Chapter 2

A Rapid and Selective Mass Spectrometric Method for the Identification of Nitrated Proteins

Angela Amoresano, Giovanni Chiappetta, Piero Pucci, and Gennaro Marino

Abstract

The nitration of protein tyrosine residues represents an important posttranslational modification during development, oxidative stress, and biological aging. The major challenge in the proteomic analysis of nitroproteins is the need to discriminate modified proteins, usually occurring at substoichiometric levels, from the large amount of nonmodified proteins. Moreover, precise localization of the nitration site is often required to fully describe the biological process. Identification of the specific targets of protein oxidation was previously accomplished using immunoprecipitation techniques followed by immunochemical detection. Here, we report a totally new approach involving dansyl chloride labeling of the nitration sites which relies on the enormous potential of MS^n analysis. The tryptic digest from the entire protein mixture is directly analyzed by MS on a linear ion trap mass spectrometer. Discrimination between nitro- and unmodified peptide is based on two selectivity criteria obtained by combining a precursor ion scan and a MS3 analysis. The novel labeling procedure was successfully applied to the identification of 3-nitrotyrosine residues in complex protein mixtures.

Key words: Protein nitration, proteomics, dansyl chloride, MS3, mass spectrometry, 3-nitrotyrosine, precursor ion scan.

1. Introduction

Oxidative stress is now recognized as accountable for redox regulation involving reactive oxygen species (ROS) and reactive nitrogen species (RNS). Its role is pivotal for the modulation of critical cellular functions, notably for neurons astrocytes and microglia, such as apoptosis program activation, ion transport, and calcium mobilization involved in excitotoxicity *(1)*.

Oxidative stress is caused by an imbalance in the pro-oxidant and antioxidant systems. Classically, oxidative stress is described as an imbalance between generation and elimination of ROS and RNS. Oxidative stress may cause reversible and/or irreversible modifications on sensitive proteins leading to structural, functional, and stability modulations *(2, 3)*. Protein modifications such as carbonylation, nitration, and protein–protein cross-linking are generally associated with loss of function and may lead to either the unfolding and degradation of the damaged proteins, or aggregation leading to accumulation as cytoplasmic inclusions, as observed in age-related neurodegenerative disorders *(4)*.

Proteomics has the potential to identify novel targets of tyrosine nitration in cells and tissues and depict the nitro- and phosphoproteome, or to identify proteins undergoing S-nitrosylation in vivo. The nitration of a tyrosine residue to form 3-nitrotyrosine (the addition of a nitro ($-NO_2$) group to position 3 of the phenolic ring of a tyrosine residue *(5)*) is an important post-translational protein modification that is associated with different biological processes and pathologies *(6, 7, 8)* including cancer, Parkinson's disease, Alzheimer's disease, Huntington's disease, lung infection, retinal ischemia, aging, and oxidative stress.

Methods for separation, detection, and quantitation of *o*-nitrotyrosine in biological samples include immunochemical techniques using anti-*o*-nitrotyrosine antibodies, HPLC in combination with various detection systems, and GC/MS *(9)*. A combination of MS techniques had been used to identify the specific tyrosine residues nitrated *in vitro* in model proteins *(10)*. More recently, a higher throughput characterization of protein targets for tyrosine nitration in cells and several tissues, including aged tissues, has been attempted using proteomic methodologies *(11, 12)*. These approaches essentially consist of protein fractionation by two dimensional polyacrylamide gel electrophoresis (2D PAGE), partial transfer onto poly(vinylidene difluoride) membranes, and western blot analysis using anti-nitrotyrosine antibody to identify the modified proteins. However, such methods are both time-consuming and laborious, and as noted require a good guess to the identity of the protein at the beginning. Moreover, post-translational modification can sometimes change the structure of proteins, which could then prevent the formation of the appropriate antigen–antibody complex. With advances in technology, proteomics coupled to mass spectrometry has been a major methodological development that allows the identification of large number of proteins at once *(13)*.

Alignment of the western blots with the 2D PAGE gels enables identification of immunopositive protein spots. These are then excised, trypsin digested, and identified by either peptide mass fingerprinting procedures using MALDI mass spectrometry or capillary LC-MS/MS analyses *(14)*. Recently, a novel nitro-proteomics analytical system was developed for the current study. A highly specific nitrotyrosine affinity column (NTAC) was used to preferentially enrich and isolate endogenous nitroproteins and nitroprotein–protein complexes from a human pituitary tumor proteome *(15)*.

Here, a novel approach to selectively label *o*-nitrotyrosine residues in proteins using dansyl modification coupled with tandem mass spectrometry experiments in precursor ion /MS3 scan mode *(16)* is presented. The methodology, developed on the basis of a general derivatization method – the reporter ion generation tag (RIGhT) strategy *(17, 18)*, was first tested on *in vitro* nitrated BSA as a model protein and then applied to more complex matrices. Because of its operational simplicity avoiding long-lasting and time-consuming fractionation procedures, this new strategy seems to be well suited for large-scale proteomic profiling of nitration sites.

2. Materials

2.1. Equipment

1. Voyager DE-STR MALDI-TOF mass spectrometer with 337 nm nitrogen laser and reflector analyzer, Applied Biosystems, Foster City, California

2. Hybrid 4000 Q-Trap instrument with linear ion trap, Applied Biosystems, Foster City, California

3. Model 1100 nano HPLC system, Agilent Technologies, Santa Clara, California

2.2. Chemicals

Tri(hydroxymethyl)aminomethane (Tris), 5-N,Ndimethylami-nophtalene-1-sulfonyl chloride (dansyl chloride, DNSCl), ammonium hydrogen carbonate (AMBIC), and iodoacetamide were purchased from Fluka (St. Louis, MO), tetranitromethane (TNM), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), sodium dithionite ($Na_2S_2O_4$), urea, sodium acetate, trypsin, and dithiothreitol (DTT) were from Sigma (St. Louis, MO) and acetonitrile (ACN) was purchased from Baker (Phillipsburg, NJ). Trifluoroacetic acid HPLC grade was from Carlo Erba. All solvents were of the highest purity available from Baker. All other reagents and proteins were of the highest purity available from Sigma.

2.3. In Vitro Nitration of BSA

1. Bovine serum albumin (BSA) solution (10 mg/mL) from Sigma was prepared in 200 mM Tris buffer (pH 8.0); stored at −20°C.

2. 350 mM Tetranitromethane (TNM) solution was prepared in acetonitrile. Made fresh as required.

3. Sephadex G-25M size exclusion chromatography column (Amersham).

4. 50 mM AMBIC buffer (pH 8.0).

2.4. Tryptic Digestion

1. Denaturant buffer contained 6 M urea, 10 mM Tris, 125 mM EDTA, pH 8.0. Stored at room temperature.

2. 50 mM AMBIC buffer (pH 8.0). Made fresh as required.

3. Reversed-phase HPLC C4 column (100 × 4.6 mm, 5 μm; Phenomenex).

4. 0.1% TFA in water (solvent A)

5. 0.07% TFA in 95% acetonitrile (solvent B).

6. Trypsin solution was prepared dissolving the enzyme at 1mg/ml in 50 mM AMBIC buffer (pH 8.0). Made fresh as required.

2.5. Reduction of Nitrotyrosine to Aminotyrosine

1. 200 mM Tris buffer (pH 8.0). Made fresh as required.

2. 200 mM $Na_2S_2O_4$ as described by McIntyre *(19)*. Freshly prepared.

3. Samples cleaning was performed using reversed-phase Zip-Tips C18 from Millipore (Billerica, MA) using the procedure recommended by the manufacturer.

2.6. Synthesis of o-Aminodansyltyrosine

1. Reversed-phase HPLC C18 column (100 × 4.6 mm, 5 μm; Phenomenex).

2. 0.1% TFA in water (solvent A)

3. 0.07% TFA in 95% acetonitrile (solvent B).

4. 100 mM sodium acetate pH 5.0 buffer, 0.9% sodium chloride. Stored at 4°C.

5. Labeling solution was prepared by dissolving DNS-Cl at 18.5 ng/μL in ACN. Made fresh as required.

2.7. Labeling of Bovine Milk Protein Extract

1. 100 mM TNM solution was prepared and stored at room temperature.

2. Precipitation with the Amersham Clean Up kit as recommended.

3. Denaturant buffer (20 mM DTT, 40 mM iodoacetamide, 6 M urea, pH 8.0)

4. 200 mM solution $Na_2S_2O_4$ in denaturant buffer.

5. 50 mM AMBIC buffer, pH 8.0. Made fresh as required.

6. Labeling solution was prepared by dissolving DNS-Cl at 18.5 ng/μL in ACN. Made fresh as required.

2.8. MALDI Mass Spectrometry

1. 10 mg/mL solution of R-cyano-4-hydroxycinnamic acid in acetonitrile/50 mM citrate buffer (2/3, v/v). Made fresh as required and stored at 4°C for 1 day.

2.9. NanoLC-MS2 and MS3 Mass Spectrometry

1. Agilent reversed-phase precolumn cartridge (Zorbax 300 SB-C18, 5 × 0.3 mm, 5 μm) working at 10 μL/min

2. Agilent reversed-phase column (Zorbax 300 SBC18, 150 mm × 75 μm, 3.5 μm), at a flow rate of 0.2 μL/min

3. Solvent A was 0.1% formic, ACN in water acid solution

4. Solvent B was 98% ACN, 2% 0.1% formic acid solution

5. Uncoated silica tip (o.d. 150 μm, i.d. 20 μm, tip diameter 10 μm) from New Objectives (Ringoes, NJ).

3. Methods

A challenging task in the proteomic analysis of nitroproteins is the need to discriminate nitrated proteins, usually in very low concentrations, from the large amount of nonmodified proteins. Moreover, precise localization of the nitration site is often required to fully describe the biological process.

The strategy described here is based on dansyl chloride labeling and relies on the enormous potential of MSn analysis. Peptide analysis is carried out by LC-MS/MS, and the ions of interest are discriminated by two selectivity criteria based on two subsequent tandem MS experiments, a precursor ion scan followed by an MS3 scan mode, a sort of "instrumentally driven" bidimensional selection. This instrumental selectivity approach needs an appropriate derivatizing agent, like dansyl chloride, producing stable and diagnostic fragment ions (**Scheme 2.1**) to be used in the MS/MS scan modes and capable of improving ionization of modified peptides.

3.1. In Vitro Nitration of BSA

1. A BSA solution (10 mg/mL) in 200 mM Tris buffer (pH 8.0) was nitrated by addition of 350 mM TNM in acetonitrile using a Tyr/TNM ratio of 1/1 (mol/mol). The reaction mixture was stirred at room temperature for 30 min.
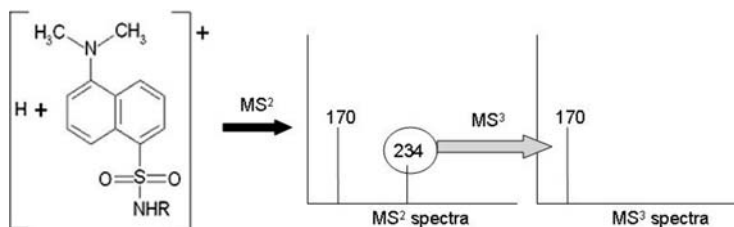
Scheme 2.1. Peculiar electrospray dansyl derivative fragmentation. Distinctive m/z 170 and 234 product ions are observed in MS2 mode and the diagnostic m/z 234→170 fragmentation is observed in the MS3 mode.

2. The excess reagent was rapidly removed by size exclusion chromatography as on a Sephadex G-25M column equilibrated and eluted with 50 mM AMBIC buffer. Protein elution was monitored at 280 and 350 nm.

3. The protein-containing fractions were manually collected, lyophilized, and stored at −20°C.

3.2. Trypsin Digestion

1. Aliquots of the BSA and N-BSA mixture were dissolved in denaturant buffer, reduced with DTT (10-fold molar excess on the cysteine residues) for 2 h at 37°C and then alkylated with iodoacetammide (5-fold molar excess on the thiol residues) for 30 min at room temperature in the dark.

2. Protein samples were desalted by reversed-phase (RP)-HPLC by means of a linear gradient from 5% to 95% of solvent B in 10 min. Fractions were lyophilized.

3. Reduced and alkylated proteins were dissolved in 50 μl of 50 mM AMBIC buffer

4. Trypsin digestion was carried out using an enzyme/substrate ratio of 1/50 (w/w) at 37°C for 18 h.

5. The resulting peptide mixtures were directly analyzed by MALDI mass spectrometry on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). Typically, 1 μL of the total mixture was mixed (1/1, v/v) with matrix solution to determine TNM-induced modifications. Spectra were acquired by monitoring positive ions using accelerating voltage of 70 kV, grid 63%, and grid wire 0.01% in respect of accelerating voltage; delay time was 100 ns. Mass range was 400–4000 m/z. Mass calibration was performed using external peptide standards by Applied Biosystems. Raw data were analyzed using the computer software provided by the manufacturer and reported as monoisotopic masses. As an example, a mass signal recorded at m/z 972.5
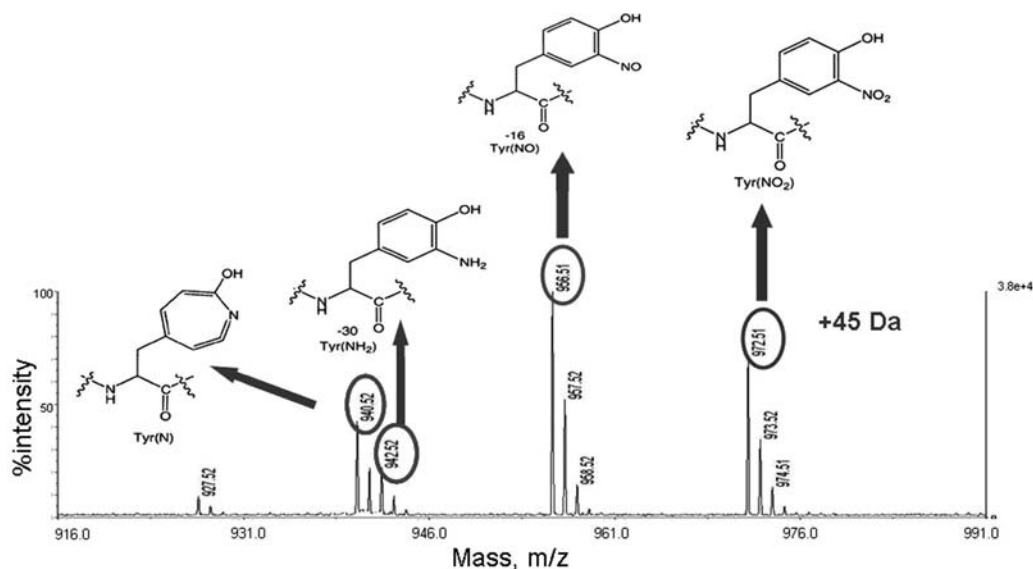
Fig. 2.1. Partial MALDI-MS spectrum of peptide mixture from *in vitro* nitrated BSA. Satellite peaks at −16 and −30 and −32 Da, revealing the typical MALDI photodecomposition pattern of nitrated peptides, are present and the corresponding structures are reported.

did not correspond to any peptide within the BSA sequence. This value occurred 45 Da higher than the signal corresponding to the peptide 137–143 suggesting the nitrated derivative of the fragment (**Fig. 2.1**).

3.3. Reduction of Nitrotyrosine to Aminotyrosine

1. Conversion of nitropeptides into their *o*-dansylamino derivatives was accomplished by $Na_2S_2O_4$ treatment. The sample was dissolved in 200 mM $Na_2S_2O_4$ solution for 1 min at room temperature.

2. Before MALDI-TOF analysis, cleaning of the samples was performed using reversed-phase Zip-Tips C18 following the procedure suggested by the manufacturer.

3. The extent of reduction was monitored by MALDI-MS performed as previously described. MALDIMS spectrum showed the disappearance of the classical *o*-nitrotyrosine photodecomposition pattern, and its replacement by a new signal at m/z 942.4 attributed to peptides m/z 137–143 containing an amino-Tyr residue.

3.4. Synthesis of o-Aminodansyltyrosine

1. The mixture of reduced BSA tryptic peptides was desalted by RP-HPLC by means of a linear gradient from 5% to 65% of solvent B in 5 min.

2. Peptide fractions were lyophilized and then dissolved in 100 mM sodium acetate buffer.

3. Samples were treated with a DNS-Cl solution (1000-fold molar excess) at 65°C for 16 h as previously reported *(17)*.

4. The extent of reaction was monitored by MALDI-MS analysis showing the presence of a new signal at m/z 1175.6 occurring 233 Da higher than the *o*-aminotyrosine-containing peptide (**Fig. 2.2**), corresponding to the expected dansyl derivative of the *o*-aminotyrosine peptide.

3.5. LC-MS/MS Analysis of o-Aminodansyl-Tyr Peptides

1. The exact location of the original nitro groups was assessed by LCMS/MS analysis of the peptide mixture using a linear ion trap 4000 Q-Trap instrument (Applied Biosystems) coupled to an 1100 nano HPLC system (Agilent Technologies).

2. Peptide mixtures were loaded onto an Agilent reversed-phase pre-column cartridge at 10 μL/min with solvent A using a loading time of 7 min.

3. Peptides were then separated on a Agilent reversed-phase column, at a flow rate of 0.2 μL/min.

4. The elution was accomplished by a 5–65% linear gradient of solvent B in 60 min.

5. A micro-ionspray source was used at 2.5 kV with liquid coupling, with a declustering potential of 50 V using an uncoated silica tip.
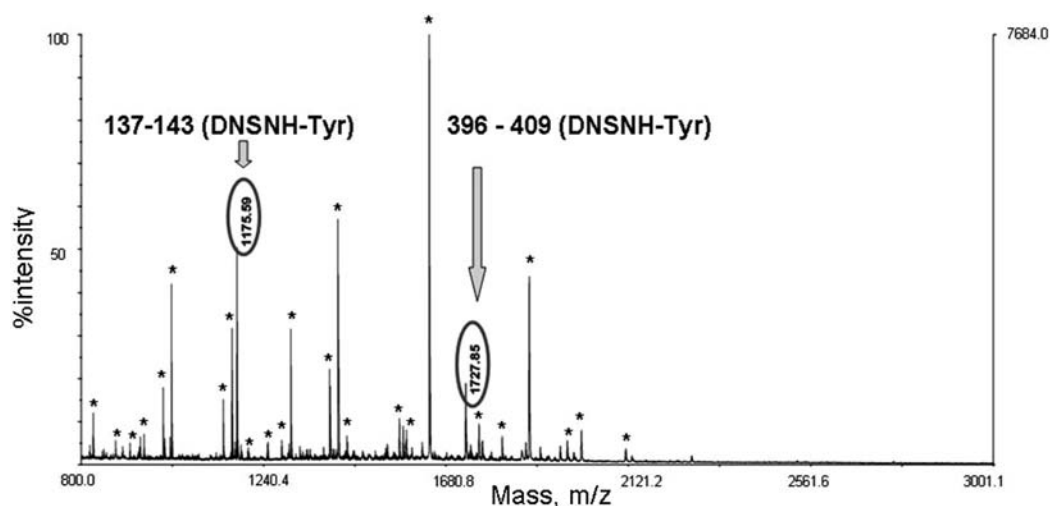

Fig. 2.2. Partial MALDI-MS spectrum of dansylated *o*-aminotyrosine-containing peptides. The modified peptides are indicated by arrows. Other peaks present in the spectrum and labeled with asterisks correspond to theoretical peptide fragments within BSA sequence.

6. Spectra acquisition was based on a survey precursor ion scan for m/z 170. The Q1 quadrupole was scanned from m/z 500 to 1000 in 2 s with resolution "low", and the precursor ions were fragmented in q2 using a linear gradient of collision potential from 30 to 70 V. Finally, Q3 was set to transmit only ions at m/z 170 with resolution "unit".

7. The survey precursor ion scan was followed by an enhanced resolution experiment for the ions of interest and then by MS3 and MS2 acquisitions of the two most abundant ions. The entire cycle duration was 5.3 s.

8. MS2 spectra were acquired using the best collision energy calculated on the basis of m/z values and charge state (rolling collision energy).

9. MS3 spectra were performed on the fragment ion at m/z 234 and acquired using Q0 trapping, with a trapping time of 150 ms and an activation time of 100 ms, scanning from m/z 160 to 210. The MS3 TIC (**Fig. 2.3A**) showed the presence of only three peaks related to doubly charged ions at m/z 588.2, 792.9, and 986.8. The corresponding MS2 fragmentation spectra led to the determination of the entire sequence, identifying the fragments m/z 137–143,
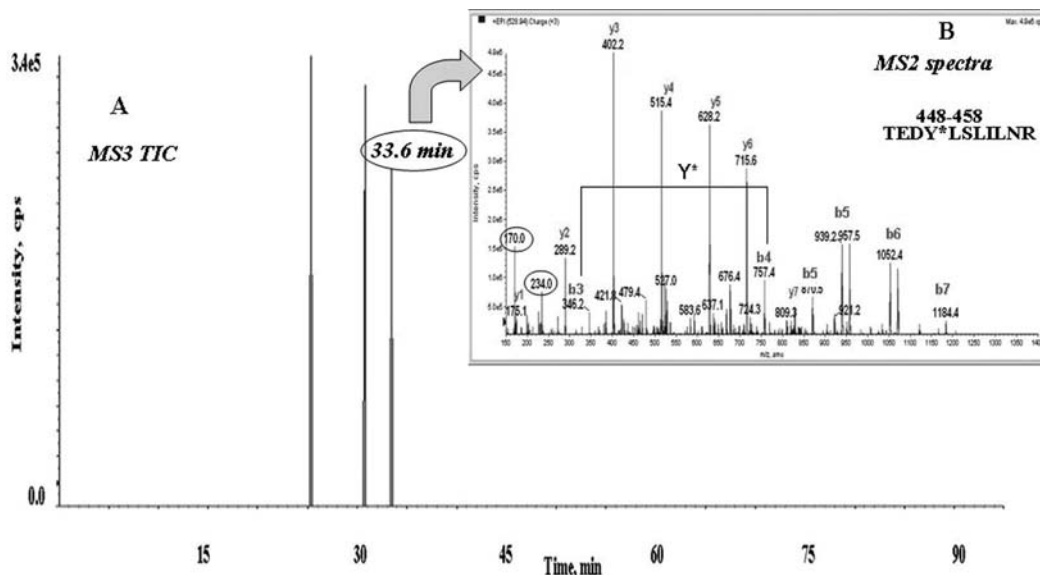

Fig. 2.3. MS3 TIC profile of tryptic nitrated BSA digest. As indicated, the MS3 analysis showed the presence of only three peaks related to doubly charged ions at m/z 588.2, 792.9, and 986.8 (Panel **A**). The corresponding MS2 fragmentation spectra led to the determination of the entire sequence of these species. The insert (**B**) showed the MSMS spectrum, properly annotated, leading to the reconstruction of the sequence 445–448. This sequence is related to the fragment 448–458, modified at level of Tyr 451 allowing for the exact identification of the original nitration site.

396–409, and 445–458, respectively, containing *o*-aminodansyl-Tyr residues.

10. Identification of nitrated tyrosine residues was carried out by taking advantage of the flexibility of the MASCOT software. In fact, a variable modification of 411.0 Da corresponding to *o*-aminodansyltyrosine residues was introduced into the Modification File within the MASCOT software.

11. The peak list used for database search only consisted in the MS2 spectra of the peptide species that had generated a signal both in the precursor ion TIC and in the MS3 ion scan.

12. Modified Tyr residues could easily be detected, by the interpretation of the MS2 spectra, allowing for the exact identification of the original nitration sites. As an example, **Fig. 2.3B** shows the MS/MS spectrum of the peptide m/z 445–458 carrying a dansyl moiety. The modified ion is stable during collision-induced dissociation. Both the y and b fragment ions still retained the modifying group linked to the amino Tyr residue, thus localizing the original nitration site.

3.6. Applications

3.6.1. Location of BSA o-Aminotyrosine-containing Peptides in Complex Protein Mixtures

1. The feasibility of the developed strategy was probed by mixing 100 µg of the mixture of BSA and nitrated BSA with 10 mg of the entire cellular extract from Escherichia coli.

2. Nitrotyrosines were reduced with dithionite, as described before.

3. The modified protein mixture was digested with trypsin as described.

4. The newly generated amino Tyr-containing peptides were labeled with dansyl chloride at pH 5.0, and an aliquot of peptide digest containing 100 fmol of nitropeptide mixture was submitted to the bidimensional mass spectrometry analysis. The precursor ion scan mode still showed the occurrence of a large number of signals, most of which not related to 3-NT-containing peptides. In fact, the corresponding MS2 spectra do not show the occurrence of fragment ion at m/z 234. The further selection based on the MS3 scan removed a large number of false positives leading to a simple ion chromatogram essentially dominated by three intense signals (**Fig. 2.4A, B**). The analysis revealed the occurrence of the three BSA 3-NT-containing peptides previously detected in the analysis of single protein.

3.6.2. Labeling and Analysis of a Nitrated Protein Extract of Bovine Milk

1. Bovine milk was reacted with a 100 mM solution of TNM for 30 min at room temperature.

2. Modified milk proteins were purified by precipitation with the Amersham Clean Up kit and dissolved in denaturant buffer.
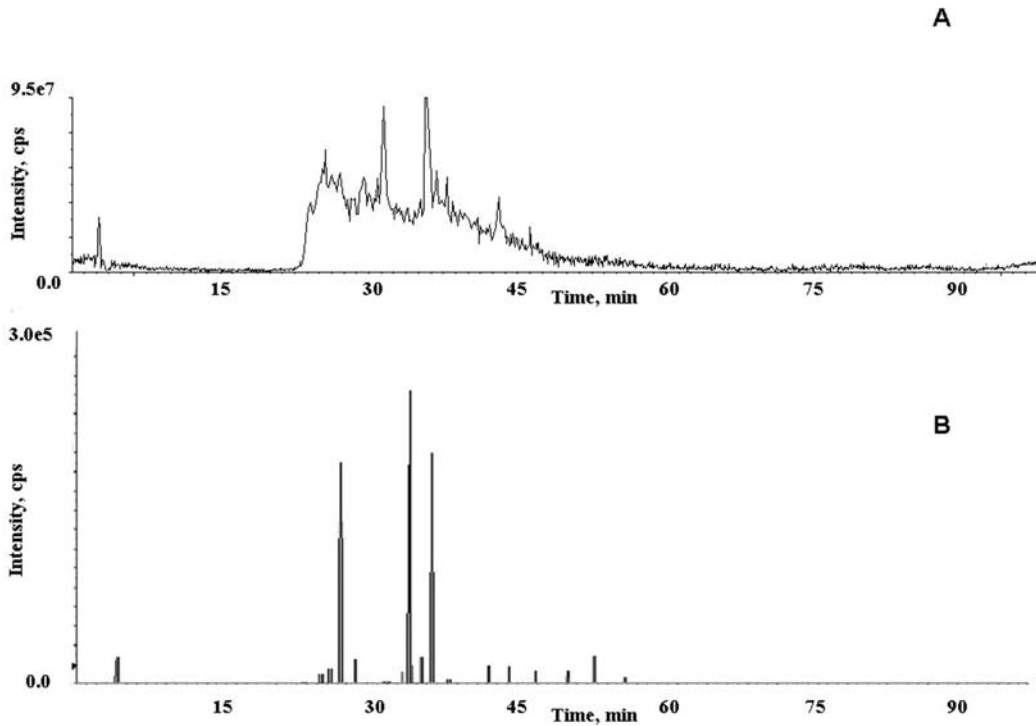
Fig. 2.4. LC-MS/MS (Panel **A**) and MS3 (Panel **B**) traces of bidimensional mass spectrometry experiment performed on the entire *E. coli.* cellular extract spiked with a mixture of BSA and nitrated BSA.

3. Reduction of both the SH and nitro groups of the protein mixture was carried out in "one pot" using 20 mM DTT, 40 mM iodoacetamide, and a 200 mM solution of $Na_2S_2O_4$ in the dark for 30 min.

4. The protein mixture was purified by size exclusion chromatography on a Sephadex G-25 M column equilibrated and eluted with 50 mM AMBIC.

5. Protein fractions were concentrated and then digested with trypsin as already described.

6. The resulting peptide mixture was selectively labeled with dansyl chloride solution.

7. 150 fmol of the labeled peptide mixture was directly submitted to LC-MS/MS analyze using the double selectivity criteria. As an example, **Fig. 2.5** (panels A and B) reports the MS, and MS3 profiles for the milk mixture.

8. Identification of nitrated milk proteins was carried out using the modified MASCOT software as previously described (*see* **Fig. 2.5B**).
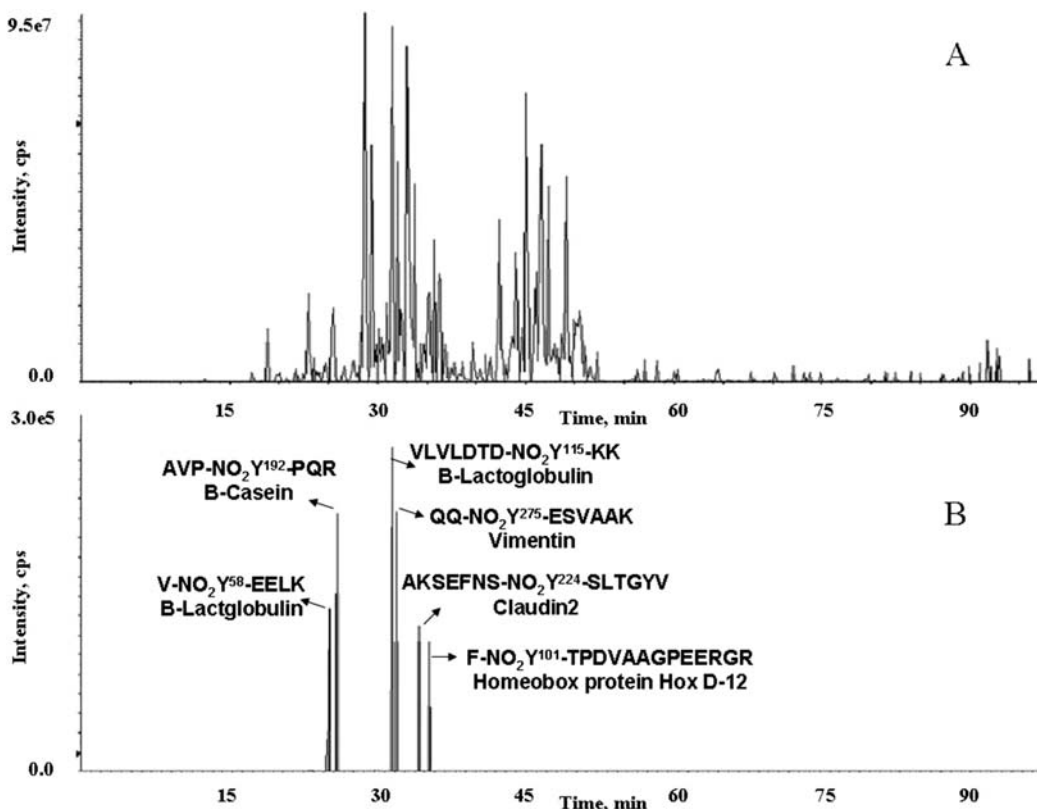
Fig. 2.5. Reconstructed ion chromatogram for the precursor ion scan (Panel **A**) and the selective dansyl transition in MS3 mode (Panel **B**) of bidimensional mass spectrometry experiment performed on *in vitro* nitrated bovine milk proteins. Nitrated proteins identified and localization of the nitration sites are reported.

4. Notes

1. Pay attention when handling TNM. We generally use two couples of gloves.

2. All solutions were prepared by using bi-distilled water by using Milli-Q system (Waters).

3. Aliquots of standard protein solution contain about 1 mg of intact protein.

4. Aliquots of 10 pmol and 150 fmol of tryptic digest were used for MALDIMS and LCMSMS analyses, respectively.

5. A sample of commercially available bovine milk was used for the labeling experiments.

6. The analysis is carried out at the level of peptides following tryptic digest of the whole protein mixture rather then at intact protein level.

7. Discrimination between nitropeptides and unmodified peptides is achieved by taking advantage of the instrumental features of a hybrid (triple quadrupole/linear ion trap) mass spectrometer.

8. The presence of a possible nitropeptide in the MALDIMS analysis is suggested by satellite peaks at −16 and −32 Da, revealing the typical photodecomposition pattern of nitrated peptides previously described to occur during the MALDI analysis of nitropeptides.

9. Check pH during Na_2SO_4 treatment. A slightly acid value could give rise to dithionite dismutation.

10. Aminotyrosine residues were chemoselectively labeled with DNS-Cl at pH 5.0 exploiting the different pKa values of the o-aminotyrosine (4.7), 15 which is partly deprotonated and is therefore amenable to reaction with DNS-Cl at variance with aliphatic (10.4–11.1) and N-terminal amino groups (6.8–8.0), which are largely protonated.

11. In the MALDIMS analysis, the occurrence of signals corresponding to the unmodified o-aminotyrosine peptides indicated that the extent of dansyl reaction was about 60% as estimated by the relative intensity of the corresponding peaks.

12. No modification at the N-terminus or Lys residues should be detected when operating in this condition.

13. The LC-MS/MS analysis revealed the occurrence of a further nitration site within the peptide m/z 448–458 at level of Tyr451, which escaped previous MALDI analysis, probably due to the different chemical-physical properties of the peptide under different ionization conditions.

14. Analysis of chemically nitrated BSA following tryptic digestion confirmed the ability of the bidimensional selection to simplify the peptide ion chromatogram, leading to selective identification of o-nitrotyrosine-containing peptides

15. The set up strategy underlined the usefulness of the two selectivity criteria in that the precursor ion scan still showed the presence of nonnitrated peptide ions that were completely ruled out in the MS3 scan mode. In fact, the precursor ion scan mode still showed the occurrence of a large number of signals, most of which not related to 3-NT-containing peptides. (the corresponding MS2 spectra do not show the occurrence of fragment ion at m/z 234). The further selection based on the MS3 scan removed a large number of false positives leading to a simple ion chromatogram essentially dominated by three intense signals.

Acknowledgments

References

1. Emerit, J., Edeasc, M. and Bricairea, F. (2004) Neurodegenerative diseases and oxidative stress. *Biomed. Pharmacother.* **58**, 39–46.

2. Nakamura, K., Hori, T., Sato, N., Sugie, K., Kawakami, T. and Yodoi, J. (1993) Redox regulation of a src family protein tyrosine kinase p56lck in T cells. *Oncogene* **8**, 3133–3139.

3. Staal, F.J., Anderson, M.T., Staal, G.E., Herzenberg, L.A. and Gitler, C. (1994) Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3619–3622.

4. Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R. and Milzani, A. (2005) Proteins as biomarkers of oxidative/ nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom. Rev.* **24**, 55–99.

5. Zhan, X. and Desiderio, D.M. (2004) The human pituitary nitroproteome: detection of nitrotyrosyl-proteins with two-dimensional Western blotting, and amino acid sequence determination with mass spectrometry, *Biochem. Biophys. Res. Commun.* **325**, 1180–1186.

6. Haddad, I.Y., Pataki, G., Hu, P., Galliani, C., Beckman, J.S. and Matalon, S. (1994) Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury, *J. Clin. Invest.* **94**, 2407–2413.

7. Tsukahara, H. (2007) Biomarkers for oxidative stress: clinical application in pediatric medicine. *Curr. Med. Chem.* **14**, 339–351.

8. Bian, K., Ke, Y., Kamisaki, Y. and Murad, F. (2006) Proteomic modification by nitric oxide. *J. Pharmacol. Sci.* **101**, 271–279.

9. Soderling, A.S., Ryberg, H., Gabrielsson, A., Larstad, M., Toren, K., Niari, S. and Caidahl, K. (2003) A derivatization assay using gaschromatography/negative chemical ionization tandem mass spectrometry to quantify 3-nitrotyrosine in human plasma. *J. Mass Spectrom.* **38**, 1187–1196.

10. Lee, S.J., Lee, J.R., Kim, Y.H., Park, Y.S., Park, S.I., Park, H.S. and Kim, K.P. (2007) Investigation of tyrosine nitration and nitrosylation of angiotensin II and bovine serum albumin with electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **21**, 2797–2804.

11. Kanski, J., Alterman, M.A. and Schoöneich, C. (2003) Proteomic identification of age-dependent protein nitration in rat skeletal muscle. *Free Radic. Biol. Med.* **35**, 1229–1239.

12. Kanski, J. and Schoneich, C. (2005) Protein nitration in biological aging: proteomic and tandem mass spectrometric characterization of nitrated sites. *Meth. Enzymol.* **396**, 160–171.

13. Butterfield, D.A., Perluigi, M. and Sultana R. (2006) Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. *Eur. J. Pharmacol.* **545**, 39–50.

14. Aulak, K.S., Koeck, T., Crabb, J.W. and Stuehr, D.J. (2004) Proteomic method for identification of tyrosine-nitrated proteins. *Methods Mol. Biol.* **279**, 151–165.

15. Zhan, X. and Desiderio, D.M. (2006) Nitroproteins from a human pituitary adenoma tissue discovered with a nitrotyrosine affinity column and tandem mass spectrometry. *Anal. Biochem.* **354**, 279–289.

16. Amoresano, A., Monti, G., Cirulli, C. and Marino, G. (2006) Selective detection and identification of phosphopeptides by dansyl MS/MS/MS fragmentation. *Rapid Commun. Mass Spectrom.* **20**, 1400–1404.

17. Cirulli, C., Marino, G. and Amoresano, A. (2007) Membrane proteome in Escherichia coli probed by MS3 mass spectrometry: a preliminary report. *Rapid Commun. Mass Spectrom.* **21**, 2389–2397.

18. Amoresano, A., Chiappetta, G., Pucci, P., D'Ischia, M. and Marino, G. (2007) Bidimensional tandem mass spectrometry for selective identification of nitration sites in proteins. *Anal. Chem.* **79**, 2109–2117.

19. McIntyre, J.C., Schroeder, F. and Behnke, W.D. (1990) Synthesis and characterization of the dansyltyrosine derivatives of porcine pancreatic colipase. *Biochemistry* **29**, 2092–2101.