Analytical Biochemistry 446 (2014) 76–81

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Inhibition of protein carbamylation in urea solution using ammonium-containing buffers

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ARTICLE INFO

Article history:
Received 19 August 2013
Received in revised form 13 October 2013
Accepted 14 October 2013
Available online 23 October 2013

Keywords:
Carbamylation
Urea
Ammonium-containing buffer
Proteomics
Mass spectrometry

ABSTRACT

Urea solution is one of the most commonly employed protein denaturants for protease digestion in proteomic studies. However, it has long been recognized that urea solution can cause carbamylation at the N termini of proteins/peptides and at the side chain amino groups of lysine and arginine residues. Protein/peptide carbamylation blocks protease digestion and affects protein identification and quantification in mass spectrometry analysis by blocking peptide amino groups from isotopic/isobaric labeling and changing peptide charge states, retention times, and masses. In addition, protein carbamylation during sample preparation makes it difficult to study in vivo protein carbamylation. In this study, we compared the peptide carbamylation in urea solutions of different buffers and found that ammonium-containing buffers were the most effective buffers to inhibit protein carbamylation in urea solution. The possible mechanism of carbamylation inhibition by ammonium-containing buffers is discussed, and a revised procedure for the protease digestion of proteins in urea and ammonium-containing buffers was developed to facilitate its application in proteomic research.

Urea is the most widely used denaturant in proteomic studies, and it is used to increase protease efficiency in protein digestion. Urea can also solubilize proteins by preventing protein precipitation and aggregation [1,2]. However, the digestion of proteins in urea solution causes the carbamylation of proteins/peptides. Although it has been used in proteomic quantification [3], carbamylation has several disadvantages for proteomic research. First, it blocks the N termini of proteins and the side chain amino groups of lysine and arginine residues in proteins/peptides and prevents them from further use such as coupling to solid-phase support or iTRAQ (isobaric tag for relative and absolute quantitation)[1] labeling [4–6]. Second, it prevents proteins from many enzymatic digestions, resulting in incompletely digested peptides. Third, it leads to unexpected chromatographic retention time in protein/peptide separation and unpredicted masses, thereby increasing the complexity of samples [7]. Fourth, it affects peptide and protein identification and accurate quantification by reducing the ionization efficiency and signal intensity of the detected peaks [8]. Fifth, it affects the study of in vivo carbamylation [9], which has been recognized as an important protein modification associated with uremia and severe renal and cardiovascular disorders [10–12]. Therefore, reducing and preventing peptide carbamylation resulting from urea is necessary to improve the quality of quantitative proteomic data and the analysis of in vivo protein modification by carbamylation.

Carbamylation is caused by isocyanic acid derived from urea [13]. Urea spontaneously dissociates to form cyanate and ammonia in aqueous solutions [14]. Temperature, incubation time, and pH are factors that are known to affect the rate of urea dissociation and the degree of protein/peptide carbamylation [14,15]. Based on this knowledge, strategies have been employed to protect peptides from urea carbamylation by either reducing the generation of cyanates or removing active cyanates from solution. For example, most proteomic procedures involving the use of urea solution suggest that it should be freshly prepared and further deionized prior to use to reduce cyanate from the beginning of the sample preparation procedure. Some proteomic protocols indicate that either the urea needs to be removed from the sample before digestion or the sample should be maintained at low temperature to reduce the decomposition rate of urea [14,15]. Acidifying the sample is another method to drive the equilibrium to favor urea over the formation of isocyanic acid [14,16]. These strategies, however, require a long handling time and might not be compatible with many enzymatic digestion procedures. In addition, removing urea before protein digestion may result in the precipitation of many readily denatured proteins. Numerous amino-containing reagents, such as methylamine, ethanolamine, ethylenediamine, Tris–HCl,

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[1] *Abbreviations used:* iTRAQ, isobaric tag for relative and absolute quantitation; NH_4HCO_3, ammonium bicarbonate; PB, phosphate buffer; TCEP, tris(2-carboxyethyl) phosphine; LC, liquid chromatography; MS, mass spectrometry; ACN, acetonitrile; TFA, trifluoroacetic acid; FA, formic acid; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; CHCA, α-cyano-4-hydroxycinnamic acid; TEAB, triethylammonium bicarbonate; MS/MS, tandem$^{MS;\ PSM,\ peptide\ spectrum\ match}$.

0003-2697/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2013.10.024

and 1,2-ethylenediamine, have been suggested for use as cyanate scavengers for protein digestion in urea solution [9,17]. These reagents work by competing with peptides for cyanates, thereby minimizing protein or peptide carbamylation. Still, protein/peptide carbamylation occurs in nearly all conditions of commonly used sample preparation procedures involving urea [9].

In this article, we report the use of ammonium-containing buffers to inhibit protein/peptide carbamylation in urea solution. Ammonium bicarbonate (NH_4HCO_3) and two other ammonium-containing buffers showed better carbamylation inhibition efficiency than phosphate buffer (PB) and Tris–HCl buffer. A high concentration of NH_4HCO_3 buffer (1 M) inhibited nearly all carbamylation on proteins/peptides and human serum with no effect on trypsin digestion. More important, NH_4HCO_3 solution is a commonly used buffer in many enzymatic digestion protocols and, therefore, is very applicable for proteomic research.

Materials and methods

Chemicals and reagents

Standard peptides angiotensin and neurotensin, standard protein bovine fetuin, human sera (frozen liquid), tris(2-carboxyethyl) phosphine (TCEP), iodoacetamide, ammonium bicarbonate, ammonium acetate, triethylammonium bicarbonate buffers, and Tris–HCl buffer (pH 7.6) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing-grade urea, liquid chromatography–mass spectrometry (LC–MS)-grade acetonitrile (ACN), trifluoroacetic acid (TFA), and formic acid (FA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). The peptide standard kit for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) was purchased from AB SCIEX (Framingham, MA, USA). Sep-Pak C18 columns (1 cc) were obtained from Waters (Milford, MA, USA). C18 ZipTips were purchased from Millipore (Bedford, MA, USA). α-Cyano-4-hydroxycinnamic acid (CHCA) matrix was purchased from Agilent Technologies (Palo Alto, CA, USA).

Carbamylation on standard peptides

Here, 5 μl of a 1-mM equimolar solution of angiotensin and neurotensin was diluted 10 times with 45 μl of 1.6 M urea in one of the following buffers: 0.1 M PB (pH 8.0), 0.2 M Tris–HCl (pH 7.6), 0.2 M NH_4HCO_3, 0.1 M NH_4HCO_3, 0.5 M NH_4HCO_3, 1 M NH_4HCO_3, 1 M ammonium acetate, or 1 M triethylammonium bicarbonate (TEAB). After incubation at 37 °C for 18 h, the samples were cooled down on ice and 1/10 of each sample was desalted by C18 ZipTips. Samples were eluted from the C18 tips with 1.5 μl of 80% ACN/0.1% TFA and directly spotted onto a MALDI plate with 0.5 μl of CHCA matrix according to the manufacturer's recommendations. Samples were air-dried and analyzed by a MALDI–TOF mass spectrometer (Applied Biosystems 4800, AB SCIEX).

Carbamylation on a standard protein

Bovine fetuin (40 μg) was denatured and incubated in 20 μl of 8 M urea in one of the following buffers: 0.1 M PB (pH 8.0), 0.2 M Tris–HCl (pH 7.6), 0.2 M NH_4HCO_3, or 1 M NH_4HCO_3 at room temperature for 30 min. The protein was reduced with 10 mM TCEP at 37 °C for 1 h and alkylated using 15 mM iodoacetamide at room temperature for 30 min in the dark. The samples were diluted 5-fold with the respective buffers as mentioned above (final concentration of urea was 1.6 M) and were digested via trypsin (protein/enzyme, 50:1, w/w) at 37 °C for 18 h. Then, 10% of each sample was desalted by C18 ZipTips prior to MALDI–TOF–MS analysis.

Carbamylation on human serum

Here, 2 μl of human serum (Sigma) was diluted 10-fold with 8 M urea in one of the following buffers: PB (pH 8.0), 0.2 M Tris–HCl, 0.2 M NH_4HCO_3, or 1 M NH_4HCO_3. After being reduced and alkylated as indicated above, the samples were diluted 5-fold with each of the buffers followed by digestion with trypsin (protein/enzyme, 50:1, w/w) at 37 °C for 18 h. The samples were desalted by employing 1-cc C18 columns and dried in a SpeedVac. The dried peptides were resuspended in 0.1% TFA for LC–MS/MS (tandem mass spectrometry) analysis.

MALDI–TOF–MS and LC–MS/MS analysis

For MS analysis, the peptides obtained above were analyzed by MALDI–TOF–MS (Applied Biosystems 4800). Spectra were acquired in the reflector mode. The relative carbamylation ratio using the peak area of the carbamylated peptide divided by the peak area of non-carbamylated peptides was used for quantification of the carbamylated peptides. For LC–MS/MS analysis, 1 μg of tryptic peptides generated from human sera was analyzed by an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides were separated on a Dionex Ultimate 3000 RSLCnano system (Thermo Scientific) consisting of a 75 μm × 15-cm Acclaim PepMap100 separating column (Thermo Scientific) downstream of a 2-cm guard column (Thermo Scientific). The mobile phase flow rate was set to 300 nl/min and was composed of 0.1% FA in water (A) and 0.1% FA in 95% ACN (B). The gradient profile was established as follows: 4 to 35% B for 70 min, 35 to 95% B for 5 min, 95% B for 10 min, and equilibration at 4% B for 15 min. MS analysis was performed using an Orbitrap Velos Pro mass spectrometer (Thermo Scientific). The spray voltage was set at 2.2 kV. Orbitrap MS1 spectra (AGC, 1×10^6) were acquired from 400 to 1800 m/z at a resolution of 60,000 followed by data-dependent HCD MS/MS (resolution of 7500, collision energy of 45%, activation time of 0.1 ms) of the 10 most abundant ions using an isolation width of 2.0 Da. Charge state screening was enabled to reject unassigned and singly charged ions. A dynamic exclusion time of 35 s was used to discriminate against previously selected ions.

Database search

The LC–MS/MS data were searched against an IPI human protein database (v3.87) via Sequest (Proteome Discoverer, Thermo Scientific). The database search parameters were set as follows. Two missed protease cleavage sites were allowed for trypsin digests with 10 ppm precursor mass tolerance and 0.06 Da fragment mass tolerance. Carbamidomethylation (C, +57 Da) was set as a static modification, whereas oxidation (M, +16 Da) and carbamylation (N terminal, K, +43 Da) were set as dynamic modifications. In addition, 1% FDR was appointed as a filter for peptide identification.

Results and discussion

Carbamylation of peptide in different buffers with urea

To determine the extent of carbamylation of the α-amino group at the N terminal of peptides and the ε-amino group at the side chain of lysine residues, we chose two standard peptides, angiotensin and neurotensin, and tested the extent of peptide carbamylation using urea solutions in different buffers (Table 1).