



A Strategy for Direct Identification of Protein S-nitrosylation Sites by Quadrupole Time-of-Flight Mass Spectrometry

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Abstract

S-nitrosylation of proteins serves an important role in regulating signal transduction. Identification of the S-nitrosylation sites is crucial for understanding the significance of this post-translational modification in modulating the function of a protein. However, it is very challenging to identify S-nitrosylation sites directly by mass spectrometric (MS) methods due to the labile nature of the S-NO bond. Here we describe a strategy for direct identification of protein S-nitrosylation sites in an electrospray ionization (ESI) quadrupole time-of-flight (QTOF) mass spectrometer without prior chemical derivatization.

Introduction

The covalent modification of a cysteine sulfhydryl group with nitric oxide (NO) is called S-nitrosylation or S-nitrosation. It may function as an important regulatory mechanism for fine-tuning protein activities within diverse cellular processes and biochemical pathways, including signal transduction, DNA repair, ion channel regulation and apoptosis. Several methods have been developed to detect S-nitrosylated proteins, including the use of a SNO-specific antibody to detect *in situ* protein S-nitrosylation by immunohistochemistry; the use of biotin switch method coupled with immunoblotting, 2D-gel electrophoresis, or fluorescence gel electrophoresis to detect changes in protein S-nitrosylation status. However, none of these methods provide the information on specific S-nitrosylation sites within the proteins. MS methods can be used downstream for S-nitrosylation site identification. However, the labile nature of NO attachment to cysteine thiol presents unique challenges for MS analysis, since NO easily falls off the precursor ions during ionization. Here we describe a strategy for the direct identification of protein S-nitrosylation sites using a QTOF MS.

Methods

Synthetic peptides (insulin B (9-23) **SHLVEALYLVCGERG** and β -amyloid peptide fragment **CFRHDSGY**) or thioredoxin (Trx1) was treated by nitroglutathione (GSNO) in an aqueous solution (Table 1). The reaction was carried out at 37° C for 30 min in the dark. The resulting peptide solutions were analyzed directly on a Waters QTOF API US MS. To identify S-nitrosylation site in Trx1, the protein was either analyzed directly by LC/MS, or digested by trypsin prior to LC/MS/MS analysis. The ESI capillary voltage was set at 3,000 V. Both cone and collision energy voltages were optimized to achieve maximal ion intensities of the S-nitrosylated peptide ions in the MS mode. The MS spectra (m/z 400 – 1900) were acquired in the positive ion mode.

Results

1. Effects of pH and metal ion chelators on S-nitrosylation

Experiment	S-nitrosylation reaction solution	MS analysis solution
I	1 mM EDTA and 0.1 mM neocuproine (pH 6.8)	1 mM EDTA and 0.1 mM neocuproine (pH 6.8)
II	1 mM EDTA and 0.1 mM neocuproine (pH 6.8)	1 mM EDTA, 0.1 mM neocuproine and 0.1% formic acid, (pH 3.0)
III	H ₂ O, (pH 6.8)	H ₂ O, (pH 6.8)
IV	1 mM EDTA, 0.1 mM neocuproine and 0.1% formic acid, (pH 3.0)	1 mM EDTA, 0.1 mM neocuproine and 0.1% formic acid, (pH 3.0)

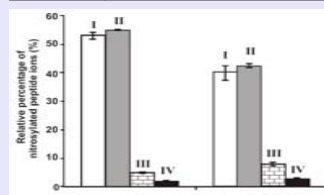


Fig. 1 Effects of pH and metal chelators on MS sensitivities for detecting S-nitrosylated peptide ions. The acidification from pH 6.8 to pH 3.0 after the S-nitrosylation reaction had little effect on S-nitrosylated peptides ions observed in MS. The peptide S-nitrosylation efficiency was reduced in the acidic environment. EDTA and neocuproine were used as chelating agents to sequester metal ions during S-nitrosylation reactions, which has the stabilizing effects on S-NO bonds.

2. Optimization of QTOF MS parameters for direct analysis of S-nitrosylated peptides

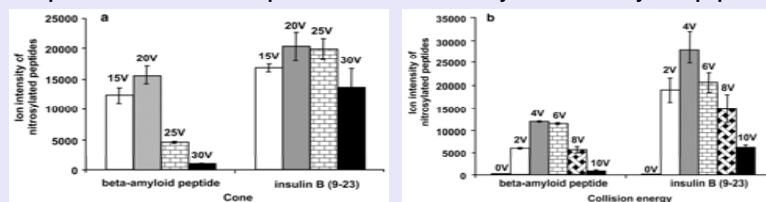


Fig. 2 Effects of QTOF MS cone and collision energy voltages on MS ion intensities of S-nitrosylated peptides. (a) With the collision energy set at 4 V, the effects of varied cone voltages on S-nitrosylated peptide ion signals were analyzed. (b) With the cone voltage set at 20 V, the effects of varied collision energy voltages were evaluated. The optimal cone voltage and collision energy were 20V and 4V, respectively.

3. Identification of S-nitrosylation sites in model peptides

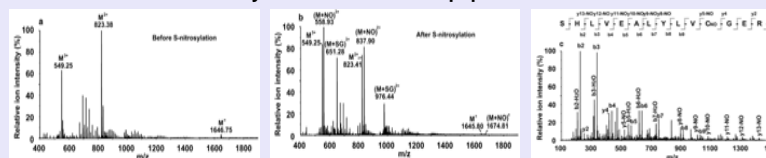


Fig. 3 MS and MS/MS analysis of S-nitrosylated insulin B (9-23). (a) MS spectrum of unmodified peptide. (b) MS spectrum of the peptide after GSNO treatment. (c) MS/MS spectrum of the doubly-charged S-nitrosylated peptide ion of m/z 837.90. The S-nitrosylation site was located on Cys19. All the y-series ions containing the S-nitrosylated cysteine were found to have a neutral loss of 29 Da. The cone voltage was 20 V and the collision energy was 37 V for the MS/MS analyses.

4. Identification of an S-nitrosylation site in Trx1

GSSHHHHHSS SGLVPRGSHM VKQIESK**TAF** QEALDAAGDK LVVVDFSATW
CGPCMKIKPF FHSLSSEKYSN VIFLEVDVDD **QDVASECEV** **KCMPTFGFFK**
KGQKVGVEFSG ANK**EKLEATI** **NELV**

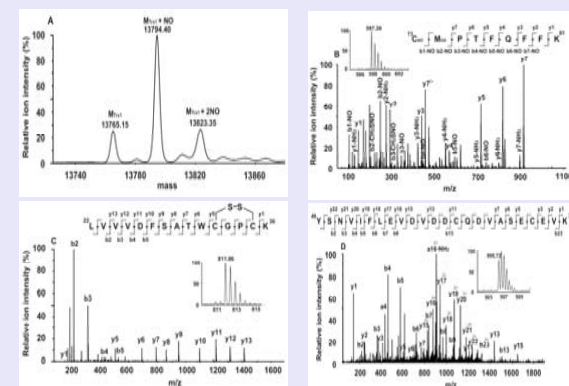


Fig. 4 Identification of Trx1 S-nitrosylation site. (A) The deconvoluted spectrum of S-nitrosylated Trx1 protein after GSNO treatment. (B) MS and MS/MS spectra of a S-nitrosylated tryptic Trx1 peptide. The doubly-charged ion (m/z 597.26, see insert) in the MS spectrum corresponded to S-nitrosylated ⁷³**CMPTFGFFK**⁸¹, with methionine oxidized. In the MS/MS spectrum, both y-series and b-series ions confirmed the peptide sequence as CMPTFGFFK, with its S-nitrosylation site located at Cys73. (C) A doubly-charged ion (m/z 811.86) in the MS spectrum (insert) corresponding to ²²**LVVVDFSATWCGPCK**³⁶, with a disulfide bond formed between the redox catalytic site Cys32 and Cys35. (D) A triply-charged ion (m/z 906.72) corresponding to ⁴⁹**YSNVIFLEVDVDDCCQDVAS** **ECEVK**⁷², with both Cys62 and Cys69 in reduced states.

Summary

We developed an effective LC/MS/MS strategy that enables us to directly identify S-nitrosylation sites in proteins using ESI-QTOF MS. Considering the labile nature of the S-NO bond, the sample solution should include both EDTA and neocuproine, and be maintained at neutral pH to prevent protein denitrosylation during protein extraction, digestion and analysis. Both cone and collision energy voltages in QTOF MS should also be fine-tuned to preserve the S-NO bonds. Using this method, we found that Cys73 in human Trx1 was specifically S-nitrosylated by GSNO treatment. A similar approach could be adapted for the identification of S-NO sites in biological samples.

Acknowledgement

This research is supported by a NIH grant, NS046593 to HL. This study has been published in J Am Soc Mass Spectrom. 2008, 19(9):1353-60