Top-Down Mass Spectrometric Analysis of Glycosylated and Phosphorylated Proteins

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Nannan Tao1, Anja Resemann2, Lars Vorwerg2 and Detlev Suckau2

1Bruker Daltonics, Billerica, MA, USA
2Bruker Daltonik, Bremen, Germany

Introduction

• Top-Down approaches allow the analysis of intact proteins for the purpose of their identification and characterization.
• In contrast to Bottom-Up methods, labile PTM’s like phosphorylation remain stable in top-down analysis and allow direct determination of modification sites.
• MALDI Top-Down Sequencing (TDS) typically delivers long N- and C-terminal sequence readouts of 80 amino acid residues, allowing for the detection of PTM’s such as N-linked glycan even at the core region of 15 kDa proteins.
• Negative mode ISD - as expected - is an additional option for negatively charged proteins such as the multi-phosphorylated caseins.

Methods

Samples: Technical casein containing α-S1-casein, β-S2-casein and γ-casein, RNase B (all bovine), horseradish peroxidase (Sigma-Aldrich) and a therapeutic antibody (Vectibix, Amgen).

Matrices: 1,5-diaminonaphthalene (DAN) and sDHB.

Preparation: 2 µl of sample were spotted to a MTP BigAnchorChip 384 sample plate (Bruker Daltonik) and dried. The dry sample spot was rinsed with 10 µl 0.1 % TFA and 1 µl of matrix solution was added to each sample spot. (Fig. 1) The casemins were RP-LC separated prior to preparation. The antibody was digested by IgZERO and Fabricator (both Genovis, Sweden) providing Fc fragments with cleaved chitobiase core.

Instrumentation: An ultraflextreme (Bruker Daltonics) was operated in positive and negative reflector mode for the detection of TDS spectra that were externally calibrated by BSA in-source decay (ISD) fragments. Spectra were analyzed using BioTools 3.2 Sk4 and Mascot 2.3.

Results

Phosphorylation: In positive ion mode, ISD fragment peak intensities decrease with every phosphorylated residue. After 2 phosphorylation sites, the N-terminal ISD ion series is typically abrogated (data not shown). Negative mode instead provides high intensity fragment ions with sequence calls including 5 phosphorylation sites in the case of γ-casein (Fig. 2). In case of α-S1-casein, the positive ion series already revealed Ser-41 not being phosphorylated as suggested in the SwissProt database. Positive and negative TDS spectra emerged to provide complementary information of phosphorylated proteins. (Fig. 2)

Glycosylation: N-linked glycosylation is a posttranslational modification (PTM) with high molecular weight and was therefore difficult to analyse in the previously achievable TDS mass range up to 4 kDa. The new ultraflextreme extended the mass range with isotopic resolution in reflector ISD spectra to at least 9 kDa.

Summary

MALDI Top-Down Sequencing of phosphorylated and glycosylated proteins were achieved by:

• In situ purification on MALDI target
• DAN and sDHB as matrices
• Positive and negative ion mode MALDI-TDS
• Newest MALDI-TDF/TOF instrumentation providing monoisotopic resolution beyond m/z 9000

High-mannose glycans in RNase B were previously not accessible using MALDI-TDS as the modified Asn-34 was bracketed by a disulfide bridge. The application of DAN as ISD matrix with reductive properties allowed to read through such SS-bonds allowing for detection of the main glycans of RNase B (Fig. 3). In case of theFc fragment of the therapeutic mAb Vectibix, isosotopically resolved ISD fragments were detected up to m/z 9000 Da. The chitobiase fragment of the fucosylated glycan forms was clearly identified. In horseradish peroxidase, 2 modifications were confirmed including the plant specific glycan (Fig 3).

Conclusions

• MALDI-TDS is a powerful new tool for PTM detection in intact proteins
• Simple sample prep, fast analysis
• The charge of a terminal protein sequence determines the best ion mode for MALDI-TDS analysis
• Negative mode TDS detects multiple phosphorylation sites
• Even large modifications such as glycans are accessible now due to enlarged isotope resolving mass range

MalDI-TDS

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