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Identification of Four Novel Types of *in Vitro* Protein Modifications

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Abstract

In vitro chemical modifications in proteins, introduced during sample preparation, can complicate mass spectra and increase the potential for false-positive identifications. While several *in vitro* protein modifications have been described previously, additional types of such modifications may exist. Here, we report discovery of four types of *in vitro* protein modifications, identified by HPLC/ MS/MS analysis and nonrestrictive protein sequence alignment by PTMap, an algorithm recently developed in our laboratory. These novel *in vitro* modifications included ethylation of aspartate and glutamate (+28 Da), esterification of aspartate and glutamate by glycerol (+74 Da), loss of 19 Da from lysine, and addition of 108 Da to cysteine. We confirmed that these modifications occurred *in vitro* and not *in vivo* in control experiments designed to avoid conditions likely to induce the modifications. We propose a plausible molecular mechanism for the −19 Da modification of lysine. Our study therefore conclusively identifies several novel *in vitro* protein modifications, suggests ways to avoid these modifications, and highlights the possibility of misidentification of peptides because of *in vitro* modifications.

Keywords

protein modifications; PTMap; automated database searching

Introduction

Over the past two decades, mass spectrometry technologies have advanced such that protein modifications of low stoichiometry, produced *in vitro* or *in vivo*, can now be detected. Analysis of proteolytic peptides by mass spectrometers with high resolution and high mass accuracy makes it possible to correlate detected peptide masses to known protein modifications.[1-3] In addition, the recent parallel development of protein sequence alignment algorithms for tandem mass spectrometry (MS/MS) data allows researchers to identify all possible protein modifications, whether known or previously undescribed.[4-6] At present, more than 300 types of protein modifications have been described,[2] some of which are generated *in vitro*.

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Supporting Information Available: Supplemental information on MS/MS spectra of *in vitro* modified peptides identified in BSA and Histone H4; figure of an *in vitro* modified peptide with a mass shift of −19 at lysine, and tables listing the number of peptides identified in BSA with either Glumethylation or Glu-ethylation induced by various destaining/ washing solutions during in-gel digestion, and the number of peptides identified with any one of four types of modifications following in-gel or in-solution tryptic digestion. This material is available free of charge via the Internet at http://pubs.acs.org.

Several types of protein modifications are known to be induced *in vitro* under specific protein environments:[7]

(1) Acrylamide adduct. SDS-PAGE, a popular method for resolving proteins, induces formation of covalent protein adducts between proteins and chemicals in the gel. The cysteine side chain can react with unpolymerized free acrylamide in the matrix to form cysteinyl-*S*-*β*-propionamide, with a mass increase of 71 Da.[8-10] Acrylamide adduction at the N-terminal amine has also been reported.[10]

(2) *β*-mercaptoethanol adduct. The cysteine side chain can also be modified *in vitro* by *β*-mercaptoethanol, leading to a mass increase of 76 Da.[11]

(3) Oxidation. Some amino acid residues, such as methionine, tryptophan, and cysteine, are easily oxidized *in vitro*.[12]

(4) Methylation. When methanol is used in staining and destaining buffers following SDS-PAGE, methylation can be induced at the side chains of aspartate and glutamate.[13]

(5) Deamidation. Deamidation can happen at the side chains of asparagine and glutamine, leading to the formation of aspartate and glutamate, respectively. The rate of this reaction is dramatically increased at high pH (pH > 10).[14]

Here, we report systematic analysis of protein modifications in bovine serum albumin (BSA) and histone H4 by HPLC/MS/ MS in combination with protein sequence alignment by PTMap, a recently developed program for comprehensive identification of protein modifications with high accuracy. Our analysis discovered four previously unreported types of *in vitro* protein modifications: ethylation of aspartate and glutamate, esterification of aspartate and glutamate by glycerol, loss of 19 Da from lysine, and addition of 108 Da to cysteine. Using various control experiments, we confirmed that the four modifications originated *in vitro*. Our study enriches our understanding of protein *in vitro* modifications, provides a procedure to avoid such modifications during protein handling, and provides a caution against assuming that protein modifications matching these mass shifts originate *in vivo*.

Material and Methods

Proteins

BSA was purchased from Sigma (St. Louis, MO) and calf thymus core histones were purified according to a procedure described previously.[15] Briefly, cold, fat-free calf thymus (200 g) was sliced into 1-2 cm^3 cubes, soaked in 160 mL of 0.5 M sucrose solution for 3 min, and then mixed with 1.44 L of homogenizing buffer (0.25 M sucrose and 3.3 mM $CaCl_2$ solution). This solution was homogenized in 250 mL portions for 30 s twice in an Oster 12-speed blender at the speed of “easy clean”. The homogenate was filtered through two layers of cheesecloth. The filtrate was centrifuged at 1000*g* for 10 min to obtain a wet cell pellet. The pellet was resus-pended in 4 vol of hypotonic buffer (50 mM Tris-Cl, pH 7.9, 2.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF) with slow stirring for 30 min. The suspension was centrifuged at 1600*g* for 10 min to collect a pellet containing nuclei. The core histones were extracted from the nuclear pellet overnight using two washes of ~4 vol of 0.4 N H_2SO_4. The extract was dialysed sequentially against H_2O and 50 mM Tris buffer (pH 7.3) for 8 h each. The core histones were resolved using 15% SDS-PAGE and visualized by colloidal Coommasie blue staining. To isolate histone H4 protein, the core histone preparation was subjected to HPLC separation using a C4 column. The histone H4 peak was collected, dried in a SpeedVac, and resolubilized in water.

Protein Tryptic Digestion

Three sets of experiments were designed to compare the effects of various reagents used in protein storage and in-gel digestion solutions on *in vitro* protein modifications. (1) BSA was incubated in 50 mM ammonium bicarbonate, pH 8, with or without 20% glycerol at 4 °C for 7 days, followed by in-solution digestion using porcine trypsin (Promega, Madison, WI) at a 1:50 enzyme/ substrate ratio. (2) BSA or calf thymus core histone H4 was digested either in solution or in gel. (3) BSA was separated by SDS-PAGE and in-gel digested following a standard protocol with varied washing solutions. SDS-PAGE gels were stained overnight with a colloidal Coomassie staining solution composed of 9 vol of G-250 stain solution (ProtoBlue, National Diagnostics, Atlanta, GA) and 1 vol of ethanol. Before in-gel digestion, gels were destained with water.

For protein in-gel digestion, the protein bands of interest were washed for 8 h, with 3 buffer exchanges, with a destaining solution in 1.5 mL microcentrifuge tubes on a Tomy MT-360 microtube mixer (Tomy Digital Biology, Japan) at medium speed. In our series of experiments, four different destaining buffers were used: destaining buffer I contained ethanol/water (50:50); destaining buffer II contained acetic acid/ethanol/water (10:50:40); destaining buffer III contained methanol/water (50:50); and destaining buffer IV contained acetic acid/methanol/ water (10:50:40).[16] After destaining, protein bands were first rehydrated with a 20-min wash with 1 mL of water, then cut into 1 mm^3 cubes, dehydrated with acetonitrile, and dried in a SpeedVac for 20 min. The dried gel pieces were swelled by covering with 50 mM ammonium bicarbonate containing 10 ng/μL trypsin and subjected to overnight digestion at 37 °C. The resulting tryptic peptides were extracted, dried, and cleaned in a C18 Zip-tip (Millipore, Bedford, MA) as previously described.[16] Protein in-solution digestion was carried out by adding trypsin stock solution into a protein solution (in 50 mM ammonium bicarbonate, pH 8.0) at a 1:50 enzyme/substrate ratio and incubating overnight at 37 °C.

Nano-HPLC/MS/MS Analysis

Tryptic peptides cleaned by a C18 Zip-tip (Millipore, Bedford, MA) were reconstituted in buffer A solution (0.1% acetic acid, 2% acetonitrile, 97.9% H_2O, v/v/v). Mass analyses were performed in an LTQ-2D ion trap mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ionization source, coupled with an Agilent 1100 nanoflow HPLC system. Two microliters of a peptide sample in buffer A was manually loaded onto a capillary column (10 cm length × 75 μm i.d.) home-packed with Jupiter C12 resin (4 μm particle size, 90 Å pore diameter; Phenomenex, Torrance, CA). Peptides were eluted from the column using a 100-min gradient from 8% to 90% buffer B (0.1% acetic acid, 90% acetonitrile, 9.9% H_2O, v/v/v). Eluted peptides were directly electrosprayed into the LTQ mass spectrometer with spectra acquired in a data-dependent mode that cycled between MS and MS/MS of the 10 strongest precursor ions of the previous MS spectrum.

Protein Sequence Alignment and Manual Validation of Peptide Hits

Each LC/MS data set was searched against the corresponding protein sequence with PTMap, software developed in-house to identify all possible protein modifications, including previously undescribed PTMs.[6] PTMap allows confident identification of protein modifications with mass shifts ranging from −100 Da to +200 Da in 1-Da increments. When searching, trypsin was specified as the proteolytic enzyme and 3 missed cleavages were allowed. Mass errors of precursor and product ions were set at (4 Da and (0.6 Da, respectively. All peptide identifications were manually validated with high stringency according to previously published criteria, and each modification site was exclusively localized in the peptide sequence by PTMap.[17]

Results

We report four types of protein modifications that occur *in vitro* during staining and destaining gels, in-gel digestion, or protein storage. These modifications were identified by HPLC/ MS/MS analysis of tryptic peptides using the PTMap algorithm, recently developed software that enables nonrestrictive sequence alignment for identifying all possible PTMs.[6] Thus, the algorithm enables us to identify previously undescribed protein modifications in addition to known ones. Comparing protein modifications under different digestion methods (in-gel or in-solution digestion) or using different sample solutions (e.g., various buffers for gel staining and destaining, and protein storage solutions) enables us to distinguish *in vitro* protein modifications from ones originating *in vivo*.

To study possible *in vitro* protein modifications, we resolved BSA and histone H4 by SDS-PAGE. The gel was stained and destained with buffers containing ethanol to avoid possible *in vitro* methylation. The resulting tryptic peptides were analyzed by nano-HPLC/MS/MS followed by nonrestrictive sequence alignment by PTMap. Here, we highlight four types of novel *in vitro* modifications identifieds in BSA and histone H4 during the course of this study.

Ethylation of Aspartate and Glutamate Residues

Methanol is usually included in staining buffer for visualization of proteins on SDS-PAGE gels and in destaining buffer before ingel digestion. Methanol can cause methylation of aspartate and glutamate residues.[13] To distinguish between *in vitro* methylation and *in vivo* methylation of acidic residues, ethanol was used to replace methanol in the staining and destaining buffers.[13,18] However, this change induces *in vitro* ethylation.

As an example, Figure 1 shows the MS/MS spectrum of a tryptic peptide from BSA that was ethylated *in vitro*. The ethylated peptide was identified in a sample that was destained with buffers containing ethanol, but not in samples destained without ethanol (Supporting Information T1), suggesting that ethylation happens *in vitro* instead of *in vivo*. The presence of acetic acid in the destaining buffer increases the number of ethylated tryptic peptides observed (Supporting Information T1), consistent with the ability of acidic conditions to induce esterification between alcohols and carboxylic acid moieties. We believe that the detected ethylation in tryptic peptides of BSA was produced *in vitro* because no ethylated peptides were present in samples that were destained with buffers devoid of ethanol (Supporting Information T1).

Likewise, the presence of acetic acid in destaining buffer containing methanol increases the number of tryptic peptides of BSA that were methylated (Supporting Information T1).

Esterification of Aspartate and Glutamate Residues by Glycerol

Glycerol is a solvent commonly used in buffers for purification and storage of proteins. Like methanol and ethanol, glycerol contains hydroxyl groups that may participate in esterification reactions with the side chains of aspartate and glutamate residues, leading to a mass shift of 74 Da. As expected, glycerol-modified tryptic peptides were identified when BSA was incubated with a buffer containing 20% glycerol (for examples, see Figure 2). The detected glycerol modifications must have occurred *in vitro* and not *in vivo*, because glycerol-modified peptides were not detected in BSA samples that were not treated with glycerol-containing buffer (data not shown).

Addition of 108 Da to Cysteine Residues

Cysteine residues are susceptible to diverse *in vitro* modifications, such as oxidation, or addition of molecules such as iodoacetamide, acrylamide, dithiothreitol, and *β*-mercaptoethanol.[19,20] It remains unknown whether additional, undescribed modifications can occur at cysteine residues. Availability of the PTMap algorithm in our laboratory enables us to identify all possible modifications at cysteine residues, produced *in vivo* or *in vitro*.

Our mass spectrometric analysis and subsequent nonrestrictive sequence alignment identified 6 cysteine residues within peptides of histone H4 that were modified by a chemical that leads to a mass shift of 108 Da (Figure 3, Supporting Information T2). To our knowledge, such a modification has not been described at cysteine before. Because the modification was not detected in tryptic peptides produced by in-solution digestion, the modification was considered to be an *in vitro* modification that happens during SDS-PAGE and/or gel-staining/destaining steps. The mass shift was determined with low mass accuracy that did not allow elemental composition to be determined. The structure of the modification moiety remains unknown.

Loss of 19 Da from Lysine Residues

The side chain of lysine is subject to several *in vivo* post-translational modifications, including methylation, acetylation, biotinylation, ubiquitination, and sumoylation, that have pivotal roles in cell physiology and pathology. Our study of tryptic peptides from histone H4 identified 8 lysine residues that were modified by loss of 19 Da (Figure 4, Supporting Information F1). Because the modification was not identified in peptides that were produced by in-solution digestion, we believe the modification happens *in vitro*. To the best of our knowledge, an *in vitro* modification with this mass shift has not been reported before.

To explore the mechanism of this modification, we compared the peptide fragmentation pattern and retention time for the peptide of interest with and without the modification. The MS/MS spectra of the histone H4 peptide “DNIQITK*PAIR” with or without K7 modification (Figure 4A,B) showed changes in intensity among a series of y ions containing the modified lysine residue. The ion intensities of y_6, y_8, $y9^{2+}$, $Y10^{2+}$, $Y11^{2+}$ are much higher for the unmodified peptide (Figure 4A) than for the K-19 Da modified peptide (Figure 4B). In addition, the y_5 and b_7 ions are the most intense peaks in the spectrum of the modified peptide. Next, we noticed that the very strong y_4 ion in the unmodified peptide's spectrum, produced by breakage at the K–P peptide bond, became much weaker in the modified peptide's spectrum, suggesting some significant structural change of the K–P residues. On the other hand, y_5 became the strongest ion in the MS/MS spectrum of the modified peptide (Figure 4B). This change suggests that the most fragile peptide bond in the sequence is no longer the K–P bond but is the T–K bond, implying an increase in steric hindrance of the T–K bond upon modification. The HPLC retention time of the modified peptide was about 49.4 min, which is significantly delayed relative to that of the unmodified peptide (~39.5 min), suggesting an increase in hydrophobicity. Similar fragmentation pattern changes and a retention time delay were also observed for other histone H4 peptides with the Lys −19 Da modification.

Taken together, our results suggest several changes in the properties of peptides affected by this modification. First, the decrease in intensity of the y ions containing the unknown modification on the lysine side chain suggests that the modification causes the loss of basicity of the *ε*-amine group. Second, the change in identity of the most fragile peptide bond indicates that this modification probably introduced strong steric hindrance on the T–K peptide bond, which may lead to the same effect as proline. Third, the increase in retention

time shows that the modified Lys side chain is more hydrophobic. On the basis of these observations, the known mass shift (−19 Da), and the cellular Lys metabolism pathway, we propose a reaction mechanism for the Lys −19 Da modification (Figure 4C). In the first step, the ε-amine group of lysine is oxidized by a free-radical reaction,[12] which may be initiated by radicals in the SDS-PAGE gel. The reactive ε-aldehyde group would then be nucleophilically attacked by the R-amine group of the peptide backbone in a mechanism similar to glutamine or asparagine deamidation through a succinimide intermediate.[21] The resulting 2-hydroxyl piperidine homologue would lose one molecule of water to form a more stable structure with a double bond. The −19 Da mass shift is the net effect of the loss of one nitrogen and five hydrogen atoms. While this model remains to be confirmed, it can explain four experimental observations arising from the modification: (1) the mass of the peptide is decreased by 19 Da; (2) the peptide becomes more hydrophobic; (3) the modified lysine residue introduces an additional steric constraint in a fashion similar to proline, thereby facilitating fragmentation; and (4) due to facile fragmentation N-terminal to the modified lysine, fragmentation N-terminal to proline is significantly reduced.

Discussion

We report discovery of four types of *in vitro* protein modifications. Addition of 108 Da to cysteine and removal of 19 Da from lysine have not been reported before. Modifications by glycerol and ethylation increase the masses of the substrate residues by 74 and 28 Da, respectively, which was reported previously for glutamate,[22] but not for aspartate.

The identified *in vitro* modifications could be confused with other known protein modifications and, therefore, could result in misassignment during protein identification and mapping of modification sites by automatic sequence alignment. For example, ethylation of aspartate and glutamate leads to a mass shift of +28 Da, which is the same as formylation or the sum of two methylations. Therefore, when methylation is included in a search as a variable modification, ethylation of aspartate and glutamate could be misassigned as two methylation sites by protein sequence database algorithms. Such misassignment would happen more easily when the candidate methylation residues are adjacent to the ethylated aspartate and glutamate residues. Likewise, the loss of 19 Da from lysine has a similar mass shift to protein modifications associated with loss of water, and to changes from serine to dehydroalanine, from cysteine to formylglycine, from glutamate to pyroglutamate, and from aspartate to succinimide, all of which have a mass shift of −18 Da. Accordingly, these 6 types of protein modifications could be mistaken for one another due to their similar mass shifts. Two peptides that have the same sequence but undergo different modifications that have the same mass shift will have similar fragmentation patterns and will probably lead to similar statistical scores when identified by statistics-based protein alignment algorithms. Therefore, exclusive mapping of the protein modification site, complete assignment of all major product ions in the MS/MS spectrum, and exact matching of mass shifts are critical to ensure the accuracy of peptide identification and mapping of modification sites. This argument also suggests the importance of emphasizing unmatched peaks to remove false peptide identifications, as we described previously.[17]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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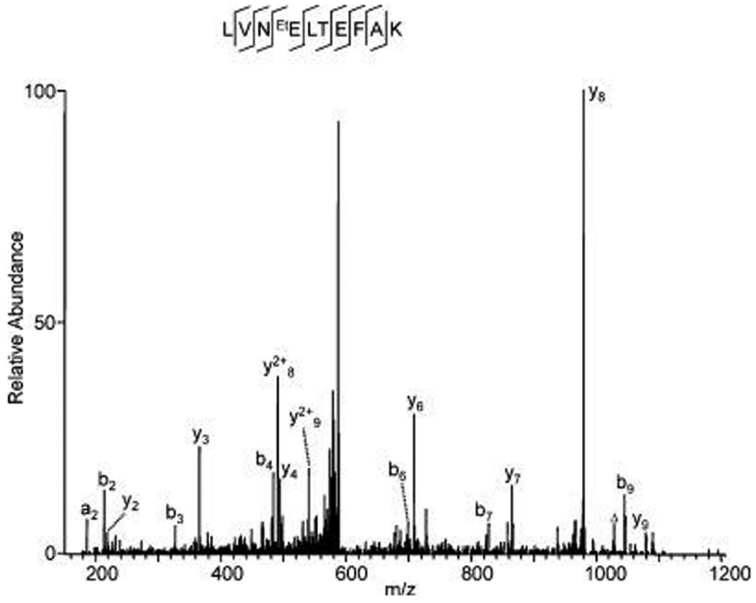

Figure 1.

Identification of ethylated glutamate in BSA. BSA (~300 ng) was resolved by SDS-PAGE and stained with colloidal Coomassie staining solution composed of 9 vol of G−250 stain solution (ProtoBlue, National Diagnostics, Atlanta, GA) and 1 vol of ethanol. The BSA protein band was destained with water and washed with a buffer containing acetic acid/ethanol/water (10: 50:40, v/v/v) before in-gel digestion and analysis by HPLC/MS/ MS. The labels “b” and “y” designate the N- and C-terminal fragment ions, respectively, of the peptide produced by breakage at the peptide bond in the mass spectrometer. The label “a” designates N-terminal fragments produced by breakage at the C-C bond adjacent to the peptide bond. The number represents the number of N- or C-terminal residues present in the peptide fragment. The label “Δ” designates “b”, “y” or “a” ions with water and/or ammonia loss. The same nomenclature system is used for all subsequent figures.

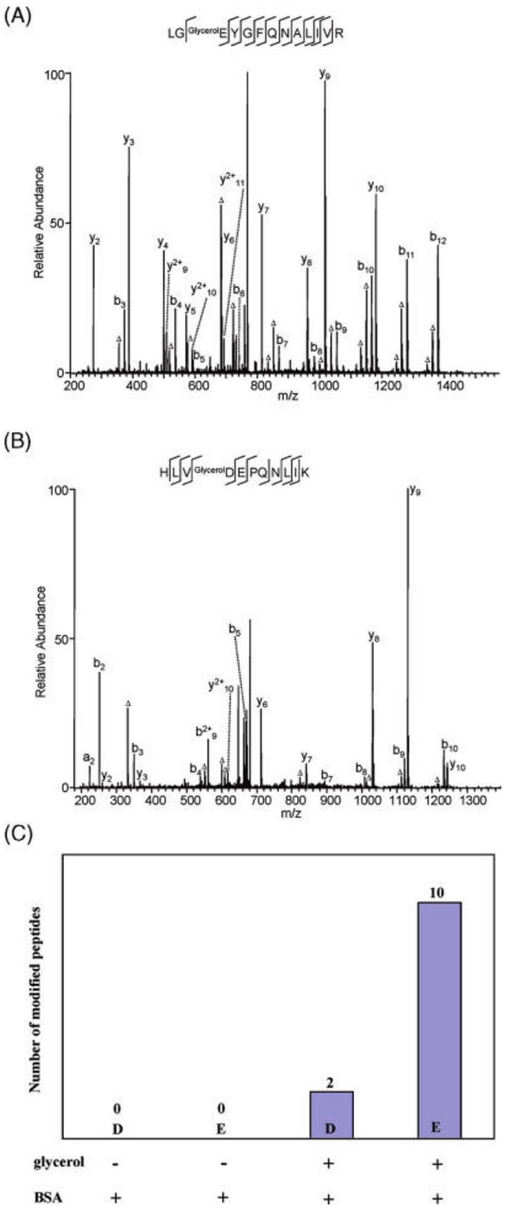

Figure 2.

Modification of acidic residues by glycerol. BSA was incubated in 50 mM ammonium bicarbonate, pH 8, containing 20% glycerol at 4 °C for 7 days before in-solution digestion by trypsin. The tryptic peptides were analyzed by HPLC/MS/MS in an LTQ mass spectrometer. (A) A peptide modified by glycerol at a glutamate residue. (B) A peptide modified by glycerol at an aspartate residue. (C) The number of glycerol-modified peptides from BSA identified in our analysis, in the presence or absence of glycerol incubation.

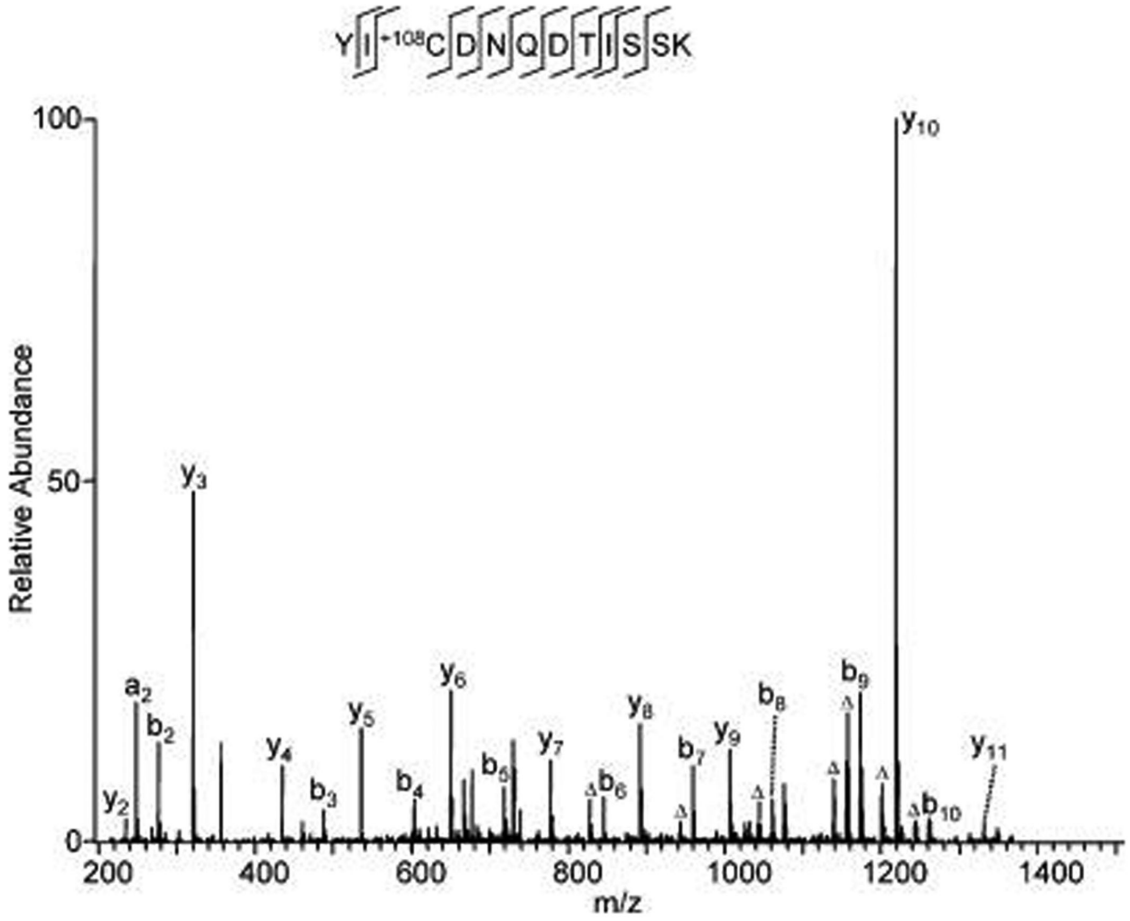

Figure 3.

An *in vitro*-modified peptide with a mass shift of +108 at cysteine. BSA (300 ng) was resolved by SDS-PAGE and stained with colloidal Coomassie staining solution composed of 9 vol of G−250 stain solution (ProtoBlue, National Diagnostics, Atlanta, GA) and 1 vol of ethanol. The gel band was destained with buffer composed of ethanol/water (50:50, v/v) before in-gel digestion and HPLC/MS/MS analysis.

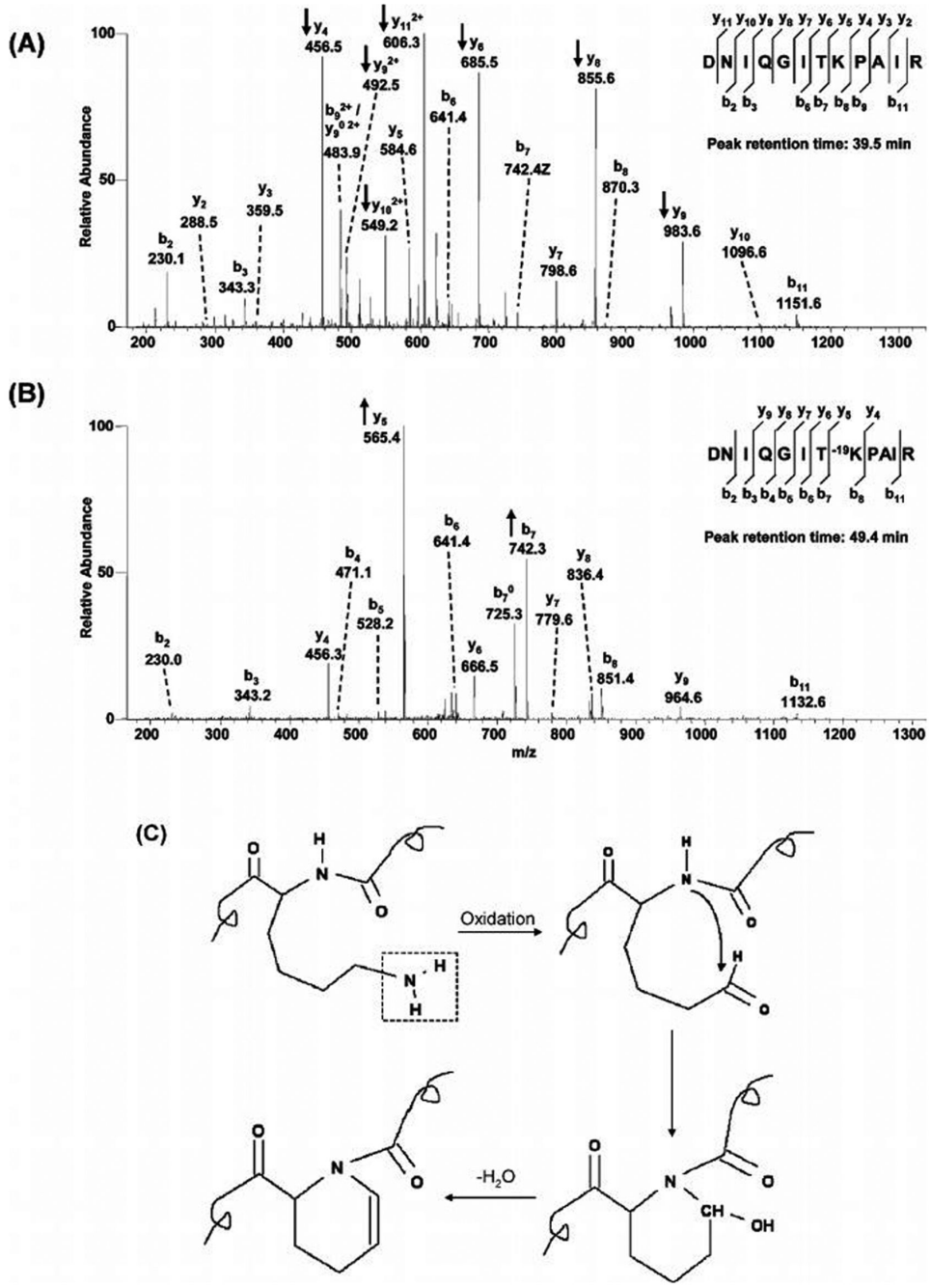

Figure 4.

Mechanistic study of the *in vitro* modification with a mass shift of −19 at lysine. (A) MS/MS of the unmodified histone H4 peptide, DNIQGITKPAIR. (B) MS/MS spectrum of the modified histone H4 peptide with loss of 19 Da at lysine, DNIQGIT^{-19}KPAIR. (C) A possible molecular mechanism of *in vitro* protein modification resulting in a mass shift of −19 Da at lysine.