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Quantitative *O*-glycomics based on improvement of the one-pot method for nonreductive *O*-glycan release and simultaneous stable isotope labeling with 1-(d_0/d_5)phenyl-3-methyl-5-pyrazolone followed by mass spectrometric analysis

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ABSTRACT

Rapid, simple and versatile methods for quantitative analysis of glycoprotein *O*-glycans are urgently required for current studies on protein *O*-glycosylation patterns and the search for disease *O*-glycan biomarkers. Relative quantitation of *O*-glycans using stable isotope labeling followed by mass spectrometric analysis represents an ideal and promising technique. However, it is hindered by the shortage of reliable nonreductive *O*-glycan release methods as well as the too large or too small inconstant mass difference between the light and heavy isotope form derivatives of *O*-glycans, which results in difficulties during the recognition and quantitative analysis of *O*-glycans by mass spectrometry. Herein we report a facile and versatile *O*-glycan relative quantification strategy, based on an improved one-pot method that can quantitatively achieve nonreductive release and *in situ* chromophoric labeling of intact mucin-type *O*-glycans in one step. In this study, the one-pot method is optimized and applied for quantitative *O*-glycan release and tagging with either non-deuterated (d_0-) or deuterated (d_5-) 1-phenyl-3-methyl-5-pyrazolone (PMP). The obtained *O*-glycan derivatives feature a permanent 10-Da mass difference between the d_0- and d_5-PMP forms, allowing complete discrimination and comparative quantification of these isotopically labeled *O*-glycans by mass spectrometric techniques. Moreover, the d_0- and d_5-PMP derivatives of *O*-glycans also have a relatively high hydrophobicity as well as a strong UV adsorption, especially suitable for high-resolution separation and high-sensitivity detection by RP-HPLC-UV. We have refined the conditions for the one-pot reaction as well as the corresponding sample purification approach. The good quantitation feasibility, reliability and linearity of this strategy have been verified using bovine fetuin and porcine stomach mucin as model *O*-glycoproteins. Additionally, we have also successfully applied this method to the quantitative *O*-glycomic comparison between perch and salmon eggs by ESI-MS, MS/MS and online RP-HPLC-UV-ESI-MS/MS, demonstrating its excellent applicability to various complex biological samples.

Biological significance: *O*-Linked glycoproteins, generated via a widely existing glycosylation modification process on serine (Ser) or threonine (Thr) residues of nascent proteins, play essential roles in a series of biological processes. As a type of informational molecule, the *O*-glycans of these glycoproteins participate directly in these biological mechanisms. Thus, the characteristic differences or changes of *O*-glycans in expression level usually relate to pathologies of many diseases and represent an important opportunity to uncover the functional mechanisms of various glycoprotein *O*-glycans. The novel strategy introduced here provides a simple and versatile analytical method for the precise quantitation of glycoprotein *O*-glycans by mass spectrometry, enabling rapid evaluation of the differences or changes of *O*-glycans in expression level. It is attractive for the field of quantitative/comparative *O*-glycomics, which has great significance for exploring the complex structure-function relationship of *O*-glycans, as well as for the search of *O*-glycan biomarkers of some major diseases and *O*-glycan related targets of some drugs.

Abbreviations: d_0/d_5-PMP, 1-(d_0/d_5)phenyl-3-methyl-5-pyrazolone; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Gal, galactose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Bio-MS, biological mass spectrometry; Asn, asparagine; Ser, serine; Thr, threonine; PEOGs, perch egg *O*-glycans; SEOGs, salmon egg *O*-glycans.

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1. Introduction

Glycosylation is one of the most common and complex forms of post-translational modifications (PTM) of proteins [1]. Typically, there are two types of glycosylation, namely *N*-linked glycosylation on asparagine (Asn) residues and *O*-linked glycosylation on serine (Ser) or threonine (Thr) residues. Both of them have been proved to be related with a wide range of biological processes, such as cell adhesion, molecular recognition, and cell signaling [2–7]. Moreover, it is worth noting that tumorigenesis and malignant transformation are associated with abnormal *O*-glycosylation alterations in both *O*-glycan structures and their expression amount [2,8]. The *O*-glycosylation is commonly formed via attachment of the core GalNAc residues of *O*-glycans to the protein backbones. It occurs in large amount on mucin-type proteins at the surface of mucous membrane, and thus the corresponding carbohydrate chains are named mucin-type *O*-glycans [9,10]. Therefore, qualitative and quantitative analysis of mucin-type *O*-glycans is of great interests to glycomic studies for its considerable significance in search for useful information on some important biomechanisms and major diseases.

Analytical techniques are often faced with difficulties in structural elucidation and quantification of glycans, due to their micro-amount, micro-heterogeneity and structural complexity in the nature. Traditionally, multiple analytical methods often need to be combined together to define and quantify a glycan structure. In the past three decades, however, various biological mass spectrometry (Bio-MS) techniques such as ESI-MS and MALDI-MS have provided a more powerful device for glycomic studies, owing to their superiority in rapid glycan sequencing and quantification [11]. On the basis of Bio-MS, relative quantitation using stable isotope labeling has become a convenient and versatile strategy for the quantitative analysis of various glycan samples. Following this strategy, the abundance of each glycan in one sample can be promptly, accurately determined in terms of relative quantity compared with the identical glycan in the other sample as an internal standard. In recent years, much progress has been made in methodological studies of the relative quantitation of *N*-glycans [12]. In contrast, there are still many difficulties in developing relative quantitation methods of *O*-glycans.

The quantitative MS analysis of *O*-glycans is typically achieved according to the following strategy: *O*-linked glycans are released from glycoproteins by β-elimination reactions under an alkaline condition and then reduced to alditols *in situ* by a reducing reagent to prevent peeling reactions; two glycan alditol samples with different sources are derivatized with the light and heavy forms of a stable isotope reagent, respectively; the two glycan samples are mixed in an equal sample amount ratio and finally subjected to MS analysis. As a result, the relative quantity of each glycan in one sample compared with the identical glycan in the other sample can be obtained according to the intensity ratio between the corresponding light and heavy isotopic MS signals [13]. For example, the methods based on permethylation of *O*-glycan alditols using CD_3I [14] (or $^{13}CH_3I$ [15,16]) and CH_3I as isotopic regents is widely used for relative quantitation of *O*-glycans at present. They are especially suitable for the analysis of micro-amount *O*-glycan samples, as the permethylated glycans have a more stable ionization efficiency and a higher MS detection sensitivity than their native forms [17,18]. However, the large, inconstant *m*/*z* shifts between the heavy and light forms of each glycan perplex the analysis of unknown glycan mixtures, making those methods inefficient to identify mass pairs corresponding to differentially labeled glycans [19]. Botelho and coworkers [19] as well as Atwood III et al. [20] improved those methods using $^{13}CH_3I$ and $^{12}CH_2DI$ as isobaric methylation reagents, the mass difference between which ($\Delta m = 0.002922$ Da) is much smaller. But that method requires high-resolution mass spectrometer and is not suitable for the analysis of glycans with fewer methylation sites by conventional mass spectrometer. The isotopic labeling of *O*-glycans can also be accomplished by reductive β-elimination in alkaline $NaBH_4$ and $NaBD_4$ solutions, and the obtained glycans exhibit a 2-Da mass deference between their light and heavy forms [18]. Nevertheless, this mass difference can hardly be distinguished using conventional mass spectrometer, making this strategy seldom used in glycomic studies. Moreover, those existing methods cause the reducing end of *O*-glycans to be destroyed and unsuitable for further tagging with chromophoric or fluorescent reagents to satisfy high-resolution and high-sensitivity chromatographic analysis. Therefore, isotopic tagging approaches of reducing *O*-glycans at the reducing terminus deserve to be investigated. Due to the lack of reliable methods for quantitative preparation of intact reducing *O*-glycans from glycoproteins [21], however, precise *O*-glycan quantitation based on this isotopic labeling strategy remains a challenge in glycomics studies.

Recently, we have developed a one-pot method for nonreductive release and simultaneous 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization of *O*-glycans [21]. In addition, a relative quantitation approach has also been established by our group for quantitative comparison of free reducing glycans using non-deuterated and pentadeuterated PMP (d_0/d_5-PMP) as stable isotope labels [22]. Herein, we describe a facile, versatile *O*-glycan relative quantitation strategy that combines the both methods, to facilitate studies of quantitative *O*-glycomics. In this study, the reaction conditions for the one-pot method are refined, to ensure quantitative generation of bis-PMP labeled *O*-glycans from glycoproteins, with peeling side reactions conspicuously diminished. The purification approach of the obtained *O*-glycan samples is also improved, to avoid undesired glycan loss during sample post-treatment. Subsequently, the both significantly modified methods are employed to quantitatively prepare d_0- and d_5-PMP derivatives of intact *O*-glycans from glycoproteins. When the d_0- and d_5-PMP derivatives of *O*-glycans are mixed in an equal sample amount ratio and then detected by ESI-MS, LC-MS and MS/MS, each *O*-glycan exhibits a permanent 10-Da mass difference between the MS peaks of its d_0- and d_5-PMP derivatives, and the MS signal intensity ratio between the light and heavy isotope derivatives of each glycan can be utilized for rapid and high-throughput quantitative analysis of *O*-glycans. The quantitation feasibility, reliability and linearity of this new strategy have been verified using bovine fetuin and porcine stomach mucin as model *O*-glycoproteins. Additionally, we have also successfully applied this method to the quantitative *O*-glycomic comparison between perch and salmon eggs by ESI-MS and online RP-HPLC-UV-ESI-MS/MS, demonstrating its excellent applicability to various complex biological samples.

2. Materials and methods

2.1. Materials

Porcine stomach mucin (type III), asialofetuin, maltopentaose (Glc_5), 1-phenyl-3-methyl-5-pyrazolone (d_0-PMP, ≥99%), sodium hydroxide (NaOH), and RIPA lysis buffer (strong type) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine fetuin was from Merck (Darmstadt, Germany). Chicken ovomucin is isolated from eggs in the lab. Sep.-Pak C18 SPE cartridges (100 mg/1 mL) were products of Waters (Milford, MA, USA). MD34 dialysis membrane (MW 8000–14,000) was the product of Union Carbide Co. (Danbury, CT, USA). HPLC grade ACN and methanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Analytical grade dichloromethane (DCM), glacial acetic acid, ammonia (26–28%, vol/vol), and ammonium acetate were obtained from Tianli Chemical Reagent Co. Ltd. (Tianjin, China). PMSF was from Beijing Dingguo Changsheng Biotech. Co. Ltd. (Beijing, China). 1-(pentadeutero)phenyl-3-methyl-5-pyrazolone (d_5-PMP, 99%) was synthesized in our laboratory [22]. *Micropterus salmoides* (perch) and *Oncorhynchus keta*