International Journal of Mass Spectrometry 333 (2013) 8–14

Contents lists available at SciVerse ScienceDirect

International Journal of Mass Spectrometry

journal homepage: www.elsevier.com/locate/ijms

Advantages of pyrene derivatization to site-specific glycosylation analysis on MALDI mass spectrometry

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ARTICLE INFO

Article history:
Received 17 February 2012
Received in revised form 2 August 2012
Accepted 2 August 2012
Available online 10 August 2012

Keywords:
Glycopeptide
Glycoform
MALDI
DHBA
Signal localization

ABSTRACT

Glycoproteomics involving the analysis of glycopeptides by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a new and attractive technique. However, quantitative performance in MALDI-MS is hampered by its poor reproducibility among laser shots. 2,5-Dihydroxybenzoic acid (DHBA) is a useful matrix for glycopeptides but forms highly heterogeneous crystals. In this study, we have investigated the distribution of significant signals generated from a sample of glycopeptides on the target plate using a MALDI imaging technique. MALDI images of glycopeptides, which have different glycans on the same peptide, in the Lys-C digests of bovine ribonuclease B were identical. Thus, all glycoforms on a given peptide can be detected at the same laser irradiation spot simultaneously, which offers a significant advantage over other techniques. A similar result was observed with glycopeptides of human serum immunoglobulin G. Interestingly, distinct MALDI images were observed for glycopeptides having different amino acid sequences, despite having an identical glycan structure. The common peptides, which were glycosylated or non-glycosylated, or sialylated or desialylated gave similar MALDI images. Taken together, our results suggest that sweet spot localization of glycopeptides is dependent on the peptide moiety rather than the glycan structure. Furthermore, introduction of pyrene group to glycopeptides which have different peptides result in a uniform MALDI image. It suggested that pyrene derivatization in MALDI-MS facilitates straightforward analysis of a glycopeptide mixture because the same mass spectrum can be obtained at every sweet spot in addition to increase in signal intensity. Thus, this study validates the use of MALDI-MS for site-specific glycoprofiling at the glycopeptide level.

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1. Introduction

In recent years, glycoproteomics has become an important field of post proteomics approaches. A glycoprotein exists as a mixture of different glycosylated species named glycoforms. To clear this microheterogeneity is indispensable to characterization of the glycoprotein because glycoforms vary biological activities. Furthermore, if a glycoprotein has multi glycosylation sites, site-specific glycoprofiling, which provides a quantitative map of glycans attached to a given protein glycosylation site, is an important approach to clearly defining the function of a glycoprotein. It is essential to analyze glycopeptides without releasing glycans to prevent from destruction of the protein moiety. It is the best that all the glycoforms can be measured at once to know quantitative contents of glycoforms.

Mass spectrometry (MS) combined with a soft ionization technique has proven to be a powerful analytical tool for the analysis of glycopeptides [1,2]. Both electrospray ionization mass spectrometry (ESI-MS) [3] and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [4–7] have been used to ionize fragile glycopeptides. MALDI-MS offers several advantages over ESI-MS for the analysis of a small amount of sample. In particular, MALDI-MS facilitates (i) relatively simple spectral interpretation; (ii) rapid analysis and (iii) repeated measurements of the same sample. Thus, MALDI-MS has been widely used in a variety of applications for the analysis of glycans and glycopeptides [8–12].

Although MALDI-MS is a very sensitive and precise method, it is difficult for quantification because ionization efficiency of analytes depends on their specific physicochemical properties. The ionization reaction of a glycopeptide, including protonation and deprotonation, mostly occurs on the peptide moiety. Thus, the signal intensity of a glycopeptide largely is dependent on the nature of the peptide portion, but only weakly dependent on the nature of glycan. Therefore, the glycoprofiling of glycopeptides based on their signal intensities should be possible because glycans are attached onto the same peptide. In fact, compared with different methods from MS, good correlation between signal intensities of glycopeptides and the actual amount of corresponding glycoform has been reported [13–15]. However, poor shot-to-shot and

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1387-3806/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.ijms.2012.08.006

sample-to-sample reproducibility in MALDI-MS may cause a problem in such quantitative measurements.

The poor reproducibility is considered to be particularly prominent when the matrix crystallizes heterogeneously. Although 2,5-dihydroxy benzoic acid (DHBA) is one of the most widely used matrices for ionizing glycans and glycopeptides, the matrix gives highly heterogeneous crystals. Typically, the long needle-like crystals exist mostly at the periphery, whereas thin microcrystals are distributed in the central region under standard dried-droplet preparation method. The appearance and intensity of analyte signal is highly dependent on the spot of laser irradiation over the crystallized matrix. Hence, significant variation in signal intensity is often observed. We have to measure all area of the matrix crystal and the signals obtained by all laser shots should be summed. To overcome the signal heterogeneity, many approaches have been suggested, including the production of a microcrystalline matrix [16], the fast evaporation of matrix solvent [17] and the use of liquid matrices [18]. However, these approaches can often promote undesirable alkali-metal adduction to the analyte. Bouschen and Spengler have suggested that relatively slow co-crystallization with matrix is an efficient sample-cleaning step in dried-droplet preparation [19].

Here, we used a MALDI imaging technique to investigate the distribution of signal spots of glycopeptides within MALDI samples. Originally, the MALDI imaging technique was applied to thin-layer chromatographic separation procedures [20] and for studying biological samples such as tissues [21]. Recently, several researchers have used MALDI imaging or a secondary ion mass spectrometry (SIMS) based imaging technique to investigate signal distribution in a sample prepared using the dried-droplet technique [19,22–24]. The results of these studies using lipids and peptides as analytes suggest that analyte signal distribution in DHBA matrix is highly heterogeneous, caused by segregation of each analyte in its physicochemical property.

In this study, we found that the MALDI images of glycoforms on the same peptide are identical to each other. We also demonstrate that pyrene derivatization of glycopeptides improves homogeneity and reproducibility of shot-to-shot spectra.

2. Experimental

2.1. Materials

Human serum albumin and bovine pancreatic ribonuclease B (RNaseB) were purchased from Sigma–Aldrich (Steinheim, Germany). Human immunoglobulin G (IgG) was purchased from WAKO Pure Chemical, Inc. (Osaka, Japan). Trypsin Gold (Mass Spectrometry Grade) and Lysyl Endpeptidase (Lys-C, Mass Spectrometry Grade) were purchased from Promega (Madison, WI) and WAKO Pure Chemical, Inc., respectively. Chymotrypsin from bovine pancreas (Sequencing Grade) was purchased from Roche (Penzberg, Germany). Cellulose fibrous medium was purchased from Sigma–Aldrich. The highly purified MALDI matrix chemicals, DHBA was purchased from Shimadzu-Biotech (Kyoto, Japan). Acetonitrile (LC/MS grade) and trifluoroacetic acid (TFA, HPLC grade) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). 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. Digestion of glycoproteins and protein

Albumin, RNaseB or IgG was incubated in 10 mM ammonium bicarbonate containing 10 mM dithiothreitol at 55 °C for 45 min. After cooling, 5 μL of 135 mM iodoacetamide was added to the mixture, which was then kept in the dark for 45 min. After adding RapiGest SF (Waters, Milford, MA) to a final concentration of 0.1% in 50 mM ammonium bicarbonate, the mixture was heated at 100 °C for 5 min. After cooling, the mixture was incubated with 1 μg of trypsin or Lys-C at 37 °C overnight. For chymotrypsin digestion, IgG was incubated in 100 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ and 10 mM dithiothreitol at 55 °C for 45 min. After cooling, the sample was carbamidomethylated and denatured using RapiGest SF as described above. The mixture was incubated with 2 μg of chymotrypsin at 25 °C overnight.

The tryptic digest of albumin was desalted using a PepClean™ C-18 Spin Column (Pierce, Rockford, IL), and the digests of RNaseB and IgG were subjected to enrichment by hydrophilic interaction using cellulose fibrous medium [12,25].

For desialylation, enriched glycopeptide fractions derived from IgG were heated in 0.8% TFA at 80 °C for 45 min, and then dried using a Speed Vac.

2.3. Preparation of glycopeptides NA2-IRNKS and A2-I*RNKS

Disialylglycopeptide A2-KVANKT, A2-IRNKS, NA2-IRNKS, IRN(GlcNAc)KS and IRNKS were prepared as described previously [12]. Stable isotope-labeled A2-I*RNKS was prepared using Fmoc-Ile*-OH instead of Fmoc-Ile-OH. Ile*, in which six ^{13}C and one ^{15}N were substituted, is 7.017 Da heavier than non-labeled Ile.

2.4. On-plate pyrene derivatization

Some samples were directly derivatized by 1-pyrenyl diazomethane (PDAM, Molecular Probes, Inc., Eugene, OR) on the target plate as described previously [12]. The plate was well rinsed with xylene to remove excess amount of PDAM.

2.5. Mass spectrometry and MALDI imaging

Samples for MALDI-MS were prepared using standard dried-droplet technique. Analyte solution was first deposited on a mirror-polished stainless steel MALDI target. DHBA was dissolved in 60% acetonitrile/H_2O to a concentration of 10 mg/mL and 0.6 μL was applied onto the samples with or without on-plate pyrene derivatization and then left to dry without active air flow. All the above procedures were done in a clean room, where the temperature (23°) and humidity (50%) are controlled. Before measuring MS, the samples on the target plate were observed using a confocal laser microscope with 50× objective, LEXT OLS3100 (Olympus, Tokyo, Japan). MALDI-TOF mass spectra were acquired using an AXIMA-QIT instrument consisting of a quadrupole ion trap and reflector time-of-flight analyzer (Shimadzu Biotech, Manchester, UK). A nitrogen laser (337 nm) was used to irradiate the sample for ionization. Spectra in positive- and negative-ion modes were obtained with higher laser fluence than the threshold fluence for $[M+H]^+$ or $[M-H]^-$ ion detection. The samples were scanned by successive 10 laser shots with a spot-to-spot center distance of 50 μm in each direction to obtain a MALDI image. The MS data was converted to comma-separated values (CSV) and was visualized as MALDI image using graphical software Graph-R.

3. Results and discussion

3.1. Heterogeneity of signals in a sample of peptide mixture using DHBA as a matrix

DHBA forms small and large needle-shaped crystals from the rim of the target spot toward the center and the crystals is non homogeneity as shown in Fig. 1. This MALDI sample contains tryptic digest of human serum albumin consisted of more than 10 different peptides. Mass spectra a, b and c in Fig. 1 were obtained from

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