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A novel, simplified strategy of relative quantification *N*-glycan: Quantitative glycomics using electrospray ionization mass spectrometry through the stable isotopic labeling by transglycosylation reaction of mutant enzyme Endo-M-N175Q

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ABSTRACT

The lack of a highly sensitive and simple method for the quantitative analysis of glycan has impeded the exploration of protein glycosylation patterns (glycomics), evaluation of antibody drug stability, and screening of disease glycan biomarkers. In this study, we describe a novel and simplified quantitative glycomics strategy. Quantitation by mutant enzyme reaction stable isotope labeling (QMERSIL) to label the *N*-glycans with either a nondeuterated (d0-) or deuterated (d8-) 4-(2,4-Dinitro-5-piperazin-1-yl-phenyl)-1,1-dimethyl-piperazin-1-ium (MPDPZ)-Boc-asparaginyl-*N*-acetyl-D-glucosamine (Boc-Asn-GlcNAc) acceptor of a positive charge structure through the glycosynthase (Endo-M-N175Q) transglycosylation reaction with mass spectrometry facilitates comparative glycomics. The sialylglycopeptide (SGP) of the complex type was used to demonstrate that QMERSIL facilitates the relative quantitation over a linear dynamic range (up to d0/d8 = 0.02:20) of 3 orders of magnitude. The area ratios of the *N*-glycan peaks from the QMERSIL method showed a good linearity (d0/d8, R^2 = 0.9999; d8/d0, R^2 = 0.9978). The reproducibility and accuracy assay precisions were all less than 6.12%, and the mean recoveries (%) of SGP spiked in the human plasma were 97.34%. Moreover, the QMERSIL using LC–MS/MS was evaluated with various molar ratios (1:1, 1:5, 5:1) of d0(d8)- MPDPZ-Boc-Asn-GlcNAc-labeled glycans from ribonuclease B, bovine fetuin, and ovalbumin. The ratios of the relative intensity between the isotopically MPDPZ-Boc-Asn-GlcNAc labeled *N*-glycans were almost equal a close to the theoretical values (1:1, 1:5, 5:1). Finally, this method was used for the relative quantitative comparison of the *N*-Linked oligosaccharides in human plasma.

1. Introduction

Glycosylation is one of the most prevalent post-translational modifications [1]. In particular, alterations in the glycosylation appear as early as carcinogenesis [2–5]. Acquired diseases that altered the protein glycosylation may represent potential biomarkers for diagnosis [6–8]. Therefore, the quantitative determination of the glycan for the study of screening for glycan biomarkers of diseases, urgently requires fast and simple relative quantitative methods.

Quantitative protein glycosylation and site specific glycosylation analysis still remains challenging due to the complexity of the structure of the glycan, the non-uniformity of their micro-structures, and the difficulty in synthesizing the standard glycans. Without internal standards or external calibration, the absolute glycan quantification is difficult. Chemical stable isotope labeling combined with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) detecting glycans is a

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potential method for the relative quantitative analysis of glycans. For the quantification of the same glycan species between two or more different biological states, various isotopic labeling methods have been used for the relative glycan quantitation [9–11]. The isotope-coded reagents, such as using $^{12}CD_3I/^{12}CH_3I$ [12], $^{12}CH_3I/^{13}CH_3I$ [13], $^{13}CH_3I/^{12}CH_2DI$ [13,14], $^{12}C_6/^{13}C_6$-aniline [15,16], $^{12}C6/^{13}C6$-2-aminobenzoic acid (2-AA) [17,18], (d0/d6-) 2-aminopyridine (2-AP) [19], $^{12}C_6/^{13}C_6$-4-phenethyl benzohydrazide [20], 1-phenyl-3-methyl-5-pyrazolone (d0/d5-PMP) [21], (d0/d5-) girard's reagent P (GP) [22] and $^{16}O+H/^{18}O+D$ labeling [23]. Furthermore, the presence of adduct formation (Na, K, etc.) is greatly reduced upon the etherification, can be employed for confirmation of the parent compound and can be driven towards a particular ion by spiking in of that substance. However, chemical labeling will produce additional side-chemical reactions during the derivation process. It will not only influence the efficiency of the labeling and its purity, but also change the original structure of the glycan.

To avoid these drawbacks, and facilitate studies of glycomics, we reported an enzymatic fluorescent and mass acceptors labeling by an end-β-*N*-acetylglucosaminidase (Endo-M) [24–27,28]. The transglycosylation activity of the permanently positively charged MPDPZ-Boc-Asn-GlcNAc, was relatively high [25,29]. However, the yield of product and the detectivity by the mass spectrometer are very low, due to of the inherent hydrolysis activity of the endo glycosidases. Recently, in order to solve the problem of the hydrolysis of Endo-M to the product, Umekawa et al. developed a novel glycosynthase, an Endo-M-N175Q mutant enzyme of Endo-M [30–32]. The Endo-M-N175Q mutant significantly diminished the hydrolysis activity for the *N*-glycan. Kinetic studies showed that the Km value of N175Q for the *N*-glycan during the hydrolysis was indeed extraordinarily high. The extension of the side chain of this residue (Asn to Gln) by one carbon might disturb the hydrogen bonding between the amide group of this residue and the acetamide of GlcNAc in the substrate. This characteristic is sufficient for preventing quick re-hydrolysis of the transglycosylation product once formed and may also allow its much faster release [31]. However, the Endo-M-N175Q enzyme has been used only as a synthesis tool of the homogeneous glycoproteins with complex-type *N*-glycans to the best of our knowledge. Enzyme reaction stable isotope labeling, which offers advantages for glycomics similar to those by the glycosynthase (Endo-M-N175Q) transglycosylation reaction labeling for quantitative glycomics, has not yet been developed. Therefore, we synthesized new light and heavy stable isotope labeling permanently positively charged d0/d8-MPDPZ-Boc-Asn-GlcANc acceptors for quantitatively analyzing the *N*-linked oligosaccharides. Our objective was to develop a novel, highly sensitive, and simplified method of quantitative glycomics using ESI–MS/MS through the stable isotopic labeling by the transglycosylation reaction of the mutant enzyme Endo-M-N175Q.

In the present study, we describe a relative quantitation method using enzyme reaction stable isotope labeling. The d0/d8-MPDPZ-Boc-Asn-GlcANc acceptors have a permanently positively charged structural formula. We have optimized the transglycosylation reaction conditions for the labeling of sialylglycopeptide (SGP) with do/d8-MPDPZ-Boc-Asn-GlcNAc, and examined the feasibility of the novel method. Furthermore, we demonstrated that this new method could also be used to analyze a human plasma sample.

2. Experimental section

2.1. Materials and chemicals

The recombinant glycosynthase (Endo-M-N175Q), *endo*-β-*N*-acetylglucosaminidase (Endo-M) and Boc-asparaginyl-*N*-acetyl-D-glucosamine (Boc-Asn-GlcNAc) was from Tokyo Kasei Co., Ltd. 100 mU Endo-M-N175Q dissolve in 250 μL water solution, stored below −80 °C until it was used. Sialylglycopeptide (SGP) was purchased from FUSHIMI Pharmaceutical Co., Ltd. (Kagawa, Japan). The d0/d8-4-(2,4-Dinitro-5-piperazin-1-yl-phenyl)-1,1-dimethyl-piperazin-1-ium (MPDPZ)-Boc-Asn-GlcNAc acceptors were synthesized according to a previous paper [25]. Ovalbumin, ribonuclease B (Tokyo, Japan), and pronase E (Merck) were used as received. Disodium hydrogenphosphate dodecahydrate (Na_2HPO_4), ammonium formate ($HCOONH_4$), potassium dihydrogen phosphate (KH_2PO_4), formic acid (HCOOH), chloroform, acetonitrile (CH_3CN) and methanol (MeOH) were of special reagent grade (Wako, Japan). The deionized water was obtained using a PURELAB flex3 automatic water distillation apparatus (ELGA LabWater, United Kingdom).

Table 1
UHPLC-ESI–MS/MS conditions.

System	Shimadzu Nexera UHPLC
Column	ACQUITY BEHC18 (2.1 × 150 mm, 1.7 μm)
Mobile phase A	0.1% Formic acid/H_2O (v/v)
Mobile phase B	0.1% Formic acid/CH_3CN (v/v)
Gradient	B% = 10-60% (0–10 min)
Flow rate	0.4 mL/min
Column temperature	40 °C
System	Shimadzu EIS-MS/MS 8040
Ion mode	ESI positive
Nebulizer gas flow	3.0 L/min
Drying gas flow	15.0 L/min
Interface voltage	4.5 kV
Interface current	11.8 μA
DL temperature	250 °C
Heat block temperature	400 °C
CID gas	230 kPa

2.2. LC-ESI–MS/MS analysis

The UHPLC was performed using a Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) coupled to a Shimadzu LCMS 8040 triple quadrupole mass spectrometer with an electrospray ionization (ESI) source interface. A BEH C18 (1.7 μm, 150 × 2.1 mm i.d.) was used at the flow rate of 0.4 mL/min. The other conditions are shown in Table 1.

2.3. Transglycosylation reaction of sialylglycopeptide

Twenty microliters of 2.5 mM SGP and an equal volume of the 4.0 mM d0/d8-MPDPZ-Boc-Asn-GlcNAc (d0/d8 = 1:1; 3:1; 5:1; 1:3; 1:5 mix) acceptor in H_2O were mixed in a 1.0 mL tube. 10 μL of 0.1 M potassium phosphate buffer (pH 6.5) and 10 μL of Endo-M-N175Q (400 mU/mL) were added to the tube. The solution was then vigorously mixed and incubated at 30 °C for 4.0 h. Chloroform (200 μL) was added to the mixture, and the chloroform layer carefully removed. The same procedures for washing the reaction mixture with chloroform were repeated three times. After the solution with water was diluted 50 times, it was stored at −80 °C just before the UHPLC-ESI–MS/MS analysis.

2.4. Validation of the method

2.4.1. Calibration curve preparation and limit of detection

Two groups of SGPs in equal quantities were labeled with d0- and d8- acceptors, the solution was reacted and determined using the procedures previously described in section 2.3., respectively, and mixed in different molar ratios (d0/d8 = 20:1, 10:1, 5:1, 10:3, 2:1, 1:1, 1:2, 3:10, 1:5, 1:15, 1:50; d8/d0 = 10:1, 10:3, 2:1, 1:1, 1:2, 3:10, 1:5, 1:15,1:20, 1:40, 1:50), followed by ESI–MS analysis. The calibration curves were developed by plotting the peak area of the