Substrate specificity of α-1,6-mannosyltransferase that initiates *N*-linked mannose outer chain elongation in *Saccharomyces cerevisiae*

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Abstract **Yeast *Saccharomyces cerevisiae OCH1* gene encodes the mannosyltransferase that is essential for the outer chain elongation of *N*-linked oligosaccharides. Mannosyltransferase activity of *OCH1* gene product (Och1p) was measured on HPLC by using pyridylaminated $Man_8GlcNAc_2$ ($Man_8GlcNAc_2$-PA) as an acceptor and the reaction product was observed at the retention time corresponding to $Man_9GlcNAc_2$-PA. ^{1}H-NMR and fast atom bombardment mass spectrometry (FAB-MS) fragmentation analysis of $Man_9GlcNAc_2$-PA showed that the additional mannose was attached with an α-1,6 linkage at the site where mannose outer chain elongation initiates. Substrate specificity of Och1p was investigated by using various high mannose-type oligosaccharides as acceptors. $Man_8GlcNAc_2$ was the best acceptor for Och1p. The loss of one or two α-1,2-mannoses from $Man_8GlcNAc_2$ reduced the mannosyltransferase activity and the $Man_5GlcNAc_2$ completely lacking α-1,2-mannose residues did not serve as an acceptor. $Man_8GlcNAcOH$ that involves an open sugar ring by reduction of reducing terminal GlcNAc residue did not serve as an acceptor for Och1p. The loss of three mannoses at the α-1,6-branch also reduced the Och1p activity. These results suggest that Och1p is an initiation specific α-1,6-mannosyltransferase that requires the intact structure of $Man_8GlcNAc$ for efficient mannose outer chain initiation.**

Key words: High mannose oligosaccharide; Mannosyltransferase; Substrate specificity; (*Saccharomyces cerevisiae*)

1. Introduction

The *Saccharomyces cerevisiae och1* mutant was isolated as a glycosylation deficient strain in mannose outer chain elongation by ^{3}H suicide method [1]. The *OCH1* gene was isolated by the complementation of temperature-sensitive phenotype of *och1* mutant and this gene product (Och1p) showed a mannosyltransferase activity [2]. The structure of *N*-linked sugar chains from *och1* gene disruptant cells revealed that this strain completely lacks the poly-mannose outer chain [3]. These results suggest that the *OCH1* gene may encode the initiation specific α-1,6-mannosyltransferase.

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Abbreviations: Man, mannose; GlcNAc, *N*-acetylglucosamine; GlcNAcOH, opened form of *N*-acetylglucosamine; PA, 2-aminopyridine; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry; GDP, guanosine-5′-diphosphate; HPLC, high-performance liquid chromatography

In this report, to obtain a direct evidence that Och1p is an initiation specific α-1,6-mannosyltransferase, we measured the mannosyltransferase activity of Och1p using pyridylaminated $Man_8GlcNAc_2$ ($Man_8GlcNAc_2$-PA) as an acceptor and GDP-mannose as a donor and the reaction product ($Man_9GlcNAc_2$-PA) was analyzed by the high resolution ^{1}H-NMR and FAB-MS. These results indicated that the Och1p is identical with the initiation specific α-1,6-mannosyltransferase reported previously [4,5]. We also investigated the substrate specificity of the Och1p for various high mannose-type oligosaccharides to identify the essential sugar structure for recognition by Och1p.

2. Materials and methods

2.1. Strains and media

Saccharomyces cerevisiae strains YS52-1-1B (*och1::LEU2*) [2] and YN5-2C (*och1::LEU2 mnn1 alg3*) [3] were used for preparation of mannoproteins. KK4 [6] was used for preparation of microsomal membranes and for overproduction of Och1p. *S. cerevisiae* cells were grown in YPD (2% Bacto peptone, 1% yeast extract and 2% glucose), SD−leu (0.67% Bacto-yeast nitrogen base w/o amino acids, 2% glucose and 20–400 μg/ml amino acids mixture lacking leucine) [7] and SG+0.5% glucose (0.67% Bacto-yeast nitrogen base w/o amino acids, 2% galactose, 0.5% glucose and 20–400 μg/ml amino acids mixture).

2.2. Preparation of oligosaccharides

Mannoproteins were obtained by hot citrate buffer extraction followed by precipitation with ethanol [8]. *N*-linked oligosaccharides were released from YS52-1-1B and YN5-2C mannoproteins by endoglycosidase H (Seikagaku Kogyo Co.) or glycopeptidase A (Seikagaku Kogyo Co.). Oligosaccharides were used for pyridylamination or separation by high-performance liquid chromatography (HPLC).

The oligosaccharides were labeled with 2-aminopyridine [9,10] by using the commercially available reagent kit (Takara Shuzo Co.). After pyridylamination, the samples were purified by HPLC.

2.3. High-performance liquid chromatography

Separation of oligosaccharides were carried out by HPLC using a Shimadzu LC-6A chromatograph system equipped with a UV spectromonitor or fluorescence spectromonitor RF-530.

Size-fractionation HPLC was performed with Asahipak NH2P-50 (0.46×25 cm) at a rate of 1.0 ml/min [11]. The column was equilibrated with 95% of solvent A (200 mM acetic acid/triethylamine (pH 7.3): acetonitrile = 3:7) and 5% of solvent B (200 mM acetic acid/triethylamine (pH 7.3): acetonitrile = 7:3). After the sample injection, the proportion of solvent B was increased linearly up to 45% for 50 min. PA-oligosaccharides were detected by fluorescence. In separation of non-labeled oligosaccharides, H_2O was used instead of 200 mM acetic acid/triethylamine (pH 7.3) and detected by absorbance of 215 nm.

2.4. Assay of mannosyltransferase activity

Yeast membrane fractions were obtained as described previously [2]. Mannosyltransferase activity was analyzed by the modification method of Nakajima and Ballou [12]. A 200 μg protein of HSP was

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incubated in 50 μl of 50 mM Tris-HCl (pH 7.5), 10 mM $MnCl_2$, 0.6% Triton X-100, 0.5 mM 1-deoxy-mannojirimycin, 0.06 mM acceptor, GDP-[^{14}C]mannose (740 Bq) at 30°C for 30 min. Excess GDP-[^{14}C]mannose was removed by passing the solution through a Dowex 1-X8 column (1 ml). The neutral products were eluted with 1 ml of water and radioactivity was counted in 10 ml of Clear-sol II. In the assay using PA-oligosaccharide as an acceptor, 20 pmol PA-labeled sugar chain and 1 mM GDP-mannose were added to reaction mixture instead of 0.06 mM acceptor and GDP-[^{14}C]mannose. PA-labeled sugar chains were analyzed by size-fractionation HPLC as described above.

2.5. Structural analysis of oligosaccharides

FAB-MS was carried out as described by Reason et al. [5] using a JMS-HX110 mass spectrometer (JEOL Co., Tokyo) equipped with an FAB ion source operated with xenon at 10 kV of accelerating voltage. Peracetylation of $Man_9GlcNAc_2$-PA (reaction product by Och1p) and that of authentic $Man_8GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA were carried out as described by Reason et al. [5] using mixture of trifluoroacetic anhydride and acetic acid (2:1) and subjected to FAB-MS. 3-Nitrobenzyl alcohol was used as a matrix.

^{1}H-NMR (400 MHz) spectrum were measured on a JNM-GX400 (JEOL Co., Tokyo) as described by Nakanishi-Shindo et al. [3]. α-1,2-Mannosidase digestion was carried out as described previously [3].

3. Results and discussion

3.1. Structural analysis of reaction product from $Man_8GlcNAc_2$-PA by Och1p

Pyridylaminated $Man_8GlcNAc_2$ from *och1* disruptant (*Δoch1*) mannoproteins [3] was purified by HPLC and incubated by using yeast microsomal membranes prepared from Och1p overproducing cells (KK4/YEp51-OCH1) [2] as an enzyme source, and GDP-mannose as a mannose donor. The reaction mixture was fractionated by amino column NH2P-50, and $Man_9GlcNAc_2$-PA containing one additional mannose to the ER-core form $Man_8GlcNAc_2$ was observed. This reaction product $Man_9GlcNAc_2$-PA was analyzed by high resolution ^{1}H-NMR (Fig. 1). One additional anomeric proton signal was observed at δ4.922 ppm which is characteristic of α-1,6-linked mannose [13], and anomeric proton signals of No. 9 and No. 11 were shifted from δ5.044 ppm to δ5.043 ppm and from δ5.044 ppm to δ5.035 ppm, respectively, in addition to the other anomeric proton signals whose assignment was shown in Fig. 1 (also see Fig. 3 in [3]). These assignments were identical with the report of Romero and Herscovics [4].

The reaction product $Man_9GlcNAc_2$-PA was subjected to α-1,2-mannosidase digestion and analyzed on size fractionation HPLC. $Man_9GlcNAc_2$-PA peak was shifted to $Man_6GlcNAc_2$-PA (data not shown). This result indicated that all three α-1,2-mannoses which existed on acceptor molecule ($Man_8GlcNAc_2$-PA), were released by α-1,2-mannosidase digestion and that α-1,6-mannose transferred by Och1p was not attached to these α-1,2-mannose residues.

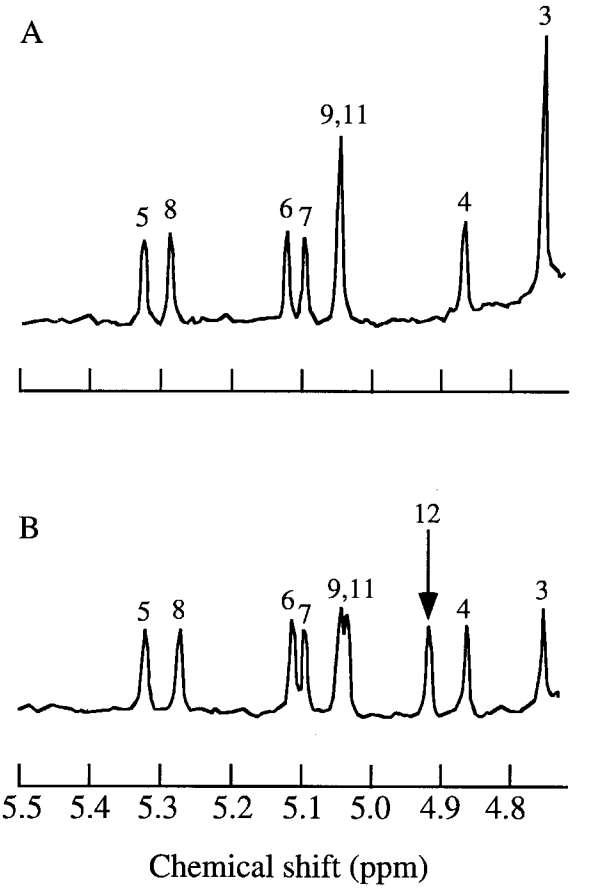


Fig. 1. 400 MHz ^{1}H-NMR chemical shifts of anomeric proton. A: $Man_8GlcNAc_2$-PA (acceptor). B: $Man_9GlcNAc_2$-PA (Och1p reaction product). The individual numbers indicate the anomeric proton signals of mannoses shown in Fig. 2. Arrow in panel B indicates chemical shift of additional mannose by Och1p (δ4.922).

FAB-MS fragmentation analysis of the peracetylated reaction product ($Man_9GlcNAc_2$-PA) was carried out as described by Reason et al. [5]. It was reported that the cleavage of α-1,3 linkage (α-1,3-branch) and α-1,6 linkage (α-1,6-branch) occurred at No. 3 mannose that was linked to GlcNAc with β-1,4 linkage (see Fig. 2) under the measurement conditions [5]. Therefore fragment ions were expected either to contain both 3-hexose ion peak due to α-1,3-branch cleavage and 5-hexose ion peak due to α-1,6-branch cleavage, or to contain only 4-hexose ion peak due to α-1,3- and α-1,6-branch cleavages, dependent on the mannose addition either at α-1,6-branch or at α-1,3-branch, respectively. FAB-MS of reaction product showed 4-hexose ion peak (m/e at 1175 and 1235), but neither 3-hexose ion peak (m/e at 887, 907 and 947) nor 5-hexose ion peak (m/e at 1447, 1463 and 1523) (Table 1).

Table 1
FAB-MS fragment ions of peracetylated authentic $Man_8GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA together with reaction product ($Man_9GlcNAc_2$-PA by Och1p in vitro

	Hex_3[a]			Hex_4			Hex_5		
$Man_8GlcNAc_2$-PA[c]	887	907	947	1159	1175	1235	N.D.[b]	N.D.	N.D.
$Man_9GlcNAc_2$-PA[c]	887	907	947	N.D.	N.D.	N.D.	1447	1463	1523
Reaction product	N.D.	N.D.	N.D.	N.D.	1175	1235	N.D.	N.D.	N.D.

[a]Hex, hexose. Peracetylated Hex_3, Hex_4 and Hex_5 ion peaks were assigned by report of Reason et al. [5].
[b]N.D., not detected.
[c]The structures of authentic $Man_8GlcNac_2$-PA (M8) and $Man_9GlcNac_2$-PA (M9) are shown in Table 3.

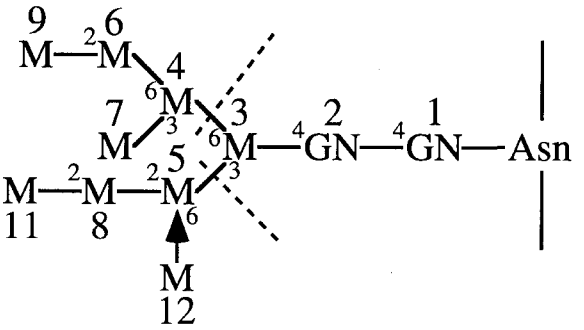


Fig. 2. The position of additional mannose by Och1p and cleavage site by FAB-MS fragmentation. A dotted lines indicate the cleavage site by FAB-MS fragmentation in Table 1. No. 12 mannose is an additional mannose with α-1,6-linkage by Och1p.

This result indicated that α-1,6-mannose residue formed by Och1p was attached to the mannose that was located on the α-1,3-branch (Fig. 2). These combined results indicate the additional mannose should be attached to No. 5 mannose in Fig. 2 with an α-1,6-linkage, the position of which is the same where the initiation of mannose outer chain elongation occurs in vivo [14]. This confirmed that the Och1p is identical to the initiation specific α-1,6-mannosyltransferase reported by Romero and Herscovics [4,5] and further purified by Romero et al. [15].

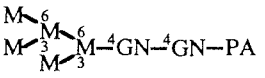
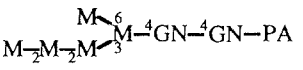
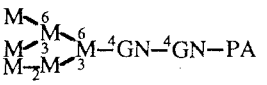
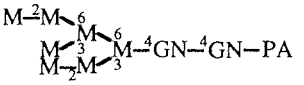
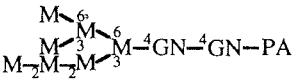
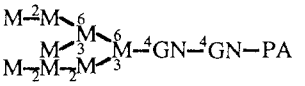
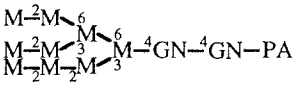
Table 2
Assay of mannosyltransferase activity

Acceptor (0.06 mM)	*Δoch1*	KK4	KK4/YEp51-OCH1
	(pmol mannose/h/mg protein)		
$Man_8GlcNAcOH$	86	78	55
$Man_8GlcNAc$	2	74	524
$Man_8GlcNAc_2$	1	69	501

3.2. *Assay of mannosyltransferase activity using GDP–[^{14}C]mannose as a donor*

To examine further the substrate recognition around the reducing terminus of oligosaccharide, mannosyltransferase activity by Och1p was compared using microsomal membranes obtained from *Δoch1* (*OCH1* gene disruptant), KK4 (single copy of *OCH1* chromosomal gene), and KK4/YEp51-OCH1 (multi-copy of *OCH1* gene) cells [2]. [^{14}C]Mannose was transferred from GDP–[^{14}C]mannose to $Man_8GlcNAc$ and $Man_8GlcNAc_2$ dependent on the amount of supplied Och1p. When $Man_8GlcNAcOH$ (Genzyme Co.) that contains open sugar ring by reduction at the reducing terminus and was described as '$Man_8GlcNAc$' previously [2] was used as an

Table 3
Assay of mannosyltransferase activity

acceptor		% of product formed
M5-1		0
M5-2		8.5
M6		32.1
M7-1		26.3
M7-2		59.5
M8		99.0
M9		98.8

acceptor, the same level of mannosyltransferase activity was observed between these cells (Table 2). Because this mannosyltransferase activity was observed in any cells including *Δoch1*, this may be due to other mannosyltransferase activity, for example, non-specific α-1,2- or α-1,3-mannosyltransferase. These results indicate that the presence of at least one pyranose form of GlcNAc residue attached to β-1,4-linked mannose is essential for the recognition by Och1p.

3.3. *Comparison of mannosyltransferase activity using PA-labeled oligosaccharides as an acceptor*

We have used various pyridylaminated oligosaccharides (PA-labeled oligosaccharides) as an acceptor and assayed mannosyltransferase activity using microsomal membranes prepared from Och1p overproducing cells to examine the substrate specificity. Although each structure of these reaction products was not analyzed except for M8 acceptor, all reaction products were confirmed to be dependent on the amount of Och1p, by using microsomal membranes prepared from wild-type and *Δoch1* cells (data not shown).

One mannose residue was transferred to M8 and M9 more than 98% efficiency during 30 min incubation at 30°C, indicating that M8 and M9 acted as a good acceptor for Och1p (Table 3). The almost same results were obtained by using $Man_9GlcNAc_2$-PA and $Man_{10}GlcNAc_2$-PA, that attached one or two α-1,3-mannoses to $Man_8GlcNAc_2$-PA, as an acceptor (data not shown). The results also showed that the presence of additional α-1,2- or α-1,3-mannose attached to $Man_8GlcNAc_2$-PA did not inhibit the recognition by Och1p. In contrast, M5-1, which completely lacks the α-1,2-mannose residues, was not used as an acceptor (Table 3). However, the M5-2 containing α-1,2-mannose residues at the α-1,3-branch, that was prepared from *alg3 Δoch1 mnn1* triple mutant mannoproteins [3], was weakly recognized by Och1p (the ratio was only 9% of M8). The M6 oligosaccharide, that lacks each α-1,2-mannose residue from both α-1,6- and α-1,3-branches, respectively, was partially recognized by the Och1p (the ratio was 32% of M8). While the M7-1 lacking one α-1,2-mannose residue in the α-1,3-branch was almost the same ratio as the M6 (27% of M8), the mannose transfer ratio in M7-2, that lacks one α-1,2-mannose at the α-1,6-branch, was larger than that of M6 (60% of M8) (Table 3). These results indicate that α-1,2-mannose residue in $Man_8GlcNAc_2$-PA is necessary for the recognition by Och1p, and that α-1,2-mannoses at α-1,3-branch were more important than that at α-1,6-branch for the recognition by Och1p. Since M5-2 was weakly recognized by Och1p, three mannoses at the α-1,6-branch in M8 also contribute some how to the recognition by Och1p.

The existence of GlcNAc, α-1,2-mannoses at α-1,3-branch and several mannoses at α-1,6-branch in $Man_8GlcNAc$ was necessary for a full activity of Och1p (Fig. 2). This indicates that Och1p requires the whole structure of $Man_8GlcNAc$ for the efficient acceptor recognition. This acceptor recognition by Och1p is significantly different from that by other glycosyltransferases so far studied. For example, while mammalian β-1,4-galactosyltransferase recognizes GlcNAc residue and transfers a galactose to this GlcNAc [16], Och1p recognizes not only the residue to which the α-1,6-mannose is added but also the several surrounding residues including GlcNAc to which the β-1,4-linked mannose is added.

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