Published in final edited form as:

J Am Soc Mass Spectrom. 2016 December ; 27(12): 2041–2053. doi:10.1007/s13361-016-1487-9.

Distinguishing aspartic and isoaspartic acids in peptides by several mass spectrometric fragmentation methods

Nick DeGraan-Weber^a, Jun Zhang^b, and James P. Reilly^{a,*}

^aDepartment of Chemistry, Indiana University, 800 East Kirkwood Avenue, Bloomington, Indiana 47405

^bPre-Pivotal Drug Product Technologies, Amgen Inc., Thousand Oaks, California 91320

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, 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.

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

* Address reprint requests to James P. Reilly, Department of Chemistry, Indiana University, 800 East Kirkwood Avenue, Bloomington, Indiana 47405, reilly@indiana.edu.

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 **Scheme S1**, the isoaspartic acid can transfer a hydrogen to the oxygen and form a five membered ring to the carbon of the amide. Electrons rearrange and carbon monoxide is released to form b+H₂O and y-46 product ions [22-24]. While the terminal carboxylic acid typically initiates the loss of the C-terminal residue, observation of b+H₂O 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]. **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.

157 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+H₂O 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].

Free-radical initiated peptide sequencing (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 **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 RAFVXHLYT, 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. The matrix, 4-chloro-α-cyanocinnamic acid (Cl-CCA), was synthesized in-house. (N-Succinimidylloxycarbonylmethyl)tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP) was purchased from Sigma. o-TEMPO-Bz-NHS was obtained from Diatech 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 μL of 0.2 M NaHCO₃ at pH 9 with 20% acetonitrile. The mixture was vortexed and reacted for 30 minutes at room temperature. 200 μL of water were added to the sample to hydrolyze the succinimidyl ester. The mixture reacted for another 30 minutes.

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 resolubilized 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 were 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 through its collision cell, analogous to a previous modification of an ABI 4700 mass spectrometer [48]. 157 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 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. 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 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 seconds.

A Thermo MALDI-LTQ mass spectrometer was employed to analyze α -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_iYR and AFVD_iSLYR were MALDI ionized and fragmented by PSD in a TOF/TOF mass spectrometer. The resulting qualitatively similar mass spectra are displayed in **Figures 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_iSLYR. Although its intensity is low, the expanded regions from 600-625 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, while 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 **Figures 1c** and **1d**. Because of the N-terminal arginines, b-type ions dominate the spectra. A b₄+H₂O ion, which is nearly as intense as the b₄ 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 whose intensity exceeds that of y-46 ions that are formed with arginine at the C-terminus [23]. In the aspartic acid spectrum, a low abundance b₄+H₂O peak at 492 Da appears as intense as the c₄ 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 b₄+H₂O peak, the intensities of the diagnostic ion can be compared with those of neighboring fragments, such as the b₄, c₄, and b₅ 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 **Figures 2a** and **b**. The complete mass spectra are in **Figures S2a** and **b**. Spectra are qualitatively similar to PSD data. However, now aspartic acid also generates a low intensity y₅-46 peak. This may be attributed to a side chain fragmentation of the aspartic acid in the y₅ ion as shown in **Scheme 2**. Unfortunately, this photofragmentation process inhibits identification of isoaspartic acid based on the y₅-46 ion. Interestingly, the side chain fragment ions, w_{a5} and v₅, are observed with low intensity 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 are observed [50]. Although there are differences between the two acids, the effects described are generally of low abundance.

Expanded mass spectra from 400-600 Da for the peptides, RAFVDSL_T and RAFVD_iSL_T, are shown in **Figures 2c** and **d** and the complete spectra are in **Figures S2c** and **d**. The N-terminal arginine facilitates production of a-type ions. a₅ and d_{a5} ions appear in both spectra and cannot distinguish the two isomers. However, similar to PSD, b₄ and b₄+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 succinimide 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.

Peptides AFVDSL_IYR and AFVD_ISLYR were labeled at the N-terminus with TMPP and fragmented by PSD. The mass spectra displayed in **Figures 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 **Scheme S1** that requires no proton to initiate fragmentation. These peptides were also fragmented by photodissociation and their far richer spectra are in **Figures 3c** and **d**. Photodissociation produces a complete series of a-type ions, many d-type ions, and a few b- and c-type ions. While 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 of charge tagged peptides, TMPP-AFVDSL_IYK and TMPP-AFVD_ISLYK, (**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 RAFVDSL_IT and RAFVD_ISLT were also TMPP labeled and fragmented in PSD and photodissociation. Their mass spectra are in **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 **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 AFVD_ISLYR were each electrosprayed; the +2 charged ions were isolated in an ion trap and fragmented by CID. The mass spectra in **Figures 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 y_6^{2+} ion. Other examples displaying fragmentation of multiply charged ions are presented below.

The fragmentation spectra for the doubly charged RAFVDSL^T and RAFVD_iSL^T in **Figures 4c** and **d** are also qualitatively similar. The intensity of the $b_4 + H_2O$ ion in the isoaspartic acid spectrum is lower than that of the adjacent c_4 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 (eg. b_5^+ and b_7^{2+}) again suggesting that there could be energetic differences between these two.

The fact that intense y -46 or $b + H_2O$ 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 $b + H_2O$ and y -46 ions, we 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. **Figures S9** and **S10** shows these results, in which minimal improvements to the $b + H_2O$ 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 **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 y_6^{2+} and y_5 ions are more intense in the isoaspartic acid spectrum in **Figure 4a** than in the aspartic acid spectrum **4b**. Other examples displaying more intense y ions for isoaspartic acid are the y_5 and y_6 ions in **Figures S1c-d**, the y_5 ion in **S11a-b** and **S11c-d**, the y_{12}^{2+} and y_{11} ions in **S13a-b**, and the y_{12}^{2+} ions in **S13c-d**. Likewise, b -type ions that contain aspartic acid tend to be more intense, such as b_5 and b_7^{2+} in **Figures 4c-d**. Other b -type fragments that exhibit this are the b_5 and b_6 ions in **Figures 1a-b**, the b_4 , b_6 , and b_7 ions in **4a-b**, the b_5 , b_6 , b_7 ions in **S1a-b**, the b_2 ion in **S11a-b** and **S11c-d**, the b_{10} , b_{11} , b_{12} , and b_{13} ions in **S12a-b**, and the b_8 , b_9 , $b_{10}/y_9^{\ddagger}$, and $b_{11}/y_{10}^{\ddagger}$ **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 when 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 $b + H_2O$ 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, AFVDSL_YAFVSLYR and AFVD_iSL_YAFVSLYR, by ETD in a Synapt G2s mass spectrometer yielded spectra shown in **Figures 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 **Figures 5c** and **d**, peptides AFVSL_YAFVDSL_YR and AFVSL_YAFVD_iSL_YR produce similar c- and z[•]-type ions, with the addition of z₅-57 and c₆[•]+57 ions in the isoaspartic acid spectrum. This observation is consistent with previous research [27, 29]. Although these c[•]+57 and z-57 ions appear to be low intensity when compared to the MH³⁺ and charge reduced MH²⁺ 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 **Figures 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 (**Figures 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 **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 **Figures 6a** and **b** display some spectral differences. First, a very low intensity z₅-57 peak is present for isoaspartic acid that is not observed for aspartic acid. The z₅[•] ion is also much more intense for isoaspartic acid, which also agrees with previous ETD results [27]. As shown in **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 z₅[•] ion. Mechanistically, there is little difference if aspartic acid is present in the sequence. However, the low abundance z₅[•] ion for aspartic acid may have resulted in a more intense 593 Da z₅[•]-CO₂ fragment which is consistent with a more significant CO₂ neutral loss. The isoaspartic acid peptide forms a 619 Da z₅[•]-H₂O ion instead of losing CO₂. Formation of x₄-H₂O 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⁺-CO₂, z₅[•], and z₅[•]-CO₂ ions were further corroborated in 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 **Figures 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]. **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 630-675 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, d₄₅, 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, RAFVDH_LT and RAFVD_iH_LT, 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 **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 $b+H_2O$ 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 acid and isoaspartic acid containing peptides should be studied to measure variances between fragment ions. This can be obtained from synthesizing relevant peptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are thankful to Diotech Korea Inc. (<http://www.diotech.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 has been supported by the National Institutes of Health grant R01GM103725, the National Science Foundation grant CHE-1012855, and the Amgen Corporation 2014585127.

References

1. Reissner KJ, Aswad DW. Deamidation and isoaspartate formation in proteins: unwanted alterations or surreptitious signals? *Cell. Mol. Life Sci.* 2003; 60:1281–1295. [PubMed: 12943218]
2. Wang W, Singh S, Zeng DL, King K, Sandeep N. Antibody structure, instability, and formulation. *J. Pharm. Sci.* 2007; 96:1–26. [PubMed: 16998873]
3. Roher AE, Lowenson JD, Clarke S, Wolkow C, Wang R, Cotter RJ, Reardon IM, Zürcher-Neely HA, Heinrikson RL, Ball MJ, Greenberg BD. Structural alterations in the peptide backbone of β -amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Bio. Chem.* 1993; 268:3072–3083. [PubMed: 8428986]
4. Kim E, Lowenson JD, MacLaren DC, Clarke S, Young SG. Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc. Natl. Acad. Sci.* 1997; 94:6132–6137. [PubMed: 9177182]
5. Aswad DW, Paranandi MV, Schurter BT. Isoaspartate in peptides and proteins: formation, significance, and analysis. *J. Pharm. Biomed. Anal.* 2000; 21:1129–1136. [PubMed: 10708396]
6. Shimizu T, Watanabe A, Ogawara M, Mori H, Shirasawa T. Isoaspartate formation and neurodegeneration in Alzheimer's disease. *Arch. Biochem. Biophys.* 2000; 381:225–234. [PubMed: 11032409]
7. Shimizu T, Matsuoka Y, Shirasawa T. Biological significance of isoaspartate and its repair system. *Biol. Pharm. Bull.* 2005; 28:1590–1596. [PubMed: 16141521]

8. Fonseca MI, Head E, Velazquez P, Cotman CW, Tenner AJ. The presence of isoaspartic acid in β -amyloid plaques indicates plaque age. *Exp. Neurol.* 1999; 157:277–288. [PubMed: 10364440]
9. Ritz-Timme S, Collins MJ. Racemization of aspartic acid in human proteins. *Ageing Res. Rev.* 2002; 1:43–59. [PubMed: 12039448]
10. Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, Shire SJ, Bjork N, Totpal K, Chen AB. Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J. Chromatogr. B.* 2001; 752:233–245.
11. Cacia J, Keck R, Presta LG, Frenz J. Isomerization of an aspartic acid residue in the complementarity determining regions of a recombinant antibody to human IgE: Identification and effect on binding affinity. *Biochem.* 1996; 35:1897–1903. [PubMed: 8639672]
12. Meinwald YC, Stimson ER, Scheraga HA. Deamidation of the asparaginyl-glycyl sequence. *Int. J. Pept. Protein Res.* 1986; 28:79–84. [PubMed: 3759344]
13. Stephenson RC, Clarke S. Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. *J. Bio. Chem.* 1989; 264:6164–6170. [PubMed: 2703484]
14. Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. *J. Bio. Chem.* 1987; 262:785–794. [PubMed: 3805008]
15. Lura R, Schirch V. Role of peptide conformation in the rate and mechanism of deamidation of asparaginyl residues. *Biochem.* 1988; 27:7671–7677. [PubMed: 3207697]
16. Tyler-Cross R, Schirch V. Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *J. Bio. Chem.* 1991; 266:22549–22556. [PubMed: 1939272]
17. Clarke S. Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int. J. Pept. Protein Res.* 1987; 30:808–821. [PubMed: 3440704]
18. Capasso S, Mazzarella L, Sica F, Zagari A. Deamidation via cyclic imide in asparaginyl peptides. *Pept. Res.* 1989; 2:195–200. [PubMed: 2520758]
19. Patel K, Borchardt RT. Chemical pathways of peptide degradation. II. Kinetics of deamidation of an asparaginyl residue in a model hexapeptide. *Pharmaceut. Res.* 1990; 7:703–711.
20. Robinson NE, Robinson AB. Prediction of protein deamidation rates for primary and three-dimensional structure. *Proc. Natl. Acad. Sci.* 2001; 98:4367–4372. [PubMed: 11296285]
21. Robinson NE, Robinson AB, Merrifield RB. Mass spectrometric evaluation of synthetic peptides as primary structure models for peptide and protein deamidation. *J. Peptide. Res.* 2001; 57:483–493. [PubMed: 11437952]
22. Schindler P, Müller D, Märki W, Groseebacher H, Richter W. Characterization of a β -Asp33 isoform of recombinant Hirudin sequence variant 1 by low-energy collision-induced dissociation. *J. Mass. Spectrom.* 1996; 24:967–974.
23. Yamazaki Y, Fujii N, Sadakane Y, Fujii N. Differentiation and semiquantitative analysis of an isoaspartic acid in human α -crystallin by postsource decay in a curved field reflectron. *Anal. Chem.* 2010; 82:6384–6394. [PubMed: 20669993]
24. González LJ, Shimizu T, Satomi Y, Betancourt L, Besada V, Padrón G, Orlando R, Shirasawa T, Shimonishi Y, Takao T. Differentiating α - and β -aspartic acids by electrospray ionization and low-energy tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2000; 14:2092–2102. [PubMed: 11114015]
25. Cournoyer JJ, Pittman JL, Ivleva VB, Fallows E, Waskell L, Costello CE, O'Connor PB. Deamidation: differentiation of aspartyl from isoaspartyl products in peptides by electron capture dissociation. *Prot. Sci.* 2005; 14:452–463.
26. Cournoyer JJ, Lin C, O'Connor PB. Detecting deamidation products in proteins by electron capture dissociation. *Anal. Chem.* 2006; 78:1264–1271. [PubMed: 16478121]
27. O'Connor PB, Cournoyer JJ, Pitteri SJ, Chrisman PA, McLuckey SA. Differentiation of aspartic and isoaspartic acids using electron transfer dissociation. *J. Am. Soc. Mass Spectrom.* 2006; 17:15–19. [PubMed: 16338146]
28. Sargaeva NP, Lin C, O'Connor PB. Identification of aspartic and isoaspartic acid residues in amyloid β peptides, including A β 1–42, using electron-ion reactions. *Anal. Chem.* 2009; 81:9778–9786. [PubMed: 19873993]

29. Ni W, Dai S, Karger BL, Zhou ZS. Analysis of isoaspartic acid by selective proteolysis with Asp-N and electron transfer dissociation mass spectrometry. *Anal. Chem.* 2010; 82:7485–7491. [PubMed: 20712325]
30. Spengler B. Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules. *J. Mass. Spectrom.* 1997; 32:1019–1036.
31. Papayannopoulos IA. The interpretation of collision-induced dissociation tandem mass spectra of peptides. *Mass Spectrom. Rev.* 1995; 14:49–73.
32. Wysocki VH, Tsaprailis G, Smith LL, Brei LA. Mobile and localized protons: a framework for understanding peptide dissociation. *J. Mass Spectrom.* 2000; 35:1399–1406. [PubMed: 11180630]
33. Gu C, Tsaprailis G, Brei L, Wysocki VH. Selective gas-phase cleavage at the peptide bond C-terminal to aspartic acid in fixed-charge derivatives of Asp-containing peptides. *Anal. Chem.* 2000; 72:5804–5813. [PubMed: 11128940]
34. Thorne GC, Ballard KD, Gaskell SJ. Metastable decomposition of peptide $[M+H]^+$ ions via rearrangement involving loss of the C-terminal amino acid residue. *J. Am. Soc. Mass Spectrom.* 1990; 1:249–257.
35. Zubarev RA, Kelleher NL, McLafferty FW. Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J. Am. Chem. Soc.* 1998; 120:3265–3266.
36. Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proc. Natl. Acad. Sci.* 2004; 101:9528–9533. [PubMed: 15210983]
37. Reilly JP. Ultraviolet photofragmentation of biomolecular ions. *Mass Spectrom. Rev.* 2009; 28:425–447. [PubMed: 19241462]
38. Thompson MS, Cui W, Reilly JP. Fragmentation of singly charged peptide ions by photodissociation at $\lambda=157$ nm. *Angew. Chem. Int. Ed.* 2004; 43:4791–4793.
39. Cui W, Thompson MS, Reilly JP. Pathways of peptide ion fragmentation induced by vacuum ultraviolet light. *J. Am. Soc. Mass Spectrom.* 2005; 16:1384–1398. [PubMed: 15979330]
40. Zhang L, Reilly JP. De Novo Sequencing of Tryptic Peptides Derived from *Deinococcus radiodurans* Ribosomal Proteins Using 157 nm Photodissociation MALDI TOF/TOF Mass Spectrometry. *J. Proteome Res.* 2010; 9:3025–3034. [PubMed: 20377247]
41. Zhang L, Reilly JP. Peptide de Novo Sequencing Using 157 nm Photodissociation in a Tandem Time-of-Flight Mass Spectrometer. *Anal. Chem.* 2010; 82:898–908. [PubMed: 20058881]
42. He Y, Parthasarathi R, Raghavachari K, Reilly JP. Photodissociation of charge tagged peptides. *J. Am. Soc. Mass Spectrom.* 2012; 23:1182–1190. [PubMed: 22532332]
43. Lee M, Kang M, Moon B, Oh HB. Gas-phase peptide sequencing by TEMPO-mediated radical generation. *Analyst.* 2009; 134:1706–1712. [PubMed: 20448941]
44. Lee CS, Jang I, Hwangbo S, Moon B, Oh HB. Side chain cleavage in TEMPO-assisted free radical initiated peptide sequence (FRIPS): Amino acid composition information. *Bull. Korean Chem. Soc.* 2015; 36:810–814.
45. Tao Y, Julian RR. Identification of Amino Acid Epimerization and Isomerization in Crystallin Proteins by Tandem LC-MS. *Anal. Chem.* 2014; 86:9733–9741. [PubMed: 25188914]
46. Jeon A, Lee JH, Kwon HS, Park HS, Moon BJ, Oh HB. Charge-directed peptide backbone dissociations of o-TEMPO-Bz-C(O)-peptides. *Mass Spectrom. Lett.* 2013; 4:71–74.
47. Jeon, A., Hwangbo, S., Ryu, ES., Lee, J., Yun, KN., Kim, JY., Moon, B., Oh, HB. Guanidination of lysine residue improves the sensitivity and facilitates the interpretation of free radical initiated peptide sequencing (FRIPS) mass spectrometry.. *Int. J. Mass Spectrom.* 2015. <http://dx.doi.org/10.1016/j.ijms.2015.06.019>
48. Zhang L, Reilly JP. Peptide photodissociation with 157 nm light in a commercial tandem time-of-flight mass spectrometer. *Anal. Chem.* 2009; 81:7829–7838. [PubMed: 19702244]
49. Paizs B, Suhai S. Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* 2005; 24:508–548. [PubMed: 15389847]
50. Carr SA, Hemling ME, Bean MF, Roberts GD. Integration of mass spectrometry in analytical biotechnology. *Anal. Chem.* 1991; 63:2802–2824. [PubMed: 1789448]

51. He Y, Reilly JP. Does a charge tag really provide a fixed charge? *Angew. Chem. Int. Ed.* 2008; 47:2463–2465.
52. Sun Q, Nelson H, Ly T, Stoltz BM, Julian RR. Side chain chemistry mediates backbone peptide fragmentation in hydrogen deficient peptide radicals. *J. Proteome Res.* 2009; 8:958–966. [PubMed: 19113886]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Scheme 1.

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

Scheme 2.

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

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

Figure 1.

PSD mass spectra for singly charged (A) AFVDSLYR (970.5 Da), (B) AFVD_iSLYR (970.5 Da), (C) RAFVDSLT (908.5 Da), and (D) RAFVD_iSLT (908.5 Da) precursor ions. * denotes loss of NH_3 and $\ddagger$ denotes loss of H_2O .

Figure 2. 157 nm photodissociation expanded mass spectra for singly charged (A) AFVDSLYR (970.5 Da), (B) AFVD_iSLYR (970.5 Da), (C) RAFVDSLTL (908.5), and (D) RAFVD_iSLTL (908.5) precursor ions. * denotes loss of NH_3 .

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

Figure 4.

CID of the doubly charged peptides, (a) AFVDSL YR (485.8 Da), (b) AFVD_iSL YR (485.8 Da), (c) RAFVDSL T (454.7 Da), and (d) RAFVD_iSL T (454.7 Da). ⁺ denotes loss of H₂O.

Figure 5.

ETD mass spectra for triply charged (a) AFVDSLYAFVSLYR (551.0 Da), (b) AFVD_iSLYAFVSLYR (551.0 Da), (c) AFVSLYAFVDSLYR (551.0 Da), and (d) AFVSLYAFVD_iSLYR (551.0 Da) precursor ions. MH^{3+} and MH^{2+} ions are off scale.

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