Characterization of N-linked oligosaccharides assembled on secretory recombinant glucose oxidase and cell wall mannoproteins from the methylotrophic yeast

_Hansenula polymorpha_

Moo Woong Kim²,³, Sang Ki Rhee², Jeong-Yoon Kim³, Yoh-ichi Shimma⁴, Yasunori Chiba⁴, Yoshifumi Jigami⁴, and Hyun Ah Kang¹,²

¹Metabolic Engineering Laboratory, Korea Research Institute of Bioscience and Biotechnology, Oun-dong 52, Yusong-gu, Daejeon 305-600, Korea; ²Department of Microbiology, Chungnam National University, Daejeon 305-764, Korea; and ³Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-4 Higashi, Tsukuba, Ibaraki, 305-8566, Japan

Presently almost no information is available on the oligosaccharide structure of the glycoproteins secreted from the methylotrophic yeast _Hansenula polymorpha_, a promising host for the production of recombinant proteins. In this study, we analyze the size distribution and structure of _N_-linked oligosaccharides attached to the recombinant glycoprotein glucose oxidase (GOD) and the cell wall mannoproteins obtained from _H. polymorpha_. Oligosaccharide profiling showed that the major oligosaccharide species derived from the _H. polymorpha_-secreted recombinant GOD (rGOD) had core-type structures (Man₅₀±2₀₀GlcNAc₂). Analyses using anti-α₁,3-mannose antibody and exoglycosidases specific for α₁,2- or α₁,6-mannose linkages revealed that the mannose outer chains of _N_-glycans on the rGOD have very short α₁,6 extensions and are mainly elongated in α₁,2-linkages without a terminal α₁,3-linked mannose addition. The _N_-glycans released from the _H. polymorpha_ mannoproteins were shown to contain mostly mannose in their outer chains, which displayed almost identical size distribution and structure to those of _H. polymorpha_-derived rGOD. These results strongly indicate that the outer chain processing of _N_-glycans by _H. polymorpha_ significantly differs from that by _Saccharomyces cerevisiae_, thus generating much shorter mannose outer chains devoid of terminal α₁,3-linked mannosas.

Key words: cell wall mannoproteins/glycan profiling/_Hansenula polymorpha_/N-linked oligosaccharides/recombinant glucose oxidase

Introduction

As a eukaryotic microorganism, yeast can carry out _N_-linked glycosylation, a posttranslational modification of proteins that involves the attachment of oligosaccharides to newly synthesized polypeptides. Initial _N_-glycan processing in the endoplasmic reticulum (ER) is very well conserved among eukaryotes but subsequent processing of oligosaccharides in the Golgi apparatus is variable, even between yeast species. In the traditional yeast _Saccharomyces cerevisiae_, the core oligosaccharides assembled on secretory proteins are elongated by addition of mannoses, which often leads to the formation of hypermannose structures (Man₅₀±2₀₀GlcNAc₂) with outer chains that may contain up to 200 mannose units. The linear backbone of the outer chain, frequently composed of 50 or more α₁,6-linked mannoses, is highly branched by the presence of α₁,2-linked mannoses and is terminally capped with α₁,3-linked mannoses (Dean, 1999). Hypermannosylated glycoproteins, especially those with terminal α₁,3-linked mannose residues, produced from _S. cerevisiae_ have been reported to be highly antigenic in humans (Ballou, 1990). On the other hand, in the methylotrophic yeast _Pichia pastoris_, the mannose outer chains of _N_-linked oligosaccharides have been shown to be generally much shorter than those found in _S. cerevisiae_ (Brethauer and Castellino, 1999), although extensive hyperglycosylation has also been reported in a few cases (Grinnin and Tschopp, 1989; Scorer et al., 1993). The major oligosaccharide species in _P. pastoris_ are reported to be Man₈₋₁₄ GlcNAc₂ forms with short α₁,6 extensions (Trimble et al., 1991). More significantly, _P. pastoris_ oligosaccharides have been reported to have no hyperimmunogenic terminal α₁,3 glycan linkages (Montesino et al., 1998; Trimble et al., 1991). However, phosphomannose has been detected in both elongated and core oligosaccharides on some recombinant proteins of _P. pastoris_ (Miele et al., 1997; Montesino et al., 1998), as observed in _S. cerevisiae_ oligosaccharides.

The thermotolerant methylotrophic yeast _Hansenula polymorpha_ has recently emerged as a promising host system for the production of recombinant proteins, ranging from industrial enzymes to therapeutic proteins (Gellissen, 2000, 2002). As the numbers of the reports of foreign gene expression using _H. polymorpha_ increases, interest in the structures of oligosaccharides attached to the polypeptide chains produced by this yeast have been raised. A few studies on the expression of heterologous glycoproteins in _H. polymorpha_ have indicated that the recombinant glycoproteins obtained appear to be less hyperglycosylated than those from _S. cerevisiae_ (Gellissen et al., 1995; Kang et al., 1998). However, currently almost no information is available on the structural characteristics of the _N_-linked oligosaccharides of _H. polymorpha_.

Received on August 27, 2003; revised on October 27, 2003; accepted on October 30, 2003

Advance Access publication December 23, 2003

DOI: 10.1093/glycob/cwh030

Glycobiology vol. 14 no. 3 © Oxford University Press 2004; all rights reserved.
H. polymorpha-derived glycoproteins. In this study, we analyze the structure of the N-linked oligosaccharides derived from recombinant Aspergillus niger glucose oxidase (GOD) secreted from H. polymorpha and compared it with that of the oligosaccharides from S. cerevisiae. To obtain more general information on the structure of N-glycans of the secretory pathway in H. polymorpha, we also analyze the N-linked oligosaccharides from H. polymorpha cell wall mannoproteins. This is the first report on the structure of N-linked glycans derived from H. polymorpha to show that most oligosaccharide species attached to glycoproteins secreted from H. polymorpha have core-type structures (Man$_{n=12}$GlcNAc$_2$) without terminal α1,3-linked mannose residues.

Results

Glycosylation pattern of rGOD expressed by H. polymorpha

Our previous studies on the heterologous expression of A. niger GOD, a glycoprotein with eight potential N-linked glycosylation sites, have shown that the recombinant GOD (rGOD) was expressed in highly mannosylated forms by S. cerevisiae wild-type strains (Kim et al., 2000; Ko et al., 2002). To obtain information on the N-linked glycosylation in H. polymorpha, we expressed A. niger GOD, as a model glycoprotein, in two H. polymorpha wild-type strains, DL1-L and A16, and compared the glycosylation patterns of rGOD expressed with those of S. cerevisiae strains. Western blot analysis of the purified rGOD from the antibody specific for GOD revealed that the electrophoretic mobility of H. polymorpha-derived rGOD was much faster than that of S. cerevisiae-derived rGOD but slower than that of authentic GOD from A. niger (Figure 1B). This result suggested that the rGOD secreted from H. polymorpha appeared to be hyperglycosylated compared to authentic GOD but less hyperglycosylated than the rGOD from S. cerevisiae. After digestion with peptide/N-glycanase F (PNGase F), rGOD from all yeast strains displayed the same mobility, appearing as a discrete band (data not shown), and thus demonstrating that the difference in electrophoretic mobility was due to a difference in the extent of the glycosylation of N-linked oligosaccharides attached to the rGOD.

To determine whether the terminal α1,3-mannose epitope was present in H. polymorpha-expressed rGOD, the purified rGOD was further analyzed using an antibody specific for terminal α1,3-linked mannoses. As shown in Figure 1C, the anti-α1,3-mannose antibody bound to the rGOD secreted from wild-type S. cerevisiae (lanes 5 and 6) but not to that of the S. cerevisiae mnn1Δ mutant (lanes 7 and 8), which is defective in adding the terminal α1,3-mannose residues on both N- and O-linked oligosaccharides due to the absence of α1,3-mannosyltransferase activity (Yip et al., 1994). It was notable that rGOD from the H. polymorpha strains DL1-L (lanes 1 and 2) and A16 (lanes 3 and 4) was not recognized by the anti-α1,3-mannose antibody, implying that the N-linked oligosaccharides on H. polymorpha-derived rGOD are apparently devoid of terminal α1,3-linked mannoses.

Size analysis of N-glycans assembled on rGOD

To gain more detailed information on the size of N-glycans assembled on the rGOD secreted by H. polymorpha, oligosaccharides were enzymatically released from purified rGOD, labeled with the fluorophore 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS), and then analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) and high-performance liquid chromatography (HPLC).
For FACE analysis, the ANTS-labeled oligosaccharide pools from *H. polymorpha*–derived rGOD and from *S. cerevisiae*–derived rGOD were run in a parallel on a polyacrylamide gel with ANTS-labeled oligomannosides of known size and structure, used as standards for size estimation (Figure 2A). The electrophoretic migration pattern of the *H. polymorpha* oligosaccharide pool was found to be quite different from that of the *S. cerevisiae* oligosaccharide pool, especially in terms of the sizes of the major oligosaccharides. In the sample from *S. cerevisiae*, large oligosaccharide species containing nine or more mannose residues (Man_{9-14} GlcNAc_{2}) were abundantly detected (Figure 2A, lane 1), whereas in the sample from *H. polymorpha* relatively short oligosaccharides containing eight or nine mannoses (Man_{8-9} GlcNAc_{2}) were the major forms (Figure 2A, lane 2). This is in a good agreement with our immunoblot result, that is, that *H. polymorpha*–secreted rGOD had much lower molecular weight forms than *S. cerevisiae*–secreted rGOD, which strongly supports the notion that the overall length of the outer mannose chain attached to glycoproteins is generally shorter in *H. polymorpha*.

The ANTS-labeled N-linked oligosaccharides were also fractionated by size on NH2P-50 HPLC column (Figures 2Ba and 2Ca). Oligosaccharide profiles of the samples from *H. polymorpha* and *S. cerevisiae* reflected the results of the FACE analysis, displaying evident differences in the relative proportions of oligosaccharide species generated by these two yeasts. In the profile from *H. polymorpha*, the core form oligosaccharide *Man*_{9}GlcNAc_{2} was the predominant species. Nearly 80% of the *H. polymorpha* oligosaccharides were distributed among oligosaccharide species containing 8–10 mannoses (Man_{8-10}GlcNAc_{2}), and only minor fractions were shown to have structures larger than Man_{10-}GlcNAc_{2} (Figure 2Ba). In contrast, the profile from *S. cerevisiae* showed a more even distribution of oligosaccharide species, ranging from *Man*_{n}GlcNAc_{2} to *Man*_{14-}GlcNAc_{2}. Almost 50% of the *S. cerevisiae* oligosaccharides contained more than 10 mannose residues (Man_{11-14} GlcNAc_{2}, Figure 2Ca). Interestingly, *H. polymorpha* oligosaccharides containing nine or more mannose residues exhibited slightly longer retention times on HPLC than the corresponding *S. cerevisiae* oligosaccharides, which is consistent with the relatively faster electrophoretic migration of the *H. polymorpha* oligosaccharides observed during FACE analysis. These results indicate that there might be significant differences between these two yeasts in generating the oligosaccharides larger than *Man*_{8}GlcNAc_{2}.

**Structural analysis of rGOD-derived N-glycans by exoglycosidase digestion**

The use of specific α-mannosidases has proved useful in the examination of the structural configurations of oligosaccharides from glycoproteins (Ichishima et al., 1999; Wong-Madden and Landry, 1995). To confirm the immunoblot result indicating the absence of terminal α1,3-linked mannose residues on the N-glycans of the *H. polymorpha*–derived rGOD, the ANTS-labeled oligosaccharide pool was treated with α1,2-mannosidase from *Aspergillus saitoi*. This exoglycosidase is highly specific for nonreducing terminal α1,2-mannose linkages. The digested products

---

**Fig. 2.** FACE and HPLC analysis of N-glycans assembled on rGOD. (A) The electrophoretic mobility of ANTS-labeled oligosaccharide pools derived from *S. cerevisiae* (lane 1) and *H. polymorpha* DL1-L (lanes 2, 5, and 6). The samples were fractionated by electrophoresis with the *Man*_{n}GlcNAc_{2} oligomannoside-ANTS (lanes 3 and 4) used as size standards. Ladder of maltooligosaccharides-ANTS is marked (lane L). Lanes 4 and 6 contain the samples after the exoglycosidase digestion by α1,2-mannosidase of the *Man*_{n}GlcNAc_{2}-ANTS oligomannoside standard (lane 4) and the ANTS-labeled oligosaccharide pool of *H. polymorpha* DL1-L (lane 6), respectively. Note that the *Man*_{n}GlcNAc_{2} oligomannoside standard having the structure of the precursor form assembled in the ER generated *Man*_{n}GlcNAc_{2} after α1,2-mannosidase digestion. (B, C) HPLC analysis of the N-linked oligosaccharides released from rGOD. The ANTS-labeled oligosaccharide pools from *H. polymorpha*–derived rGOD (B) and from *S. cerevisiae*–derived rGOD (C) before (a) and after (b) α1,2-mannosidase digestion. Size-fractionation HPLC was performed with Shodex Asahipak NH2P-50 column using two solvents, A and B. Solvent A was glacial acetic acid (6% v/v) in a 70:30 (v/v) mixture of acetonitrile-water adjusted to pH 5.5 with triethylamine. Solvent B was glacial acetic acid (6% v/v) adjusted to pH 5.5 with triethylamine. The gradient used was 5–50% B solvent in 90 min at a flow rate of 0.7 ml/min. The elution times of authentic ANTS mannose oligomers are indicated by arrows.
obtained were subjected to FACE and HPLC. As shown in Figures 2A, 2Bb, and 2Cb, after digestion with α1,2-mannosidase, virtually all of the *H. polymorpha*-derived oligosaccharides were converted to two small oligosaccharide forms, Man₅GlcNAc₂ (M₅) and Man₆GlcNAc₂ (M₆). Some tiny peaks not shifted to M₅ or M₆, indicated by arrowheads in Figure 2Bb, were detected after a 1,2-mannosidase digestion. However, these peaks were resistant to the subsequent digestion with α1,2,3-mannosidase, implying that they do not contain the terminal α1,3-linked mannose residues (data not shown). On the other hand, after the digestion of the oligosaccharide pool from *S. cerevisiae*-derived rGOD, which was expected to contain terminal α1,3-linked mannose residues, substantial fractions of the digested products still remained as large oligosaccharide species containing eight or more mannose residues and only minor fractions were converted to Man₅GlcNAc₂ and Man₆GlcNAc₂ (Figure 2Cb). Therefore, the susceptibility of *H. polymorpha* oligosaccharides to α1,2-mannosidase digestion strongly indicates the absence of terminal α1,3-mannose residues on these oligosaccharides. Moreover this result also suggests that the outer chains of the oligosaccharides on *H. polymorpha*-secreted rGOD are elongated mostly by the addition of α1,2-linked mannoses.

We further analyzed the structure of the oligosaccharide species, Man₅GlcNAc₂ and Man₆GlcNAc₂, which were generated from the *H. polymorpha* oligosaccharide pool after α1,2-mannosidase treatment, by digestion with α1,6-mannosidase from *Xanthomonas manihotis* (Figure 3). This highly specific exoglycosidase removes the terminal α1,6-linked mannose residues that are linked to a nonbranched sugar. After digestion with α1,6-mannosidase, the peak corresponding to Man₅GlcNAc₂ was completely converted to a peak corresponding to Man₆GlcNAc₂, indicating the presence of an extra α1,6-linked mannose in the Man₆GlcNAc₂ species. Considering that the structure of the core oligosaccharide Man₈GlcNAc₂ synthesized in the ER is the same in all eukaryotes examined so far (Munro, 2001), one can speculate that the Man₅GlcNAc₂ species is the final product of specific α1,2-mannosidase digestion of Man₉±14GlcNAc₂, in which the core oligosaccharide Man₈GlcNAc₂ is elongated by a single α1,6-linked mannose addition and branched with a variable number of α1,2-linked mannose units (Figure 3C). Therefore the results shown in Figure 3 strongly suggest that the outer chains of the N-glycans on *H. polymorpha*-secreted rGOD are mainly elongated in α1,2-linkages without terminal α1,3-linked mannose addition and have only one α1,6-linked mannose extension.

---

Fig. 3. HPLC analysis of the exoglycosidase digestion products of oligosaccharides obtained from the *H. polymorpha*-secreted rGOD. (A) The digestion products by α1,2-mannosidase of the ANTS-labeled oligosaccharide pool. (B) The final product after a sequential digestion by α1,6-mannosidase. (C) A proposed demannosylation reaction of the *H. polymorpha* oligosaccharides. The structures of Man₅GlcNAc₂ and Man₆GlcNAc₂ are based on information from *S. cerevisiae* N-linked oligosaccharides (Dean, 1999). Mannose linkages susceptible to α1,2- or α1,6-mannosidase are indicated by arrowheads or an arrow, respectively.
Oligosaccharide structure of H. polymorpha

To determine whether the glycosylation of the rGOD reflect the general pattern of N-glycosylation in H. polymorpha, we investigated the structure of oligosaccharides derived from cell wall mannoproteins. The oligosaccharides prepared from the cell wall mannoproteins of H. polymorpha DL-1 strain were labeled with 2-aminopyridine (PA) and fractionated by HPLC under the elution condition that can separate neutral oligosaccharides from negatively charged ones in a chromatogram. As a control sample to check the HPLC condition, the oligosaccharides of the cell wall mannoproteins from S. cerevisiae mnn1Dvan1D were analyzed. Due to its defects in extensive polymerization of α1,6-linked mannosae and in terminal addition of α1,3-linked mannosae on N-glycans, the S. cerevisiae mnn1Δvan1Δ strain generates much shorter oligosaccharides, ranging from Man₈GlcNAc₂ to Man₁₂GlcNAc₂, compared to the S. cerevisiae wild-type strain (Shimma, unpublished data). As shown in Figure 4A, the oligosaccharides from S. cerevisiae mnn1Δvan1Δ were separated into three distinctive pools, depending on the phosphorylation status. The oligosaccharides of pool I corresponded to the N-glycans free of net negative charge, whereas those of the pools II and III corresponded to mono- or dimannosylphosphorylated glycans, respectively (Wang et al., 1997). The same HPLC analysis of the oligosaccharides derived from H. polymorpha mannoproteins revealed that the majority of the H. polymorpha oligosaccharides were of Man₈-12GlcNAc₂ sizes with the retention time of neutral glycans (Figure 4B). Almost identical oligosaccharide profiles were also obtained from mannoproteins of the other H. polymorpha strains, CBS4732 andNCYC495 (data not shown). The relatively small Man₈GlcNAc₂ and Man₉GlcNAc₂ oligosaccharides were commonly present as major species in H. polymorpha mannoproteins, contrast to S. cerevisiae mannoproteins with much larger oligosaccharides as dominant species. The oligosaccharide profile of H. polymorpha mannoproteins was in fact quite similar to that of H. polymorpha-derived rGOD (Figure 4C), implying that small oligosaccharide species (Man₈-12GlcNAc₂) without mannosylphosphorylation might be the general features of N-glycans of the secretory pathway in H. polymorpha.

Further analysis of the oligosaccharide structure of H. polymorpha mannoproteins was carried out by exoglycosidase

Fig. 4. HPLC profiles of oligosaccharides from H. polymorpha mannoproteins. The PA-labeled N-linked oligosaccharides released from S. cerevisiae mnn1Δvan1Δ cell wall mannoproteins (A), H. polymorpha cell wall mannoproteins (B), and H. polymorpha-derived rGOD (C) were separated on Shodex Asahipak NH2P-50 column using two solvents, C and D. Solvent C was composed of 200 mM acetic acid adjusted with triethylamine (pH 7.3) and acetonitrile (10:90, v/v). Solvent D was 200 mM glacial acetic acid adjusted to pH 7.3 with triethylamine. The gradient used was 20–100% D solvent in 40 min at a flow rate of 1.0 ml/min. The PA-labeled oligosaccharides from H. polymorpha cell wall mannoproteins were digested by α1,2-mannosidase (D) and then sequentially treated with α1,6-mannosidase (E), or subjected to digestion by jack bean α-mannosidase (F) and then separated by HPLC. The unidentified peak x appears to be a contaminant generated during the preparation of cell wall materials.
digested. After treatment with α1,2-linkage-specific mannosidase, all of the oligosaccharides were converted to Man₈GlcNAc₅ and Man₉GlcNAc₆ (Figure 4D). Sequential digestion with α1,6-mannosidase corresponded to Man₉GlcNAc₆ into a peak corresponding to Man₈GlcNAc₅ (Figure 4E). The results strongly indicate that N-linked oligosaccharides of *H. polymorpha* mannoproteins were not terminally capped by α1,3-linked mannose residues and extended mainly by α1,2-linkages, as observed in the glycan assembled on the *H. polymorpha*-derived rGOD. During the HPLC analysis, we observed an unknown peak, designated x, in the *H. polymorpha* oligosaccharide samples. The elution pattern of peak x was not changed even after the treatment of jack bean α-mannosidase, which cleaves all three (α1,2, α1,3, and α1,6) types of nonreducing terminal α-mannose linkages. The x peak was dominantly detected especially in the samples of *H. polymorpha* cell wall mannoproteins, implying that it may correspond to a contaminant generated during the preparation of cell wall materials (Figure 4F).

*S. cerevisiae* and *P. pastoris* are reported to add only mannose to their glycoprotein glycans, whereas *Schizosaccharomyces pombe* (Gemmill and Trimble, 1999). Monosaccharides contents of the N-linked oligosaccharides prepared from *H. polymorpha* mannoproteins were analyzed by using anion-exchange column. The peak corresponding to PA-Man was detected as one major peak, whereas the peaks to PA-Glc and PA-GlcNAc as minor peaks (Figure 5B). No peaks corresponding to PA-galactose or PA-fucose were detected. Although the possibility that the outer chains of *H. polymorpha* contain glucose as a minor structural component can not be excluded, it is more likely that glucose was generated from the contaminated cell wall glucan during monosaccharide preparation (Peat et al., 1961). The presence of glucose inhibits the digestion of oligosaccharides by α-mannosidases. Judging from our results of α1,2 and α1,6-mannosidase digestion experiments, it appears that *H. polymorpha* also add only mannose to their glycoprotein glycans, as do the other yeasts *S. cerevisiae* and *P. pastoris*.

**Discussion**

Yeasts have been considered for the production of glycoprotein biopharmaceuticals (Lehle and Tanner, 1995). As an alternative to the conventional yeast *S. cerevisiae*, the methylotrophic yeast *H. polymorpha* is rapidly gaining favor for the production of recombinant proteins (Gellissen, 2000, 2002; van Dijk et al., 2000). However, little is known about the structure of the *N*-glycans assembled on glycoproteins secreted from this methylotrophic yeast. In this study, we determined the size distribution and the structures of the N-linked oligosaccharides attached on *H. polymorpha*-secreted rGOD and cell wall mannoproteins. The results of oligosaccharide profiling showed that the small oligosaccharides with core-type structures (Man₈₁₂ GlcNAc₂) were present as major species in the *H. polymorpha* oligosaccharide (Figures 2 and 4), demonstrating that the *H. polymorpha* N-glycans are much less hypermannosylated than the *S. cerevisiae* N-glycans. More significantly, the inability of the anti-α1,3-mannose antibody to bind to *H. polymorpha*–secreted rGOD (Figure 1C), and the susceptibility of the *H. polymorpha* N-glycans from rGOD and mannoproteins to α1,2-mannosidase digestion (Figures 2B and 4D) strongly indicated that the *H. polymorpha* oligosaccharides were not terminally capped by α1,3-linked mannose residues and were extended mainly in α1,2-mannose linkages. These structural characteristics suggest that *H. polymorpha* has some advantages over *S. cerevisiae* for the production of recombinant glycoproteins. In particular, the highly immunogenic α1,3-terminal linkages are common on *S. cerevisiae* oligosaccharides and present a major problem for the use of *S. cerevisiae* as a host strain for the production of human pharmaceuticals (Romanos et al., 1992).

The results of the sequential digestion with α1,2- and α1,6-mannosidase (Figures 3B and 4E) further indicate that the outer chains of the *H. polymorpha* N-linked oligosaccharides from rGOD and mannoproteins had very short α1,6 extensions, mainly composed of a single α1,6-linked mannose. The outer chain of the budding yeast *S. cerevisiae* has a long α1,6-linked mannose backbone, which is often composed of more than 50 mannose residues and branched extensively by addition of α1,2- and α1,3-linked mannoses (Dean, 1999). In contrast, *P. pastoris* N-linked oligosaccharides have relatively short α1,6 extensions. The oligosaccharides assembled on the recombinant proteins secreted from *P. pastoris* were reported to contain one to four α1,6-linked mannose units in their outer chains (Kalidas et al., 2001; Miele et al., 1997). Therefore the N-linked glycosylation pathways in both methylotrophic yeasts *H. polymorpha* and *P. pastoris* are different from that of *S. cerevisiae*.
and *P. pastoris* appear to be quite similar but significantly different from that in *S. cerevisiae*, with the addition of much shorter α,6 outer chain backbone to the core and no α,1,3 outer extensions.

Further modification of N-linked oligosaccharides with the addition of mannnosylphosphate often occurs both in *S. cerevisiae* (Jigami and Odani, 1999) and *P. pastoris* (Bretthauer and Castellino, 1999). It is known that the core oligosaccharide Man$_5$GlcNAc$_2$ also contains two potential phosphorylation sites (Hernandez et al., 1989). However, we could not yet observe intensively phosphorylated oligosaccharides in the samples prepared for the *H. polymorpha*–derived mannoproteins and rGOD (Figures 4B and 4C). The digestion of most fractions of *H. polymorpha* oligosaccharides to Man$_5$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ neutral oligomannosides by α,1,2-mannosidase treatment (Figures 2Bb and 4D) also supports the absence of phosphate in the major species of *H. polymorpha* (Figures 2Bb and 4D) also supports the absence of the possibility that some tiny peaks not shifted completely to the small oligosaccharide M5 and M6 species after α,1,2-mannosidase digestion (Figure 2Bb) could be minor oligosaccharide species containing phosphate residues, the present results imply that the mannosphosphorylation of the N-linked glycans may not occur actively in *H. polymorpha*. However, the extent of mannosphosphorylation is known to depend on culture conditions, such as media and cultivation periods, and a more detailed study should be carried out to discuss the possibility of mannosphosphorylation as another form of oligosaccharide modification in *H. polymorpha*.

Increasing evidence shows that oligosaccharides have profound effects on critical properties of glycoprotein products for human therapeutic use, such as plasma clearance rate, antigenicity, and specific activity. Therefore the way to get correct glycosylation has been important issues in the field of biotechnology industry (Jenkins et al., 1996; Koeller and Wong, 2000). Further studies on the structural characteristics of *H. polymorpha* N-linked oligosaccharides, especially those derived from mutant strains defective in glycosylation, should facilitate the delineation of the *H. polymorpha*–specific N-glycosylation pathway. This would provide valuable information for the development of glycoengineering strategies in *H. polymorpha* to achieve the optimal glycosylation of recombinant proteins.

### Materials and methods

#### Yeast strains and plasmids

The *H. polymorpha* strains, DL1-L (leu2) and A16 (leu2), used in this study were a derivative of DL-1 (ATCC26012) and CBS4732 (ATCC34438), respectively. The *S. cerevisiae* mnn1Δ strain (MATa ura3-52 leu2-3,112 his4-34 mnn1:::LEU2) was derived from *S. cerevisiae* L3262a (Kim et al., 2000). The *S. cerevisiae* mnn1Δ van1Δ (MATa leu2 ura3 trpl1 ade8 his3 mnn1::URA3 van1::LEU2) was derived from *S. cerevisiae* RA1-1B (Y. Shimma, unpublished data). To express *A. niger* GOD tagged with six residues of histidine (GOD-His) in *H. polymorpha* under the MOX promoter, the 1.9-kb EcoRI/Sall DNA fragment encoding GOD-His was obtained from the *S. cerevisiae* GOD expression vector pYGOD-His (Kim et al., 2000) and exchanged with the 0.7-kb EcoRI/XhoI fragment coding for hirudin in pDLMOX-HIR (Kang et al., 1998), generating pDLMOX-GOD(H).

#### Media and general techniques

Yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or SD minimal medium (0.67% yeast nitrogen without amino acids, 2% glucose) containing appropriate nutritional supplements. Yeast transformation was performed by the modified dimethyl sulfoxide–lithium acetate method (Hill et al., 1991). General DNA manipulations were performed as described previously (Sambrook and Russell, 2001). To induce the expression of *A. niger* GOD in *H. polymorpha*, the transformants harboring pDLMOX-GOD(H) were precultured overnight in a synthetic complete medium lacking leucine and transferred to flasks containing YPM medium (1% yeast extract, 2% peptone, 2% methanol) for 36 h at 37°C. For the induction of GOD expression in the *S. cerevisiae* transformants harboring pYGOD-His, YPDG medium (1% yeast extract, 2% peptone, 1% glucose, 1% galactose) was used. Immunoblot analysis was carried out using the polyclonal antibodies raised against *A. niger* GOD (Accurate Chemical & Scientific, Westbury, NY) and against α,1,3-linked mannose (provided by R. Schekman, University of California, Berkeley, CA) as described previously (Kim et al., 2000).

#### Purification of rGOD

Culture supernatants containing His$_6$-tagged GOD were concentrated by ultrafiltration (YM30 membrane, Millipore, Bedford, MA). The 100-fold concentrated culture supernatants were dialyzed against 50 mM sodium phosphate (pH 6.0) and 300 mM NaCl, and His$_6$-tagged GOD was purified using AÈ KTA$^{	ext{prime}}$ chromatography system (Amersharm Pharmacia Biotech AB, Uppsala, Sweden).

#### Preparation of cell wall mannoproteins

Yeast cells were cultivated in YPD medium supplemented with 0.3 M KCl and harvested at early stationary phase (OD$_{600}$ = 10). Total cell wall mannoproteins were extracted by hot citrate buffer (0.1 M citrate buffer, pH 7.0) followed by precipitation with ethanol (Peat et al., 1961). The precipitates were desalted by a PD-10 column containing Sephadex G-25 (Amersharm Pharmacia Biotech). The eluent was further purified by a concanavalin A–agarose column (Amersharm Pharmacia Biotech), which was equilibrated with concanavalin A buffer (0.1 M Tris-HCl buffer [pH 7.2] containing 0.15 M NaCl, 1 mM MnCl$_2$, and 1 mM CaCl$_2$). The column was eluted by concanavalin A buffer containing 1 M methyl α-d-mannoside. The mannoprotein fraction was dialyzed against water and lyophilized.

#### Oligosaccharide preparation

N-linked oligosaccharides were released from the purified rGOD and cell wall mannoproteins by PNGase F (New England Biolabs, Beverly, MA) or Glycanase F (Takara Biomedical).
Shuzo, Shiga, Japan) following the manufacturer’s instructions. Oligosaccharides were labeled covalently with fluorogenic compounds ANTS (Glyko, Novato, CA) or PA (Takara Shuzo) at their reducing ends (Jackson, 1990; Kondo et al., 1990). Digestion of fluorophore-labeled oligosaccharides with α1,2-mannosidase from A. saitoi, Glyko), α1,2,3-mannosidase (from X. manihotis, New England Biolabs), α1,6-mannosidase (from X. manihotis, New England Biolabs), or α-mannosidase (from jack bean, Sigma, St. Louis, MO) was carried out according to the manufacturer’s instructions.

**FACE and HPLC of oligosaccharides**

The ANTS-labeled oligosaccharides were separated on high-resolution polyacrylamide gels using FACE N-linked oligosaccharide profiling kit. The electrophoretic migration of a band was compared to an ANTS oligomannoside standard (Man₉GlcNAc₂-ANTS) or ladder of maltoligosaccharides-ANTS (Glyko). Size-fractionation HPLC of ANTS-labeled or PA-labeled oligosaccharides was carried out with Shodex Asahipak NH2P-50 column (Showa Denko, Tokyo, 0.46 × 25 cm) using a Waters 247 chromatography system (Waters, Milford, MA, USA). Fluorescence was measured using a Waters 2475 fluorescence detector (Waters) for ANTS at λₑₓ 353 nm and λₑₘ 535 nm and for PA at λₑₓ 320 nm and λₑₘ 400 nm, respectively.

**HPLC analysis of monosaccharides**

Monosaccharides from N-linked oligosaccharides attached on H. polymorpha cell wall mannoproteins were prepared by acid hydrolysis as described previously (Takasaki et al., 1992). Twenty microliters of oligosaccharide samples (100 pmol) were hydrolyzed with 40 ml 6 M trifluoroacetic acid at 100°C for 3 h using a gas-phase hydrazinolysis apparatus (Hydracrub S-204, Honen Oil, Tokyo). After hydrolysis, the resulting monosaccharides were N-acetylated twice as described previously (Nakanishi-Shindo et al., 1993). The monosaccharides were labeled with PA and analyzed using anion-exchange column, PALPAK Type A (Takara Shuzo, 0.46 × 15 cm) at 65°C. The products were identified with the authentic PA-mono- saccharides standard (Takara Shuzo).

**Acknowledgments**

This study was supported by grants form the Korean Ministry of Science and Technology (Korea-Japan International Cooperation Program, 21st Century Frontier R&D program) and the Japan Society for the Promotion of Science. The authors deeply appreciated Drs. M. Kainuma and K. Nakayama for their helpful discussions.

**Abbreviations**

- ANTS, 8-aminonaphthalene-1,3,6-trisulphonic acid; ER, endoplasmic reticulum; FACE, fluorophore-assisted carbohydrate electrophoresis; GOD, glucose oxidase; HPLC, high-performance liquid chromatography; PA, 2-aminopyridine; PNGase F, peptide:N-glycanase F.

**References**


Nakanishi-Shindo, Y., Nakayama, K., Tanaka, A., Toda, Y., and Jigami, Y. (1993) Structure of the N-linked oligosaccharides that show the complete loss of alpha-1,6-polymannose outer chain from och1, och1 mnn1, and och1 mnn1 alg3 mutants of Saccharomyces cerevisiae. *J. Biol. Chem.*, 268, 26338–26345.


