Comprehensive Study of O-Linked Glycans of Erythropoeitin

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Anja Resemann, [2] *Nannan Tao*[1], Ulrike Schweiger-Hufnagel [2], Kristina Marx [2], Stephanie Kaspar [2]
[1] Bruker Daltonics, Fremont, CA
[2] Bruker Daltonik GmbH, Bremen, Germany

Introduction

Erythropoietin (EPO) is a glycoprotein with hormone activity that controls the production of red blood cells in the bone marrow. Recombinant human EPO is produced on a large scale in cell culture and therefore, available as therapeutic agent for treating anemia related to different diseases. It is also known as blood doping agent in endurance sports. Human EPO is an approximately ~30 kDa glycoprotein with one *O*- and three *N*-glycosylation sites (Fig. 2). In pharmaceutical drug production, glycosylation heterogeneity of EPO is an important quality characteristic providing functionality as well as bioavailability of the therapeutic protein. Here, we describe a mass spectrometric approach including dedicated software tools to automatically identify *O*-linked glycosylation patterns of tryptic glycopeptides from human EPO of 2 different origins.

Methods

EPO BRP (LGC Standards) and recombinant human EPO expressed in HEK 293 cells (Sigma-Aldrich) were reduced, alkylated and subjected to trypsin digestion. The resulting peptides and glycopeptides were separated by nano-HPLC (nano-Advance, Bruker) and further analyzed by MALDI-TOF/TOF-MS (ultrafleXtreme, Bruker). Fractions were collected on a Bruker MTP Anchorchip target (800 µm hydrophilic anchors on a hydrophobic surface) with a sheath flow of matrix solution (DHB in water/acetonitrile) providing co-crystallization of sample and matrix. MS spectra were acquired in linear and in reflectron mode. MS/MS spectra were imported to ProteinScape 3.1 and there automatically screened for a glycopeptide fragmentation pattern specific for O-glycans. Typical pattern for core 1 structures is -18/+203/+162.
The fragmentation pattern was used to determine the peptide mass of each glycopeptide. Subsequently, protein and glycan database searches were performed using Mascot 2.4 for peptide identification and GlycoQuest (Bruker) for glycan identification in GlycomeDB (non-redundant combined glycan database of all relevant glycan databases) and in a custom-made EPO database containing also acetylated structures not present in GlycomeDB.

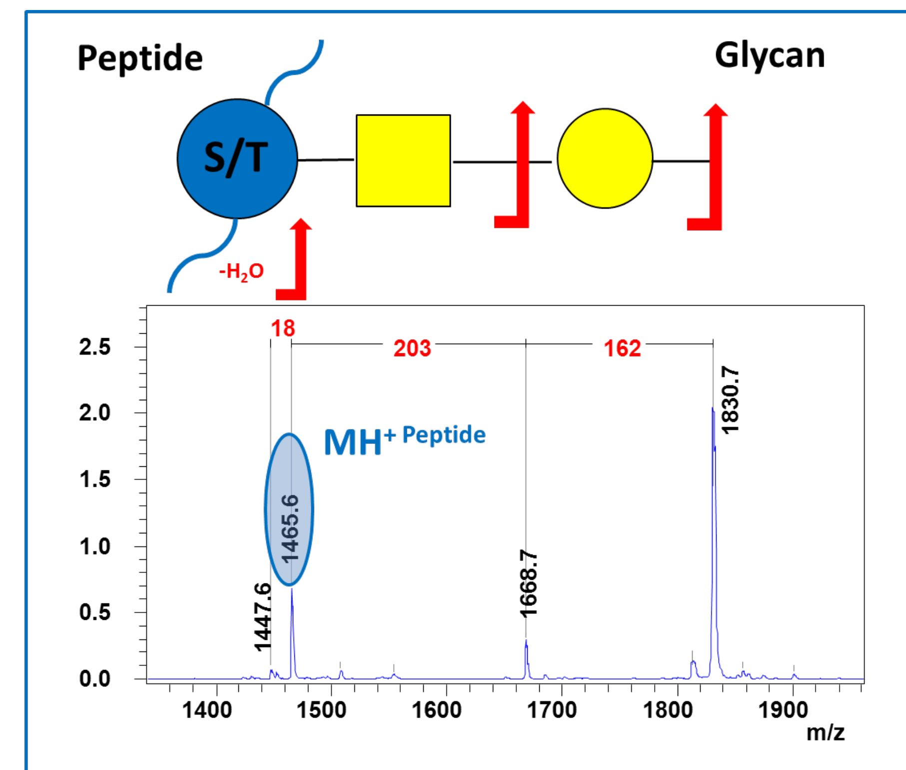
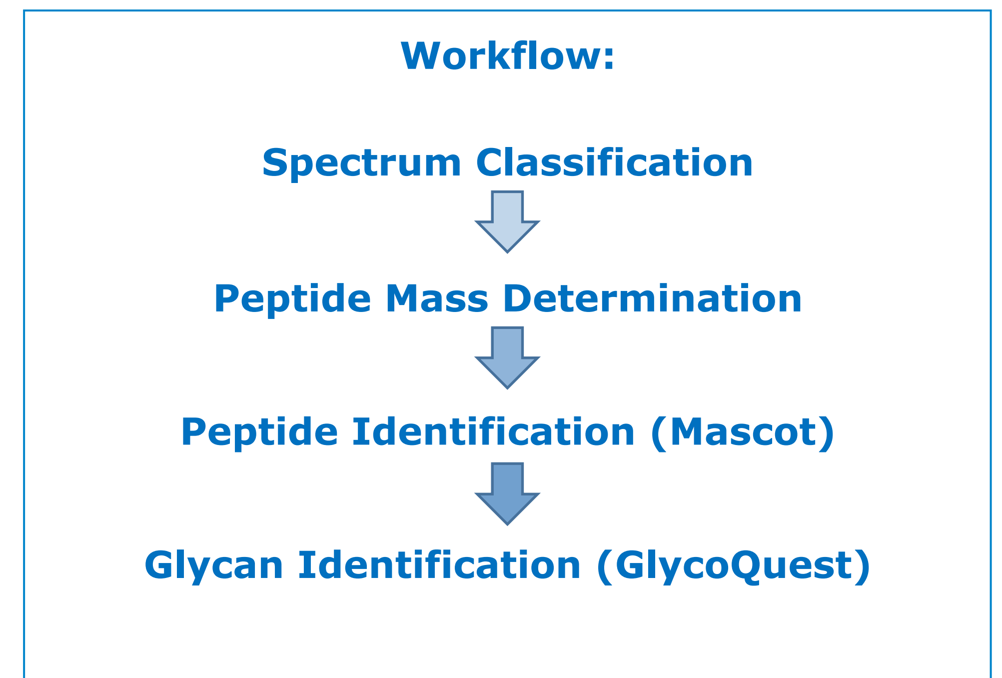


Fig. 1 Scheme of a fragmentation pattern of O-linked core 1 glycopeptides. Cleavage is observed between galactose, N-acetyl-galactosamine and the peptide with an additional loss of water. This pattern was used for EPO MS/MS-spectrum classification and peptide mass (MH+) determination.

Results

- A core 1 specific fragmentation pattern was identified for EPO: **-18/+203/+162** (Fig. 1)
- ProteinScape 3.1 provides a spectrum classifier using any fragmentation pattern defined by the user
- Glycan searches via GlycoQuest can be performed using all available glycan databases (either separately or combined in GlycomeDB)
- Since not all acetylated EPO glycans are integrated in standard databases, an individual EPO database was created
- O-linked glycans of EPO BRP and EPO HEK 293 cells are significantly different (Fig. 4)
- In the BRP standard, mainly 3 glycoforms (core 1 with 1 or 2 sialic acids) were identified
- BRP standard was intensively acetylated
- O-linked glycans from HEK 293 cells were more heterogeneous with poly-N-lactosamines and up to 2 sialic acids without acetylation



Summary

MALDI-TOF/TOF technology combined with an integrated software solution was used to analyze and compare O-linked glycosylation of 2 EPO samples. MALDI specific fragmentation patterns were used to successfully find the glycopeptide spectra in a complete LC-MALDI dataset and identify glycan and peptide moieties. ProteinScape 3.1 provides the spectrum classifier, the glycan search engine GlycoQuest and the tools for result interpretation and spectrum visualization. The approach is interesting for research and for quality control purposes of O-linked glycosylated proteins.

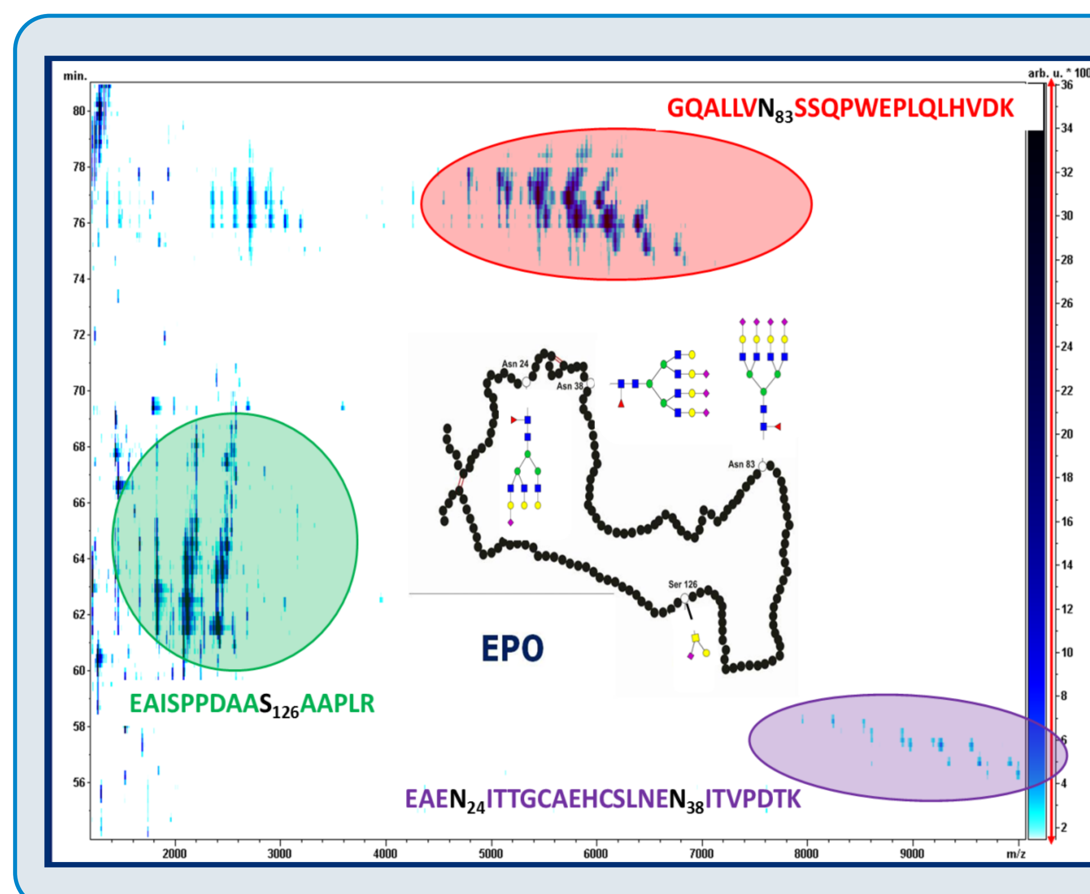


Fig. 2 Survey view of an LC-MALDI-MS experiment of human EPO-BRP tryptic digest prepared with DHB as matrix and acquired in linear positive ion mode : Singly charged peptides (m/z) are displayed against retention time (min). 3 glycopeptide fractions are visible: 2 N-linked glycopeptide fractions (red and purple) and 1 O-linked glycopeptide fraction (green).

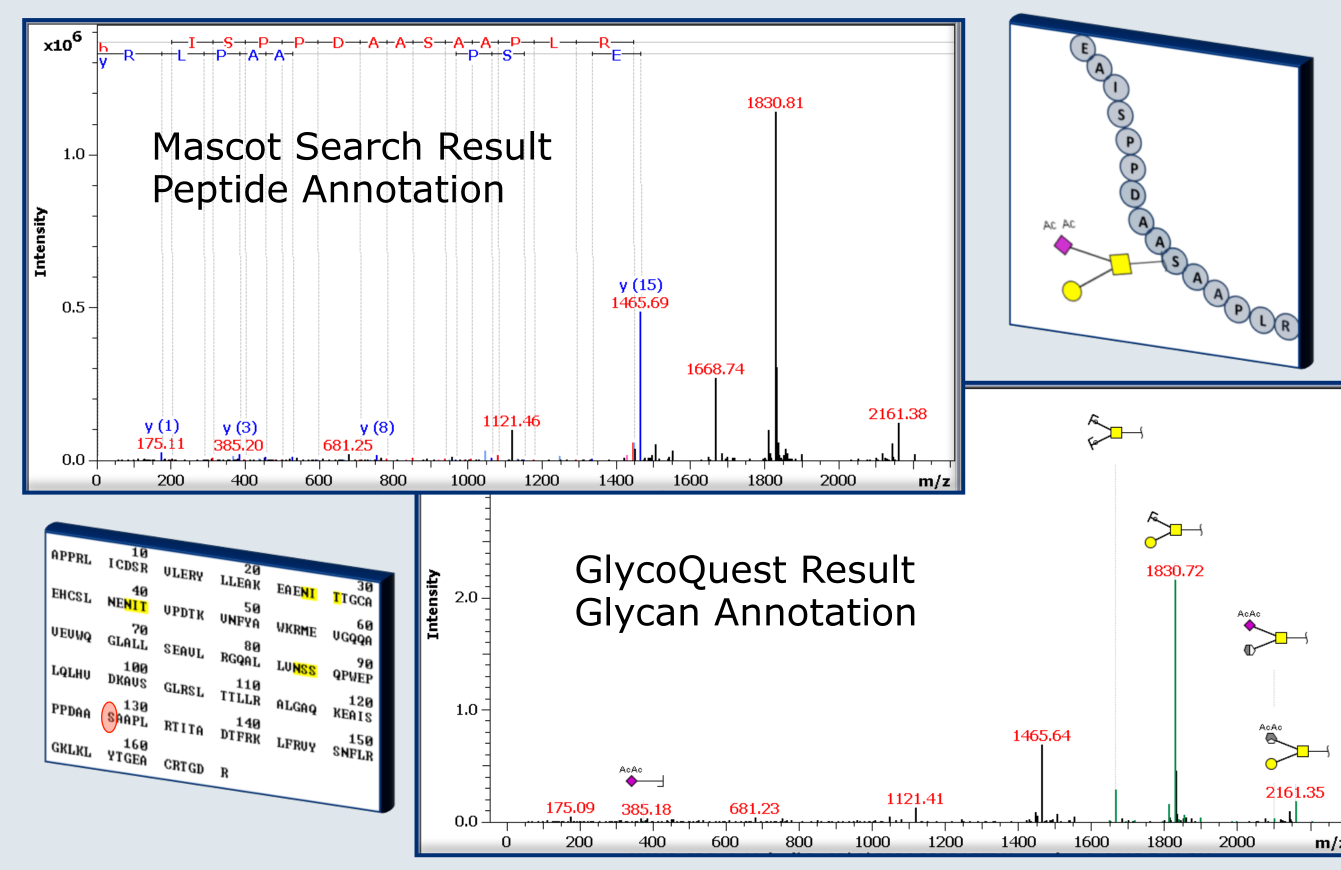
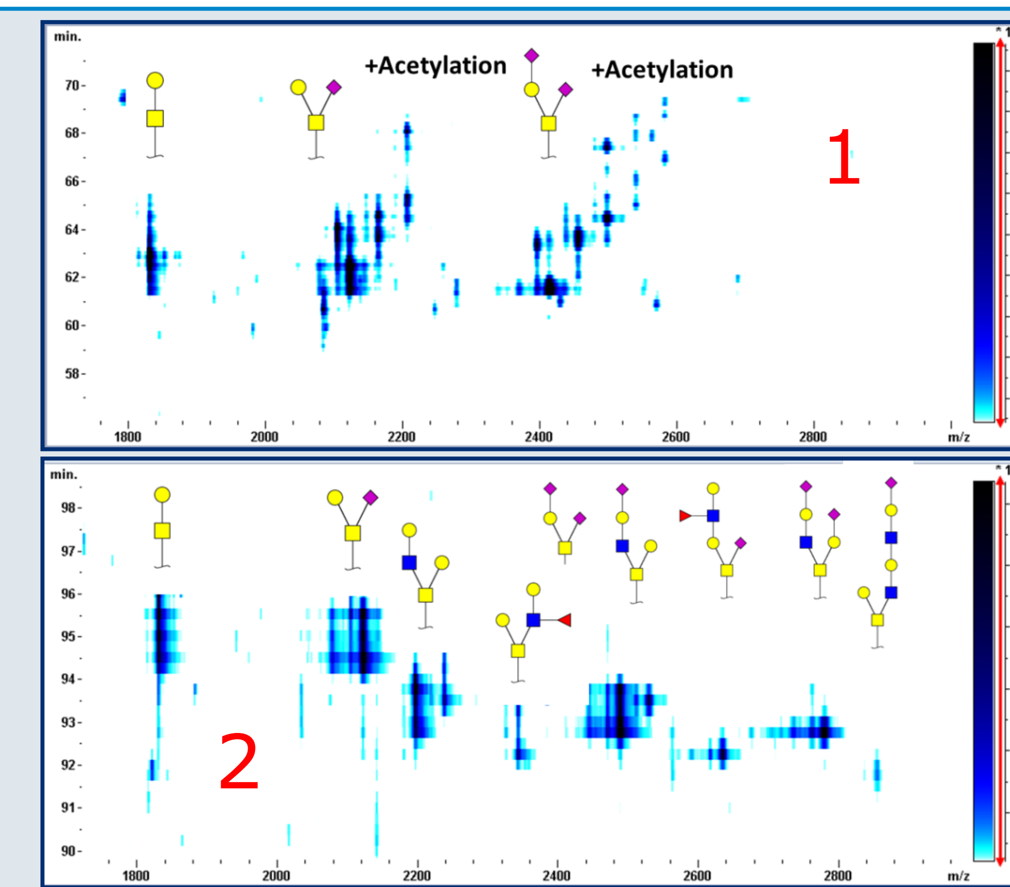


Fig. 3 MALDI TOF/TOF spectra of O-linked glycopeptides contain both – peptide and glycan fragments. An MS/MS spectrum of an O-linked glycopeptide of 2206 m/z was analyzed using ProteinScape 3.1. The results of the peptide search using Mascot and the glycan search using GlycoQuest were visualized, respectively.



EPO BRP-Standard:

- 3 main glycan forms
- High acetylation grade of sialic acids

EPO HEK 293 Cells:

- Many glycan forms
- No acetylation

Fig. 4 ProteinScape 3.1 provides a survey view for visualization of complete LC-MALDI datasets. Here, a zoom-in of the O-linked glycopeptide fractions of EPO BRP-Standard (1) and of EPO HEK 293 cells (2) allow for comparison at a glance. Main differences were found in terms of glycan forms and acetylation grade.

Conclusions

- MALDI-TOF/TOF-MS is very well suited for the analysis of O-linked glycopeptides
- ProteinScape 3.1 is able to classify and identify O-linked glycopeptide spectra
- Different EPO samples can be easily distinguished in terms of O-linked glycosylation using this approach

MALDI TOF/TOF

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