Comprehensive Study of O-Linked Glycans of Erythropoietin

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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 and 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 (LLG 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 positive ion mode : human EPO-BRP tryptic digest prepared with DHB as matrix and acquired in linear positive ion mode.

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).

Conclusions
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 and spectrum visualization. The approach is interesting for research and for quality control purposes of O-linked glycosylated proteins.

Workflow:
- Spectrum Classification
- Peptide Mass Determination
- Peptide Identification (Mascot)
- Glycan Identification (GlycoQuest)

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 and spectrum visualization. The approach is interesting for research and for quality control purposes of O-linked glycosylated proteins.

EPO BRP-Standard:
- 3 main glycan forms
- High acetylation grade of sialic acids

EPO HEK 293 Cells:
- Many glycan forms
- No acetylation

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