Winning The Last Battle Against Edman Degradation: Reliable Leucine/Iso-leucine Differentiation In Peptide Sequencing Using an Orbitrap Fusion Mass Spectrometer

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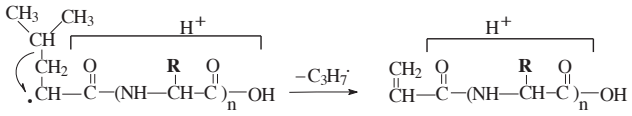
Introduction

The problem of differentiation between isomeric leucine and isoleucine has been the most relevant for *de novo* sequencing of peptides by means of mass spectrometry. The most successful approaches involve secondary fragmentation of the odd electron z·-ions with formation of *w* ions in MS2 and MS3 experiments (SCHEME 1).



SCHEME 1 z_n w_n

Discrimination of isomeric residues deals with the characteristic losses from the side chains: -43 Da (Leu) and -29 Da (Ile). R. Zubarev et al. used ECD[1] and HECD[1] (ē energy up to 11 eV) for this purpose in MS2 experiments. Unfortunately, further fragmentation of z· ions does not have a general character and the corresponding *w* ions are often not present in the spectra. McLuckey et al.[2] and Balaram et al.[3] carried out MS3 experiments when ETD formation of z ions was followed by CID. The efficiency of the targeted fragmentation increased, however there were too many fragment ions in the spectra while the process of radical site migration[4] along the backbone often resulted in uncertainties when both Leu and Ile residues were present in the original z-ion. Here we report new results on reliable and straightforward discrimination of Leu/Ile in natural peptides using the power of the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer (ETD/HCD).

Methods

Sample Preparation

Six natural peptides were isolated from the skin secretion of Russian frog *Rana ridibunda.* Their length was in the range between 15 and 37 amino acids in the backbone, the number of basic residues (lysine and arginine) – between 3 and 6, and the number of targeted isomeric (leucine and isoleucine) residues – between 1 and 7 (24 cases altogether).

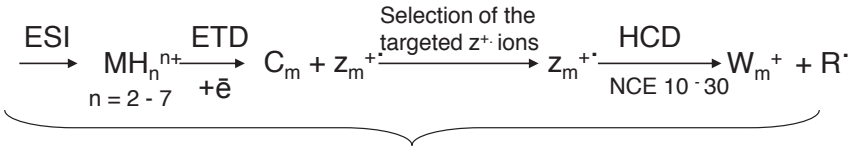
Natural peptides used in the experiments:

Name	Sequence	Mass, Da	Comment
Ranatuerin 2R	AVNIPFKVKFRCKAAFC	1939.0325	Confirmed structure
Brevinin 1Ra	VIPFVASVAAEMMQHVYCA ASRRC	2636.2485	Confirmed structure
Brevinin 1E	FLPLLAGLAANFLPKIFCKIT RKC	2674.5220	Confirmed structure
Brevinin 2Ec	GILLDKLKNFAKTAGKGVLQ SLLNTASCKLSC	3516.9161	Confirmed structure
Ranatuerin2a	KXXXNPKFRCKAAFC	1748.9583	Not confirmed structure
Esculentin 2R	GXXSXVKGVAKXAGKTFAK EGGKFGXEFXAKVTNQC	3823.0893	Not confirmed structure

Mass Spectrometry

Experiments were carried out with an Orbitrap Fusion mass spectrometer using the standard Thermo Scientific™ Tune™ 1.0 instrument software and a Thermo Scientific™ EASY-Max NG™ ion source in infusion mode. Selection of different peptide ions for MS2(ETD) fragmentation as well as *z*-ions selection for MS3(HCD) fragmentation was performed manually in Tune page.

SCHEME 2. The sequence of mass spectrometry stages

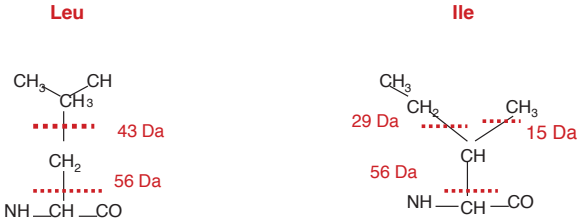


Sequencing in Orbitrap Fusion MS

The principal features of the experiment
1. Precise extraction of the targeted $z^{\cdot}$ ions (with N-terminal Leu/Ile) is quite easy due to the rich choice of the required $z^{\cdot}$ ions with the charges from +2 to +7.
2. HCD collision energy is varied in the range 10 - 40 NCE.

Outcome: Extremely selective fragmentation of the targeted $z^{\cdot}$ ions is achieved. MS3 spectra as a rule contain exclusively precursor $z^{\cdot}$ ions and *w* - product ions.

SCHEME 3. Possible MS3 losses from side chain of Leu/Ile in the method.

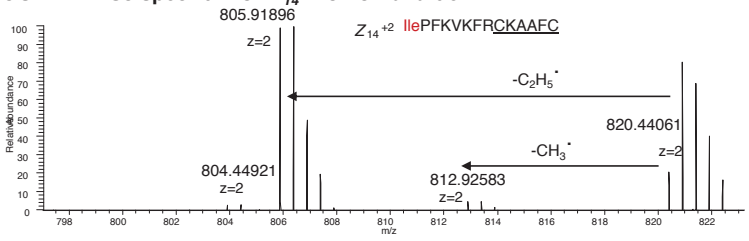


Results

Single Ile in $z^{\cdot}$ ions

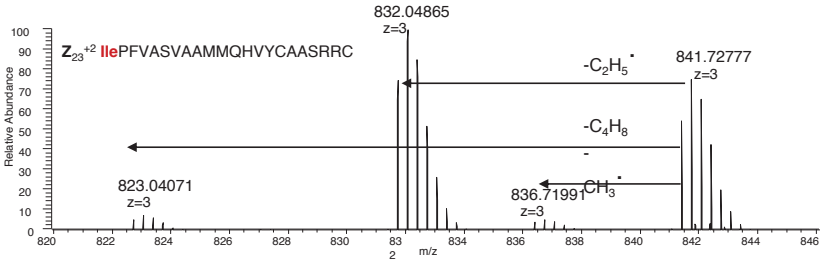
Figures 1-3 exemplify identification of Leu/Ile for solitary residues within a peptide.

FIGURE 1. MS3 spectrum of z_{14}^{+2} ion of ranatuerin 2R



The difference of 14.519 Da between the precursor and the main product ion corresponds to the loss of $C_2H_5^{\cdot}$ and identifies ^{4}Ile. There is no trace of an alternative for Leu loss of isopropyl radical (43.0546 Da). Formation of w ions in the case of Ile involves the loss of the ethyl group (Scheme 3). However alternative elimination of the methyl group is also possible. The intensity of this ion is usually low according to the maximal alkyl loss rule. Nevertheless, due to very pronounced fragmentation in ETD/HCD mode the loss of methyl becomes visible as well.

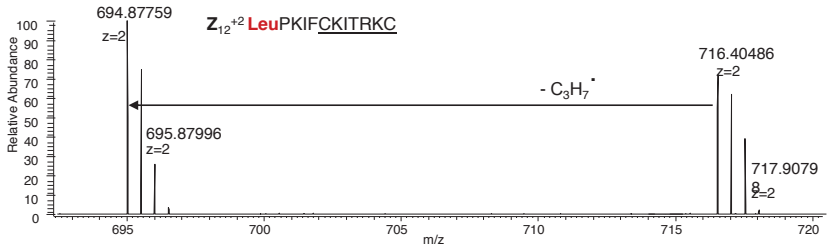
FIGURE 2. MS3 spectrum of z_{23}^{+3} ion of brevinin 1Ra



$CH_3^{\cdot}$, $C_2H_5^{\cdot}$ and C_4H_8 losses from the triply charged z_{23} ion generated by ETD from $[M+4H]^{4+}$ of brevinin 1Ra confirm the presence of ^{2}Ile. In all other cases with one Leu/Ile residue in the backbone the results were similar.

The presence of another Ile/Leu residue in the remote position from the N-terminus of z· ion

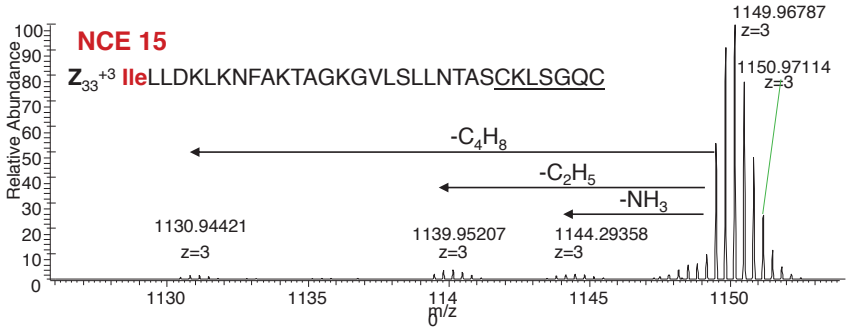
FIGURE 3. MS3 spectrum of z_{12}^{+2} ion of brevinin 1E



The difference 21.5273 Da between the precursor and the main product ion corresponds to the loss of C_3H_7· and identifies reliably ^{13}Leu. This fact demonstrates the extraordinary selectivity of the proposed method. There are no even traces of the ethyl group loss, due to the presence of ^{16}Ile residue. Radical migration is less pronounced in ETD/HCD mode: no other product ions are formed.

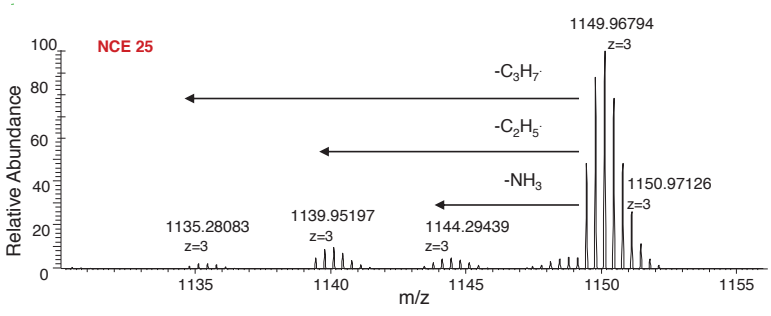
Neighboring ^{2}Ile-^{3}Leu-^{4}Leu in brevinin 2Ec

FIGURE 4. MS3 spectrum of z_{33}^{+3} ion of brevinin 2Ec at NCE15



Due to possible radical site migration the losses of both Et and iPr radicals were expected in ETD/HCD experiment with z_{33}^{+3} ion. The application of the minimal collision energy (NCE 10) resulted in the selective fragmentation of the side chain of N-terminal ^{2}Ile. There were no even traces of the alternative iPr losses at NCE 15 (Figure 4).

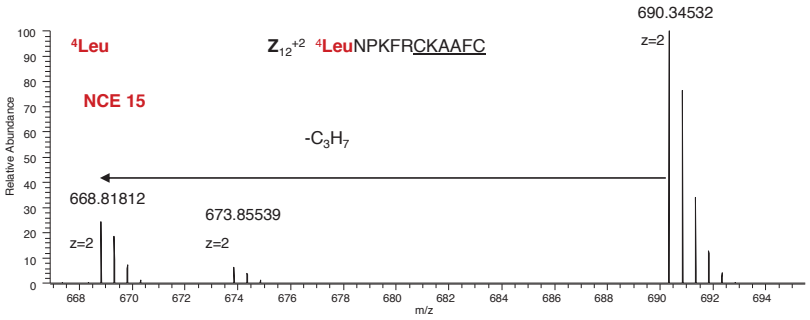
FIGURE 5. MS3 spectrum of z_{33}^{+3} ion of brevinin 2Ec at NCE 25



At NCE 25 (Figure 5) the signal of *m/z* 1135 representing the loss of C_3H_7· arises due to the neighboring ^{3}Leu and ^{4}Leu. Therefore, radical site migration is also possible in the proposed method. However, the process does not interfere with the correct identification of Leu/Ile pairs even in neighboring positions.

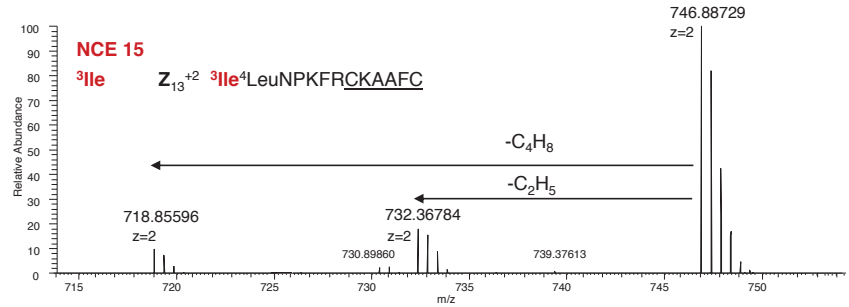
Discrimination of ^{2}Xle, ^{3}Xle, ^{4}Xle in Ranatuerin 2Ra

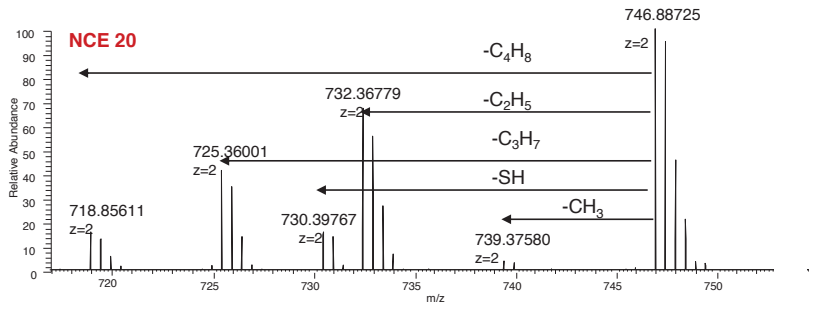
FIGURE 6. MS3 confirmation of ^{4}Leu in Ranatuerin 2Ra



^{4}Leu may be unequivocally identified using MS3 spectrum of z_{12}^{+2} ion.

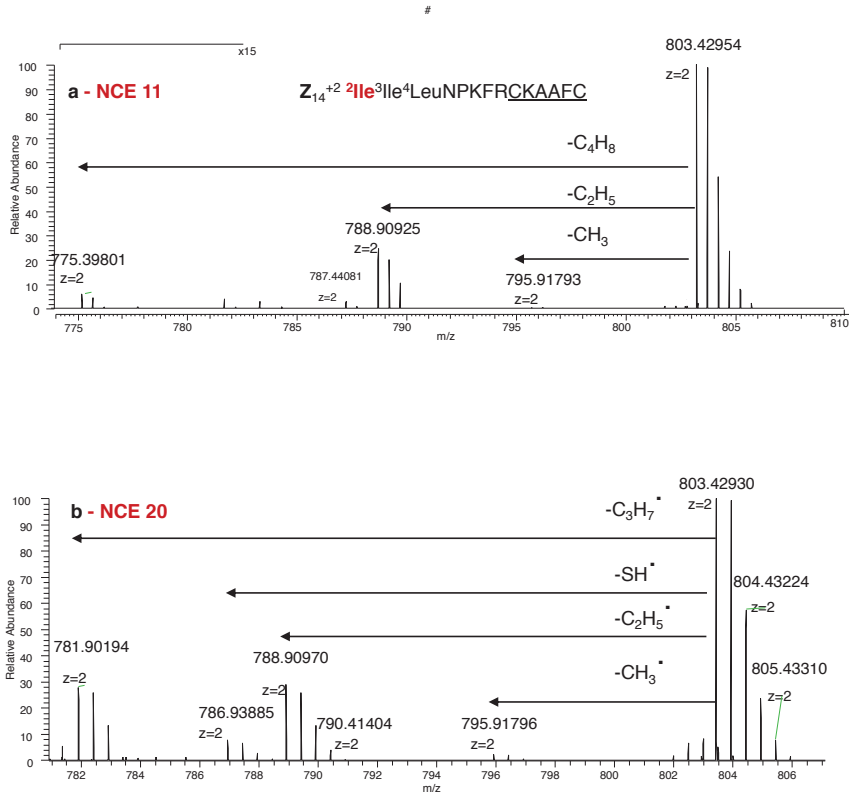
FIGURE 7. MS3 confirmation of ^{3}Ile in Ranatuerin 2Ra





^{3}Ile was easily and reliably identified using MS3 spectrum of z_{13}^{+2}, with ion registered at NCE 15. All the observed losses are due to Ile side chain (Figure 7). With the increase of NCE to 20 radical site migration becomes visible. MS3 spectrum of z_{13}^{+2} ion (Figure 8) represents the loss of $C_3H_7^{\cdot}$ from the neighboring ^{4}Leu.

FIGURE 8. Identificaion of ^{2}Ile in Ranatuerin 2Ra at NCE: a - 11; b- 20



The loss of Et is obvious in MS3 spectrum of z_{14}^{+2} at NCE 10 (Figure 8a). With the increase of NCE to 20 radical site migration becomes quite pronounced. MS3 spectrum (Figure 8b) besides Et elimination represents an alternative loss of C_3H_7· due to ^{4}Leu. Nevertheless MS3 spectra of the targeted z ions at small NCE allowed for reliable identification of all three linked isomeric residues in Ranatuerin 2Ra: KIILNPKFRCKAAFC.

Conclusion

- ETD/HCD approach with the Orbitrap Fusion MS is the key to the successful identification of Leu/Ile in non-tryptic natural peptides with chain length up to 37 residues.
- The resulting MS3 spectra are very selective with 1-4 secondary fragment ions, with targeted *w* ions usually being the most abundant.
- The effects of radical site migration are significantly less pronounced than in the case of the ion trap techniques reported earlier.
- The proposed approach may be used in high throughput proteomics experiments and allows creating automated methods for the complete *de novo* sequencing exclusively by means of mass spectrometry.

References

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