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Identification of aspartic and isoaspartic acid residues in amyloid β peptides, including A β 1-42, using electron-ion-reactions

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Abstract

Amyloid β peptides are the major components of the vascular and plaque amyloid filaments in individuals with Alzheimer's disease (AD). Although it is still unclear what initiates the disease, isomerization of aspartic acid residues in A β peptides is directly related to the pathology of AD. The detection of isomerization products is analytically challenging due to their similar chemical properties and identical molecular mass. Different methods have been applied to differentiate and quantify the isomers, including immunology, chromatography and mass spectrometry. Typically, those methods require comparative analysis with the standard peptides and involve many sample preparation steps. To understand the role of A β isomerization in AD progression, a fast, simple, accurate, and reproducible method is necessary. In this work, Electron Capture Dissociation (ECD) Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR MS) was applied to detect isomerization in A β peptides. ECD generated diagnostic fragment ions for the two isomers of A β 17-28: $[M+2H-60]^+$ and z_6^+-44 when aspartic acid and z_6^+-57 when isoaspartic acid were present. Additionally, the z_n^+-57 diagnostic ion was also observed in the Electron Ionization Dissociation (EID) spectra of the modified A β 17-28 fragment. ECD was further applied towards A β 1-40 and A1- β 42. The diagnostic ion c_6^+-57 was observed in the ECD spectra of the A1- β 42 peptide demonstrating isomerization at residue 7. In conclusion, both ECD and EID can clearly determine the presence and the position of isoaspartic acid residues in Amyloid β peptides. The next step, therefore, is to apply this method to analyze samples of Alzheimer's patients or healthy individuals in order to generate a better understanding of the disease.

Introduction

Amyloid beta (A β) peptides are the major components of the vascular and plaque amyloid filaments in individuals with Alzheimer's disease (AD). Various forms of A β are proteolytically cleaved from the A β precursor protein, with A β 1-40 and A β 1-42 being the most abundant forms found in Amyloid deposits.¹ Ever since A β was first purified and characterized, it has been strongly associated with the pathology of AD,^{2, 3} although it remains unclear what initiates the disease. According to the most widely accepted hypothesis, cerebral A β accumulation is the primary cause in AD. The rest of the disease process stems from imbalance between A β production and clearance.⁴ Many attempts have been made to measure the concentration of A β peptides in biological fluids, but it is difficult to correlate A β levels with disease stage and, thus, to use it as an AD biomarker.⁵ Further

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research and development of analytical methods is necessary for early AD diagnosis, disease progression, monitoring, and better understanding of the disease.

The current research has focused on A β isomerization. Isomerization of aspartic acid is one of the most common post translational modifications (PTMs) in all proteins that accumulate with age in long-lived proteins, especially in tooth, bone, cartilage, lens, and brain tissues.⁶ The isomerization product is isoaspartic acid (isoAsp). It can also be formed from asparagine deamidation (scheme 1). Both reactions proceed via an entropy driven⁷ formation of the five-membered succinimide ring intermediate followed by a rapid hydrolysis. As a result aspartic and isoaspartic acid residues are formed in a ratio of 1:3.^{8, 9} Moreover, at pH ~7.4, IsoAsp formation is preferential due to the higher acidity of isoAsp side chain residue.⁷ Under physiological conditions, both Asp isomerization and Asn deamidation are spontaneous nonenzymatic reactions.^{6–11} The reaction rates depend mainly on the nature of the adjacent residues, the higher order structure of the protein, and the molecular environment.^{6, 8, 12} Formation of isoAsp is speculated to change protein structure as it introduces an additional methylene group into the polypeptide backbone. This can change protein function and activity, or trigger aggregation.^{6, 13, 14} In addition, proteins containing isoAsp may not fully degrade as isoaspartate residue hinders proteolytic degradation.¹⁵ Nonetheless, harmful effects of isomerization can be partially repaired by the intracellular enzyme, called protein isoaspartyl methyltransferase (PIMT), which selectively converts isoAsp residues back to the succinimide intermediate.^{6, 10}

Isomerization of aspartic acid is directly related to the pathology of Alzheimer's disease. A β peptides have 3 aspartic acids in the sequence at the 1st, 7th, and 23^d residues and formation of isoaspartate is enhanced in A β peptides in AD. Roher *et al.* found that Asp1 and Asp7 were isomerized in the cerebral plaque samples of Alzheimer patients.¹³ Recently isoAsp7 and isoAsp23 were found in the core of senile plaques and Amyloid-bearing vessels as was shown with anti-isoasp7 and anti-isoasp23 antibodies.¹⁰ Moreover, the Iowa (Asn23)¹⁶ and Japanese-Tottori mutations (Asn7)¹⁷ in familial AD have a potential to accelerate formation of isoAsp, presumably due to asparagine deamidation. Accordingly, isoAsp23 containing A β peptides were preferentially detected in vascular deposits in Iowa cerebral Amyloid angiopathy brain.¹⁸ It was further suggested that spontaneous isomerization at position 23 induces the conformational change to form a β -turn of the polypeptide chain. This, in turn, plays a pathogenic role in the deposition of A β peptides in sporadic AD.¹⁰ In vitro experiments showed increased fibrillogenesis and enhanced neurotoxicity of isoAsp23 containing A β peptides,^{10, 19} however other results suggested that A β aggregative ability and neurotoxicity were not enhanced by this modification.²⁰ Similar studies of A β with isoAsp7 revealed that it is primarily deposited in the core of senile plaques. IsoAsp7 accumulation was associated with the age of the plaque as the number of isoAsp positive plaques increased in parallel with the disease severity.²¹

There have been many studies of isomerization events in A β peptides, but its role in AD pathogenesis is still unclear. Formation of isoAsp in A β might be a reason for its impaired degradation leading to accumulation. This aggregation could be a way for biological systems to reduce the toxicity of the non-degradable A β peptides. Further research is needed to better understand the role of isoAsp formation.

The detection of isomerization products is analytically challenging due to their similar chemical properties and identical molecular mass. Nevertheless, various methods have been applied to differentiate and quantify the isomers. Those include immunological methods based on detection by specific isoAsp antibodies,^{10, 18, 21} as well as liquid chromatography,^{11, 13} PIMT enzyme utilizing assays,^{11, 13} and tandem mass spectrometry (MS) analysis.^{22–24} New methods addressing isoAsp detection are quickly emerging in the

field to better understand the harmful effects isoAsp accumulation can generate in biological systems and pharmaceutical applications. These new methods often include already known methods, such as high-performance liquid chromatography (HPLC) separation followed by MS analysis.^{25, 26} In addition, immunochemistry analysis, capillary electrophoresis, or HPLC coupled to MS, or tandem MS alone, can be applied to analyze *l*-Asp, *l*-isoAsp, *d*-Asp, and *d*-isoAsp isomerized/racemized peptides.^{27–29} HPLC upgraded to ultra performance liquid chromatography (UPLC) system could further separate isoAsp and Asp located directly at the *N*-terminus.³⁰

Edman degradation is a chemical cleavage mechanism useful for IsoAsp identification because it does not cleave the isopeptide bond. Similarly, enzymatic digestion with the endoprotease Asp-N does not cleave *N*-terminal to the isoAsp acid. This was utilized in combination with ¹⁵N isotope labeling and MS for the detection of IsoAsp.³¹ The ¹⁸O labeling coupled to MS can also be used for detection of Asn deamidation and Asp isomerization;^{32, 33} however, this can only be applied to detection of modification sites in the protein, but not to identify modifications already existing in biological samples prior the analysis. In addition, Alfaro *et. al.* recently introduced a new method for the affinity enrichment of isoaspartyl proteins, where chemo-enzymatic detection using the PIMT enzyme and hydrazine trapping were applied.³⁴

Many of the methods for studying deamidation include mass spectrometry, which over several decades, has become one of the most powerful tools in the biological sciences. Although MS alone cannot normally distinguish the isomers - the molecular masses of the isomers are identical and represent a single peak in mass spectrum - tandem MS have shown some successful results. Low energy collisionally activated dissociation (CAD) was applied based on the difference in the abundance of immonium and *b*- and *y*- ions,^{23, 24} and also *b* + *H*₂*O* and *y*-46 ions (*N*-terminal to the isoAsp residue).²² These methods can be used to distinguish isoAsp from Asp containing peptides, although the abundance of all mentioned ions was strongly influenced by the sequence of the peptides; in some cases diagnostic ions were not observed. Therefore, such methods require comparative analysis with standard peptides. This is often impossible for the analysis of biological systems.

An alternative tandem MS method was recently developed, where isoaspartic residues in peptides³⁵ and proteins³⁶ were unambiguously differentiated and quantified³⁷ by Electron Capture Dissociation (ECD). ECD was first introduced by Zubarev³⁸ and since then has been widely implemented for routine structural analysis of biological molecules.³⁹ ECD provides fragments (mostly *c*- and *z*-) complementary to those (*b*- and *y*-) generated by other tandem MS methods such as CAD⁴⁰ or infrared multiphoton dissociation (IRMPD).⁴¹ The ECD fragmentation mechanism is believed to be fundamentally different from other tandem methods. It is sometimes referred to as a 'non-ergodic' method,³⁸ *viz.* energy gain upon electron capture is used directly to cleave the *N*-*C*_α bond rather than being randomized along the molecule to break the weakest bond as in CAD,⁴⁰ an interpretation which is the subject of intense debate.^{42–44} In the ECD experiment multiply charged gas phase molecular ions produced by electrospray ionization (ESI) react with low energy electrons traditionally in a Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS),^{45, 46} and recently implemented in a radio frequency ion trap.⁴⁷ Electron capture is believed to occur in proximity to the protonated site of the molecule followed by the hydrogen-bonded carbonyl group H⁺ abstraction which results in the *N*-*C*_α bond cleavage producing *c* and *z*[•] type fragments (scheme 2).^{38, 48} Furthermore, radicals created by electron capture can initiate multiple free radical rearrangements and secondary fragmentation, which generate additional backbone as well as side chain cleavages.^{44, 49} These can result in the cleavage of *C*_α-*C*_β, or *C*_β-*C*_γ bonds.^{50, 51} Specifically, *C*_α-*C*_β cleavage will lead to the formation of specific fragments in Asp and isoAsp containing peptides (scheme 2). Thus, in addition to

c_m and $z_{n-m}^{\bullet}$ fragments, a loss of 60 Daltons from the reduced species of the parent ion, $[M + nH - 60]^{(n-1)+\bullet}$, will be observed; however, in the case of isoaspartic acid cleavage of C_{α} - C_{β} bond will result in a breakage of polypeptide chain and therefore $c_m + 57$ and $z_{n-m-57}^{\bullet}$ fragment ions will be generated (scheme 2).

Since the implementation of ECD, several new fragmentation techniques based on similar electron-molecular ion interactions were developed. For instance, electron transfer dissociation (ETD),⁵² was introduced as a substitute for ECD in instruments other than FTICR mass spectrometers. In ETD, the electron is transferred to the molecular ion from a radical anion to produce fragmentation pattern similar to ECD. This method, now widely used in the biological analysis,^{53, 54} was successfully applied in differentiation of aspartic and isoaspartic acid in peptides.⁵⁵ Furthermore, the electron ionization dissociation (EID)⁵⁶ technique was recently developed. In EID, ions of interest are irradiated with energetic electrons (>30 eV) further ionizing the excited radical ion species, which undergoes rapid dissociation. This method was successfully applied to standard singly and multiply-charged peptides and proteins. Additionally, EID was implemented in a radiofrequency linear ion trap instrument along with ECD and other fragmentation techniques for complementary analysis.⁵⁷

In the present study, ECD in FTICR MS was applied towards the analysis of Amyloid β peptides and their isomerized forms. Application of the top-down technique is a clever approach to distinguish isoAsp in a bigger A β (1-40 and 1-42) as it requires no extra chemical steps that could complicate the analysis, increase analysis time, and can lead to the loss of a significant fraction of the sample. Minimization of the sample preparation is particularly advantageous for A β , as the peptide is very 'sticky' due to its high hydrophobicity and can be completely lost during additional steps of the analysis. In addition to ECD, EID was also probed for its ability to distinguish A β isomers.

Experimental

Sample preparation

Standard A β 1-40 was purchased from Sigma Aldrich (St. Louis, MO, USA). The isomerized form of A β 17-28 (LVFFAEisoDVGSNK custom synthesized), and A β 1-42 with Tottori - Japanese Mutation at [Asn7] were obtained from AnaSpec (San Jose, CA, USA). A β 1-40 was digested with sequencing grade trypsin (Roche Diagnostics, Indianapolis, IN, USA) in 100mM Ammonium Bicarbonate buffer at 1:50 enzyme:substrate ratio in $\mu\text{g}/\mu\text{l}$ concentration, overnight at 37°C with prior incubation at 65°C for 20 min. A β 1-42 [Asn7] was deamidated overnight at 37°C in 0.4% aqueous Ammonium Hydroxide with pH >10.2 in $\mu\text{g}/\mu\text{l}$ concentration. After digestion or deamidation, samples were dried in a SpeedVac system to stop the reaction and evaporate volatiles.

Mass Spectrometry

Mass spectra were acquired on a custom built qQq-FTICR MS with a nanospray source and a 7T actively shielded magnet.^{58, 59} Samples were electrosprayed at 1–5 μM concentration in 50:50 MeOH:H₂O with 1% formic acid. Ions were isolated in the first quadrupole Q₁, accumulated in the second quadrupole Q₂, and transmitted into the ICR cell where they were irradiated with the electrons emitted from an indirectly heated dispenser cathode (Heatwave, Watsonville, CA, USA) for ion fragmentation. The following ECD and EID parameters were employed: electron irradiation time 35 – 100 ms, cathode potential -0.2 - 1.2 V (ECD), - 17 – 27 V (EID).

Results and Discussion

Distinguishing the isomers

ESI FTICR MS of the trypsin digested A β 1-40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV, hereafter abbreviated as A β 40) revealed good ionization efficiency of the peptide 17-28 (Figure 1a, LVFFAEDVGSNK, abbreviated as A β 17-28). The doubly charged precursor ion of this peptide was isolated, accumulated, and irradiated with low energy electrons for the ECD event as described in the experimental section. ECD mass spectra of this peptide showed abundant fragmentation with 95% sequence coverage (Figure 1a). In addition, specific neutral losses from the reduced species of the doubly-charged tryptic peptide were observed, similar to those detected earlier:⁶⁰ the loss of (C₃H₄O₂+NH₃) from glutamic acid, (C₃H₇+NH₃) from leucine, CH₃NO from asparagine, and C₂H₄O₂ loss from aspartic acid, as well as NH₃ from the N-terminus.

In order to perform a comparative analysis of peptide isomers, the same A β 17-28 peptide was custom-synthesized with isoaspartic acid at position 23 [isoD23] (Figure 1b, LVFFAEisoDVGSNK, abbreviated as isoA β 17-28). ESI FTICR MS spectra of the isolated doubly charged A β 17-28 and isoA β 17-28 precursor ions showed identical m/z (data not shown). ECD of isoA β 17-28 generated the $z_6^{\bullet}$ -57 diagnostic fragment ion which was not found in the A β 17-28 peptide ECD spectrum. In contrast, carbon dioxide (CO₂=43.9898) was lost from the same $z_6^{\bullet}$ fragment ion of A β 17-28, but not from isoA β 17-28 (Figure 1a, b insets), indicating that CO₂ loss is a diagnostic for the presence of Asp, but not isoAsp at a particular residue. While the utility of CO₂ loss as a diagnostic ion has been previously shown,³⁷ it is not normally as reliable as the $c_n+57/z_{m-n}-57$ diagnostic peaks as isoAsp can also lose CO₂. However, loss of CO₂ from isoAsp appears to be a minor dissociation channel (as evident by the small peak in a 100% isoAsp containing peptide³⁷). In current study, loss of CO₂ from $z_6^{\bullet}$ is completely eliminated (<1% relative abundance) from isoA β 17-28 indicating that $z_6^{\bullet}$ -44 is another diagnostic fragment ion for Asp containing A β peptides. The nominal loss of 60 was also detected from the charged reduced molecular ion species of the isomerized peptide, but its exact mass corresponds to the leucine side chain plus ammonium (60.0813=C₃H₇+NH₃), not loss of the sidechain of aspartic acid (60.0211=C₂H₄O₂), indicating that aspartic acid was not present in the modified peptide. Interestingly, as shown in the inset of the $c_{10}^{\bullet}$ radical fragment, this ion was of a lower abundance in the isomerized version of the peptide. This could be explained by the fact that isoaspartic acid has a shorter side chain, which may affect hydrogen bond formation, and the hydrogen transfer within the complex of c and $z^{\bullet}$ fragments.^{44, 61-64} It could be assumed that a weaker hydrogen bonded complex will have a shorter lifetime, leading to a lower degree of intracomplex hydrogen atom transfer ($c + z^{\bullet} \rightarrow c^{\bullet} + z$), and fewer number of $c^{\bullet}$ radicals would be generated.

Furthermore, the fragmentation pattern of the isoA β 17-28 peptide produced by EID was also investigated to determine whether it could be used to establish the presence and location of an isoaspartic acid. EID spectrum of the isoA β 17-28 contained many a , b and y , as well as c and $z^{\bullet}$ type fragments (Figure 1c). The $z^{\bullet}$ -57 diagnostic fragment was also present in the spectrum (with an error of 1.2ppm, indicating a confident assignment), although with lower abundance compared to the ECD spectra, correlating with a general reduction in abundance of all c and $z^{\bullet}$ type fragments as competing reaction channels were accessed. Nevertheless, this experiment demonstrated that EID can be applied in isoaspartomics research along with ECD. The EID technique will be of particular benefit in the analysis of singly charged molecular ions, when small molecules are studied in ESI or when MALDI is used as the ionization technique.

Generally, the isomers were distinguished by means of ECD and EID using the diagnostic ions $[M+2H-60]^+$ and z_6^*-44 when aspartic acid and z_6^*-57 when isoaspartic acid were present.

ECD of the full length Amyloid beta protein fragment 1-40

The analysis of A β 40 is rather challenging due to its high hydrophobicity at its C-terminus and its tendency to aggregate. The ESI spectrum of its tryptic digest showed only one peptide A β 17-28 with high abundance (data not shown). The other three tryptic peptides were either suppressed or had lower ionization efficiency, which made it difficult to isolate them and to perform MS/MS analysis. In addition, top-down analysis of A β is advantageous because digestion solutions are usually of elevated pH relative to physiological conditions, which can introduce undesired structural changes as well as additional deamidation and isomerization.^{9, 65, 66} In biological fluids or tissues, Amyloid beta is present as a mixture of various length fragments from 13 to 42 amino acids long.⁶⁷ Digestion of this mixture will complicate the analysis and will create biases in the identification of the origins of the resulting digest peptides. Thus it is important to create a top down approach for the analysis of A β peptides. At this stage of the research, an entire A β 40 peptide was electrosprayed into the mass spectrometer and analyzed by ECD. The 3⁺ to 5⁺ charged ions were observed in the spectra and the highest charge state $[M+5H]^{5+}$ ions were isolated, and accumulated. The ECD spectrum of A β 40 showed extensive fragmentation (Figure 2). Insets show the charge-reduced species $[M+5H]^{4+}$ and $[M+5H]^{3+}$, as well as their neutral losses. Importantly, the complementary fragment pairs c_6/z_{34}^* , and c_{22}/z_{18}^* adjacent to aspartic acids at positions 6 and 23 respectively are present in the spectra. It is likely that these fragment ions would generate diagnostic fragments in isoAsp containing peptides, which could be used to determine the presence of isoaspartic acid.

Detecting isoAsp in Amyloid beta 1-42

The amyloid beta fragment 1-42 (A β 42: which includes 2 more hydrophobic amino acids at the C-terminus compared to A β 40 DAE...GAIIGLMVGGVVIA⁴²) is the most abundant in the plaque deposits. Its level is greatly enhanced in AD brain, and it is believed to be the most toxic of the A β peptides.^{68, 69} It is worthwhile, therefore, to develop a method for the analysis of this particular fragment, even though it is notoriously difficult to analyze due to its high hydrophobicity and tendency to aggregate. In addition, longer peptides generally have higher probability for significant secondary structure in the gas phase and form numerous hydrogen bonds. Unless such ions are activated prior to ECD analysis to disrupt secondary structure, strong hydrogen bonds tend to keep c/z^* fragment pairs in a complex, reducing the net efficiency of ECD analysis. Nevertheless, successful analysis of A β 42 would create a method suitable for different length A β peptides with the same or fewer number of amino acids.

Formation of isoaspartic acid in a standard A β 42 was induced to create a relevant test-case for development of this method. Because the rate of aspartate isomerization is ~40 times slower than the rate of deamidation,⁹ the variant containing Japanese –Tottori mutation [Asn7] was chosen to accelerate formation of isoAsp by deamidating the asparagine residue. The sample was incubated in ammonium hydroxide at pH >10.2, overnight at 37°C and analyzed in ESI FTICR MS using ECD. $[M+5H]^{5+}$ molecular ions were isolated, accumulated and irradiated with low-energy electrons. An ECD spectrum of the peptide is shown in Figure 3. The mass of the parent ion increased by one or two units (de-convoluted mass) indicating the presence of single and double deamidation correspondingly (see insets). Double deamidation was observed due to the presence of a second asparagine in the peptide sequence at position 27. Partial deamidation of Asn27 was also observed, which is explained by the fact that the rate of HNS deamidation is faster than the rate of SNK deamidation. In

particular, according to the experimental data acquired at near-physiological conditions, the first order deamidation half-life of GHNSG and GSNKG model pentapeptides are 15.7 and 55.5 days.¹² For the purpose of this experiment, however, deamidation of Asn27 was of a lesser interest, as was the completeness of the deamidation reaction in general. Deamidation of asparagine [Asn7] was performed to induce the formation of isoaspartic acid at this particular residue because in Amyloid beta peptides found in the cerebral plaque samples of Alzheimer patients aspartic acid is 75% isomerized to isoAsp at the residue position 7.¹³ As a result of ECD analysis of the deamidated A β 42 peptide (Figure 3 upper spectrum), the diagnostic fragment c_6+57 was observed (at 2.2 ppm mass accuracy), demonstrating the formation of isoaspartic acid at the 7th residue and the ability of the current method to detect and localize the modification. It should be noted that this model system experienced a highly non-physiological (pH >10) environment, so that the deamidation mechanism is unlikely to follow the *in vivo* mechanism as the pH would have been likely to unfold the peptide, thus perturbing the native deamidation rate. However, the results of this experiment provide a needed baseline for future studies. It was clearly demonstrated that ECD can be used to detect the isomerized aspartate in a top down experiment of the longest A β peptide. Exploring these studies further in terms of quantitative analysis and combining this technique together with HPLC separation will present a powerful tool for the analysis of physiological fluids in Alzheimer's disease patients. Comparative studies of isoAsp containing A β peptides in normal and diseased samples of different stages might reveal a novel perspective on Alzheimer's disease initiation and progression.

Conclusions

The ECD diagnostic ions $[M+2H-60]^{*+}$ and z_6^{*-57} were observed and successfully applied to differentiate the isomeric forms of the Amyloid β tryptic peptide 17-28. Differentiation of aspartic and isoaspartic acid residues is also possible using EID using the same diagnostic ion z_6^{*-57} as in ECD. Although the abundance of the diagnostic peak was relatively low compare to ECD, EID could be particularly useful in the analysis of singly charged precursor ions, where ECD is not applicable. Amyloid β 1-40 and 1-42 peptides can be analyzed by a top-down ECD approach without prior digestion and provide substantial sequence coverage. Isomerized Amyloid β 1-42 at the residue 7 can be differentiated by ECD using the c_6+57 diagnostic ion. Both ECD and EID can clearly define the presence and the position of isoaspartic acid residues in Amyloid β peptides; therefore, they could be applied to the samples of Alzheimer's patients or healthy individuals for better understanding of the disease.

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Figure 1.

a. ECD of Aβ 17-28; b. ECD of isoAβ 17-28; c. EID of isoAβ 17-28.

Figure 2.
ECD of A β 40.

Figure 3.
ECD of A β 42 deamidated at Asn7 and Asn27

Scheme 1.
Isomerization of Aspartic and Isoaspartic acids, and deamidation of Asparagine via succinimide intermediate

Scheme 2.

a. ECD major pathway produces *c*- and *z*- fragment ions with identical *m/z* but different chemical structure of *z*- fragments-isomers; b. proposed ECD mechanism for the formation of diagnostic fragment ions.