Detection of β-1,2-mannosyltransferase in *Candida albicans* cells

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Abstract **A particulate insoluble fraction from *Candida albicans* J-1012 (serotype A) strain cells was obtained as the residue after extracting a 105,000 × *g* pellet of cell homogenate with 1% Triton X-100. Incubation of this fraction with a mannopentaose, Manβ1→2Manα1→(2Manα1→)$_2$2Man (αβMan$_5$), in the presence of GDP-mannose followed by high performance liquid chromatography showed the formation of a mannohexaose. Analysis of the product by ^{1}H NMR indicates that αβMan$_5$ was changed to Manβ1→2Manβ1→2Manα1→(2Manα1→)$_2$ 2Man (αβMan$_6$). This β-1,2-mannosyltransferase (ManTase) II activity was completely inhibited by Zn^{2+} and was not restored by the addition of EDTA. The corresponding enzyme fraction from *C. albicans* NIH B-792 (serotype B) strain cells, the mannan of which does not possess both the αβMan$_5$ and αβMan$_6$ side chains, also exhibited the same β-1,2-ManTase II activity.**

Key words: *Candida albicans*; β-1,2-Linked mannose; Mannosyltransferase

1. Introduction

Candidiasis is an infectious disease frequently seen in early childhood and in adults with predisposing conditions such as diabetes, cancer, AIDS, and immuno-suppressive therapy [1,2]. *Candida albicans* strains were divided into two serotypes, A and B, by Hasenclever and Mitchell [3] from the antigenicity of cell wall mannans. A structural study of the mannans of the *C. albicans* serotype A and B strains has been achieved during the last several years [4,5], and we have demonstrated the presence of phosphodiesterified β-1,2-linked oligomannosyl moieties as a group of common epitopes throughout the two serotype strains [6,7]. The major difference between the structural features of mannans from *C. albicans* serotype A and B strain cells is the presence of a β-1,2-linked mannose unit attached to an α-1,2-linked one in the side chains of the former mannan strain [8,9], but lacking in the latter ones. We also reported in recent papers that the serotype A-specific β-1,2-linked mannose unit in the mannans of *C. albicans* disappeared by cultivation of the cells under acidic pH [10] or high temperature [11] conditions. Although a single β-1,2-linked mannose unit is present in the cell wall polysaccharides of several bacteria, e.g., *Serratia narcescens* [12] and *Salmonella thompson* [13], consecutive β-1,2-linked mannose units are contained only in the genus *Candida* and several other yeasts. Thus, the detection of the circulating mannan antigen containing β-1,2-linkages in patients' sera by immunological procedures is important for the diagnosis of invasive candidiasis. Furthermore, several workers reported that the β-1,2-linkage-containing side chains participate in adherence of *C. albicans* cells to mammalian cells in the initial step of *Candida* infection [14,15]. Therefore, we tried to detect and characterize a β-1,2-mannosyltransferase (ManTase) which is responsible for the biosynthesis of the serotype A specific epitope of *C. albicans* mannan. The biosynthetic pathway of the serotype A-specific side chain can be depicted as shown in Fig. 1 based on the structural analysis findings of *C. albicans* mannans [4]. Namely, the first transfer of the β-1,2-linked mannose unit from GDP-mannose to the non-reducing terminal site of the α-1,2-linked mannotetraosyl side chain takes place by participation of the β-1,2-ManTase I. However, since β-1,2-ManTase I and α-1,3-ManTase compete for the acquisition of the α-1,2-linked mannotetraosyl side chain as the common acceptor [16], it appears difficult to easily detect the activity of the β-1,2-ManTase I. Therefore, we first attempted to detect a β-1,2-ManTase II by taking advantage that Manβ1-2Manα1-(2Manα1-)$_2$ 2Man (αβMan$_5$) works as the substrate of only the β-1,2-ManTase II.

2. Materials and methods

2.1. Materials

2-Aminopyridine was obtained from Nakarai Tesque (Kyoto, Japan) and was recrystallized from *n*-hexane. Pyridylaminated (PA) mannose was purchased from Takara Shuzo Co. (Kyoto, Japan).

The *C. albicans* J-1012 (serotype A), NIH B-792 (serotype B), and *Candida stellatoidea* IFO 1397 (synonym of *C. albicans* serotype B) strain cells were the same specimens used in the previous studies [6,17]. GDP-mannose was obtained from Sigma Products. The TSK-Gel Amide-80 column (0.46 × 25 cm) and TSK-Gel HW-40 were obtained from Tosoh Co. (Tokyo, Japan).

2.2. Preparation of the substrates

Manα1-(2Manα1-)$_2$2Man (αMan$_4$) was prepared from the mannan of *C. albicans* J-1012 strain cells by acetolysis under the conventional conditions [18]. Manβ1-2Manα1-(2Manα1-)$_2$2Man (αβMan$_5$), and Manβ1-2Manβ1-2Manα1-(2Manα1-)$_2$2Man (αβMan$_6$), Manβ1-2Manα1-2Man (αβMan$_3$), Manβ1-2Manα1-3Manα1-2Manα1-2Man (αβSMan$_5$) were prepared from the mannans of *C. albicans* J-1012 [9], *Pichia pastoris* IFO 0948 [19], and *Saccharomyces kluyveri* IFO 1685 [20] strain cells, respectively, by acetolysis under mild conditions followed by α-mannosidase digestion. β-1,2-Linked mannooligosaccharides, Manβ1-2Man (βMan$_2$), Manβ1-2Manβ1-2Man (βMan$_3$), and Manβ1-(2Manβ1-)$_2$2Man (βMan$_4$) were prepared from the mannan of *C. albicans* NIH B-792 strain cells by treatment with 10 mM HCl at 100°C for 60 min [7,21]. PA-oligosaccharides were prepared and purified in accordance with the description by Hase et al. [22].

2.3. Enzyme preparation

C. albicans J-1012 strain cells were grown to the stationary phase in 500 ml of YPD medium (0.5% (w/v) yeast extract, 1% (w/v) peptone,

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Abbreviations: Tris, tris-hydroxymethylaminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; PA, 2-aminopyridine.

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and 2% (w/v) glucose) at 28°C, and then about 20-ml aliquots of the culture were used to inoculate each of the 14 flasks containing 250 ml of YPD medium. The culture was grown with agitation to the mid-logarithmic growth phase (OD_{600} = ~6). The medium was removed by centrifugation, and the harvested cells were washed once with saline and then washed twice with 5 mM Tris-HCl buffer (pH 7.5) by centrifugation. The washed cells (about 40 g wet weight) were resuspended in 15 ml of 5 mM Tris-HCl buffer (pH 7.5), containing 3 mM $MgCl_2$, 0.5% glycerol, 1.0% β-mercaptoethanol, 1 mM PMSF and homogenized by a Bead Beater (Biospec Products, Bartlesville, OK) with 50 g of glass beads. The homogenate was centrifuged for 20 min at 15,000 × *g*, and the supernatants were centrifuged for 1 h at 105,000 × *g*. The supernatant was discarded, and 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. A prolonged extraction period did not afford higher enzyme activity in the supernatant. 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 ManTase activity.

2.4. ManTase assay

The assay mixture containing fraction P (80 μg protein), 5 mM PA-oligosaccharide, and 20 mM GDP-mannose donor, and 50 mM Tris-maleate buffer (pH 7.0), containing 20 mM $MnCl_2$ and 0.3% (w/v) Triton X-100 in a total volume of 25 μl, and 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 PA-mannose as a standard.

2.5. Analysis of products by HPLC with PA-oligosaccharides

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

2.6. Preparation of products for NMR analysis

For NMR analysis, the enzyme reaction was carried out in a total volume of 500 ml, containing 5 mM of $\alpha\beta Man_5$, fraction P (about 3.2 mg of protein), 50 mM Tris-maleate buffer (pH 7.0), 20 mM $MnCl_2$, 20 mM GDP-mannose, 0.3% (w/v) Triton X-100, and 1 mM PMSF. The reaction was stopped after 36 h of incubation at 30°C by boiling. After removal of the denatured protein by centrifugation, the reaction mixture was fractionated by HPLC. The eluate corresponding to the reaction product was pooled, freeze-dried and used for NMR analysis.

2.7. Nuclear magnetic resonance spectroscopy

^1H NMR and 2D-HOHAHA spectra of the oligosaccharides were recorded using a JEOL JNM-GSX 400 spectrometer operating at 400 MHz at a probe temperature of 45°C. Each mannooligosaccharide was dissolved in D_2O and acetone was used as the internal standard (2.217 ppm).

2.8. Another method

The protein was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co.) [23] with bovine serum albumin as the standard.

3. Results and discussion

Although several reports on ManTases concerning the synthesis of cell wall mannoprotein of *S. cerevisiae* [24–27] have been published, all of these reports were investigated for characterization of α-ManTases. On the contrary, this report can be regarded as prior evidence for the existence of β-1,2-ManTase throughout all yeasts. In order to find the first clue of a series of biosynthetic studies of β-1,2-linkage-containing fungal mannans, we first tried to detect the β-1,2-ManTase II, the substrate of which does not work like that of the other transferases (Fig. 1). The membrane preparations, fractions S and P from the *C. albicans* J-1012 strain cells, were incubated with GDP-mannose and free $\alpha\beta Man_5$. Each reaction mixture was analyzed by HPLC. Both reaction systems gave the same single product corresponding to mannohexaose. Furthermore, another reaction system using PA-$\alpha\beta Man_5$ gave a similar result, producing the corresponding PA-mannohexaose as the sole product. Since the β-1,2-ManTase II activity was observed mainly in fraction P, we used this fraction as the enzyme preparation for further studies.

To determine the structure of the enzyme reaction product,

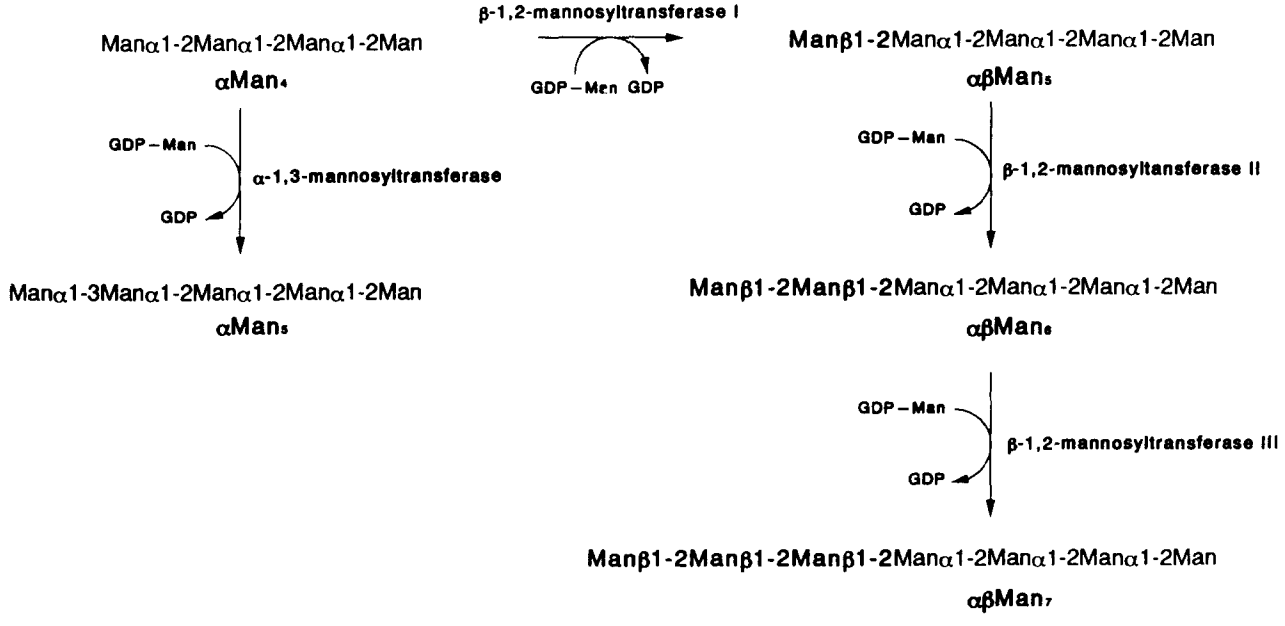

Fig. 1. Biosynthetic process of mannan side chains. The pathway for the synthesis of the side chain of *C. albicans* mannan was deduced from the findings of structural analysis obtained in our laboratory.

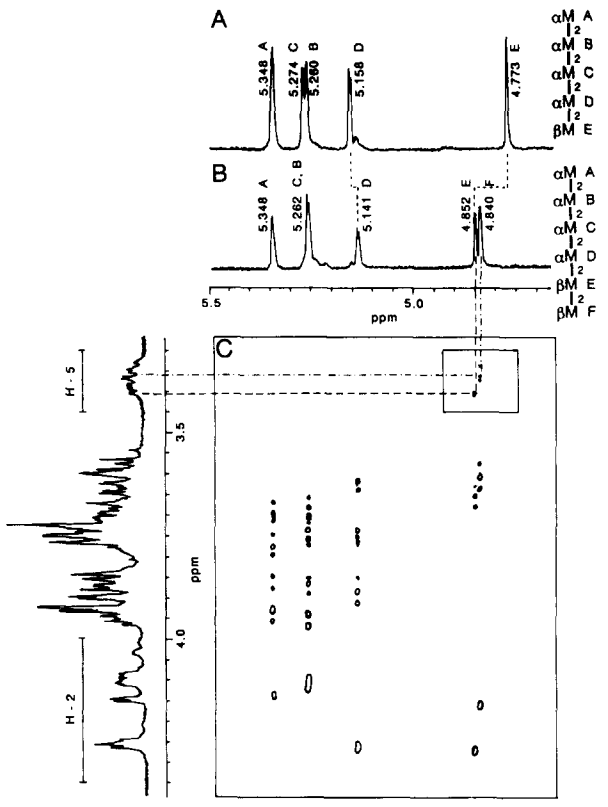

Fig. 2. ^{1}H NMR spectra of $\alpha\beta$Man$_5$ and the enzyme reaction product. 1D-NMR of $\alpha\beta$Man$_5$ (A), the reaction product (B), and 2D-HOHAHA of the reaction product (C) are shown. Boxed cross-peaks indicate the H-1-H-5 correlated ones of β-D-mannose units [23,24]. The reaction was carried out at 30°C for 36 h by using $\alpha\beta$Man$_5$ as the substrate and fraction P prepared from *C. albicans* J-1012 (serotype A) strain cells as the enzyme. Spectra were recorded using a JEOL JNM-GSX 400 spectrometer in D_2O solution at 45°C using acetone as the standard (2.217 ppm).

mannohexaose, by ^{1}H NMR, the large scale reaction mixtures were incubated for 36 h. The HPLC profile of the reaction products indicated that ca. 50% of the $\alpha\beta$Man$_5$ had been transformed into the hexaose. The H-1 proton chemical shifts in normal NMR (Fig. 2B) and H-1-H-2 and H-1-H-5 correlated

Table 1
Effect of divalent metal ions and EDTA on the β-1,2-mannosyltransferase activity in fraction P

Metal salt (20 mM)	Mannose incorporated (nmol/mg protein/h)
None	13 (100)*
$MnCl_2$	15 (115)
$MgCl_2$	16 (123)
$ZnCl_2$	0 (0)
$CaCl_2$	14 (108)
$NiCl_2$	12 (92)
EDTA	13 (100)
$ZnCl_2$, EDTA**	0 (0)

Note: The reaction conditions were the same as those given under 'Materials and methods' except that the additional divalent metal ion to the reaction mixture was varied as indicated.
*Relative activity (%).
**After 3 h incubation at 30°C with 20 mM $ZnCl_2$, the same amount of 40 mM EDTA was added.

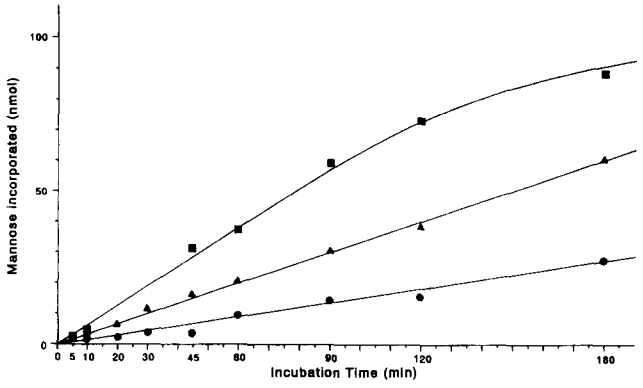

Fig. 3. Effect of protein concentration and incubation time on β-1,2-ManTase II activity. Assay conditions are described in the 'Materials and methods' except for the concentration of the enzyme protein and the incubation time. Enzyme protein: 80 μg (●), 130 μg (▲), 300 μg (■).

cross-peaks in 2D-HOHAHA (Fig. 2C) of the reaction product completely consistent with those of $\alpha\beta$Man$_6$ [9,20], prepared from the mannan of *C. albicans* J-1012 strain cells by mild acetolysis. Especially the downfield shifted H-2 proton chemical shifts of Man-D and Man-E, 4.26 ppm, indicate that these two mannose units were substituted by β-1,2-linkage. Therefore, it is apparent that a signal at 4.773 ppm in the $\alpha\beta$Man$_5$ acceptor was downfield shifted to 4.852 ppm by the addition of a β-1,2-linked mannose unit. The hexaose structure has also been confirmed to be $\alpha\beta$Man$_6$ by its inertness to the digestion with an exo-α-mannosidase. Since these findings indicate that this enzyme activity was that of the β-1,2-ManTase II, we further examined the properties of this enzyme.

The β-1,2-ManTase II in fraction P exhibits maximum activity at pH 7.0 in 50 mM Tris-maleate buffer (data not shown). This enzyme has no absolute requirement for metal ions similar to those previously observed in some other glycosyltransferases [28,29], i.e., the enzyme activity was not affected by the addition of EDTA and/or divalent cations except for Zn^{2+} to the incubation mixture. The enzyme activity was completely inhibited by the addition of 20 mM $ZnCl_2$, and the lost activity could not be recovered by the addition of EDTA (Table 1). This result suggests that there is the possibility that a thiol residue is close to the active site of this enzyme. These properties of the enzyme are different from those previously reported for various ManTases from *S. cerevisiae* which catalyze the addition of α-1,2-, α-1,3- and α-1,6-linked mannose units to various other oligosaccharide substrates [30]. Under standard conditions, the enzyme activity is proportional to protein concentrations of up to about 300 mg, and the enzyme activity is linear for at least 90 min at 30°C (Fig. 3).

Fig. 4A illustrates the effect of substrate concentration on the β-1,2-ManTase II activity. The plot of the reaction velocity versus different PA-$\alpha\beta$Man$_5$ concentrations gave Michaelis-Menten kinetics. The enzyme activity is proportional to the substrate concentration up to 5 mM. The Lineweaver-Burk plots indicate that the apparent K_m value is 10 mM for PA-$\alpha\beta$Man$_5$ (Fig. 4B).

To assess the substrate specificity of the enzyme, PA derivatives of several acceptor oligosaccharides prepared from the mannan of *C. albicans* were tested (Table 2). The β-1,2-Man-

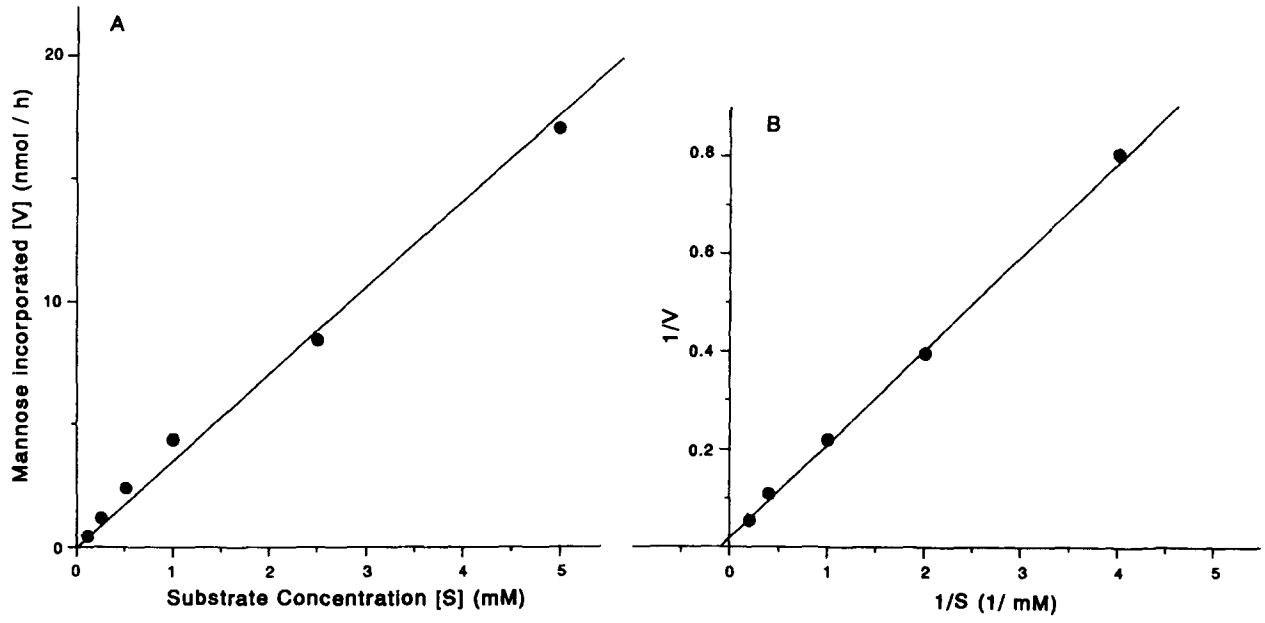

Fig. 4. Effect of substrate concentration on β-1,2-ManTase II activity. Assay conditions are the same as described in the 'Materials and methods' except for the concentration of PA-αβMan$_5$. (A) concentration of PA-αβMan$_5$ vs. velocity of PA-αβMan$_6$ generated, (B) Lineweaver-Burk plots.

Tase II showed high activity to PA-αβMan$_3$ and PA-αβSMan$_5$ as well as PA-αβMan$_5$, but not to PA-αβMan$_6$, PA-αMan$_4$, or any PA derivatives of the β-1,2-linked mannooligosaccharides. This result indicates that the enzyme requires not only the non-reducing terminal β-1,2-linked mannose unit but also a penultimate α-1,2- or α-1,3-linked mannose unit as the substrate. Furthermore, the result that the PA-αβMan$_3$, the reducing terminal of the mannotriose of which is changed to mannitol, also works as a substrate of this enzyme indicates that the third mannose unit from the non-reducing terminal is not essential. Namely, the minimum structural requirement of β-1,2-ManTase II is as follows: Manβ1-2Manα1-.

The β-1,2-ManTase II activity in the cells of *C. albicans* serotype B strain was examined. The enzyme fractions prepared from the homogenate of *C. albicans* NIH B-792 (serotype B) and *C. stellatoidea* IFO 1397 strain cells were incubated in the same assay mixture as described under 'Materials and methods', and the enzyme activity was determined. Since the mannans of these strains do not contain serotype A-specific epitopes corresponding to αβMan$_5$ and αβMan$_6$, as demonstrated in the preceding papers [7,17,31–33], it was unexpected to detect the β-1,2-ManTase II activity in the cells of these strains (Table 2). These results suggest that the lack of the serotype A-specific epitope in the serotype B strain cells is due to the absence of or unreactiveness of the key enzyme, β-1,2-ManTase I activity, and that the origin of the *C. albicans* species was the serotype A strain, and only the β-1,2-ManTase I, the first enzyme for the biosynthesis of serotype A-specific epitope, was changed so as to be unable to act at a point of evolution to separate the serotype B strains. Since the density of the β-1,2-linked mannose units in *C. albicans* mannans is readily affected by morphological changes [31], cultivation conditions, pH [10], temperature [11], etc., comparison with that of the α-linked mannose units, it is reasonable to speculate that the serotype B strains occur using the same mutational change in a transport system which is responsible for the transfer of the β-1,2-Man-

Table 2
Substrate specificity of the β-1,2-mannosyltransferase activity in fraction P

Abbreviation	Substrate (5 mM)	Mannose incorporated (nmol/mg protein/h)		
	Structure	*C. albicans* J-1012	*C. albicans* NIH B-792	*C. stellatoidea* IFO 1397
PA-βMan$_2$	Manβ1-2Man-PA	0 (0)*	–**	–
PA-βMan$_3$	Manβ1-2Manβ1-2Man-PA	0 (0)	–	–
PA-αβMan$_3$	Manβ1-2Manα1-2Man-PA	20 (69)	–	–
PA-βMan$_4$	Manβ1-2Manβ1-2Manβ1-2Man-PA	0 (0)	–	–
PA-αMan$_4$	Manα1-2Manα1-2Manα1-2Man-PA	0 (0)	–	–
PA-αβMan$_5$	Manβ1-2Manα1-2Manα1-2Manα1-2Man-PA	29 (100)	56 (193)	35 (121)
PA-αβSMan$_5$	Manβ1-2Manα1-3Manα1-2Manα1-2Man-PA	29 (100)	–	–
PA-αβMan$_6$	Manβ1-2Manβ1-2Manα1-2Manα1-2Manα1-2Man-PA	0 (0)	–	–

Note: the reaction conditions were the same as those given under 'Materials and methods' except that the substrate was varied as indicated.
*Relative activity (%).
**Not determined.

Tase I to an appropriate membrane, or for the activation of the enzyme. Namely, *C. albicans* serotype B strains can be regarded as a mutant of serotype A strain being affected by the biosynthetic mechanisms of the epitopes containing β-1,2-linked mannose units.

Ballou and his coworkers [34] developed many mutant strains of *S. cerevisiae* species. From the results of the structural and biosynthetic studies of the mannans of the several mutant strains, they reported that these mutants are not simply deficient of ManTases but a mutant for transportation of the same enzymes [25]. In the present study, it was unexpected that only $\alpha\beta Man_5$ was found to act as the substrate of the β-1,2-ManTase to yield $\alpha\beta Man_6$, and αMan_4 did not behave as the substrate of any β-1,2-ManTase. This finding led us to the assumption that β-1,2-ManTase I requires some additional structure(s) to act as the substrate of this enzyme such as α-1,6-linked mannose unit(s) in the core moiety.

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