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PROTOCOL

Structural analysis of *N*-/*O*-glycans assembled on proteins in yeasts

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Protein glycosylation, the most universal and diverse post-translational modification, can affect protein secretion, stability, and immunogenicity. The structures of glycans attached to proteins are quite diverse among different organisms and even within yeast species. In yeast, protein glycosylation plays key roles in the quality control of secretory proteins, and particularly in maintaining cell wall integrity. Moreover, in pathogenic yeasts, glycans assembled on cell-surface glycoproteins can mediate their interactions with host cells. Thus, a comprehensive understanding of protein glycosylation in various yeast species and defining glycan structure characteristics can provide useful information for their biotechnological and clinical implications. Yeast-specific glycans are a target for glyco-engineering; implementing human-type glycosylation pathways in yeast can aid the production of recombinant glycoproteins with therapeutic potential. The virulence-associated glycans of pathogenic yeasts could be exploited as novel targets for antifungal agents. Nowadays, several glycomics techniques facilitate the generation of species- and strain-specific glycome profiles and the delineation of modified glycan structures in mutant and engineered yeast cells. Here, we present the protocols employed in our laboratory to investigate the *N*- and *O*-glycan chains released from purified glycoproteins or cell wall mannoproteins in several yeast species.

Keywords: yeast, protein glycosylation, *N*-/*O*-glycans, structure analysis

Overview

Post-translational modifications of proteins are important biochemical events involved in regulating various cellular functions (Pejaver *et al.*, 2014). The most ubiquitous post-translational modification is protein glycosylation: the addition of glycans to amino acid residues of proteins, which is estimated to be present in more than half of all proteins in nature. Protein glycosylation is subdivided into two main types: *N*-glycosylation, in which glycans are covalently attached to the nitrogen atom of an asparagine residue present in the consensus sequon Asn-X-Ser/Thr, where X might be any amino acid except proline; and *O*-glycosylation, in which glycan attachment occurs at the hydroxyl group of either serine or threonine residues in proteins (Butler *et al.*, 2003; Ben-Dor *et al.*, 2004; Ohtsubo and Marth, 2006; Morelle and Michalski, 2007). The early stages of protein *N*-glycosylation are evolutionarily conserved from yeast to humans in eukaryotes (Willer *et al.*, 2003). *N*-glycosylation of proteins in eukaryotic cells is accomplished in two distinct organelles: the endoplasmic reticulum (ER) and the Golgi (Helenius and Aebi, 2001). Once proteins are glycosylated and folded in the ER, they are moved to the Golgi complex for the subsequent maturation of *N*-linked glycans, which varies considerably depending on the species and cell type (Dean, 1999). In mammalian cells, some mannose residues in the core form, $Man_8GlcNAc_2$, are trimmed during protein transport from the ER to the Golgi apparatus, in which several different sugars such as *N*-acetylglucosamine, galactose, sialic acid, and/or fucose are added to achieve complexity. In contrast, in yeast, the core $Man_8GlcNAc_2$ glycans are generally further extended by the addition of mannose or mannosylphosphate residues without mannose trimming, leading to hypermannosylation (Dean, 1999). Most eukaryotic *O*-biosynthetic events take place in the Golgi, although some classes of *O*-glycans, for example, *O*-mannose linked glycans, are initiated in the ER (Dell *et al.*, 2010). Because an enormous variety of sequences can be attached, *O*-glycosylation is very diverse in the eukaryotic domain (Haltiwanger and Lowe, 2004). The structure of *O*-glycans is thus quite diverse among different eukaryotic organisms and even within yeast species. In most yeasts, an α-1,2-linked mannotriose is present as the major common core structure of *O*-glycans, which is differentially expanded according to the species (Gemmill and Trimble, 1999).

The traditional yeast *Saccharomyces cerevisiae* has been used as a model for investigating fugal *N*-/*O*-glycan synthesis. In *S. cerevisiae*, elongation of the *N*-glycan outer chain in the Golgi is initiated by the mannosyltransferase Och1p, which adds a single α-1,6-linked mannose residue to the core *N*-linked oligosaccharides (Nakanishi-Shindo *et al.*, 1993). Following the extension of the α-1,6-linked polymannose back-

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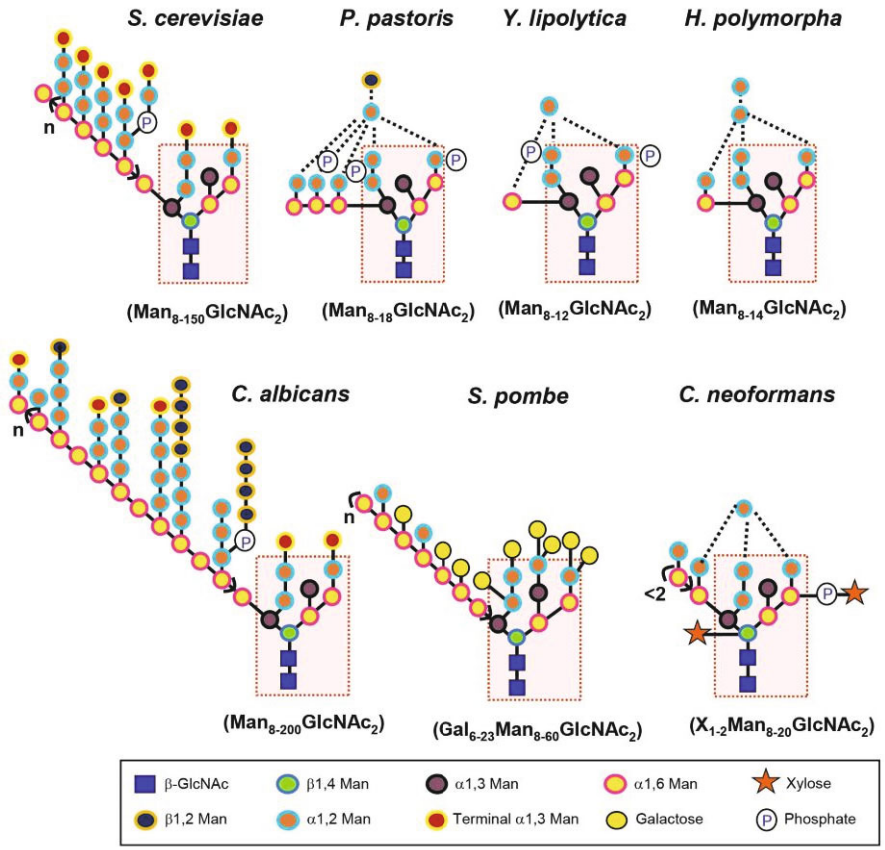

Fig. 1. *N*-glycan structures of various yeast species. Dotted lines indicate putative sites for mannose addition. $M_nGlcNAc_2$ (n = number of mannose residues). Shaded box indicates the core structure of *N*-glycans conserved in yeast species. *N*-linked mannans of *S. cerevisiae* and *C. albicans* have a long α-1,6-linked mannosyl backbone. These backbones are decorated with α-1,2-and α-1,3-linked mannosyl side chains and mannosylphosphate in *S. cerevisiae* (Varki *et al.*, 2009), and also β-1,2-linked mannosyl side chains in *C. albicans* (Hall and Gow, 2013).

bone, α-1,2- and α-1,3-linked mannoses are added to the core oligosaccharide in *S. cerevisiae*. Other yeast species, including *Pichia pastoris*, *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, *Candida albicans*, and *Cryptococcus neoformans* also use the Och1 protein to initiate the mannose outer chain biosynthesis of *N*-glycans (Yoko-o *et al.*, 2001; Vervecken *et al.*, 2004; Wildt and Gerngross, 2005; Kim *et al.*, 2006; Song *et al.*, 2007; Park *et al.*, 2012). However, each yeast species exhibits quite different *N*-linked outer chain processes, generating *N*-glycans that vary considerably in the linkage and composition of their side chains (Fig. 1 and Table 1). The human pathogenic yeast *C. albicans* has extensively hypermannosylated *N*-glycans, as shown in *S. cerevisiae*, but *C. albicans* *N*-glycans possess β-1,2-linked mannose residues that are not present in *S. cerevisiae* glycans. Notably, non-conventional yeasts including *H. polymorpha*, *P. pastoris*, and *Y. lipolytica* have *N*-linked core structures ($Man_{8-14}GlcNAc_2$) with α-1,2-linked mannose as a major form, but do not contain immunogenic terminal α-1,3-linked mannose residues. In general, the outer chains of *N*-glycans

Table 1. Comparison of *N*-glycan structures in various yeast species

Species	Type	α-1,6 extension in outer chain	Terminal α-1,3 mannose epitope	β-1,2-linked mannose	Mannosyl-phosphorylation
S. cerevisiae	Polymannose type (M_{8-150})	*OCH1* (^{N}average > 10)	*MNN1*	None	Mannosylphosphorylation (*Mnn6*, *MNN4*, *MNN14*)
P. pastoris	Core type (M_{8-18})	*OCH1* (^{N}average = 3-4)	None	Yes	Mannosylphosphorylation (*PNO1*, *MNN4A*, *B*, *C*)
Y. lipolytica	Core type (M_{8-12})	*OCH1* (^{N}average = 1)	None	None	Mannosylphosphorylation (*MPO1*)
H. polymorpha	Core type (M_{8-14})	*OCH1* (^{N}average = 1)	None	None	Not detected
C. albicans	Polymannose type (M_{8-200})	*OCH1* (^{N}average = 10)	*MNN1* family	Yes	Mannosylphosphorylation (*MNN4*)
*S. pombe**	Moderate high mannose type ($Gal_{6-23}M_{8-60}$)	*OCH1* (^{N}average = 3-4)	None (α1,2-, α1,3- galactose)	None	Addition of pyruvate
*C. neoformans***	Moderate high mannose type (M_{8-20})	*OCH1* (^{N}average > 2)	None	None	Xylosylphosphorylation

* Some *S. pombe* glycans contain α-1,2-linked galactose caps that may also be pyruvylated
** Most *C. neoformans* glycans contain xylose β-1,2-linked to the trimannosyl core

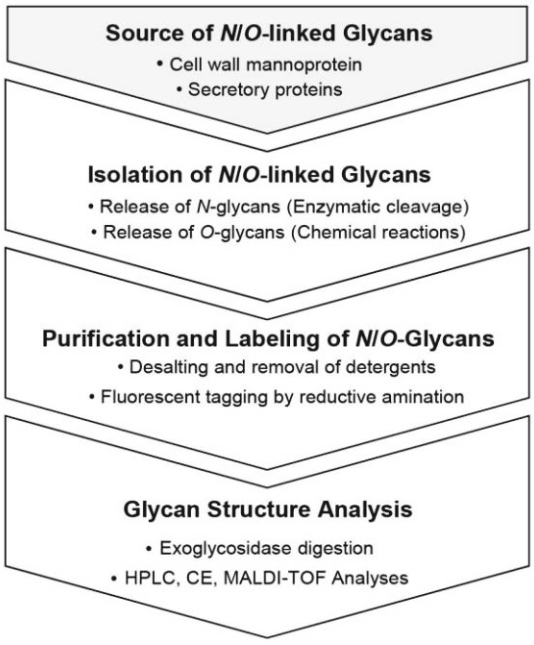

Fig. 2. General scheme for yeast glycome analyses. The first step of glycan structure analysis is the isolation of glycan samples. Since most yeast cell wall proteins are glycoproteins, the cell wall mannoproteins can be analyzed to obtain information on the structure of whole yeast glycans. The next step is the release of glycans from glycoproteins by enzymatic and chemical methods. Following desalting and purification, glycans are subjected to structural analysis by various approaches based on chromatography and mass spectrometry, coupled with exoglycosidase digestion.

in non-conventional yeasts have very short α-1,6-extensions, composed of either only one or few mannose residues (Kim *et al.*, 2006; Song *et al.*, 2007; Park *et al.*, 2012). In the case of *P. pastoris*, the presence of β-1,2 mannose residues has been reported (Vinogradov *et al.*, 2000). Some unique sugars are found in *N*-/*O*-glycans of a few yeast species: Galactose caps that may also be pyruvylated are found as a terminal sugar of *N*-glycans in *S. pombe* (Gemmill and Trimble, 1996), while xylose and xylosyl phosphate residues are present in *N*-/*O*-glycans of *C. neoformans* (Park *et al.*, 2012; Lee *et al.*, 2015).

Cell-surface proteins and secreted proteins of yeasts are mostly glycoproteins, and glycosylation can affect protein conformation, function, and immunogenicity. Detailed structural analyses of glycan chains are required to correlate functional features with defined protein frameworks. Among several important roles of protein glycosylation in various biological processes, two aspects of protein glycosylation are particularly important issues for applying yeast research to the human health field: the effect of glycan structure on recombinant proteins produced from yeasts and the impact of glycan structure on virulence of pathogenic yeasts (Leach and Brown, 2012). The presence of yeast-specific hypermannosylation has been a primary hindrance to exploiting yeast as a host for producing therapeutic glycoproteins. In various yeast species, glycoengineering of yeast host cells has been attempted to produce glycoproteins with human-compatible glycan structures without immunogenic yeast-specific glycans (Jigami and Odani, 1999; Choi *et al.*, 2003; Oh *et al.*, 2008; Cheon *et al.*, 2012; De Pourcq *et al.*, 2012). On the other hand, glycans can modulate the activity of virulence factors involved in adhesion and host infection. Studies carried out in *C. albicans* and *C. neoformans* have reported the impact of protein glycosylation in virulence and interaction with host cells (Bai *et al.*, 2006; Lee *et al.*, 2015). Therefore, a comprehensive understanding of protein glycosylation in pathogenic yeasts would provide useful information for identifying targets for antifungal drugs. Analyzing protein glycans can be difficult due to the structural diversity of glycans and the large number of potential glycosylation combinations. Therefore, detailed characterization of glycans requires the use of several methods. Here, we present the protocols employed in our laboratory to investigate the *N*- and *O*-glycan chains from purified glycoproteins or cell wall mannoproteins in several yeast species. The following steps are used to characterize protein glycosylation: first, the attached glycans are released by enzymatic (for *N*-glycans) or chemical reaction (for *O*-glycans); second, the released glycans are purified and labeled with fluorescent compounds; third, glycan samples are analyzed through the sequential employment of several analytical techniques (Fig. 2).

Table 2. Representative exoglycosidases used for structural analysis of yeast glycans

Exoglycosidase	Specificity	Buffer	Source	Analysis
α-Sialidase, (Neuraminidase)	non-reducing terminal α-2,3-, α-2,6-, or α-2,8-linked sialic acid residues	50 mM sodium acetate, pH 5.0	*Arthrobacter ureafaciens*	Humanized glycans
β-Galactosidase	non-reducing terminal β-Gal	50 mM ammonium formate, pH 4.6	Bovine testes	Humanized glycans
α-Galactosidase	non-reducing terminal α-Gal	50 mM sodium phosphate, pH 6.5	Coffee bean	Yeast glycans
β-N-Acetylglucosaminidase (Hexosaminidase)	non-reducing terminal β-(1-2,3,4,6)-linked N-GlcNAc and N-GalNAc residues	50 mM ammonium formate, pH 4.6	Jack bean	Humanized glycans
α-1,2-Mannosidase	non-reducing terminal α1,2-linked mannose from oligosaccharides	50 mM sodium acetate, pH 5.0	*Aspergillus saitoi*	Yeast glycans
α-1,2-, -1,3- Mannosidase	non-reducing terminal α-1,2-, -1,3 linked mannose from oligosaccharides	5 mM $CaCl_2$, 50 mM sodium acetate, pH 5.5	*Xanthomonas manihotis*	Yeast glycans
α-1,6-Mannosidase	non-reducing terminal α-1,6-linked mannose from oligosaccharides	5 mM $CaCl_2$, 50 mM sodium acetate, pH 5.5	*Xanthomonas manihotis*	Yeast glycans
β-1,2-Xylosidase	terminal xylose β-1,2-linked to the β-mannose of *N*-linked oligosaccharides	5 mM $CaCl_2$, 50 mM sodium acetate, pH 5.5	*Xanthomonas* species	Yeast glycans

Applications

'Omics' data are providing comprehensive descriptions of nearly all cellular components and their interactions. Glycomics, defined as a systems or integrated approach to glycan investigation, has now emerged as a fundamental field, providing an important dimension to systems biology approaches. The complete structural elucidation of glycans requires the sequential employment of several analytical techniques. The protocols employed here have been successfully used in our laboratory to delineate the detailed structure of the *N*-/*O*-glycan chains released from purified glycoproteins or cell wall mannoproteins in several yeast species, including *S. cerevisiae*, *H. polymorpha*, *Y. lipolytica*, and *C. neoformans*. A comprehensive understanding of the main factors and events implicated in protein glycosylation can be obtained by combining glycomics data on glycan structure with other omics data, such as genomics data on glycosyltransferase genes, proteomics data on the glycosylation products, and further integration of metabolomics data on the nucleotide precursors and sugar-phosphates. With the advances of systems and synthetic glycobiology based on glycomics analysis, yeast glycans are expected to be sweet spots with great potential for therapeutic applications, which will provide valuable information on rational design for pathway engineering to produce recombinant proteins with designed glycan structure and on potential targets for antifungal drugs.

Methods

Preparation of *N*-/*O*-linked glycan samples for structural analysis

Release of *N*-/*O*-linked glycans : Since most yeast cell wall proteins are glycoproteins, we usually analyze the cell wall mannoproteins to ascertain the structure of whole glycans of yeast strains. In the case of protein-specific glycosylation pattern analysis, the glycans are released from purified glycoproteins before structural analysis. Several enzymes are available to release *N*-linked glycans. PNGase F (peptide: *N*-glycanase F) is the most popular deglycosylating enzyme that can hydrolyze *N*-glycan moieties from glycoproteins or glycopeptides. This enzyme cleaves the glycan between the innermost N-acetylglucosamine (GlcNAc) attached to the protein and the asparagine residue with an optimum pH of 8.0 to 8.5 (Nuck *et al.*, 1990). Since the catalytic domain of PNGase F interacts directly with the reducing end of the asparagine-linked N-GlcNAc residue of the glycan motif, denaturation prior to PNGase F treatment highly improves the efficiency of deglycosylation. Although PNGase F has a broad substrate spectrum and can release all types of *N*-linked oligosaccharide chains including high mannose, hybrid, and complex biantennary oligosaccharides, but cannot release *N*-glycans with an α-1,3-fucose linkage to the asparagine-linked GlcNAc of the di-N-acetylchitobiose core, a modification that is commonly found in glycoproteins from plants and insects. PNGase A can release all types of *N*-glycans, including polysaccharides with a core α-1,3-fucose structure (Altmann *et al.*, 1995). An endoglycosidase termed Endo H can be used for cleaving high mannose and hybrid *N*-linked glycans (Trimble and Tarentino, 1991). In general, PNGase F is sufficient to isolate *N*-glycans from fungi carrying high mannose and polymannose type *N*-glycans.

The enzymatic methods to release *O*-glycans are very limited, and only the mammalian type *O*-linked Gal-β-1,3-GalNAc is capable of being cleaved by endo-GalNAc-ase (Merry and Astrautsova, 2003). Other methods to release *O*-glycans include chemical reactions, such as hydrazinolysis and alkaline borohydride treatment (*β*-elimination). Hydrazinolysis can release both *N*/*O*-linked glycans at the same time, or they can even be released separately under controlled conditions, specifically by controlling the reaction temperature. *N*-linked glycans are released at 95°C, while *O*-linked glycans are released at 60°C (Patel *et al.*, 1993; Geyer and Geyer, 2006). Hydrazinolysis produces intact *O*-glycans with an intact reducing end. Although yeast oligosaccharides are generally liberated from cell wall proteins by hydrazinolysis followed by *N*-acetylation, this method's popularity has decreased because of its chemical toxicity. Another method for *O*-glycan release is reductive alkaline *β*-elimination processing, in which only *O*-linked glycans are released from proteins under carefully controlled conditions with sodium borohydride ($NaBH_4$), which prevents glycan degradation (Goetz *et al.*, 2009).

Purification and labeling of *N*-/*O*-linked glycans : Since glycans released from proteins usually contain salts, detergents, peptides, and other compounds, glycans must be purified by several methods such as the graphitized carbon desalting method (Packer *et al.*, 1998). The *N*-glycans released by PNGase F treatment need purification to remove the detergents used for protein denaturation, and the *O*-glycans released by chemical reactions require purification to desalt the solution. The purified *N*-/*O*-glycans can be labeled at the free reducing ends by reductive amination with fluorophores, such as 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) or 8-aminopyrene-1,3,6-trisulfonic acid (APTS), 2-aminobenzoic acid (2-AA), and pyridylamine (PA). While sensitivity is achieved via fluorescent tagging by reductive amination, this requires chemical modification of the analyte, thus affecting retention time during chromatography-based separation. The fluorescent-labeled glycans should be then purified to remove excess fluorophores before structural analysis by HPLC or MALDI-TOF.

Analysis of glycan structures

Structural analysis by FACE and HPLC : Fluorescent-labeled *N*-/*O*-glycans can be separated based on their size by high-performance liquid chromatography (HPLC), which is likely the best current method for quantitative *N*-/*O*-glycomics in various yeast species. However, isomer separation is often not achieved by this method, and complex samples may result in incomplete derivatization and/or co-elution of glycans, thereby compromising the quantitation. The glycan profiles from HPLC provide structural information based on their similarity to well-characterized standards, requiring authentic, fluorophore-labeled oligosaccharides as reference compounds. Reversed-phase chromatography is also often used for glycan separation, which is particularly useful for the detailed structural analysis of small oligosaccharides. Depending on the column properties, eluents, and run time, separation of isomeric and isobaric structures can

be accomplished with reversed-phase chromatography (Vreeker and Wuhrer, 2017). Monosaccharide contents of the *N*-/*O*-linked oligosaccharides prepared from glycoproteins can be analyzed by HPLC using an anion-exchange column, providing complementary data on the monosaccharide composition of glycans assembled on glycoproteins (Kim *et al.*, 2004).

Using fluorophore-assisted carbohydrate electrophoresis (FACE) analysis, the labeled oligosaccharides can be also quantified to obtain the molar ratio and degree of glycosylation. This system is based on the use of polyacrylamide gel electrophoresis to separate and quantify the *N*-linked oligosaccharides.

Exoglycosidase digestion of *N*-/*O*-glycans, in combination with HPLC or MALDI-TOF, is useful to determine the monosaccharides present and their anomeric configurations, and to confirm tentative sequences of glycans. Exoglycosidases remove monosaccharides from the non-reducing termini of glycan chains with high specificity to the stereochemistry, the anomeric configuration and linkage site of monosaccharides with respect to the remainder of the glycan chain. Exoglycosidases useful for the structural analysis of endogenous glycans or designed glycans in yeast are listed in Table 2. After enzymatic treatment, the mixture is analyzed by HPLC or MALDI-TOF to determine the number of monosaccharides released by enzyme treatment. Using several well-defined enzymes in sequence, changes of elution position in HPLC and molecular mass in MALDI-TOF mass spectrometry after each digestion step thus reveal the sequence of the monosaccharide constituents of the glycan chains with linkage specificity (Jacob and Scudder, 1994; Morelle and Michalski, 2007).

Structural analysis by mass spectrometry : In mass spectrometry (MS) analysis, the structural assignments of glycans are based on molecular weight, fragment ion formation, susceptibility to exoglycosidase digestion, and linkage data. In recent years, matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF-MS) and nano-electrospray ionization mass spectrometry/mass spectrometry (nano-ESI-MS-MS) have been employed as the major methods for analyzing oligosaccharides (Harvey, 2003; Zaia, 2004). This robust and highly sensitive mass spectrometric approach permits highly sensitive rapid profiling and detailed characterization of heterogeneous structures as well as their relative quantitation. There are several modifications that stabilize the negative charge of glycans by neutralization; permethylation derivatization is currently the most popular method for MALDI-TOF analysis (Morelle and Michalski, 2007). Isolated *N*- and/or *O*-glycans are derivatized by permethylation and the methylated derivatives are analyzed by MS for linkage analysis before and after sequential exoglycosidase digestions. After MS data is obtained, automated software tools that determine glycan composition may be used (Woodin *et al.*, 2013). MALDI-TOF-MS analysis is generally employed to characterize glycans assembled on yeast glycoproteins, which have relatively simple structures and compositions. Neutral glycans are analyzed in the reflective positive ion mode, whereas the negatively charged glycans, such as glycans containing mannoylphosphate or xylosylphosphate residues, are analyzed in linear negative ion mode.

High-throughput structural analysis of glycans using DNA-sequencers : Along with HPLC, capillary electrophoresis (CE) is another very useful technique to provide quantitative information with detailed linkage information. A quantitative analytical method for *N*-/*O*-glycans using a DNA sequencer has been developed, in which all steps, including PNGase F treatment, glycan preparation, and exoglycosidase digestion, were optimized for high-throughput applications using 96-well format procedures and automatic analysis on a DNA sequencer (Lee *et al.*, 2009).

Structural analysis by lectins and glycan structure-specific antibodies : Because of their glycan-recognizing specificities, antibodies and lectins are generally used to detect glycoproteins with specific glycan structures (Cummings *et al.*, 2017). Lectins not only exist in plants, but also animals and mushrooms, and are classified by their specificity to bind monosaccharides. Since mannose is a common monosaccharide present in all glycans from yeast to humans, lectins recognizing mannose residues are usefully exploited to detect the presence of *N*-glycosylation on proteins. For example, concanavalin A (Con A) can bind to α-mannose and α-glucose in *N*-glycans either strongly or weakly depending on structure, whereas *Galanthus nivalis* agglutinin (GNA), it recognizes terminal α-1,2-, α-1,3-, and α-1,6-linked mannose residues (Lee *et al.*, 2015). There are lectins that bind specifically to sialic acid (Siglecs), fucose (F-type lectins), galactose (Galectins), *N*-acetylgalactosamine, and *N*-acetylglucosamine (Ficolins) (Kumar *et al.*, 2012), while *Helix pomatia* agglutinin (HPA), a lectin of animal origin, recognizes and binds terminal GalNAc. Antibodies against glycan antigen, such as antibodies specific to α-1,3-terminal mannose residues, are also useful tools to detect specific structures through immunoblotting (Kim *et al.*, 2004).

Materials

Reagents

- 2-Aminobenzoic acid (2-AA; Sigma, Synonym:Anthranilic acid)
- 2-Aminopyridine (PA; TaKaRa Shuzo)
- 2,5-Dihydroxybenzoic acid (DHB; Bruker Daltonics Inc.)
- 2 × loading buffer (Glyko)
- 6-Aza-2-thiothymine (ATT; Sigma)
- 8-Aminopyrene-1,3,6-trisulfonic acid (APTS; Sigma)
- Acetic anhydride ($(CH_3CO)_2O$; Sigma)
- Acetonitrile (Fisher)
- Ammonium acetate ($NH_4CH_3CO_2$; Sigma)
- Boric acid (H_3BO_3; Sigma)
- Bovine RNase B (Sigma)
- Butanol (Sigma)
- Calcium chloride ($CaCl_2$; Sigma)
- Citric acid (Sigma)
- Dimethylsulfoxide (DMSO; Sigma)
- EDTA (JUNSEI)
- Ethanol (Millipore)
- Glacial acetic acid (J.T. Baker)
- Glucose (JUNSEI)
- HPLC water (Fisher)
- Hydrazine monohydrate ($NH_2NH_2{\cdot}H_2O$; Tokyo Chemical Industry Co.)

- Jack bean α-mannosidase (Prozyme)
- Liz-labeled Genescan (Thermo Fisher Scientific)
- Maltodextrose ladder (Glyko)
- Manganese(II) chloride ($MnCl_2$; Sigma)
- Methanol (Fisher)
- Methyl-α-D-mannopyranoside (Sigma)
- Oligo Labeling Dye (ANTS; Glyko)
- OLIGO Ladder Standard (a mixture of glucose polymers ranging from $Glucose_1$ to $Glucose_{16}$; Glyko)
- OLIGO Quantitation Control (unlabeled maltotetraose; $Glucose_4$; Glyko)
- PA-monosaccharides standard (TaKaRa Shuzo)
- Peptide: *N*-glycanase F (PNGase F; New England Biolabs, 500 U/μl)
- Peptone (BD Difco)
- Reducing Agent ($NaBH_3CN$; Glyko)
- Sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$; Sigma)
- Sodium bicarbonate ($NaHCO_3$; Sigma)
- Sodium chloride (NaCl; Duchefa)
- Sodium citrate (Sigma)
- Sodium cyanotrihydridoborate ($NaBH_3CN$; Sigma)
- Tetrahydrofuran (THF; Sigma)
- Triethylamine (TEA; Sigma)
- Trifluoroacetic acid (TFA; Sigma)
- Tris (Duchefa)
- Yeast extract (BD Difco)
- α-1,2-mannosidase (Prozyme)
- α-1,6-mannosidase (New England Biolabs)
- β-1,2-xylosidase (Calbiochem)

Solutions and buffers

- 10 × GlycoBuffer 2 (New England Biolabs): 0.5 M sodium phosphate, pH 7.5.
- 10 × glycoprotein denaturing buffer (New England Biolabs): 5% SDS, 0.4 M DTT.
- 10 × NP-40 (New England Biolabs): 10% NP-40.
- 2-AA solution: 30 mg of 2-AA and 30 mg of $NaBH_3CN$ in 1 ml of methanol containing 4% of $CH_3COONa{\cdot}3H_2O$ (w/v) and 2% H_3BO_3 (w/v).
- 25% acetonitrile: Acetonitrile/HPLC water (1:3, v/v).
- 60% acetonitrile: Acetonitrile/HPLC water (6:4, v/v).
- 70% ethanol: Ethanol/water (7:3, v/v).
- 96% acetonitrile: Acetonitrile/HPLC water (96:4, v/v).
- Basic buffer: 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl.
- $CH_3COONa{\cdot}3H_2O$ solution: 1% $CH_3COONa{\cdot}3H_2O$ (w/v) in HPLC water.
- Citrate buffer: 0.1 M sodium citrate (Adjust pH 7.0 by adding 0.1 M citric acid).
- Column activating solution: Acetonitrile/HPLC water (8:2, v/v) with 0.1% TFA (v/v).
- Con A binding buffer: 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$.
- Con A elution buffer: 1 M methyl-α-D-mannopyranoside.
- Matrix solution: Saturated ATT, and DHB (v/v, 1:1) in 25% acetonitrile (v/v) containing 0.075% TFA (v/v).
- *N*-glycan elution buffer: Acetonitrile/HPLC water (1:3, v/v) with 0.075% TFA (v/v).
- PBS buffer (iNtRON): 137 mM NaCl, 2.7 mM KCl, Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2-7.4.
- Solvent A: 2% glacial acetic acid (v/v) and 1% THF (v/v) in acetonitrile.
- Solvent B: 5% glacial acetic acid (v/v), 3% TEA (v/v), and 1% THF (v/v) in HPLC water.
- Solvent C: 100 mM acetic acid/TEA, pH 4.0.
- Solvent D: solvent C + 0.5% 1-butanol, 98:2 v/v, pH 4.0.
- TBE buffer: 89 mM Tris, 89 mM borate, and 2.2 mM EDTA.

Equipment

- 0.45 μm filter membrane (Millipore)
- 2475 fluorescence detector (Waters)
- 2690 HPLC (Waters)
- 30 kDa ultrafiltration membranes (Millipore)
- ABI3130 sequencer (Applied Biosystems)
- Amicon-15 (30 kDa molecular weight-cutoff, Millipore)
- Anion-exchange column, PALPAK Type A (0.46 × 15 cm; TaKaRa Shuzo)
- Carbograph Extract-Clean column (150 mg/4 ml; GRACE)
- Con A Sepharose beads (concanavalin A Sepharose beads; GE Healthcare)
- Cyano Base cartridge (Bond Elut-CN-E; 100 mg; Agilent)
- Dialysis membrane (17 kDa molecular weight-cutoff; Sigma)
- Dowex 50WX8–400 resins (H^+ form; Sigma)
- Empower 2 chromatography data software (Waters)
- Gas-phase hydrazinolysis apparatus (Hydraclub S-204; Hohnen Oil)
- GENESCAN 3.1 software (Applied Biosystems)
- Microcon-10 kDa (Millipore)
- Microflex mass spectrometer (Bruker Daltonics Inc.)
- MSP 96 target polished steel (Bruker Daltonics Inc.)
- Multi-Screen-HV (Millipore)
- Poly-prep chromatography columns (Bio-Rad)
- Reversed-phase column (TSK-GEL ODS-80Ts; 0.46 × 25 cm; Tosoh Corp.)
- Sephadex G10 (GE Healthcare)
- Size-fractionation column (Shodex Asahipak NH2P-50 4E; 0.46 × 25 cm; Showa Denko)
- Size-fractionation column (TSKgel Amide-80 column; 0.46 × 25 cm; Tosoh Corp.)
- Spectrophotometer (NanoDrop; CellTAGen)
- Stirred ultrafiltration cell (Millipore)
- Syringe filter (Sartorius Stedim Biotech)
- Vacuum evaporator (Hanil Scientific)

Protocols

A. Preparation of cell wall mannoproteins (cwMPs)

1. Inoculate yeast cells, freshly grown on YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) plates, in YPD medium for seed cultivation, and incubate at the strain's optimum temperature overnight with shaking.
2. Transfer the seed culture of yeast cells into YPD medium and cultivate until the stationary phase.
3. Harvest yeast cells by centrifugation (3,400 × *g*, 10 min, 4°C) to save the cell pellet.
4. Wash the cell pellets twice with HPLC water.
   ➢ **NOTE: In case of yeast species with capsule**
   (i) To reduce the possibility of capsular polysaccharide

contamination, incubate the washed cells twice for 30 min at room temperature in an equal volume of DMSO with mild rotation.
(ii) After centrifugation, wash the cell pellets with HPLC water.

5. Resuspend the washed cells in citrate buffer (see Solutions and buffers) in a ratio of 1:1 (w/v), autoclaved at 121°C for 90 min.
6. Vortex and centrifuge the solution at 3,400 × *g* for 30 min at 4°C.
7. Save the supernatants (mixture of crude cell wall proteins).
   - **NOTE:** If you have concerns about capsular polysaccharide contamination, precipitate capsular polysaccharides from the supernatants by slowly adding an equal volume of cold ethanol to the supernatants and remove the precipitates by filtration with a 0.45 µm syringe filter.
8. Precipitate the total cwMPs from the supernatant by adding 3 volumes of 100% cold ethanol and incubating at 4°C overnight.
9. Collect crude cwMPs by centrifugation at 3,400 × *g* for 30 min at 4°C and wash cwMPs with 5 ml of 70% ethanol (see Solutions and buffers).
10. Dry the pellet at room temperature overnight.
11. Dissolve the dried cwMPs with 9 ml of Con A binding buffer (see Solutions and buffers).
12. Pour 1 ml of Con A Sepharose beads into open column (poly-prep chromatography columns) and then wash with 10 ml of basic buffer (see Solutions and buffers).
13. Incubate the mixture of cell wall mannoproteins with 1 ml Con A Sepharose beads in a column for 4 h at room temperature with slow rotation.
14. Discard the flow-through and wash with 10 volumes of Con A binding buffer.
15. Elute the cwMPs by adding 5 ml of Con A elution buffer (see Solutions and buffers).
16. Dialyze the eluted cwMPs with 3rd DW using dialysis membrane at 4°C or at room temperature for 24–36 h.
17. Dry the purified cwMPs using a vacuum evaporator and dissolve in HPLC water.
18. Quantify the purified cwMPs with a spectrophotometer.

B. Preparation of secretory glycoproteins

1. Harvest yeast cells at mid-log phase by centrifugation (3,400 × *g*, 10 min, 4°C) and save the cell supernatant.
2. Filter the culture supernatants using a nitrocellulose 0.45 µm filter membrane.
3. Concentrate the culture supernatants with PBS buffer (see Solutions and buffers) using Stirred Ultrafiltration Cell with a 30 kDa ultrafiltration membranes.
4. Concentrate and change the buffer to 10 ml Con A binding buffer (see Solutions and buffers) using an Amicon-15.
5. Purify secretory glycoproteins from the concentrated culture supernatants using Con A, as described above for the purification of cwMPs, but use PBS buffer for washing.
6. If necessary, carry out further protein-specific purification steps to isolate protein-specific glycans.
7. Dialyze the eluted glycoproteins or a specific glycoprotein with 3rd DW for 24–36 h, and dry using a vacuum evaporator.
8. Dissolve the dried glycoproteins in HPLC water and quantify the protein concentration by a spectrophotometer.

C. Release of *N*-glycans by PNGase F treatment

1. Treat the purified cwMP or secretory glycoproteins (100 µg) with 10 × glycoprotein denaturing buffer (see Solutions and buffers) and denature glycoprotein at 100°C for 10 min.
2. Chill glycoprotein on ice and add 10 × GlycoBuffer 2 (see Solutions and buffers) and 10 × NP-40 (see Solutions and buffers).
3. Add 3-5 µl of PNGase F and incubate at 37°C for 16 h.
4. Purify the separate *N*-linked glycans using a Carbograph Extract-Clean column (150 mg/4 ml).
   - **NOTE:** The column should be activated in advance with 10 ml of column activating solution (see Solutions and buffers).
5. Wash the column with 10 ml of HPLC water and then load *N*-linked glycans.
6. Wash the column with 10 ml of HPLC water and elute the *N*-linked glycans with 1 ml of *N*-glycan elution buffer (see Solutions and buffers).
7. Dry the purified *N*-linked glycans using a vacuum evaporator.

D. Release of *O*-glycans by hydrazinolysis

1. Resuspend the purified cwMPs or secretory glycoproteins (100 µg) in 100 µl of hydrazine monohydrate.
   - **NOTE:** Dry the protein samples completely using a vacuum evaporator before reaction with hydrazine.
   2. Incubate the mixture at 60°C for 4–6 h.
3. Cool at room temperature and dry the reactant using a vacuum evaporator to remove the hydrazine monohydrate without heating.
4. Dissolve the pellets in 100 µl of saturated $NaHCO_3$, mix with 10 µl of $(CH_3CO)_2O$, and incubate on ice for 30 min without shaking.
5. Purify *O*-glycans using Dowex 50WX8–400 resins to remove other contaminants, such as peptides and salts.
   - **NOTE:** Pour the 0.5 ml of Dowex 50WX8–400 resins into an poly-prep chromatography columns and wash the resins twice with 0.5 ml of HPLC water.
6. Add 0.5 ml of HPLC water to the *O*-glycan samples and load into column.
7. Collect the dripping solution, and load 0.5 ml of HPLC water to collect the residual *O*-glycan samples.
8. Dry the glycan samples using a vacuum evaporator.

E. Labeling of *N*-/*O*-glycans with 2-AA

► 2-AA, carrying a negative charge, is one of the most frequently used labeling materials for HPLC and either positive or negative mode of MALDI-TOF analysis to detect neutral and negative charged glycans (Ruhaak *et al.*, 2010).

1. Resuspend the isolated glycans in 50 µl of fresh $CH_3COONa \cdot 3H_2O$ solution Con A binding buffer (see Solutions and buffers).
2. Incubate with 100 µl of 2-AA solution (see Solutions and buffers) at 80°C for 45 min.
   - **NOTE**: Pay attention to keep the reaction tubes closed during the reaction at high temperature.
3. Purify 2-AA labeled glycans using a Cyano Base cartridge

to remove excess 2-AA.
4. Wash the column with 1 ml 25% acetonitrile (see Solutions and buffers) and equilibrate with 0.5 ml 96% acetonitrile (see Solutions and buffers) twice.
5. Mix the samples with 100% acetonitrile, load into the Cyano column and wash repeatedly with 0.5 ml 96% acetonitrile.
6. Elute the labeled glycans with 0.35 ml 60% acetonitrile (see Solutions and buffers) twice and dry using a vacuum evaporator.

F. Size-fractionation of 2-AA-labeled *N*-glycans by normal-phase HPLC

► Since fluorescence tagging by reductive amination affects retention time on chromatography-based separation, 2-AA-labeled authentic oligosaccharides should be used as reference when analytes are labeled with 2-AA.

1. Equilibrate a size-fractionation column (Shodex Asahipak NH2P-50 4E) with a solution comprising 70% Solvent A (see Solutions and buffers) and 30% Solvent B (see Solutions and buffers).
2. After sample injection, maintain the proportion of solvent B at 30% for 5 min and then increase linearly to 70% over 80 min at a rate of 1.0 ml/min.
3. Analyze 2-AA-labeled-oligosaccharides with a Waters 2690 HPLC system and a 2475 fluorescence detector with excitation and emission wavelengths of 360 and 425 nm, respectively.
4. Collect data using Empower 2 chromatography data software.

G. Separation of acidic and neutral *N*-glycans by normal-phase HPLC

1. Equilibrate a size-fractionation column (Shodex Asahipak NH2P-50 4E) with a solution comprising 90% solvent A (see Solutions and buffers) and 10% solvent B (see Solutions and buffers).
2. After sample injection, maintain the proportion of solvent B at 10% for 5 min and then increase in a linear fashion up to 90% for 60 min at a rate of 1.0 ml/min.
3. Detect 2-AA-labeled-oligosaccharides with a Waters HPLC system as described above.

H. Glycan separation by reversed-phase HPLC

1. Equilibrate a reversed-phase column (TSK-GEL ODS-80Ts) with solvent C (see Solutions and buffers).
2. After sample injection, run with solvent D (see Solutions and buffers) for 5 min.
3. Increase the proportion of solvent D in a linear fashion up to 20% over 60 min at a flow rate of 1.0 ml/min.
4. Detect 2-AA-labeled-oligosaccharides with a Waters HPLC system as described above.

I. HPLC analysis of *O*-glycan profiles

1. Equilibrate a size-fractionation column (TSKgel Amide-80 column) with 90% solvent A (see Solutions and buffers) and 10% solvent B (see Solutions and buffers).
2. After sample injection, maintain the proportion of solvent B at 10% for 5 min.
3. Increase the proportion of solvent B in a linear fashion up to 90% over 60 min at a flow rate of 1.0 ml/min.
4. Detect 2-AA-oligosaccharides with a 2475 fluorescence detector at excitation and emission wavelengths of 360 and 425 nm, respectively.

J. MALDI-TOF analysis

1. Mix the oligosaccharide samples with an equal volume of the matrix solution (see Solutions and buffers).
   - **NOTE:** Each ATT and DHB solution should be saturated in solvent with 10 min sonication, 10 min vortexing, and then 10 min centrifugation.
2. Spot 1 µl of mixed glycan sample on a MSP 96 target polished steel.
3. Allow the mixture to air-dry at room temperature.
4. Analyze the crystallized glycan samples using a Microflex mass spectrometer in the reflective positive mode for neutral glycan analysis, or in the linear negative mode for acidic glycan analysis.
   - **NOTE:** Oligosaccharide samples can be analyzed by MALDI-TOF without fluorescence-labeling if more than 100 µg of purified glycoproteins are available.

K. High-throughput *N*-glycan analysis using DNA sequencer

1. Load the purified glycoproteins or cwMPs samples on the 96-well plates.
2. Add PNGase F reaction mixtures to release *N*-glycans from the purified glycoproteins or CWPs (cell wall proteins) as described above.
3. Purify the released *N*-glycans using non-porous graphitized carbon resin, packed in 96-well plates of Multi-Screen-HV.
   - **NOTE:** The resin should be activated in advance by washing five times with 200 µl of column activating solution (see Solutions and buffers) and finally washing with 200 µl of HPLC water.
4. Wash the 96-well plates five times with 200 µl of HPLC water.
5. Elute *N*-glycans three times via the addition of 200 µl *N*-glycan elution buffer (see Solutions and buffers) and centrifugation at 750 × *g*.
6. Label the eluted *N*-glycans with the fluorophore APTS by reductive amination.
7. Transfer labeled *N*-glycans to a Sephadex G10 packed plate for clean-up and collection.
8. After drying the collected elutes, if exoglycosidase treatment is required, add appropriate glycan-processing enzymes.
9. Dry and reconstitute the incubated samples in 10 µl of water containing a Liz-labeled Genescan standard.
   - **NOTE:** On each gel, include *N*-glycans of bovine RNase B and a maltodextrose ladder as references.
10. Analyze the glycan profile using capillary electrophoresis (CE) equipped with an ABI3130 sequencer by using a 12% polyacrylamide gel and TBE buffer (see Solutions and buffers).
11. Detect glycans at the end of the gel with an Ar laser.
12. Analyze data using the GENESCAN 3.1 software.

L. Exoglycosidase treatment

1. Transfer the cleaned-up labeled *N*-/*O*-glycans to small reaction tubes for exoglycosidase enzyme digestion.
2. Add 200 µl of the appropriate buffer (see Table 2) with the appropriate amount of exoglycosidase, such as α-1,2-mannosidase, α-1,6-mannosidase, β-1,2-xylosidase, or jack bean α-mannosidase, according to the manufacturer's instructions.
3. Incubate the mixture at 37°C for 18 h.
4. Stop the reaction by heating the solution at 100°C for 10 min.
5. Load the mixture onto Microcon-10 kDa, and centrifuge at 13,800 × *g* for 20 min to remove exoglycosidases by filtration.
6. Dry the glycans in a vacuum evaporator and reconstitute the sample with 50 µl of water for HPLC or MALDI-TOF analysis.

M. FACE analysis of *N*-linked oligosaccharide

1. Isolate *N*-glycans from the glycoprotein sample (generally 50–200 µg of glycoprotein is required for analysis) by PNGase F treatment as described above.
2. Add 5 µl of reconstituted Oligo Labeling Dye (ANTS) to each dried oligosaccharide pellet.
3. Mix well by vortexing and add 5 µl of reconstituted Reducing Agent ($NaBH_3CN$) and incubate the sample at 45°C for 3 h (or overnight at 37°C).
4. Dry the samples using a vacuum evaporator until the sample reaches a viscous gel stage.
5. Add water to samples, then add 2 × loading buffer.
6. Load sample, OLIGO Quantitation Control and OLIGO Ladder Standard onto high-resolution polyacrylamide gels.
   - ➢ **NOTE:** OLIGO Ladder Standard (a mixture of glucose polymers ranging from $Glucose_1$ to $Glucose_{16}$), OLIGO Quantitation Control (unlabeled maltotetraose, $Glucose_4$)
7. Separate ANTS-labeled oligosaccharides on high-resolution polyacrylamide gels at a temperature range of 5–8°C.
   - ➢ **NOTE:** FACE electrophoresis buffer should be prepared up to one day in advance of the electrophoresis run and stored at 4°C (the use of recirculating is recommended).

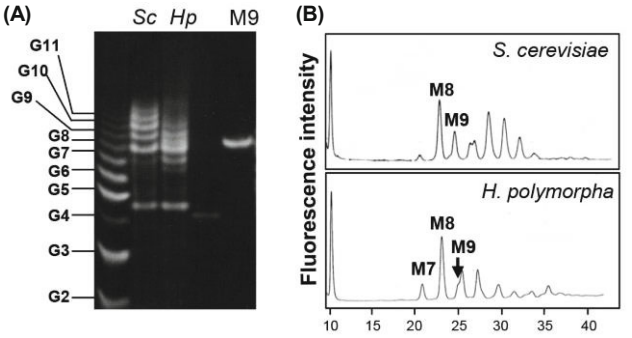

Fig. 3. Comparative analysis of fluorophore-labeled glycans of *H. polymorpha* and *S. cerevisiae*. (A) FACE analysis of ANTS-labeled glycans. Lanes: 1, oligoglucose standard ladder; 2, *S. cerevisiae* wild-type; 3, *H. polymorpha* wild-type; 4, oligo quantitation control maltotetraose; 5, oligomannose 9 ($Man_9GlcNAc_2$). (B) *S. cerevisiae* and *H. polymorpha* *N*-glycan profiles in HPLC. The same glycan samples analyzed in A were subjected to HPLC analysis. The figure was modified from Fig. 2 in Kim *et al.* (2004).

N. HPLC analysis of monosaccharides

1. Isolate the cell wall mannoproteins according to the procedure described above.
2. Hydrolyze the oligosaccharide samples (200 µg) in 100 µl of 4 M TFA at 100°C for 3 h using a gas-phase hydrazinolysis apparatus.
3. Evaporate to dryness under reduced pressure to remove TFA.
4. After hydrazinolysis, *N*-acetylate the oligosaccharides at room temperature for 30 min by adding 250 µl of 0.2 M ammonium acetate and 25 µl of acetic anhydride.
5. Repeat the *N*-acetylation process twice.
6. Label the monosaccharides with PA and analyze using an anion-exchange column, PALPAK Type A, at 65°C.
7. Identify the products with the authentic PA-monosaccharides standard.

Expected Results

Structural analysis of glycans by FACE and HPLC

The electrophoretic migration pattern of the *H. polymorpha*

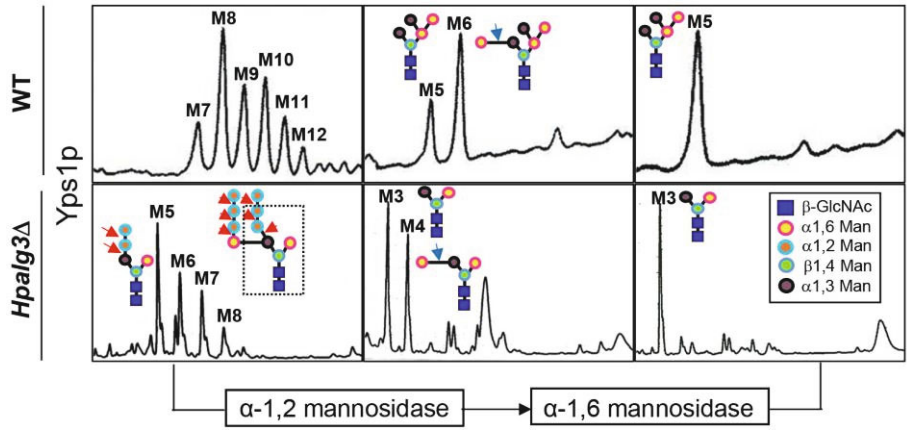

Fig. 4. Sequential exoglycosidase digestions of the wild-type and *alg3*Δ mutant strain of *H. polymorpha*. *N*-glycans of cwMPs from the wild-type and *Hpalg3*Δ mutant strain were treated serially with α-1,2 mannosidase and α-1,6 mannosidase. The glycan profiles were analyzed with HPLC. The figure was modified from Fig. 5 in Oh *et al.* (2008).

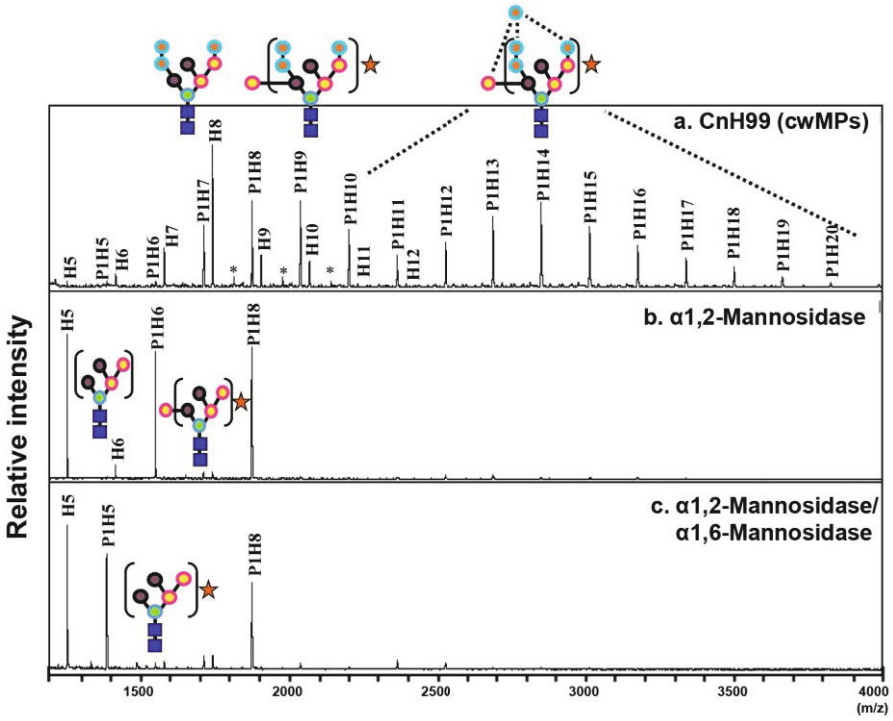

Fig. 5. *N*-Linked oligosaccharide profiles of *C. neoformans* serotype A. *N*-glycans of cwMPs from the serotype A H99 strain, cultivated up to the stationary phase, were analyzed by MALDI-TOF mass spectrometry in the positive mode. (a) no mannosidase treatment. (b) α-1,2-mannosidase treatment. (c) α-1,6-mannosidase treatment of α-1,2-mannosidase-treated *N*-glycans. The figure was modified from Fig. 1 in Park *et al.* (2012).

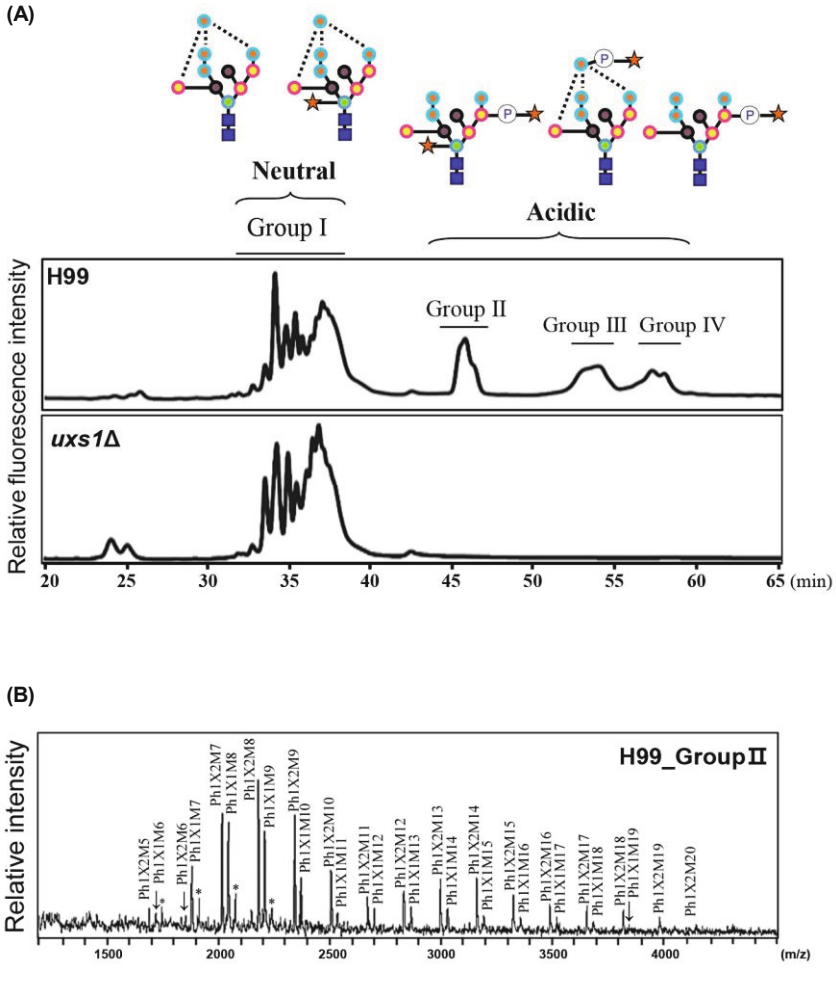

Fig. 6. Acidic *N*-glycan analysis of *C. neoformans* mutant strains. (A) Total *N*-glycan profiles of *C. neoformans* H99 wild-type and *Cnuxs1*Δ strains by HPLC analysis. (B) Acidic *N*-glycan (Group II) profile by MALDI-TOF in negative mode. The figure was modified from Fig. 6 in Park *et al.* (2012).

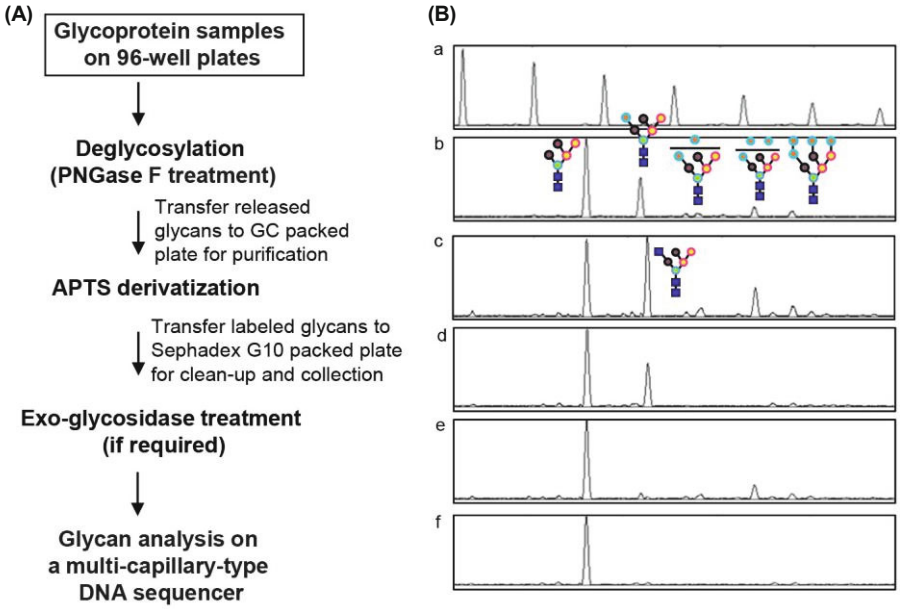

Fig. 7. High-throughput *N*-glycan analysis of glycoengineered *H. polymorpha* strains. (A) Workflow scheme for high-throughput *N*-glycan analysis using a DNA sequencer. (B) APTS-labeled glycans of oligomaltose hydrolysate (a) and Bovine RNase B (b) that serve as a size reference. APTS-labeled *N*-glycans released from the CWPs of the glycoengineered *Hpoch1Δ* strain expressing MsdS and human β-1,2-N-acetylglucosamintransferase I (c), treated with α-1,2 mannosidase (d), treated with β-N-hexosamidase (e), and treated with β-N-hexosamidase and subsequently with α-1,2 mannosidase (f). The figure was modified from Fig. 3 in Cheon *et al*. (2012).

oligosaccharide pool was compared with that of the *S. cerevisiae* oligosaccharide pool using FACE (Kim *et al.*, 2004). The fast migration of *H. polymorpha N*-glycans indicates that less hypermannosylated *N*-glycans are present in this species (Fig. 3A), which is consistent with the glycan profiles analyzed by HPLC (Fig. 3B). Exoglycosidase digestion of *N*-/*O*-glycans is useful to determine the kind of monosaccharides present and their anomeric configurations, and to confirm tentative sequences of glycans. Sequential digestions with α-1,2-mannosidase and α-1,6-mannosidase and HPLC analysis of *N*-glycans from wild-type and *alg3*-null mutant strains of *H. polymorpha* clearly showed that *H. polymorpha N*-glycans are extended mostly in α-1,2-mannose linkages with a very short α-1,6 extension and that the *alg3*-null mutant exhibits defective core *N*-glycan biosynthesis, generating a truncated form of *N*-glycans (Fig. 4) (Oh *et al.*, 2008).

Structural analysis of glycans by mass spectrometry

The MALDI-TOF-MS analysis of *C. neoformans N*-glycans in combination with exoglycosidase digestions clearly revealed the uniqueness of *C. neoformans N*-glycans, particularly the presence of a pentose residue (Fig. 5), which was identified as a β-1,2-xylose residue attached to the trimannosyl core by subsequent experiments (Park *et al.*, 2012). Fractionation by HPLC under specific elution conditions can separate neutral oligosaccharides from negatively charged ones in a chromatogram. After fractionation, the peaks separated by HPLC can be further identified by MALDI-TOF MS using the linear negative mode. The combined structural analysis provided a piece of data strongly supporting the presence of mannosylphosphate residues in *C. neoformans* (Fig. 6). By HPLC fractionation, *C. neoformans N*-glycans were separated into a large neutral pool and three acidic pools. The oligosaccharides of pool I corresponded to the *N*-glycans free of net negative charge, whereas those of the pools II, III, and IV corresponded to mono- or di-mannosylphosphorylated glycans, respectively (Park *et al.*, 2012).

High-throughput structural analysis of glycans using DNA-sequencers

This technique allowed high-throughput glycan analysis and screening for glycoengineered *H. polymorpha* host strains designed for producing recombinant proteins with human-compatible glycans (Fig. 7) (Cheon *et al.*, 2012).

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