Analysis of *N*-linked Glycans by CE/LIF Using Various Glycoproteomic Protocols

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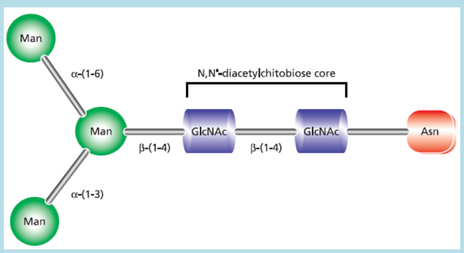
INTRODUCTION

Glycosylation occurs in the production of many protein-based biologics and can have a major impact on their biological, clinical and pharmacological properties.

Glycosylation:
- **the covalent linkage of an oligosaccharide side chain to a protein**
- **protein post-translational modification (PTMs)**
- **affects the physico-chemical properties of a protein**
- **alter essential biological functions**
- **four main categories of enzymatic glycosylation, depending on the linkage between the amino acid and the sugar (glycan):**
  - ***N*-linked glycosylation**
  - **O-linked glycosylation**
  - **C-mannosylation**
  - **glycophosphatidylinositol (GPI) anchor attachments**



Analysis and characterization of glycans is an increasingly important area for both the medical and biopharmaceutical industries as it provides indispensable information, thus understanding glycan profiles helps in the development of new therapeutic agents.

THE AIM OF THIS STUDY

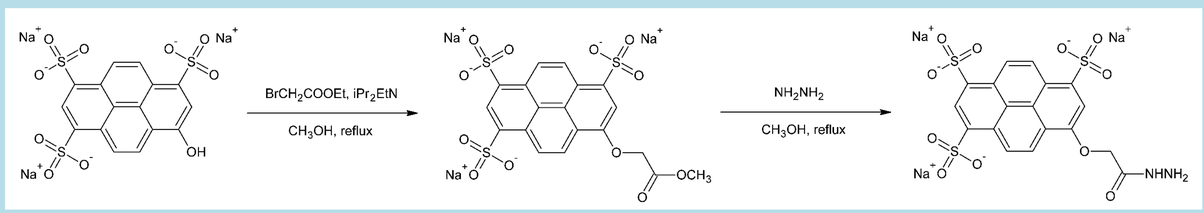
- **Synthesis of Cascade Blue hydrazide (CBH)**
- **Comparison of labeling chemistries - CBH vs. APTS**
- **Optimization of proteomic protocols for glycan analysis (comparison of reducing agents: DTT vs. TCEP)**
- **Analysis of *N*-linked glycans by CE/LIF**

EXPERIMENTAL

Synthesis of Cascade Blue hydrazide → **Optimization of different proteomic protocols** → **Purification and MALDI-TOF analysis** → **CE/LIF analyses** → **Spectra interpretation**

SYNTHESIS OF CASCADE BLUE HYDRAZIDE

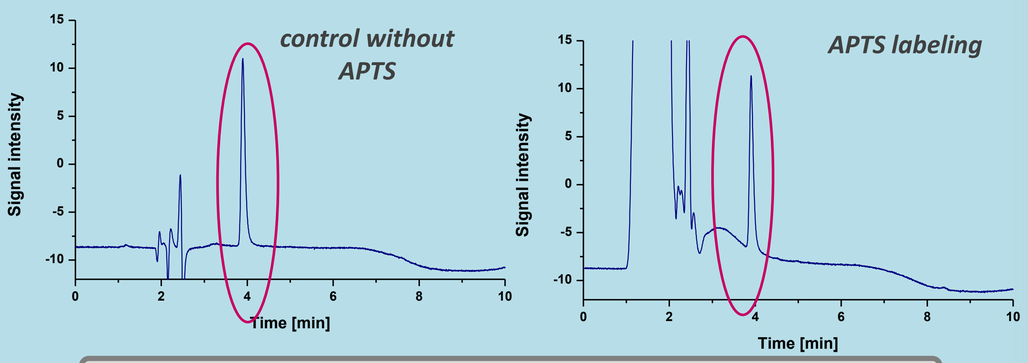
Cascade Blue hydrazide was synthesized from trisodium 8-hydroxypyrene-1,3,6-trisulfonate (pyranine) using a two-step protocol outlined in the scheme. Pyranine was treated with ethyl bromoacetate in the presence of a base to afford *O*-methoxycarbonylmethyl derivative. The obtained ester was then converted to hydrazide via addition of hydrazine in methanol to yield the desired product.



RESULTS

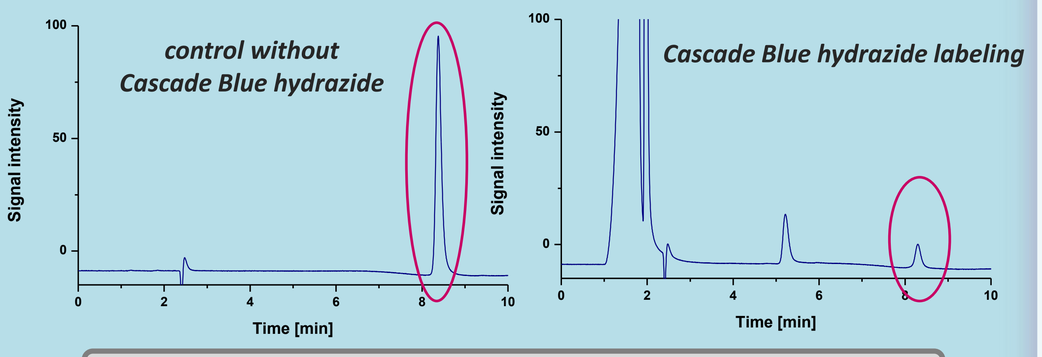
COMPARISON OF LABELING CHEMISTRIES

Reductive amination



4-hydroxybenzylalcohol quantification = *labeling yield: 10.6%*

Hydrazone formation



4-hydroxybenzaldehyde quantification = *labeling yield: 89.4%*

LC/UV analysis

Agilent 1100 Series HPLC - Poroshell 120 SB-AQ (150 × 2.1 mm; 2.7 µm), 35 °C, 0.2 mL/min, gradient elution: 10%-70% acetonitrile in water/10 min, UV detection at 280 nm

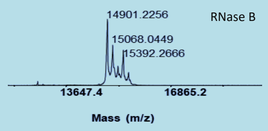
RESULTS

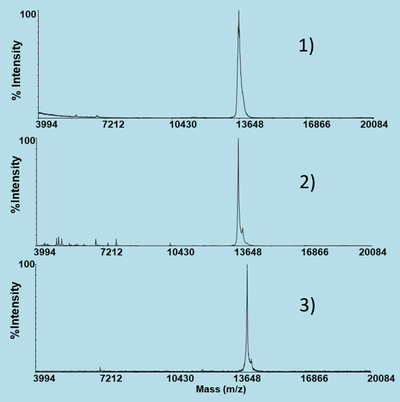
OPTIMIZATION OF PROTEOMIC PROTOCOLS

MALDI/MS analysis

Individual RNase B samples were purified using C18 Zip Tips before MALDI/MS analysis.
MS measurements were carried out using a 5800 Proteomics Analyzer (AB Sciex).

Intact protein measurements were performed in the positive linear (+LIN) mode with SA as a matrix.

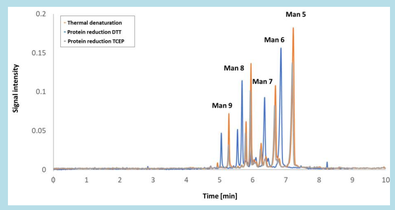




***MALDI/MS spectra of RNase B after individual deglycosylation conditions*:**
1) thermaly denatured protein 90°C
2) reduction by DTT, 80°C
3) reduction by TCEP, 80°C

CE/LIF analysis

Sciex P/ACE MDQ Plus CE instrument
CE: 50 mm ID NCHO-coated capillary (48.5/38.5 cm), carbohydrate separation gel buffer (Sciex), -25 kV
LIF: 405 nm laser/425 nm long pass filter

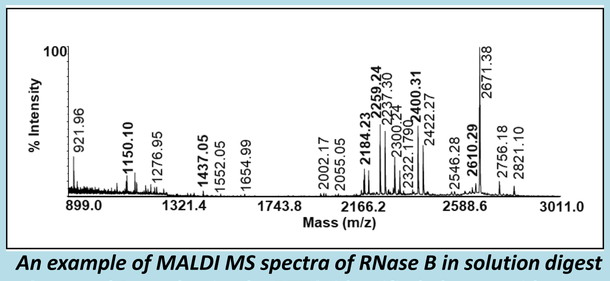


Labeling

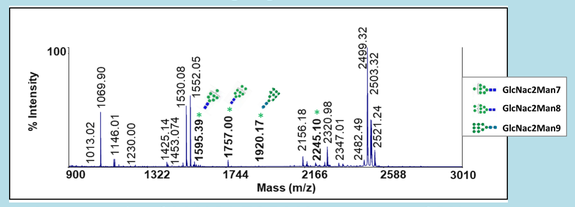
Glycans released from RNase B Cascade blue hydrazide label optimized conditions:

- **1:10 molar ratio,**
- **7.5 % acetic acid,**
- **50 °C, 20 h,**
- **no reduction agent**

MALDI-TOF MS spectra of RNase B tryptic in solution digest were obtained in positive ion reflectron mode, a solution of α-cyano-4-hydroxycinnamic acid (10 mg/mL in acetonitrile/0.1% TFA, 1:1, v/v) for dried-droplet preparation was used for mass spectrometric analysis of peptides.



An example of MALDI MS spectra of RNase B in solution digest (protocol 2 – reduction by DTT), identified glycopeptides are highlighted in bold.



An example of MALDI-TOF MS spectrum of RNase B glycans obtained after deglycosylation protocol 2 – reduction by DTT.

CONCLUSION

- **This work compares the efficiency of glycan labeling by reductive amination (APTS) and hydrazone formation (CBH) chemistries**
- **CBH labeling provides: a) single product formation, b) higher labeling yield, c) no competitive reaction**
- ***N*-linked glycans were released from RNase B after individual deglycosylation protocols (denaturation, reduction steps)**
- **The most successful protocol was based on DTT reduction during thermal denaturation, no significant results were found by comparing the reducing agents**
- **The effective hydrazone formation technique was applied for labeling of released *N*-linked glycans followed by CE/LIF analysis**
- **In the next step, the research will focus on the use of the optimized glycoproteomic protocol at different concentrations of glycoproteins and in-gel enzymatic treatment**

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