Video Article

LERLIC-MS/MS for In-depth Characterization and Quantification of Glutamine and Asparagine Deamidation in Shotgun Proteomics

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Abstract

Characterization of protein deamidation is imperative to decipher the role(s) and potentialities of this protein posttranslational modification (PTM) in human pathology and other biochemical contexts. In order to perform characterization of protein deamidation, we have recently developed a novel long-length electrostatic repulsion-hydrophilic interaction chromatography-tandem mass spectrometry (LERLIC-MS/MS) method which can separate the glutamine (Gln) and asparagine (Asn) isoform products of deamidation from model compounds to highly complex biological samples. LERLIC-MS/MS is, therefore, the first shotgun proteomics strategy for the separation and quantification of Gln deamidation isoforms. We also demonstrate, as a novelty, that the sample processing protocol outlined here stabilizes the succinimide intermediate allowing its characterization by LERLIC-MS/MS. Application of LERLIC-MS/MS as shown in this video article can help to elucidate the currently unknown molecular arrays of protein deamidation. Additionally, LERLIC-MS/MS provides further understanding of the enzymatic reactions that encompass deamidation in distinct biological backgrounds.

Video Link

The video component of this article can be found at https://www.jove.com/video/55626/

Introduction

Deamidation is a protein posttranslational modification (PTM) that introduces a negative charge to the protein backbone through modification of asparagine (Asn) and/or glutamine (Gln) residues[1]. This modification while affecting Asn residues generates the isomeric products isoaspartic acid (isoAsp) and *n*-aspartic acid (Asp) at a common 3:1 ratio[2]. Notwithstanding, this ratio can be altered by the intervention of the repairing enzyme L-isoaspartyl methyltransferase (PIMT)[3,4]. Similarly, deamidation of Gln residues generates the isomeric gamma-glutamic acid (γ-Glu) and alpha-glutamic acid isoforms (α-Glu) at an expected 1:7 ratio[3,5], but this ratio can be shifted by the action of the ubiquitous enzyme transglutaminase 2 and other transglutaminases, including transglutaminase 1, an enzyme recently identified as associated with extracellular vesicles in the brain[6].

The origin of deamidation can be either spontaneous or enzymatic, the former is especially common on Gln residues in which transglutaminases and other enzymes mediate inter/intra-molecular crosslinking via transamidation (see [3] for further details on Gln transamidation and its implications in several chronic and fatal human diseases). Therefore, deamidation is a PTM that has a crucial repercussion on the structure and function of affected molecules[4,7,8] and requires an in-depth chemical characterization[3] in the light of its diverse biochemical consequences including its service as molecular clock of aging[9].

Although deamidation of Asn residues has been relatively well-characterized by bottom-up shotgun proteomics[1,10], deamidation of Gln residues still does not have a suitable characterization method beyond the challenging analysis of model compounds by electron-based radical fragmentation[11]. We have recently developed a novel one-dimension shotgun proteomics strategy (LERLIC-MS/MS)[3] that enables separation of Gln and Asn deamidation isoforms from complex biological samples and model compounds in a single analysis. LERLIC-MS/MS is based on the separation of tryptic digested peptides using a long-length (50 cm) ion exchange column (LAX) working on electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) mode and coupled to tandem mass spectrometry (LC-MS/MS). This new analytical strategy has been used to characterize and relatively quantitate the extent of each deamidated residue in human brain tissues[3]. Nevertheless, the protocol outlined here will provide video imaging of LERLIC-MS/MS aimed to study the peculiarities of protein deamidation in the biochemical context of interest.

ETHICS STATEMENT

All procedures of this protocol have been approved by the institutional review board of the Nanyang Technological University in Singapore and have been performed in accordance to the institutional guidelines.

Protocol

1. Packing the Long-length Anion-exchange (LAX) Capillary Column

(Note: Although the LAX column can be in-home packed as we describe in this protocol, LAX columns are also commercially available, see **Table of Materials and Reagents** for further details).

1. Suspend 50 mg of weak anion exchange packing material in 3.5 mL of *packing buffer* (**Table 1**) to prepare the slurry.
2. Assemble the end of the capillary column (50 cm length - 200 µm internal diameter (ID) tubing) using a female-to-female fitting, a ferule and a female nut. Place a screen 1/16" OD of 1 micron pore size inside the female-to-female fitting. (Note: The screen used here must have smaller pore size than the particle size of the packing material to prevent leakage of the material from the column).
3. Pack the capillary with the slurry using a pressure pump operated at 4,500 psi. (Note: Pack the column until the packing material is visible at the entry of the column).
4. Assemble the other end of the capillary column as described in point 1.2.

2. Sample Preparation

This protocol outlines the application of LERLIC-MS/MS to analyze human brain tissues as model proteome. (Note: In case to use other tissues or proteomic samples, the sample preparation procedures should be adapted.)

1. Tissue homogenization:
   a. Wash brain tissues (50 to 100 mg) with 1x phosphate buffer solution for five minutes thrice.
   b. Homogenize the tissue at 1:1:2.5 tissue/metallic beads/*SDC homogenization buffer* (**Table 2**) ratio (w/w/v) for 5 min at 4 °C in safe-lock tubes at maximum intensity using a tissue homogenizer. (Note: SDC homogenization buffer may include protease inhibitors as previously indicated in[3])
   c. Centrifuge the tissue homogenate, obtained from the previous step, at 10,000 × g, 4 °C for 10 min and collect the supernatant into a new 1.5 mL tube.
   d. Repeat steps *b-c* for the remaining pellets and combine the supernatants as many times as necessary until no pellet is observed. (Note: If you have to repeat the steps *b-c* more than two times, move the sample and the beads to a new safe-lock tube to prevent the loss of sample during the centrifugation step).
   e. Quantify the protein concentration of the obtained homogenates by Bicinchoninic Acid assay (BCA)[12].
2. Sodium deoxycholate (SDC)-assisted in-solution tryptic digestion[13] of brain homogenates.
   a. Add a final concentration of 10 mM dithiothreitol (DTT) (using the stock solution indicated in *Solution 5* of **Table 3**) to the obtained homogenate to initiate the reduction of protein disulfide bonds.
   b. Incubate the homogenate during 30 min in a bath pre-set at 60 °C.
   c. Add to the homogenate a final concentration of 20 mM iodoacetamide (IAA) using the stock solution indicated in *Solution 7* of **Table 4**.
   d. Incubate the homogenate containing IAA at room temperature in the dark during 45 min.
   e. Dilute the brain homogenate two-folds using *dilution buffer* (*Solution 6* of **Table 3**).
   f. Incubate the sample for a second reduction step during 30 min at 37 °C.
   g. Add sequencing-grade-modified trypsin dissolved in *Solution 2* of **Table 2** at protein-to-enzyme ratio 1:50 (w/w).
   h. Digest the homogenate with trypsin by overnight incubation at 30 °C.
   i. Quench the enzymatic digestion on the next day by adding 0.5% final concentration of formic acid (FA). (Note: Addition of FA will cause precipitation of the SDC salts under acidic conditions.)
   j. Gently vortex the samples containing SDC precipitates.
   k. Pellet down the SDC salts by centrifuging the samples at 12,000 × g, 4 °C during 10 min.
   l. Collect the supernatant and transfer the liquid to a clean new tube. (Note: Take care during the pipetting of this step to do not re-suspend and/or collect salts from the SDC pellet.)
   m. Re-dissolve the SDC pellet in *SDC re-dissolving buffer* (**Table 5**) under vigorous vortexing for 1 min.
   n. Repeat steps *j-l* twice to recover precipitated peptides and combine the supernatants. (Note: See Serra *et al.* 2016[13] for further details on the SDC-assisted in-solution tryptic digestion protocol adapted here.)
3. Desalting of tryptic digested samples.
   a. Perform desalting of digested samples using a 1g C-18 cartridge. (Note: The use of a big volume cartridge (5 mL) independently of the amount of protein obtained guarantees proper cleaning of remaining SDC salts in the samples prior to LC-MS/MS injection.)
   b. Perform conditioning of the 1g C-18 cartridge with 5 mL of acetonitrile (ACN).
   c. Pass 5 mL of *clean-up buffer* (**Table 6**) by the conditioned 1g C-18 cartridge to remove any remaining organic solvent.
   d. Load the sample to the 1g C-18 cartridge.
   e. Perform 3 - 5 clean-up steps to the sample in the 1g C-18 column using 5 mL of *clean-up buffer* each step.
   f. Elute the desalted peptides from the 1g C-18 cartridge using 5 mL of *elution buffer* (**Table 7**).
   g. Dry the eluted peptides in a vacuum concentrator.
   c. Reconstitute the dried sample in a final volume of 200 µL of *elution buffer*. (Note: Use vigorous and long (> 10 min) vortexing followed by subsequent sonication in a sonication bath during 30 min to completely re-suspend the dried peptides in injection buffer.)
   d. Adjust the injection volume of the sample for LERLIC-MS/MS to analyze between 1 to 3 µg of protein.

3. One-dimension LERLIC-MS/MS Separation

1. Liquid chromatography conditions:

Mobile phases:
A: 0.1% FA in water (**Table 8**).
B: 0.1% FA in ACN (**Table 9**)
Flow rate: 0.4 µL/min
Column: LAX capillary column (50 cm length - 200 µm ID)
a. Use the following 1200 min-gradient: 95% B for 40 min, 95 − 85% B for 434 min, 85 − 70% B for 522 min, 70 − 35% B for 124 min, 35 − 3% B for 45 min, isocratic at 3% B for 5 min, 3 – 95% B over 7 min and kept isocratic at 95% B for 23 min.
b. Perform separation of peptides using the LAX capillary as indicated in Serra & Gallart-Palau *et al.*[3] using ultra-high pressure liquid chromatography.

2. Mass spectrometer configuration:
a. Configure the scan event details to perform data acquisition alternating between full Fourier transform-mass spectrometry (FT-MS) and Fourier transform-tandem mass spectrometry (FT-MS/MS) with the following parameters:
a.1. Data acquisition mode: positive
a.2 FT-MS parameters:
Mass range: 350 − 2,000 m/z
Resolution: 60,000
Microscans: 1 per spectrum
Automatic gain control: 1×10^6
a.3 FT-MS/MS parameters:
Fragmentation mode: High-energy collisional dissociation (HCD)
a.4 Mass range: 150 − 2,000 m/z
a.5 Resolution: 30,000
a.6 Top N ions: 10
Microscans: 1 per spectrum
Charge state: > 2+
Isolation width: 2 Da
a.7 Automatic gain control: 1×10^6
b. Perform mass spectrometry detection of peptides as indicated in[3] using an orbitrap mass spectrometer equipped with a nanoelectrospray ion source working at 1.5 kV.

4. Data Analysis

1. Perform protein database search to the LERLIC-MS/MS obtained data using the following parameters using a specific proteomic software: (Note: See[3] for further details.)
a. Ion tolerance: 10 ppm
b. Fragment ion tolerance: 0.05 Da
c. False Discovery Rate (FDR): 1%
d. Database: UniProt Human database
e. Fixed modifications: Carbamydomethylation at Cys
f. Variable modifications (if required by the software): Oxidation (Met), Deamidation (Asn and Gln).
2. Confident characterization and quantification of isomeric deamidated products in LERLIC-MS/MS data:
a. Export database search results from LERLIC-MS/MS to an spreedsheet software for analysis.
b. Find and extract the whole list of deamidated peptides and their non-deamidated counterparts.
c. Using the list of the obtained peptides as reference, extract the ion chromatograms (XICs) of these peptides using an appropiate software at 5 ppm of mass tolerance. (Note: See[3] for further details on the software used for the extraction of XICs.)
d. Visually inspect the obtained XICs to identify the separated double-peak elution of the isomeric products as indicated[3]. (Note: Isomeric products of those peptides identified at MS/MS level can be easily found guided by the presence of two different retention times in the database search result.)
e. Relative quantification of the isomeric products from each deamidated site/peptide has to be performed based on the peak area of each identified isomer in the obtained XICs.

Representative Results

Deamidation of Gln and Asn residues is considered a degenerative protein modification (DPM) implicated in several chronic and fatal diseases[14]. It has been demonstrated that this PTM can predict the half-life and degradative states of antibodies and other molecules in the human body and similar biological backgrounds[1,15]. The significance of protein deamidation, in fact, goes beyond the biomedical context, hence this modification is present in diverse proteomes[16,17] and some studies have demonstrated the potential of deamidation at the time to perform accurate dating of paintings and archeological remains[18,19]. Although significance and functionality of protein deamidation in diverse backgrounds have been affirmed for long time, there was until very recently a lack of technical advance on the way to achieve an in-depth characterization of this PTM[3].

Application of LERLIC-MS/MS[3], as depicted in **Figure 1,** provides for the first time and in a single run resolved separation of Gln and Asn deamidated isoforms from complex proteomes or model compounds even for those peptides showing two independent Asn and Gln deamidated proteoforms.

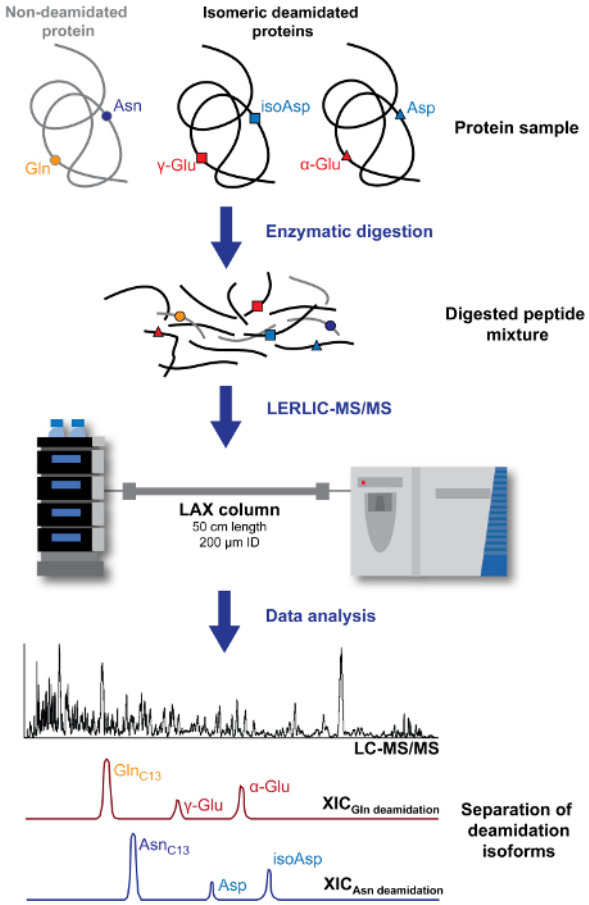

Figure 1: Diagram of LERLIC-MS/MS Workflow for the Separation and Quantification of Gln and Asp Deamidation Products from Complex Samples by Shotgun Proteomics. LERLIC-MS/MS involves protein extraction and enzymatic digestion prior to one-dimension separation of peptides by LC-MS/MS using the LAX capillary column. Analysis of XICs allow the identification of deamidation isomeric products eluted at different retention times based on their different acidity[3,20,21]. MS/MS allows discrimination between deamidated and non-deamidated peptides and confident identification of the deamidated residue. Please click here to view a larger version of this figure.

Enzymatically modified intermediate Gln residues of transamidation displaying an inverted γ/α-glutamyl ratio (**Figure 2**) can be uncovered and characterized by LERLIC-MS/MS, which might have significant implications on the study of proteinopathy in neurodegenerative diseases[3,22,23,24]. Furthermore, as previously reported in Serra & Gallart-Palau 2016, several unknown PIMT substrate proteins presenting an inverted isoAsp/Asp ratio in deamidated Asn residues can also be identified in human tissues by LERLIC-MS/MS[3].

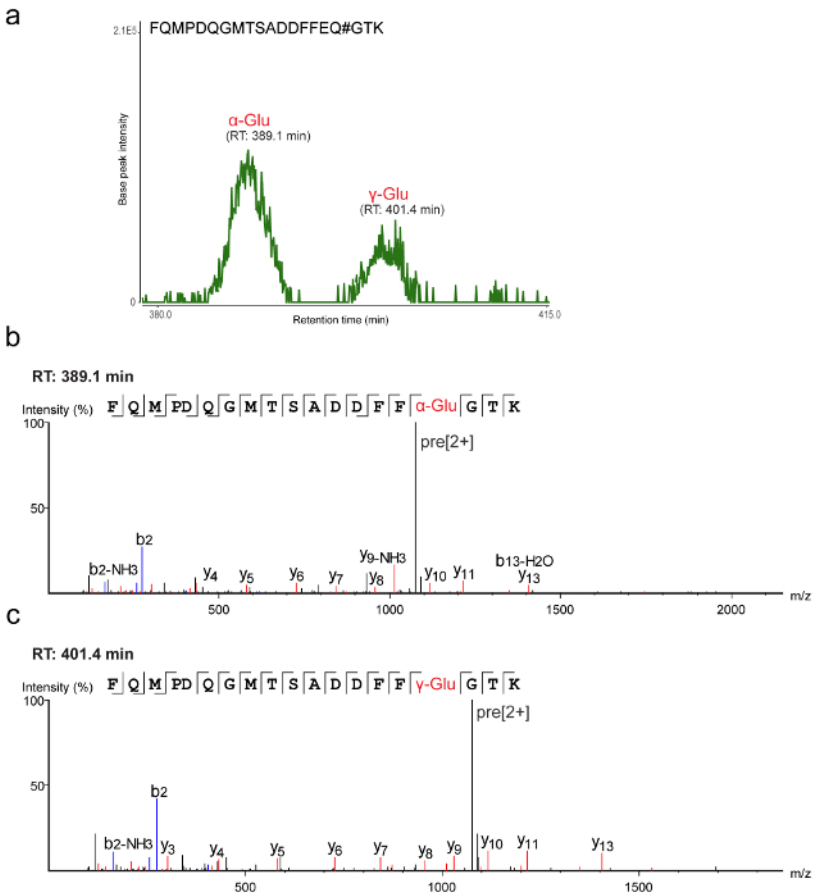

Figure 2: Inspection of XICs and Identification of Double Retention Times at the MS/MS Level for the Deamidated Peptides Allow Characterization of Gln and Asn Deamidated Isomers and Identification of Products from Crucial Enzymatic Reactions that Take Place in Deamidation. a. Detail of the XIC showing the two peaks corresponding to the γ/α-glutamyl isomers of the peptide FQMPDQGMTSADDFFEQ#GTK from the brain protein DPYL2 dihydropyrimidinase related-protein 2. The inverted γ/α-glutamyl ratio detected in that peptide indicates the potential implication of the γ-glutamyl-containing specie in the transglutamination reaction as a transglutaminase γ-glutamyl intermediate. The deamidated residue was verified by MS/MS at both retention times, **b.** at 389.1 min corresponding to the α-glutamyl-containing peptide and **c.** at 401.4 min corresponding to the γ-glutamyl-containing peptide. Please click here to view a larger version of this figure.

As a novelty of this paper, we found that LERLIC-MS/MS allows characterization of the succinimide intermediate (**Figure 3**). The use of mild acidic pH during sample processing stabilizes the succinimide intermediate in modified Asn residues[25]. Therefore, LERLIC-MS/MS allows proteome-wide study of the succinimide intermediate, a labile and poorly understood modification with significant physiological and pathological implications[26].

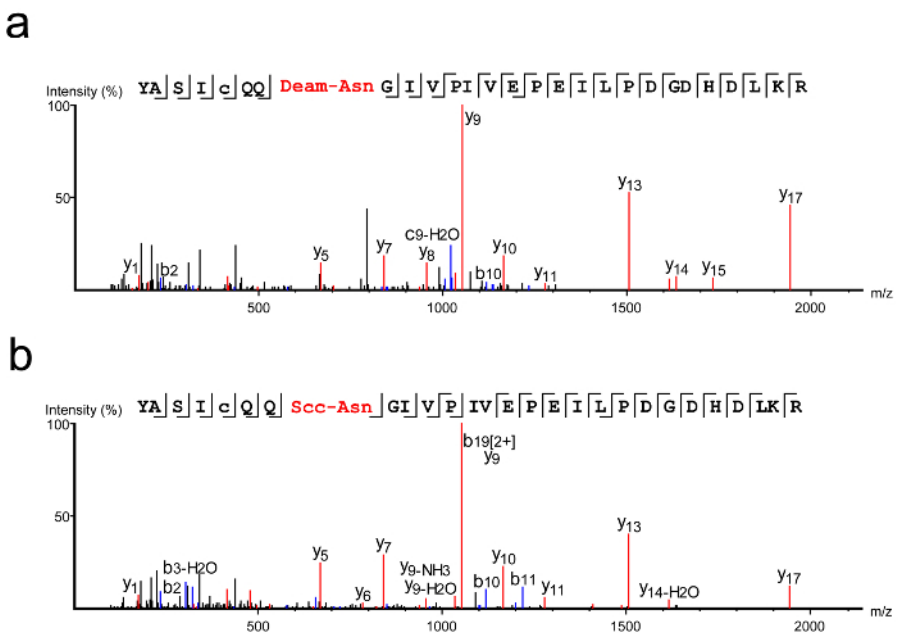

Figure 3: Identification of the Succinimide Intermediate in Asn Residues by LERLIC-MS/MS. a. Spectrum of the Asn deamidated peptide YASICQQN#GIVPIVEPEILPDGDHDLKR from the brain protein ALDO C Fructose-bisphosphate aldolase C. Deamidated residue is indicated as Deam-Asn. **b.** Spectrum of the peptide YASICQQN#GIVPIVEPEILPDGDHDLKR, which in this case shows a mass shift of -17 Da at the previously deamidated Asn residue (Scc-Asn) due to the ammonium loss that take place during the formation of the succinimide intermediate. Please click here to view a larger version of this figure.

Component	**Amount for 100 mL**
Isopropanol	90 mL
Water	10 mL

Table 1. Packing Buffer Composition (90% isopropanol).

Solution 1: 1 M of ammonium acetate.	
Component	**Amount for 50 mL**
Ammonium acetate	3.86 g
Water (make up to 50 mL)	~ 50 mL
Solution 2: 100 mM of ammonium acetate.	
Component	**Amount for 50 mL**
Solution 1	5 mL
Water	45 mL
Solution 3: 10% Sodium deoxycholate.	
Component	**Amount for 1 mL**
Sodium deoxycholate	0.1 g
Water	1 mL
Solution 4: SDC homogenization buffer (100 mM ammonium acetate containing 1% Sodium deoxycholate).	
Component	**Amount for 10 mL**
Solution 2	9 mL
Solution 3	1 mL

Table 2. SDC Homogenization Buffer Composition (100 mM Ammonium Acetate Containing 1% Sodium Deoxycholate). Prepare the stock solution of 1 M of ammonium acetate (Solution 1) and dilute to get 100 mM of ammonium acetate (Solution 2). Additionally, prepare the stock solution of 10% sodium deoxycholate (Solution 3). Prepare the SDC homogenization buffer as described in Solution 4.

Solution 5: 1 M DTT	
Component	**Amount for 10 mL**
Dithiothreitol	77 mg
Solution 2 (See **Table 2**)	0.5 mL
Solution 6: Dilution buffer	
Component	**Amount for 10 mL**
Solution 5	0.1 mL
Solution 2 (See **Table 2**)	9.9 mL

Table 3. Dilution Buffer Composition (100 mM Ammonium Acetate Containing 10 mM Dithiothreitol (DTT)). Prepare the stock solution of 1 M DTT (Solution 5) and subsequently prepare the dilution buffer as detailed in Solution 6.

Solution 7: 1 M IAA	
Component	**Amount for 10 mL**
IAA	93 mg
Solution 2 (See **Table 2**)	0.5 mL

Table 4. Alkylation Buffer Composition (100 mM Ammonium Acetate Containing 20 mM Iodoacetamide (IAA)). Prepare the stock solution of 1 M IAA (Solution 7).

Component	**Amount for 50 mL**
Ammonium hydroxide	0.25 mL
Water	24.75 mL

Table 5. SDC Redissolving Buffer Composition (0.5% Ammonium Hydroxide).

Component	**Amount for 100 mL**
Trifluoroacetic acid	0.1 mL
Water	99.9 mL

Table 6. Clean-up Buffer (0.1% Trifluoroacetic Acid).

Component	**Amount for 100 mL**
Acetonitrile	75 mL
Formic acid	0.1 mL

Table 7. Elution Buffer (75% Acetonitrile, 0.1% Formic Acid).

Component	**Amount for 1,000 mL**
Formic acid	1 mL
Water	999 mL

Table 8. Mobile Phase A Composition.

Component	**Amount for 1,000 mL**
Formic acid	1 mL
Acetonitrile	999 mL

Table 9. Mobile Phase B Composition.

Discussion

In this video-article we present a step-by-step protocol of LERLIC-MS/MS[3], a method to perform in-depth characterization and to accurately determine the extent of protein deamidation and the enzymatic processes involved on this protein modification. LERLIC-MS/MS is based on the use of a long-length (50 cm) LAX under the principle of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC)[27]. The use of a long-length column, as shown in our study[3], maximizes the potential of ERLIC to separate peptides according to their isoelectric point[27].

LERLIC-MS/MS represents a novel shotgun one-dimensional separation strategy, a workflow that saves time-on-hands during sample processing although it requires a 1,200 min gradient as indicated in[3] to obtain optimal results on highly complex proteomes. A 60 min gradient in LERLIC-MS/MS can also achieve optimal separation of Gln deamidation isoforms on model compounds for the first time in shotgun proteomics[3].

Based on these two applications, we speculate that second dimension separation performed by LAX capillary could be coupled to a first dimension reverse-phase chromatography under a multidimensional chromatography approach for the analysis of samples of middle complexity, a workflow previously used by our group[10].

Sample preparation for the analysis of protein deamidation is a crucial step that requires careful attention to prevent the occurrence of artefactual deamidation on Asn residues at a great extent[1]. Hao *et al.* found that tryptic digestion under mild acidic pH significantly prevents apparition of artefactual deamidation during sample preparation[28]. We have used in this study our SDC-assisted in solution tryptic digestion[13], a method where sample processing is carried out at pH 6. On the other hand, stronger acidic conditions might challenge the digestion ability of trypsin when the pH is kept lower than 6[29]. Addressing this issue, very recently Liu *et al*. have reported an optimal digestion strategy that allows successful digestion and prevention of the occurrence of artefactual deamidation on Asn residues by substituting trypsin by Glu-C at pH 4.5[30]. Implementation of the digestion method reported by Liu *et al.*[30] to analyze Asn deamidation by LERLIC-MS/MS could be a potential strategy that requires experimental verification.

The protocol outlined in this video-article has a great potential to further characterize and quantitate currently unknown isoform ratio arrays that define the involvement of protein deamidation in aging and human diseases. Furthermore, application of LERLIC-MS/MS in other biological backgrounds will help to determine the potentialities and risks of protein deamidation as a molecular clock modification.

Disclosures

The authors have nothing to disclose.

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