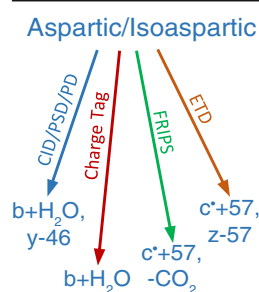


RESEARCH ARTICLE

Distinguishing Aspartic and Isoaspartic Acids in Peptides by Several Mass Spectrometric Fragmentation Methods

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Abstract. Six ion fragmentation techniques that can distinguish aspartic acid from its isomer, isoaspartic acid, were compared. MALDI post-source decay (PSD), MALDI 157 nm photodissociation, tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) charge tagging in PSD and photodissociation, ESI collision-induced dissociation (CID), electron transfer dissociation (ETD), and free-radical initiated peptide sequencing (FRIPS) with CID were applied to peptides containing either aspartic or isoaspartic acid. Diagnostic ions, such as the $y-46$ and $b+H_2O$, are present in PSD, photodissociation, and charge tagging. $c+57$ and $z-57$ ions are observed in ETD and FRIPS experiments. For some molecules, aspartic and isoaspartic acid yield ion fragments with significantly different intensities. ETD and charge tagging appear to

be most effective at distinguishing these residues.

Keywords: Peptide fragmentation, Isoaspartic acid, CID, ETD, FRIPS, PSD, Charge tagging, Photodissociation

Received: 8 July 2016/Revised: 17 August 2016/Accepted: 19 August 2016/Published Online: 9 September 2016

Introduction

Protein modifications, such as the conversion of aspartic acid or asparagine residues to isoaspartic acid, can lead to conformational changes, reduced function, and aggregation [1–9]. Their occurrence in the complementarity determining regions (CDRs) of antibodies can decrease the affinity of antigen binding. For example, rhuMAb HER2 is a therapeutic monoclonal antibody for metastatic breast cancer that contains an aspartic acid in a heavy chain CDR. Isomerization of this residue reduces the activity by 79%–91% of the unmodified antibody [10]. In another example, the monoclonal antibody, E25, contains an aspartic acid in a light chain CDR that readily undergoes isomerization. If the aspartic acid in one light chain converts to isoaspartic acid, the binding affinity of $F(ab')_2$ drops by 58%, whereas isomerization of both residues in the two light chains lowers it by 85% [11]. The formation of isoaspartic acid from asparagine deamidation or aspartic acid isomerization and the conditions under which this occurs have been previously reported [12–21].

Mass spectrometry has been applied to distinguish asparagine, aspartic acid, and isoaspartic acid [22–29]. Differentiating

asparagine from the acids is trivial because of their 0.984 Da mass difference. However, distinguishing aspartic acid from isoaspartic acid is more challenging since they are isomeric. Fragmentation of peptide ions containing aspartic or isoaspartic acid has been investigated and several important features in post-source decay (PSD) [23], collision-induced dissociation (CID) [22, 24], electron capture dissociation (ECD) [25, 26], and electron transfer dissociation (ETD) [27–29] have been reported. Two other methods not previously applied are 157 nm vacuum ultraviolet (VUV) photodissociation in a MALDI-TOF/TOF mass spectrometer and free-radical initiated peptide sequencing (FRIPS) using the reagent, *o*-Tempo-Bz-NHS.

PSD following matrix-assisted laser desorption/ionization (MALDI) and CID are two low-energy peptide fragmentation techniques that generate b - and y -type product ions [30, 31]. PSD and CID produce an enhanced cleavage on the C-terminal side of aspartic acid. Wysocki et al. showed that the side chain of this residue folds over to form a seven-membered hydrogen bonded ring with the C-terminal amide oxygen in the peptide backbone to initiate cleavage [32, 33]. Herein, this process will be referred to as the “D-effect.” Analogously, the side chain of isoaspartic acid can form a hydrogen bonded seven-membered ring to the amide oxygen on its N-terminal side. In contrast, aspartic acid would form a larger, less favorable eight-membered ring. As shown in Supplementary Scheme S1, the isoaspartic acid can transfer a hydrogen to the oxygen and form

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1487-9) contains supplementary material, which is available to authorized users.

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a five-membered ring to the carbon of the amide. Electrons rearrange and carbon monoxide is released to form $b+H_2O$ and $y-46$ product ions [22–24]. While the terminal carboxylic acid typically initiates the loss of the C-terminal residue, observation of $b+H_2O$ ions at locations other than the C-terminus is unusual [34].

ECD and ETD both produce c - and z^* -type fragments from multiply charged peptide ions [35, 36]. Supplementary Scheme S2 shows the effect of isoaspartic acid in ECD and ETD. After electron attachment on a lysine or arginine residue, rearrangement of the electrons can break the bond between the α -carbon and the methylene group in the backbone. This produces c^*+57 and $z-57$ ions [25–29]. Since aspartic acid does not have a methylene group in the backbone, these methods only yield normal c - and z^* -type ions.

One hundred fifty-seven nm photodissociation is a high energy fragmentation technique that generates b -, y -, a -, x -, d -, v -, and w -type ions. Initially, a 7.9 eV photon is absorbed by the peptide ion and subsequent homolytic cleavage of C_α -CO bonds occurs to create $a+1$ and $x+1$ radical ions [37]. These radical species rearrange to even-electron a -, x -, d -, v -, and w -type ions [37–39]. The multitude of product ions can facilitate de novo sequencing [40, 41]. Likewise, this technique has the potential to distinguish isoaspartic acid. Charge tagging has been shown to improve the $b+H_2O$ intensity in electrospray ionization (ESI) studies [24]. While previous charge tagging studies with PSD demonstrated very little fragmentation of these derivatized peptides due to the absence of a mobile proton, 157 nm photodissociation yields extensive sequence coverage [42].

FRIPS enables the production of high energy fragment ions with a low energy activation method, CID [43, 44]. An analogous radical-labeling method has previously been applied to distinguishing aspartic and isoaspartic acids [45]. This process involves modifying a peptide with a small reagent that contains a succinimidyl ester, *o*-TEMPO-Bz-NHS, as displayed in Supplementary Scheme S3. When the labeled peptide is ionized and collisionally activated, a relatively stable radical ion is formed. Subsequent collisional activation of this radical species produces a -, x^* -, c -, and z^* -type fragments, as well as neutral losses from some residue side chains [43, 44]. Although this method is somewhat analogous to ECD and ETD, it does not require multiple charges for fragmentation to occur. In fact, the presence of more charges than the number of basic groups can actually inhibit the radical pathways and facilitate charge-induced fragmentation [46].

In the present work, peptides containing aspartic or isoaspartic acid are fragmented using previously studied techniques, PSD, CID, and ETD, along with 157 nm photodissociation, PSD and photodissociation of charge tagged peptides, and FRIPS. The most important observations and conclusions are discussed below. Additional details about some of the experiments are provided in the Electronic Supplementary Material.

Experimental

Materials

Peptides, AFVXSLYR, AFVXSLYK, AFVXHLYR, AFVXSLYAFVSLYR, AFVSLYAFVXSLYR, RAFVXSLT, KAFVXSLT, and RAFVXHLT, where X is either aspartic acid (D) or isoaspartic acid (D_i), were synthesized in-house. Hydrophobic amino acids are commonly found in the CDRs of monoclonal antibodies and these sequences contain a few of them. The residue on the C-terminal side of aspartic or isoaspartic acid is either serine or histidine because these two residues are known to promote isomerization and deamidation [13, 16, 17, 20, 21]. The matrix, α -cyano-4-hydroxycinnamic acid (CHCA), was obtained from Fluka (St. Louis, MO, USA). The matrix, 4-chloro- α -cyanocinnamic acid (Cl-CCA), was synthesized in-house. (N-succinimidyl)oxycarbonylmethyl tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) was purchased from Sigma (St. Louis, MO, USA). *o*-TEMPO-Bz-NHS was obtained from Diotech Korea (Seoul, Korea).

Sample Preparation

Charge tagging with TMPP at the N-terminus of peptides was performed as described by He et al. [42]. A molar ratio of approximately 1:10 of peptides to TMPP reagent was used in a total volume of 100 μ L of 0.2 M $NaHCO_3$ at pH 9 with 20% acetonitrile. The mixture was vortexed and reacted for 30 min at room temperature; then 200 μ L of water were added to the sample to hydrolyze the succinimidyl ester. The mixture reacted for another 30 min.

The *o*-TEMPO-Bz-NHS derivatization was adapted from previous procedures [43, 47]. Peptides were mixed with *o*-TEMPO-Bz-NHS reagent in an approximate ratio of 1:100 in a solution of anhydrous dimethylsulfoxide (DMSO). The reaction proceeded overnight and the solution was removed by vacuum. Derivatized peptides were re-solubilized in water.

Peptides analyzed by MALDI were either directly spotted onto a MALDI plate or first loaded onto micro-C18 pipette tips to remove salts and excess reagents before spotting. For both CHCA and Cl-CCA, 10 mg/mL of matrix was dissolved in 50:50 acetonitrile:water with 0.1% trifluoroacetic acid. The matrix was either spotted on top of dried peptide samples or used to elute peptides from C18 columns.

Mass Spectrometry

Peptide samples were analyzed by PSD and 157 nm photodissociation in an ABI 4800 MALDI-time of flight/time of flight (TOF/TOF) mass spectrometer. The intensity of the 355 nm ionization laser was adjusted for optimal peptide signal. Results from 500 shots were signal averaged to produce each spectrum. To obtain photodissociation spectra, the instrument was previously modified to pass 157 nm VUV light from a Lambda Physik CompexPro laser (Göttingen, Germany) through its collision cell, analogous to a previous modification of an ABI 4700 mass

spectrometer [48]. One hundred fifty-seven-nm light is generated by a mixture of 5% fluorine in helium. A single 2–4 mJ laser pulse irradiates the passing ions. Resulting spectra include contributions from both PSD and photodissociation.

A Thermo LTQ linear ion trap mass spectrometer (Waltham, MA, USA) with an ESI source was utilized to fragment peptides by CID. All peptides were first individually separated by reversed-phase liquid chromatography with an Eksigent 2D Nano LC (Dublin, CA, USA). A self-packed C18 capillary column was used to separate the sample from impurities in the peptide synthesis or labeling reactions. The eluent from the LC separation was directly infused into the ESI source of the LTQ. CID of a peptide ion was performed with a 3 Da isolation width, 35 eV collision energy, 0.250 activation Q, and 30 ms activation time. CID of fragment ions used the same parameters, except with a 2 Da isolation width.

Peptides were directly infused into the ESI source of a Waters Synapt G2-S mass spectrometer (Milford, MA, USA) for analysis by ETD. The capillary voltage was set to 0.65 kV. The isolation width for peptide ions was 1.5 Da and the electron transfer reagent was dicyanobenzene. A single mass spectrum is the average of signal over 30 s.

A Thermo MALDI-LTQ mass spectrometer was employed to analyze *o*-TEMPO samples. The matrix and peptide spotting procedure was the same as used for MALDI-TOF/TOF experiments. The MALDI laser energy was optimized for each sample. CID was performed with the same parameters described above. Data from 200 shots were signal averaged into one spectrum.

Results and Discussion

MALDI PSD

Peptides AFVDSL_YR and AFVD_iSL_YR were MALDI ionized and fragmented by PSD in a TOF/TOF mass spectrometer. The resulting qualitatively similar mass spectra are displayed in Figure 1a and b. Intense y_4 ions due to the aspartic acid side chain (the D-effect) appear in both spectra. The unusual y_5 -46 fragment ion is in the spectrum of AFVD_iSL_YR. Although its intensity is low, the expanded regions from 600 to 625 Da show that the ion is formed with isoaspartic acid and not with aspartic acid. Most significantly, isoaspartic acid leads to a much more intense y_5 ion. This is due to its shorter side chain. As illustrated in Scheme 1, isoaspartic acid forms a seven-membered hydrogen bonded ring with the oxygen of the amide N-terminal to it, whereas aspartic acid forms a less favorable eight-membered ring [49].

PSD mass spectra of the peptides, RAFVDSL_T and RAFVD_iSL_T, are shown in Figure 1c and d. Because of the N-terminal arginines, b-type ions dominate the spectra. A b_4 +H₂O ion, which is nearly as intense as the b_4 ion, is the diagnostic ion in the isoaspartic acid spectrum. Consistent with previous research, peptides with arginine at the N-terminus produce b+H₂O ions, the intensity of which exceeds that of y -46 ions that are formed with arginine at the C-terminus [23].

In the aspartic acid spectrum, a low abundance b_4 +H₂O peak at 492 Da appears as intense as the c_4 ion at 491 Da. However, this could be due to either an internal fragment, AFVDS-28, or some of the aspartic acid having already isomerized from the time of peptide synthesis to analysis. Since both peptides yield a b_4 +H₂O peak, the intensities of the diagnostic ion can be compared with those of neighboring fragments, such as the b_4 , c_4 , and b_5 ions. Such relative measurements may improve the ability to quantitate the presence of aspartic and isoaspartic acids.

MALDI 157 nm Photodissociation

The peptides studied with PSD were photodissociated in a modified MALDI-TOF/TOF mass spectrometer. The 500–700 Da regions of the photodissociation mass spectra of AFVDSL_YR and AFVD_iSL_YR are shown in Figure 2a and b. The complete mass spectra are in Supplementary Figure S2a and b. Spectra are qualitatively similar to PSD data. However, now aspartic acid also generates a low intensity y_5 -46 peak. This may be attributed to a side-chain fragmentation of the aspartic acid in the y_5 ion as shown in Scheme 2. Unfortunately, this photofragmentation process inhibits identification of isoaspartic acid based on the y_5 -46 ion. Interestingly, the side chain fragment ions, w_{a5} and v_5 , are observed with low intensity for the aspartic acid-containing peptide and not for isoaspartic acid. Standard w- and v-type ions for isoaspartic acid are not the same mass as those for aspartic acid and neither of these is observed [50]. Although there are differences between the two acids, the effects described are generally of low abundance.

Expanded mass spectra from 400 to 600 Da for the peptides, RAFVDSL_T and RAFVD_iSL_T, are shown in Figure 2c and d, and the complete spectra are in Supplementary Figure S2c and d. The N-terminal arginine facilitates production of a-type ions; a_5 and d_{a5} ions appear in both spectra and cannot distinguish the two isomers. However, similar to PSD, b_4 and b_4 +H₂O ions are more intense with isoaspartic acid.

Charge Tagging

Since b+H₂O and y -46 ions are formed by side chain interactions with the peptide backbone that are most readily observable during charge-remote fragmentation, fixing a charge on the N-terminus of a peptide could facilitate their observation. Peptides were charge tagged with a TMPP label and fragmented by PSD and photodissociation. TMPP was selected for this application for two reasons. First, charge tags containing quaternary amines can generate mobile protons by side reactions [51]. Second, the TMPP charge tag contains a succinimidyl ester, which enables derivatization to amines at N-termini and lysines. With the charge tag on the N-terminus, fragmentation yields N-terminal ions that are preferable because b+H₂O ions are good isoaspartate markers, as noted in both PSD and photodissociation experiments above.

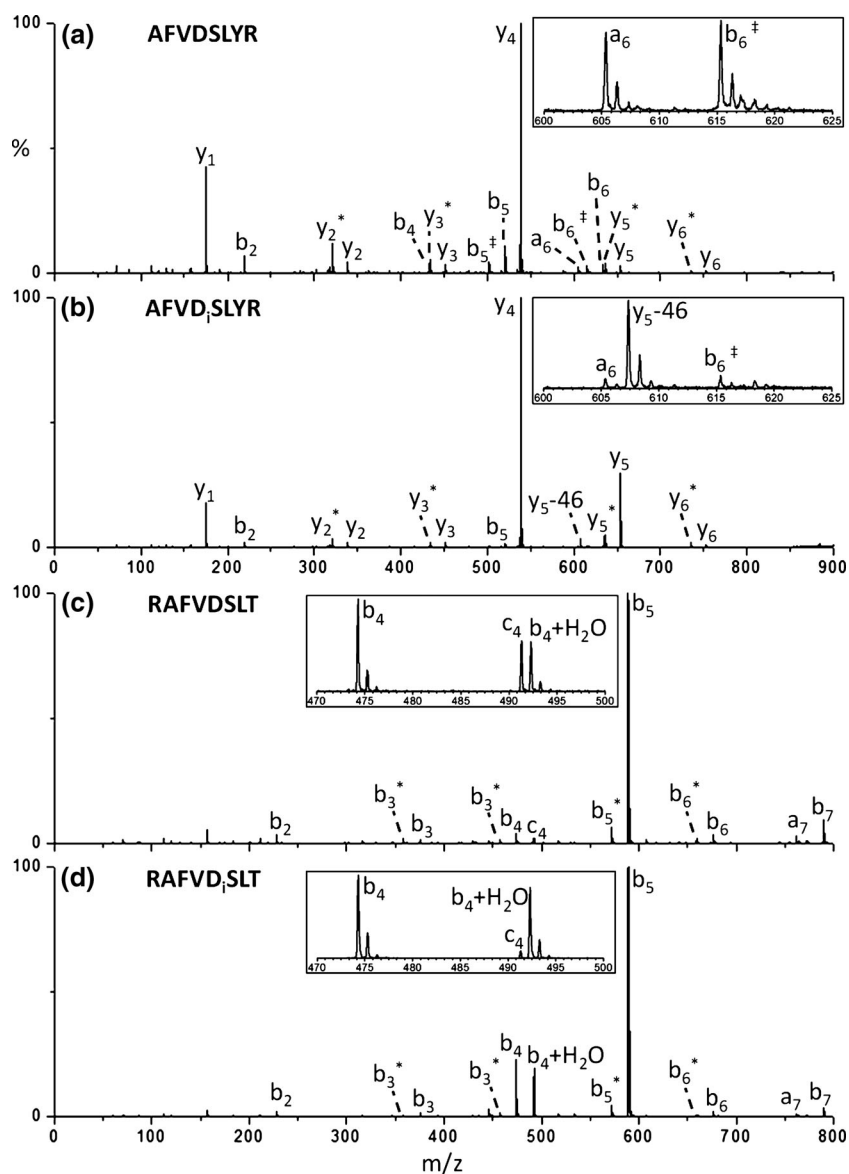
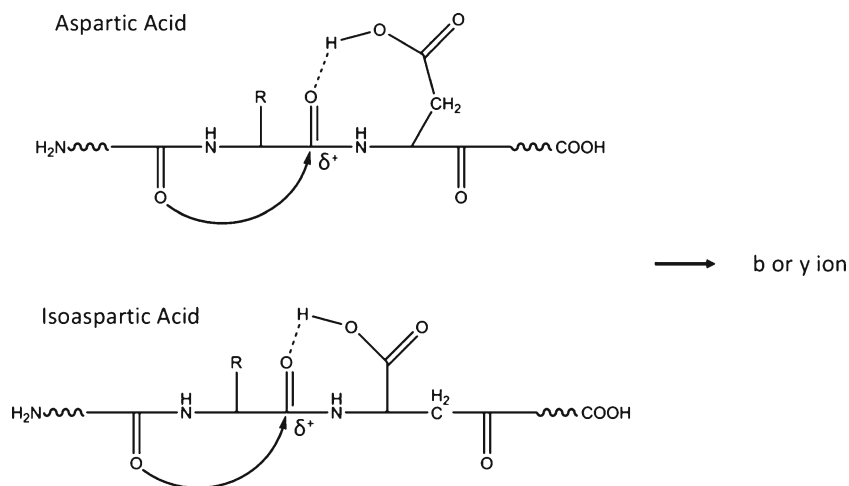


Figure 1. PSD mass spectra for singly charged (a) AFVDSL⁺LYR (970.5 Da), (b) AFVD_iSL⁺YR (970.5 Da), (c) RAFVDSL⁺T (908.5 Da), and (d) RAFVD_iSL⁺T (908.5 Da) precursor ions. *Denotes loss of NH₃ and †denotes loss of H₂O



Scheme 1. Enhanced b- or y-type ion on the N-terminal side of isoaspartic acid

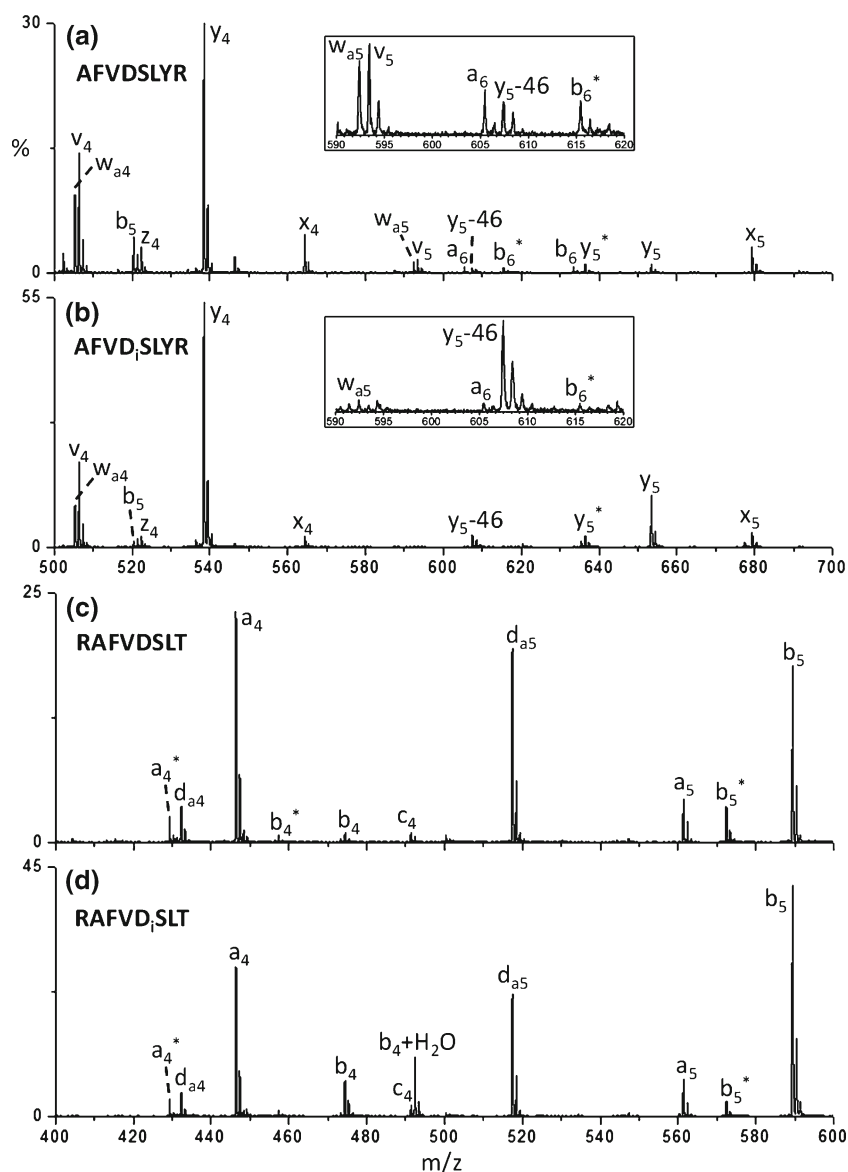
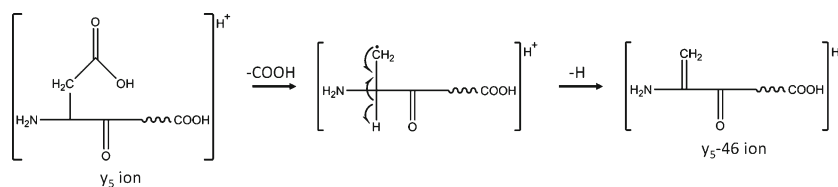


Figure 2. 157 nm photodissociation expanded mass spectra for singly charged (a) AFVDSLRYR (970.5 Da), (b) AFVD_iSLRYR (970.5 Da), (c) RAFVDSLTL (908.5), and (d) RAFVD_iSLTL (908.5) precursor ions. *Denotes loss of NH₃

Peptides AFVDSLRYR and AFVD_iSLRYR were labeled at the N-terminus with TMPP and fragmented by PSD. The mass spectra displayed in Figure 3a and b contain relatively few fragment ions. The most intense ion, b₇+H₂O, results from the carboxylic acid at the C-terminus interacting with the backbone to release the terminal amino acid [34, 42]. The intense b₄ ions in both spectra are from the D-effect. The b₃+H₂O and b₃ ions are more prominent in the isoaspartic acid-containing peptide.

This is consistent with the mechanism in Supplementary Scheme S1 that requires no proton to initiate fragmentation. These peptides were also fragmented by photodissociation and their far richer spectra are in Figure 3c and d. Photodissociation produces a complete series of a-type ions, many d-type ions, and a few b- and c-type ions. Although the spectra are similar, the b₃+H₂O ion from isoaspartic acid points to the presence of this residue. Not surprisingly, the PSD and photodissociation mass spectra



Scheme 2. Proposed mechanism for obtaining a y₅-46 ion in photodissociation with a y₅ ion precursor

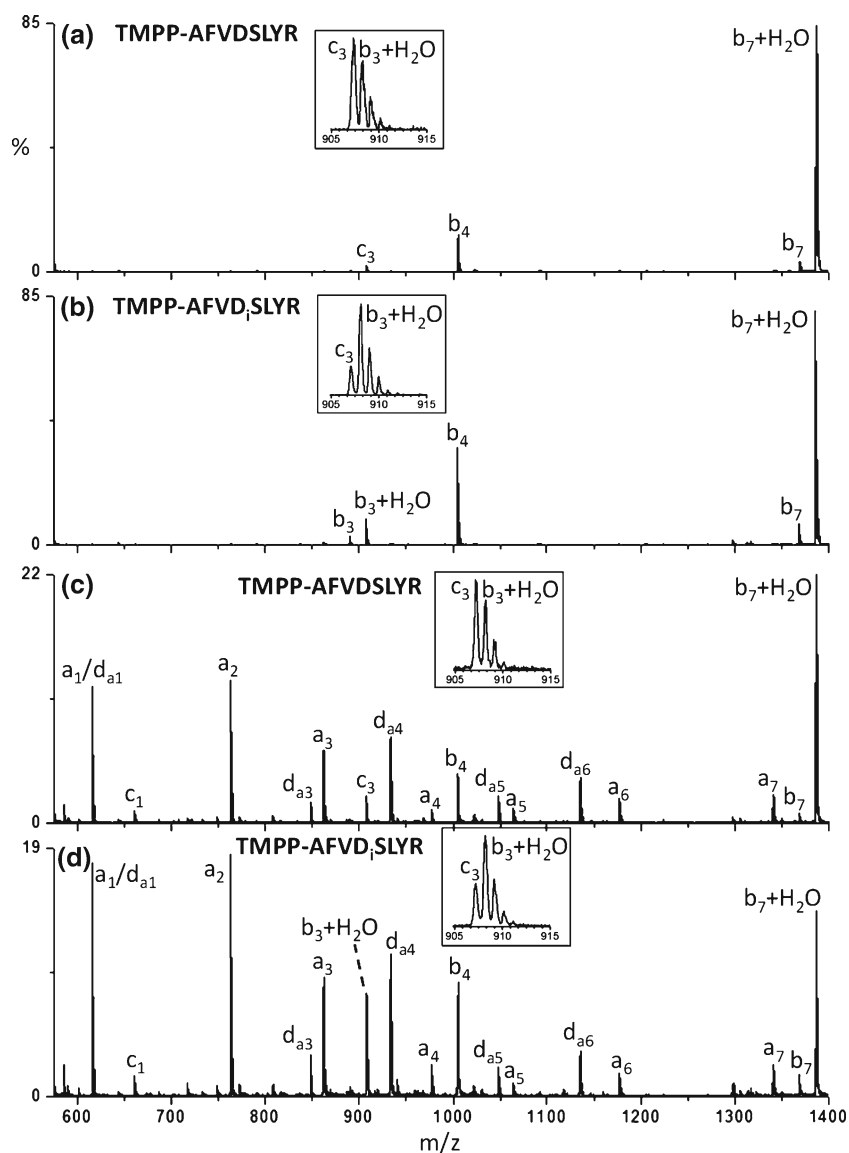


Figure 3. PSD of singly charged TMPP derivatized peptides, (a) AFVDSLRYR (1542.5 Da) and (b) AFVD_iSLYR (1542.5 Da); 157 nm photodissociation of these peptides is in (c) and (d), respectively.

of charge tagged peptides, TMPP-AFVDSLRYK and TMPP-AFVD_iSLYK, (Supplementary Figure S5) are nearly identical to those in Figure 3. With the charge sequestered at the N-terminus, charge remote pathways are not influenced by the terminating basic group. A significant improvement in the b₃+H₂O ion intensity is observed compared with the unlabeled peptides that only produce a low intensity y-46 ion.

Peptides RAFVDSLTL and RAFVD_iSLTL were also TMPP labeled and fragmented in PSD and photodissociation. Their mass spectra are in Supplementary Figure S6. With arginine located at the N-terminus, a significant decrease in fragmentation efficiency is observed. This observation has been previously reported and attributed to an interaction between the arginine side chain and the TMPP label [42]. This interaction does not occur when lysine is at the N-terminus. As a result, significant

b₄+H₂O ions are obtained from both PSD and photodissociation for the peptide KAFVD_iSLT, as shown in Supplementary Figure S7. This fragmentation is a vast improvement compared with the unlabeled peptides that suffered from mobile protons inhibiting the formation of the b₄+H₂O ion.

In summary, these experiments produce one diagnostic ion in either PSD or photodissociation. Charge tagging avoids issues with mobile protons that inhibit the production of b+H₂O and y-46 ions. By fixing the charge at the N-terminus, the more intense b+H₂O ion is produced. Furthermore, PSD spectra are simple to interpret, since only fragmentation via a carboxylic acid mediated interaction is predominately observed. If in-depth sequence information is desired, photodissociation can provide many a- and d-type fragment ions. Nevertheless, the large size of the charge tag may reduce the photofragmentation efficacy of larger peptides.

ESI CID

The peptides AFVDSL_IYR and AFVDSL_IYR were each electrosprayed; the +2 charged ions were isolated in an ion trap and fragmented by CID. The mass spectra in Figure 4a and b show similar fragment distributions containing mainly b- and y-type ions. In contrast with singly charged ions, y₄ ions are now low in intensity. The y₅-46 ion is not observed in the isoaspartic acid spectrum, which has been noted in previous research [24]. Although these results show no unique diagnostic isoaspartic acid ions, the y₆²⁺ ion is significantly more intense for isoaspartic acid than aspartic acid. Since this cleavage is not adjacent to isoaspartic or aspartic acid, there may be energetic considerations that affect fragmentation. Perhaps, the isoaspartic acid-containing peptide is more favorable to producing the y₆²⁺ ion. Other examples displaying fragmentation of multiply charged ions are presented below.

The fragmentation spectra for the doubly charged RAFVDSL_IT and RAFVDSL_IT in Figure 4c and d are also qualitatively similar. The intensity of the b₄+H₂O ion in the isoaspartic acid spectrum is lower than that of the adjacent c₄ ion peak, which contrasts with PSD results from fragmenting singly charged isoaspartic acid-containing ions. Although there are no diagnostic ions present, there are some interesting variations in the intensities of fragments (e.g., b₅⁺ and b₇²⁺) again suggesting that there could be energetic differences between these two.

The fact that intense y-46 or b+H₂O ions are not observed in Figure 4 makes it difficult to distinguish the two isomers. This is presumably because the charge-remote processes that generate these fragment ions cannot compete with charge-induced processes enabled by mobile protons. Since PSD fragmentation of singly charged ions yielded b+H₂O and y-46 ions, we

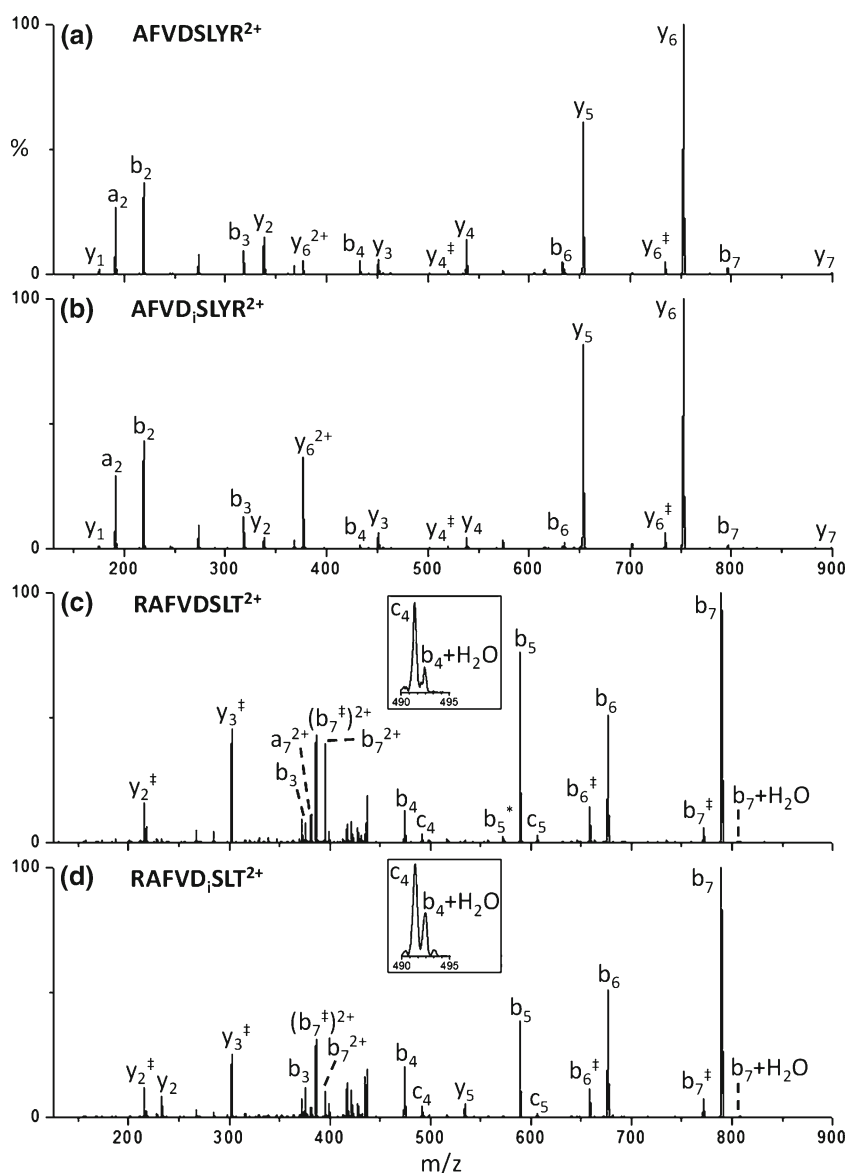


Figure 4. CID of the doubly charged peptides, (a) AFVDSL_IYR (485.8 Da), (b) AFVDSL_IYR (485.8 Da), (c) RAFVDSL_IT (454.7 Da), and (d) RAFVDSL_IT (454.7 Da). ⁺Denotes loss of H₂O

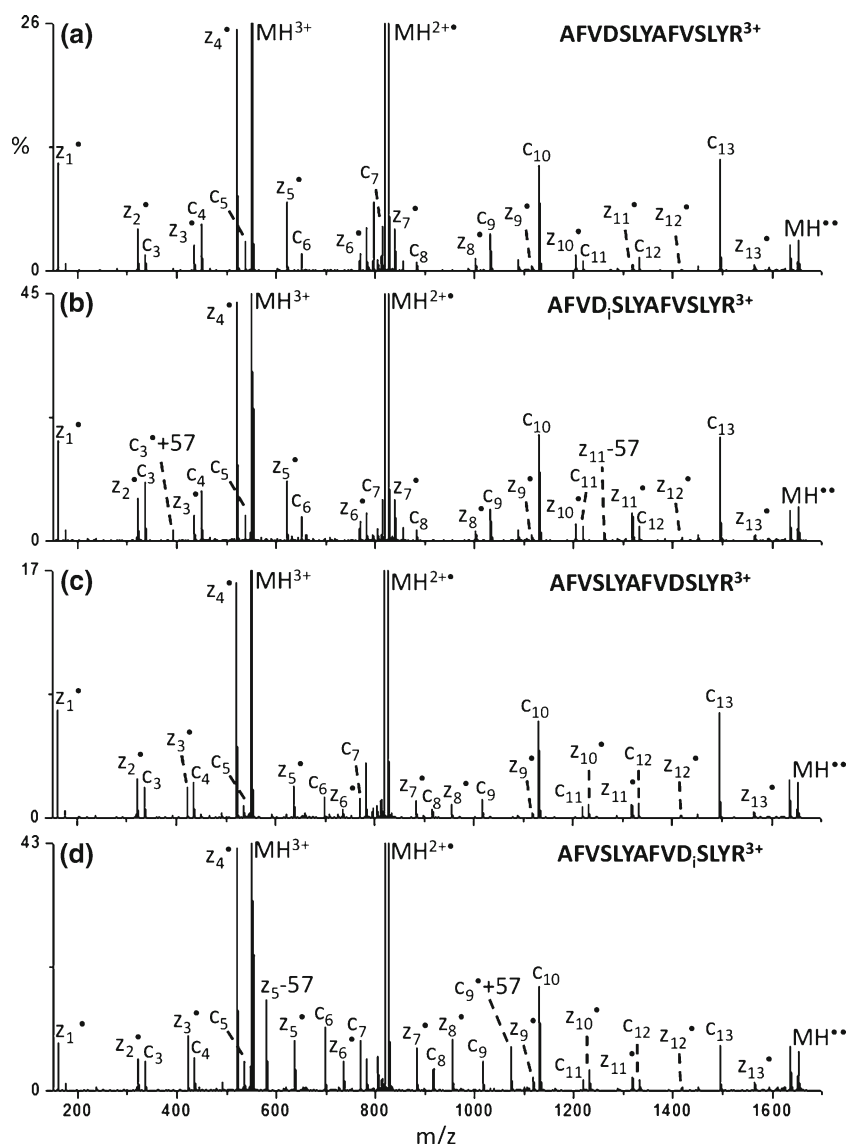


Figure 5. ETD mass spectra for triply charged (a) AFVDSLAFVSLYR (551.0 Da), (b) AFVD_iSLYAFVSLYR (551.0 Da), (c) AFVSLYAFVDSLRYR (551.0 Da), and (d) AFVSLYAFVD_iSLYR (551.0 Da) precursor ions. MH³⁺ and MH²⁺ ions are off scale

considered the possibility that MS/MS of singly charged *fragment* ions generated by CID of multiply charged ions might produce better results, since singly charged precursor ions are sparingly produced by ESI. This is not exactly comparable to the MALDI experiment because singly charged *fragments* formed by collisional activation of multiply charged ions may have more internal energy than MALDI precursor ions. Supplementary Figures S9 and S10 show these results, in which minimal improvements to the b+H₂O and y-46 ion intensities were obtained. This may be a factor of fragment ion size, in which there are structural limitations for this charge sequestered pathway to occur. As exemplified in Supplementary Figure S10, larger fragment ions produce more intense diagnostic ions. A further discussion of these results can be found in Electronic Supplementary Materials.

Spectra of peptides containing aspartic and isoaspartic acid residues display some unanticipated peak intensity observations. In particular, y ions that contain isoaspartic acid tend to be more intense than those that contain aspartic acid. For example, as mentioned above, the y₆²⁺ and y₅ ions are more intense in the isoaspartic acid spectrum in Figure 4a than in the aspartic acid spectrum Figure 4b. Other examples displaying more intense y ions for isoaspartic acid are the y₅ and y₆ ions in Supplementary Figure S1c, d; the y₅ ion in S11a, b and S11c, d; the y₁₂²⁺ and y₁₁ ions in S13a, b; and the y₁₂²⁺ ions in S13c, d. Likewise, b-type ions that contain aspartic acid tend to be more intense, such as b₅ and b₇²⁺ in Figure 4c, d. Other b-type fragments that exhibit this are the b₅ and b₆ ions in Figure 1a, b; the b₄, b₆, and b₇ ions in 4a, b; the b₅, b₆, and b₇ ions in S1a, b; the b₂ ion in S11a, b, and S11c, d; the b₁₀, b₁₁, b₁₂, and b₁₃ ions

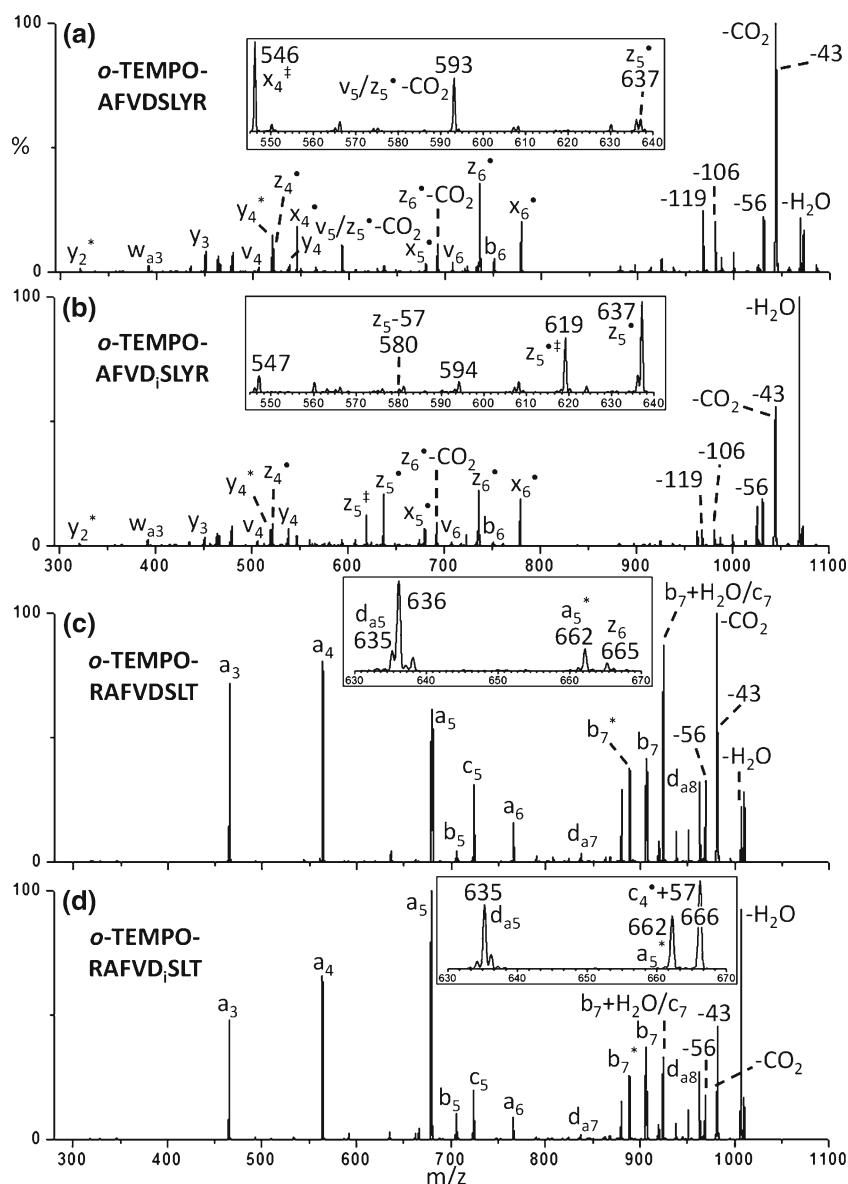


Figure 6. CID mass spectra of the singly charged radical *o*-TEMPO derivatized peptides, (a) AFVDSLRYR (1087.5 Da), (b) AFVD_iSLRYR (1087.5 Da), (c) RAFVDSLTL (1025.5 Da), and (d) RAFVD_iSLTL (1025.5 Da). *Denotes loss of NH₃ and †denotes loss of H₂O

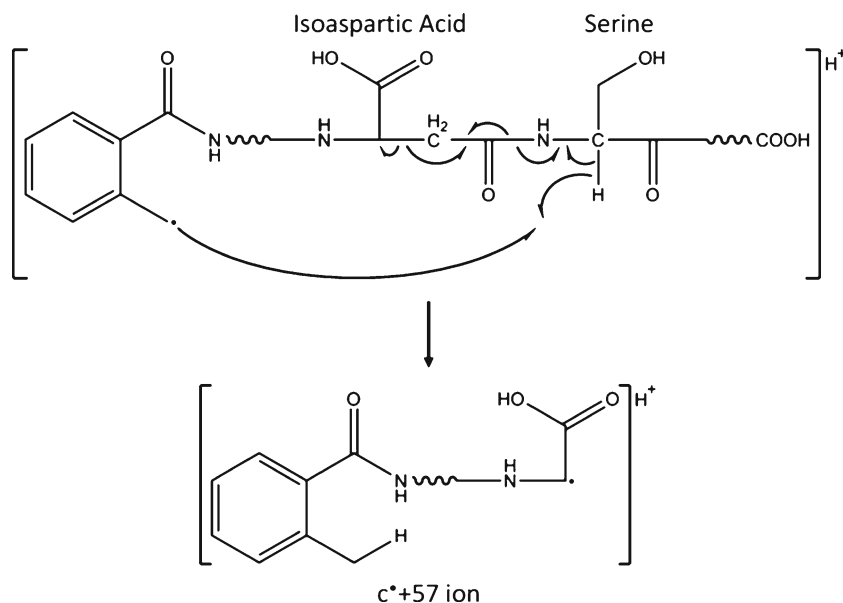
in S12a, b; and the b₈, b₉, b₁₀/y₉[†], and b₁₁/y₁₀[†] in S13a, b. In these examples, it should be noted that not all b-type ions that include aspartic acid are more intense than their isoaspartic acid counterparts and some of the intensity variations are small. Nevertheless, these observations of b-type ions being more intense if they contain aspartic acid and y-type ions being more intense if they contain isoaspartic acid are consistent across all these spectra.

In summary, CID of multiply charged ions produces poor y-46 and b+H₂O diagnostic ion intensities. However, several examples demonstrated differences in the intensities of fragment ions containing aspartic and isoaspartic acid that could potentially be exploited to differentiate the two isomers. Preliminary results suggest that fragmentation of +1 and +2 charged ions tends to produce more intensity differences than

fragmentation of +3 charged peptides, but further investigations are needed to corroborate these examples.

ETD

Fragmentation of larger peptides, AFVDSLRYAFVSLRYR and AFVD_iSLRYAFVSLRYR, by ETD in a Synapt G2s mass spectrometer yielded spectra shown in Figure 5a and b. Intense peaks for the precursor, MH³⁺, and charge reduced peptide, MH²⁺, are off-scale, which is not untypical for ETD [36]. Lower intensity peaks correspond to c- and z[•]-type ions. Two unusual ions, c₃[•]+57 and z₁₁-57, are identified from the isoaspartic acid-containing peptide. These ions are not produced by the aspartic acid peptide. As seen in Figure 5c and d, peptides AFVSLRYAFVDSLRYR and AFVSLRYAFVD_iSLRYR produce similar c- and z[•]-type ions, with the



Scheme 3. Formation of a c^*+57 ion for isoaspartic acid

addition of z_5-57 and c_9^*+57 ions in the isoaspartic acid spectrum. This observation is consistent with previous research [27, 29]. Although these c^*+57 and z_5-57 ions appear to be low intensity compared with the MH^{3+} and charge reduced MH^{2+} peaks, they are usually comparable to neighboring c - and z^* -type fragments. The limiting factor of using ETD is that peptides ideally need to be multiply charged ions (+3 or greater) to yield sufficient fragment ions [36], and many peptides obtained from standard trypsin digestions are not large enough to accommodate three charges.

Free-Radical Initiated Peptide Sequencing (FRIPS)

The *o*-TEMPO derivatized peptides, AFVDSL_YR and AFVD_iSL_YR, were fragmented by CID in a MALDI-LTQ ion trap mass spectrometer to form radical *o*-TEMPO peptides. These radical species were subsequently fragmented to produce MS³ spectra that are displayed in Figure 6a and b. More details about this experiment are included in Electronic Supplementary Material. x^* -, y -, z^* -, and w -type ions were all observed. Neutral losses, such as -18 (H₂O), -43 (leucine), -44 (CO₂), -56 (leucine), -106 (tyrosine), and -119 (*o*-TEMPO label with two hydrogens) also occurred [44]. The first striking observation is that the ratio of the neutral loss peaks, -18 and -44, is very different for aspartic and isoaspartic acid. The loss of H₂O is attributed to the presence of serine, since this peak is small for the same peptides with serine replaced by histidine (Supplementary Figure S15a and b). The loss of CO₂ is notable because there are two sources of CO₂: the C-termini and the side chains of aspartic or isoaspartic acid. The mechanism for losing CO₂, which is based on previously reported radical chemistry [52], is shown in Supplementary Scheme S4. The hydrogen in the carboxylic acid is first abstracted by the radical on the *o*-TEMPO

modification and then the radical subsequently rearranges to lose CO₂. Since this loss is more intense for the aspartic acid-containing peptide, the side chain of aspartic acid must be more susceptible to this fragmentation than that of isoaspartic acid, possibly because its longer length may facilitate interaction with the radical. Although this assumes that C-terminal contributions are similar for these peptides, differences in how they fold could affect this loss.

The expanded regions between 545 and 640 Da in Figure 6a and b display some spectral differences. First, a very low intensity z_5-57 peak is present for isoaspartic acid that is not observed for aspartic acid. The z_5^* ion is also much more intense for isoaspartic acid, which also agrees with previous ETD results [27]. As shown in Supplementary Scheme S5, the radical from the *o*-TEMPO modification can remove a hydrogen from the tertiary carbon of the valine side chain [52]. The radical can then break the bond between the C_α and CO to form a - and x^* -ions. The x^* ion can release CONH to form a z_5^* ion. Mechanistically, there is little difference if aspartic acid is present in the sequence. However, the low abundance z_5^* ion for aspartic acid may have resulted in a more intense 593 Da $z_5^*-CO_2$ fragment, which is consistent with a more significant CO₂ neutral loss. The isoaspartic acid peptide forms a 619 Da $z_5^*-H_2O$ ion instead of losing CO₂. Formation of x_4-H_2O requires a radical abstraction of the hydrogen at the β-carbon in the side chain of aspartic acid and the loss of water from the serine residue. For isoaspartic acid, this hydrogen exchange would actually occur at the α-carbon in order to initiate the fragmentation to produce an x^* ion. This site may be less accessible than one located on an amino acid side chain, limiting formation of an x -type ion from isoaspartic acid. The observation of intense MH^+-CO_2 , z_5^* , and $z_5^*-CO_2$ ions were further corroborated in

Table 1. Summary of Fragmentation Results

Method	Isoaspartic acid identification
MALDI PSD	b+H ₂ O/y-46, enhanced b-/y-type ion N-terminal to iso-D, depending on location of basic residue
MALDI photodissociation	b+H ₂ O/y-46, enhanced b-/y-type ion N-terminal to iso-D, depending on location of basic residue
MALDI PSD or photodissociation with charge tagging	b+H ₂ O, enhanced b-type ion N-terminal to iso-D
ESI CID	No diagnostic ions, b/y fragment intensities can vary depending on presence of D or iso-D
ESI ETD	c ⁺ +57/z-57 ions
MALDI CID FRIPS	Enhanced z ⁻ -type ion N-terminal to iso-D with a basic group at the C-terminus; c ⁺ +57 ion only appears with iso-D and a basic group at the N-terminus. Neutral loss of CO ₂ is enhanced for aspartic acid and is less apparent for isoaspartic acid

Electronic Supplementary Material by the peptides AFVDHLYR and AFVD_iHLYR. In contrast, in their radical directed dissociation work, Tao and Julian primarily observed b- and y-type ions [45].

The radical *o*-TEMPO derivatized peptides, RAFVDSL_T and RAFVD_iSL_T, were fragmented to produce MS³ spectra shown in Figure 6c and d, respectively. Intense a-type ions are observed throughout the spectra, which is consistent with previous research [43] and is similar to photodissociation data [39]. Supplementary Scheme S5 shows an example of a pathway to form an a-type ion initiated by hydrogen transfer from the β-carbon of valine. A neutral loss of CO₂ is more intense for aspartic acid. The expanded spectra from 630 to 675 Da shows some noticeable differences. First, the c₄⁺+57 diagnostic ion is observed in the isoaspartic acid spectrum and is virtually unobservable in the aspartic acid spectrum. To explain the formation of this ion, the mechanism proposed in Scheme 3 initially shows the radical on the *o*-TEMPO modification removing hydrogen from the α-carbon of the serine residue. The radical can then rearrange to break the bond between the α-carbon and the methylene group of isoaspartic acid producing a c⁺+57 ion. Because more than one bond is broken, a z-57 ion cannot be formed by this mechanism. In fact, z-57 ions were not detected in these experiments. The side-chain fragment, d_{a5}, is more intense for isoaspartic acid, but for aspartic acid, a peak at 636 Da is more intense. This could indicate an extra hydrogen, suggesting an alternative pathway for forming a d-type ion. However, it should be noted that the peptides, RAFVDHL_T and RAFVD_iH_L_T, did not exhibit these differing ratios, as shown in Electronic Supplementary Material. These peptides also produced a very low intensity c⁺+57 ion, which supports the mechanism proposed in Supplementary Scheme S5 because the C-terminal residue (histidine) may influence the initial hydrogen exchange, impeding formation of the c⁺+57 ion.

These preliminary results demonstrate that the *o*-TEMPO radical fragmentation produces some features that can distinguish aspartic- and isoaspartic acid-containing peptides. For all peptides investigated, the loss of CO₂ is more facile for aspartic acid than isoaspartic acid. Likewise for peptides with arginine at the C-terminus, the N-terminal side of aspartic acid produces a more intense z⁻-CO₂ ion, whereas the N-terminal side of isoaspartic acid produces an intense z⁻-type ion. Peptides with arginine at the N-

terminus produce the ETD diagnostic ion, c⁺+57, but its intensity can depend on the residue on the C-terminal side of isoaspartic acid. Further investigations should be performed to verify these results in a variety of peptides in which sequences and locations of aspartic and isoaspartic acids are varied.

Conclusions

Table 1 summarizes the six fragmentation experiments performed in this work and lists the noteworthy features for distinguishing aspartic and isoaspartic acids. In future experiments, the most useful method will depend on the type of sample and techniques available. In the present work, the best methods for identifying isoaspartic acid are ETD and charge tagging. ETD can fragment high mass peptides efficiently and produce c⁺+57 and z-57 ions for isoaspartic acid. Charge tagging in PSD or photodissociation should be mainly used for low mass peptides; it produces a diagnostic b+H₂O ion for isoaspartic acid. However, if these techniques are not available, differences in ion intensities may be observed in fragmenting multiply charged ions with low energy CID. Quantitation of isoaspartic acid can be performed through many of these methods, but isolated aspartic- and isoaspartic acid-containing peptides should be studied to measure variances between fragment ions. This can be obtained from synthesizing relevant peptides.

Acknowledgments

The authors thank Diatech Korea Inc. (<http://www.diatech.co.kr>) for the generous donation of *o*-TEMPO-Bz-C(O)-NHS, and David Smiley from the Richard DiMarchi lab for synthesizing the peptides. This work was supported by the National Institutes of Health grant R01GM103725, the National Science Foundation grant CHE-1012855, and the Amgen Corporation 2014585127.

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