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Discrimination of Leucine and Isoleucine in Peptides Sequencing with Orbitrap Fusion Mass Spectrometer

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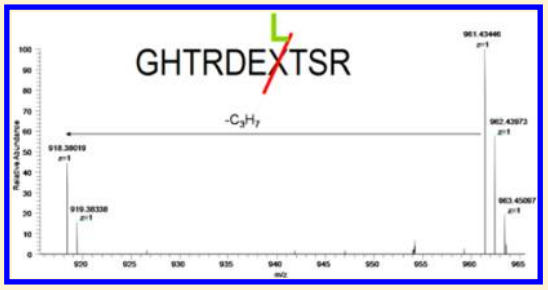
Supporting Information

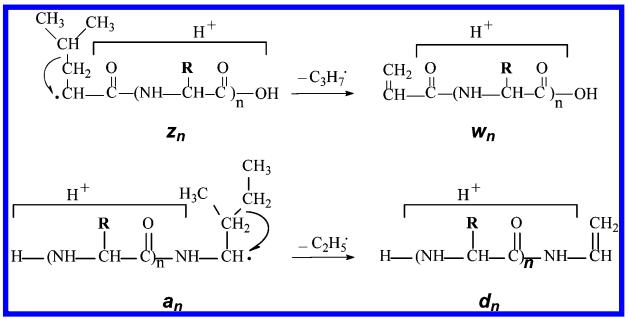
ABSTRACT: An efficient approach to easy and reliable differentiation between isomeric leucine and isoleucine in peptide sequencing utilizes multistage electron transfer dissociation and higher energy collision activated dissociation in the Orbitrap Fusion mass spectrometer. The MS^3 method involves production and isolation of primary odd-electron $z^{\bullet}$ ions, followed by radical site initiation of their fragmentation with formation of w-ions, characteristic of the isomeric amino acid residues. Six natural nontryptic peptides isolated from the secretion of frog *Rana ridibunda* were studied. Their lengths were in the range between 15 and 37 amino acids and the number of targeted isomeric (Leu/Ile) residues varied between 1 and 7. The experiments were successful in all 22 cases of Leu/Ile residues, leaving no doubts in identification. The method is extremely selective as the targeted w-ions appear to be the most intense in the spectra. The proposed approach may be incorporated into shotgun proteomics algorithms and allows for the development of an exclusively mass spectrometric method for automated complete *de novo* sequencing of various peptides and proteins.



For about 50 years, Edman degradation was considered to be the most reliable method of peptide sequencing.[1] However, by 1990, mass spectrometry established itself as a preferential analytical tool for this purpose being more sensitive, able to work with mixtures and longer and modified peptides. Besides, mass spectrometry is extremely faster and even cheaper. Despite the clear and substantial advantages offered by mass spectrometry, the use of Edman degradation persists in part because of its ability to discriminate isomeric Leu and Ile residues. As the masses of these residues are the same, they generally cannot be identified using an MS/MS approach with collision-induced dissociation (CID) of the protonated molecules. Quite often, the sequences of peptides developed *de novo* by mass spectrometry are marked with L/I or X signs, meaning that the results could not be used to distinguish between these isomeric residues.[2]

To address this deficiency, an additional step may be applied in tandem mass spectrometry methods. The most straightforward method deals with observation of ions belonging to the d or w series (Scheme 1), respectively, formed from odd-electron $a^{\bullet}$ and $z^{\bullet}$ ions.[3] The masses of the d and w ions for Leu (*i*Pr loss) and Ile (Et loss) are different. Therefore, identification of Ile vs Leu may be carried out quite reliably. Although $z^{\bullet}$ ions are rarely formed under CID conditions, they appear occasionally due to the statistical possibility of the cleavages of the corresponding bonds of the peptide chain, making this CID-based approach successful to some extent.[3,4] However, the resulting spectra are very complex, while the targeted fragment ions peaks are of very low intensity or not observed at all.

Scheme 1. Formation of w and d Ions



Distinction of isomeric amino acids (Leu vs Ile) or their corresponding residues in dipeptides may be achieved by MS/MS using copper(II)-bound complexes[5,6] or preliminary derivatization.[7] The application of metastable atom-activated dissociation allows differentiation of Leu/Ile residues in longer peptides.[8] However, tandem mass spectra of the whole protonated molecules appear to be very complex, while the losses of characteristic moieties (29 vs 43 mass units) proceed from many precursors. If both Ile and Leu residues are present in the molecule, determination of their exact positions may not be possible.

Immonium ions may be used to differentiate Leu and Ile residues.[9] This procedure is successful in the case where the

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peptide sequence contains only a single amino acid of this type (I or L). To achieve the goal, the authors[9] proposed using thermolysin at the first stage of the analysis. This enzyme cleaves amide bonds at the N-termini of Leu and Ile. Armirotti[10] proposed an MS^4 approach, based on the selection of an immonium ion formed from b- or y-precursor with only one Leu/Ile residue. The approach showed good results for a number of tryptic peptides; however, the authors mentioned several limitations and did not recommend it for peptides heavier than 1600 Da.[10]

Utilization of electron capture dissociation (ECD)[11] or electron transfer dissociation (ETD)[12] results in predominant fragmentation of the protonated molecules of peptides with formation of c and odd-electron $z^\bullet$ ion series. The latter are less stable than even-electron cations.[13] To induce the target fragmentation with the loss of an alkyl radical, one should just add energy to the $z^\bullet$ ion. Zubarev et al.[14a] proposed so-called hot ECD (HECD) for this purpose. The method is based on the capture of a high energy electron (~11 eV) by protonated molecules of polypeptides. The forming $z^\bullet$ ions have a certain excess of energy and may fragment further, leading to the generation of w ions (Scheme 1). The method may be very helpful.[14b] Unfortunately, this reaction does not have a general character and the corresponding w ions are often not present in HECD spectra.

Fung and Chan[14c] studied the losses of side chains in model peptides RGGGXGGGR under ECD conditions. They demonstrated different losses from Leu and Ile residues of the primary $z^\bullet$ ions. However, since side-chain loss may be initiated by remote radical centers, there is an ambiguity dealing with unknown peptides and using w ions this way.

Two articles deal with application of MALDI for the L/I discrimination.[15a,b] The results are good for the peptides with a single targeted residue. However, for longer peptides with several L/I residues, the spectra are very complex and the required peaks may be absent. McLuckey et al.[16] used a quadrupole mass filter-ion trap mass spectrometer to generate odd-electron $z^\bullet$ ions from various synthetic peptides with ETD. Ion trap CID was applied to 44 $z_n^\bullet$ ions. If Leu or Ile residues were present in the $z^\bullet$ ion, the CID spectrum contained ion peaks due to the losses of Et or *i*Pr, respectively. The selection of $z^\bullet$ ions formed due to the cleavage of the N−C_α bond in Leu or Ile residues was not required. The targeted fragment w ions were also observed when Leu or Ile residues occupied rather remote positions from the original N−C_α bond cleavage. This effect was rationalized by radical site migration.[17] However, if both residues (Leu and Ile) are present in the selected $z^\bullet$ ion, correct assignment cannot be reliably inferred from the CID spectra. The authors[16] showed an example demonstrating that when Ile was present at the cleavage site with Leu adjacent to it, the loss of Et appeared to be more pronounced than the alternative loss of *i*Pr. Therefore, fragmentation without preliminary radical migration is preferred, but if both residues are present in $z^\bullet$-ions but not at the ETD cleavage site, the loss of *i*Pr is preferred, even if Ile is closer to the ETD cleavage site. The latter observation may be treated as a drawback, since mistakes in assignment become possible in *de novo* sequencing experiments. Gupta et al.[18] used MS^3 (ETD-CID) experiment with an HCT Ultra ETD II ion trap to discriminate Leu and Ile in longer peptides. The process of radical migration was important as quite abundant losses of characteristic radicals were observed when the targeted residues were situated 1−3 positions away from the original ETD cleavage.[18]

Frog peptides represent a unique class of natural compounds. The secretion of peptides, in addition to performing regulatory functions, has a protective function against microorganisms (antifungal, antimicrobial, antiviral activity) and/or predators (being venomous or inducing vomiting reflex). Furthermore, frog peptides appear to be very useful compounds for the development of new mass spectrometry approaches for peptide sequencing. Frog peptides generally consist of 4−46 aa residues, comprising both short peptides that may be used to study cyclization processes in the ion source,[19] and long peptides that may be treated as small proteins and used to develop methods for their top-down sequencing.[20] Frog peptides usually contain several basic amino acids (up to 8), being very different from the tryptic ones.[21] Another challenge for their *de novo* sequencing arises from the presence of several L/I residues and a disulfide bond at the C-terminus due to two cysteines in the chain.[21]

Here we report new results demonstrating reliable and easy discrimination of Leu and Ile in several rather long and "difficult" frog peptides using the power of the Orbitrap Fusion mass spectrometer. As discussed above, earlier studies of *de novo* sequencing of frog peptides relied on Edman degradation[19d,22] or HECD[20b] to discriminate these isomeric residues.

EXPERIMENTAL SECTION

Skin Secretion: HPLC Purification and Isolation. A male species of *Rana ridibunda* was caught in the Kolomna district of Moscow region (Russia) in summer 2013. Preparation of frog skin secretion was accomplished as described earlier.[23] Purification and separation of individual peptides from crude secretion were performed with HPLC while the fractions were collected and lyophilized as mentioned above.[24] Six individual peptides were isolated this way (Table S1 in the Supporting Information). Four of them had the sequence including I/L positions reliably proved earlier, while two others contained uncertain identifications.

Mass Spectrometry. Experiments were carried out with an Orbitrap Fusion mass spectrometer (Thermo Scientific, San Jose, CA) using the standard instrument software (Tune 1.0) and EASY-Max NG ion source (Thermo Scientific, San Jose, CA) in infusion mode. A description of the instrument and its operation may be found elsewhere.[25] Selection of different peptide ions for MS^2 (ETD) fragmentation as well as $z^\bullet$-ions selection for MS^3 (HCD) fragmentation was performed manually in Tune page.

RESULTS AND DISCUSSION

Mass spectrometric analysis involved the generation and selection of multiprotonated molecules of the isolated peptides, triggering their fragmentation using ETD to achieve N−C_α bonds cleavage and formation of odd-electron $z^\bullet$ ions, isolation of particular $z^\bullet$ ions with targeted residues at the N-terminus and inducing their fragmentation by higher-energy collisional dissociation (HCD). The MS^3 sequence of ETD-HCD events was implemented on a standard Orbitrap Fusion mass spectrometer without special modifications.

Since multiprotonated molecules readily arise in electrospray ionization (ESI) conditions, it is possible to use any charge state of these species for the first MS/MS stage (ETD). In the present study, charges from 2 to 7 were observed. Any $z^\bullet$ ion (irrespective of its charge) may be selected for the second stage. Ions with charges from 1 to 4 were used in the present study.

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Simple arithmetic estimates demonstrate that the target fragmentation with formation of certain w ions may be realized with about 20 combinations of molecular and $z^{\bullet}$ ions. Because of this fact, in all the experiments we strived to work precisely with the required $z^{\bullet}$ ion, obtained due to the cleavage of the N−C_{α} bond at the N-terminus of Leu/Ile.

Figure 1 represents the HCD spectrum of isolated z_{14}^{2+} ion generated by ETD from $[M + 4H]^{4+}$ of ranatuerin 2R. The

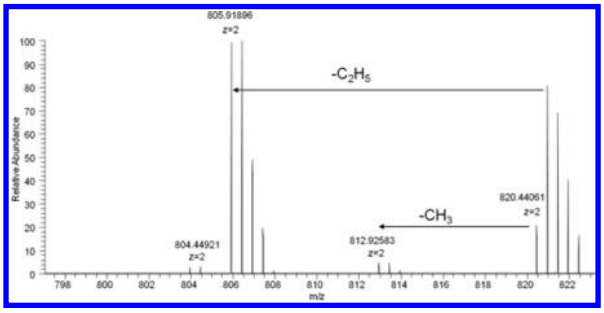

Figure 1. HCD spectrum of z_{14}^{2+} ion of ranatuerin 2R.

difference of 14.519 Thomson between the precursor and the main product ion corresponds to the loss of ethyl radical and reliably identifies ^{14}Ile. The spectrum shows no traces of alternative loss of *i*Pr characteristic for Leu. Two other fragment ions are due to the loss of methyl and SH radicals. It is worth especially mentioning the loss of CH_3 radical. Formation of w ions in the case of Ile involves the loss of Et (Scheme 1). However, alternative elimination of Me is also possible. The intensity of this ion is usually low according to the maximal alkyl loss rule.[13] Nevertheless, because of the very pronounced fragmentation in HCD mode depicted in Figure 1, the loss of methyl becomes visible as well.

Figure 2 represents the HCD spectrum of the isolated z_{23}^{3+} ion generated by ETD from $[M + 4H]^{4+}$ of brevinin 1Ra. The

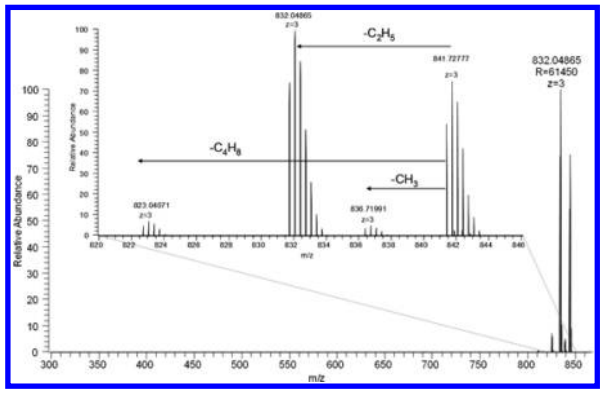

Figure 2. HCD spectrum of z_{23}^{3+} ion of brevinin 1Ra.

complete isotopic clusters of both precursor and product ions are present. The loss of ethyl radical identifies reliably ^{2}Ile. Two other product ions are due to the losses of methyl radical and C_4H_8 molecule, confirming the presence of ^{2}Ile. Another important issue is the absence of any other peaks in the full mass scale spectrum (Figure 2). The same results were obtained in all the experiments discussed below. This result demonstrates that the applied version of ETD-HCD mode provides very clean spectra answering exclusively the question concerning the identity of isomeric residues.

Brevinin 1E contains seven isomeric residues in the chain. However, ^{20}Ile resides inside the S−S loop and is not visible when dealing with spectra of an intact peptide. The other six Leu/Ile residues were reliably identified using the ETD-HCD approach. Again, all the spectra were clean, while the main product ions belong to targeted w ions. Figure 3 represents the

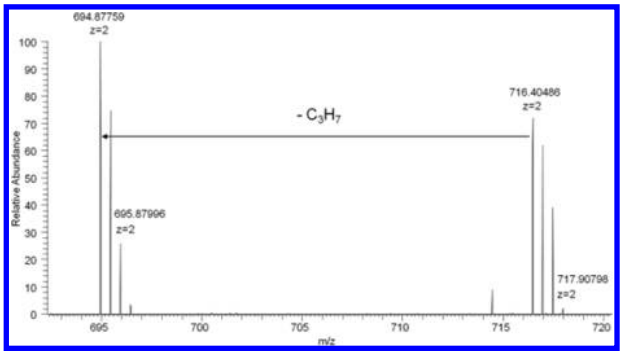

Figure 3. HCD spectrum of z_{12}^{2+} ion of brevinin 1E.

HCD spectrum of isolated z_{12}^{2+} ion generated by ETD from $[M + 4H]^{4+}$ of brevinin 1E. The loss of *i*Pr identifies reliably ^{13}Leu. This fact demonstrates once again the extraordinary selectivity of the ETD-HCD. The intensive radical migration was mentioned earlier[16−18] in similar experiments with ion traps; however, there are no detectable traces of Et loss, due to radical migration to the neighboring ^{16}Ile residue. Therefore, these processes are less pronounced in ETD- HCD mode.

A more difficult case involves confirmation of ^{5}Leu. Figure 4 represents isolation of the z_{20}^{2+} ion generated by ETD from [M

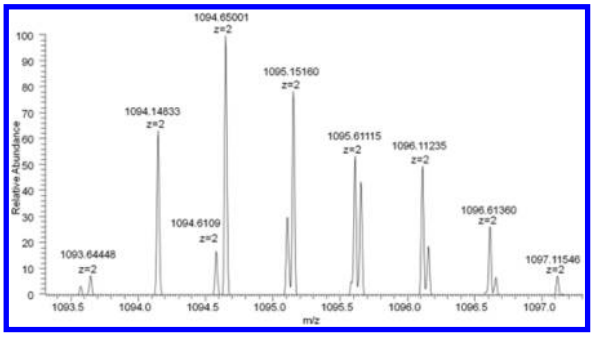

Figure 4. Isolated cluster of the z_{20}^{2+} ion of brevinin 1E with interference of a cluster of another ion.

+ 4H]$^{4+}$. There are two nonseparable clusters, while the targeted ion with a monoisotopic mass of 1094.6109 is of lower abundance. Figure 5 represents the HCD spectrum of the z_{20}^{2+} ion. In spite of interference with the major impurity, complete isotopic cluster of product ions are observable. The loss of C_3H_7 radical identifies ^{5}Leu in brevinin 1E. Another fragment ion is due to the loss of SH radical from the interfering precursor ion with monoisotopic mass 1094.14835. The latter ion does not interfere with identification of isomeric residues. The other L/I cases in brevinin 1E spectra are obvious (Figures.S1−S4 in the Supporting Information). Therefore, the sequence of brevinin 1E FLPLLAGLAANFLPKIFCKITRKC was reliably confirmed by the ETD-HCD method.

Brevinin 2Ec contains eight isomeric residues in the chain including ^{30}Leu inside the S−S loop, which is not visible when dealing with spectra of an intact peptide. The other seven Leu/

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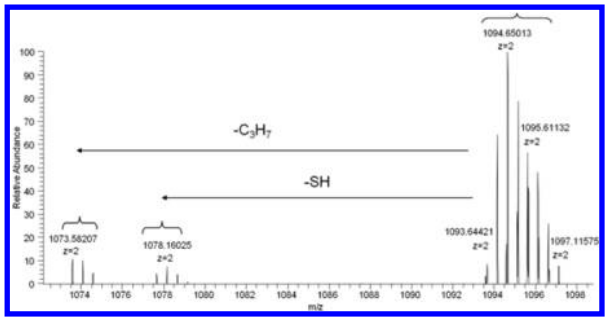

Figure 5. HCD spectrum of the z_{20}^{2+} ion of brevinin 1E.

Ile residues were reliably confirmed by selective and clean ETD- HCD spectra. The main product ions belong to the targeted w ions. All the spectra are available in the Supporting Information (Figures.S5−S11).

One particular illustrative example is confirmation of ^{2}Ile with HCD of z_{33}. The problem comes from the presence of neighboring ^{3}Leu and ^{4}Leu. Because of radical migration,[17b] the loss of *i*Pr was expected to accompany the loss of Et characteristic for ^{2}Ile. A scarcely visible ion due to the loss of Et appears at normalized collision energy (NCE) 10. Figures 6

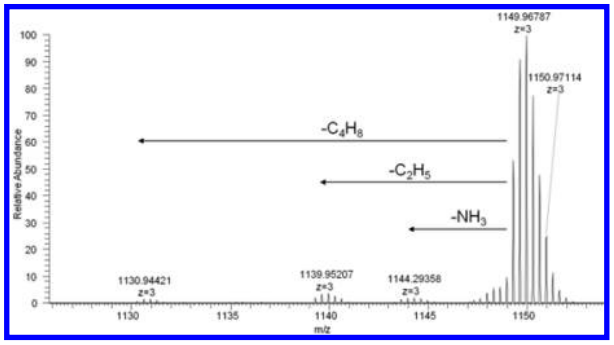

Figure 6. HCD spectrum of z_{33}^{3+} ion of brevinin 2Ec at NCE15.

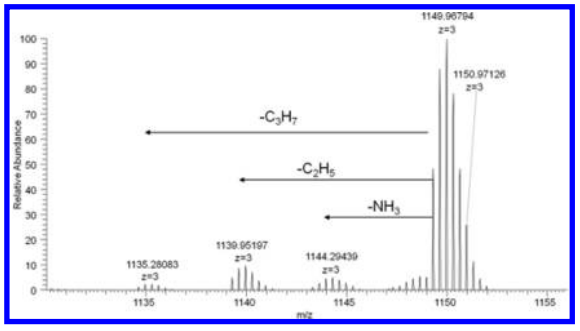

Figure 7. HCD spectrum of z_{33}^{3+} of brevinin 2Ec at NCE 25.

and 7 demonstrate the spectral changes observed with an increase of NCE from 15 to 25. At NCE 15 (Figure 6), the ions corresponding to the losses of NH_3 and C_4H_8 molecules appear, in addition to the w_{33} ion (*m*/*z* 1139). At NCE 25 (Figure 7), the signal of *m*/*z* 1135 representing the loss of *i*Pr due to neighboring ^{3}Leu and ^{4}Leu arises. Therefore, radical site migration[17b] is also possible in the utilized version of ETD-HCD. However, the process does not interfere with the correct identification of Leu/Ile pairs even in neighboring positions by acquiring spectra at lower energies. The confirmed sequence of brevinin 2Ec is GILLDKLKNFAKTAGKGVLQSL-LNTASCKLSGQC.

Since the results with the peptides with known sequence were encouraging, we carried out the same ETD-HCD experiments with two other peptides (ranatuerin 2Ra and esculentin 2R) with uncertain Leu/Ile residues.

Only three Leu/Ile residues are present in 15-member ranatuerin 2Ra. However, the difficulty involves the fact that all three of them are linked to one another. Figure 8 represents the

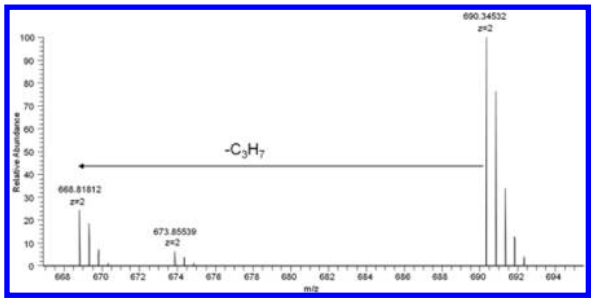

Figure 8. HCD spectrum of z_{12}^{2+} ion of ranatuerin 2Ra.

HCD spectrum of the doubly charged z_{12} ion generated by ETD from $[M + 4H]^{4+}$ of ranatuerin 2Ra. Complete isotopic clusters of the precursor and product ions are present. The difference 21.527 Da between the precursor and the major product ion corresponds to the loss of *i*Pr and identifies ^{4}Leu. Another fragment ion is attributable to the loss of the SH radical.

At NCE 15, the HCD spectrum of z_{13}^{2+} ion (Figure 9) demonstrates the major peak of *m*/*z* 732 with complete

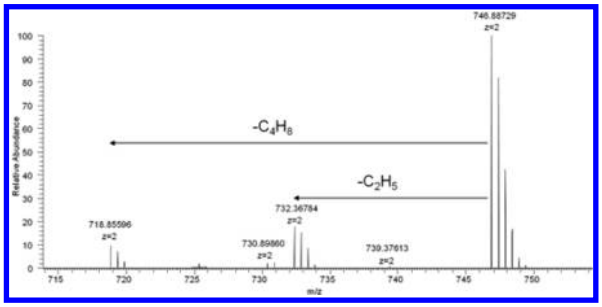

Figure 9. HCD spectrum of z_{13}^{2+} of ranatuerin 2Ra at NCE 15.

isotopic cluster corresponding to the loss of C_2H_5 radical, characteristic for Ile. Another isotopically resolved peak illustrates the loss of the C_4H_8 molecule (*m*/*z* 718). However, at higher NCE (Figure 10), three additional clusters become visible. The smaller one (*m*/*z* 739) represents the loss of Me,

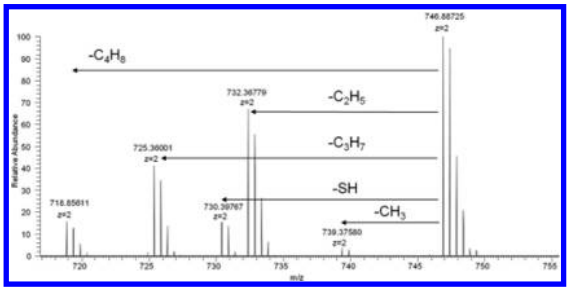

Figure 10. HCD spectrum of ranatuerin 2Ra z_{13}^{2+} ion at NCE 20.

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which confirms the presence of ^{3}Ile (see above). The ion of m/z 730 is attributable to the loss of the SH radical. The rather intense peak of m/z 725 is formed as a result of *i*Pr loss. Therefore, at higher NCE, radical site migration becomes more pronounced and (in addition to the loss of C_2H_5 from ^{3}Ile) the loss of C_3H_7 from ^{4}Leu takes place. It is noted that at lower collision energy, there are no problems in identification of the residue as ^{3}Ile.

Identification of a residue in position 2 was challenging. At NCE 10, only traces of Et loss are visible. At NCE 13 (Figure 11), an isotopically resolved cluster of the targeted ion appears in the spectrum accompanied by some other very small singlets. This result allows positing the presence of ^{2}Ile.

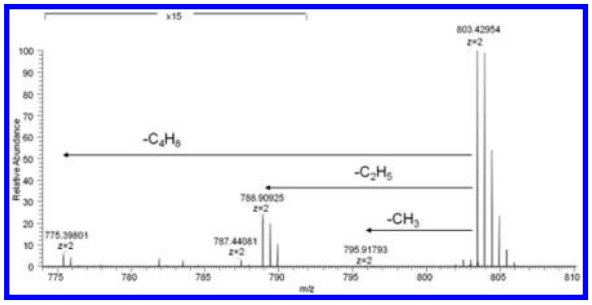

Figure 11. HCD spectrum of ranatuerin 2Ra z_{14}^{2+} ion at NCE 13.

Nevertheless, at higher energies, the alternative peak of m/z 781 appears and grows rapidly (Figure 12). Therefore, radical

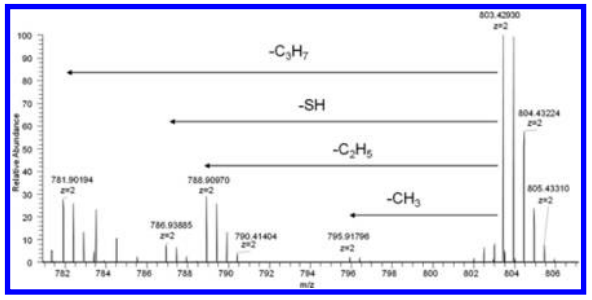

Figure 12. HCD spectrum of z_{14}^{2+} of ranatuerin 2Ra at NCE 20.

site migration[17b] is quite pronounced. It was the most difficult example of Leu/Ile differentiation in the studied samples. In any case, ETD-HCD experiments enabled the following sequence for ranatuerin 2R to be proposed, KIILNPKFRCK-AAFC. Again, the necessity to record spectra at lower energy should be emphasized.

Esculentin 2R contains 37 amino acid residues including six Leu/Ile. The spectra of z_9, z_{12}, z_{26}, z_{33}, and z_{35} ions (Figures.S12–S16 in the Supporting Information) showed only one type of fragmentation (either C_2H_5 or C_3H_7 losses) and allowed reliably identifying residues ^{29}Leu, ^{26}Leu, ^{12}Leu, ^{5}Ile, and ^{3}Leu. Taking into account the possibility of radical site migration, we took particular care recording the z_{36} ion HCD spectrum. The z_{36}^{4+} ion formed from $[M + 5H]^{5+}$ was isolated (Figure.S17 in the Supporting Information). Although two other ions were interfering, their abundance was lower than that of the targeted ion with a monoisotopic mass 938.518 08. Its HCD spectrum contains only one intense fragment ion cluster due to the loss of Et, identifying ^{2}Ile (Figure 13). The only other fragment ion is attributable to the loss of Me thereby confirming that conclusion.[13] Radical site migration does not

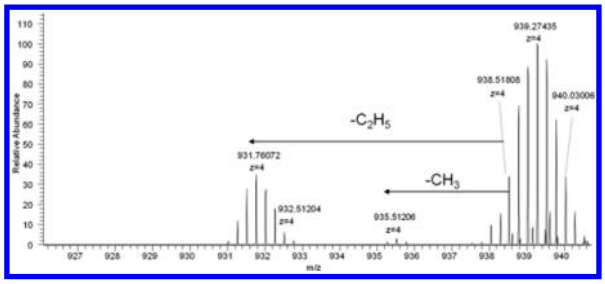

Figure 13. HCD spectrum of z_{36}^{4+} ion of esculentin 2R.

take place in this case. The esculentin 2R sequence is GILSIVKGVAKLAGKTFAKEGGKFGLEFLACKVTNQC.

To check the possibility of using radical site migration, 6 z-ions of brevinin-2Ec with L/I residues in the second or 3d position from N-terminus were subjected to further fragmentation. Evidence of radical site migration was observed but not in every case. Usually the process produces peaks of low intensity. Two figures (Figures.S18,19 in the Supporting Information) demonstrate the cases of the presence and the absence of radical site migration. Therefore, one may conclude that radical site migration is not pronounced in ETD-HCD, does not interfere with L/I identification, and may help obtaining results in certain cases.

CONCLUSIONS

Identification of isomeric leucine and isoleucine residues during mass spectrometric sequencing may be easily, selectively, and reliably carried out using an MS^3 (ETD-HCD) approach on the Orbitrap Fusion mass spectrometer. The proposed method demonstrated uniformly successful results with 6 natural nontryptic peptides with chain length up to 37 residues. The resulting 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. Since the targeted characteristic w ions may be calculated *a priori*, their search may be conducted automatically with the corresponding software. Taking into account the reliability and selectivity of the proposed approach, it may be used in high throughput proteomics experiments and allows creating automated methods for the complete *de novo* sequencing of new peptides exclusively by means of mass spectrometry.

ASSOCIATED CONTENT

Supporting Information

All the spectra mentioned in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) (a) Edman, P. *Arch. Biochem.* **1949**, *22*, 475−476. (b) Edman, P.; Begg, G. *Eur. J. Biochem.* **1967**, *1*, 80−91.
(2) http://www.uniprot.org/uniprot.
(3) Johnston, R. S.; Martin, S. A.; Biemann, K.; Stults, J. T.; Watson, J. T. *Anal. Chem.* **1987**, *59*, 2621−2625.
(4) Martin, S. A.; Biemann, K. *Mass Spectrom. Rev.* **1987**, *6*, 1−78.
(5) Seymour, J. L.; Turecek, F. *J. Mass Spectrom.* **2000**, *35*, 566−571.
(6) Tao, W. A.; Wu, L. M.; Cooks, R. G. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 490.
(7) (a) Borisov, R. S.; Rychkov, A. A.; Vas'kovskii, B. V.; Zaikin, V. G. *Mass Spectrom. (in Russian)* **2004**, *1*, 196−206. (b) Borisov, R. S.; Zaikin, V. G.; Vas'kovskii, B. V.; Sklyarov, L.Yu. *Russ. Chem. Bull.* **2006**, *55*, 2285−2290.
(8) Cook, S. L.; Collin, O. L.; Jackson, G. P. *J. Mass Spectrom.* **2009**, *44*, 1211−1223.
(9) Nakamura, T.; Nagaki, H.; Ohki, Y.; Kinoshita, T. *Anal. Chem.* **1990**, *62*, 311−313.
(10) Armirotti, A.; Millo, E.; Damonte, G. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 57−63.
(11) Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1998**, *120*, 3265−3266.
(12) Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (26), 9528−9533.
(13) Turecek, F.; McLafferty, F. W. *Interpretation of Mass Spectra*, 4th ed.; University Science Books, Mill Valley, CA, 1993; p290.
(14) (a) Kjeldsen, F.; Haselmann, K. F.; Sørensen, E. S.; Zubarev, R. A. *Anal. Chem.* **2003**, *75*, 1267−1274. (b) Williams, J. P.; Creese, A. J.; Roper, D. R.; Green, B. N.; Cooper, H. J. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1707−1713. (c) Fung, Y. M. E.; Chan, T.-W. D. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1523−1535.
(15) (a) Nachman, R. J.; Russell, W. K.; Coast, G. M.; Russell, D. H.; Predel, R. *Peptides* **2005**, *26*, 2151−2156. (b) Soltwisch, J.; Dreisewerd, K. *Anal. Chem.* **2010**, *82*, 5628−5638.
(16) Han, H.; Xia, Y.; McLuckey, S. A. *J. Proteome Res.* **2007**, *6*, 3062−3069.
(17) (a) Leymarie, N.; Costello, C. E.; O'Connor, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 8949−8958. (b) O'Connor, P. B.; Lin, C.; Cournoyer, J. J.; Pittman, J. L.; Belyayev, M.; Budnik, B. A. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 576−585.
(18) Gupta, K.; Kumar, M.; Chandrashekara, K.; Krishnan, K. S.; Balaram, P. *J. Proteome Res.* **2012**, *11*, 515−522.
(19) (a) Vachet, R. W.; Bishop, B. M.; Erickson, B. W.; Glish, G. L. *J. Am. Chem. Soc.* **1997**, *119*, 5481−5488. (b) Jia, C.; Qi, W.; He, Z. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 663−678. (c) Harrison, A. G. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 2248−2253. (d) Samgina, T. Y.; Kovalev, S. V.; Gorshkov, V. A.; Artemenko, K. A.; Poljakov, N. B.; Lebedev, A. T. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 104−111.
(20) (a) Liu, J.; Jiang, J.; Wu, Z.; Xie, F. *J. Proteomics* **2012**, *75*, 5807−5821. (b) Samgina, T. Y.; Vorontsov, Y. A.; Gorshkov, V. A.; Artemenko, K. A.; Zubarev, R. A.; Lebedev, A. T. *J. Am. Soc. Mass Spectrom.* **2013**, *24*, 1037−1044.
(21) Pukala, T. L.; Bowie, J. H.; Maselli, V. M.; Musgrave, I. F.; Tyler, M. J. *Nat. Prod. Rep.* **2006**, *23*, 368−393.
(22) (a) Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Ogourtsov, S. V.; Zubarev, R. A.; Lebedev, A. T. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 3517−3525. (b) Samgina, T. Y.; Artemenko, K. A.; Gorshkov, V. A.; Ogourtsov, S. V.; Zubarev, R. A.; Lebedev, A. T. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1241−1248.
(23) Tyler, M.; Stone, D.; Bowie, J. *J. Pharmacol. Toxicol. Methods* **1992**, *28*, 199−200.
(24) Samgina, T. Y.; Vorontsov, E. A.; Gorshkov, V. A.; Hakalehto, E.; Hanninen, O.; Zubarev, R. A.; Lebedev, A. T. *J. Proteome Res.* **2012**, *11*, 6213−6222.
(25) Senko, M. W.; Remes, P. M.; Canterbury, J. D.; Mathur, R.; Song, Q.; Eliuk, S. M.; Mullen, C.; Earley, L.; Hardman, M.; Blethrow, J. D.; Bui, H.; Specht, A.; Lange, O.; Denisov, E.; Makarov, A.; Horning, S.; Zabrouskov, V. *Anal. Chem.* **2013**, *85*, 11710−11714.