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Towards the Identification/Verification (Search for Molecular Substructure Markers) of recombinant EPOs Applied in Doping using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

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Introduction

Since erythropoietin (EPO) and derivatives (e.g. NESP) are available as recombinant therapeutic agents (e.g. Neorecormon, Erypo, Aranesp), they became increasingly misused as doping agents to improve aerobic performances in sports. Several analytical methods have been examined for the detection of EPO in biological fluids [1-4]. It is proposed, that primary structure differences exist between urinary (uhEPO), serum EPO (shEPO) and recombinant human EPO (rhEPO), and these variations are attributed mainly to the microheterogeneity of the glycan structures of these proteins [5-7].

Our aim is now to develop a glycoproteomic based approach to distinguish rhEPO and u/shEPO. Molecular substructure markers on the glycoprotein or on the peptide/carbohydrate level will be searched for by means of the most sensitive mass spectrometric technique, namely matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).

Methods

Mass spectrometry

MALDI mass spectra in the positive ion linear mode were acquired on an AXIMA-LNR instrument (Shimadzu Biotech Kratos Analytical, Manchester, UK) using the mass resolution enhanced DE (delayed extraction) mode. Tandem MS (MS^2) experiments were performed on an AXIMA-QIT mass spectrometer by low energy CID (collision induced dissociation) of selected precursor ions in the quadrupole ion trap part of the instrument. Sinapinic and ferulic acid were used for intact glycoprotein measurements [8], whereas α-cyano-4-hydroxy

cinnamic acid (CHCA) and 2,5-dihydroxy benzoic acid (DHB) [9,10] were used as matrices for peptide mapping and CID experiments, respectively.

Sample preparation

All proteins used for mass spectrometric calibration were purchased from Sigma (St. Louis, MO, USA): serum albumin (bovine), trypsin (bovine) and enolase (baker's yeast). The cleavage enzymes, recombinant peptide-N-glycosidase F (PNGaseF) from *Flavobacterium meningosepticum*, neuraminidase from *Athrobacter urefaciens* and trypsin from *bovine pancreas* (modified, sequencing grade) were obtained as lyophilized preparations from Roche (Mannheim, Germany). Sample amounts in the range of 0.5-1 µg were subjected to enzymatic treatment by either of the enzymes. Digestion buffers and conditions were adjusted according to the manufacturer's instructions. After enzymatic digestion the samples were purified and concentrated by ZipTip® technology [11] prior to MALDI-MS experiments. Sample solutions (0.3 µl) containing 1-3 pmol of protein were usually applied for MALDI analyses. For the measurements on sinapinic acid, ferulic acid and DHB, the dried-droplet technique was used, whereas for peptide preparation on CHCA matrix, the thin-layer technique was applied [9,10]. In general, stainless-steel targets with 384 sample spots were used as MALDI targets, while for high sensitivity measurements of the intact proteins a DropStop® foil was used as sample support [12].

Results

Our experiments show, that it was possible to detect different types of rhEPO (EPO-α, EPO-β and NESP) using MALDI-TOF-MS in the linear mode. EPO-α and EPO-β showed quite similar molecular weights, while NESP could be clearly discriminated due to the mass of its additional glycan structures already by means of external calibration (Fig.1). Rather broad signal distributions showing a microheterogeneity that could not be conclusively resolved on the intact protein level were observed. An exact molecular weight determination was achieved by internal calibration using trypsin and enolase as protein standards (Fig.2). Detection limits for the purified, intact glycoproteins were achievable in the low fmol range (25-50 fmol on target) using a sample preparation method applying a hydrophobic sample support (DropStop®) as MALDI target surface. These results appeared very promising for the development of highly sensitive detection methods for a direct identification of rhEPO from human body fluids. The extension to surface-enhanced laser desorption ionization (SELDI)-TOF-MS might be therefore feasible.

During our investigation we were also able to detect molecular substructure markers of EPO molecules by specific enzymatic reactions. Most of the microhetreogeneity of the intact glycoproteins could be attributed to their huge sialic acid content. A direct detection of the different N- or O-glycoforms from rhEPOs using MALDI-MS was possible by removal of sialic acids from the glycan structures of the intact molecules and after de-N-glycosylation. Differences between the main isoforms of EPO-α, EPO-β and NESP based on their N-glycan composition could be observed from MALDI mass spectra. Mass increments of 365 Da between the signals indicated different contents of N-acetyllactosamine units that are characteristic for the antennary structures of complex N-glycans. Comparison with the calculated masses of distinct glycoforms revealed that structures predominately containing 3-5 tri- and tetraantennary N-glycans could be observed in rhEPO molecules. Glycoforms bearing tetraantennary structures with additional lactosamine repeats (1-2) contribute to the higher molecular weight peaks of the signal distributions (Fig.3). A microheterogeneity of the molecules based mainly on the degree of sialylation was observable from the de-N-glycosylated molecules (Fig. 4). The investigation of tryptic digests from the different rhEPO samples clearly confirmed these observations. Thereby, beside sialylation also further post-translational modification (e.g. acetylation) of the O-glycan structures could be realized (Fig.5). Using MALDI-QIT-MS characteristic fragment ions from isolated glycopeptides corresponding to loss of monosaccharide residues clearly confirmed the core-1 structure (Gal-GalNAc) of the rhEPO O-glycan (Fig.6). Now, a further investigation of the individual glycoforms of rhEPO and u/shEPO for their differentiation after chromatographic separation using MALDI- and ESI-MS is currently in progress.

Conclusion

MALDI-TOF-MS turned out to be a very promising tool for the detection and structural characterization of rhEPO molecules. Employing a careful matrix preparation strategy, a highly sensitive detection of the intact sialo-glycoproteins was possible. The limit of detection for intact rhEPOs was found to be in the low fmol range (25-50 fmol). An identification of N- and O-glycoforms was possible after removal of sialic acids from the glycan structures and de-N-glycosylation of the intact molecules. In the course of our study, differences between EPO-α, β and NESP based on their N-glycan composition could be realized. Removal of sialic acid residues by enzymatic treatment led to distinct glycoforms, which could be correlated mainly to tri- and tetraantennary complex N-glycan structures. Also from the de-N-glycosylated molecules a microheterogeneity based on the degree of sialylation and additional

post-translational modifications of the O-glycan (particularly acetylation) could be observed. Such molecular substructures may serve as biomarkers for the future development of novel detection strategies of rhEPO administration in doping analysis.

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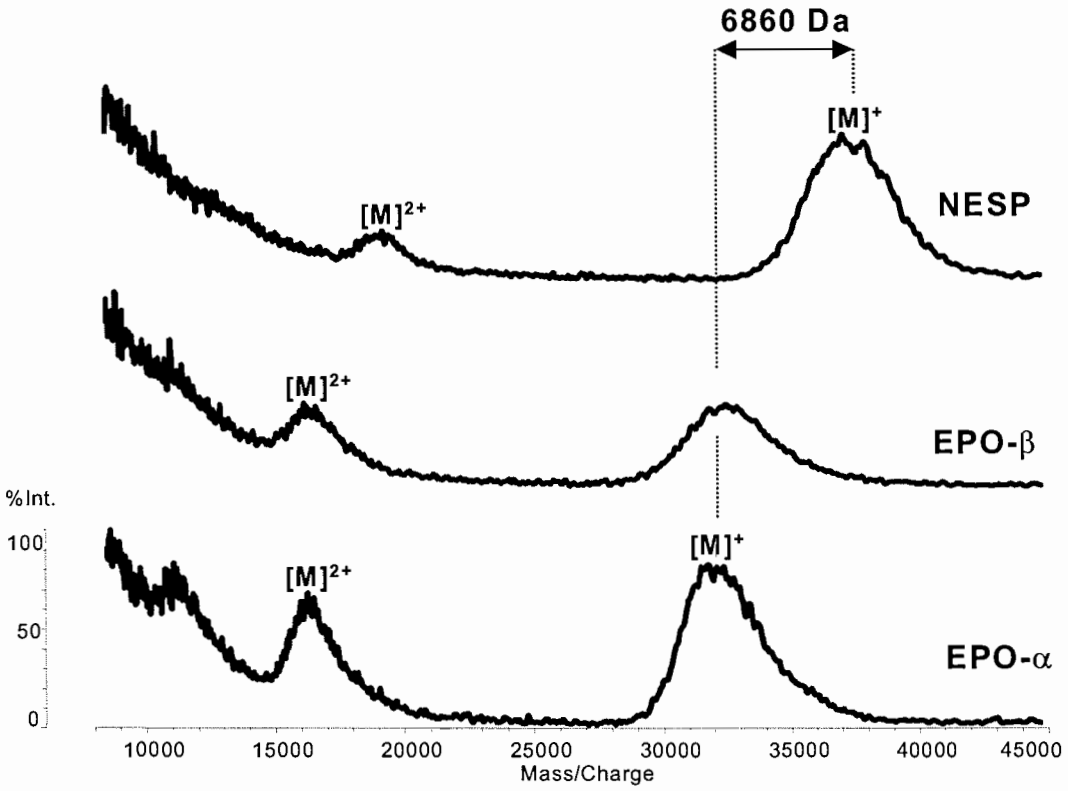

Fig.1 Detection of intact rhEPO by MALDI-TOF-MS (samples were externally calibrated by BSA, matrix: sinapinic acid)

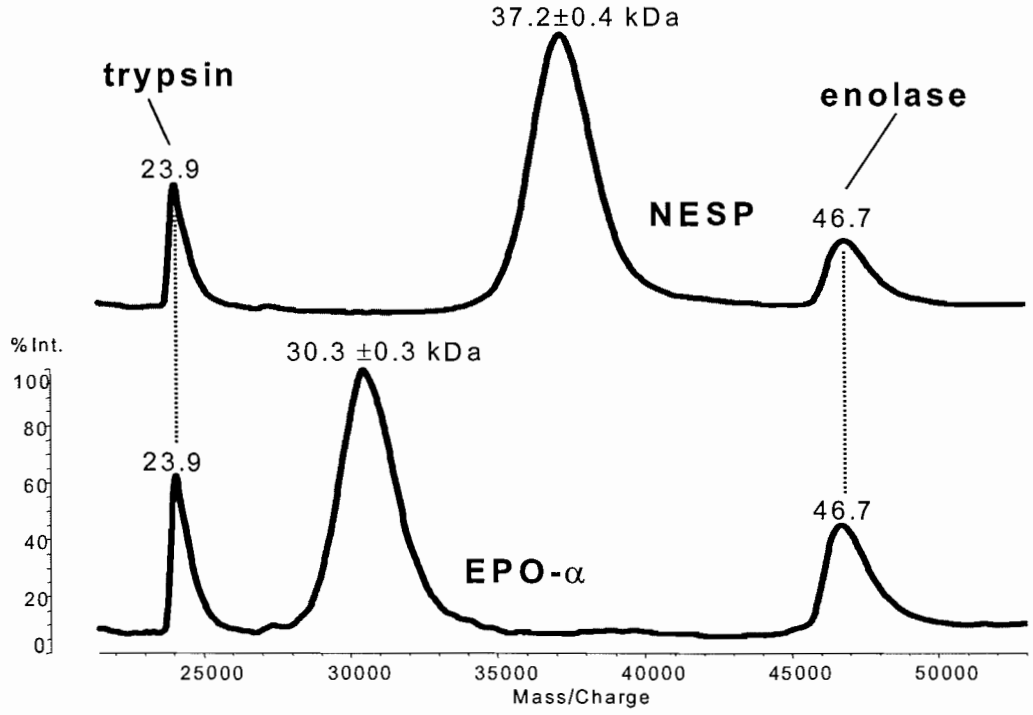

Fig.2 Exact molecular weight determination of rhEPO (bovine trypsin and enolase from baker's yeast were used as internal calibrants, matrix: sinapinic acid)

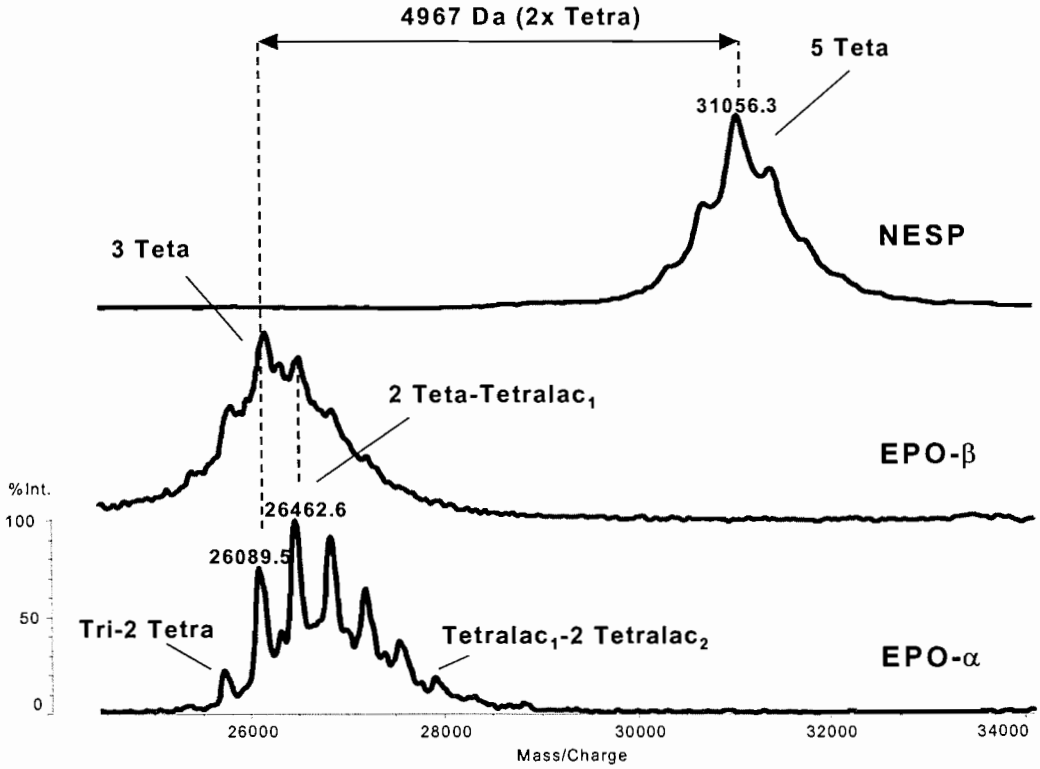

Fig.3 MALDI mass spectra of de-sialylated rhEPO (N-glycan structures: Tri = triantennary, Tetra = tetraantennary, lac1 and lac2 = number of additional N-acetyllactosamine repeats; glycan structure assignments are only indicative based on comparison with calculated theoretical glycoform masses)

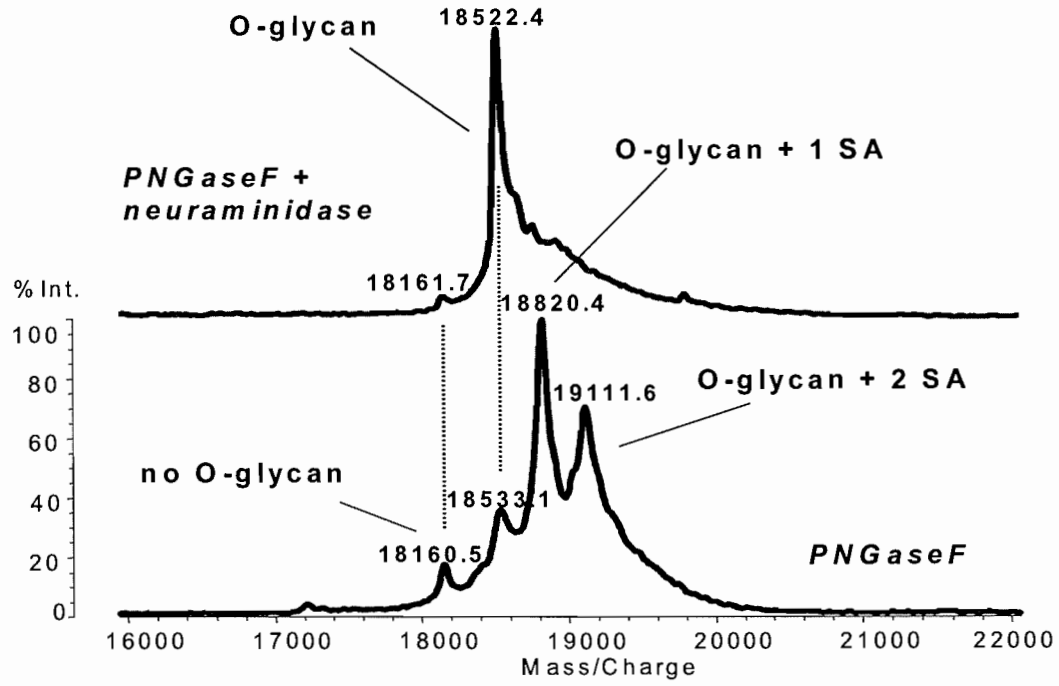

Fig.4 MALDI mass spectrum of de-N-glycosylated EPO-α (mass spectra show enzymatic treatments with PNGaseF and neuraminidase; SA: sialic acid)

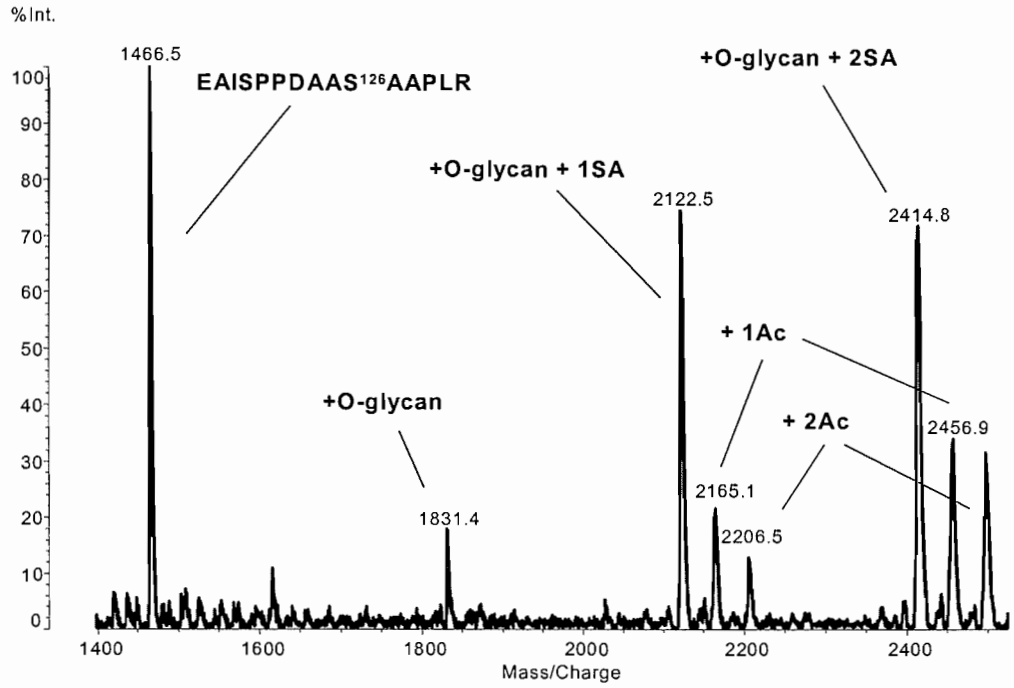

Fig.5 MALDI mass spectrum of O-glycopeptides of NESP (m/z 1466 is the non-O-glycosylated peptide sequence including serine126; m/z 1831, 2122 and 2414 represent peptides with different types of O-glycoforms; SA: sialic acid, Ac: acetylation)

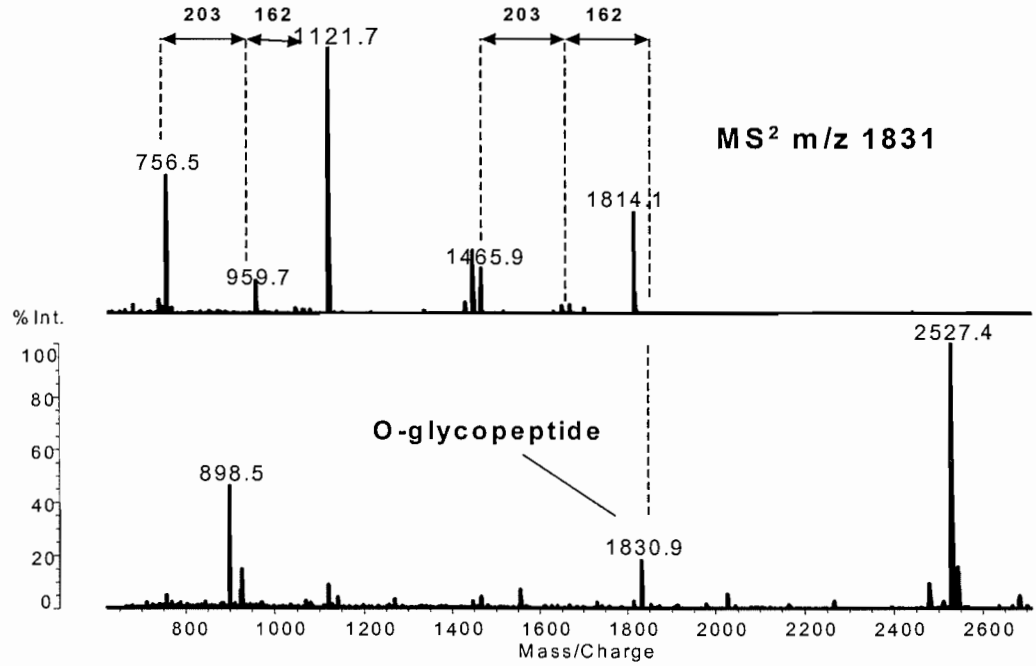

Fig.6 O-glycan structure analysis by MALDI-QIT-MS (shown is the scan range between m/z 700-3000 with the selected precursor ion m/z 1830.9 and the corresponding MS^2 spectrum exhibiting fragment ions related to loss of the monosaccharide residues; mass increments of 162 and 203 Da indicate Gal and GalNAc residues from the core-1 structure while m/z 1121 represents a sequence fragment ion containing the O-glycan)