2'-E2. Therefore, we assumed that Man-D of SagMan6 is substituted by a β-1,2-linked mannose unit, Man-E. It has been shown that the H-1 signal of an α-linked mannose unit substituted by a single β-1,2-linked unit causes a downfield shift (Δδ = 0.09–0.12 ppm) (10, 11, 17) and that the β-1,2-linked mannose units gave H-1-H-5-correlated cross-peaks in a characteristic region (14, 17, 18). In the spectrum of SagMan6, the H-1 proton of Man-D at 5.239 ppm appeared at 0.1 ppm more downfield than that of SaMan5 at 5.129 ppm, and there is a β-mannose specific H-1-H-5-correlated cross-peak. Moreover, SagMan6 resisted digestion with a jack bean α-mannosidase. These data also supported the fact that Man-D of SagMan6 is substituted by the β-1,2-linked mannose unit. Therefore, we determined the structure of SagMan6 to be the following.

\[
\text{Man} \beta_1-2 \text{Man} \alpha_1-3 \text{Man} \beta_1-2 \text{Man} \alpha_1-2 \text{Man} \\
\uparrow 6 \\
\text{Man} \alpha_1
\]

**Structure 1**

The same hexaoside has been found in O-linked oligosaccharides (16) and acetylosate (17) of the mannan of *S. kluyveri*.

Since these findings indicate that the assay system is able to detect the β-1,2-mannosyltransferase IV, we further examined some properties of this enzyme.

**Optimum pH**—The effect of buffer pH on the transferase activity was studied over the pH range of 3.5–8.5. The β-1,2-mannosyltransferase IV exhibits maximum activity at about pH 6.0 in 50 mM Tris/maleate (Fig. 3).

**Metal Ion Requirement**—The effect of several divalent cations, Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\), on the enzyme activity was studied using their respective chlorides. As shown in Table I, the enzyme activity was enhanced by the addition of Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\). The enzyme activity was completely inhibited by the addition of 20 mM ZnCl\(_2\), and the last activity could not be recovered by the addition of EDTA.

**Enzyme Kinetics**—The enzyme activity is linear for at least 1.5 h at 30 °C under the standard conditions. The Lineweaver-Burk plot of the β-1,2-mannosyltransferase IV for pyridylamino-SaMan5 is shown in Fig. 4. The \(K_m\) and the \(V_{max}\) values for pyridylamino-SaMan5 calculated from this figure were about 18 mM and 200 nmol-mg protein\(^{-1}\) h\(^{-1}\), respectively.

**Substrate Specificity**—To assess the substrate specificity of β-1,2-mannosyltransferase IV, we used the pyridylamino-derivatives of the oligosaccharides prepared from the mannans of *C. albicans*, *C. krusei*, and *S. cerevisiae* and of enzymatically synthesized oligosaccharides as described under “Experimental Procedures.” Table II shows that the oligosaccharides containing an α-1,3-linked mannose unit at the nonreducing terminal can serve as acceptors. Although KaMan4, AaMan4, and SaMan4 can also possibly act as the acceptors of both the β-1,2-mannosyltransferase IV and an α-1,2-mannosyltransferase judging from the structure of *C. guilliermondii* mannan, their enzyme reaction products, designated as KaMan5, AaMan5, and SaMan5, exhibited a signal at about 4.76 ppm in the \(^1\)H NMR spectra (data not shown). Therefore, it is apparent that the transferred mannose unit is attached to each substrate with a β-1,2 linkage. Since these β-1,2 linkage-containing branched oligosaccharides have not been found in the acetylosate of yeast mannans, it is reasonable to say that we could prepare novel oligosaccharides using the β-1,2-mannosyltransferase IV of *C. guilliermondii*. These results indicate that the β-1,2-mannosyltransferase IV requires the nonreducing terminal α-1,3-linked mannose unit as the substrate.

Furthermore, to investigate whether an elongation reaction by the action of the β-1,2-mannosyltransferase IV proceeds, we carried out the enzyme reaction using the β-1,2 linkage-containing oligosaccharide, SagMan5, as the substrate, and confirmed the structure of enzyme reaction product, SaggMan7. The H-1 and H-2 signals of SaggMan7 were sequentially assigned from Man-A to Man-P, A2-A2'-B2-B2'-C3-C3'-D2-D2'-E2-E2'-F2. It has been shown that the H-1 signal of an α-linked mannose unit substituted by two consecutive β-1,2-linked units causes an upfield shift (Δδ = 0.02 ppm) (10, 11, 17, 18). The H-1 proton of the Man-D of SaggMan7 at 5.216 ppm appeared at about 0.02 ppm upfield from that of SagMan5 at 5.239 ppm. Moreover, SaggMan7 exhibits a signal at about 4.84 ppm, which corresponds to two consecutive β-1,2-linked mannose units (10, 11, 17, 18). The elongation of the β-mannose unit was
also confirmed by α-mannosidase treatment. Therefore, we determined the structure of SagMan₆ and SagMan₇ to be the following.

$$\text{Manβ1→2Manβ1→2Manα1→3Manα1→2Manα1→2Man} \quad \uparrow 6$$

$$\text{Manα1}$$

**Structure 2**

The assignment results of SagMan₆ and SagMan₇ are shown in Table III

**Modification of Mannan by the β-1,2-Mannosyltransferases**—On the basis of these results, we performed a large scale enzyme reaction using the acid-treated mannan of *C. albicans* serotype B strain, which possess the nonreducing terminal β-1,2-linked mannose unit, but do not contain the β-1,2-linked mannose unit in the side chains. To confirm the addition of the β-1,2-linked mannose unit to the mannan, we compared the structures of the parent and enzyme modified mannans using two-dimensional HOHAHA (Fig. 5). As shown in Fig. 5B, the reaction product gave cross-peaks 4 and 5, which correspond to the α-1,3-linked mannose units substituted by one and two β-1,2-linked mannose units, respectively (17, 18), concomitant with the appearance of cross-peaks 8, 9, and 10, corresponding to the β-1,2-linked mannose units. However, the reaction product did not give cross-peaks 6 and 7, corresponding to the α-1,2-linked mannose units substituted by one and two β-1,2-linked mannose units, respectively (17, 18). These results demonstrate that the mannan was changed to give complex structure by the action of the β-1,2-mannosyltransferases, i.e. the α-1,3 linkage-containing side chains in the mannan were substituted by the β-1,2-linked mannose units.

**DISCUSSION**

Most of the studies on mannosyltransferases concerning the synthesis of the cell wall mannoprotein of yeast have been done using Saccharomyces cerevisiae (32–47), and some of the structural genes coding for yeast mannosyltransferases have been isolated. OCH1 encodes the initiating mannosyltransferase that adds the first α-1,6-linked mannose unit to a Man₉GlcNAc₃ core oligosaccharide (38, 42). KRE2/MNT1 is an α-1,2-mannosyltransferase gene (39), and Kre2p/Mnt1p is responsible for the addition of mannose units to both N- and O-linked carbohydrate chains (40, 41). The MNN1 gene encodes an α-1,3-mannosyltransferase (43), and Mnn1p is also responsible for the synthesis of N- and O-linked carbohydrate chain (36, 43). Recently, YUR1, KTR1, and KTR2 genes were also found to correspond to mannosyltransferases (45, 46). However, all of these enzymes were for α-mannosyltransferases. In a preceding paper (19), we have reported the β-1,2-mannosyltransferase II of *C. albicans*, the enzyme which transfers one β-1,2-linked mannose unit to a β-1,2 linkage-containing side chain. Since there is no other study on the β-1,2-mannosyltransferase, this is the first report of the β-1,2-mannosyltransferase responsible for the introduction of β-1,2-linked mannose unit to an α-linked manno oligosaccharide.

The substrate specificity study indicates that the β-1,2-mannosyltransferase IV requires the nonreducing terminal α-1,3-linked mannose unit. However, the linkage of the penultimate mannose unit does not affect the substrate activity of the oligosaccharides. A similar substrate specificity has been found on the α-1,6-mannosyltransferase responsible for the synthesis of α-1,6-branched side chains (23). It is of interest to compare

**FIG. 4. Effect of substrate concentration on β-1,2-mannosyltransferase activity.** Assay conditions are the same as those described under “Experimental Procedures” except for the concentration of GDP-mannose, 100 mM, and pyridylamino-SaMan₅.

**TABLE II**

| Substrate (5 mM) | Structure | β-1,2-Mannosyltransferase | α-1,6-Mannosyltransferase |
|-----------------|-----------|---------------------------|--------------------------|
| Pyridylamino-KMan₆ | Man₁→3Man₁→2Man-pyridylamine | 13 | 149 |
| Pyridylamino-AMan₄ | Man₁→2Man₁→2Man₁→2Man-pyridylamine | 0 | 0 |
| Pyridylamino-SMan₄ | Man₁→3Man₁→2Man₁→2Man-pyridylamine | 1 | 41 |
| Pyridylamino-KaMan₄ | Man₁→3Man₁→2Man₁→2Man-pyridylamine | 52 | |
| Pyridylamino-AMan₅ | Man₁→3Man₁→2Man₁→2Man₁→2Man-pyridylamine | 1 | 49 |
| Pyridylamino-SMan₅ | Man₁→3Man₁→2Man₁→2Man₁→2Man-pyridylamine | 6 | 97 |
| Pyridylamino-SaMan₆ | Man₁→3Man₁→2Man₁→2Man₁→2Man-pyridylamine | 60 | |
| Pyridylamino-SaMan₆ | Man₁→3Man₁→2Man₁→2Man₁→2Man-pyridylamine | 48 | |

*The β-1,2-mannosyltransferase and α-1,6-mannosyltransferase activities were calculated from the amount of the jack bean α-mannosidase-resistant and susceptible enzyme reaction products, respectively.*
the substrate recognition mechanisms of the two transferases or with α-1,3-mannosidase (48) or with the α-1,3-linked mannos-specific lectin (28). Since C. guilliermondii cells contain the β-1,2- and α-1,6-mannosyltransferases judging from the structure of its mannan (18), it is predictable that if we incubate an α-1,3 linkage-containing linear oligosaccharide with the enzyme prepared from this strain, we obtain β-1,2 linkage-containing linear oligosaccharides.

In this study, we could synthesize a pure β-1,2 linkage-containing oligosaccharide, Manβ1→2Manα1→3(Mana1→6)-Manα1→2Man, using fraction P prepared from C. guilliermondii cells as the enzyme and Manα1→3(Mana1→6)-Manα1→2Man as the substrate in the presence of MnCl₂ and GDP-mannose. Since the synthesis of the oligosaccharides using specific glycosyltransferases does not require a complicated protecting procedure and does not produce by-products, it is an effective tool for the synthesis of oligosaccharides.

We will be able to synthesize many novel oligosaccharides by taking advantage of the substrate specificity of each enzyme obtained from several Candida species. The novel oligosaccharides seem to be useful for studying not only the substrate specificity of mannosyltransferases but also the specificity of lectins and mannosidases. Furthermore, this approach will become important for the synthesis of the sugar chains of medically important glycoproteins or glycolipids.

The requirement for Mn²⁺, Mg²⁺, or Ca²⁺ by the enzyme was similar to those previously reported for α-mannosyltransferases of S. cerevisiae (32, 33, 35). However, this property of

![Image](212x302 to 558x508)

**FIG. 5. Partial two-dimensional HOHAHA spectra of C. albicans NIH B-792 (serotype B) strain mannan (A) and the enzyme reaction product (B).** Cross-peaks 1, 2, and 3 indicate the presence of α-1,6-linked branching mannose units (23–25), cross-peaks 4–10 indicate the presence of β-1,2-linked mannose units (17–19).
the enzyme is different from that previously reported β-1,2-mannosyltransferase II of C. albicans, which has no absolute requirement for metal ions (22).

In the preceding study (22), we could not detect the β-1,2-mannosyltransferase I that is responsible for the introduction of the first β-1,2-linked mannose unit to an α-1,2-linked mannotetraose side chain of C. albicans. Although the β-1,2-mannosyltransferase IV recognizes only the nonreducing terminal α-1,3-linked mannose unit as identified in this study, the β-1,2-mannosyltransferase I does not seem to require only a nonreducing terminal α-1,2-linked mannose unit. This is because the α-1,2-linked oligomannosyl moiety of the β-1,2 linkage-containing side chains of C. albicans mannan is only tetraose and that of the β-1,2 linkage-containing side chains of the mannans of C. glabrata (13) and Candida lusitaniae are predominantly triose. Therefore, the β-1,2-mannosyltransferases of these species seem to recognize the length of the α-1,2-linked oligomannosyl side chains from the α-1,6-linked backbone mannose unit. Similar correlation seems to be present for the recognition system of the α-1,3-mannosyltransferases of C. albicans and C. guilliermondii or S. cerevisiae.

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