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ARTICLE

Overexpression of a homogeneous oligosaccharide with ^{13}C labeling by genetically engineered yeast strain

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Abstract This report describes a novel method for overexpression of ^{13}C-labeled oligosaccharides using genetically engineered *Saccharomyces cerevisiae* cells, in which a homogeneous high-mannose-type oligosaccharide accumulates because of deletions of genes encoding three enzymes involved in the processing pathway of asparagine-linked oligosaccharides in the Golgi complex. Using uniformly ^{13}C-labeled glucose as the sole carbon source in the culture medium of these engineered yeast cells, high yields of the isotopically labeled $Man_8GlcNAc_2$ oligosaccharide could be successfully harvested from glycoprotein extracts of the cells. Furthermore, ^{13}C labeling at selected positions of the sugar residues in the oligosaccharide could be achieved using a site-specific ^{13}C-enriched glucose as the metabolic precursor, facilitating NMR spectral assignments. The ^{13}C-labeling method presented provides the technical basis for NMR analyses of structures, dynamics, and interactions of larger, branched oligosaccharides.

Keywords Oligosaccharide · Stable isotope labeling · Engineered yeast · *Saccharomyces cerevisiae* · NMR structural glycobiology

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Oligosaccharides attached to proteins and lipids play important roles in cell–cell communications and viral infections (Varki 1993). Most of these carbohydrate functions are exerted through interactions with a variety of sugar-binding proteins collectively termed lectins (Sharon 2007). Knowledge of the 3D structures of these oligosaccharides is indispensable not only to gain understanding of the molecular mechanisms underlying such biological events but also to design drugs targeting the carbohydrate–protein interaction systems (Fadda and Woods 2010; Gabius et al. 2011; Kamiya et al. 2011; von der Lieth et al. 1998).

However, the conformational characterization of oligosaccharides remains a challenge because of their flexible properties, which hamper crystallization and interpretation of electron density. Consequently, the structural information available for oligosaccharides remains extremely limited (Skrisovska et al. 2010; Yamaguchi 2008). NMR approaches have great potential for conformational

analyses of oligosaccharides and the complexes they form with lectins in solution (Angulo et al. 2006; Kato et al. 2008; Säwén et al. 2010). Therefore, it is essential to obtain large amounts of homogeneous oligosaccharides. Furthermore, stable isotope labeling of oligosaccharides is highly desirable for detailed NMR analyses (Kato et al. 2010).

Chemically synthesized di- or trisaccharides with ^{13}C labeling at selected positions have previously been subjected to conformational characterization by NMR (Bose et al. 1998; Duker and Serianni 1993; Jonsson et al. 2011; Olsson et al. 2008; Zhang et al. 2009). However, there have been only few reports of chemical synthesis for stable isotope-labeling of larger, branched oligosaccharides of biological interest (Matsuo et al. 2003).

Another approach for preparing isotopically labeled oligosaccharides is metabolic labeling using eukaryotic cells (Kato et al. 2010). Stable isotope labeling of recombinant glycoproteins for NMR studies has so far been reported for the expression systems using baculovirus-infected insect Sf9 cells, methylotrophic yeast *Pichia pastoris* cells, and *Dictyostelium discoideum*, as well as mammalian cell lines (Arya et al. 2008; Blanchard et al. 2008; Kato et al. 2010; Lustbader et al. 1996; Skrisovska et al. 2010; Strauss et al. 2005; Walton et al. 2006; Weller et al. 1996; Yamaguchi and Kato 2010). It should, however, be noted that glycosylation heavily depends on the host species, giving rise to glycans with varying microheterogeneities. Although glycans can be made homogeneous by enzymatic treatments in vitro, the resultant structures are, in general, not tailored (Kato et al. 2010; Yamaguchi et al. 2006).

In this communication, we report a novel method for producing NMR quantities of homogeneous oligosaccharides with ^{13}C-labeling by using genetically engineered yeast cells. In particular, we focus on high-mannose-type oligosaccharides, which modify proteins in the early secretory pathway and control their fates in cells i.e., folding, translocation, and degradation (Aebi et al. 2010; Kamiya et al. 2009; Kato and Kamiya 2007; Lederkremer 2009). In this pathway, the trianntenary oligosaccharide $Glc_3Man_9GlcNAc_2$, a common precursor of asparagine-linked glycans, is trimmed by the actions of a series of glucosidases and mannosidases, giving rise to varying high-mannose-type oligosaccharides (Fig. 1). These processing intermediates are recognized by a variety of intracellular lectins, operating as molecular chaperones, cargo receptors, and ubiquitin ligases in mammalian cells (Aebi et al. 2010; Kamiya et al. 2009; Kato and Kamiya

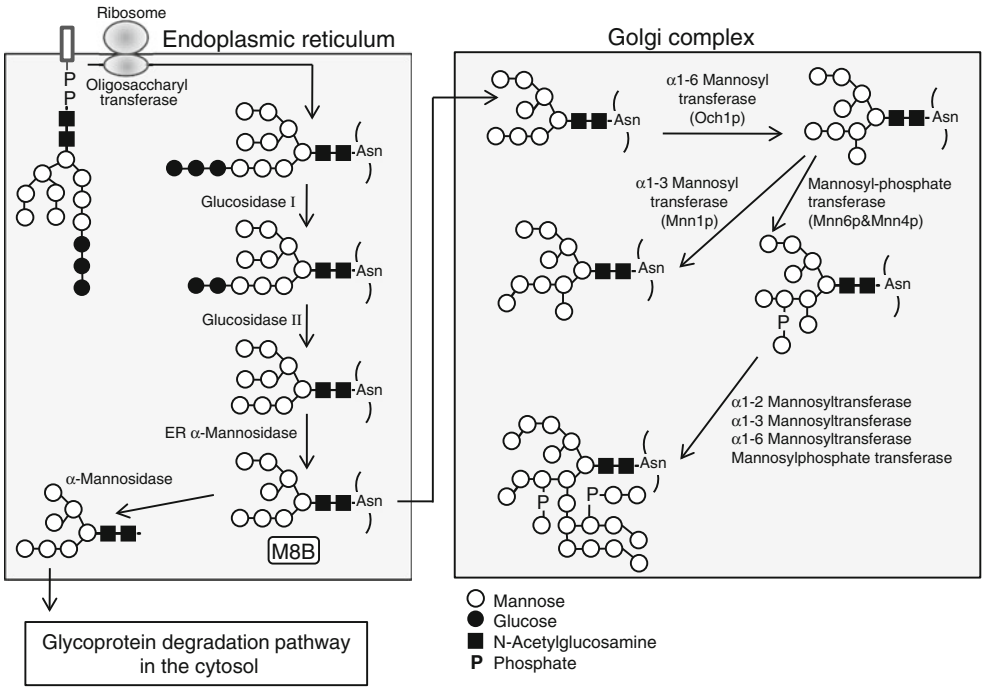

Fig. 1 Scheme showing the processing pathway of asparagine-linked oligosaccharide. The $Glc_3Man_9GlcNAc_2$ oligosaccharide is attached to a polypeptide and then trimmed by the ER enzymes. Trimming of the outermost mannose residue on the middle branch in oligosaccharides by ER α1-2-mannosidase gives rise to a glycoprotein carrying the $Man_8GlcNAc_2$ (M8B) oligosaccharide, which is translocated from the ER to the Golgi complex. In the Golgi complex, the oligosaccharide is modified by attachment of an α1-6 linked mannose residue by the action of mannosyltransferase (Och1p), and subsequently undergoes further mannosylation as well as mannosylphosphorylation catalyzed by Och1p, α1-3-mannosyltransferase (Mnn1p), α1-2-mannosyltransferase, and mannosylphosphate-transferase (Mnn4p and Mnn6p), generating diverse hypermannosylated oligosaccharides

2007; Lederkremer 2009). In yeast, the glycan processing pathways in the endoplasmic reticulum (ER) eventually converge to generate glycoproteins carrying only the Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc (abbreviated as M8B) glycoform, which are subsequently translocated from the ER to the Golgi complex and therein are hypermannosylated by α1-2-, α1-3-, and α1-6-mannosyltransferases and phosphorylated by mannosylphosphate-transferases (Herscovics and Orlean 1993). It has been demonstrated that engineering *Saccharomyces cerevisiae* by knocking out *OCH1*, *MNN1*, and *MNN4* genes, which encode an α-1,6-mannosyltransferase, an α-1,3-mannosyltransferase, and a positive regulator of mannosylphosphate-transferase (Mnn6p), respectively, results in an almost exclusive accumulation of the M8B glycoform (Fig. 1; Chiba et al. 1998; Nakanishi-Shindo et al. 1993). In this study, we employed this engineered yeast as a model system to overexpress ^{13}C-labeled M8B oligosaccharide.

Instead of the conventional culture method using YPAD medium, the Δ*och1* Δ*mnn1* Δ*mnn4* triple mutant cells (Takamatsu et al. 2004) were grown in medium containing 0.5% D-[UL-$^{13}C_6$] glucose (Cambridge isotope laboratories, Inc.), 0.67% yeast nitrogen base without amino acids (Difco), supplemented with 0.3 M KCl as an osmotic stabilizer for 72 h at 30°C. The *S. cerevisiae* cells were collected and suspended in 100 mM citrate buffer, pH 7.0, and subsequently lysed by autoclaving at 121°C for 120 min (Peat et al. 1961). After collection of the supernatant by centrifugation at 8,000*g* for 10 min, three volumes of cold ethanol were added to precipitate the glycoproteins. Approximately 600 mg of lyophilized glycoproteins were obtained from 1 l of culture. Asparagine-linked oligosaccharides were released from glycoproteins by hydrazinolysis and subsequently re–*N*-acetylated using acetic anhydride, then fluorescently labeled with 2-aminopyridine (Wako Pure Chemical Industries, Osaka, Japan). A single peak was observed in the elution profile from a TSK-gel Amide-80 column (Tosoh, Tokyo, Japan; Fig. 2). This oligosaccharide solution was further subjected to HPLC using a Shim-pack HRC ODS column (Shimadzu, Kyoto, Japan). The major oligosaccharide was identified as M8B based on the elution times on the two columns (Takahashi and Kato 2003), and this was confirmed by MALDI-TOF–MS analysis. Thus, homogeneous ^{13}C-labeled high-mannose-type oligosaccharide was successfully produced by the engineered yeast cells, with a yield of 400 nmol per liter of culture. Figure 3a shows the ^{1}H–^{13}C HSQC spectrum of the pyridylamino derivative of uniformly ^{13}C-labeled M8B recorded at a proton observation frequency of 920.7 MHz with a JEOL ECA-920-MHz NMR spectrometer.

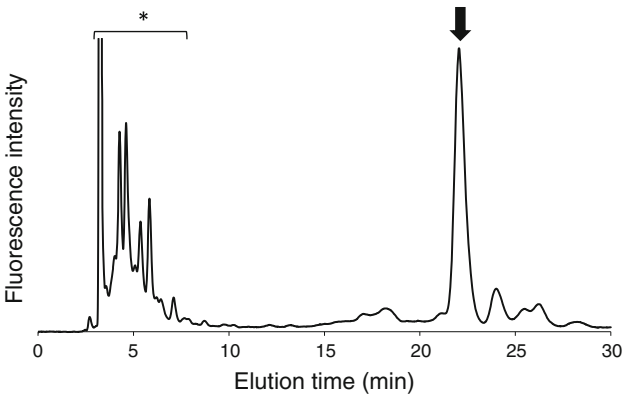

Fig. 2 The elution profile of the pyridylamino derivative of asparagine-linked oligosaccharides derived from the engineered *S. cerevisiae* cells on an Amide-80 column. The fraction indicated by an *arrow* corresponds to the pyridilaminated M8B oligosaccharide, while those indicated by an *asterisk* contained no detectable oligosaccharides

We further attempted to obtain M8B labeled with ^{13}C at selected carbon positions by culturing the yeast cells using appropriate metabolic precursors. Figure 3b shows a superposition of ^{1}H–^{13}C HSQC spectra of the pyridylamino derivatives of M8B produced in the engineered yeast cells grown in minimum medium that contains glucose labeled with ^{13}C at carbon position C1, C2, C3, C4, C5, or C6 (Cambridge isotope laboratories, Inc.). The spectral data demonstrate that selective ^{13}C enrichment can be achieved at specific positions of each sugar residue, accelerating peak classification in spectral assignments of the oligosaccharide, despite isotope scrambling during the metabolic labeling with D-[1-^{13}C]glucose, which resulted in a modest ^{13}C enrichment at the C2 position (Supplementary Figure S1). Based on these data in conjunction with ^{1}H–^{13}C HSQC-TOCSY and ^{1}H–^{13}C HSQC-NOESY data of the uniformly ^{13}C-labeled oligosaccharide, the ^{1}H and ^{13}C resonances originating from the CH groups were assigned, confirming the previously reported ^{1}H assignments of M8B (Berman et al. 1981; González et al. 2000; Vliegenthart 1980).

We have thus established a method for expressing triantennary high-mannose-type oligosaccharide uniformly and selectively labeled with ^{13}C using engineered *S. cerevisiae* cells. The resultant M8B oligosaccharide can be further derivatized by in vitro mannosidase treatments, giving rise to a series of the processing intermediates of high-mannose-type oligosaccharides, which can be easily separated by chromatographic methods (Kamiya et al. 2005; Tomiya et al. 1991). Yeast has several advantages in terms of convenience in cell culture and cell engineering in comparison with higher eukaryotic cells (Chiba and Akeboshi 2009). It has recently been reported that sialylated complex-type oligosaccharides (Hamilton and Gerngross 2007) and mucin-type glycopeptides (Amano et al.

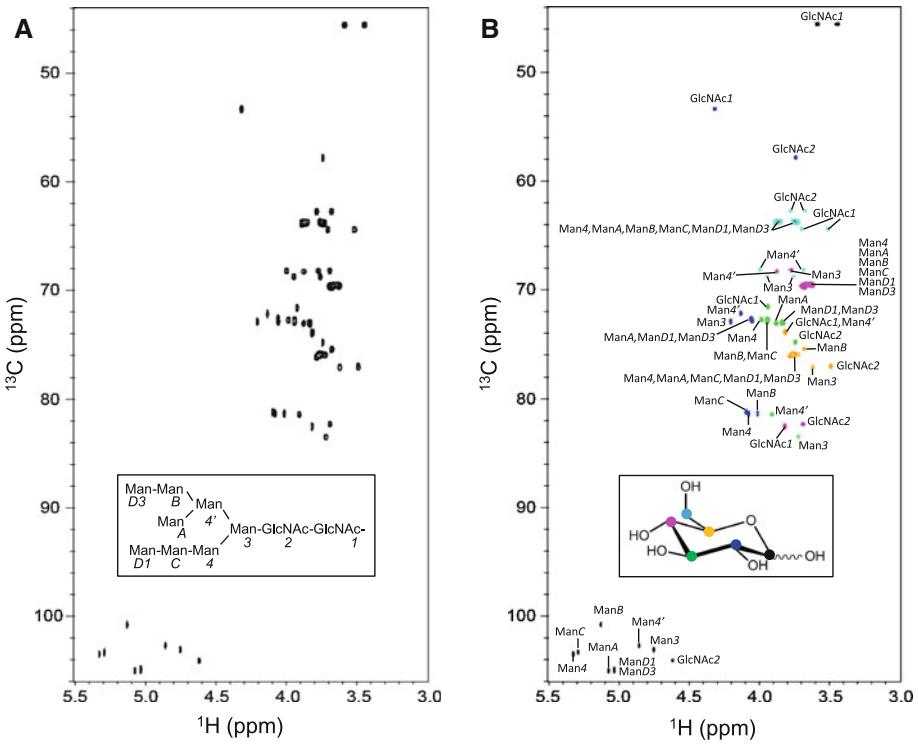

Fig. 3 ^{1}H–^{13}C HSQC spectra of the pyridylamino derivative of M8B, metabolically ^{13}C-labeled using **a** D-[UL-$^{13}C_6$]glucose and **b** D-[1-^{13}C]glucose (*black*), D-[2-^{13}C]glucose (*blue*), D-[3-^{13}C]glucose (*green*), D-[4-^{13}C]glucose (*magenta*), D-[5-^{13}C]glucose (*orange*), or D-[6-^{13}C]glucose (*cyan*). The carbohydrate sequence of M8B is shown in **a**. In **b**, the six spectra were superposed and the ^{13}C-labeled positions in the glucose isotopomers used as metabolic precursors are shown with *circles* in the same colors as the corresponding spectra.

2008) can be produced in engineered yeast cells by disrupting yeast-specific glycosyltransferases and introducing genes responsible for sugar-nucleotide synthesis as well as glycosyltransferases. This line of approach combined with the metabolic labeling method described will open up new possibilities for production of ^{13}C-labeled mammalian-type oligosaccharides. In conclusion, the metabolic ^{13}C labeling of oligosaccharides assisted by yeast engineering facilitates NMR studies of the conformations, dynamics, and interactions of complicated oligosaccharides.

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NMR experiments were performed with a JEOL ECA-920-MHz NMR spectrometer at 30°C. The concentration of the ^{13}C-labeled M8B analog was 0.3 mM in 99.96% D_2O. ^{1}H–^{13}C HSQC spectra were recorded at a proton observation frequency of 920.7 MHz with 256 (t_1) × 1,024 (t_2) complex points and 16 scans per t_1 increment. The spectrum width was 16.2 kHz for the ^{13}C dimension and 17.3 kHz for the ^{1}H dimension. Data were processed using Delta (JEOL) and NMRPipe (Delaglio et al. 1995)

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