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# Functional Characterization and Application of the *HpOCH2* Gene, Encoding an Initiating α1,6-Mannosyltransferase, for *N*-glycan Engineering in the Methylotrophic Yeast *Hansenula polymorpha*

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## Abstract

The α1,6-mannosyltransferase encoded by *Saccharomyces cerevisiae* OCH1 plays a key role for the outer chain initiation of the *N*-linked oligosaccharides. A search for *Hansenula polymorpha* genes homologous to *S. cerevisiae OCH1* (*ScOCH1*) has revealed seven open reading frames (ORF100, ORF142, ORF168, ORF288, ORF379, ORF576, ORF580). All of the seven ORFs are predicted to be a type II integral membrane protein containing a transmembrane domain near the amino-terminal region and has a DXD motif, which has been found in the active site of many glycosyltransferases. Among this seven-membered *OCH1* gene family of *H. polymorpha*, we have carried out a functional analysis of *H. polymorpha* ORF168 (*HpOCH2*) showing the highest identity to *ScOCH1*. Inactivation of this protein by disruption of corresponding gene resulted in several phenotypes suggestive of cell wall defects, including hypersensitivity to hygromycin B and sodium deoxycholate. The structural analysis of *N*-glycans synthesized in *HpOCH2*-disrupted strain (*Hpoch2Δ*) and the *in vitro* α1,6-mannosyltransferase activity assay strongly indicate that HpOch2p is a key enzyme adding the first α1,6-mannose residue on the core glycan $Man_8GlcNAc_2$. The *Hpoch2Δ* was further genetically engineered to synthesize a recombinant glycoprotein with the human compatible *N*-linked oligosaccharide, $Man_5GlcNAc_2$, by overexpression of the *Aspergillus saitoi* α1,2-mannosidase with the "HDEL" ER retention signal.

## Introduction

The thermotolerant methylotrophic yeast, *Hansenula polymorpha*, has emerged as a promising host for the high-level expression of heterologous genes, due to its well established expression toolboxes along with the feasibility of high cell density culture in methanol-containing media (Gellissen and Veenhuis, 2001; Gellissen, 2002). Furthermore, the relatively less extensive hyperglycosylation of glycoproteins from *H. polymorpha* than those from *S. cerevisiae* may be another favorable factor for the production of mammalian cells-originated proteins (Rodriguez *et al.*, 1996; Kang *et al.*, 1998), since most therapeutic proteins

originated from mammalian cells require the co- or post-translational addition of proper glycans for the function or stability of the proteins. Recently, we have found out that most *N*-linked glycans synthesized in *H. polymorpha* have core-type structures ($Man_{8-12}GlcNAc_2$) and that the core oligosaccharide $Man_8GlcNAc_2$ is elongated by a single α1,6-linked mannose addition and mainly branched with α 1,2-linkages without hyper-immunogenic terminal α1,3-linked mannose residues (Kim *et al.*, 2004). However, there are limited number of studies on the genes and enzymes involved in *N*-linked glycosylation pathway of *H. polymorpha*.

Here, we describe the isolation and characterization of *H. polymorpha* homolog of *S. cerevisiae* OCH1 and application of the isolated gene to synthesize a recombinant glycoprotein with the human compatible *N*-linked oligosaccharide, $Man_5GlcNAc_2$.

## Results and Discussion

### 1. Identification of the *H. polymorpha* homolog of *S. cerevisiae* OCH1

When open reading frames (ORFs) in the genome of *H. polymorpha* (Ramezani-Rad *et al.*, 2003) were searched for homology to *S. cerevisiae* Och1 protein (ScOch1p), seven ORFs (ORF100, ORF142/OCH1, ORF168, ORF288, ORF379/HOC1, ORF576, ORF580) were found. Among seven ORFs, *H. polymorpha* ORF168 (*HpOCH2*) displayed the highest homology to *ScOCH1* (37% amino acid identity and 54% amino acid similarity). The *Hpoch2Δ* disruptant did not show the growth retardation but exhibited a temperature sensitive growth phenotype. The mutant strain also showed several characteristics associated with defects in cell wall integrity such as hypersensitivity to hygromycin B and sodium deoxycholate (Fig. 1). HPLC analysis on the *N*-glycan structures of recombinant glucose oxidase (rGOD) secreted from the wild type and *Hpoch2Δ* strains showed that the proportion of the larger oligosaccharides corresponding to $Man_{9-14}GlcNAc_2$ was greatly reduced in the *Hpoch2Δ* mutant compared in the wild type strain species (Fig. 2 (a) and (d)). Whereas all of the *N*-glycan were converted to $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ with α1,2-mannosidase digestion in the wild type strain, all the *N*-glycans were converted to $Man_5GlcNAc_2$ in the *Hpoch2Δ* mutant (Fig. 2 (b) and (e)). We have also analyzed the structures of *N*-glycans assembled on the *H. polymorpha* Yps1 protein, which is an aspartic protease predicted to be localized on cell surface by mediation of GPI anchor (Ash *et al.*, 1995), and obtained the same pattern of *N*-glycan profiles as detected with the recombinant GOD (data not shown). These results clearly indicate that the *Hpoch2Δ* mutant has a defect in addition of an α 1,6-mannose onto the core oligosaccharide

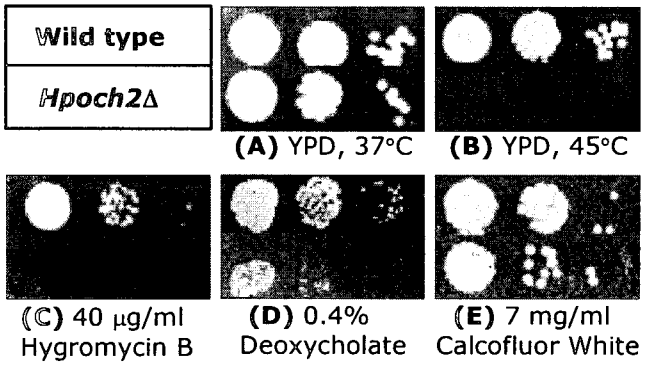


**Fig. 1. Phenotypic analysis of the *Hpoc2hΔ* mutant strain.** YPD plates were incubated at 37°C (A) or at 45°C (B) for 2 days. YPD supplemented with 40 μg/ml hygromycin B (C), YPD with 0.4% sodium deoxycholate (D), and YPD with 7 mg/ml Calcofluor White (E) were incubated at 37°C for 2 days. Yeast cultures ($OD_{600}$=0.1) were diluted serially by 10-fold (from left to right) and spotted.

$Man_8GlcNAc_2$, which is catalyzed by α1,6-mannosyltransferase. Therefore, it is highly likely that HpOch2p functions as an initiating α1,6-mannosyltransferase acting on the initiation of outer chain elongation.

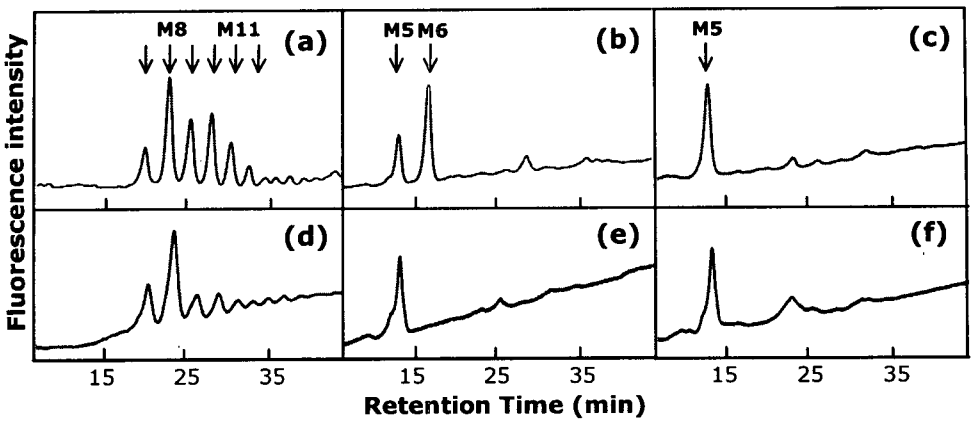


**Fig. 2. HPLC analysis of *N*-linked oligosaccharides assembled on rGOD expressed from the *H. polymorpha* wild type (a, b, and c) and *Hpoch2Δ* (d, e, and f).**
Chromatogram of the *N*-glycan profiles released from rGOD before any treatment (a and d), after α 1,2-mannosidase treatment (b and e), and after subsequent α1,6-mannosidase treatment (c and f). The elution times of authentic PA-sugar chains were indicated by arrows. M5, $Man_5GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA; M11, $Man_{11}GlcNAc_2$-PA.

### 2. HpOch2p has an initiating α1,6-mannosyltransferase activity

We carried out the *in vitro* activity assay for an initiating α1,6-mannosyltransferase using the solubilized membrane franction from the *Scoch1Δmnn1Δmnn4Δ* cells expressing the *HpOCH2* gene. The membrane fraction generated a peak corresponding to $Man_9GlcNAc_2$ from the acceptor oligosaccharide, $Man_8GlcNAc_2$-PA (data not shown), indicating that *HpOCH2* encodes an α1,6-mannosyltransferase as *ScOCH1* does. Moreover, the α1,6-mannosyltransferase activity was also measured with the solubilized membrane fractions prepared from the *H. polymorpha* wild type and *Hpoch2Δ* mutant strains. While a peak corresponding to $Man_9GlcNAc_2$-PA was produced by the membrane fraction from the wild type strain, almost no $Man_9GlcNAc_2$-PA was produced as a reaction product from the *Hpoch2Δ* mutant (data not shown). While a peak corresponding to $Man_6GlcNAc_2$-PA of wild type strain was generated after the α 1,2-mannosidase digestion, all of the oligosaccharide constituents of the *Hpoch2Δ* mutant were changed to $Man_5GlcNAc_2$-PA after the α1,2-mannosidase digestion (data not shown). It suggests that HpOch2p is highly like to be the only initiating α1,6-mannosyltransferase in *H. polymorpha*. At present, only single α 1,6-mannosyltransferase has been reported to be responsible for adding the initiating α1,6-mannose in the outer chain elongation in *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Pichia pastoris* (Nakayama *et al.*, 1992; Yoko-o *et al.*, 2001; Choi *et al.*, 2003). However, we cannot exclude the possibility that other *OCH1* homologs might be involved in the first addition of α1,6-mannose onto the core glycan of certain specific glycoprotein or under certain conditions.

### 3. Expression of the ER-targeted α1,2-mannosidase in *Hpoch2Δ*

As the first step to produce recombinant glycoproteins carrying humanized *N*-glycans in *H. polymorpha*,

the *Aspergillus saitoi* α1,2-mannosidase with the "HDEL" endoplasmic reticulum retention/retrieval signal was introduced into the *Hpoch2Δ* mutant having a defect in the outer chain initiation on the core glycan $Man_8GlcNAc$. The major *N*-glycans on the rGOD produced in the *Hpoch2Δ* mutant without α 1,2-mannosidase-HA-HDEL were heterogeneous, mostly consisting of 8 or more mannoses (data not shown). On the contrary, those produced in the recombinant *Hpoch2Δ* mutant strain expressing the active α 1,2-mannosidase-HA-HDEL were $Man_{5-10}GlcNAc_2$ with the $Man_5GlcNAc_2$ oligosaccharide as a major component (data not shown). When the $Man_{5-10}GlcNAc_2$ oligosaccharides were subjected to *in vitro* α 1,2-mannosidase treatment, the $Man_5GlcNAc_2$ species was generated as the only product of reaction (data not shown). However, $Man_6GlcNAc_2$ in addition to $Man_5GlcNAc_2$ was generated from the *N*-glycan profiles of the wild type strain expressing the active α1,2-mannosidase (data not shown), suggesting that the deletion of the *HpOCH2* gene is required to produce glycoproteins with the human compatible *N*-glycan, $Man_5GlcNAc_2$. The results of our study present the potential of *H. polymorpha* to be developed as a host for the production of therapeutic glycoproteins with humanized oligosaccharides.

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