Improved Deglycosylation of N-linked Glycoproteins and Sample Preparations for MALDI-TOF MS Analysis

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Overview

An acid-labile surfactant (RapiGest™ SF) was used to denature N-linked glycoproteins prior to enzymatic deglycosylation. This anionic surfactant was shown previously to denature proteins without inhibiting the activity of proteolytic enzymes (*Anal. Chem.* 75, 6023-28, 2003). This surfactant was easily decomposed via direct acidification, therefore minimizing the subsequent sample preparations for MALDI-TOF MS or LC/MS analysis of the oligosaccharides released. A Hydrophilc Interaction Chromatography (HILIC) Solid Phase Extraction micro elution plate was used to extract the oligosaccharides and remove salt and other contaminants from the glycans. Our method requires less time for deglycosylation and minimizes the sample clean up process drastically.

Introduction

Glycosylation accounts for a majority of the post translational modifications of proteins in eukaryotic cells. Due to the extensive heterogeneity of the glycans, characterization of the glycoproteins is very challenging. Standard methods for glycan analysis do not exist due to the complexity of the glycans. The existing methods require extended periods of enzymatic or chemical deglycosylation and laborious sample clean up prior to mass spectrometry analysis.

We developed a deglycosylation method for N-linked glycoproteins using an acid-labile surfactant (RapiGest™ SF) that is useful for denaturing the proteins prior to an enzymatic deglycosylation reaction. Complete deglycosylation of proteins can be achieved within a couple of hours. This surfactant was decomposed via addition of a strong acid (TFA or HCl) to the sample (Figure 1). After the surfactant was decomposed, the glycans released by PNGase F were extracted using a HILIC 96 well micro-elution plate. Salts and other contaminants were washed away, and the eluted oligosaccharides were analyzed by MALDI-TOF MS. This procedure enables fast analysis of glycans without compromising the quality of the data.

Methods

Deglycosylation reaction

- Glycoproteins were solubilized in 0.05% or 0.1% (w/v) RapiGest™ SF solution containing 20 mM DTT
- The protein sample was heated at 55 °C for 40 minutes and cooled to room temperature
- The sample was pH buffered with 100 mM NH_4HCO_3 (1:1, v/v)
- 5 units of PNGase F was added to the protein sample and incubated at 37°C for 1-2 hrs
- The deglycosylation reaction was quenched by boiling the sample for 5 minutes
- RapiGest™ SF was decomposed by adding TFA to the sample to a final concentration of 0.5% (v/v) and incubated at 37 °C for 30 minutes
- The sample was centrifuged and the precipitate was discarded

SPE of Oligosaccharides

Extracting oligosaccharides from deglycosylated proteins (Figure 1, 3)

- ACN was added to the deglycosylated protein sample to a final concentration of 80 to 90% (v/v)
- The sample was loaded onto a HILIC micro elution plate operated *via* a vacuum manifold
- The SPF cartridge was washed with 90% ACN (50 µl) and eluted with 10% ACN (25 µl)

Extracting oligosaccharides from deglycosylated tryptic peptides (Figure 5)

- The deglycosylated tryptic peptides (generated from the N-linked glycoproteins) were loaded onto a Waters Oasis® HLB micro elution plate (reversed phase). The oligosaccharides remained in the break-through while the tryptic peptides were retained on the SPE device.
- The oligosaccharides was extracted and desalted from the break-through using the same HILIC SPE protocol (shown above)

Intrumentation

- MALDI TOF MS: Waters MALDI® *LR* TOF MS, Reflectron mode
- MALDI matrix: 2, 5- DHB (10 mg/ml in EtOH)
- RP HPLC/MS: Waters CapLC® and Q-TOF *micro*™

Results and Discussion

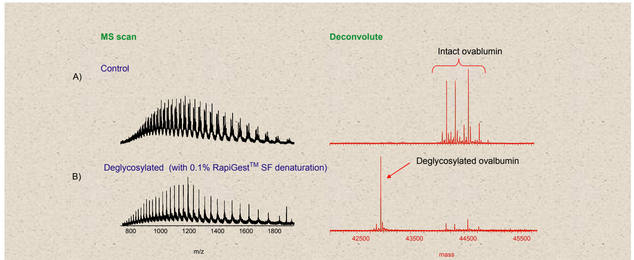

Figure 1: The intact ovalbumin (A) and the deglycosylated ovalbumin (B) were analyzed via LC/MS. The MS scans were deconvoluted to the MW of the protein. RapiGest™ SF was able to facilitate the enzymatic deglycosylation process. Only trace amount of ovalbumin remained undeglycosylated after 1.5 hrs incubation with PNGase F.

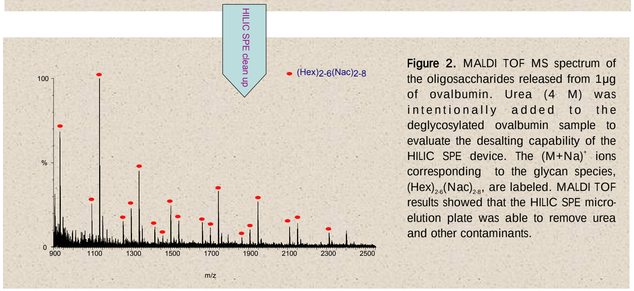

Figure 2. MALDI TOF MS spectrum of the oligosaccharides released from 1µg of ovalbumin. Urea (4 M) was intentionally added to the deglycosylated ovalbumin sample to evaluate the desalting capability of the HILIC SPE device. The $(M+Na)^+$ ions corresponding to the glycan species, $(Hex)_{2-6}(Nac)_{2-8}$, are labeled. MALDI TOF results showed that the HILIC SPE micro-elution plate was able to remove urea and other contaminants.

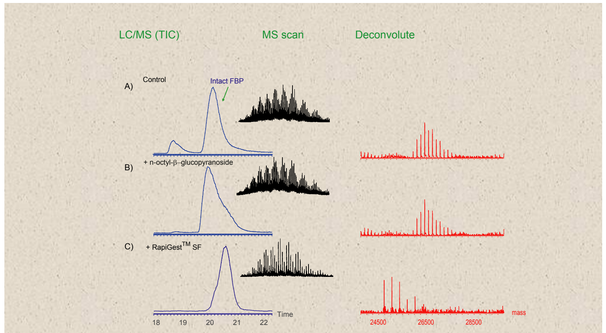

Figure 3. Left panel shows the LC/MS TIC of the deglycosylated bovine Folate Binding Protein (FBP) after 2.5 hrs deglycosylation under the labeled conditions: the unreacted control (A) the 0.1% n-octyl-β-glucopyranoside (non ionic surfactant) denatured FBP (B) and the 0.1% RapiGest™ SF denatured FBP (C). The multiply charged ions were deconvoluted to the molecular mass of the protein (right panel). The deconvoluted spectra show that n-octyl-β-glucopyranoside did not improve the deglycosylation of FBP since no mass shift from the intact FBP was observed, while RapiGest™ SF denatured FBP was deglycosylated extensively.

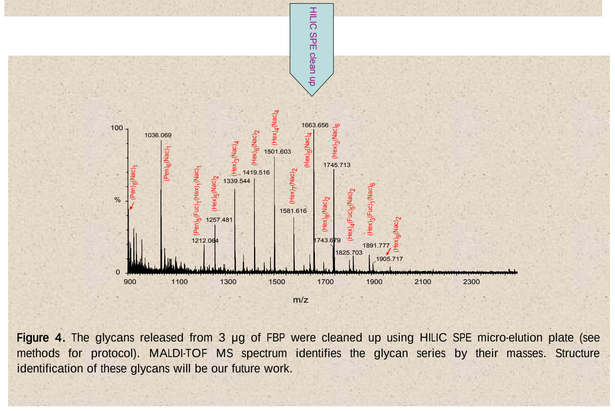

Figure 4. The glycans released from 3 µg of FBP were cleaned up using HILIC SPE micro-elution plate (see methods for protocol). MALDI-TOF MS spectrum identifies the glycan series by their masses. Structure identification of these glycans will be our future work.

Glycans released from tryptic peptides can also be isolated using two SPE devices. A reversed-phase SPE micro-elution plate (Oasis® HLB) was used to separate the glycans (in the break through) from the peptides. The glycans were further desalted using the HILIC SPE device.

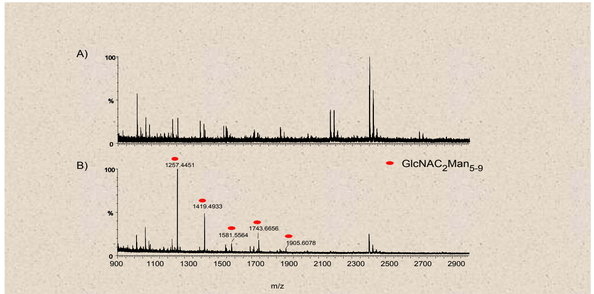

Figure 5. A) MALDI TOF MS of the deglycosylated RNase B tryptic peptides without sample clean up. The glycans released from RNase B were greatly suppressed. B) MALDI TOF MS of the same sample that was cleaned up using a reversed phase SPE micro elution plate (Oasis® HLB) and a HILIC SPE micro-elution plate.

Conclusions

- Enzymatic deglycosylation on N-glycoproteins was improved using an acid-labile surfactant (RapiGest™ SF) to denature the glycoproteins. Complete deglycosylation can be achieved within a couple of hours
- RapiGest™ SF promotes the denaturation of proteins more than n-octyl-β-glucopyranoside
- The glycans released from N-linked proteins were desalted using a 96 well HILIC SPE micro-elution plate, hence, high quality MALDI-TOF MS spectra can be generated
- Glycans released from the tryptic peptides can also be isolated and cleaned up using the combination of a reversed phase SPE micro elution plate and a HILIC SPE micro elution plate