

# A Rapid and Improved Chemical Method for Deglycosylation of Glycoproteins

Asgar Electricwala,<sup>1</sup> Ian Wright,<sup>1</sup> Elnor B. Rathbone<sup>1</sup> and Steven L. Cockrill<sup>2</sup>

<sup>1</sup>Sigma-Aldrich Corporation, Poole, UK <sup>2</sup>Sigma-Aldrich Corporation, St. Louis, USA

---

## Overview

### Purpose

- An improved chemical method is described for the removal of carbohydrates from glycoproteins using trifluoromethanesulfonic acid for specific solvolysis of glycosidic bonds, without destruction of the peptide backbone

### Methods

- Dry glycoproteins treated with pre-cooled, anhydrous TFMS
- Incubation on ice for 30 minutes
- Addition of Bromophenol Blue indicator
- Neutralization with pyridine solution, monitored *in situ* by indicator
- Desalt, digestion and/or analysis by SDS-PAGE, MALDI-TOF MS, etc

### Results

- Glycan removal can be accomplished in 30 minutes using the improved method
- Deglycosylation is demonstrated by shifts to lower mass in TFMS-treated glycoproteins, monitored by SDS-PAGE and MALDI-TOF MS
- N-terminal sequencing confirms integrity of peptide backbone after deglycosylation
- Applicability to glycoproteins from diverse sources demonstrated, including plant species resistant to deglycosylation by PNGase F
- Comparative MALDI-TOF MS analysis of native and deglycosylated bovine fetuin following digestion with trypsin shows improved sequence coverage for the deglycosylated sample
- Confirmed identification of N-linked glycosylation sites by PSD

---

## Introduction

Glycosylation represents one of the most common protein post-translational modifications. Glycans attach to the peptide backbone through either amide or glycosidic bonds (N-linked or O-linked) and may be removed either enzymatically or chemically. Enzymatic modes offer mild conditions, but are specific for either N- or O-linked glycans. Chemical methods are non-specific, capable of removing the entire glycan complement under appropriate reaction conditions. Two methods, hydrazinolysis<sup>1</sup> and  $\beta$ -elimination<sup>2</sup> are widely utilized, but result in complete destruction of the protein. An alternative procedure for deglycosylation using trifluoromethanesulfonic acid (TFMS) has been reported with minimal degradation of the protein.<sup>3</sup> However, the methods employed are cumbersome and time-consuming. An improved method is presented here that achieves rapid, non-selective deglycosylation without compromise of the protein moiety, permitting subsequent analysis for proteomics and other applications.

## Materials

### Glycoproteins

- Ribonuclease B, bovine ([R 1153](#), Sigma-Aldrich)
- Fetuin, bovine ([F 3004](#), Sigma-Aldrich)
- Horseradish Peroxidase ([H 8375](#), Sigma-Aldrich)

### Deglycosylation

- GlycoProfile™ Chemical Deglycosylation Kit ([PP0510](#), Sigma-Aldrich)
- Sephadex G25 ([G-25-80](#), Sigma-Aldrich)

### Digestion & Mass Spectrometry

- Trypsin, Proteomics Grade ([T 6567](#), Sigma-Aldrich)
- ProteoMass™ Calibration Kit ([MSCAL1](#), Sigma-Aldrich)
- 4-Sulfophenyl isothiocyanate ([857823](#), Sigma-Aldrich)
- Sinapinic acid ([S 8313](#), Sigma-Aldrich)
- -Cyano-4-hydroxycinnamic acid ([C 8982](#), Sigma-Aldrich)

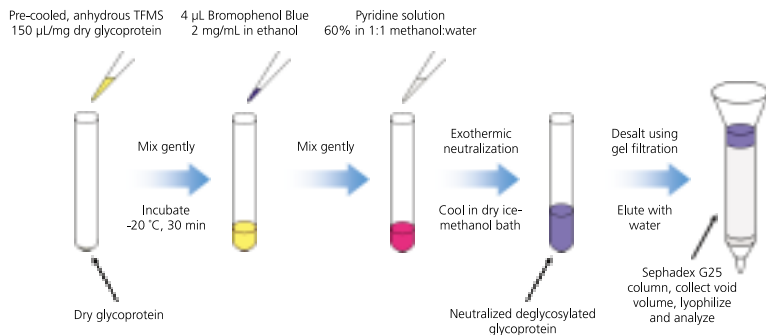
---

## Methods — Analysis

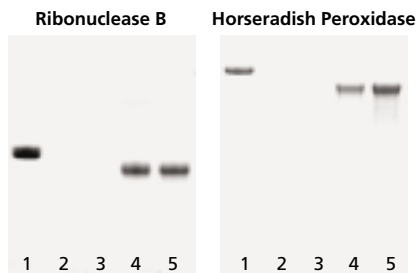
Following deglycosylation and processing to desalt by gel filtration, the deglycosylated proteins were analyzed by N-terminal sequencing, SDS-PAGE and MALDI-TOF MS. In order to assess comparability of searchable protein sequence coverage, aliquots of both native and TFMS-treated bovine fetuin were subjected to proteolytic digestion with trypsin. In addition, tryptic peptides were derivatized at the N-terminus using 4-sulfophenyl isothiocyanate. Subsequent post-source decay (PSD) analysis of the T<sub>12</sub> tryptic peptide was used to identify a glycosylation site.

MALDI-TOF MS data were acquired using a Kratos Axima CFR+ instrument in positive ion linear, reflectron or PSD modes. Tryptic peptides were purified prior to analysis using C18 ZipTips®, and eluted directly using matrix solution. For intact proteins, sinapinic acid was used as matrix, whereas -cyano-4-hydroxycinnamic acid was utilized for digest samples. Both matrices were prepared at 10 mg/mL in 70% acetonitrile, 0.03% TFA. Aliquots (1  $\mu$ L) of the sample-matrix mix were spotted on the MALDI target.

## Methods — Deglycosylation



## Results — SDS-PAGE



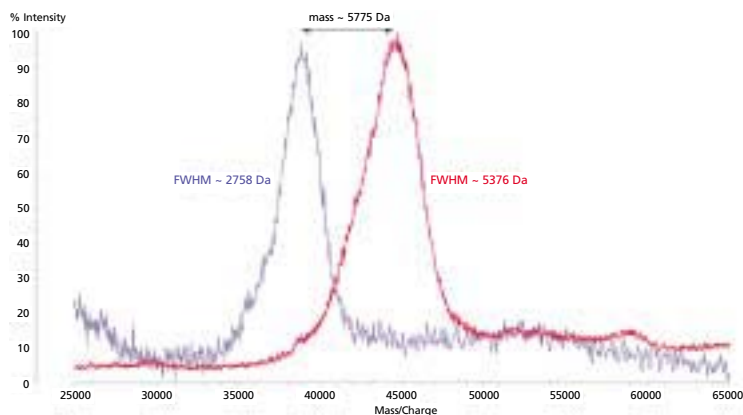
**Figure 1.** Separation of native and deglycosylated proteins by SDS-PAGE. Lane 1 is native glycoprotein, lanes 2–5 are fractions collected following gel filtration of TFMS-treated glycoprotein.

## Results — N-Terminal Sequencing

**K-E-T-A-A-A-K-F-E-R-Q-H-M-D-**  
**K-E-T-A-X-X-K-F-E-R-N-H-M-D-**

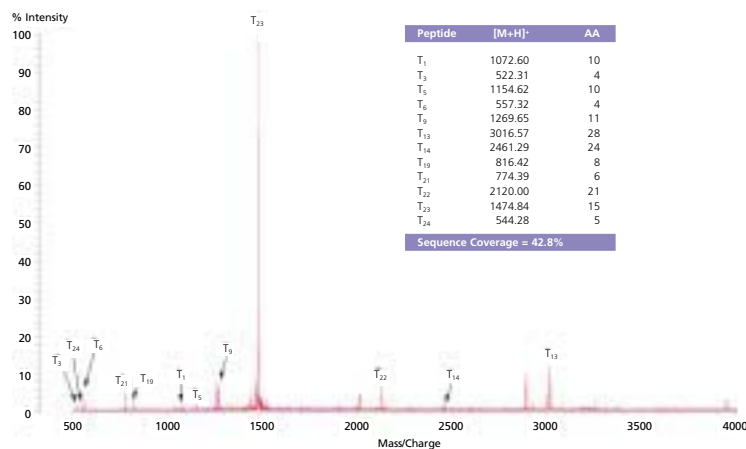
**Figure 2.** N-terminal sequencing of Ribonuclease B. NCBI database sequence (top), experimentally determined sequence (bottom). “X” symbolizes unidentified amino acid.

## Results — MALDI-TOF MS of Intact Protein

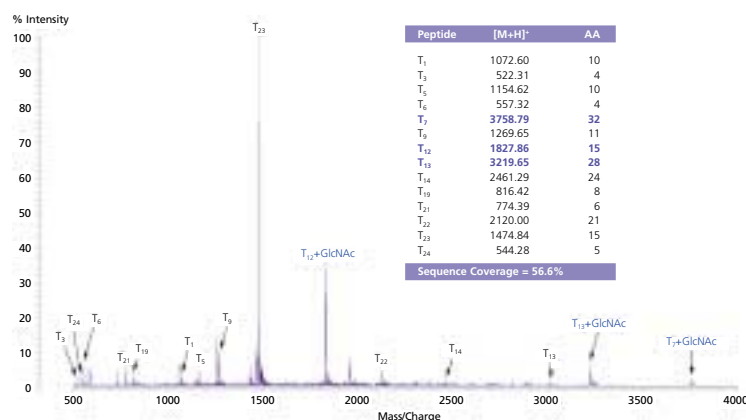


**Figure 3.** MALDI-TOF mass spectrum of native (red) and TFMS-treated (blue) bovine fetuin. Note shift in apparent mass and narrowing of distribution upon deglycosylation.

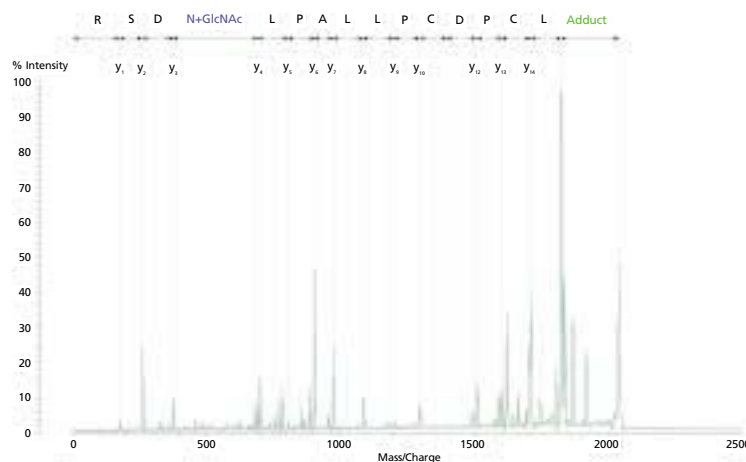
## Results — MALDI-TOF MS of Tryptic Digest



**Figure 4.** MALDI-TOF mass spectrum of native bovine fetuin tryptic digest.



**Figure 5.** MALDI-TOF mass spectrum of deglycosylated bovine fetuin tryptic digest.



**Figure 6.** MALDI-PSD mass spectrum of derivatized T12 peptide: LCPDCPLLAPLNDSR.

## Results

A time-course study of chemical deglycosylation using Ribonuclease B as a model glycoprotein was undertaken, with mass shifts resulting from the reaction monitored by SDS-PAGE. The investigation demonstrated that complete carbohydrate removal was accomplished in 30 minutes with minimal degradation of the protein (<5%, based on densitometric analysis). SDS-PAGE images for Ribonuclease B and Horseradish Peroxidase are shown in **Figure 1**.

Preservation of the peptide backbone was confirmed by N-terminal sequencing, and comparison of the experimentally determined sequence to that obtained from the NCBI database (accession NRBO). The results of this study are summarized in **Figure 2** and show the observed sequence in excellent agreement with the literature values for the first 14 amino acid residues, indicating that the protein is not compromised during deglycosylation with TFMS. Analysis of other TFMS-treated glycoproteins also produced the expected N-terminal sequences.

To assess the impact on subsequent mass spectrometric analysis of TFMS-treated glycoproteins, chemically-deglycosylated bovine fetuin was compared with native bovine fetuin. The mass spectra shown in **Figure 3** demonstrate the mass shift following deglycosylation. In addition, the observed width of the deglycosylated protein peak (FWHM ~ 2758 Da) is significantly reduced from that of the native glycoprotein (FWHM ~ 5376 Da). This is attributable to the highly heterogeneous glycoforms of the native glycoprotein compared to the TFMS-treated sample.

The native and deglycosylated bovine fetuin samples were also subjected to tryptic digestion in parallel in order to assess the impact of TFMS treatment in proteomic applications. As illustrated in **Figures 4 and 5**, significantly improved sequence coverage is realized for the deglycosylated protein digest due to the reduction in heterogeneity of the glycoprotein to a single isoform. The mass spectra obtained from both native and deglycosylated protein are comparable, indicating the method does not compromise the quality of data.

The peptide mass fingerprint data also permit identification of all three tryptic N-linked glycopeptides – T<sub>12</sub>, T<sub>13</sub> and T<sub>7</sub>. Analysis of the deglycosylated digest following in-solution derivatization with 4-sulfophenyl isothiocyanate to promote fragmentation and generation of  $\gamma$ -ion series is shown in **Figure 6**. These data confirm that the T<sub>12</sub> peptide contains a single N-linked glycosylation site (indicated).

This study showed that solvolysis using anhydrous TFMS preferentially cleaved the susceptible glycosidic bonds between adjacent monosaccharide residues, but left the innermost GlcNAc residue attached through a more resistant amide bond to an Asn residue. This provides utility for confirmation that a potential site for N-linked glycosylation (identified by the amino acid consensus sequence) is indeed occupied by a carbohydrate moiety.

## Conclusions

An improved method is detailed for the use of TFMS for deglycosylation of glycoproteins. Removal of the glycan moieties was accomplished in as little as 30 minutes, and inclusion of an indicator dye facilitated monitoring of the neutralization reaction. Solubilization of the glycoprotein was maintained throughout the reaction by the use of an aqueous-based reagent, and post-reaction clean-up/desalting is accomplished by routine methods.

Amenability of the TFMS-treated glycoprotein to mass spectrometric analysis and proteomics applications was demonstrated through processing of the deglycosylated protein by digestion with trypsin and subsequent analysis by MALDI-TOF MS using bovine fetuin as a model glycoprotein. Improved sequence coverage from the peptide mass fingerprint data was achieved compared to the native glycoprotein, without compromising data quality. Subsequent higher-order mass spectrometric analysis permitted confirmation of the sequence and site of glycosylation.

In the analysis of other glycoproteins, protein degradation and recovery was found to be variable. This is likely a result of the enormous variation in physico-chemical properties of different glycoproteins. Hence, reaction conditions (incubation time, temperature control, inclusion of "scavenger" species, etc.) for each glycoprotein may require empirical manipulation for optimization.

---

## References

1. T. Patel, J. Bruce, A. Merry, C. Bigge, M. Wormald, A. Jaques, R. Parekh, *Biochemistry* 1993, **32**, 679-693.
2. R. Dwek, C. Edge, D. Harvey, M. Wormald, R. Parekh, *Annu. Rev. Biochem.* 1993, **62**, 65-100.
3. A. Edge, C. Faltynek, L. Hof, L. Reichert, P. Weber, *Anal. Biochem.* 1981, **118**, 131-137.

