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Microwave-assisted nonspecific proteolytic digestion and controlled methylation for glycomics applications

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Dedicated to Professor Yongzheng Hui on the occasion of his 70th birthday

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ABSTRACT

Nonspecific proteolytic digestion of glycoproteins is an established technique in glycomics and glycoproteomics. In the presence of pronase E, for example, glycoproteins are digested to small glycopeptides having one to six amino acids residues, which can be analyzed with excellent sensitivity using mass spectrometry. Unfortunately, the long digestion times (1–3 days) limit the analytical throughput. In this study, we used controlled microwave irradiation to accelerate the proteolytic cleavage of glycoproteins mediated by pronase E. We used ESI-MS and MALDI-MS analyses to evaluate the microwave-assisted enzymatic digestions at various digestion durations, temperatures, and enzyme-to-protein ratios. When digesting glycoproteins, pronase E produced glycopeptides within 5 min under microwave irradiation; glycopeptides having one or two amino acids were the major products. Although analysis of peptides containing multiple amino acid residues offers the opportunity for peptide sequencing and provides information regarding the sites of glycosylation, the signals of Asn-linked glycans were often suppressed by the glycopeptides containing basic amino acids (Lys or Arg) in MALDI-MS experiments. To minimize this signal-to-content dependence, we converted the glycopeptides into their sodiated forms and then methylated them using methyl iodide. This controlled methylation procedure resulted in quaternization of the amino group of the N-terminal amino acid residue. Using this approach, the mass spectrometric response of glyco-Asn was enhanced, compensating for the poorer ionization efficiency associated with the basic amino acids residues. The methylated products of glycopeptides containing two or more amino acid residues were more stable than those containing only a single Asn residue. This feature can be used to elucidate glycan structures and glycosylation sites without the need for MS/MS analysis.

1. Introduction

Protein glycosylation is a common post-translational modification in many biological processes.[1,2] For example, glycosylation alteration is associated with several inflammatory diseases,[3] cancers,[4,5] and congenital disorders of glycosylation (CDG).[6,7] Although electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) are often the most powerful and useful analytical tools for studying glycoproteins and protein glycosylation, the variability of glycosylation sites and glycoforms (glycan microheterogeneity) at corresponding glycosylation sites complicates the structural analysis of targeted glycoprotein and demands more selective and sensitive analytical methodologies.[8,9] Most MS-based methods for glycoprotein characterization require the use of sophisticated sample preparation techniques. Typically, glycoproteins are first digested by specific endoproteinases (e.g., trypsin) to produce peptides and glycopeptides, from which the glycopeptides are enriched using high-performance liquid chromatography (HPLC) or affinity chromatography. The enriched glycopeptides are then digested using exoglycosidases (e.g., PNGase F) or other chemical methods to obtain the free glycans and peptides, which can be analyzed using LC–ESI-MS and/or LC–MALDI-MS. Alternatively, the information on the glycosylation sites and glycan structures can be obtained by analyzing the tryptic glycopeptides directly without releasing their glycans.[10] Trypsin digestion might not be appropriate, however, for many highly post-translationally modified proteins.[11,12] Furthermore, tryptic glycopeptides often exhibit low mass spectrometric responses and complicated MS/MS fragmentation spectra because of their large sizes.

To overcome those limitations, a promising alternative protein digestion method using pronase E was developed for the analysis of glycoproteins.[13–16] Pronase E cleaves peptide bonds randomly and usually produces glycopeptides ranging in length from two to six amino acid residues. Higher enzyme-to-protein ratios and longer digestion times can be used to produce glycopeptides containing a single amino acid residue.[17,18] Although mass

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spectrometric analysis of pronase E-generated glycopeptides is relatively simple and rapid, 2 days or 3 days are usually required for complete digestion of the targeted glycoproteins.[17] Such extended digestion times limit the throughput of large-scale glycoprotein identification and screening. Therefore, there is a need to develop rapid and reproducible glycoprotein digestion methods using pronase E.

Recently, Temporini et al.[19] reported an automated analytical method for the simultaneous determination of glycan and peptide moieties using a pronase E immobilized enzyme reactor. By coupling the bioreactor to a chromatographic column, on-line selective glycopeptide enrichment prior to normal-phase liquid chromatography–mass spectrometry was performed. The authors demonstrated that a reasonable degree of digestion could be achieved within 20 min for three main glycopeptides containing slightly longer peptide moieties (five to eight amino acids). An alternative method for accelerating protein digestion is the use of microwave irradiation, which has proven to be a rapid and robust technique in proteomics studies.[20] Microwave irradiation speeds up the hydrolyses of peptides and proteins in concentrated HCl,[21,22] formic acid,[23] and trifluoroacetic acid[24] solutions. The Li group discovered that proteins exposed briefly to microwave irradiation were readily hydrolyzed to form predominantly two series of polypeptide ladders: one containing the N-terminal amino acid and the other containing the C-terminal amino acid.[22] Unfortunately, these methods do not provide any structural details related to the sites of protein glycosylation, because oligosaccharides are themselves acid-labile.[25]

Microwave-assisted protein enzymatic digestion (MAPED) is often an improvement over conventional enzymatic digestion, providing protein digests suitable for peptide analysis within minutes.[26,27] Most recently, Sun et al.[28] extended the application of MAPED to gel-based proteomics. Compared with the standard protocol, the in-gel MAPED method can shorten the total analysis time for protein mixture preparation, digestion, and peptide extraction to 25 min and provide higher peptide recovery efficiencies. We expected that the pronase E-mediated digestion of glycoproteins could be accelerated using the MAPED strategy and that would be an attractive tool for use in glycomics and glycoproteomics studies.

In this paper, we describe the application of microwave technology to the study of nonspecific proteolysis digestion of glycoproteins using pronase E. The MS response bias due to the existence of basic amino acid residues was reduced significantly through controlled methylation of complex glycopeptides, for example, glyco-Asn, glycodipeptides, or glycotripeptides. This rapid and sensitive method allows the unambiguous identification of glycan structures and glycosylation sites.

2. Results and discussion

2.1. Microwave-assisted digestion

Incomplete digestion of glycoproteins by pronase E can produce glycopeptide microheterogeneity for otherwise identical glycans. In addition to the information related to the glycan structures, the peptide residues can also provide some structural information regarding the sites of glycosylation. On the other hand, however, several glycopeptides that share the same glycan structure can display lower detection sensitivity and lead to complicated mass interpretation. Increasing the enzyme-to-glycoprotein ratio and prolonging the digestion time can be used to overcome those

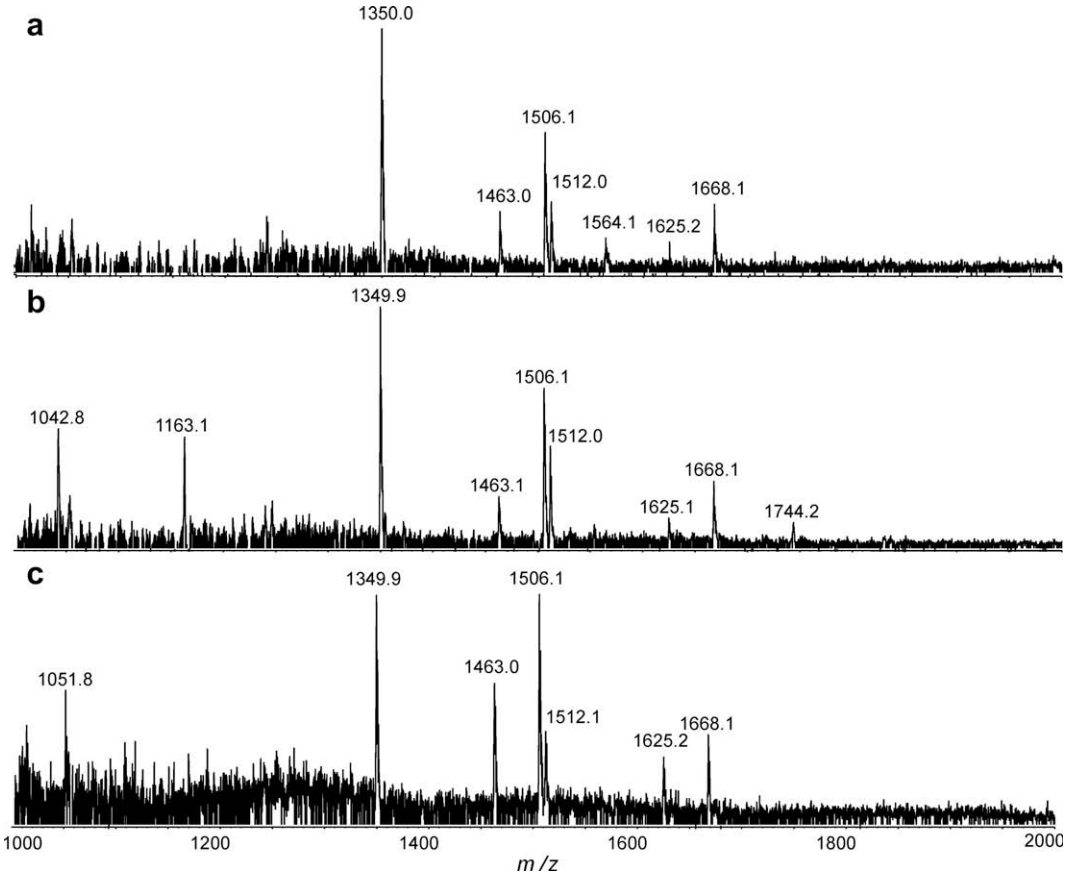

Figure. 1. Positive-ion ESI-MS spectra of glycopeptides from RNase B digested with pronase E at a protease-to-protein ratio of 2:1 (w/w). Microwave irradiation was applied for (a) 1, (b) 2, and (c) 5 min.

shortcomings.[16] In this study, we attempted to establish a rapid and simple approach based on microwave technology that would provide the greatest extent of complete digestion of glycoproteins.

We chose RNase B as our first model glycoprotein for studying microwave-assisted digestion. We employed the same ratio of pronase E to RNase B as that described in the previous report,[17] but in this case utilized microwave irradiation. Figure 1 illustrates the effect of the microwave irradiation time. When the sample was irradiated with microwaves for 1 min, glycopeptides containing Asn, Arg-Asn^{60}, Asn^{60}-Leu, and Asn^{60}-Leu-Thr were the major products. Based on tandem MS analysis, we detected protonated ions at *m/z* 1350.0, 1512.0, 1463.0, 1625.2, 1506.1, 1668.1, and 1564.2, corresponding to Asn-linked $GlcNAc_2Man_5$, $GlcNAc_2Man_6$, Asn^{60}-Leu-linked $GlcNAc_2Man_5$, $GlcNAc_2Man_6$, Arg-Asn^{60}-linked $GlcNAc_2Man_5$, $GlcNAc_2Man_6$, and Asn^{60}-Leu-Thr-linked $GlcNAc_2Man_5$, respectively. At this limit of detection, we did not observe any glycopeptides containing the glycans $GlcNAc_2Man_{7-9}$. After applying microwave irradiation for 2 min, we did not observe the tripeptide-containing glycopeptides at *m/z* 1564.2 (Fig. 1b). The signal-to-noise ratios of each peak, in particular those of the Asn-glycans worsened significantly when microwave irradiation was performed for 5 min. Using a thermocouple, we found that the temperatures within the reaction mixtures subjected to microwave irradiation for 1, 2, and 5 min were 41, 63, and 89 °C, respectively. It has been reported that the reduction in the enzyme digestion time is due to the higher temperature resulting from the microwave irradiation.[20] In this present study, however, we found that too much heat resulted in a decrease in the digestion efficiency. It is worth mentioning that repeated microwave irradiation did not significantly improve the yields of single amino acid-containing glycopeptides. Therefore, we chose a microwave irradiation time of 2 min for our subsequent experiments.

We also investigated the effects of five different enzyme-to-protein ratios (1:50; 1:10; 1:5; 1:1, and 2:1) on the digestion efficiency of RNase B. Enzyme-to-protein ratios ranging from 1:10 to 2:1 provided similar digestion mixtures. When the enzyme-to-protein ratio was 1:50, however, we observed two new glycopeptides incorporating the tripeptides, Asn^{60}-Leu-Thr and Ser-Arg-Asn^{60}, respectively (data not shown). These findings suggest that low enzyme-to-protein ratios are preferred if more information is needed regarding the sites of glycosylation. Because our objective in this study was to develop a simple and sensitive method for glycan analysis, we selected an enzyme-to-protein ratio of 1:1 for our subsequent experiments.

2.2. MALDI-TOF MS analysis of glycopeptides

MALDI-MS has become a major tool for carbohydrate analysis. Relative to ESI-MS, MALDI-MS has the advantage of allowing direct analysis of complex mixtures with low sample consumption and high sensitivity. Moreover, MALDI-MS results in predominantly singly charged ions, which is advantageous for rapid interpretation of mass spectra. Figure 2a displays the MALDI-TOF spectrum of the same glycopeptide mixture as that in Figure 1a, the dominant protonated ions appear at *m/z* 1505.2, 1667.1, 1829.0, and 1991.5, corresponding to the series of Arg-Asn-$GlcNAc_2Man_{5-8}$

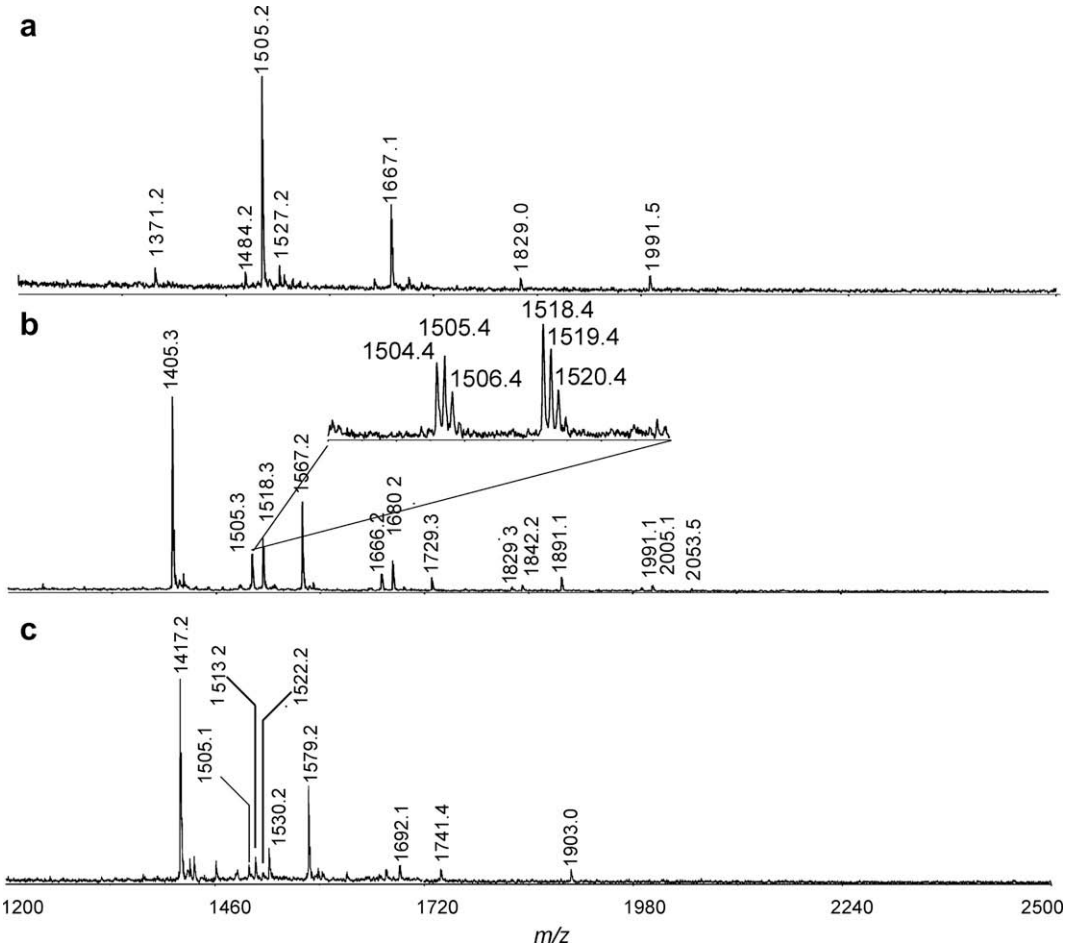

Figure. 2. MALDI-TOF mass spectra of glycopeptides from RNase B after microwave irradiation for 2 min. (a) No derivatization; (b) after controlled methylation using CH_3I; (c) after controlled methylation using CD_3I. The sample amount loaded on target was equivalent to 50 ng of RNase B for each spot. Matrix: 10 mg/mL DHB in 50% MeCN.

with a mass difference of 162 Da. Much higher ionization efficiencies were observed for the glycopeptides containing basic amino acid residues. Meanwhile, the glycopeptides lacking basic amino acid residues (Lys or Arg) resulted in multiple peaks due to the formation of alkali metal ion adducts, usually those of Na^+ and K^+. For the Asn-glycans and the Asn-(Leu)-glycans, we detected two weak peaks at *m/z* 1371.2 and 1484.4 for their sodiated ions when the carbohydrate moiety was $GlcNAc_2Man_5$. The ESI-MS and MALDI-TOF mass spectral responses were significantly different for glycopeptides containing basic amino acids: Asn-glycans were the major products observed in the ESI mass spectra (e.g., at *m/z* 1350.0 and *m/z* 1371.2), whereas Arg-Asn-glycans predominated in the corresponding MALDI-TOF mass spectra (e.g., at *m/z* 1505.2 and 1527.2).

2.3. Methylation

Previously, we reported a controlled methylation strategy for the analysis of Asn-glycans,[18] namely a simple methylation to produce the methyl esters of sialic acids and generate permanent charges on Asn moieties. If a permanent charge can be generated on the C-terminal amino acid of a glycopeptide, its ionization efficiency would improve, perhaps reducing the MS response bias.

Using the same protocol as that described in a previous report,[18] we methylated glycopeptides using CH_3I after converting the carboxyl groups into their corresponding sodiated carboxylate forms. This controlled methylation protocol resulted in a significant increase in the peak intensities related to the glycopeptides containing Asn and Asn^{60}-Leu residues (Fig. 2b). The observed mass spectrum displayed three predominant series of ions. The first series of ions appeared at *m/z* 1405.3, 1567.2, 1729.3, 1891.1, and 2053.1, corresponding to the methylated derivatives of Asn-$GlcNAc_2Man_{5-9}$. The second series of ions, *m/z* 1518.3, 1680.2, 1842.1, and 2005.1, corresponded to the methylation derivatives of Asn^{60}-(Leu)-$GlcNAc_2Man_{5-8}$, as is evident by the mass increment of 113 Da relative to each signal of Series 1. The third series of ions, *m/z* 1505.3, 1666.2, 1829.3, and 1991.1, represent the signals of Arg-Asn-$GlcNAc_2Man_{5-8}$ in Figure 2a. An expanded view of the MALDI-TOF spectrum revealed the presence of an ion at *m/z* 1504.4 (inset to Fig. 2b); although the origin of this ion could be assigned to the methylated derivative of Asn^{60}-(Leu)-$GlcNAc_2Man_5$, the observation of a signal at *m/z* 1505.4 suggests that methylation did not occur readily when the glycopeptides contained a basic amino acid.

The formation of methyl esters of carboxylic acid moieties often occurs in the presence of CH_3I under basic conditions. In other words, the methyl esters should also be formed for Arg-Asn^{60}-$GlcNAc_2Man_{5-8}$ to result in detectable ions at *m/z* 1519, 1681, 1843, and 2006, respectively. We also observed a mass difference of only 1 Da between the molecular ions of the methyl ester derivatives of Arg-Asn^{60}-$GlcNAc_2Man_{5-8}$ and methyl-ester-plus-permanent-charge derivatives of Asn^{60}-(Leu)-$GlcNAc_2Man_{5-8}$. This small mass difference could lead to incorrect assignment of each ion in a low-resolution MALDI-TOF spectrum. Although, a high-resolution mass spectrometer, for example, a Fourier transform mass spectrometer, would be able to solve this problem, its use is not always feasible or practical.

To this end, we employed CD_3I in the methylation reaction to further distinguish the signals of the glycopeptide derivatives. In this case, we detected a series of ions having a mass shift of 12 Da at *m/z* 1417.2, 1579.2, 1741.4, and 1903.0, corresponding to the trideuteromethylated derivatives of Asn-$GlcNAc_2Man_{5-8}$ (Fig. 4). This mass increment of 12 Da corresponds to the addition of three CD_3 groups to form a quaternary ammonium ion, as well as a CD_3 ester group at the Asn residues. In addition, we also detected the ions corresponding to the formation of a CD_3 ester in the glycopeptides containing Arg-Asn^{60} (Fig. 2c). We infer that the ion at *m/z* 1505.1 corresponded to the native Arg-Asn^{60}-$GlcNAc_2Man_5$, whereas the ion at *m/z* 1522.2 was the methylated form arising from the addition of one CD_3 group. Because the isoelectric point of Arg is 12.5, the cation resin cannot convert the carboxyl groups into their corresponding sodiated carboxylate forms, which precludes the formation of a permanent charge on the Arg-Asn residues. The ion at *m/z* 1513.2 represented the incompletely methylated product of Asn^{60}-(Leu)-$GlcNAc_2Man_5$, and we assign the ion at *m/z* 1530.2 to the completely trideuteromethylated derivatives. The calculated *m/z* values for the derivatives of

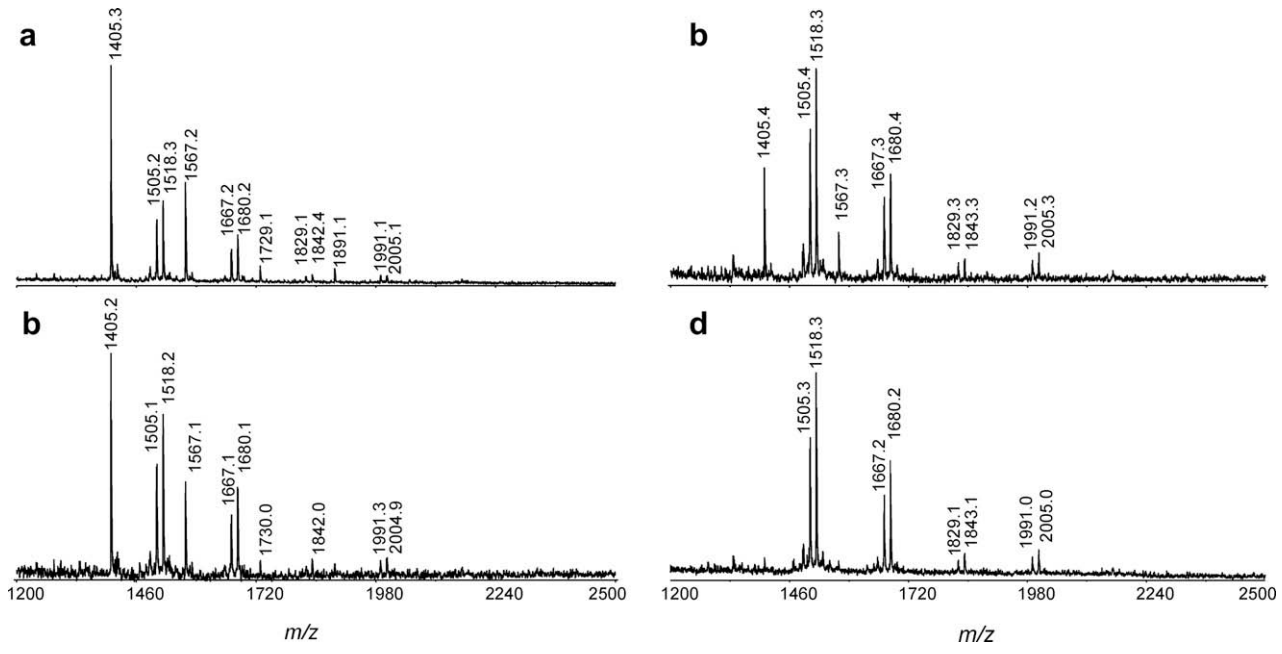

Figure. 3. MALDI-TOF spectra of CH_3I-methylated glycopeptides from RNase B after treatment at (a) 24 °C for 60 min, (b) 37 °C for 60 min, (c) 50 °C for 60 min, and (d) 50 °C for 120 min. Other conditions as in Figure 2.

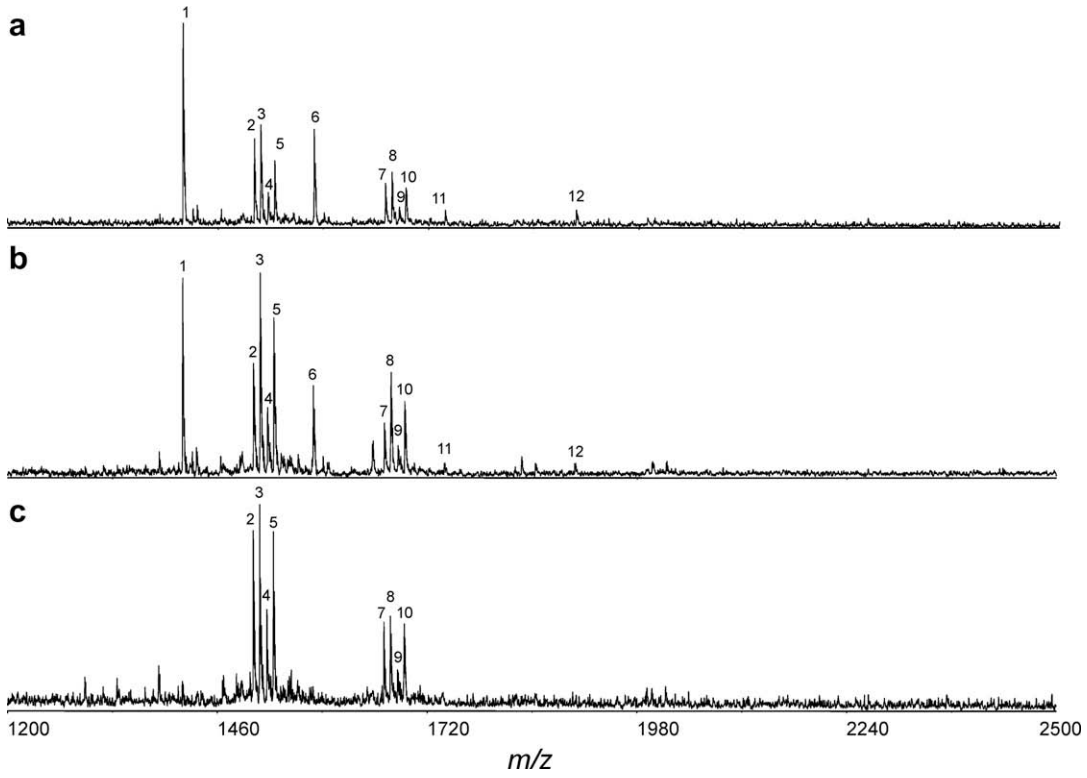

Figure. 4. MALDI-TOF mass spectra of CD_3I-methylated glycopeptides from RNase B that were heated at 50 °C for (a) 20, (b) 60, and (c) 120 min. Peak assignment: 1 = *m/z* 1417.2; 2 = *m/z* 1505.1; 3 = *m/z* 1513.2; 4 = *m/z* 1522.2; 5 = *m/z* 1530.2; 6 = *m/z* 1579.2; 7 = *m/z* 1667.0; 8 = *m/z* 1675.2; 9 = *m/z* 1684.1; 10 = *m/z* 1692.1; 11 = *m/z* 1741.4; 12 = *m/z* 1903.0. Other conditions as in Figure 2.

Asn^{60}-(Leu)-$GlcNAc_2Man_5$ methylated incompletely and completely with CH_3I are 1504 and 1518, respectively. Although such methylation of glycopeptides can complicate the mass spectral analysis, unambiguous information related to the glycosylation sites is achievable, especially with the combined analysis of CD_3I- and CH_3I-methylated products.

We investigated the stability of the methylated derivatives for different periods of time at 24, 37, and 50 °C. As expected, increasing the temperature accelerated the degradation of the methylated mixtures. Surprisingly, the methylated derivatives of the glycodipeptides were more stable than the Asn-glycans. As indicated in Figure 3, the ratio of the ion intensities at *m/z* 1405.3 and 1505.3 decreased by 20% and 50% after treating the sample for 60 min at 24 and 37 °C, respectively. When we heated the sample at 50 °C, the ratios decreased to 35% and 78% after 20 and 60 min, respectively. The peaks of the Asn-glycan derivatives completely disappeared after 120 min, but we observed only a slight decrease in the intensities of the glycodipeptide derivatives. In addition, the glycodipetide derivatives also disappeared when the incubation time was prolonged to 24 h at 50 °C. These results suggest that Asn-glycans can be identified in complicated MALDI-TOF spectra simply by comparing the ion intensities after different incubation times at higher temperature (e.g., 50 °C). We observed similar results for the products methylated with CD_3I (Fig. 4), where the signals of the glycodipeptides were almost the only ones detectable after the methylation products had been heated at 50 °C for 2 h.

2.4. Analysis of sialylated glycoproteins

We also used transferrin to evaluate our proposed microwave-assisted digestion method for its application to the analysis of sialylated glycoproteins. Transferrin has two potential glycosylation sites and contains a major glycoform of two biantennary glycans with a total number of four sialic acid residues. After microwave irradiation for 2 min, we analyzed the glycopeptide mixtures using CE-MS operated in the negative mode (Fig. 5a). Most of the detected ions belonged to the biantennary glycans associated with the different peptide residues. The deprotonated glycopeptide ions were detected at *m/z* 1167.9, 1211.4, 1232.0, and 1240.4, corresponding to the species incorporating the peptide moieties of Asn, Ser-Asn^{611}, Asn^{413}-Lys, and Gly-Ser-Asn^{611}, respectively. The existence of the $[M+Na-2H]^-$ ions of these glycopeptides resulted in an even more complicated spectrum (Fig. 5a).

After the purified glycopeptides were subjected to an additional period of microwave-assisted digestion, we detected only the Asn-biantennary glycan and the Ser-Asn^{611}-biantennary glycan (Fig. 5b). We methylated the two transferrin glycopeptide mixtures and then analyzed the mixtures using MALDI-TOF. The signal at *m/z* 2421.7 in Figure 5c and d reveals the presence of the ion of the Asn-biantennary glycan. A low-abundance signal at *m/z* 2117.3 corresponding to the Asn-biantennary glycan with a monosialic acid unit was detected when we increased the degree of digestion (Fig. 5d). A noteworthy initial observation is the formation of the complete and partial methylated derivatives of the biantennary glycan associated with other peptide residues, including Ser-Asn^{611}, Asn^{413}-Lys, and Gly-Ser-Asn^{611}, as indicated in Figure 5c. Based on their molecular masses, the first pair of ions at *m/z* 2493.4 and 2507.9 represent the glycodipeptides containing Ser-Asn^{611}; the second pair of ions at *m/z* 2519.4 and 2532.8 contain an Asn^{413}-Lys dipeptide; and the third pair of ions at *m/z* 2551.3 and 2556.8 represent the glycotripeptide-containing Gly-Ser-Asn^{611}. The decrease of 17 Da for the ions corresponding to Asn^{413}-Lys dipeptide methylation derivatives (e.g., *m/z* 2519.4 and 2532.9) suggested that deamination occurred which resulted in removing one amino group.[29]

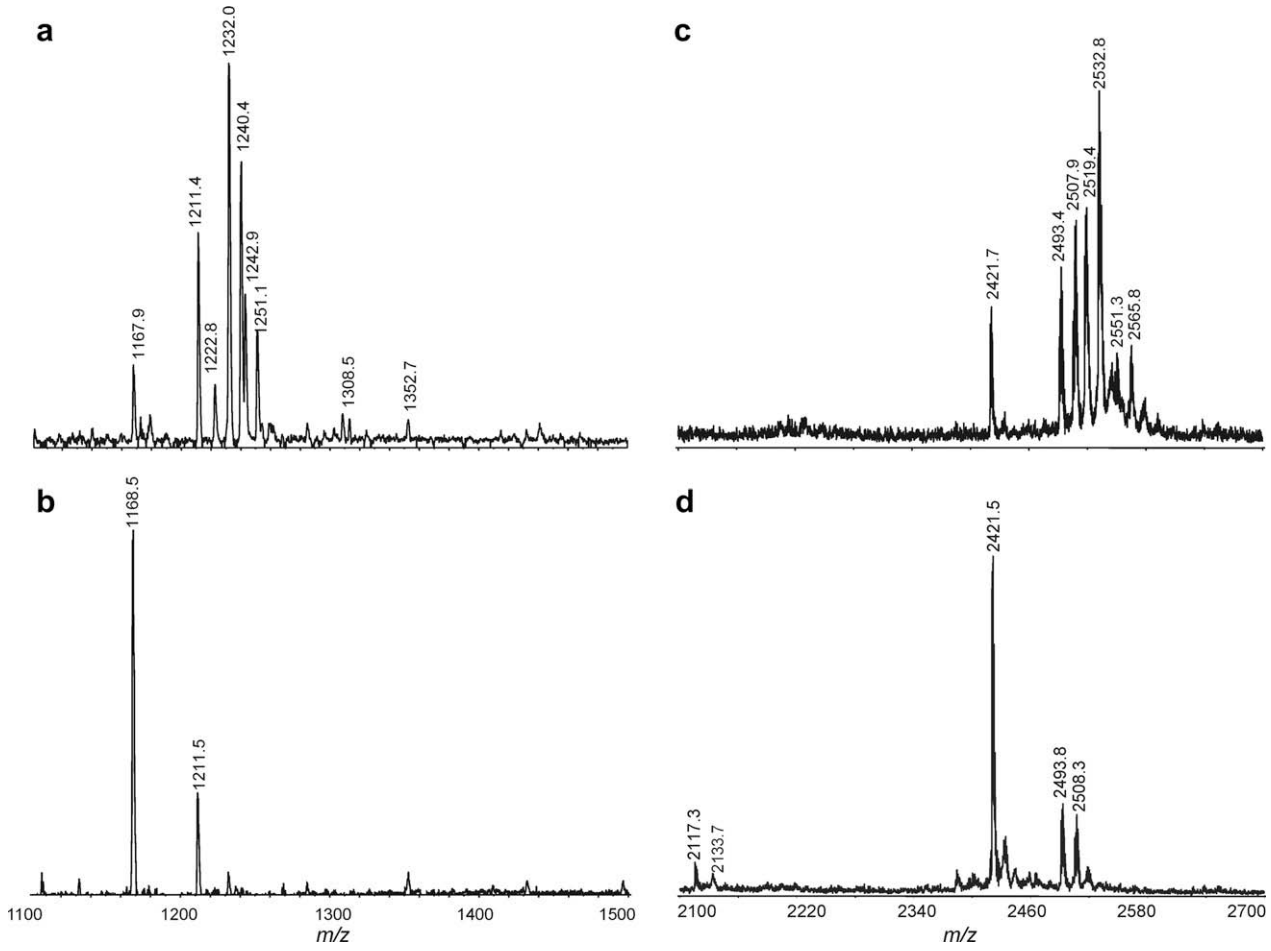

Figure. 5. ESI-MS and MALDI-TOF mass spectra of native glycopeptides and methylated glycopeptides from transferrin: (a) native glycopeptides after microwave irradiation for 2 min; (b) native glycopeptides after microwave irradiation twice for 2 min each time; (c) derivatized glycopeptides after microwave irradiation for 2 min; (d) derivatized glycopeptides after microwave irradiation twice for 2 min each time.

To elucidate further and assign the glycopeptide structures, a number of the ions were selected to perform tandem MS (Fig. 6). Due to the existence of a permanent charge, all the selected ions have similar fragmentation characteristics, in which a series of Y ions were detected. In addition, $^{0,2}X_0$ fragments with peptide residues attached can be used as diagnostic ions to determine peptide sequences. For example, fragment ions at *m/z* 272.1, 359.1, 416.0, and 383.1 represent Asn, Ser-Asn, Gly-Ser-Asn, and Asn-Lyn residues, respectively. The difference of 57 between signals at *m/z* 359.1 and 416.0 indicates a Gly residue. As expected, the methylation also stabilized sialic acid residues, as loss of sialic acid was not observed in the MALDI-TOF mass spectra. Interestingly, protonated fragment ions (e.g., *m/z* 204, 671) were also detected in the tandem mass spectra. We are now investigating the effects that various methylated substituents have on the fragmentation mechanism of the methylated glycopeptide cations.

In conclusion, this is a novel approach to glycoprotein analysis using a combination of microwave-assisted pronase E digestion and controlled methylation. Compared to conventional analytical techniques used in glycomics, our proposed method has many advantageous features. First, the application of microwave accelerates the enzymatic reaction and thereby improves the rate of sample preparation. Second, methylation enhances the analytical sensitivity for those glycopeptides lacking basic amino acid residues and reduces the response bias associated with the peptide contents of glycopeptides. Third, the different stabilities of the methylated glycopeptides can be exploited to simplify the mass spectra for improved data interpretation. Finally, the glycopeptide sequences can be determined readily through MS and MS/MS analyses. This proposed method is suitable for application to the high-throughput screening of glycoproteins, in particular for the quality control of recombination proteins.

3. Experimental

3.1. Chemicals and materials

Ribonuclease B (RNase B), *holo*-transferrin, pronase E, DMSO, and other solvents were purchased from Sigma–Aldrich (St. Louis, MO). Ultra-clean nonporous graphitic carbon (PGC) cartridges were obtained from Alltech Associates, Inc. (Deerfield, IL). All aqueous solutions were prepared using water purified through a Milli-Q purification system (Millipore, Bedford, MA).

3.2. Microwave-assisted digestion with pronase E

The glycoprotein (0.5–1.0 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.5, 100 μL) in a 1.5 mL polypropylene centrifuge vial, and then pronase E (10 mg/mL, 100 μL) was added. The capped sample vial was placed in a domestic microwave oven (Panasonic, ON, Canada) having a maximum power of 1200 W; the power level was set at 'Level 2'. A beaker of water (250 mL) was placed beside the sample vial to absorb extra microwave energy. After being subjected to microwave irradiation for a pre-programmed length

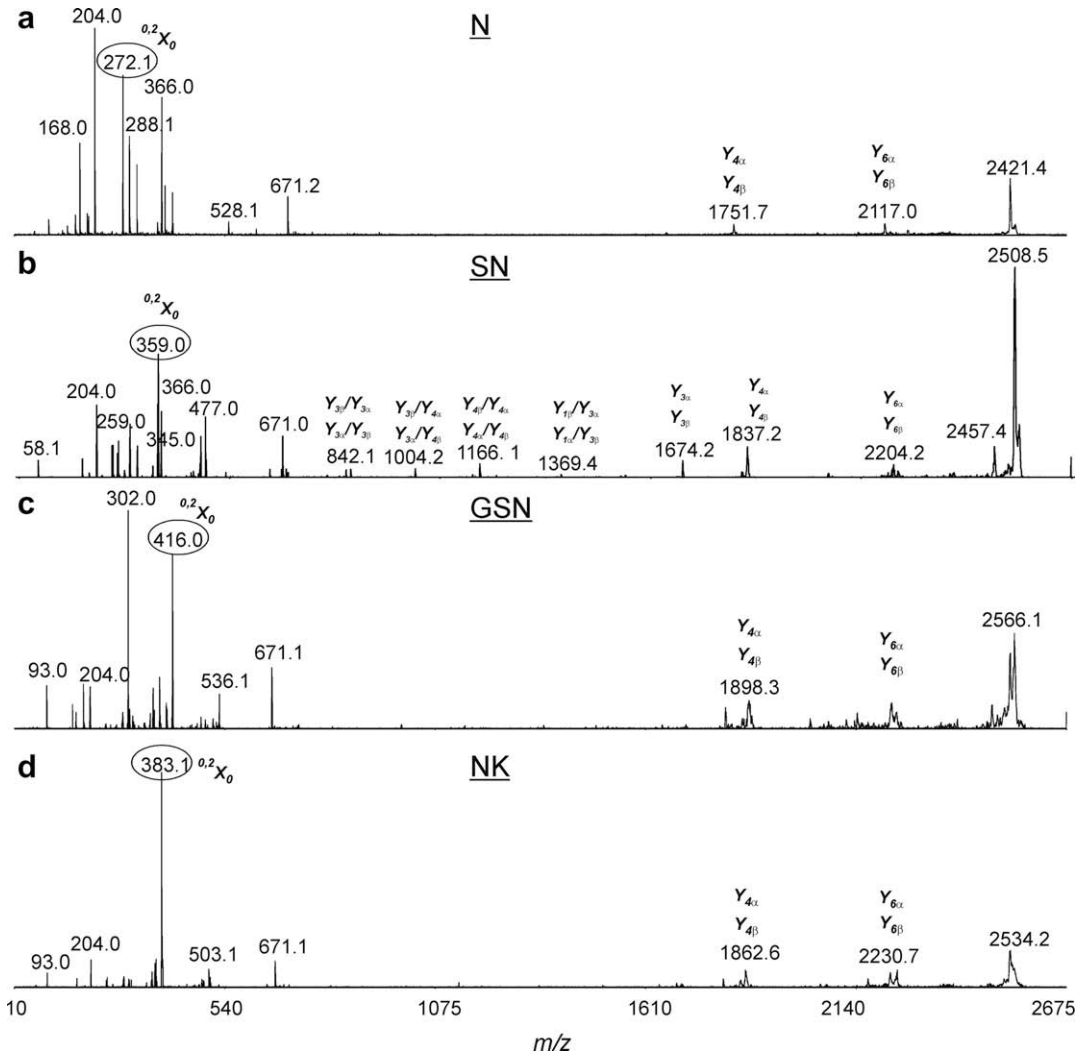

Figure. 6. MALDI-TOF/TOF tandem mass spectra of the derivatized glycopeptides from transferrin. The selected precursor ions were (a) *m*/*z* 2421.9, (b) *m*/*z* 2508.9, (c) *m*/*z* 2566.8, and (d) *m*/*z* 2532.9.

of time, the sample vial was removed from the microwave oven and the digested glycopeptides were purified using a porous graphitic carbon (PGC) cartridge.

3.3. Purification using PGC

PGC cartridges were washed with a solution (3.0 mL) of 80% (v/v) MeCN containing 0.1% TFA and then with deionized water (3.0 mL). The glycopeptides obtained after pronase E digestion were loaded into PGC cartridges and washed with water (3.0 mL) to remove buffer and salts. The glycopeptides were first eluted with 25% MeCN in 0.1% TFA and then with 50% MeCN in 0.1% TFA. Each fraction was collected and dried for analysis or further processing.

3.4. Methylation

An AG50W × 8 resin column was preconditioned with 1.0 M NaOH and then rinsed with water. The glycopeptides dissolved in water (100 μL) were applied into the column and eluted with water (2.0 mL) to convert the carboxylic acid moieties into their sodiated carboxylate forms. Dry DMSO (10 μL) and methyl iodide (10 μL) were added to the lyophilized sample, and then the solution was stirred for 1 h at room temperature. After removing excess methyl iodide under a stream of nitrogen, the residue was purified using hydrophilic chromatography.[18]

3.5. ESI-MS and ESI-MS/MS analysis

Mass spectra were acquired as described previously using a 4000 Q-Trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a capillary electrophoresis interface as described previously, except that no separation voltage was applied.[17] The MS data were acquired with dwell times of 2.0 ms per step of 0.1 *m*/*z* units in full-mass-scan mode. In the MS^2 experiments (enhanced product ion scan or EPI), the scan speed was set at 4000 Da/s, with Q_0 trapping. The trap fill time was set at 'dynamic' and the resolution of Q1 was set at 'unit'.

3.6. MALDI-TOF and MALDI-TOF/TOF analysis

MALDI-TOF spectra were acquired on a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser (337 nm). The spectrometer was operated in the positive reflectron mode by delayed extraction with a voltage of 20 kV as the accelerating voltage, a pulse delay time of 210 ns, and a grid voltage of 72%. The spectra were accumulated from 100 laser shots. Tandem mass spectra were obtained using

a 4800 MALDI-TOF/TOF spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada). Dihydroxybenzoic acid (DHB, 10 mg/mL) in 50% MeCN was used as the matrix.

Acknowledgments

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