In-depth peptide mapping of biopharmaceuticals using an electron-transfer/higher-energy collision dissociation (EThcD) implemented on a modified Orbitrap hybrid MS

Reiko Kiyonami[1], Cong Wang[2], Peter Krueger[2], Roberto Gamez[1], Heiner Koch[2], Min Du[1]

[1]Thermo Fisher Scientific, Lexington, MA; [2]Thermo Fisher Scientific, Bremen, Germany

Abstract

Purpose: Develop an in-depth peptide mapping method using the EThcD technique, which enables high protein sequence coverage, characterization of low-abundant post-translational modifications (PTMs), and detection of isomeric amino acids in a single LC-MS run.

Methods: We utilized EThcD on the new Thermo Scientific™ Orbitrap™ Excedion Pro BioPharma mass spectrometer, coupled with a Thermo Scientific™ Vanquish™ Horizon UHPLC system, to perform in-depth peptide mapping of a NISTmAb sample. The NISTmAb was digested with trypsin and analyzed using a conventional flow LC-MS/MS setup. EThcD fragmentation was optimized to ensure comprehensive peptide fragmentation.

Results: The EThcD technique, implemented on the new Orbitrap Excedion Pro BioPharma mass spectrometer, enabled in-depth peptide mapping with high sequence coverage, confident PTM characterization, and unambiguous differentiation of isomeric amino acids.

Introduction

Peptide mapping is a widely used method for therapeutic protein characterization. Although the higher-energy collisional dissociation (HCD) technique is primarily used for peptide mapping, it can sometimes cause the loss of labile PTMs such as phosphorylation and glycosylation due to the high energy involved in the fragmentation process. Additionally, HCD lacks the ability to determine peptide side chain differences and disulfide linkage locations. Electron-transfer/higher-energy collision dissociation (EThcD) combines the softer electron transfer dissociation (ETD) with HCD and has emerged as an important tool for peptide mapping in biotherapeutic protein characterization, including labile PTM analysis, sequence variant analysis, and de novo sequencing. EThcD is particularly beneficial for identifying and locating PTMs such as asparagine deamidation, aspartic acid isomerization, glycosylation, and disulfide linkages, as it can simultaneously fragment both the peptide backbone and the modified site, providing valuable structural details. EThcD is also very useful for differentiating isomeric amino acids. The new Orbitrap Excedion Pro BioPharma mass spectrometer implements ETD (Figure 1) on a hybrid Orbitrap mass spectrometer platform for the first time, enabling EThcD fragmentation. In this study, we demonstrate that EThcD enabled on the new Orbitrap Excedion Pro BioPharma mass spectrometer allows for complete sequence coverage, precise PTM characterization, and differentiation of isomeric amino acid residues in biotherapeutic proteins.

Materials and methods

Sample preparation

A commercially available NISTmAb (RM8671) was used as a model biotherapeutic protein. 10 µL of NISTmAb standard (10 mg/mL) was denatured using 7 M guanidine HCl and 100 mM Tris, and adjusted to a final concentration of 1 mg/mL. The denatured NISTmAb was reduced using TCEP and alkylated using iodoacetamide, then digested with trypsin. The digestion process was stopped by adding water containing 10% formic acid. The final volume of the digest was adjusted to 120 µL.

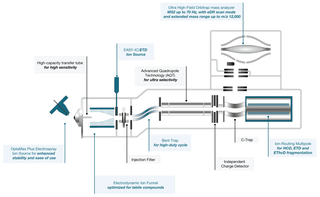
HPLC conditions

The Thermo Scientific™ Vanquish™ Horizon UHPLC system performed separations. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The column was a Thermo Scientific™ Hypersil Gold™ Peptide UHPLC column (2.1 mm x 150 mm, 1.9 µm) that operated at 45 ° C and a flow rate of 250 µL/min. The gradient condition used was listed in Table 1. The injection volume was 5 µL (4.2 µg on column).

Table 1. HPLC gradient condition

Time (min)	%B
0	1
5	1
6	10
70	35
72	90
77	90
77.1	1
83	1

Figure 1. Schematic of Orbitrap Excedion Pro mass spectrometer



MS Conditions

All the data was collected on a Orbitrap Excedion Pro BioPharma mass spectrometer. MS/MS data acquisition was carried out using a data dependent MS/MS set up. The ESI and mass spectrometer set ups are shown in Table 2 and 3, respectively.

Table 2. ESI source parameter set ups

ESI source settings	
Sheath gas (a.u.)	40
Aux. gass (a.u.)	10
Sweep gas (a. u.)	0
spray voltage (+V)	3400
Capillary Temp. (°C)	320
Vaporizer Temp. (°C)	250

Table 3. MS parameter set ups

General	
Application mode	Peptide
Pressure mode	Standard
RF lens (%)	40
Full MS	
Scan range (m/z)	200 - 2000
Resolution	12,000 at m/z 200
AGC target value (%)	300
Max injection time (ms)	50
dd MS/MS (cycle time: 1.5 sec)	
Resolution	30,000 at m/z 200
Isolation window (m/z)	2
Activation type	ETD
Use calibrated charge dependent ETD parameters	True
ETD supplemental activation	True
SA collision energy (%)	25
AGC target value (%)	100
Max injection time (ms)	250

Data Analysis

All data were processed using a beta version of Thermo Scientific™ BioPharma Finder™ 5.3 software for sequence confirmation, PTM characterization, and differentiation of isomeric amino acid residues. A 5ppm precursor ion mass search window was used for protein database search.

Results

As shown in Figure 1, the Orbitrap Excedion Pro BioPharma mass spectrometer performs ETD/EThcD in the ion routing multipole where precursor ions and fluoranthene radical anions are mixed for electron transfer and c/z fragment ion generation. For performing EThcD, the generated *c/z* ions are transferred to the C-trap and accelerated back into the ion routing multipole for supplemental higher-energy collisional activation, further improving *c/z* fragmentation efficiency and allowing generation of additional HCD type ions (*b/y*) based on the chosen activation energy. This new design enables EThcD fragmentation with a short reaction time while keeping good fragmentation efficiency. Figure 2 shows the base peak chromatogram of the NISTmAb digest and the sequence coverage from a single HPLC-EThcD MS/MS run. 100% sequence coverage was achieved.

Less than 0.1% low abundant PTMs were detected with high confidence by the HPLC- EThcD MS/MS experiment. Figure 3 highlights the detection of a very low-abundant deamidation on the peptide VYACEVTHQGLSSPVTK, confidently identified by EThcD fragmentation. The relative quantification ratio of the deamidation was determined to be 0.026% using the integrated peak areas of native and modified peptide pairs (Figure 3A). The 0.984 amu difference of the fragment ions between the native and modified peptide pairs was clearly detected in the highlighted fragment ion range of *m/z* 670–1400 (Figure 3B).

Figure 2. Basepeak chromatogram and 100% sequence coverage of the NISTmAb digest from a single peptide mapping experiment using EThcD

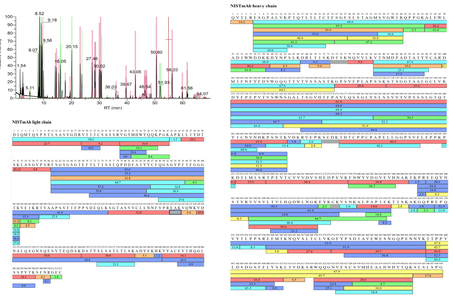
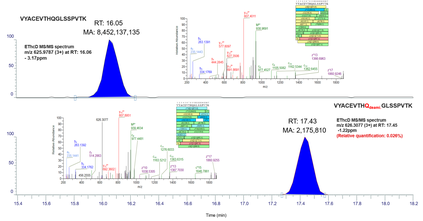
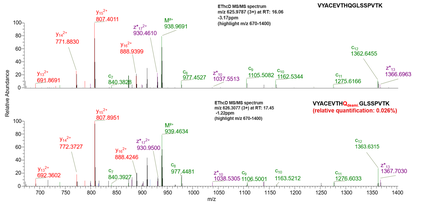


Figure 3. Low abundant PTM characterization and relative quantification

A: XICs and MS/MS assignment results of peptide VYACEVTHQGLSSPVT without/with deamidation



B: Representative MS/MS fragment ion comparison of peptide VYACEVTHQGLSSPVTK without/with deamidation



One benefit that EThcD offers is its capability to differentiate isomeric amino acid residues. For example, the formation of odd-electron *z-type* ions from multi-protonated peptide ions by ETD and their subsequent HCD fragmentation leads to the formation of secondary *w-type* ions. Since the *z-type* ions lose 43.055 mass units (isopropyl radical) in the case of leucine and 29.039 mass units (ethyl radical) in the case of isoleucine, the masses of the corresponding *w-type* ions are different for leucine and isoleucine, allowing reliable identification of these residues[1] (Figure 4).The EThcD fragmentation on the Orbitrap Excedion Pro BioPharma mass spectrometer was highly sensitive and efficient, even for precursor ions with low charge states. Figure 5 shows that the diagnostic *w-type* ions (*z*-43 and *z*-29) were clearly detected from a doubly charged precursor ion of the peptide ALPAPIEK, enabling the distinction between leucine and isoleucine.

Figure 4. Diagnostic *w-type* ions for differentiating Leu and Ile

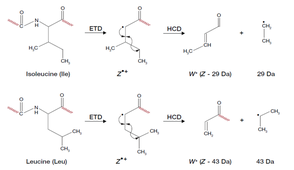
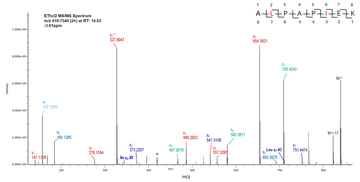


Figure 5. EThcD MS/MS spectrum of peptide ALPAPIEK. The leucine and isoleucine can be clearly differentiated by the diagnostic *w-type* ions (*z*-43 and *z*-29)



EThcD also provides diagnostic fragment ions to distinguish the aspartic acid and isoaspartic acid isomers. In the case of isoaspartic acid (isoAsp), EThcD fragments the peptide backbone at the Cα-Cβ bond within the isoAsp residue, leading to the formation of *c*+57 and *z*-57 ions[2] (Figure 6). While in case of aspartic acid (Asp), EThcD only yield normal *c*- and *z*• *type* ions because Asp does not have a methylene group in the backbone. Figure 7 shows that the diagnostic *c*+57 and *z*-57 ions were detected clearly from a triply charged precursor ion of peptide WQQGNVFSCSVMHEALHDHYTQK, enabling confident identification of this low abundant isoAsp peak (0.18%) and its modification location.

Figure 6. Diagnostic secondary fragment ions for identification of isoAsp acid

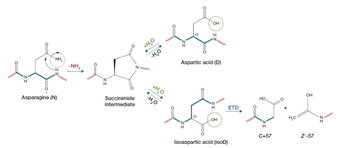
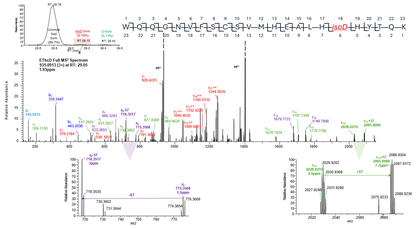


Figure 7. EThcD MS/MS spectrum of peptide WQQGNVFSCSVMHEALHDHYTQK. The diagnostic *c*+57 and *z*-57 fragment ions were clearly detected, enabling confident isoAsp identification



Conclusions

- EThcD on the Orbitrap Excedion Pro BioPharma mass spectrometer offers combined fragment ion types from both ETD and HCD with high sensitivity and fast acquisition rates, enabling extensive sequence coverage. 100% sequence coverage was observed for NISTmAb trypsine digest with a single HPLC-MS/MS run using EThcD.
- The high sensitivity of the EThcD on the Orbitrap Excedion Pro BioPharma mass spectrometer enables excellent quality of EThcD data even for low abundant peptides with PTMs, allowing confident identification and precise localization of low abundant post-translational modifications. Down to 0.26% low abundant deamidation was identified unambitiously from the NISTmAb digest with the EThcD fragment ions.
- EThcD is particularly useful for differentiate isomeric amino acid residues such as leucine and isoleucine, aspartic acid and isoasparic acid by providing unique diagnostic fragment ions. EThcD on the Orbitrap Excedion Pro BioPharma mass spectrometer was able to generate the diagnostic *w-type* ions (*z*-43 and *z*-29) to differentiate leucine and isoleucine even for a small double charge peptide. Very low abundant (0.18%) isoasparic acid was confidently identified from the NISTmAb digest by detection of diagnostic fragment ions (*c*+57, *z*•-57).
- Overall, EThcD enhances the depth and accuracy of peptide mapping, making it a powerful technique for in-depth peptide mapping analysis.

References

1. Kjeldsen, F. et. Al., Distinguishing of I le/Leu Amino Acid Residues in the PP3 Protein by (Hot) Electron Capture Dissociation in Fourier Transform Ion Cyclotron Resonance Mass Spectrometry. Anal. Chem. 2003, 75, 1267-1274.
2. Hurtado, P.P. et. Al., Differentiation of isomeric amin acid residues in proteins and peptides using mass spectrometry. Mass Spectrometry Reviews, 2012, 31, 609–625.

Conflict of interest disclosure

The authors declare no competing financial interest.

Trademarks/licensing

Learn more at **thermofisher.com/orbitrapexcedionpro**