ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/45658639

Analysis of Isoaspartic Acid by Selective Proteolysis with Asp-N and Electron Transfer Dissociation Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2010

Impact Factor: 5.64 · DOI: 10.1021/ac101806e · Source: PubMed

CITATIONS
36

READS
76

4 AUTHORS, INCLUDING:

Shujia Dai
Sanofi Aventis Group
26 PUBLICATIONS **509** CITATIONS
SEE PROFILE

Barry Karger
Northeastern University
363 PUBLICATIONS **16,808** CITATIONS
SEE PROFILE

Sunny Zhou
Northeastern University
44 PUBLICATIONS **944** CITATIONS
SEE PROFILE

Available from: Shujia Dai
Retrieved on: 29 January 2016

NIH Public Access
Author Manuscript
Anal Chem. Author manuscript; available in PMC 2011 September 1.

Published in final edited form as:
Anal Chem. 2010 September 1; 82(17): 7485–7491. doi:10.1021/ac101806e.

Analysis of Isoaspartic Acid by Selective Proteolysis with Asp-N and Electron Transfer Dissociation Mass Spectrometry

Wenqin Ni[‡], **Shujia Dai**[‡], **Barry L. Karger**, and **Zhaohui Sunny Zhou**[*]
Barnett Institute of Chemical and Biological Analysis and Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, USA

Abstract

A ubiquitous yet underappreciated protein post-translational modification, isoaspartic acid (isoAsp, isoD or β-Asp), generated *via* the deamidation of asparagine or isomerization of aspartic acid in proteins, plays a diverse and crucial role in ageing, as well as autoimmune, cancer, neurodegeneration and other diseases. In addition, formation of isoAsp is a major concern in protein pharmaceuticals, as it may lead to aggregation or activity loss. The scope and significance of isoAsp have, up to now, not been fully explored, as an unbiased screening of isoAsp at low abundance remains challenging. This difficulty is due to the subtle difference in the physicochemical properties between isoAsp and Asp, e.g., identical mass. In contrast, endoprotease Asp-N (EC 3.4.24.33) selectively cleaves aspartyl peptides but not the isoaspartyl counterparts. As a consequence, isoaspartyl peptides can be differentiated from those containing Asp and also enriched by Asp-N digestion. Subsequently, the existence and site of isoaspartate can be confirmed by electron transfer dissociation (ETD) mass spectrometry. As little as 0.5 % of isoAsp was detected in synthetic beta amyloid and cytochrome c peptides, even though both were initially assumed to be free of isoAsp. Taken together, our approach should expedite the unbiased discovery of isoAsp.

Ubiquitous in biological systems and protein pharmaceuticals, isoaspartic acid (isoAsp, isoD or β-Asp) in peptides and proteins arises from either deamidation of asparagine (Asn) or isomerization of aspartic acid (Asp) *via* a common succinimide intermediate, as depicted in Scheme 1.[1–3] Formation of isoAsp inserts an extra methylene group into the protein backbone, resulting in a beta-peptide linkage.[1–4] As a consequence, a “kink” in the protein conformation is generated, potentially leading to altered functions of the protein. For example, isoAsp has been implicated in β-amyloid aggregation and neurodegenerative disorders.[4–6] In most cases, isoAsp formation is associated with loss in protein function. Typically accumulated over time, isoAsp is naturally associated with ageing, perhaps acting as a molecular clock.[2,7] For example, the age-dependent accumulation of isoAsp in numerous proteins, e.g. alpha-A crystallin in the eye lens, has been observed.[8] On the other hand, signaling and regulatory functions have also been reported for isoAsp formation, for example, in the case of fibronectin.[9] Another well studied system is the deamidation of proteins of the Bcl-2 family, critical apoptotic regulators following DNA damage and cancer therapy.[10–11] Furthermore, even present at low abundance, isoAsp may also trigger autoimmune responses,[12–13] likely due to altered antigen presentation.[14] Hence, it is not surprising that all organisms possess mechanisms to reduce the levels of isoAsp, either *via* the action of protein isoaspartic acid methyltransferase (PIMT or PCMT, EC

[*]To whom correspondence should be addressed. z.zhou@neu.edu.
[‡]These authors contributed equally to this work.

SUPPORTING INFORMATION AVAILABLE

Additional spectral and chromatographic data as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

2.1.1.77) or putative proteolytic pathways.[1,15–16] For example, isoAsp formation and repair is central to the survival and germination of plant seeds.[17–18] IsoAsp is certainly much more prevalent and plays a much broader role than currently realized owing to the high occurrence of Asn (~ 4 %) and Asp (~ 5 %) in proteins and the propensity for many of these residues to be converted into isoAsp. As discussed below, approaches that enable unbiased screening for low abundant isoAsp will greatly expedite the systematic analyses of isoAsp in proteomics and basic biology.

Another important area for isoAsp characterization is in the biopharmaceutical industry.[19–22] Once isolated from host cells, protein pharmaceuticals and reagents alike are devoid of repair mechanisms and are thus prone to the accumulation of isoAsp upon long-term storage. In fact, the presence of isoAsp in protein pharmaceuticals is often the norm rather than the exception.[19–22] The effects of isoAsp formation have also been examined in numerous cases, including aggregation and loss of activity.[23–24] In a few cases, isoAsp formation was eliminated by protein engineering.[25] But most commonly, for long-term storage, isoAsp formation is minimized by optimizing formulation conditions, e.g., under mildly acidic conditions and at low temperature.[19,21] However, once administered into patients and thus exposed to physiological conditions (pH ~ 7 and 37 °C), protein pharmaceuticals—particularly those with long circulation time—may generate significant amount of isoAsp; indeed, several recent investigations have shown considerable deamidation of monoclonal antibodies in serum; in one case, around 15 % over 20 days.[22,26] Clearly, these results call for comprehensive *in vivo* studies, as *in vivo* modifications and activities of protein drugs ultimately determine their clinical outcome. Because samples recovered from biological systems are much more heterogeneous and only available in limited amounts, there is again a pressing need for sensitive and facile methods to characterize isoAsp.

Approaches for isoAsp analysis range from instrumental, chemical, enzymatic to immunological.[1–4,27] Since the early days of isoAsp studies, protein isoaspartic acid O-methyltransferase, which methylates isoAsp but not Asp, has been used for the detection and quantitation of isoAsp; the process has been commercialized under the name IsoQuant.[28] We have previously extended this method by trapping the resulting methyl isoaspartate esters, which are intrinsically labile, with hydrazines and various tags, allowing affinity enrichment and orthogonal detection.[29]

Currently, the most frequently employed method for isoAsp analysis in peptides is reversed phase liquid chromatography (RPLC) combined with mass spectrometry (MS). If separation can be achieved, peak assignment is typically inferred from the elution order. However, as others have noted,[30–31] and we also report in this paper, the elution order of these peptides significantly varies depending on the chromatographic conditions, and hence by itself is insufficient for unambiguous assignment. Equally challenging can be mass spectrometric analysis since Asp and isoAsp have identical mass and formal charge. Although different fragmentation patterns have occasionally been observed for the Asp and isoAsp peptides in selected cases *via* collision induced dissociation (CID), high energy CID (HCD) or negative electrospray ionization, the reporter ions are non-specific for isoAsp and highly dependent on sequence and conditions, rendering the assignment unreliable.[27,32–35] As such, reference peptides are usually required in order to identify unambiguously each species.

A recent advance in distinguishing Asp and isoAsp is the application of electron capture dissociation (ECD) or electron transfer dissociation (ETD) mass spectrometry.[27,36–45] As shown previously and illustrated in Scheme S-1, both ETD and ECD can generate a single pair of reporter ions (c+57 and z−57) that are unique to isoAsp.[27,36–45] Using LC-ECD MS, hundreds of human isoAsp peptides have been identified from cell lines.[44] However, many of the initial hits based on the reporter ions for isoAsp were inclusive, and consequently, additional

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

constraints and manual inspection were required for definitive assignment. The difficulty is mainly due to the intrinsic limitations of ETD/ECD MS: the peak intensities for both signature ions of isoAsp are significantly lower than those for the common fragmentation ions, typically around 5 % of the nominal level in ETD/ECD MS/MS spectra.[27,36–45] Indeed, the two peaks characteristic of isoAsp are often indistinguishable from spurious noise peaks. The problem is even more exacerbated when Asp and isoAsp peptides co-elute. It is again to be emphasized that the precursor ions for both isoAsp and Asp have identical mass, so co-eluted Asp peptides essentially dilute the isoAsp species, further reducing the intensities of the characteristic peaks of isoAsp in the MS/MS spectra. As such, isoAsp peptides, particularly of low abundance, are likely to be not observed.

Given the current situation, a method to differentiate isoAsp peptides from the Asp counterparts prior to the LC-MS analysis would be highly desirable. Owing to the different chemical structure of isoAsp *vs* Asp, as illustrated in Scheme S-2, isoAsp linkage has been shown to be refractory to proteolytic cleavage by endoprotease Asp-N (EC 3.4.24.33), an enzyme widely used in protein analysis;[24,46–50] in contrast and importantly, Asp-N efficiently cuts at the N-terminal side of Asp residues.[46–52] Up to now, the resistance of isoAsp to cleavage by Asp-N protease has been explored—albeit only in a handful cases—to identify isoAsp candidates, but not with ECD or ETD analysis.[24,46–50] The underutilization of this straightforward and general sample preparation step may be attributed to several factors, such as whether all isoAsp residues are refractory to Asp-N digestion. However, the major concern up to now has been that the assignment is only tentative due to the uncertainty of incomplete digestion of the Asp species and the lack of direct identification of the isoAsp species.

Altogether, we envisaged that differentiation and enrichment of isoAsp peptides *via* Asp-N digestion (sample preparation) and detection of isoAsp by ETD MS (sample analysis) should lead to definitive identification and quantitative determination of isoAsp peptides, even at trace levels in the presence of the corresponding Asp peptides. As illustrated in Scheme 2, first, Asp-N is utilized to cleave Asp peptides into smaller peptides while the isoAsp peptides remain intact, flagging and enriching the isoAsp species. Next, with little interference from the Asp species, the isoAsp peptides are detected by ETD MS with high confidence. Indeed, as we report herein, low abundant (as low as 0.5 %) isoAsp in beta-amyloid (Aβ) and cytochrome c peptides are detected and quantified, even when isoAsp and Asp peptides are co-eluted. Moreover, for samples that were initially thought to be free of isoAsp, trace amounts of isoAsp was observed, highlighting the utility of the approach for unbiased screening and discovery.

EXPERIMENTAL SECTION

Chemicals

Sequences and abbreviations of the peptides are listed in Table 1. Aβ1-16 and Aβ1-42 were purchased from Bachem (Torrance, CA). Cyt-c, isoCyt-c and isoAβ1-42 were gifts from Drs. Mark Mamula and Hester Doyle of Yale University.[12–13] Sequencing grade endoprotease Asp-N (EC3.4.24.33) from *Pseudomonas fragi* (mutant strain) was purchased from Sigma-Aldrich (Catalog No. P3303, St. Louis, MO), and mass spectrometry grade lysyl endopeptidase (Lys-C) was from Wako (Catalog No. 121-05063, Richmond, VA).

Sample Preparation

Peptide concentrations were determined from the absorbance at 280 nm measured on a NanoDrop UV-vis spectrometer (ND-1000, Wilmington, DE), using extinction coefficients of 1490 and 5500 $M^{-1}{\cdot}cm^{-1}$ for tyrosine and tryptophan, respectively. Separately, Aβ1-42 and isoAβ1-42 were freshly dissolved in 0.16 % ammonium hydroxide at pH 9.5 to a final concentration of 111 μM. Then, each solution was diluted 4-fold into 50 mM ammonium

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

bicarbonate in water and mixed to afford different percentages of isoAsp peptide. Separately, Cyt-c and isoCyt-c were dissolved in 50 mM ammonium bicarbonate in water to a final concentration of 70 μM. Then, the above solutions of Cyt-c and isoCyt-c were mixed to generate different percentages of isoAsp peptide.

Aging of Amyloid Peptide

Aβ1-16 was dissolved in 25 mM ammonium bicarbonate in water at pH 8.3 to a final concentration of 750 μM. The peptide solution was diluted 37.5-fold into 100 mM sodium acetate at pH 4.0 to a final concentration of 20 μM. The solution was then incubated at 37 °C for 1 month and stored at −80 °C.

Protease Digestion

Lys-C and Asp-N were dissolved in 100 mM ammonium bicarbonate in water (pH 8.3) to final concentrations of 0.5 and 0.05 μg/μL, respectively. Each solution of Aβ1-42 and isoAβ1-42 was digested with Lys-C at an enzyme:peptide ratio (w/w) of 1:10 for 6 hrs at 20 °C. All other solutions containing Asp and isoAsp mixtures were treated with Asp-N at an enzyme:peptide ratio (w/w) of 1:40 in 50 mM ammonium bicarbonate in water (pH 8.0) overnight at 37 °C. The samples were then stored at −80 °C.

HPLC Separation of Asp and IsoAsp Containing Peptides

HPLC was performed on a Varian HPLC/UV-Vis system using a Vydac C18 column (4.6 mm i.d. × 250 mm, Grace, Deerfield, IL). Trifluoroacetic acid (TFA, 0.1 %) or 0.1 % formic acid in water were used as mobile phase A, and in acetonitrile as mobile phase B. The flow rate was set at 1.0 mL/min, and the wavelength for UV detection was 220 nm. A linear gradient from 2 % to 40 % mobile phase B over 30 minutes was used to separate Lys-C digest of Aβ1-42 and isoAβ1-42. A gradient from 2% to 30% mobile phase B over 56 minutes was used to separate the peptide mixture of Cyt-c and isoCyt-c.

LC-MS Analysis

LC-MS experiments were performed on an LTQ-XL with ETD mass spectrometer (Thermo Fisher, San Jose, CA), consisting of a linear ion trap with an additional chemical ionization source to generate fluoranthene anions. An Ultimate 3000 nanoLC pump (Dionex, Mountain View, CA) was used to generate the gradient, and a self-packed reversed phase (RP) column (Vydac C18, 300 Å pore and 5 μm particle size, 75 μm i.d. × 10 cm) was coupled online to the mass spectrometer through a nanospray ion source (New Objective, Woburn, MA). Mobile phase A consisted of 0.1 % formic acid in water and mobile phase B of 0.1 % formic acid in acetonitrile. Initially, a linear gradient from 2 % to 10 % mobile phase B over 2 minutes, and then from 10 % to 40 % mobile phase B over 15 minutes was used to separate the mixtures of Cyt-c peptides and Lys-C digest of Aβ1-42 and isoAβ1-42. Due to the co-elution of Asp and isoAsp isoforms using the above condition, a shallower gradient from 2 % to 40 % mobile phase B over 30 minutes, at a flow rate of 200 nL/min, was applied to achieve a near baseline separation of these isoforms. At least three blank runs were used to minimize any carryover effects between sample injections. The temperature of ion transfer tube of the linear ion trap was held at 245 °C, and the electrospray voltage at 2.2 kV. The mass spectrometer was operated in the data-dependent mode to switch automatically between MS (1st scan event), CID-MS^2 (2nd scan event), ETD-MS^2 (3rd scan event). Briefly, a full MS scan at the mass range of 400–2000 m/z was followed by 2 sequential data-dependent MS^2 scans (CID and ETD) of the most abundant precursor ion, with a 3 mass unit isolation width, from the full MS scan. Dynamic exclusion was implemented with 2 repeat counts (repeat duration of 30 seconds, exclusion list 200, and exclusion duration of 30 seconds). The normalized collision energy in CID was set at 28 %. The chemical ionization (CI) source parameters for fluoranthene, such as ion optics,

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

filament emission current, anion injection time (anion target value set at 3×10^5 ions), fluoranthene gas flow, and CI gas flow, were optimized automatically following the standard procedure for tuning the instrument. The reagent transfer multipole RF amplitude was set at 300 V. The duration time of the ion/ion reaction was maintained constant throughout the experiment at 150 ms. The supplemental activation function was integrated into the ETD data acquisition method. To detect and quantify Asp/isoAsp containing peptides, selective reaction monitoring (SRM) was applied to achieve lower detection limits. The multiply charged precursor ions of Asp/isoAsp containing peptides were selected for SRM detection based on the quality of ETD spectra, such as the relatively high response of c_n and z_{l-n} ion series and reporter ions (c_n+57 and z_{l-n}−57, where n is the position of Asp/isoAsp from the N terminus and l is the total number of amino acids in the peptide).

Data Processing

LC-MS/MS data were analyzed using the Xcalibur 2.0 software (Thermo Fisher, San Jose, CA). For all isoAsp and Asp containing peptides, c_n, z_{l-n}, c_n+57, and z_{l-n}−57 ions were extracted using Qual Browser software (Thermo Fisher, San Jose, CA). The peak areas of selected ions were extracted manually to quantify the relative amount of isoAsp peptide and its Asp counterpart.

RESULTS AND DISCUSSION

Distinguishing IsoAsp and Asp Peptides by ETD

We first characterized the synthetic peptides containing either isoAsp or Asp using LC-ETD-MS. For the Cyt-c/isoCyt-c peptides, the ETD spectra from the triply charged precursor ions [m/z = 568.3] of both the isoAsp/Asp forms showed the complete sequence coverage of the peptides by the c and z series ions. For the isoaspartyl peptide, the diagnostic ions, c_5+57 (m/z = 630.3, 1+) and z_{10}−57 (m/z = 1073.6, 1+), were observed, as shown in Figure S-1. Conversely, no such ions were detected from the corresponding aspartyl peptide, as shown in Figure S-1. Comparable results were observed for the peptide fragment [1 DAEFRH(*D/isoD*) SGYEVHHQK16] from Aβ1-42/isoAβ1-42 digested by Lys-C, as shown in Figure S-2. These observations are in agreement with those of others.[27,36–45] For all peptides studied, however, the intensity of the pair of diagnostic ions was found to be considerably lower than other fragmentation ions; for example, the intensity of the z_{10}−57 peak was only about 7 % of the z_{10} peak in the case of isoCyt-c peptide (see Figure 2).

Limit of Detection for IsoAsp Peptide when Co-eluted with Asp Peptide

To simulate complex systems, mixtures of synthetic Asp and isoAsp peptides with varied amounts of isoAsp ranging from 0 to 100 % were prepared and analyzed by LC-ETD-MS. The initial LC separation conditions led to, at best, only partial separation of all isoAsp/Asp isoforms. As discussed earlier, due to the subtle difference between the physiochemical properties of isoAsp and Asp peptide, these peptides often co-elute; one example is shown in Figure 4 in a recent paper.[44] The precursor ions for the matching isoAsp and Asp peptides have identical mass and generate identical c and z ions; hence, the co-eluted Asp peptide essentially dilutes the isoAsp species, further reducing the already low intensities of the z_n−57 and c_{l-n} +57 diagnostic ions of the isoAsp peptide. Indeed, as shown in Figure 1, the ratio of peak area for the z_{10}−57 to z_{10} ions from Aβ1-16 fragment in Lys-C digest of Aβ1-42 peptides exhibited an overall linear correlation with the percentage of the isoAsp peptide in the mixture. However, as expected, the ratio significantly varied from one run to another when isoAsp was low (see the inset in Figure 1). Similar results were obtained for the c_6 and c_6+57 ions, as well for the Cyt-c peptide, see Figures S-3 and S-4. Therefore, the large variation of the peak area ratio makes it difficult to assign isoAsp when the abundance of this form is lower than 5 %; however, this level is commonly found in biological samples or protein pharmaceuticals. [1–4,19–22]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Differentiation and Enrichment of IsoAsp Peptides via Asp-N Digestion

As depicted in Scheme 2, we devised a strategy to differentiate and enrich isoAsp-containing peptides, in which endoprotease Asp-N specifically cleaves Asp but not isoAsp peptides. Figure 2 illustrates the results for the cytochrome c peptides that contained low levels of isoAsp: the ratios of peak areas for the $z_{10}-57$ to z_{10} ions were quite low before Asp-N digestion, but markedly increased after digestion, and in fact, approached that for the synthetic isoAsp peptides (marked by the dashed line), indicating near complete digestion of the Asp species. Similar results were observed for beta-amyloid peptides (see Figure S-5). Significant lowering of the limit of detection for isoAsp makes feasible a broad array of applications, e.g., the discovery of early disease biomarkers present at low abundance and shorten the stability tests of protein pharmaceuticals.

Unbiased Identification of IsoAsp

Of importance in Figures 2 and S-5, after Asp-N digestion, isoAsp was clearly detectable for the synthetic standard Asp peptide (0 % spike) of both beta-amyloid and cytochrome c. This result initially appeared puzzling, but turned out to be an excellent example of the power of our approach. As mentioned in the previous section, the synthetic standard Asp peptides were originally assumed to be free of isoAsp, as the initial LC-ETD-MS analyses did not reveal signature ions above the noise level. In order to determine whether the low level isoAsp was generated during sample preparation, we repeated the experiments in ^{18}O water.[31,40,53–54] No peaks corresponding to ^{18}O isoAsp were detected (data not shown), suggesting isoAsp was present in the original samples and not due to sample preparation factors. The results were consistent with the observation that, under our conditions (pH above 7 and a few hours in solution), isomerization of Asp is negligible.[1–3,7,19] To ascertain the level of isoAsp in the untreated standard peptides, we used ETD MS detection as a guide to optimize LC conditions (the shallower gradient in the Experimental Section) to fully resolve the isoAsp and Asp peptides. The chromatogram for the Aβ1-16 peptide is shown in Figure 3; the small peak (B) following the main peak (A) was identified as the isoAsp form by both ETD-MS and comparison to the relative retention time of the isoAsp standard peptide. Assuming the response factor of isoAsp and Asp isomers are equal in the ETD-MS spectrum, about 0.5 %, 0.7 % and 1.0 % of isoAsp was present in the synthetic peptides, Aβ1-16, Aβ1-42 and Cyt-c (see Figures S-9 and S-10), respectively. The presence of isoAsp species in synthetic Asp peptides is not surprising, since isoAsp formation has been frequently observed in solid-phase peptide synthesis.[55–56]

Specificity of Asp-N

Considering there had been limited comparison by others of the activities of Asp-N on matching isoAsp and Asp peptides,[24,46–50] we tested and found that all synthetic isoAsp peptides were resistant to Asp-N digestion while their Asp counterparts were completely cleaved under the same conditions (see Figure S-8). As illustrated in Scheme S-2, isoAsp introduces an extra methylene group into the peptide backbone and results in a beta-peptide linkage, compared to a typical alpha-peptide linkage rendered by Asp. Moreover, relative to peptide backbones, the stereochemistry of the chiral center next to the amide is inverted when Asp is converted into isoAsp, essentially generating a D-amino acid from the L-Asp as depicted in Scheme S-2. As such, isoAsp linkages have been shown to be resistant to other proteases as well.[57–58] Hence, isoAsp in other peptides are in all likelihood resistant to Asp-N cleavage as well. It should be noted that cleavage at the N-terminal side of glutamic acid (Glu11) in Aβ1-16 was also observed, albeit less than 5 %. Slow cleavage at some glutamyl sites has been reported for Asp-N,[52] but this digestion can be minimized by shorter incubation time, and importantly, the cleavage at glutamate does not affect the analysis of isoAsp.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Elution Order of IsoAsp and Asp Peptides

As discussed in the introduction, assignment of isoAsp and Asp peptides often has been based largely on elution order. The assumption is that isoAsp peptides elute earlier than their corresponding Asp forms on reversed phase liquid chromatography (RPLC).[30–31] However, both beta-amyloid and cytochrome c isoAsp peptides eluted after their Asp counterparts under the LC-MS conditions, see Figure 3 for beta-amyloid peptides and Figure S-6 for cytochrome c peptides. To the best of our knowledge, this is the first report of such a scenario. In addition, similar to reported by others, the elution order was affected by many factors, including the ion pairing reagent in the mobile phase and the packing material.[30–31] As shown in Figures S-6 and S-7, for both pairs of peptides, the isoAsp peptides eluted earlier than the Asp forms in RPLC (monitored by UV) with 0.1% TFA in the mobile phase, while the two forms co-eluted by using 0.1% formic acid in the mobile phase using the same gradient. The above results again emphasize that it is unreliable to assign the isoAsp and Asp forms solely based on the elution order in liquid chromatography.

Detection of IsoAsp in Aged Beta-amyloid Peptide

Finally, we applied our approach to an unknown sample that closely resembled a biological system. Beta-amyloid peptides are generally considered as a major causative factor in Alzheimer's disease (AD).[4–6,58] Noticeable levels of isoAsp have been detected at several positions, e.g., Asp-1 and Asp-7, in beta-amyloid from the senile plaques in the brains of Alzheimer's patients.[4–6] To simulate aging, the Aβ1-16 peptide was incubated at pH 4, 37 °C for a total of one month. As shown in Figure 4, LC-MS analysis of this aged peptide showed three peaks. First, to establish which peptide(s) contained isoAsp, the sample was digested with Asp-N. As shown in Figure 4, peak A disappeared after digestion, indicating this peptide contained an Asp residue at position 7. Interestingly, both peaks B and C remained unchanged, suggesting isoAsp was formed at the 7th position in both peptides. Subsequently, peaks B and C, before and after Asp-N digestion, were analyzed by ETD MS and found to produce the pair of characteristic ions for isoAsp (z_{10}−57 and c_6+57; XIC for the former is shown in Figure 4), confirming both contained isoAsp at the 7th position. In addition, in one of the peptides, the first Asp had the potential to be converted to isoAsp due to the DA sequence; however, the expected signature ions (M−57 and c_1+57) were observed at the near noise level, probably due to the lower fragmentation efficiency in N-terminus of the peptide. It has been reported that isomerization of Asp7 should be faster than that of Asp1, [4–6] so peak B is likely to be D^1AEFRH*isoD*^{7}SGYEVHHQK^{16} and peak C as *isoD*^{1}AEFRH*isoD*^{7}SGYEVHHQK^{16}. In summary, even for this relative simple system, the partially-resolved peaks could not be assigned based on their elution order. On the other hand, the changes in peaks before and after Asp-N treatment were easily observed.

CONCLUSIONS

This paper presents a general workflow for isoAsp analysis that comprises the following steps. First, a sample, e.g., tryptic digest, is treated with endoprotease Asp-N that cleaves Asp. Second, the resulting mixture and the original sample are analyzed by LC-MS to determine all peptides which sequences contain Asp but are resistant to Asp-N cleavage. For this screening step, CID MS rather than ETD MS suffices and multiply charged peptides are not required. Third, candidate isoAsp peptides identified in the second step can be interrogated further by a combination of optimized separation and ETD MS. Such analysis can be performed on either the original sample or Asp-N digest, or both, to further increase the confidence of assignment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Acknowledgments

We thank Drs. Mark Mamula and Hester Doyle for providing standard peptides. This work was supported in part by NIH AI05814 to Z.S.Z. and GM 15847 to B.L.K. This is contribution number 970 from the Barnett Institute.

References

1. Clarke S. Ageing Res Rev 2003;2:263. [PubMed: 12726775]
2. Robinson, NE.; Robinson, AB. Molecular Clocks: Deamidation of Asparaginyl and Glutaminyl Residues in Peptides and Proteins. Althouse Press; Cave Junction, Oregon, USA: 2004.
3. Reissner KJ, Aswad DW. Cell Mol Life Sci 2003;60:1281. [PubMed: 12943218]
4. Shimizu T, Matsuoka Y, Shirasawa T. Biol Pharm Bull 2005;28:1590. [PubMed: 16141521]
5. Roher AE, Esh CL, Kokjohn TA, Castano EM, Van Vickle GD, Kalback WM, Patton RL, Luehrs DC, Daugs ID, Kuo YM, Emmerling MR, Soares H, Quinn JF, Kaye J, Connor DJ, Silverberg NB, Adler CH, Seward JD, Beach TG, Sabbagh MN. Alzheimers Dement 2009;5:18. [PubMed: 19118806]
6. Roher AE, Lowenson JD, Clarke S, Wolkow C, Wang R, Cotter RJ, Reardon IM, Zurcher-Neely HA, Heinrikson RL, Ball MJ, et al. J Biol Chem 1993;268:3072. [PubMed: 8428986]
7. Robinson NE, Robinson AB. Proc Natl Acad Sci U S A 2001;98:944. [PubMed: 11158575]
8. Fujii N, Shimo-Oka T, Ogiso M, Momose Y, Kodama T, Kodama M, Akaboshi M. Mol Vis 2000;6:1. [PubMed: 10706893]
9. Curnis F, Longhi R, Crippa L, Cattaneo A, Dondossola E, Bachi A, Corti A. J Biol Chem 2006;281:36466. [PubMed: 17015452]
10. Cimmino A, Capasso R, Muller F, Sambri I, Masella L, Raimo M, De Bonis ML, D'Angelo S, Zappia V, Galletti P, Ingrosso D. PLoS One 2008;3:e3258. [PubMed: 18806875]
11. Zhao R, Follows GA, Beer PA, Scott LM, Huntly BJ, Green AR, Alexander DR. N Engl J Med 2008;359:2778. [PubMed: 19109573]
12. Doyle HA, Gee RJ, Mamula MJ. Autoimmunity 2007;40:131. [PubMed: 17453712]
13. Mamula MJ, Gee RJ, Elliott JI, Sette A, Southwood S, Jones PJ, Blier PR. J Biol Chem 1999;274:22321. [PubMed: 10428801]
14. Moss CX, Matthews SP, Lamont DJ, Watts C. J Biol Chem 2005;280:18498. [PubMed: 15749706]
15. Lowenson JD, Kim E, Young SG, Clarke S. J Biol Chem 2001;276:20695. [PubMed: 11279164]
16. Kim E, Lowenson JD, MacLaren DC, Clarke S, Young SG. Proc Natl Acad Sci U S A 1997;94:6132. [PubMed: 9177182]
17. Villa ST, Xu Q, Downie AB, Clarke SG. Physiol Plant 2006;128:581.
18. Oge L, Bourdais G, Bove J, Collet B, Godin B, Granier F, Boutin JP, Job D, Jullien M, Grappin P. Plant Cell 2008;20:3022. [PubMed: 19011119]
19. Wakankar AA, Borchardt RT. J Pharm Sci 2006;95:2321. [PubMed: 16960822]
20. Liu H, Gaza-Bulseco G, Faldu D, Chumsae C, Sun J. J Pharm Sci 2008;97:2426. [PubMed: 17828757]
21. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Pharm Res 2010;27:544. [PubMed: 20143256]
22. Correia IR. mAbs 2010;2
23. Wakankar AA, Borchardt RT, Eigenbrot C, Shia S, Wang YJ, Shire SJ, Liu JL. Biochemistry 2007;46:1534. [PubMed: 17279618]
24. Teshima G, Porter J, Yim K, Ling V, Guzzetta A. Biochemistry 1991;30:3916. [PubMed: 2018763]
25. George-Nascimento C, Lowenson J, Borissenko M, Calderon M, Medina-Selby A, Kuo J, Clarke S, Randolph A. Biochemistry 1990;29:9584. [PubMed: 2271602]
26. Liu YD, van Enk JZ, Flynn GC. Biologicals 2009;37:313. [PubMed: 19608432]
27. Yang H, Zubarev RA. Electrophoresis 2010;31:1764. [PubMed: 20446295]
28. Schurter BT, Aswad DW. Anal Biochem 2000;282:227. [PubMed: 10873277]
29. Alfaro JF, Gillies LA, Sun HG, Dai S, Zang T, Klaene JJ, Kim BJ, Lowenson JD, Clarke SG, Karger BL, Zhou ZS. Anal Chem 2008;80:3882. [PubMed: 18419136]
30. Winter D, Pipkorn R, Lehmann WD. J Sep Sci 2009;32:1111. [PubMed: 19360781]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

31. Krokhin OV, Antonovici M, Ens W, Wilkins JA, Standing KG. Anal Chem 2006;78:6645. [PubMed: 16970346]
32. Andreazza HJ, Wang T, Bagley CJ, Hoffmann P, Bowie JH. Rapid Commun Mass Spectrom 2009;23:1993. [PubMed: 19489040]
33. Gonzalez LJ, Shimizu T, Satomi Y, Betancourt L, Besada V, Padron G, Orlando R, Shirasawa T, Shimonishi Y, Takao T. Rapid Commun Mass Spectrom 2000;14:2092. [PubMed: 11114015]
34. Lehmann WD, Schlosser A, Erben G, Pipkorn R, Bossemeyer D, Kinzel V. Protein Sci 2000;9:2260. [PubMed: 11152137]
35. Olsen JV, Macek B, Lange O, Makarov A, Horning S, Mann M. Nat Methods 2007;4:709. [PubMed: 17721543]
36. Chan WY, Chan TW, O'Connor PB. J Am Soc Mass Spectrom 2010;21:1012. [PubMed: 20304674]
37. Cournoyer JJ, Lin C, Bowman MJ, O'Connor PB. J Am Soc Mass Spectrom 2007;18:48. [PubMed: 16997569]
38. Cournoyer JJ, Lin C, O'Connor PB. Anal Chem 2006;78:1264. [PubMed: 16478121]
39. Cournoyer JJ, Pittman JL, Ivleva VB, Fallows E, Waskell L, Costello CE, O'Connor PB. Protein Sci 2005;14:452. [PubMed: 15659375]
40. Li X, Cournoyer JJ, Lin C, O'Connor PB. J Am Soc Mass Spectrom 2008;19:855. [PubMed: 18394920]
41. Li X, Lin C, Han L, Costello CE, O'Connor PB. J Am Soc Mass Spectrom 2010;21:646. [PubMed: 20171118]
42. O'Connor PB, Cournoyer JJ, Pitteri SJ, Chrisman PA, McLuckey SA. J Am Soc Mass Spectrom 2006;17:15. [PubMed: 16338146]
43. Sargaeva NP, Lin C, O'Connor PB. Anal Chem 2009;81:9778. [PubMed: 19873993]
44. Yang H, Fung EY, Zubarev AR, Zubarev RA. J Proteome Res 2009;8:4615. [PubMed: 19663459]
45. Mukherjee R, Adhikary L, Khedkar A, Iyer H. Rapid Commun Mass Spectrom 2010;24:879. [PubMed: 20196189]
46. Kameoka D, Ueda T, Imoto T. J Biochem 2003;134:129. [PubMed: 12944379]
47. Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, Shire SJ, Bjork N, Totpal K, Chen AB. J Chromatogr B Biomed Sci Appl 2001;752:233. [PubMed: 11270864]
48. Lapko VN, Purkiss AG, Smith DL, Smith JB. Biochemistry 2002;41:8638. [PubMed: 12093281]
49. Miesbauer LR, Zhou X, Yang Z, Sun Y, Smith DL, Smith JB. J Biol Chem 1994;269:12494. [PubMed: 8175657]
50. Zhang W, Czupryn JM, Boyle PT Jr, Amari J. Pharm Res 2002;19:1223. [PubMed: 12240950]
51. Hagmann, M-L. Handbook of Proteolytic Enzymes. 2. Barrett, AJ.; Rawlings, ND.; Woessner, JF.; Neil, RD., editors. Vol. 1. Elsevier; 2004. p. 1037
52. Ingrosso D, Fowler AV, Bleibaum J, Clarke S. Biochem Biophys Res Commun 1989;162:1528. [PubMed: 2669754]
53. Xiao G, Bondarenko PV, Jacob J, Chu GC, Chelius D. Anal Chem 2007;79:2714. [PubMed: 17313184]
54. Gaza-Bulseco G, Li B, Bulseco A, Liu HC. Anal Chem 2008;80:9491. [PubMed: 19072263]
55. Mergler M, Dick F, Sax B, Weiler P, Vorherr T. J Pept Sci 2003;9:36. [PubMed: 12587881]
56. Ruczynski J, Lewandowska B, Mucha P, Rekowski P. J Pept Sci 2008;14:335. [PubMed: 17975850]
57. Bohme L, Bar JW, Hoffmann T, Manhart S, Ludwig HH, Rosche F, Demuth HU. Biol Chem 2008;389:1043. [PubMed: 18979629]
58. Bohme L, Hoffmann T, Manhart S, Wolf R, Demuth HU. Biol Chem 2008;389:1055. [PubMed: 18979630]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

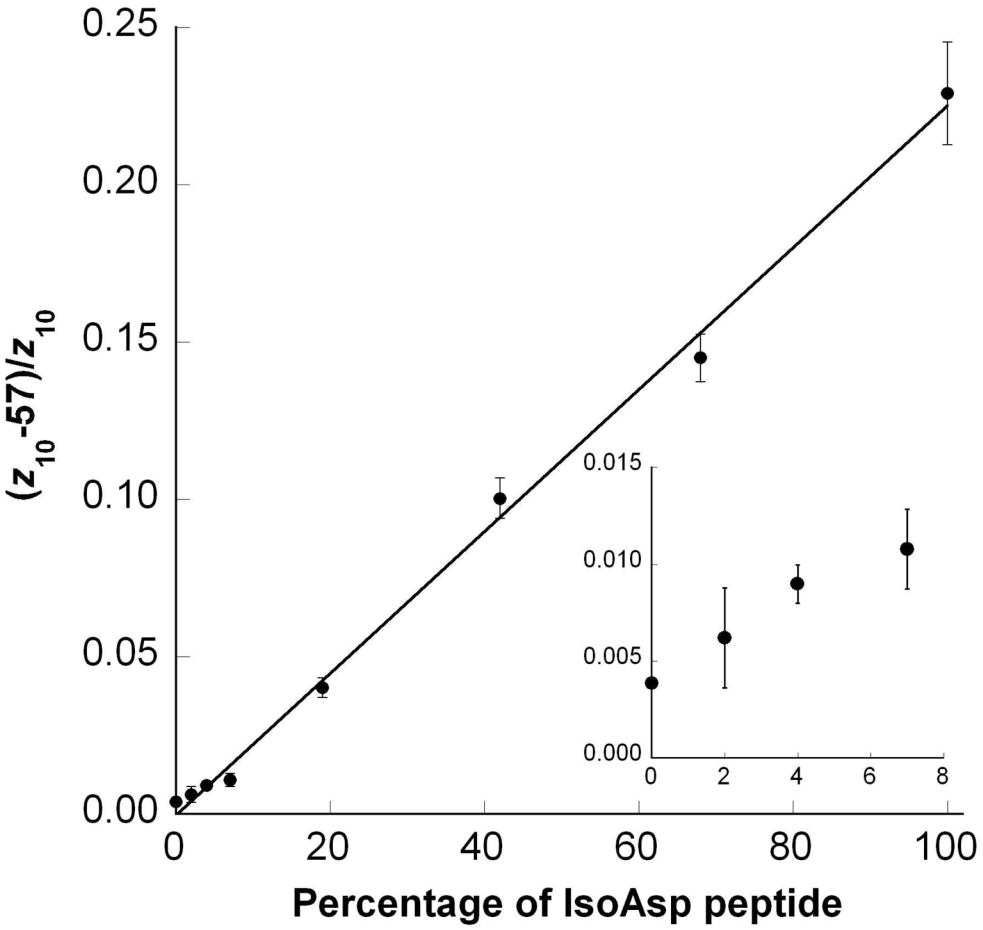


Figure 1.
Correlation between the percentage of isoAsp in Lys-C digests of Aβ1-42 and isoAβ1-42 and the peak area ratio of $(z_{10}-57)/z_{10}$. The inset shows the lower precentage region.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

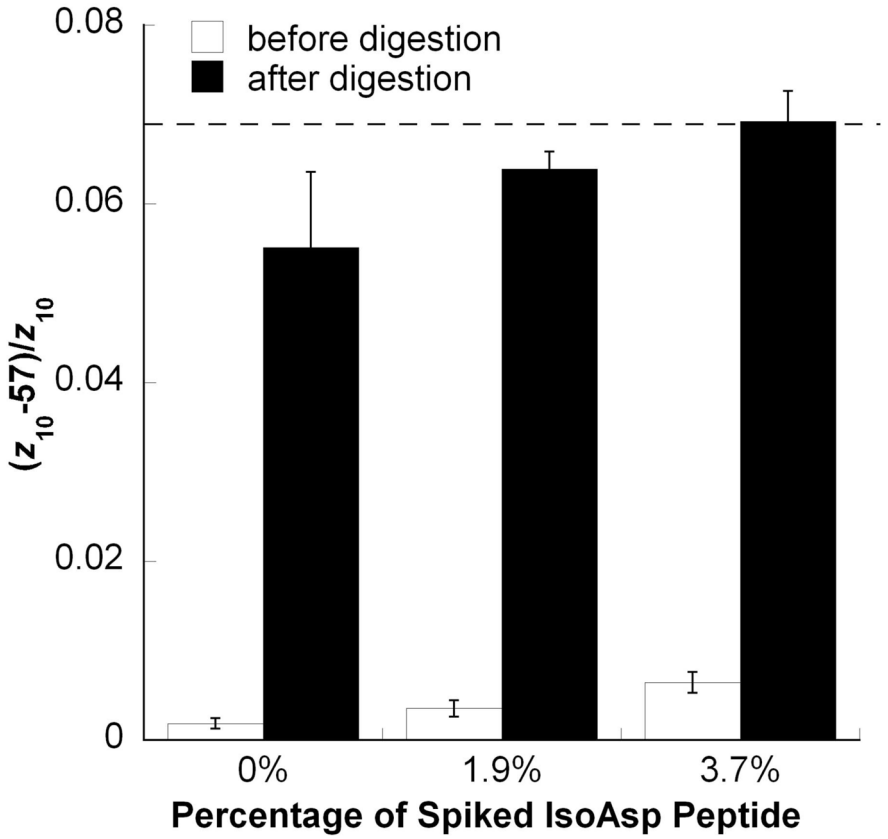


Figure 2.
Enrichment of isoAsp containing peptide in cytochrome c *via* Asp-N digestion. The dashed line indicates the average value of peak area ratios of $(z_{10}-57)/z_{10}$ observed in the synthetic isoCyt-c peptide.

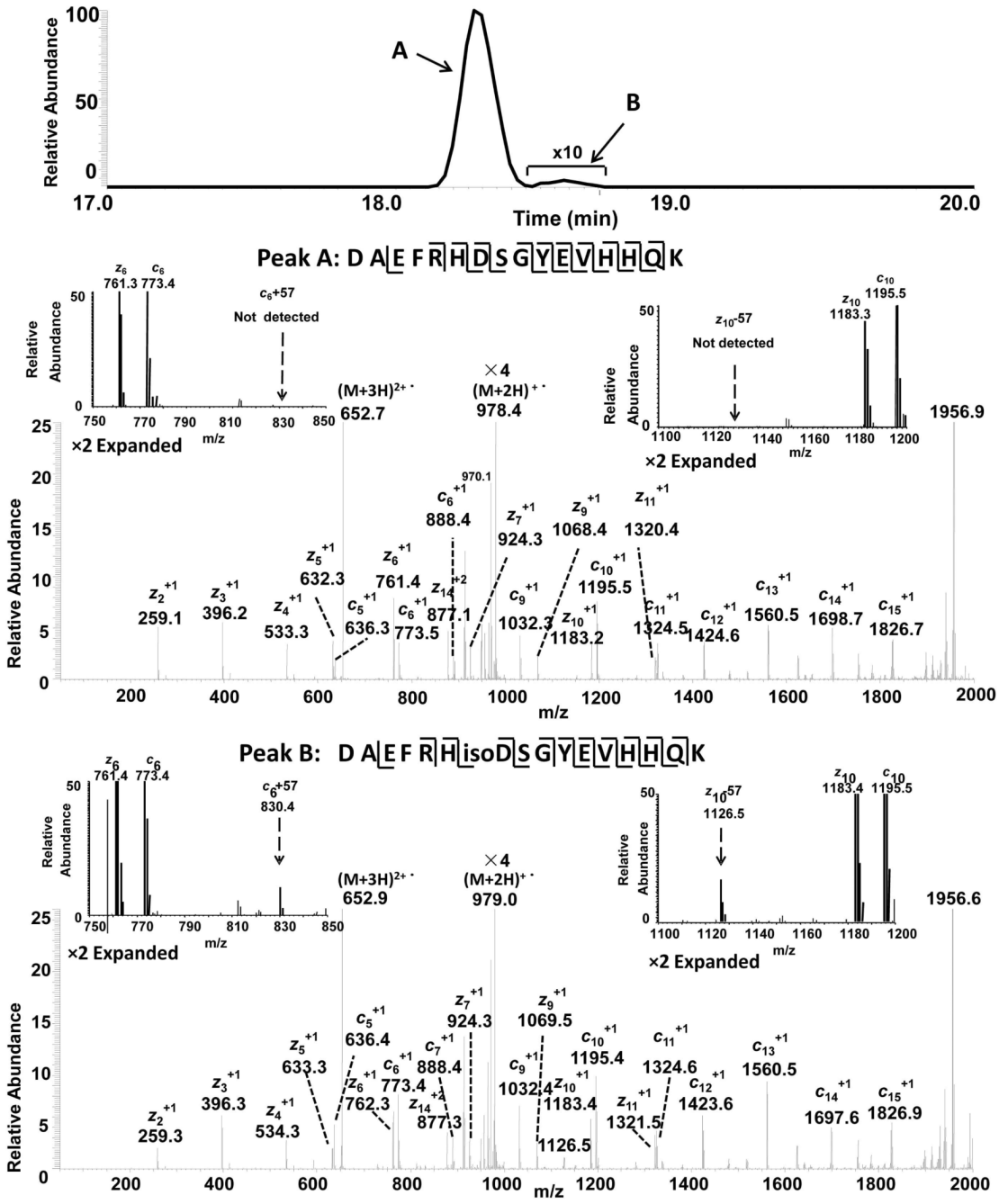


Figure 3.

Extracted ion chromatogram and ETD spectra of the Aβ1-16 peptide. The reporter ions of isoAsp (c_6+57, z_{10}−57) are observed only in Peak B, not in Peak A.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

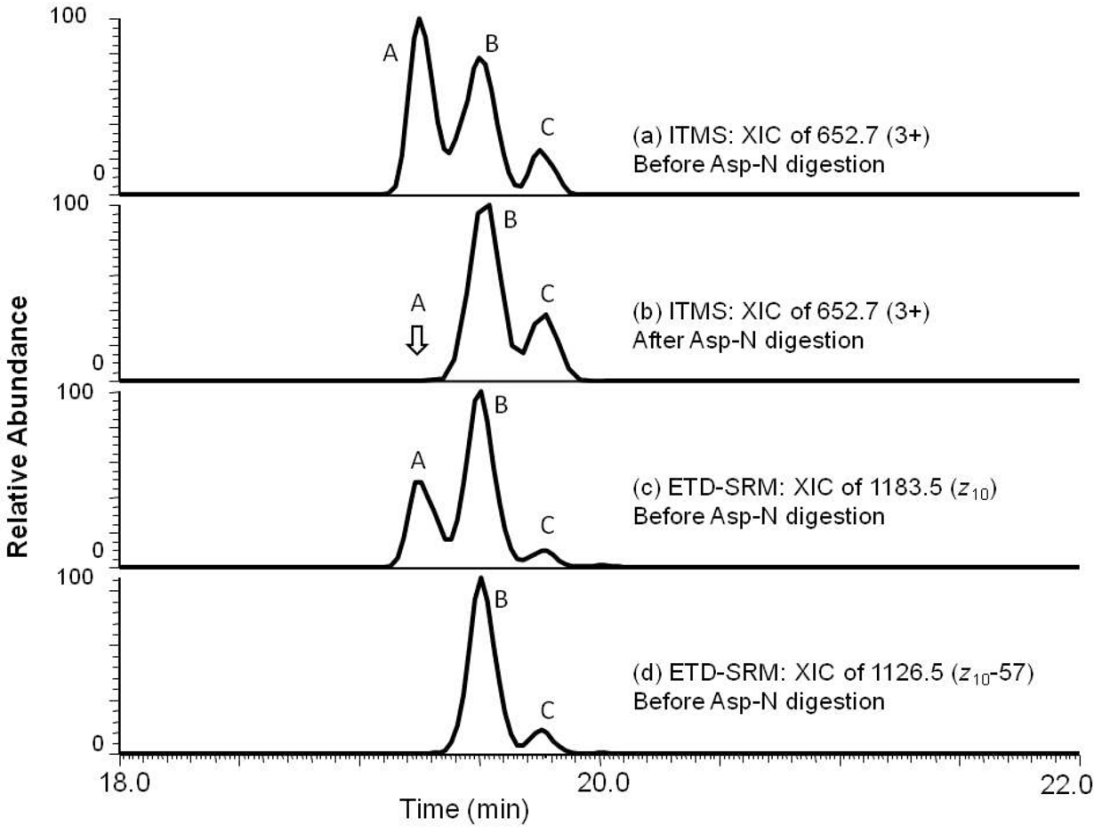
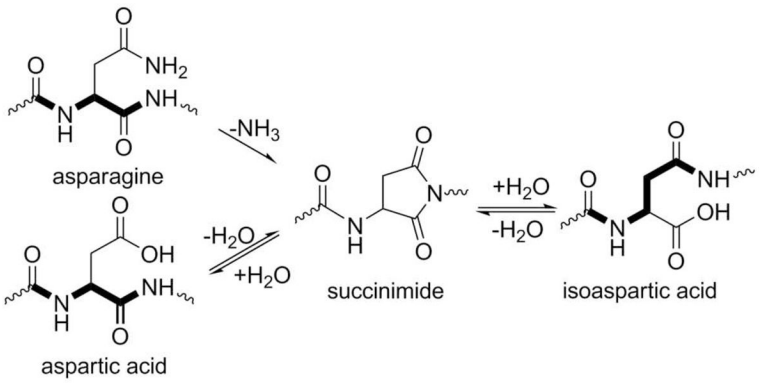


Figure 4.

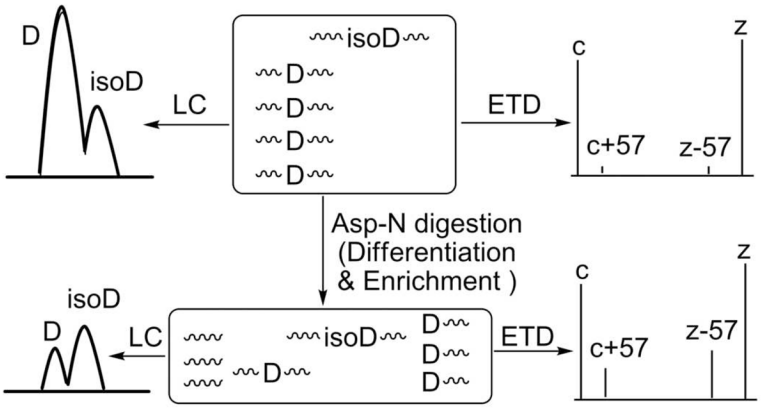
Extracted ion chromatograms of aged Aβ1-16 peptide before and after Asp-N digestion. (a) and (b) show the XICs of the triply charged precursor ion from aged Aβ1-16 peptide before and after Asp-N digestion, respectively; (c) and (d) demonstrate the XICs of product ions, z_{10} and $z_{10}-57$, of aged Aβ1-16 peptide before Asp-N digestion, respectively.



Scheme 1.

Formation of isoaspartic acid *via* deamidation of asparagine or isomerization of aspartic acid. The peptide backbones are highlighted in bold.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript



Scheme 2.

Overview of the approach combining the differentiation and enrichment of isoAsp peptides by Asp-N digestion and the identification of isoAsp species by ETD MS.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Table 1

Sequences and abbreviations of the synthetic peptides.

Peptides	Abbreviations	Sequences
Beta-amyloid (1-42) Asp7	Aβ1-42	DAEFRH**D**SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Beta-amyloid (1-42) isoAsp7	isoAβ1-42	DAEFRH**isoD**SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Beta-amyloid (1-16)	Aβ1-16	DAEFRHDSGYEVHHQK
Cytochrome c (46-60) Asp51	Cyt-c	GFSYT**D**ANKNKGITW
Cytochrome c (46-60) isoAsp51	isoCyt-c	GFSYT**isoD**ANKNKGITW