Evaluation of the Effect of Trypsin Digestion Buffers on Artificial Deamidation

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Running Title: Effect of Trypsin Digestion Buffers on Artificial Deamidation

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Abbreviations: ABC, ammonium bicarbonate; TEAB, triethylammonium bicarbonate; ERLIC, electrostatic repulsion-hydrophilic interaction chromatography; WAX, weak anion exchange; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MS, mass spectrometry; FA, formic acid; FDR, false discovery rate; MS^2, MS/MS; HCD, higher-energy collisional dissociation;

ABSTRACT

Nonenzymatic deamidation occurs readily under the condition of trypsin digestion, resulting in the identification of many artificial deamidation sites. To evaluate the effect of trypsin digestion buffers on artificial deamidation, we compared the commonly used buffers of Tris-HCl (pH 8), ammonium bicarbonate (ABC) and triethylammonium bicarbonate (TEAB), and ammonium acetate (pH 6) which was reported to reduce Asn deamidation. iTRAQ quantification on rat kidney tissue digested in these four buffers indicates that artificial Asn deamidation is produced in the order of ammonium acetate<Tris-HCl<ABC<TEAB, and Gln deamidation has no significant differences in all tested buffers. Label-free experiments show the same trend, while protein and unique peptide identification are comparable using these four buffers. To explain the differences of these four buffers in producing artificial Asn deamidation, we determined the half-life of Asn deamidation in these buffers using synthetic peptides containing –Asn-Gly- sequences. It is 51.4±6.0 days in 50 mM of ammonium acetate (pH 6) at 37ºC, which is about 23, 104 and 137 times of that in Tris-HCl, ABC and TEAB buffers, respectively. In conclusion, ammonium acetate (pH 6) is more suitable than other tested buffers for characterizing endogenous deamidation and N-glycosylation.

Keywords: trypsin digestion buffers, artificial deamidation, glycosylation, ERLIC, HCD

INTRODUCTION

Protein deamidation is reported to contribute to Alzheimer's disease and cataracts,[1-3] and it is also proposed as a molecular clock of biological events, such as in protein turnover, development and aging.[4, 5] Shotgun proteomics is powerful in detecting deamidation sites in proteome scale due to its sensitivity, accuracy and high throughput,[6, 7] and trypsin has been the most widely used protease in proteomics research. However, nonenzymatic deamidation occurs readily under the condition of trypsin digestion, i.e. prolonged incubation in mildly alkaline buffers at 37ºC, which results in the identification of many artificial Asn deamidation sites and N-glycosylation sites when glycosylation site assignment is based on the detection of Asn deamidation in the consensus sequence N-X-S/T (with X not proline).[8-10] Digestion buffers, temperature and digestion time are three major factors controlling the extent of deamidation during proteolytic digestion.[8, 9] Since overnight tryptic digestion at 37ºC is generally used in most proteomics experiments for complex samples, such as cell, tissue and plasma, it is necessary to comprehensively evaluate the effect of trypsin digestion buffers on artificial deamidation.

In proteomics studies, 25-100 mM ammonium bicarbonate (ABC) has been the most widely used trypsin digestion buffer for both in-gel digestion and in-solution digestion due to its volatility,[11] but it is reported to result in ~70-80% conversion of -Asn-Gly- into -Asp-Gly- or -βAsp-Gly during the overnight digestion.[12] Tris-HCl (pH 8) is another widely used digestion buffer and the recommended buffer from trypsin manufacturers,[13] and it is reported to be a very mild catalyst of deamidation.[14] However, it remains unclear whether Tris-HCl is good enough for reducing artificial deamidation in proteomic studies without a comprehensive evaluation. Triethylammonium bicarbonate (TEAB) is commonly used in trypsin digestion buffers when

amine-reactive isobaric tagging reagents, such as isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tag (TMT), are used to label proteins or tryptic peptides for protein quantification because it has no primary amines which would interfere with the labeling.[15, 16] Presently, there are no reports about the effect of TEAB on artificial deamidation, but it has already been used in the identification and quantification of N-glycopeptides.[17] The identified N-glycopeptides may include many false positives if TEAB is a strong catalyst of deamidation. In addition to these commonly used buffers, we suggested an improved trypsin digestion buffer, i.e. ammonium acetate (pH 6), which was shown to significantly reduce artificial Asn deamidation without affecting peptide identification using in-gel digested samples.[8] Due to the difference between in-gel and in-solution digested samples, it is necessary to conduct a comprehensive evaluation on in-solution digestion before widely applying it to proteomics studies. In this study, we comprehensively evaluated 50 mM of ammonium acetate (pH 6), Tris-HCl (pH 8), ABC and TEAB as the buffers for in-solution tryptic digestion of rat kidney tissue using both iTRAQ quantification and label-free experiments, and determined the half-life of Asn-deamidation in these buffers using synthetic peptides. Our results indicate that ammonium acetate (pH 6) significantly outperforms the commonly used trypsin digestion buffers by minimizing artificial deamidation for proteomic study of endogenous deamidation and N-glycosylation.

MATERIALS AND METHODS

Sample Preparation

Male Sprague−Dawley rats were handled in accordance with the guidelines of NTU Institutional Animal Care and Use Committee (NTU-IACUC), NTU, Singapore. Rat kidneys were snap-

frozen immediately in liquid nitrogen after collection, and kept at -80^{o}C until use. The tissue was cut into small pieces and ground into fine powders in liquid nitrogen with a pestle. The powders were then suspended in lysis buffer (8 M urea, 50 mM Tris-HCl, pH 8) with protease inhibitor (05 892 791 001, Roche) added (10 ml/tablet). The suspension was sonicated for 10 seconds thrice on ice and centrifuged at 20,000g at 4^{o}C for 20 min. The protein concentration of the supernatant was then determined by the bicinchoninic acid (BCA) assay. About 5 mg tissue lysate was diluted to 4 mg/ml using the lysis buffer, reduced with 10 mM DTT at 37^{o}C for 2 h and alkylated with 40 mM iodoacetamide for 45 min in the dark. After the concentration of urea was diluted to 1M with 50mM Tris-HCl (pH 8), trypsin (T8802, Sigma) was added at a weight ratio of 1:50. It was then incubated at 37^{o}C for 15 h. The obtained tryptic peptides were desalted using a Sep-Pak® C18 cartridge (Waters, Milford, MA, USA) and dried in a SpeedVac® (Thermo Electron, Waltham, MA, USA). The trypsin digestion using other digestion buffers was done using the same procedure as that of Tris-HCl trypsin digestion except for the lysis buffers (NH4Ace: 8 M urea, 50 mM ammonium acetate, pH 6; ABC: 8 M urea, 50 mM ABC, pH not adjusted; TEAB: 8 M urea, 50 mM TEAB, pH not adjusted) and digestion buffers (NH4Ace: 50 mM ammonium acetate, pH 6; ABC: 50 mM ABC, pH not adjusted; TEAB: 50 mM TEAB, pH not adjusted). Three technical replicates were done for each step. It should be noted that protein concentration in ammonium acetate buffer was quantified using 2D-Quant Kit (Thermo Fisher, MA) since protein precipitation at pH 6 during dilution of urea concentration interfered BCA quantification.

iTRAQ Labeling

Peptides digested in different buffers were labeled with 4-plex iTRAQ reagents (Applied Biosystems, Foster City, CA) according to the instructions from the manufacturer as follows: ammonium acetate, 114; Tris-HCl, 115; ABC, 116; TEAB, 117. The labeled peptides were pooled and desalted using Sep-Pak C18 cartridges. They were then fractionated using Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) as follows.

ERLIC Fractionation of iTRAQ Labeled Peptides

iTRAQ labeled Peptides were fractionated using a PolyWAX LP weak anion-exchange column (4.6 × 200 mm, 5 μm, 300 Å, PolyLC, Columbia, MD) on a Shimadzu Prominence UFLC system. Mobile Phase A (10 mM CH_3COONH_4 in 85% ACN/1% FA) and mobile phase B (30% ACN/0.1% FA) were used to establish a 26 min gradient of 0%–28% buffer B for 19 min, 28%–100% buffer B for 0.1 min and 100% buffer B for 6.9 min at a flow rate of 1 mL/min with 16 fractions collected. The collected fractions were then dried with vacuum centrifuge, pooled into 6 fractions by combining (1-3); (4-5); (6-7); (8-9); (10-11); (12-16), and redissolved in 3% ACN/0.1% FA for LC-MS/MS analysis. The use of ammonium acetate in the mobile phase A is to improve the solubility of iTRAQ labeled peptides since iTRAQ labeling greatly reduces the solubility of peptides in salt-free solvents.[16]

ERLIC Fractionation of Label-free Peptides

Label-free peptides from 1 mg of proteins were fractionated using a PolyWAX LP weak anion-exchange column on a Shimadzu Prominence UFLC system in triplicate. Mobile Phase A (85% ACN/0.1% FA) and mobile phase B (10% ACN/0.4% FA) were used to establish a 30 min gradient of 0%–10% buffer B for 10 min, 8%–35% buffer B for 12 min, 35%–100% buffer B for

2 min and 100% buffer B for 6 min at a flow rate of 1 mL/min with 18 fractions collected. The collected fractions were then dried with vacuum centrifuge, pooled into 5 fractions by combining (1-4); (5-6); (7-8); (9-11); (12-18), and redissolved in 3% ACN/0.1% FA for LC-MS/MS analysis.

LC-MS/MS

Peptides were separated and analyzed on a Dionex Ultimate 3000 RSLCnano system coupled to a Q Exactive (Thermo Fisher, MA). About 1 μg of the peptides from each pooled fraction were injected into an Acclaim peptide trap column (Thermo Fisher, MA) via the auto-sampler of the Dionex RSLCnano system. Peptides were separated in a capillary column (75 μm x 10 cm) packed with C18 AQ (5 μm, 300Å; Bruker-Michrom, Auburn, CA, USA) at room temperature. The flow rate was at 300 nl/min. Mobile phase A (0.1% formic acid in 5% ACN) and mobile phase B (0.1% formic acid in 90% ACN) were used to establish a 60 min gradient. Peptides were then analyzed on Q Exactive with a nanospray source (Thermo Fisher, MA) at an electrospray potential of 1.5 kV. A full MS scan (350-1600 m/z range) was acquired at a resolution of 70,000 at m/z 200 and a maximum ion accumulation time of 100 msec. Dynamic exclusion was set as 30 s. Resolution for HCD spectra was set to 17,500 at m/z 200. The AGC setting of full MS scan and MS^2 were set as 1E6 and 1E5, respectively. The 10 most intense ions above a 1000 counts threshold were selected for HCD fragmentation with a maximum ion accumulation time of 100 msec. Isolation width of 2 Th was used for MS^2. Single and unassigned charged ions were excluded from MS/MS. For HCD, normalized collision energy was set to 28%. The underfill ratio was defined as 0.1%.

Data Analysis

The raw data were first converted into .apl files with MaxQuant 1.4.1.2 using the function of "Partial processing" with step 1 to 5, and then the .apl files were converted into Mascot generic file format using an in-house Perl script. The UniProt rat protein database (release 2013_10, 28855 sequences) concatenated with cRAP contaminants (version 2012.01.01) and their reverse complement were combined and used for database searches. The database search was performed using an in-house Mascot server (version 2.4.1, Matrix Science, Boston, MA, USA) with MS tolerance of 10 ppm, #^{13}C of 2 and MS/MS tolerance of 0.02 Da. Two missed cleavage sites of trypsin were allowed. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M) and deamidation (NQ) were set as variable modifications. For iTRAQ labeled peptides, 4-plex iTRAQ was set as fixed modifications on N-terminal and lysine, and it was set as a variable modification on tyrosine. iTRAQ 4-plex quantification method embedded in Mascot was used for peptide quantification. Only peptides with an expectation value of ≤ 0.05 and a minimum length of 7 are used for statistical analysis, resulting in a FDR of less than 1%.[18] For high confidence protein identification, only protein groups identified with at least 2 unique peptides (E value ≤ 0.05) are reported, and only peptides of which the search engine rank is 1 and peptides in top scored proteins are counted. A paired Student's t-Test was used to verify the significance of the differences between each comparison.

Determination of the Half-life of Asn deamidation in the Four Buffers Using Synthetic Peptides

The peptide DGNGYISAAELR was synthesized at the Peptide Synthesis Core Facility, Nanyang Technological University. It was incubated in 50 mM Tris-HCl, 50 mM ABC and 50 mM TEAB

at 37^{o}C for 2h, 4h, 8h and 24h, respectively. Since Asn deamidation occurs slowly in 50 mM ammonium acetate (pH 6),[8] the incubation time is 1d, 3d, 7d and 10 d. After incubation, peptides were analyzed using a PolyWAX LP weak anion-exchange column on a Shimadzu Prominence UFLC system. Mobile phase A (80% ACN/0.1% FA) and mobile phase B (10% ACN/2% FA) were used to establish a 30 min gradient of 0–20% B for 10 min, 20–100% B for 15 min and 100% B for 5 min at a flow rate of 1 ml/min. The absorbance was monitored at 280 nm. The half-life of Asn deamidation was determined based on the percentage of remaining unmodified peptides over time.

RESULTS AND DISCUSSION

The suitable buffer for studying endogenous deamidation and N-glycosylation should introduce as little as possible artificial deamidation during proteomic sample preparation without significantly affecting protein and peptide identification. In this study, we comprehensively evaluated the three commonly used trypsin digestion buffers of Tris-HCl, ABC and TEAB and an improved buffer of ammonium acetate (pH 6) in these aspects. To explain the difference of these four buffers in introducing artificial Asn deamidation, we also determined the half-life of Asn deamidation in these buffers using synthetic peptides.

iTRAQ based Relative Quantification of Asn-deamidated Peptides and Gln-deamidated Peptides Digested in the Four Buffers

To evaluate the potential of these four buffers in producing artificial deamidation during the course of proteomic sample preparation, we used 4-plex iTRAQ reagents to label tryptic peptides from rat kidneys digested in these four buffers and compared the summed areas of the reporter ions from each labeling with experimental bias normalized. Only Asn-deamidated peptides and Gln-deamidated peptides with reporter ions detected in all four digestion conditions are used for statistical analysis. As shown in Figure 1, based on the results from two technical replicates, the summed areas of the reporter ions of Asn-deamidated peptides are in the order of ammonium acetate<Tris-HCl<ABC<TEAB, indicating the difference in the rate of Asn deamidation in these four buffers. In contrast, Gln deamidation is consistent in the four tested buffers. The lists of iTRAQ labeled Asn-deamidated peptides and Gln-deamidated peptides are shown in Supplemental data 1. It should be noted that the difference of Asn deamidation between different buffers from iTRAQ experiments may be underestimated due to the following two reasons: 1)

TEAB in the dissolution buffer of iTRAQ labeling Kits increases Asn deamidation in all labeled samples during the course of iTRAQ labeling and vacuum drying of the pooled samples, which reduces the difference of Asn deamidation between different digestion conditions; 2) the ratio compression problem in iTRAQ quantification. [19, 20]

Identification of Asn-deamidated Peptides, Gln-deamidated Peptides and Artificial N-glycopeptides in Label-free Experiments

Due to the limitation of iTRAQ quantification in comparing the four buffers in producing artificial deamidation, label-free experiments were used as an alternative. To clearly show the effect of different digestion buffers on artificial deamidation, we used an ERLIC gradient that could selectively enrich deamidated peptides. Therefore, the observed ratio of deamidation in this study may be higher than that from other proteomics studies without enriching deamidated peptides. Based on the label-free LC-MS/MS results from three technical replicates, the number of identified unique Gln-deamidated peptides was comparable while using 50 mM of the four digestion buffers, but the number of identified unique Asn deamidation peptides in Tris-HCl, ABC and TEAB is 2.2, 3.0 and 3.8 times of that from ammonium acetate (pH 6) ($P<0.002$ for each comparison) , respectively (Figure 2). As a result, the number of identified unique glycopeptides in Tris-HCl, ABC and TEAB is 2.3, 3.4 and 4.4 times of that from ammonium acetate (pH 6), respectively ($P<0.01$ for each comparison). As the samples were not treated with PNGase F, all of the identified N-glycopeptides were artificial ones either from endogenous or artificial Asn deamidation. Our results indicate that ammonium acetate (pH 6) is more efficient than the three commonly used trypsin digestion buffers in reducing artificial Asn deamidation, and thus is a suitable buffer for studying endogenous deamidation and N-glycosylation. It is

worth noticing that the number of unique Asn-deamidated peptides is less than that of unique Gln-deamidated peptides while using ammonium acetate buffer (pH 6), which is contrary to the results from other large-scale proteomic studies using ABC buffer.[8] It indicates that the identified Asn-deamidated peptides while using ammonium acetate buffer are mainly native ones and the rate of Asn deamidation is controlled in vivo since it occurs at a rate of 50 times faster than that of Gln deamidation in vitro.[21] Parker et al. used samples without PNGase F treatment to detect and remove false positive N-glycopeptides,[17] but it might not be an efficient way due to the low reproducibility of LC-MS/MS in analyzing complex samples. The ammonium acetate buffer (pH 6) provides a convenient and reliable alternative.

The Distribution of Asn deamidation and Gln deamidation while Using the Four Trypsin Digestion Buffers

To study the effect of different trypsin digestion buffers on nonenzymatic deamidation in details, we reported the number of Asn deamidation and Gln deamidation based on the residues on the C-terminal side of the deamidated Asn/Gln. As shown in Figure 3A, the number of Asn deamidation detected at the –N-G- sequence in Tris-HCl (pH 8), ABC and TEAB were 4.2, 5.6 and 7.7 times of that in ammonium acetate (pH 6), respectively, and similar but less remarkable trend occurred on –N-S-, -N-D- and –N-A- sequences. However, trypsin digestion buffers have a negligible effect on those sequences with long deamidation half-life, such as –N-R-, –N-Y-, –N-I- and –N-P-.[21] For Gln deamidation, the effect of trypsin digestion buffers is negligible on all sequences because of its extremely long half-life (Figure 3B).[21] These results indicate that trypsin digestion buffers affect nonenzymatic deamidation of peptides mainly on sequences with short half-life of deamidation.

Protein and Peptide Identifications and Tryptic Miscleavages in Label-free Experiments

As shown in Table 1, based on the label-free LC-MS/MS results from three technical replicates, protein and unique peptide identifications are comparable while using 50 mM of the four trypsin digestion buffers. The results of protein and peptide identifications are shown in Supplemental data 2. However, the percentages of tryptic peptides with miscleavages in ammonium acetate (pH 6) are significantly higher than that in other tested buffers ($P<0.01$ for each comparison), indicating the reduced activities of trypsin in ammonium acetate (pH 6). Surprisingly, Tris-HCl (pH 8) also produces significantly more tryptic peptides with miscleavages than ABC ($P=0.0002$) and TEAB ($P=0.0014$) although it is the recommended buffer from manufacturers. The increase of tryptic miscleavages does not affect protein and peptide identification possibly due to that many peptides that are too short to be identified with full cleavages become identifiable with miscleavages. Further studies may be necessary to evaluate the effect of digestion buffers on protein quantification before using ammonium acetate (pH 6) in quantitative proteomics.[22] Because of the increase of miscleavaged peptides, ammonium acetate (pH 6) and Tris-HCl (pH 8) may not be ideal buffers for SRM assay development in targeted MS analysis.[23] However, targeted SRM validation may still be used with caution if the control and samples are digested in ammonium acetate (pH 6) under completely same conditions. It was worth noticing that the solubility of proteins decreased greatly at pH 6 and resulted in protein precipitation when the concentration of urea was diluted to less than 0.1 M during protein quantification using BCA. However, it does not affect trypsin digestion much since it is generally done in 1M urea. Nevertheless, protein concentration was diluted to 0.5 mg/ml during trypsin digestion using all tested buffers to ensure the solubility of proteins.

Determination of the Half-life of Asn deamidation in the Four Buffers Using Synthetic Peptides

The synthetic peptide DGNGYISAAELR was used to determine the half-life of Asn deamidation in 50 mM of ammonium acetate (pH 6), Tris-HCl (pH 8), ABC and TEAB. As shown in Figure 4, the ERLIC chromatograms showed the gradual transformation of unmodified peptides into two deamidation products containing –βDG- and –DG-. The half-life of Asn deamidation was determined as 51.4±6.0 days in 50 mM ammonium acetate (pH 6), which was about 23, 104 and 137 times of that in Tris-HCl, ABC and TEAB buffers, respectively. This sufficiently explains the reduced artificial Asn deamidation in ammonium acetate buffer (pH 6) detected using both label-free qualitative experiments and iTRAQ quantification. Deamidation decreased with decreasing pH in the range of pH 12 to 5.[24] For 1 day incubation in 50 mM ammonium acetate buffer (pH 6), 0.56% of the peptides are deamidated, which has a negligible effect on proteomic studies. The peak shape of the undeamidated peptide is much broader after incubated in ammonium acetate (pH 6) than that in other buffers (Figure 4). The possible explanation is that ammonium acetate interferes with the ERLIC separation since a sharp peak was obtained in a previous study using SCX column for separation.[8] It was reported that the half-life of Asn deamidation was 1.03 days in 0.15 M Tris-HCl, pH 7.4 at 37ºC,[21] which was about half of that observed here. The possible explanation is that 3 times higher concentration of Tris-HCl than ours was used in that study, and Asn deamidation accelerated as ionic strength increased.[25] We also determined the half-life of Asn-deamidation in 25 mM ABC buffer, and it was 15.08±2.03 h, i.e. about 1.27 times of that in 50 mM ABC buffer. Thus, a lower concentration of buffers should be used in proteomic sample preparations to reduce artificial deamidation. For example, 25-50

mM may be a good choice to the keep the pH of the buffers stable during trypsin digestion. The half-life of Asn deamidation in 50 mM TEAB is about 9 hours at 37ºC. Since iTRAQ labeling is done in TEAB buffer at room temperature for 2 hrs, it may not introduce much artificial Asn deamidation, but TEAB should not be used as the digestion buffer for studying protein deamidation.

As shown in Figure 4A, it seems that a side product was eluted at 9 min. We collected the fraction at 9 min and identified it as N-terminal acetylated DGNGYISAAELR by MALDI-TOF/TOF. We then evaluated the effect of trypsin digestion buffers on introducing acetylation on tryptic peptides. As shown in Supplemental Data 3, artificial acetylation occurs in the order of ammonium acetate>Tris-HCl>ABC>TEAB, which is contrary to that of Asn deamidation. As the half-life of N-term acetylation is about half of that of Asn deamidation for DGNGYISAAELR (Figure 4A) in 50 mM ammonium acetate at 37ºC, it does not affect peptide quantification greatly. However, it is worth noticing that trypsin digestion buffers can introduce artificial acetylation on peptides in different degree.

CONCLUSIONS

This work comprehensively compared 50 mM of ammonium acetate (pH 6), Tris-HCl (pH 8), ABC and TEAB as in-solution trypsin digestion buffers. Both iTRAQ quantification and label-free results indicate that ammonium acetate (pH 6) is more suitable than other buffers for studying endogenous deamidation and N-glycosylation due to the significant decrease of artificial Asn deamidation in comparison to other buffers without affecting protein and peptide identification and Gln deamidation. Determination of the half-life of Asn deamidation in the four buffers further validates the conclusion. Our results also indicate that among the commonly used tryspin digestion buffers, ABC and TEAB are not suitable for studying Asn deamidation and N-glycosylation, but Tris-HCl may be used if trypsin digestion has to be done at around pH 8.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org. Supplemental Data 1: The lists of iTRAQ labeled Asn-deamidated peptides and Gln-deamidated peptides from two technical replicates; Supplemental Data 2: Supplemental Data2-Protein and peptide identifications from label-free experiments.

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FIGURE LEGENDS:

Figure 1. The summed area of the reporter ions of Asn-deamidated peptides and Gln-deamidated peptides under different digestion conditions from iTRAQ experiments. Artificial Asn deamidation is produced in the order of ammonium acetate<Tris-HCl<ABC<TEAB; Gln deamidation has no significant differences in all tested buffers.

Figure 2. Number of unique Asn-deamidated peptides, Gln-deamidated peptides and artificial N-glycopeptides identified from rat kidney tissue digested in 50 mM of ammonium acetate (pH 6), Tris-HCl (pH 8), ABC and TEAB from three technical replicates of label-free experiments. Significantly less Asn-deamidated peptides and artificial N-glycopeptides were identified while using ammonium acetate (pH 6) than other buffers; Gln deamidation was consistent among the four buffers.

Figure 3. Comparison of the distribution of Asn deamidation and Gln deamidation in rat kidney tissue digested in the four buffers. Experimental distribution of Asn deamidation (A) and Gln deamidation (B). The x axis lists the residue on the C-terminal side of the deamidated N- or Q-residue.

Figure 4. Determination of the half-life of Asn deamidation using the synthetic peptide DGNGYISAAELR by HPLC. Solutions of the synthetic peptide in 50 mM CH3COONH4, pH 6 were incubated for 1d, 3d, 7d and 10d at 37 °C. The incubation time in other buffers were 2h, 4h, 8h and 24h at 37°C. Peptide mixtures were resolved with an ERLIC column with UV detection at 280 nm. The half-life of Asn deamidation in 50 mM CH3COONH4, pH 6 is about 23, 104 and

137 times of that in 50 mM of Tris-HCl (pH 8), ABC and TEAB. A side product with the elution time of around 9 minutes formed during the incubation in 50 mM CH_3COONH_4, but it had no effect on the detection of endogenous Asn deamidation.

TABLES:

Table 1. Number of Protein and Peptide Identifications and Percentage of Miscleavaged Peptides from Rat Kidney Tissue Digested in the Four Buffers

	Unique peptides	Proteins	Misscleavaged peptides (%)
Ammonium acetate (pH 6)	18259±140	2337±9	34.1%±0.3%
Tris-HCl (pH 8)	18025±185	2347±24	31.9%±0.1%
ABC	17823±182	2382±35	24.8%±0.1%
TEAB	17447±875	2269±76	26.3%±0.3%

Note: This table shows the results from three technical replicates with individual sample preparations. As detailed in the section of "Materials and Methods", only protein groups with at least 2 unique peptides (E value ≤ 0.05) are reported.

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