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Relative Quantification of Glycans by Metabolic Isotope Labeling with Isotope Glucose in *Aspergillus niger*

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Abstract : Protein glycosylation is a common post-translational modification by non-template-based biosynthesis. In fungal biotechnology, which has great applications in pharmaceuticals and industries, the importance of research on fungal glycoproteins and glycans is accelerating. In particular, the importance of quantitative analysis of fungal glycans is emerging in research on the production of filamentous fungal proteins by genetic modification. Reliable mass spectrometry-based techniques for quantitative glycomics have evolved into chemical, enzymatic, and metabolic stable isotope labeling methods. In this study, we intend to expand quantitative glycomics by metabolic isotope labeling of glycans in *Aspergillus niger*, a filamentous fungus model, by the MILPIG method. We demonstrate that incubation of filamentous fungi in a culture medium with carbon-13 labeled glucose (1-$^{13}C_1$) efficiently incorporates carbon-13 into N-linked glycans. In addition, for quantitative validation of this method, light and heavy glycans are mixed 1:1 to show the performance of quantitative analysis of various N-linked glycans simultaneously. We have successfully quantified fungal glycans by MILPIG and expect it to be widely applicable to glycan expression levels under various biological conditions in fungi.

Keywords : *Aspergillus niger,* Glycans, Glycosylation, Metabolic labeling, Isotopic glucose, Quantitative glycomics, MILPIG, Mass spectrometry

Introduction

Protein glycosylation is a common post-translational modification by a non-templated dynamic process.[1,2] Glycans of glycoproteins play a crucial role in cellular responses to external stimuli, growth, and differentiation, and abnormal glycan composition is directly linked to various diseases.[3] Current use of glycoprotein biomarkers in the clinical setting is usually based on the protein level, but complex and highly dynamic protein decorators such as glycosylation require continuous developments of analytical strategies. As analytical approaches for glycoproteins and glycans, mass spectrometry has contributed greatly to understanding the physiological and pathological processes regulated by glycans and overcoming the challenges of quantitative analysis posed by the complexity of glycoconjugates.[4,5]

Most strategies for relative quantitative glycomics have been achieved with isotopic labeling of glycans through the incorporation of stable D, ^{13}C, ^{15}N, and ^{18}O isotopes. Chemical *in vitro* labeling of glycans includes permethylation of glycans by isotope-labeled[6] or isobaric iodomethane[7,8] and reductive amination by isotope-labeled[9] or isobaric tag[10,11] with an amine group as a nucleophile. As enzymatic *in vitro* labeling strategies for glycans, methods by labeling ^{18}O at the reducing end of glycans through hydrolysis of ^{18}O-water with glycosidase were introduced.[12,13] Methods for relative quantification of *N*-linked glycans utilizing transglycosylation of Endo-M with isotopically labeled acceptors have also been proposed.[14-16]

In addition to the chemical and enzymatic approaches, metabolic isotope labeling strategies of glycans with isotope-labeled monomer building blocks (glutamine or glucose) have been introduced as viable alternatives. IDAWG, isotopic detection of amino sugars using glutamine, is a pioneering report on the feasibility of metabolic incorporation for relative quantification in cell culture.[17,18] This idea was expanded into a strategy to comprehensively quantify glycomes as the metabolic incorporation of isotopes into glycans.

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MILPIG (metabolic isotope labeling of polysaccharides with isotopic glucose) was devised as a metabolic labeling strategy for glycans by adding isotopically labeled glucose (1-$^{13}C_1$) to a glucose-free medium in rice (*Oryza sativa*) cell culture.[19] A follow-up study further demonstrated the quantification of high-mannose *N*-linked glycans with a small number of aminosugars and linear *O*-Man glycans without aminosugars through baker's yeast (*Saccharomyces cerevisiae*) culture for the construction of an isotope labeling model system.[20]

In this study, the MILPIG method is used as a model for *Aspergillus niger*, the most versatile filamentous fungal platform strain, to explore the feasibility of isotope labeling and quantitative analysis of fungal glycans.[21,22] It is noteworthy that the study of glycoproteins and glycans of fungi is considerably accelerated in fungal biotechnology, which is of great importance in pharmaceutical and industrial research.[23-25] In particular, the importance of quantitative analysis of fungal glycans is emerging in the study of the overproduction of filamentous-fungal enzymes and glycosylation pathways by genetic modification.[26-28]

The biosynthesis of isotopically labeled glycan and quantitative analysis of glycan were performed in fungal culture conditions with isotopically labeled glucose (1-$^{13}C_1$) by the MILPIG method. We demonstrated that fungal *N*-linked glycans have mass differences between light and heavy glycans, depending on the number of sugars in the glycan. Based on this, a comparative quantitative analysis method was provided by obtaining a mass spectrum of a 1:1 mixture of light and heavy glycans for quantitative glycomics. From the experimental investigation with filamentous fungi, MILPIG was shown to yield excellent relative quantification of glycans by providing sufficient mass differences that increase with the number of sugars in the glycan.

Experimental

Materials and Chemicals

Peptide: *N*-glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA, USA). 1-$^{13}C_1$ glucose (98% - 99%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and sodium metabisulfite ($Na_2S_2O_5$) were purchased from Alfa Aesar (Haverhill, MA, USA). All other reagents and materials such as yeast nitrogen base (YNB), potato dextrose agar (PDA), d-glucose, trypsin, chymotrypsin, acetic acid (AcOH), methyl iodide (CH_3I), ammonium bicarbonate (NH_4HCO_3), dimethyl sulfoxide (DMSO), anhydrous DMSO, methanol (MeOH), anhydrous MeOH, chloroform, HPLC grade water, dichloromethane (DCM), isopropanol, sodium hydroxide (NaOH), acetonitrile (ACN), acetone, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), polyvinylpolypyrrolidone (PVPP), Discovery® DSC-18 SPE tube (solid-phase extraction C18 column), respectively were purchased from Sigma-Aldrich (St. Louis, MO).

Fungiculture and Protein Extraction

The isolated *Aspergillus niger* was cultured on a potato dextrose agar plate at 26°C in darkness. The fungus spores were inoculated in the potato dextrose agar medium at 28°C and incubated in a shaking incubator at 28°C for 5 days of activation. To prepare fungal culture media according to the experimental conditions, yeast nitrogen base (67 mg) and d-glucose or 1-$^{13}C_1$ glucose (50 mg or indicated amount) were dissolved with 10 mL of distilled water in 15 mL conical tube and autoclaved at 121°C for 15 min. The activated fungal spores were inoculated in conditional culture media and incubated in a shaking incubator at 28°C for 14 days or otherwise specified duration.

On the day of harvest, the fungal biomass was recovered through centrifugation and the fungi were washed three times with 3 mL of distilled water. For the glycan quantitative experiment, normal glucose and 1-$^{13}C_1$ glucose-labeled fungi were mixed in equal proportion after harvest for subsequent analytical procedures. The fungal sample was transferred to a mortar, frozen with liquid nitrogen, and then finely ground and homogenized. The homogenized fungal sample was transferred to a conical tube and resuspended in 5 mL of 50 mM HEPES (pH 7.5) buffer containing 20 mM sodium metabisulfite, 5 mM EDTA, 0.1% (w/v) SDS, and 1.7% polyvinylpolypyrrolidone to extract fungal proteins. The sample was vortexed for 5 min at room temperature and centrifuged at 5,000 × g for 15 min at 4°C. The supernatant was recovered and subjected to acetone precipitation three times at 4°C for 3 hours to remove contaminants. The pellet was collected by centrifugation and dried by a vacuum concentrator. The dried pellet was weighed and stored at -20°C until analysis.

Preparation of *N*-linked Glycans

Equal amounts of protein pellets (2.0 mg) were resuspended in 300 μL of 40 mM NH_4HCO_3 by sonication followed by boiling at 100°C for 2 min. After cooling to room temperature, 25 μL of trypsin stock (2 mg/mL in 40 mM NH_4HCO_3) was added. The samples were incubated overnight (18 h) at 37°C and boiled at 100°C for 5 min to deactivate proteases. The digested samples were loaded onto an equilibrated C18 extraction column, washed with 1 mL of 5% AcOH three times, and then eluted stepwise using 1 mL of 20% isopropanol in 5% AcOH, 40% isopropanol in 5% AcOH, and 100% isopropanol. The resulting glycopeptides were dried in a vacuum concentrator, resuspended with 29 μL of 1 × PNGase F reaction buffer and 1 μL PNGase F (500 U), and incubated for 18 h at 37°C. The released glycan mixture was reconstituted in 5% AcOH and loaded onto an equilibrated C18 extraction column. *N*-linked oligosaccharides were

eluted by 1 mL of 5% AcOH three times and dried by the vacuum concentrator for subsequent permethylation.

Permethylation of Glycans

To facilitate the analysis of oligosaccharides by mass spectrometry, released oligosaccharide mixtures were permethylated as described previously.[8] Briefly, glycans were resuspended in 200 μL of anhydrous dimethyl sulfoxide and 250 μL of freshly prepared dehydrated NaOH/DMSO reagent (mixture of 50% NaOH in 2 mL of anhydrous DMSO). After sonication and vortexing under the nitrogen gas, 100 μL of CH_3I was added and the mixtures were vortexed vigorously for 5 min. 2 mL of distilled water was added to the samples and the excess CH_3I was removed by bubbling with a nitrogen stream. 2 mL of dichloromethane was added. After vigorous mixing and phase separation by centrifugation, the upper aqueous layer was removed and discarded. The nonpolar organic phase was then extracted 4 times with distilled water. Dichloromethane was evaporated on the heating module at 45°C with a mild nitrogen stream and the permethylated glycans were stored at -20°C until analysis.

Analysis of Glycans by Mass Spectrometry

For glycome analysis via direct infusion nanospray MS, permethylated glycans were dissolved by combining 15 μL of the isotopically mixed sample in 100% methanol plus 35 μL of 1 mM NaOH in 80% methanol. They were infused directly into a Q Exactive™ Plus Orbitrap mass spectrometer (Thermo Fishier Scientific, USA) using a Nanospray Flex™ ion source with a fused-silica emitter (360 × 75 × 30 μm, SilicaTip™, New Objective) at 2.2 kV capillary voltage, 220°C capillary temperature, and a syringe flow rate of 0.8 μL/min. The full FTMS spectra of *N*-linked glycans, typically recorded at 70,000 resolution in positive ion and profile mode, were collected at 500–2,000 m/z for 30 s with 5 microscans and 150 ms maximum injection time. The MS/MS spectra following higher energy collision dissociation (HCD) for structural information were obtained at 40% normalized collision energy for *N*-linked glycans.

Data Analysis

Mass spectra of glycan samples were interpreted manually and glycan structures were built graphically using GlycoWorkbench glycoinformatics tools.[29] The area of each isotope peak was integrated and the area of the glycan peak for comparative quantification was calculated as the sum of the peak areas obtained from the isotope envelope and the error according to the purity of the isotope labeled glucose was corrected. The relative abundance ratio of the 1:1 mixture was defined as the peak area ratio between normal glycans and isotopically labeled glycans.

Results and Discussion

Incorporation of Isotopic (1-$^{13}C_1$) Glucose into Glycans

Glucose is the most important energy source for all living organisms and an indispensable precursor for glycan biosynthesis through the hexosamine biosynthetic pathway (HBP) in protein glycosylation. Therefore, in the MILPIG strategy as shown in Figure 1, isotopically labeled glucose in fungal culture media containing 1-$^{13}C_1$ glucose is converted to the isotopically labeled UDP-*N*-acetylglucosamine (UDP-GlcNAc), a nucleotide sugar donor, which is important for protein glycosylation. Isotope-labeled UDP-GlcNAc is utilized in the biosynthesis of glycans and finally leads to the synthesis of isotope-labeled glycoproteins.

Considering that intracellular glucose flux is essential for cellular glycoconjugate biosynthesis, quantitative glycomics were established from the biosynthesis of isotopically labeled glycans with isotopically labeled glucose as shown in Figure 2. Here, we applied *Aspergillus niger* as a model system to the MILPIG method of fungi and conducted a study to evaluate the labeling efficiency of isotope-labeled glycans and the feasibility of quantitative glycomics.

Isotopically Labeled Glycans in Fungi by MILPIG

In our initial experimental design, we labeled fungi for 14 days with 7.5 mg/mL of normal (light) or with 1-$^{13}C_1$ isotopic (heavy) glucose and then isolated *N*-linked glycans from glycoproteins. To reliably quantitate the

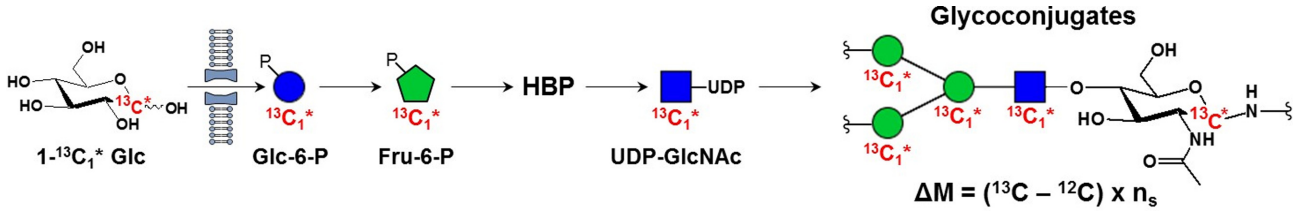


Figure 1. Biosynthetic pathway of isotopically labeled glycans by MILPIG method in fungi. Isotope labeling of glycoconjugates through the hexosamine biosynthetic pathway (HBP). An asterisk denotes an isotopically labeled species. ΔM is the mass difference between normal and isotopically labeled glycans by the sum of the number of sugars (n_s) in the glycans. The glycan structures are drawn based on the Consortium of Functional Glycomics Nomenclature Committee convention.[29]

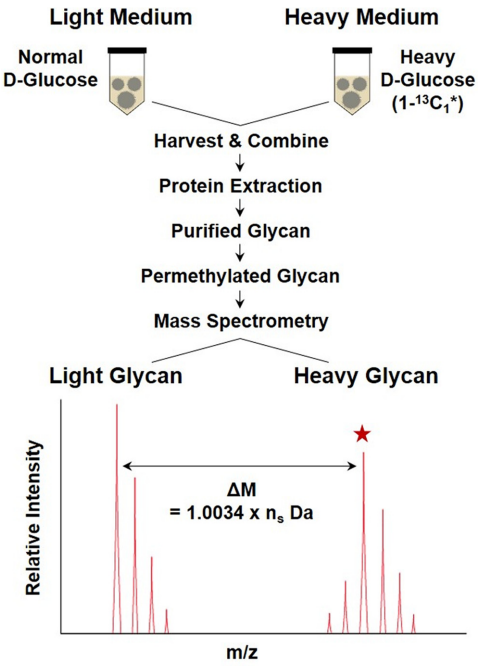


Figure 2. Schematic of the MILPIG strategy for relative quantification of glycans in fungi.

incorporation of 1-$^{13}C_1$ glucose into the *N*-linked glycans, released glycans were permethylated prior to mass spectrometry-based analysis.

Glycan synthesis of filamentous fungi reported in the literature generally follows the high mannose pathway, and as shown in Figure 3, mass spectra of high mannose *N*-linked glycans from $Man_8GlcNAc_2$ (Man8) to $Glc_3Man_8GlcNAc_2$ (Glc3Man9) were obtained. In particular, the full spectrum of heavy *N*-linked glycans isolated from fungi cultured in the presence of isotopically labeled glucose is shown. The increase in the mass of isotopically labeled glycans is correlated with the total number of sugars in their corresponding structures and the charge state.

Comparing the monoisotopic peaks of high mannose *N*-linked glycan structures obtained from the fungi grown in normal or 1-$^{13}C_1$ glucose reveal increases in mass ranging from *m/z* 5.0170 to 7.0213 Da, complying with their sugar ring numbers as well as their doubly charge status. It is noteworthy that the $^{13}C_1$-incorporation is universal added to all carbohydrate rings, including both Man and GlcNAc.

Collectively, we demonstrated that MILPIG applied to fungi can be effectively applied to simultaneously isotope labeling and quantify *N*-linked glycans released from their glycoproteins.

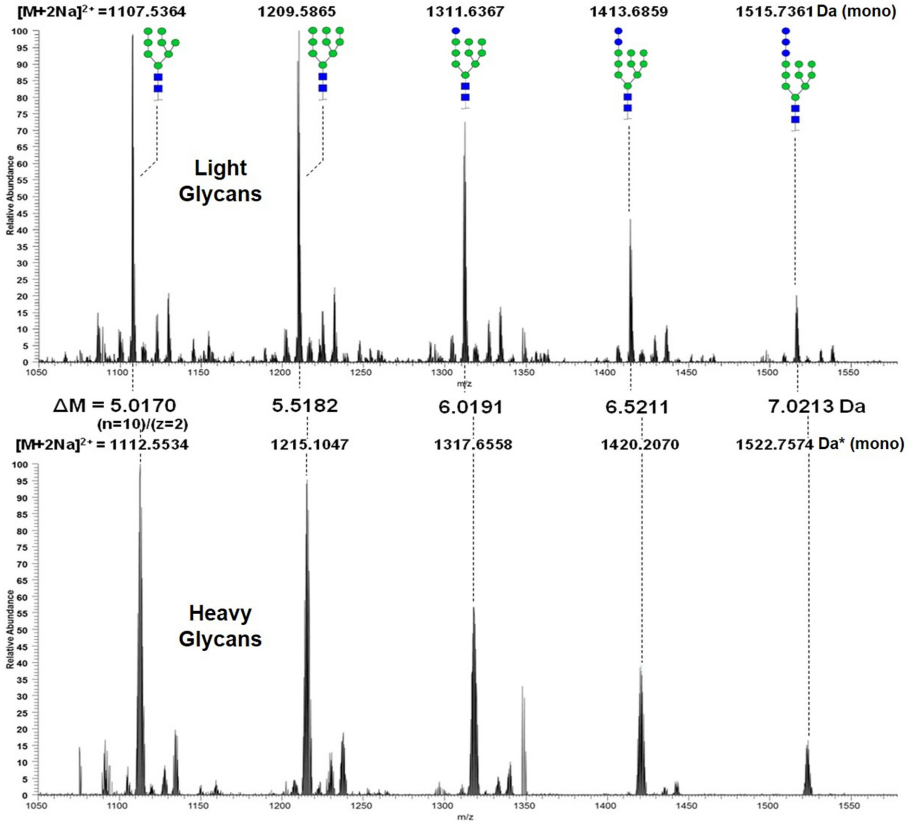


Figure 3. Full mass spectra of the light and heavy *N*-linked glycans released from fungi grown in either normal (light) or 1-$^{13}C_1$ (heavy) glucose. ΔM is the mass difference according to the number of sugar (n)/charge state (z).

Mass Shift and Isotope Distribution of Light and Heavy Glycans

Isotope labeling efficiency in *in vivo* metabolic labeling determines the success of quantitative mass spectrometry. Sufficient mass shift and proper isotope distribution in the mass spectrum between light and heavy are particularly important for quantitative analysis of large biomolecules such as *N*-linked glycans.

In the yeast model experiment of MILPIG, over-incorporation of isotope carbon of glycan was observed by glucose depletion and incubation time. Therefore, in this study, fungi were cultured for 7.5 mg/mL and 14 days, respectively, considering the relatively high glucose concentration and slow growth rate, and Figure 4 shows the light and heavy mass spectrum of a representative Glc2Man9 *N*-linked glycan.

The full spectrum of the glycan mixture containing the light and heavy glycan pairs showed the masses of *m*/*z* 1413.6806 (mono) and *m*/*z* 1420.2019 (mono) by sodiated divalent adducts $[M + 2Na]^{2+}$, as shown in the respective spectrum shown in Figure 3. The mass shift is 6.5 Da in the 13 sugar and charge states of the glycan, showing a sufficient mass difference without mass interference between the last over-incorporation peak (dotted arrow) of the light glycan and the first under-incorporation peak (dotted arrow) of the heavy glycan.

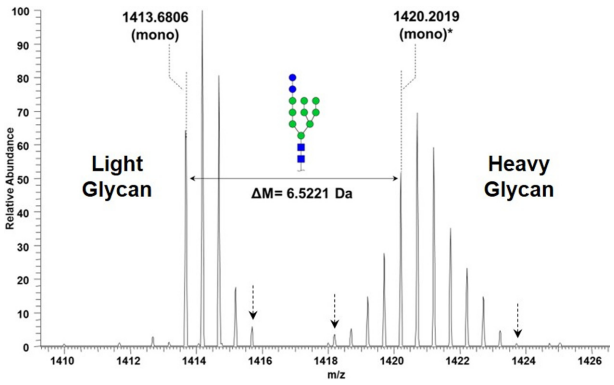


Figure 4. Mass shift and isotope distribution of light and heavy Glc2Man9 *N*-linked glycans in a 1:1 mixture of fungi grown in light and heavy culture media. The mass difference is 6.5 Da in the number of 13 sugars and $[M + 2Na]^{2+}$.

Describing the isotopic envelope of glycans, the isotopic envelope range of light glycans shows 2.5 Da from monoisotopic mass, whereas that of heavy glycans shows a relatively broad isotopic cluster of 5.5 Da. This observation can be summarized as showing a broad isotope envelope due to under- and over-incorporation centered on monoisotopic mass as ^{13}C recycling occurs frequently due to increased incubation time according to the growth rate of fungi.

As a result, although under- and over-incorporation due to the purity of isotope glucose and gluconeogenesis occurs, the sufficient mass difference between light and heavy glycans shows the advantage of quantitative glycomics without interference of mass spectrum peaks.

Relative Quantification of *N*-linked Glycans in Fungi

To provide MILPIG-based quantitative glycomics of fungi, we mixed equal amounts of normal and metabolically labeled fungal samples in culture media with light or heavy glucose. The glycans were then released from the purified glycoproteins with PNGase F, permethylated, and analyzed by mass spectrometry. A full MS spectrum of *N*-linked glycan mixtures containing both light and heavy glycan pairs for Man8, Man9, Glc1Man9, Glc2Man9, and Glc3Man9 is shown in Figure 5. As all of them are ionized as sodiated divalent adducts, each of glycan pairs exhibits

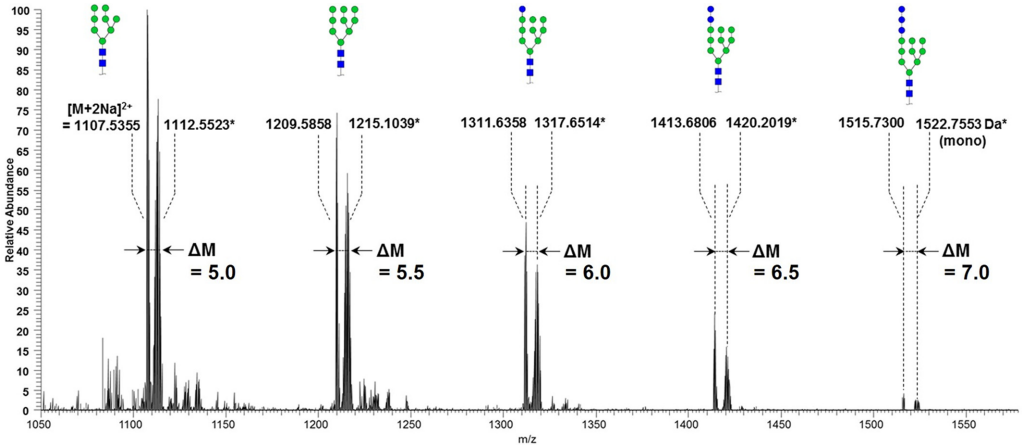
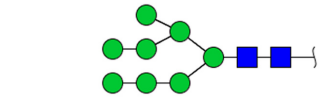
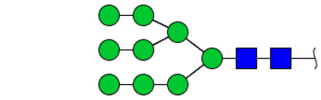
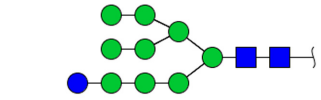
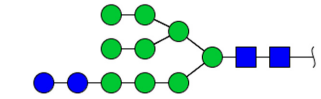
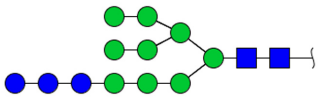


Figure 5. Full mass spectrum of *N*-linked glycans measured as a 1:1 mixture of normal (light) glycans and isotope-labeled (heavy) glycans in fungi. ΔM denotes the mass difference according to the number of sugar/charge state.

Table 1. Relative area ratios for a 1:1 mixture of light and heavy *N*-linked glycans in fungi

No.	Structure	Number of Sugars	Measured $[M + 2Na]^{2+}$ (mono)		ΔM	Ratio of Areas[a] ($^{12}C/^{13}C$)
			Light (^{12}C)	Heavy (^{13}C)		
1		10	1107.5355	1112.5523	5.0168	0.76 ± 0.04
2		11	1209.5858	1215.1039	5.5181	0.91 ± 0.01
3		12	1311.6358	1317.6514	6.0156	0.85 ± 0.03
4		13	1413.6806	1420.2019	6.5213	1.06 ± 0.01
5		14	1515.7300	1522.7553	7.0253	1.03 ± 0.02

[a]The values represent the mean ± standard deviation (SD) for biological triplicates.

their characteristic mass differences of 5.0, 5.5, 6.0, 6.5, and 7.0 Da.

The full mass spectrum shows that the higher the molecular weight, the lower the peak intensity is due to the difference in ionization efficiency and glycan expression. However, light and heavy glycan pairs show a decrease at the same rate, providing quantitative information. In general, it can be seen that the peak patterns of glycan pairs are offset from each other in area comparison of the mass peaks of glycan pairs because the peaks of heavy glycans are low and the isotope clusters are wide compared to light glycans. By enlarging the isotopic envelope pairs of light and heavy glycans as shown in Figure 4, relative quantification of glycans can be obtained from the peak area of each isotope pair without overlapping between the glycan pairs with sufficient mass increments.

To obtain quantitative information, we tabulated the ratio of the area of each isotope pair to the five glycans as shown in Table 1. The theoretical area ratio of light and heavy glycans was 1, whereas the area ratio of experimental results obtained an average of 0.92 in light/heavy, indicating that the expression of heavy glycans was relatively high. However, Man8, Man9, and Glc1Man9 glycans have relatively large peak areas, so errors due to high concentrations are relatively large, whereas Glc2Man9 and Glc3Man9 glycans provide accurate ratios at appropriate concentrations. These results support the excellent performance of the MILPIG method for quantitative glycomics in fungi with high isotope labeling efficiency.

Conclusions

Various qualitative and quantitative analysis techniques of glycans by mass spectrometry have contributed significantly to the biological information and biomarker discovery of glycoproteins. Quantitative analysis of glycans has generally evolved into methods for labeling isotope tags or isotopic elements by chemical or enzymatic *in vitro* reactions for mass spectrometry.

In addition, metabolic isotope labeling strategies of glycans have been proposed, and isotope-labeled glycans are biosynthesized by glycosylation machinery by supplying isotope-labeled biomolecules mainly needed on HBP in cell culture. By the IDAWG and MILPIG methods, the biosynthesis of isotope-labeled glycans in murine embryonic stem cells using isotope-labeled glutamine and in rice and yeast using isotope-labeled glucose were respectively demonstrated, and quantitative glycomics by mass spectrometry were established.

It is noteworthy that the study of glycoproteins and glycans of fungi is greatly accelerated in fungal biotechnology, which is of great importance in pharmaceutical and industrial research. In particular, the importance of quantitative analysis of fungal glycans is emerging in the study of overproduction of filamentous-fungal enzymes and glycosylation pathways by genetic modification. For this reason, in this study, using *Aspergillus niger* as a model system, the biosynthesis of isotope-labeled glycans and quantitative analysis of glycans in the culture conditions of isotope-labeled glucose are provided.

We showed that the fungal *N*-linked glycans by MILPIG method provide a mass difference between light glycans and heavy glycans according to the number of sugars in the glycan. It was shown that the isotopically labeled glycan provides a sufficient mass shift compared to the normal glycan, resulting in effective separation without mass overlapping interference. Based on this, a comparative quantitative analysis method was provided by obtaining mass spectrum of a 1:1 mixture of light and heavy glycans for quantitative glycomics. From the experimental investigation, it was shown that the MILPIG method can be applied to the quantitative analysis of glycans through successful isotope labeling of glycans in fungal culture systems.

Acknowledgments

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