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Negative-ion MALDI-MS^2 for discrimination of α2,3- and α2,6-sialylation on glycopeptides labeled with a pyrene derivative☆

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ABSTRACT

Here, we propose a novel method for the discrimination of α2,3- and α2,6-sialylation on glycopeptides. To stabilize the sialic acids, the carboxyl moiety on the sialic acid as well as the C-terminus and side chain of the peptide backbone were derivatized using 1-pyrenyldiazomethane (PDAM). The derivatization can be performed on the target plate for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), thereby avoiding complicated and time-consuming purification steps. After the on-plate PDAM derivatization, samples were subjected to negative-ion MALDI-MS using 3AQ-CHCA as a matrix. Deprotonated ions of the PDAM-derivatized form were detected as the predominant species without loss of sialic acid. The negative-ion collision-induced dissociation (CID) of PDAM-derivatized isomeric sialylglycopeptides, derived from hen egg yolk, showed characteristic spectral patterns. These data made it possible to discriminate α2,3- and α2,6-sialylation. In addition, sialyl isomers of a glycan with an asparagine could be discriminated based on their CID spectra. In brief, the negative-ion CID of PDAM-derivatized glycopeptides with α2,6-sialylation gave an abundant ^{0,2}A-type product ion, while that with α2,3-sialylation furnished a series of ^{2,4}A/Y-type product ions with loss of sialic acids. The unique fragmentation behavior appears to be derived from the difference of pyrene binding positions after ionization, depending on the type of sialylation. Thus, we show that on-plate PDAM derivatization followed by negative-ion MALDI-MS^2 is a simple and robust method for the discrimination of α2,3- and α2,6-sialylation on glycopeptides.

1. Introduction

Both electrospray ionization mass spectrometry (ESI-MS) [1] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [2–5] have proven to be powerful analytical tools for characterizing biomolecules. MALDI-MS has several advantages over other mass spectroscopic techniques due to the relative simplicity of spectral interpretation, rapid analysis and repeated measurement of the same sample. Thus, MALDI-MS has been used in a wide variety of applications for the analysis of glycans [6–8]. In particular, a collision-induced dissociation (CID) technique combined with MALDI-MS will be indispensable for the structural determination of glycans because of the plethora of isomeric structures.

Sialic acids exist on many glycoconjugates, such as glycoproteins and glycolipids. Given that sialylated glycans of glycoconjugates play an important role in various biological functions, determination of their precise structures, including the type of sialyl linkages, is essential. However, determination of the type of sialyl linkage by MS is still a challenging task because of preferential detachment of sialic acid residues from the glycan. This detachment usually occurs not only in the CID conditions but also in the ionization events in MALDI. As a result, structural information of sialylated glycan is lost. Various approaches have been reported to prevent loss of sialic acid, such as the use of a cool matrix [9–13] and measurement by high-pressure MALDI [14,15]. Although these approaches are effective for MS^1 experiments, the sialic acids are preferentially detached under CID, which is needed for structural determination. It is believed that a dissociative proton of the carboxyl moiety on sialic acid leads to the loss of the sialic acid unit. To eliminate the dissociative carboxylic proton, sialic acid could be converted to its ester [16,17], amide [18] or derivatized with acetohydrazide [19]. Indeed, these methods enabled us to detect sialylated glycans in positive-ion mode without a loss of sialic acid. Unfortunately, however, the type of sialyl linkage could not be identified. Permethylation [20–22] and perbenzylation [23] has been reported to be effective for discrimination of α2,3- and α2,6-sialylation by preferential formation of lactone from α2,3-linked sialic acid. Harvey et al. introduced a unique methyl ester derivatization for discrimination of α2,3- and α2,6-sialylation on the glycan from the MALDI

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mass spectrum without MS^n experiments [24]. Although these approaches may be applicable to released glycans, there are currently no reports concerning the stabilization of sialic acid and discrimination of the type of sialyl linkage on glycopeptides.

Since the original results reported by Biemann and Martin [25], positively charged ions and their product ions have been used for amino-acid sequencing of peptides and proteins. However, scant attention has been paid to negatively charged ions. The structural determination of glycans based on MS^n has also been performed in positive-ion mode because glycans usually ionize as $[M+Na]^+$ or $[M+K]^+$. Recently, CID of negatively charged ions has proved to be effective for the structural determination of glycans [26–33]. In particular, we have focused on the analysis of both neutral and acidic glycans in negative-ion mode, rather than positive-ion mode, because negative-ion CID of glycans derivatized with pyrene generate unique product ions for structural determination [29,30]. In general, negative-ion formation from neutral glycan is difficult, showing no or low ion yields. By contrast, we found that glycans derivatized with pyrene produced an abundance of ions compared with other derivatization protocols such as 2-aminopyridine [29]. In order to obtain higher yields of negative ions, on-plate pyrene derivatization for glycans has been developed as a simple and rapid method [34]. Furthermore, we have developed another unique on-plate pyrene derivatization for glycopeptides [35]. The method not only enhances ion yield of glycopeptides, but also suppresses a loss of sialic acids on glycans and glycopeptides. Previously, by using this pyrene derivatization method, we could discriminate α2,3- and α2,6-sialylation on released glycans by MALDI-MS^2 [36]. In the present study, we succeeded in discriminating glycopeptides with sialylated isomers based on their negative-ion MS^2 spectra. In contrast to the analysis of released glycans, glycopeptide analysis provides site-specific information of glycosylation in addition to the possible identification of the corresponding protein. The determination of altered patterns of sialylation in glycopeptides is indispensable for the elucidation of their biological function. This is the first report to describe a highly sensitive and straightforward method of discriminating α2,3- and α2,6-sialylation on glycopeptides by MALDI-MS.

2. Experimental

2.1. Materials

A sialylglycopeptide (SGP) was prepared from hen egg yolk [37]. α-2,3-Sialyltransferase (EC: 2.4.99.4) from *Photobacterium phosphoreum* JT-ISH-224 strain and α-2,6-sialyltransferase (EC: 2.4.99.1) from *Photobacterium damselae* JT160 strain were purchased from Japan Tobacco Inc. (Tokyo, Japan). CMP-NeuAc was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Cellulose fibrous medium was purchased from Sigma–Aldrich (Steinheim, Germany). NuTip Carbon was purchased from Glygen Corp. (Columbia, MD). Four kinds of sialylated isomers of disialylated glycans attached to an asparagine (A2-Asn) were purchased from Otsuka Chemical (Tokushima, Japan). The highly purified MALDI matrix chemicals, 2,5-dihydroxybenzoic acid (DHBA) and α-cyano-4-hydroxycinnamic acid (CHCA), were purchased from Shimadzu-Biotech (Kyoto, Japan); and 3-aminoquinoline (3AQ) was purchased from Sigma–Aldrich; 1,5-diaminonaphtalene (1,5-DAN) was purchased from WAKO Pure Chemical, Inc. (Osaka, Japan). 1-Pyrenyldiazomethane (PDAM) was purchased from Molecular Probes, Inc. (Eugene, OR). The solvents, ethanol (EtOH, LC/MS grade), acetonitrile (MeCN, LC/MS grade) and butanol (HPLC grade), were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan); toluene (HPLC grade) and xylene (HPLC grade) were purchased from Sigma–Aldrich. The water used in all experiments was purified by using a NANOpure DIAMOND Ultrapure Water System from Barnstead (Boston, MA). All reagents were used without further purification.

2.2. Preparation of glycopeptides with α2,3- or α2,6-linked sialic acid

For desialylation, a purified SGP derived from hen egg yolk was heated in 0.8% TFA at 80 °C for 45 min. Then, sialic acids were added on the non-reducing ends of desialylated SGP glycans with specific enzymes. The enzyme reaction was performed according to the protocol described by Yamamoto et al. [38,39] with slight modification. The 150 μL reaction mixture consisted of 20 mU of each enzyme solution (α-2,3-sialyltransferase or α-2,6-sialyltransferase), 500 pmol of asialo-SGP, 2.3 mM CMP-NeuAc, 20 mM bis–Tris buffer (pH 6.0), 0.5 M NaCl and 0.03% Triton X-100. The reaction was carried out at 37 °C for 4 h. Under this condition, both disialylated and monosialylated SGP were obtained and confirmed by using different type of MALDI instrument in linear mode, which can prevent loss of sialic acid. The structures of desialylated SGP and synthesized SGP are shown in Fig. 1.

The glycopeptides were isolated by using the hydrophilic affinity method [40]. Reaction products were dissolved in an organic solvent mixture containing butanol/ethanol/water (4:1:1 by volume). 30 mg of cellulose fibrous medium was washed in water, ethanol/water (1:1 by volume), and conditioned with butanol/ethanol/water (4:1:1 by volume). After conditioning, cellulose was suspended with the 400 μL of organic solvent mixture. A 20 μL aliquot of the suspension was then added to the reaction product solution. The resulting mixtures were shaking with a vortex mixer for 1 h and then centrifuged for 2 min. The supernatants were removed and the cellulose was washed five times with the 300 μL of the same organic solvent. The samples were extracted with a vortex mixer the cellulose in 200 μL of ethanol/water (1:1, by volume) for 15 min. The samples were then centrifuged, and the supernatants transferred to another microtube. The same extraction procedure was repeated four times. Finally, the supernatants were completely dried using a Speed Vac and reconstituted with 20 μL of 0.1% TFA.

Cellulose-purified reaction products were further desalted by NuTip carbon. Before loading the sample, the NuTip Carbon was conditioned by aspirating 20 μL of the releasing solution, 80% MeCN with 0.1% TFA, followed by the binding solution, 0.1% TFA. Cellulose-purified reaction products were loaded on the NuTip Carbon by aspirating 20 times. Then the NuTip Carbon was washed with 20 mL of binding solution by aspirating 20 times, and the samples were eluted by expelling the releasing solution 10 times. The same releasing procedure was repeated 10 times. The released solution were completely dried on a Speed Vac and reconstituted with water.

2.3. Pyrene derivatization on the target plate and mass spectrometry

Analyte solution was first deposited on a mirror-polished stainless-steel MALDI target and left to dry. A fresh solution of 0.25 μL of PDAM 10 nmol/μL in dimethyl sulfoxide was added onto the dried analyte and the plate was heated at 80 °C for 5 min. The plate was then rinsed with toluene or xylene to remove excess PDAM and briefly dried under vacuum. Subsequently, the matrix solution, DHBA or 3AQ-CHCA was applied. DHBA was dissolved in 60% MeCN/H_2O to a concentration of 10 mg/mL. Then the 1 μL of resulting solution was applied onto the plate and left to dry. A stock solution of 3AQ-CHCA liquid matrix was prepared by dissolving 35 mg 3AQ in 150 μL of a saturated solution of CHCA in MeOH [41]. A stock solution of 3AQ-CHCA was diluted by a factor of 10 in the 60% MeCN/H_2O and 1 μL of resulting solution was applied onto the plate. MALDI-TOF mass spectra and MS^n spec-