**TITLE:**

Nitropeptide Profiling and Identification, Illustrated by Angiotensin II

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**SUMMARY:**

Proteomic profiling of tyrosine-nitrated proteins has been a challenging technique due to the low abundance of the 3-nitrotyrosine modification. Here we describe a novel approach for nitropeptide enrichment and profiling by using Angiotensin II as the model. This method can be extended for other *in vitro* or *in vivo* systems.

**ABSTRACT:**

Protein nitration is one of the most important post-translational modifications (PTM) on tyrosine residues and it can be induced by chemical actions of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in eukaryotic cells. Precise identification of nitration sites on proteins is crucial for understanding the physiological and pathological processes related to protein nitration, such as inflammation, aging, and cancer. Since the nitrated proteins are of low abundance in cells even under induced conditions, no universal and efficient methods have been developed for the profiling and identification of protein nitration sites. Here we describe a protocol for nitropeptide enrichment by using a chemical reduction reaction and biotin labeling, followed by high resolution mass spectrometry. In our method, nitropeptide derivatives can be identified with high accuracy. Our method exhibits two advantages compared to the previously reported methods. First, dimethyl labeling is used to block the primary amine on nitropeptides, which can be used to generate quantitative results. Second, a disulfide bond containing NHS-biotin reagent is used for the enrichment, which can be further reduced and alkylated to enhance the detection signal on a mass spectrometer. This protocol has been successfully applied to the model peptide Angiotensin II in the current paper.

**INTRODUCTION:**

Nitration of tyrosine residues in proteins to form 3-nitrotyrosine regulates many biological processes. Due to the different chemical properties between tyrosine and 3-nitrotyrosine, a nitrated protein may have perturbed signaling activity1-2. Therefore, it is important to develop methods that can enrich and identify nitration sites on proteins efficiently. As 3-nitrotyrosine is a low abundance modification on proteins compared to other forms of PTM, such as phosphorylation and acetylation, it is challenging to identify endogenous nitration sites directly from cell lines or tissue samples. Nevertheless, the methodology of using mass spectrometry (MS) to characterize the fragmentation pattern of nitrotpeptide has been developed (for example, Zhan & Desiderio3), which lays the foundation for new methods of nitroproteomics.

Currently, an enrichment step followed by MS is the most powerful strategy for nitropeptide profiling4-5. The enrichment methods can be classified into two classes. One class is based on antibodies that can recognize 3-nitrotyrosine specifically, whereas the other class is based on the chemical derivation that reduces a nitro group to an amine group4-5. For the antibody-based method, nitrotyrosine affinity column is used for the enrichment, from which the eluted material is further resolved and analyzed by high resolution MS6-7. For the chemical derivation-based method, the amine groups at the N-terminus of the peptide or lysine should be blocked in the first step either by acetylation, isobaric tags for relative and absolute quantitation (iTRAQ), or tandem mass tags (TMT) reagents. Next, a reducer is used to reduce nitrotyrosine to aminotyrosine followed by modifying the newly formed amine group, which includes biotin ligation, sulphydryl peptide conversion, or other types of tagging systems8-11. Most of the protocols established so far are based on *in vitro* over-nitrated proteins, instead of endogenously nitrated proteins.

In the present study, a modified procedure of the chemical derivation of nitrotyrosine is developed for the nitropeptide enrichment and identification, which shows enhanced sensitivity during MS detection and is suitable for quantification purpose. Our recent study employing this method in biological systems identified that nitration of lymphocyte-specific protein tyrosine kinase (LCK) at Tyr394 by RNS produced from myeloid-derived suppressor cells (MDSCs) plays an important role in the immunosuppression of the tumor microenvironment12. Therefore, our method of nitropeptide identification can be applied to complex biological samples as well. Here, we describe our protocol by using the model peptide Angiotensin II, of which the fragmentation pattern is known and widely used in nitroproteomic studies8-11, as an example.

**PROTOCOL:**

1. **Nitration of Angiotensin II**
   1. To generate nitrated peptide, dilute 10 μL of Angiotensin II (DRVYIHPF) parent solution (2 mM in water) in 390 μL PBS solution (10 mM NaH2PO4, 150 mM NaCl, pH 7.4) at the final concentration of 50 μM.
   2. Add 10 μL of peroxynitrite (200 mM in 4.7% NaOH) to the Angiotensin II solution to make the final concentration of peroxynitrite 5 mM.

NOTE: Peroxynitrite is unstable and easy to resolve when it is in acid form, so parent solution of peroxynitrite is kept in basic solution and stored in -80 °C.

* 1. Adjust the pH value of the solution to 7-8 by 1 M HCl. To initiate the nitration reaction, shake the tube at 400 rpm for 5 min.
  2. Use a commercially available reverse phase SPE column (see **Table of Materials**) for the desalting.

1.4.1. Prepare 2 solutions for the desalting, 5% methanol in water for the washing and 80% methanol in water for the elution.

1.4.2. Add 500 μL of methanol, followed by 500 μL of water to pre-condition the column, put 1 mL pipettor with the tip on the top of the column, press the pipettor to accelerate the flow.

1.4.3. Load 410 μL of the sample in step 1.3 on the column, discard the flow through.

1.4.4. After washing with 500 μL of 5% methanol, use 300 μL of 80% methanol to elute the nitropeptide, which is then fully dried by speed-vac in the default setting at room temperature.

NOTE: Desalting is very important for the reactions, since the solvent left in the previous step may have a negative effect on the next steps.

1. **Alkylation of primary amines by dimethyl labeling**
   1. Reconstitute the powdered nitro-Angiotensin II in 100 μL of 100 mM triethylammonium bicarbonate (TEAB) solution.

NOTE: The pH value of the solution should be between 7 and 9, and make sure no primary amine is added in the solution.

2.2. Add 4 μL of 4% formaldehyde to the solution, mix briefly.

CAUTION: Formaldehyde vapors are toxic; perform the experiments in a fume hood.

2.3. Add 4 μL of 0.6 M NaBH3CN to the solution, shake the tube at 400 rpm for 1 h at room temperature.

2.4. Quench the labeling reaction by adding 16 μL of 1% ammonia solution, incubate at room temperature for 5 min.

2.5. Add 8 μL of formic acid to acidify the solution and perform desalting using reverse phase SPE column as described in step 1.4.

1. **Reduction of nitrotyrosine to aminotyrosine**

3.1. Reconstitute the dimethyl labeled nitro-Angiotensin II in 500 μL of PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4).

3.2. Add 10 μL of 1 M sodium dithionate to the solution and incubate at room temperature for 1 h. After the reduction reaction, the yellow color of the solution becomes clear.

NOTE: Weigh 174.1 mg sodium dithionate and dissolve it in water to make the final volume is 1 mL. This solution can be stored at -20 °C no longer than a week.

3.3. Use the reverse phase SPE column for the desalting as described in step 1.4.

1. **Biotinylation, enrichment, and detection**

4.1. Reconstitute the dimethyl labeled amino Angiotensin II in 500 μL of PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4).

4.2. Add 5 μL of 40 nM NHS-S-S-biotin, dissolved in DMSO, to the solution, after 2 h incubation at room temperature, use 1 μL of 5% hydroxylamine to quench the reaction.

4.3. In the meantime, equilibrate streptavidin agarose beads by adding 500 μL of PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4), centrifuging at 580 x g for 5 min at room temperature and discarding the supernatant. Perform the equilibration 3 times.

4.4. Add 100 μL streptavidin agarose beads to the reaction system, incubate 1 h on a rotary shaker at room temperature. Wash 4 times with PBS (10 mM NaH2PO4, 150 mM NaCl, pH 7.4). For each washing step, add 500 μL of PBS to the beads, mix well, then centrifuge at 580 x g for 5 min at room temperature and discard the supernatant.

4.5. Use 400 μL of 10 mM dithiothreitol (DTT) to incubate with agarose beads at 50 °C for 45 min, spin down at 580 x g for 5 min at room temperature and discard the beads, add 20 μL of 0.5 M idoacetamide (IAM) to the supernatant for 20 min in darkness.

NOTE: DTT is used to break the disulfide bond between the link of biotin and peptide, the latter is released from beads and further alkylated by IAM.

4.6. Use reverse phase SPE column for the desalting shown in step 1.4.

4.7. After resolving the dry peptides in 20 μL of 0.1% formic acid (FA), subject the product of each section to liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis.

4.7.1. Separate the modified Ang II peptides by a 60 min gradient elution (0-8 min, 2% B; 8-10 min, 5% B; 10-35 min, 18% B; 35-50 min, 28% B; 50-52 min, 80% B; 52-58 min, 80% B; 58-59 min, 2% B; 59-60 min, 2% B) at a flow rate 0.300 μl/min with nano-HPLC system which is directly interfaced with the high resolution mass spectrometer.

NOTE: The analytical column is in 75 μm ID, 150 mm length, packed with C-18 resin. Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid.

4.7.2. Operate the mass spectrometer in the data-dependent acquisition mode, set a single full-scan mass spectrum (300-1800 m/z, 60 000 resolution) followed by 20 data-dependent MS/MS scans at 28% normalized collision energy.

4.7.3. Open mass spectra data and identify the peaks of the product in each step to confirm the chemical reaction has been successfully performed.

1. **Quantification of nitropeptide**
   1. Prepare 3 sets of nitro-Angiotensin II solutions from step 1.4, each set containing 2 concentrations of nitro-Angiotensin II: Set #1, 20 nmol in tube A and 20 nmol in tube B, each in 100 μl TEAB solution; Set #2, 10 nmol in tube C and 20 nmol in tube D, each in 100 μl TEAB solution; Set #3, 40 nmol in tube E and 10 nmol in tube F, each in 100 μl TEAB solution.

Note: The two concentrations of nitro-Angiotensin II in each set are used for dimethyl labeling, to react with formaldehyde (light) and formaldehyde-D2 (heavy), respectively.

5.2. For each set, add 4 μL of 4% formaldehyde and formaldehyde-D2 to the sample to be labeled as light and heavy, respectively. Follow the steps 2.3 to 2.5 to finish dimethyl labeling.

5.3. Mix the light and heavy labeled samples.

5.4. Follow the steps from 3.1 to 4.6 for the reduction, biotinylation, enrichment, and detection.

**REPRESENTATIVE RESULTS:**

The flowchart for nitropeptide profiling in this manuscript is shown in **Figure1**. **Figure 2**, **3**, **4** and **5** show the mass spectra of Angiotensin II, nitro-Angiotensin II, dimethyl labeled nitro-Angiotensin II and dimethyl labeled amino Angiotensin II, respectively. The molecular weight of the compound can be reflected by the m/z values of the mono-isotope peak in each figure, indicating the chemical modification on Angiotensin II was successfully achieved for each step. **Figure 6** shows the final product in step 4.7 detected and characterized by LC-MS/MS. **Figure 7** shows the quantitative results of nitropeptide by dimethyl labeling. The relative amounts of light and heavy were determined by the comparison of the intensity of the mono-isotope peak in each group, which allows us to quantify the enriched nitropeptides from different groups.

**FIGURE AND TABLE LEGENDS:**

**Figure 1.** **The workflow of the chemical derivation method for enriching and detecting nitro- Angiotensin II.** First, Angiotensin II is nitrated by peroxynitrite, after desalting, dimethyl labeling is used to block the primary amine on the peptide. Then Sodium dithionate is used to reduce nitrotyrosine to aminotyrosine, which is further reacted with NHS-S-S-biotin. After enriched by streptavidin beads, the peptide is cut by DTT followed by alkylation and detected by LC-MS/MS. This figure has been modified from12, Figure 2.

**Figure 2. The mass spectrum of Angiotensin II.** This figure showed that the mono-isotope peak at m/z 523.78 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of Angiotensin II (bottom). This figure has been modified from12, Figure S2.

**Figure 3.** **The mass spectrum of nitro-Angiotensin II.** This figure showed that the mono-isotope peak at m/z 546.28 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of nitro-Angiotensin II (bottom). This figure has been modified from12, Figure S2.

**Figure 4.** **The mass spectrum of dimethyl labeled nitro-Angiotensin II.** This figure showed that the mono-isotope peak at m/z 560.29 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of dimethyl labeled nitro-Angiotensin II (bottom). This figure has been modified from12, Figure S2.

**Figure 5.** **The mass spectrum of dimethyl labeled amino Angiotensin II.** This figure showed that the mono-isotope peak at m/z 545.31 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of dimethyl labeled amino Angiotensin II (bottom). This figure has been modified from12, Figure S2.

**Figure 6. The mass spectrum of the final product.** This figure showed that the mono-isotope peak at m/z 617.82 matched the duple charged peptide (up) and the MS/MS fragmentation pattern of the final product (middle) and the zoom-in spectrum from 400 to 1000 m/z exhibited more detailed fragmentation pattern (bottom). This figure has been modified from12, Figure S2.

**Figure 7.** **The mass spectra of the quantification results of the nitropeptides by dimethyl labeling.** The light to heavy ratios are 1:1 (up), 1:2 (middle) and 4:1 (bottom), respectively.

**DISCUSSION:**

The protocol here describes the nitropeptide enrichment and profiling. Using Angiotensin II as the model peptide, we illustrated the procedure shown in **Figure 1**. After obtaining the nitro-Angiotensin II, the primary amine on the peptide should be blocked to avoid the further amine conjugation, which is one of the most critical steps within the protocol. In the current protocol, dimethyl labeling is used to block the primary amines for two reasons: first, it enables the acquisition of quantitative results; second, the cost for this reaction is lower than NHS-based reaction and the blocking efficiency is high13. For the quantification, the measured light to heavy ratios are very close to the expected ratios (**Figure 7**), the errors are calculated less than 10%.

Another critical step of the protocol is the reduction of the 3-nitrotyrosine on dimethyl labeled peptide to aminotyrosine by sodium dithionate, which reacts fast and efficiently. The newly generated amine group is further labeled with 4-5 folds excess of NHS-S-S-biotin. Less or more NHS-S-S-biotin would lead to incomplete labeling reaction or incomplete enrichment, respectively. DTT can fully break the disulfide bond and release the enriched peptide from streptavidin beads, which are alkylated by IAM and analyzed by LC-MS/MS.

This protocol is suitable for identifying and quantifying nitropeptides in both biochemical system and biological materials such as cells or tissues. Since biotin enrichment and IAM alkylation can significantly enhance the sensitivity, the lowly abundant nitropeptides become detectable by MS. For example, we used this protocol to profile endogenous nitropeptides of isolated infiltrating T cells from the murine prostate and lung carcinoma models12. We identified nitro-Tyr294 of the protein LCK and performed functional studies to show that this modification may play a critical role in the process of immunosuppression by MDSCs12.

The application of our technique is limited by two factors: first, the low abundance of nitropeptides in cells and tissues dictates that few nitropeptides can be identified; second, the inherent low detection sensitivity of MS compared with the highly sensitive methodologies such as single cell genomic and transcriptomic sequencing. We envision that the application of this protocol to other pathological conditions will help define more protein nitration PTMs with previously unrecognized functions in disease progression.

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**DISCLOSURES:**

The authors have nothing to disclose.

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