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Quantitation of asparagine deamidation by isotope labeling and liquid chromatography coupled with mass spectrometry analysis

Hongcheng Liu *, Fengqiang Wang, Wei Xu, Kimberly May, Daisy Richardson

Merck Research Laboratories, Union, NJ 07083, USA

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ABSTRACT

Nonenzymatic asparagine (Asn) deamidation is one of the commonly observed posttranslational modifications of proteins. Recent development of several specific analytical methods has allowed for efficient identification and differentiation of the deamidation products (i.e., isoaspartate [isoAsp] and aspartate [Asp]). Isotope labeling of isoAsp and Asp that are generated during sample preparation by ^{18}O has been developed and can differentiate isoAsp and Asp as analytical artifacts from those present in the samples prior to sample preparation for an accurate quantitation. However, the ^{18}O labeling procedure has a limitation due to the additional incorporation of up to two ^{18}O atoms into the peptide C-terminal carboxyl groups. Variability in the incorporation of ^{18}O atoms into the peptide C-terminal carboxyl groups results in complicated mass spectra and hinders data interpretation. This limitation can be overcome by the dissection of the complicated mass spectra using a calculation method presented in the current study. The multiple-step calculation procedure has been successfully employed to determine the levels of isoAsp and Asp that are present in the sample prior to sample treatment.

Liquid chromatography coupled with mass spectrometry (LC–MS)[1] is an essential approach to study asparagine (Asn) deamidation, with the ultimate goal of identifying the sites of deamidation and quantitating the levels of deamidation products (i.e. isoaspartate [isoAsp] and aspartate [Asp]). The approximate 1-Da increase of isoAsp and Asp as compared with Asn residue can be readily detected by modern mass spectrometers, and the deamidation sites can be localized through tandem mass spectrometry (MS/MS) fragmentation. IsoAsp and Asp can be differentiated by several chemical, instrumental, or enzymatic approaches. Examples of these include Edman sequencing [1,2], fragmentation by high-energy collision-induced dissociation (CID) [3,4], low-energy CID [5–7], electron capture dissociation (ECD) and electron transfer dissociation (ETD) [8–12], specific chemical and enzymatic enrichment [13], specific enzymatic cleavage [14], and protein isoaspartate methyltransferase (PIMT)-catalyzed isotope labeling [15]. However, on the other hand, although significant progress has been made, it remains a challenge to accurately quantify the levels of isoAsp and Asp due to the increased levels of isoAsp and Asp introduced during sample preparations.

Nonenzymatic Asn deamidation can readily occur under the mild conditions used to digest proteins for LC–MS analyses. Depending on the experimental conditions, various levels of isoAsp or Asp have been detected as analytical artifacts [16–20]. The common approaches to reduce the amounts of isoAsp and Asp introduced during sample preparations include shortened digestion time and digestion at lower temperature and at lower pH [18,19,21]. The goal of those approaches is to eliminate or reduce deamidation artifacts to a level that does not significantly contribute to the total isoAsp and Asp detected by LC–MS. An alternative approach, isotope labeling of deamidation products by carrying out sample preparations in ^{18}O-enriched water, has been established to differentiate deamidation artifacts [17,18]. The molecular weight increase of isoAsp and Asp introduced during sample preparation is 3 Da, whereas the molecular weight increase of isoAsp and Asp that are present in the samples originally is 1 Da. In theory, the 2-Da difference should be sufficient to differentiate isoAsp and Asp formed either prior to or during sample preparation. However, during sample preparation, proteases can simultaneously catalyze the incorporation of up to two ^{18}O atoms into the peptide C-terminal carboxyl groups [22–24], resulting in complicated mass spectra containing multiple overlapping isotopic peak series that hinder data interpretation.

Previously, a method was established to use *b*-ion mass spectra from fragmentation of the isoAsp- and Asp-containing peptides to

* Corresponding author.
E-mail address: hongcheng.liu@merck.com (H. Liu).

[1] *Abbreviations used:* LC–MS, liquid chromatography coupled with mass spectrometry; Asn, asparagines; isoAsp, isoaspartate; Asp, aspartate; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; ECD, electron capture dissociation; ETD, electron transfer dissociation; PIMT, protein isoaspartate methyltransferase; DTT, dithiothreitol; TFA, trifluoroacetic acid; EIC, extracted ion chromatogram; TIC, total ion chromatogram.

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calculate the percentage of deamidation that occurs prior to or during sample preparations [25]. Because *b*-ion mass spectra contain only amino acid information for the peptide N terminus, interference from variation in the incorporation of ^{18}O atoms into the peptide C-terminal carboxyl groups was eliminated. In the current study, the method of ^{18}O labeling was revisited. A multiple-step calculation procedure was established to determine the levels of isoAsp and Asp that are present in the sample by subtracting analytical artifacts. With the new calculation procedure, therefore, the labeling method can be generally applied to accurately determine the level of Asn deamidation in proteins.

Materials and methods

Materials

The recombinant monoclonal antibody was produced and purified at Merck (Union, NJ, USA). Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide, urea, and ^{18}O-enriched water (97.1% purity) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, formic acid, and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sequencing-grade trypsin was purchased from Worthington (Lakewood, NJ, USA).

Trypsin digestion

Antibody samples were first buffer exchanged into 100 mM ammonium bicarbonate prepared in ^{18}O-enriched water using Zeba spin desalting columns (Thermo Scientific, Rockford, IL, USA). Zeba spin columns were washed and equilibrated four times (150 µl each time) using the ammonium bicarbonate buffer before sample loading. The collected samples were buffer exchanged again using new Zeba spin columns that were also washed and equilibrated using the same buffer. Stock solutions of 1 M DTT, 8 M urea, and 0.5 M iodoacetamide were prepared in ^{18}O-enriched water. The buffer-exchanged samples were denatured and reduced at 37 °C for 30 min using 10 mM DTT in the presence of 6 M urea in the ammonium bicarbonate buffer and then alkylated using 30 mM iodoacetamide at 37 °C for 30 min. The samples were buffer exchanged again using Zeba spin columns into 100 mM ammonium bicarbonate prepared in ^{18}O-enriched water. Trypsin was added to each sample based on the assumption of 100% sample recovery at each buffer exchange step to a final ratio of 1:10 (trypsin/antibody, w/w) and then incubated at 37 °C for 8 h. A normal water control was included by treating samples following the same procedures except using buffers and reagents prepared in normal water.

LC–MS and LC–MS/MS

An Agilent Infinity 1290 HPLC device (Santa Clara, CA, USA) equipped with a Proto C18 column (250 × 1 mm i.d., 5 µm particle size, Higgins Analytical, Mountain View, CA, USA) were coupled with an Agilent 6538 UHD Q-TOF mass spectrometer to analyze the peptides. Approximately 20 µg of each sample was loaded onto the column at the initial conditions of 95% mobile phase A (0.02% TFA and 0.08% formic acid in water) and 5% mobile phase B (0.02% TFA and 0.08% formic acid in acetonitrile). The peptides were eluted off the column by first increasing mobile phase B to 10% in 50 min and then to 35% mobile phase B in 140 min. The column was washed using 95% mobile phase B and then equilibrated using 5% mobile phase B before the next injection. The flow rate was 50 µl/min. The column oven was heated at 60 °C. The mass spectrometer was operated in positive ion mode with a full mass scan from *m*/*z* 200 to 2000.

Results and discussion

Analysis of peptide 1

Mass spectra of peptide 1

The amino acid sequence of peptide 1 is SINSATHYAESVK, which contains one Asn residue and is the only potential deamidation site. A typical extracted ion chromatogram (EIC) of peptide 1 is shown in Fig. 1. Digestion of the antibody in either normal water or ^{18}O-enriched water did not impact the peak profiles or the relative percentage of various peaks. Mass spectra of the doubly charged ions from the three peaks are shown in Fig. 2. Clearly, the molecular weight of the peptides for peaks 1 and 3 is approximately 1 Da higher than that for peak 2, suggesting deamidation of the Asn residue. Deamidation at the only Asn residue was further confirmed by MS/MS experiments (data not shown). Based on their relative retention times, the peaks were assigned as isoAsp for peak 1, Asn for peak 2, and Asp for peak 3. The relative percentages of isoAsp and Asp were determined to be 5.8 ± 0.11% for isoAsp and 1.8 ± 0.07% for Asp based on integration of the EICs from three experiments. As discussed later, these percentages include isoAsp and Asp that were originally present in the samples and isoAsp and Asp that were generated during sample treatment.

Mass spectra of the doubly charged ions of the same peptide from digestion of the antibody in ^{18}O-enriched water are shown in Fig. 3. As expected, much more complicated mass spectra were obtained from each of the three peaks. The peak identities are summarized in Table 1 of the supplementary material. In brief, the mass spectrum of the Asn-containing peptide contains two overlapping peak series (i.e., incorporation of either one [*m*/*z* 704.85] or two [*m*/*z* 705.85] ^{18}O atoms into the peptide C-terminal carboxyl groups). Each mass spectrum of the isoAsp or Asp peptide contains four overlapping peaks (i.e., two peptides with the incorporation of one C-terminal ^{18}O atom with deamidation either prior to [*m*/*z* 705.34] or during [*m*/*z* 706.34] sample treatment and two peptides with the incorporation of two C-terminal ^{18}O atoms with deamidation either prior to [*m*/*z* 706.34] or during [*m*/*z* 707.34] sample treatment).

Inspection of the mass spectra in Figs. 2 and 3 clearly indicates the incorporation of either one or two ^{18}O atoms into the peptide C-terminal groups and one ^{18}O-atom to the deamidation site during sample preparation. First, the extent of incorporation of ^{18}O atoms into the peptide C-terminal group can be analyzed by comparing the Asn mass spectra from digestion of the antibody in either normal water or ^{18}O-enriched water (Figs. 2 and 3, Asn). The molecular weight increase of 2 Da (*m*/*z* difference by 1 Da) in the Asn mass spectrum from digestion in ^{18}O-enriched water (Fig. 3, Asn) compared with the Asn mass spectrum from digestion in normal water (Fig. 2, Asn) is due to the incorporation of one ^{18}O atom into the peptide C-terminal carboxyl group. Incorporation of

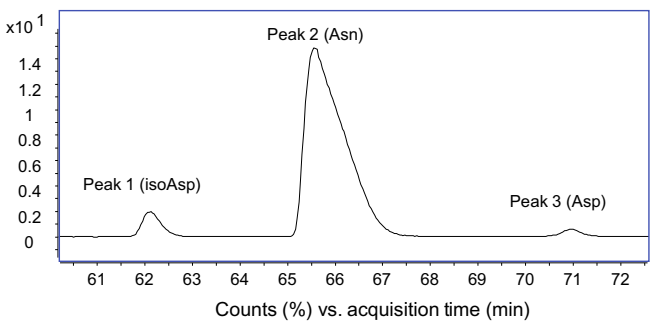

Fig.1. EIC of peptide 1 obtained from digestion of the antibody in normal water. The peaks are assigned as isoAsp (peak 1), Asn (peak 2), and Asp (peak 3).