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Bidimensional Tandem Mass Spectrometry for Selective Identification of Nitration Sites in Proteins

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Nitration of protein tyrosine residues is very often regarded as a molecular signal of peroxynitrite formation during development, oxidative stress, and aging. However, protein nitration might also have biological functions comparable to protein phosphorylation, mainly in redox signaling and in signal transduction. 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. Existing methodologies essentially rely on immunochemical techniques either using 2D-PAGE fractionation in combination with western blot analyses or exploiting immunoaffinity procedures to selectively capture nitrated proteins. Here we report a totally new approach involving dansyl chloride labeling of the nitration sites that rely 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 an MS^3 analysis. This new procedure was successfully applied to the identification of 3-nitrotyrosine residues in complex protein mixtures.

Nitration of protein tyrosine residues leading to *o*-nitrotyrosine is a most intriguing post-translational modification commonly regarded as a "footprint" of peroxynitrite formation during development, oxidative/nitrosative stress, and aging. Tyrosine nitration can compromise protein structure and function,[1,2] partially due to the shift of the phenolic pK_a value into the physiological range, i.e., 7.1,[3] entailing that a significant fraction of *o*-nitrotyrosine may exist in vivo in its deprotonated state, displaying a net negative charge. The functional consequences of protein nitration may be manifold, but the impact is mainly on the structural and conformational properties and catalytic activity. Protein nitration may also have biological functions comparable with those of protein phosphorylation, being involved in redox signaling. On the other hand, modification of the phenolic ring might impair tyrosine phosphorylation, thus inhibiting covalent modulation, signal transduction, or both.

Nitration is a relatively stable modification that can be suitably analyzed by different specific techniques. 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.[4] The chromatographic methods usually analyze *o*-nitrotyrosine released by acid or enzymatic hydrolysis of protein extracts.[5] A combination of MS techniques had been used to identify the specific tyrosine residues nitrated in vitro in model proteins[6−8] and in proteins purified from tissues.[9] 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.[10,11] 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. 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.[12] Usually, how-

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ever, the direct mass spectrometric identification of nitrated peptides is difficult and merely relies on the correct alignment of western blot analyses and 2D gels. Moreover, a prerequisite for successful identification is the presence of a single protein per gel spot. If the gel spot contains multiple proteins or protein isoforms,[13] unambiguous identification of the nitrated protein can only be obtained by tandem MS sequencing of the *o*-nitrotyrosine-containing peptide.

Recently we developed a novel approach to selectively label phospho-Ser/-Thr residues in proteins using dansyl modification coupled with tandem mass spectrometry experiments in precursor ion /MS^3 scan mode, taking advantage of a hybrid mass spectrometer.[14] This concept led to the development of a general method, reporter ion generation tag (RIGhT), of potential interest for large-scale proteomic identification of a broad range of PTMs. In this paper, we report the extension of this innovative strategy to the selective isolation and identification of *o*-nitrotyrosine-containing proteins. The methodology was first tested on in vitro nitrated BSA as a model protein and then applied to more complex matrices.

EXPERIMENTAL SECTION

Chemicals. Tri(hydroxymethyl)aminomethane (Tris), 5-*N*,*N*-dimethylaminophtalene-1-sulfonyl chloride (dansyl chloride, DNS-Cl), ammonium hydrogen carbonate (AMBIC), and iodoacetamide, were purchased from Fluka, Tetranitromethane (TNM), b;ovine 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), Acetonitrile (ACN) was purchased from Baker (Phillipsburg, NJ). SDS-PAGE and western blot chemicals were purchased from Bio-Rad. Nitrotyrosine antibody was from Alexis. 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.

In Vitro Nitration of BSA. 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 as previously described.[15] Nitrated BSA (N-BSA) was rapidly desalted by size exclusion chromatography on a Sephadex G-25M column (Amersham). Protein elution was monitored at 280 and 350 nm. The fraction containing the protein was manually collected, lyophilized, and stored at −20 °C.

Tryptic Digestion. Aliquots of the BSA and N-BSA mixture were dissolved in denaturant buffer (urea 6 M, Tris, EDTA pH 8.0), 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. Protein samples were desalted by reversed-phase (RP)-HPLC on a reversed-phase C4 column (100 × 4.6 mm, 5 μm; Phenomenex). Fractions were lyophilized and then dissolved in 50 mM AMBIC buffer (pH 8,0). Trypsin digestion was carried out using an enzyme/substrate ratio of 1/50 (w/w) at 37 °C for 18 h.

Reduction of Nitrotyrosine to Aminotyrosine. Reduction of *o*-nitrotyrosine to *o*-aminotyrosine was carried out on aliquots of the modified peptide mixtures by using $Na_2S_2O_4$ as previously reported.[16] Before MALDI-TOF analysis, cleaning of the samples was performed using reversed-phase Zip-Tips C18 from Millipore (Billerica, MA).

Synthesis of *o*-Aminodansyltyrosine. The mixture of reduced BSA tryptic peptides was desalted by RP-HPLC on a reversed-phase C18 column (100 × 4.6 mm, 5 μm; Phenomenex). Peptide fractions were lyophilized and then dissolved in 100 mM sodium acetate pH 5.0 buffer, 0.9% sodium chloride. Samples were treated with a 18.5 ng /μL solution of DNS-Cl in ACN (1000-fold molar excess), and the buffered solutions were allowed to react at 65 °C for 16 h as previously reported.[17]

Labeling of Bovine Milk Protein Extract. A sample of commercially available bovine milk was reacted with a 100 mM solution of TNM for 30 min at room temperature. Modified milk proteins were purified by precipitation with the Amersham Clean Up kit and dissolved in denaturant buffer. 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.

The protein mixture was purified by size exclusion chromatography on a Sephadex G-25 M column equilibrated and eluted with 50 mM AMBIC. Protein fractions were concentrated and then digested with trypsin as already described. The resulting peptide mixture was reacted with DNS-Cl as described above.

Mass Spectrometry. MALDI-MS experiments were performed 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 a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/50 mM citrate buffer (2/3, v/v).

NanoLC-MS^2 and MS^3 experiments were performed on a 4000 QTrap mass spectrometer (Applied Biosystems) coupled to an 1100 nanoHPLC system (Agilent Technologies). Peptide mixtures were loaded onto an Agilent reversed-phase precolumn cartridge (Zorbax 300 SB-C18, 5 × 0.3 mm, 5 μm) at 10 μL/min with solvent A (0.1% formic acid, loading time 7 min). Peptides were then separated on a Agilent reversed-phase column (Zorbax 300 SB-C18, 150 mm × 75 μm, 3.5 μm), at a flow rate of 0.2 μL/min using 0.1% formic acid, 2% ACN in water as solvent A 0.1% and formic acid, 2% water in ACN as solvent B. The elution was accomplished by a 5−65% linear gradient of solvent B in 60 min. 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 (o.d. 150 μm, i.d. 20 μm, tip diameter 10 μm) from New Objectives (Ringoes, NJ). 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".

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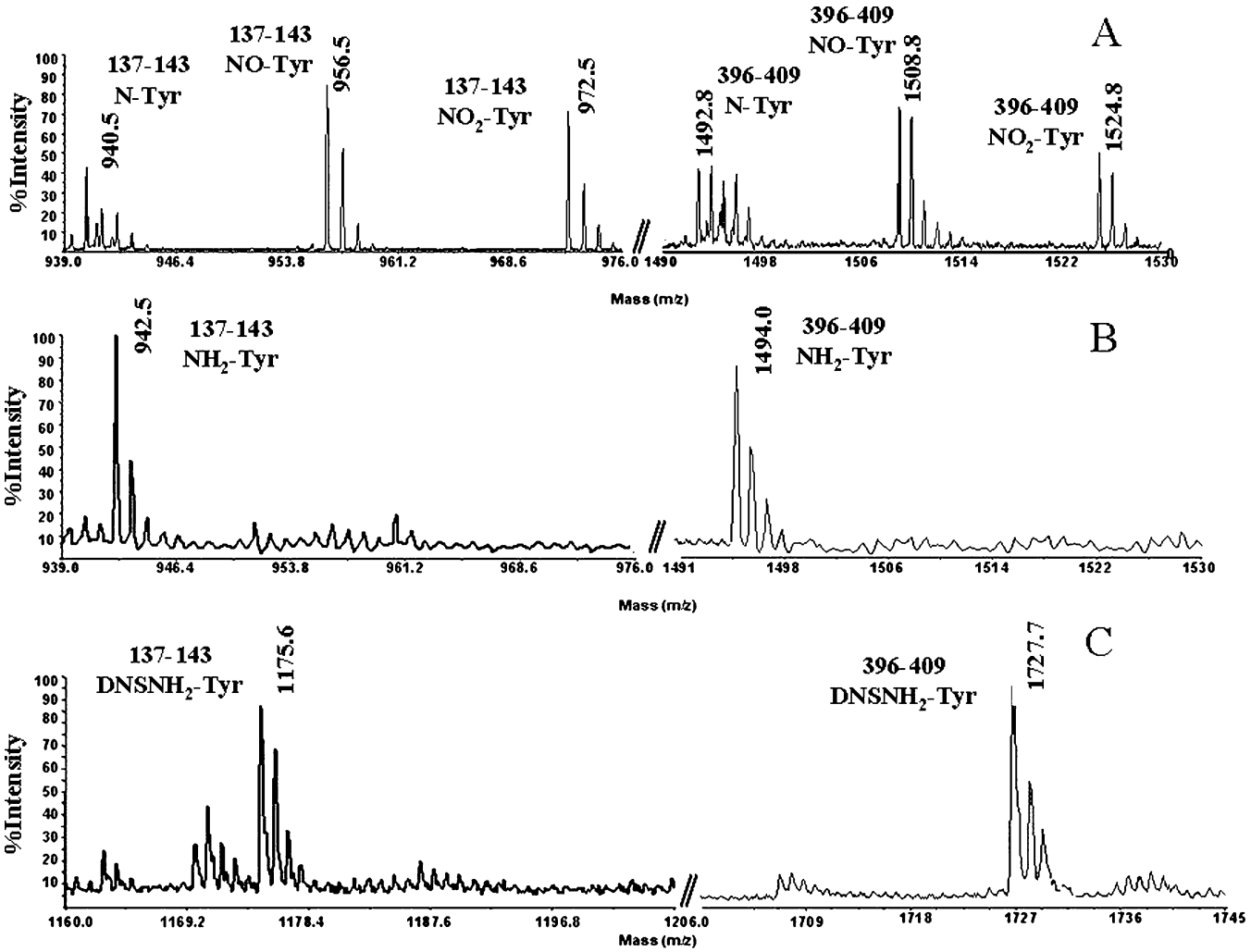

Figure 1. MALDI-MS analysis of peptide mixture from BSA. Panel A: Partial MALDI-MS spectrum of nitrated peptides. Panel B: Partial MALDI-MS spectrum of *o*-aminotyrosine-containing peptides. Panel C: Partial MALDI-MS spectrum of dansylated *o*-aminotyrosine-containing peptides.

This scan mode was followed by an enhanced resolution experiment for the ions of interest and then by MS^3 and MS^2 acquisitions of the two most abundant ions. MS^2 spectra were acquired using the best collision energy calculated on the basis of m/z values and charge state (rolling collision energy). MS^3 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 entire cycle duration is 5.3 s.

RESULTS

BSA (1 mg) was nitrated with TNM, and the excess reagent was rapidly removed by size exclusion chromatography as described in the Experimental Section. TNM-induced modifications were determined by MALDI mapping analyses. Aliquots of the BSA and N-BSA mixtures were reduced with DTT and alkylated with iodoacetamide. Proteins were then digested with trypsin, and the resulting peptide mixtures were directly analyzed by MALDI mass spectrometry. Spectral analyses led to ~85% of BSA sequence coverage, and more important, ~95% of tyrosine residues could be verified. Two mass signals recorded at m/z 972.5 and 1524.8, respectively, did not correspond to any peptide within the BSA sequence. These values occurred 45 Da higher than the signals corresponding to the peptides 137−143 and 396−409, respectively, suggesting that they originated from the nitrated derivatives of these fragments. This hypothesis was corroborated 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[1] (Figure 1A). This finding indicated that Tyr137 and Tyr399 were partially converted into the corresponding 3-NT residues following TNM treatment. These data were in perfect agreement with previous results on in vitro BSA nitration.[18,19]However, signals occurring at m/z 927.5 and 1479.7 were attributed to the theoretical fragments, m/z 137−143 and 396−409, respectively, were detected thus indicating that the nitration reaction was not quantitative.

Synthesis of *o*-Aminodansyltyrosine. Conversion of nitropeptides into their *o*-dansylamino derivatives was accomplished as indicated in Scheme 1. First, nitro groups were reduced by

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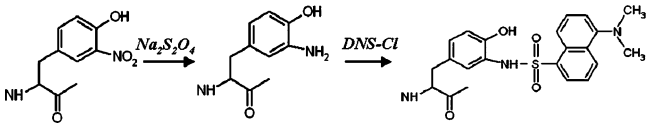
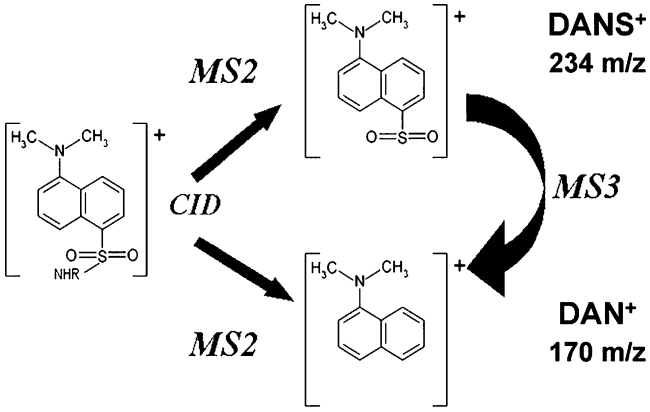
Scheme 1. Syntesis of *o*-Dansylaminotyrosine



Scheme 2. ESI-MS/MS Fragmentation Pathway



$Na_2S_2O_4$ treatment,[16] the peptide mixture was desalted by HPLC, and the extent of reduction was monitored by MALDI-MS. Mass spectral analysis showed the disappearance of the classical *o*-nitrotyrosine photodecomposition pattern, indicating nearly quantitative reduction, and its replacement by two new signals at *m/z* 942.4 and 1494.8, attributed to peptides *m/z* 137−143 and 396−409, respectively, containing an amino-Tyr residue (Figure 1B).

Aminotyrosine residues were chemoselectively labeled with DNS-Cl following the reported procedure.[16] The reaction was carried out at pH 5.0 exploiting the different pK_a value 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.

The extent of reaction was monitored by MALDI-MS analysis showing the presence of two new signals at *m/z* 1175.6 and 1727.8 occurring 233 Da higher than the *o*-aminotyrosine-containing peptides (Figure 1C). These signals corresponded to the expected dansyl derivatives of the *o*-aminotyrosine peptides. The occurrence of signals at *m/z* 942.0 and 1494.7 corresponding to the unmodified *o*-aminotyrosine peptides indicated that the extent of dansyl reaction was ∼60%. No modification at the N-terminus or Lys residues was detected.

LC−MS/MS Analysis of *o*-Aminodansyl-Tyr Peptides. The exact location of the original nitro groups was assessed by LC−MS/MS analysis of the peptide mixture using a linear ion trap 4000 Q-Trap instrument and an experimental procedure that combines precursor ion scan with MS^3 scan mode as already described.[14] In the precursor ion scan mode, only the precursor ions producing the *m/z* 170 fragment were detected. The selected precursor ions were then subjected to a combined MS^2 and MS^3 experiment to specifically detect only those ions originating the dansyl-specific m/z 234 → 170 transition in MS^3 mode as indicated in Scheme 2.

Figure 2 shows the LC−MS/MS traces corresponding to the various experiments. The chromatographic profiles reported in Figure 2B and C for the *m/z* 170 precursor ion scan and for the transition *m/z* 234/170 in MS^3 mode, respectively, showed a lower number of signals compared to the LC−MS/MS full scan profile (Figure 2A). Moreover, a significant increase in the signal/noise ratio could be achieved as the higher selectivity MS criteria were applied (Figure 2B and C). However, the precursor ion TIC exhibited a large number of ions that very unlikely could be only related to *o*-aminodansyl-Tyr peptides (Figure 2B). The MS^3 TIC (Figure 2C) showed the presence of only three peaks related to doubly charged ions at *m/z* 588.2, 792.9, and 986.8. The corresponding MS^2 fragmentation spectra led to the determination of the entire sequence of these species, identifying the fragments *m/z* 137−143, 396−409, and 445−458, respectively, all of which contain *o*-aminodansyl-Tyr residues. Moreover, modified Tyr residues could easily be detected at the level of Tyr137, Tyr399, and Tyr451, allowing for the exact identification of the original nitration sites.

As an example, Figure 3A shows the MS/MS spectrum of the peptide *m/z* 396−409 carrying a dansyl moiety. The modified ion is stable during collision-induced dissociation, providing easily interpretable daughter ion spectra. Both the y and b fragment ions still retained the modifying group linked to the amino Tyr residue, thus denoting original nitration. As indicated in the spectrum, the mass difference between the b_4 and b_3 fragment ions is 411 Da corresponding to the *o*-dansylaminotyrosine residue.

It should be noted that 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. Moreover, the daughter ion spectra of *o*-dansylamino-Tyr-containing peptides showed some peculiar features. Besides the signals at *m/z* 170 and 234, typical of dansyl derivatives, a further signal at *m/z* 384 was always observed and assigned to the immonium ion of *o*-dansylamino-Tyr.[19] This stable fragment could then be used either as an alternative diagnostic fragment in the precursor ion scan or simply to confirm the presence of an *o*-dansylamino-Tyr residue in the peptide sequence. Finally, a fragment ion related to the loss of the dansyl moiety as neutral group was always observed in the daughter ion spectra when the *o*-nitrotyrosine residue is located at N-terminal position (Figure 3B). In fact, the fragment ion at *m/z* 942.5 in Figure 3B belongs to the y series and occurs 233 Da lower than the molecular mass of the dansylated peptide *m/z* 137−143 , showing the neutral loss of the dansylated moiety.

Location of BSA *o*-Aminotyrosine-Containing Peptides in Complex Protein Mixtures. The feasibility of the developed procedure to identify *o*-nitrotyrosine peptides in proteomics analysis 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*. An aliquot of the modified proteome was fractionated by SDS-PAGE to demonstrate that BSA was indistinguishable from and in similar amount to other *E. coli* proteins (data not shown).

The total protein extract was then submitted to the procedure described above. Nitrotyrosines were reduced with dithionite, and the modified protein mixture was digested with trypsin. 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 bidimen-

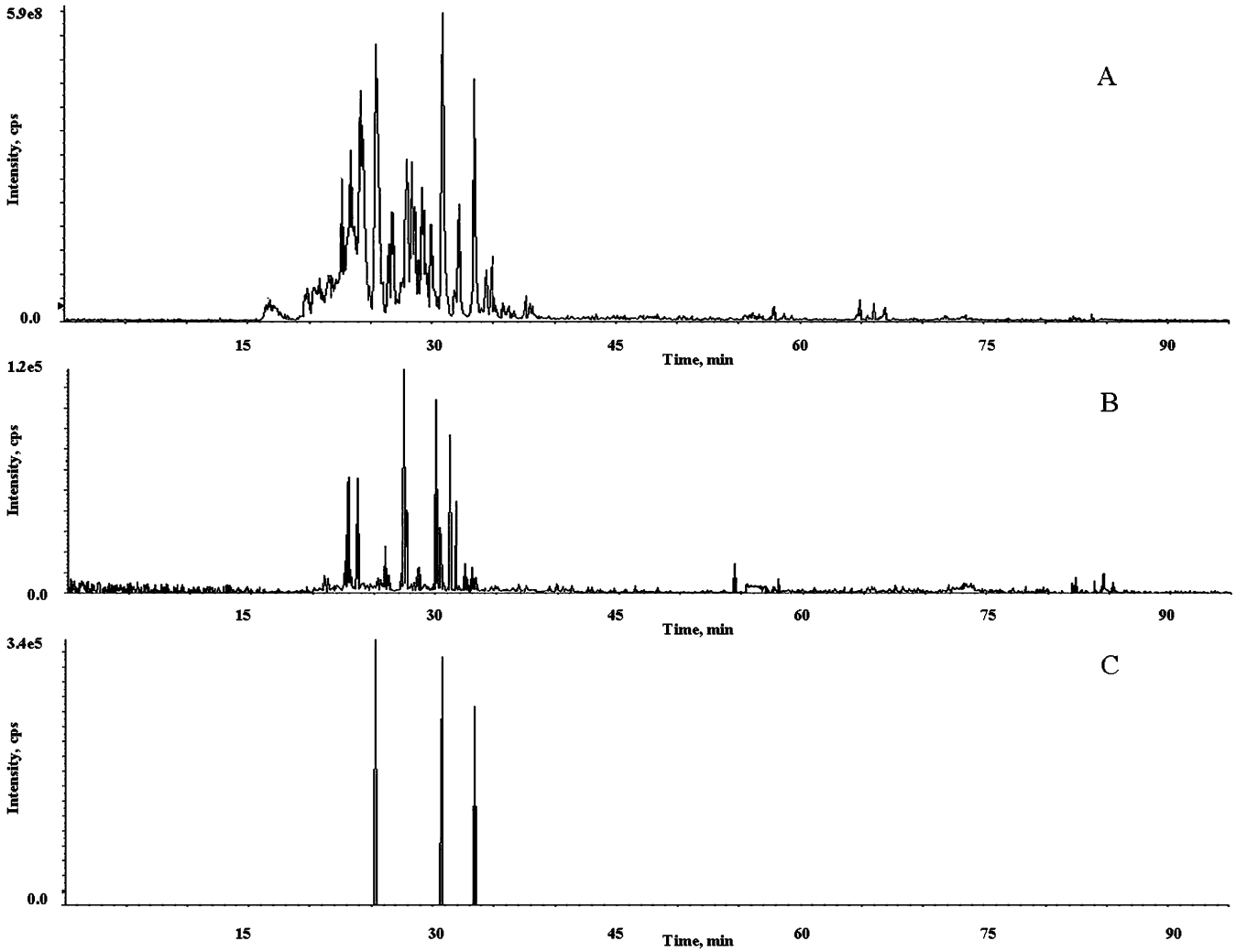


Figure 2. LC−MS/MS traces of bidimensional mass spectrometry experiment performed on the tripsin digest from BSA. Panel A: LC−MS/MS full scan profile. Panel B: Chromatographic profile of the *m*/*z* 170 precursor ion scan. Panel C: Chromatogram related to the total ion current for the transition *m*/*z* 234/170 in MS^3 mode.

sional tandem mass spectrometry procedure described above. Figure 4 shows the reconstructed ion chromatogram for the precursor ion scan (A) and the selective dansyl transition in MS^3 mode (B). As clearly indicated in the figure, 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 MS^2 spectra do not show the occurrence of fragment ion at *m*/*z* 234. The further selection based on the MS^3 scan removed a large number of false positives leading to a simple ion chromatogram essentially dominated by three intense signals. Identification of nitrated tyrosine residues was carried out by taking advantage of the flexibility of the MASCOT software, a database search utility available on the net. First, a variable modification of 411.0 Da corresponding to *o*-aminodansyltyrosine residues was introduced into the Modification File within the MASCOT software. Second, the peak list used for database search only consisted in the MS^2 spectra of the peptide species that had generated a signal both in the precursor ion TIC and in the MS^3 ion scan. The MS^2 fragmentation spectra of the three signals observed in the MS^3 scan reported in Figure 4B revealed the occurrence of three 3-NT-containing peptides originated from the BSA sequence. In particular, these peptides corresponded to the same peptides previously detected in the analysis of homogeneous nitrated BSA. Moreover, this procedure led to the identification of additional endogenous nitration sites in *E. coli* proteins that had been nitrated in vivo. Table 1 reports the nitropeptides found in the BSA−complex mixture and the MASCOT scores with the corresponding identifications of *E. coli* proteins.

Labeling and Analysis of a Nitrated Protein Extract of Bovine Milk. The proposed strategy was finally employed to identify *o*-nitrotyrosine residues in a more complex sample, the entire bovine milk. Bovine milk was in vitro nitrated with TNM, and an aliquot of milk proteins were fractionated by SDS-PAGE and submitted to a western blot analysis using *o*-nitrotyrosine antibody to confirm the extent of nitration. The presence of nitrated proteins was essentially detected in the regions between 20 and 35 and 45−60 kDa as indicated in Figure 5.

The entire milk protein extract was dissolved in denaturant buffer, and then cysteine alkylation and nitro groups reduction were performed in "one pot" as described above. The protein sample was then desalted and digested with trypsin. The resulting peptide mixture was selectively labeled with dansyl chloride and directly submitted to LC−MS/MS analyze using the double selectivity criteria.

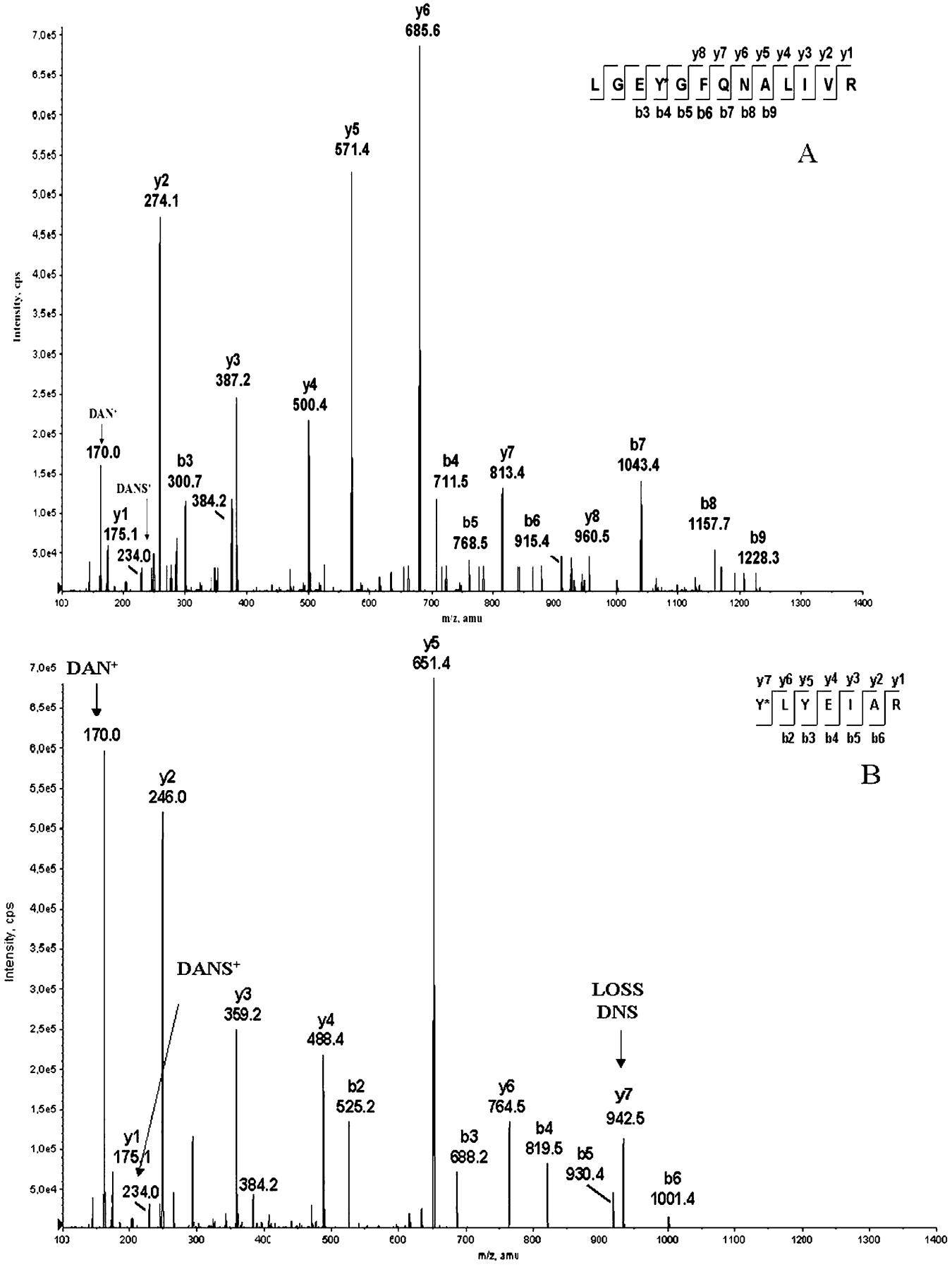


Figure 3. Panel A: MS/MS spectrum of the peptide *m*/*z* 396−409 within BSA sequence carrying a dansyl moiety. Panel B: MS/MS spectrum of the peptide *m*/*z* 137−143 within BSA sequence carrying an *o*-nitrotyrosine residue at the N-terminal position.

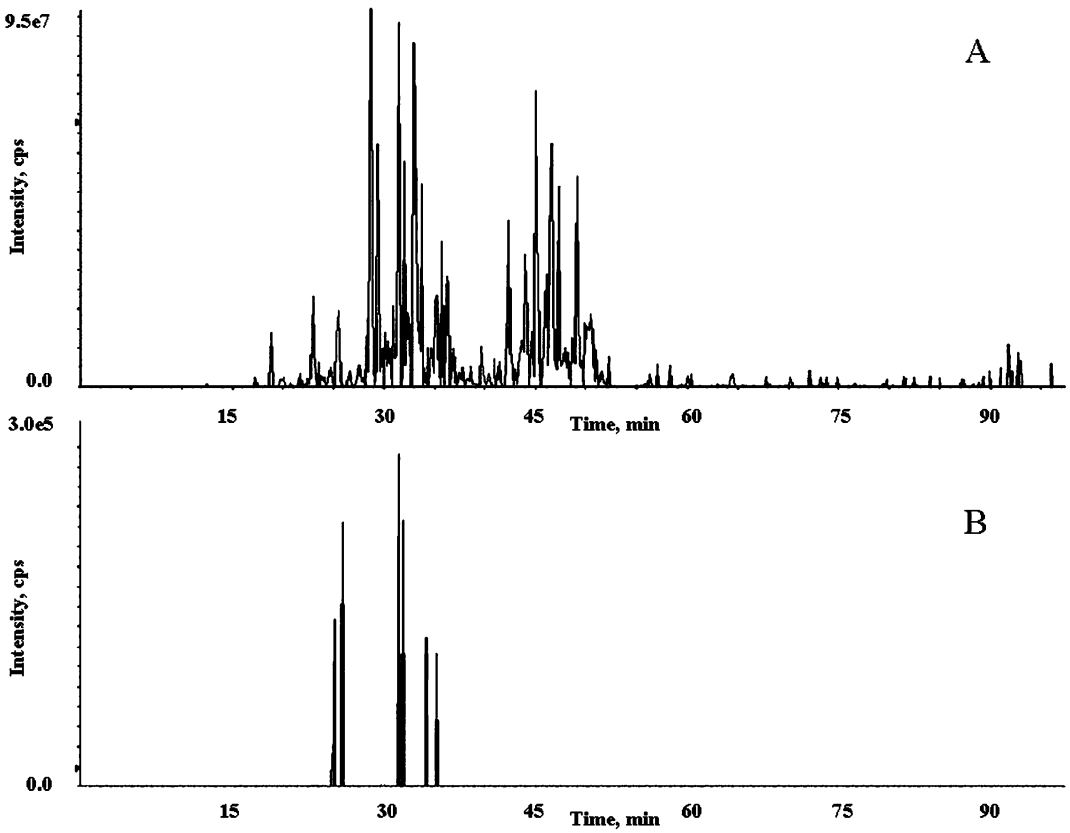


Figure 4. Reconstructed ion chromatogram for the precursor ion scan (panel A) and the selective dansyl transition in MS^3 mode (panel B) of bidimensional mass spectrometry experinent performed on the entire *E. coli.* cellular extract spiked with a mixture of BSA and nitrated BSA.

Table 1. Nitrated Proteins Identified by RIGhT Approach in *E. coli*

protein	sequence	MASCOT scores
hypothetical fimbrial chaperone yraI precursor	TRIKMF-NO_2Y^{139}-RPAQHLK	21
hypothetical protei yciQ	NQQIKHGF-NO_2Y^{10}-R	20
homeobox outer membrane usher protein yehB	IAAP-NO_2Y^{134}-R	23

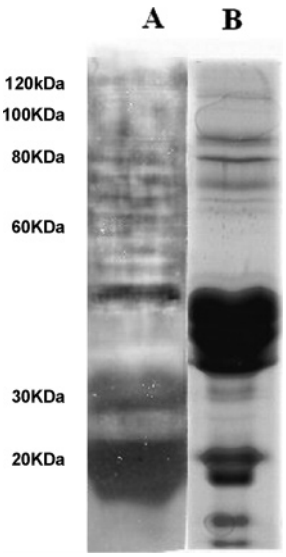


Figure 5. Fractionation of in vitro nitrated bovine milk proteins by SDS-PAGE analysis (lane 2) and western blot analysis using *o*-nitrotyrosine antibody to confirm the extent of nitration (Lane 1). The presence of nitrated proteins was detected in the regions between 20 and 25, 75−80, and 100−120 kDa.

Identification of nitrated milk proteins was carried out using the modified MASCOT software as described above. As an example, Figure 6 (panels A−C) reports the MS, MS/MS, and MS^3 spectra for the peptide fragment m/z 108−117 of β-lactoglobulin. The results of the identification procedure are reported in Table 2 together with the modified Tyr residues and MASCOT scores. Besides the high-abundant milk proteins (β-casein and β-lactoglobulin), this procedure was also able to assess the nitration sites occurring in low-abundant proteins, such as vimentin and claudin 2.

DISCUSSION

The major problem in the proteomic analysis of nitroproteins is the need to discriminate modified proteins, usually in very low concentrations, from the large amount of nonmodified proteins. Moreover, biological relevant PTMs often occur at substoichiometric level making even more difficult the identification of the *o*-nitrotyrosine-containing proteins in the presence of a larger percentage of the corresponding nonnitrated form. Finally, precise localization of the nitration site is often required to fully describe the biological process.

Existing methodologies essentially rely on immunochemical techniques, whereby nitrated proteins can be detected by alignment of the western blots with the 2D PAGE gels of the entire

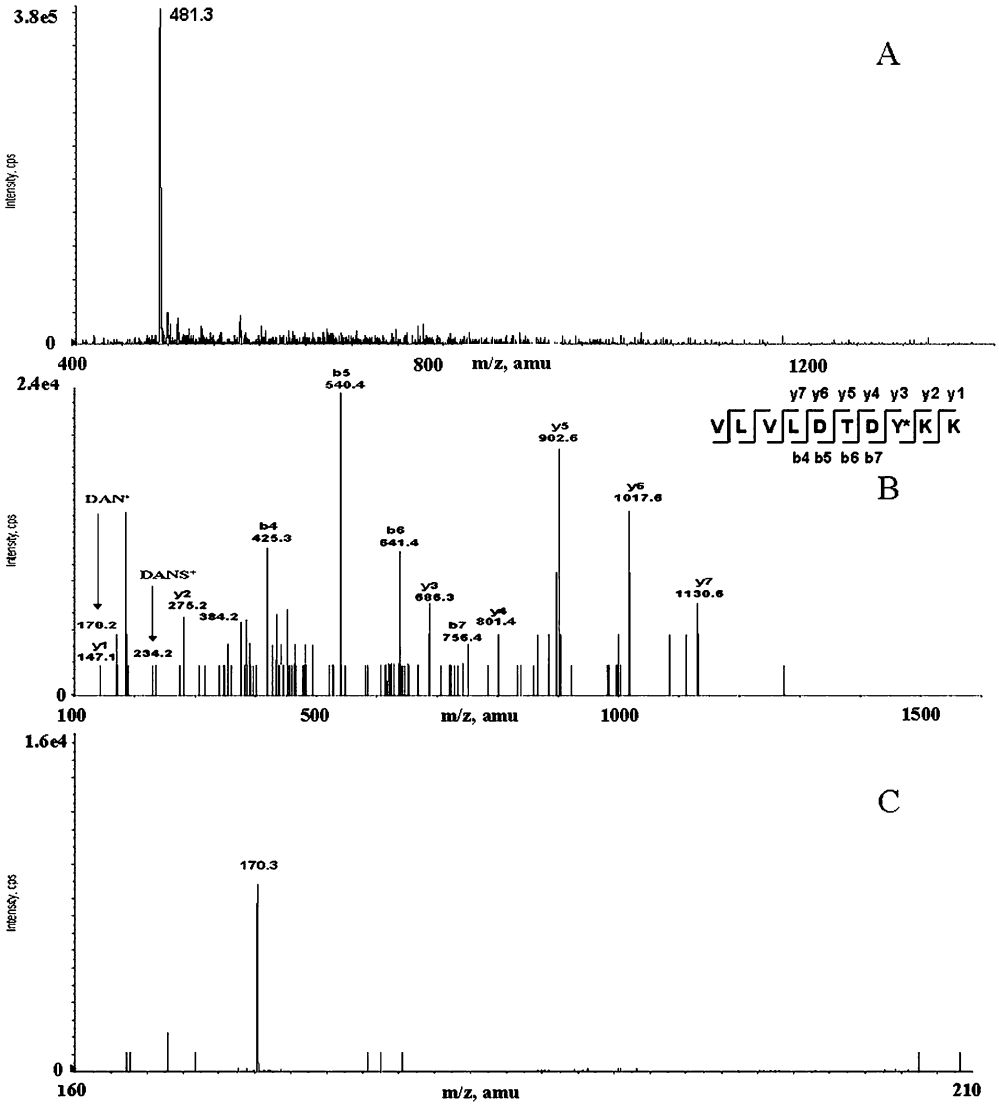


Figure 6. MS spectrum (panel A), MS/MS spectrum (panel B) and MS^3 spectrum (panel C) for the peptide fragment *m*/*z* 108−117 of β-lactoglobulin.

Table 2. Nitrated Proteins Identified by RIGhT Approach in Milk

protein	sequence	MASCOT scores
β-casein	AVP-NO_2Y^{195}-PQR	40
β-lactoglobulin	VLVLDTD-NO_2Y^{115}-KK	36
	V-NO_2Y^{115}-EELK	20
vimentin	QQ-NO_2Y^{275}-ESVAAK	25
claudin 2	AKSEFNS-NO_2Y^{224}-SLTGYV	23
homeobox protein hox-D12	F-NO_2Y^{101}-TPDVAAGPEERGR	21

cellular proteome.[20] Alternatively, specific capture of *o*-nitrotyrosine-containing proteins by immunoaffinity procedures can also be employed.[21] However, neither of these approaches completely fulfills the analytical requirements for nitro proteome analysis. The main problems of the 2D PAGE procedure are related to the low abundance of the *o*-nitrotyrosine-containing proteins, the low steady-state levels of *o*-nitrotyrosine in modified proteins, and the poor recovery of nitropeptides from the gel. Specific nitro-Tyr immunoaffinity purification can preferentially enrich and isolate nitroproteins, thus overcoming some of the above-mentioned drawbacks. However, the peptide mixture from the tryptic digest of the whole enriched nitroprotein bulk is still too complicated to be analyzed in a simple one-step process and 2D-HPLC, typically consisting of ion exchange and reversed-phase separations, in conjunction with tandem mass spectrometric methods had to be used.[22]

The strategy described in this paper is based on a totally different approach involving dansyl chloride labeling and relies on the enormous potential of MS^n analysis. According to the so-called "gel-free procedures", the analysis is carried out at level of peptides following tryptic digest of the whole protein mixture. However, discrimination between nitropeptides and nonmodified

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peptides is achieved by taking advantage of the instrumental features of a hybrid (triple quadrupole/linear ion trap) mass spectrometer. Peptide analysis is carried out by LC−MS/MS, and the peptide ions of interest are discriminated by two selectivity criteria based on two subsequent tandem MS experiments, a precursor ion scan followed by an MS^3 scan mode. This procedure can then be considered as a sort of "instrumentally driven" bidimensional selection.

This instrumental selectivity approach needs an appropriate derivatizing agent producing stable and diagnostic fragment ions to be used in the MS/MS scan modes and capable of improving ionization of modified peptides. A valuable agent that can purposefully serve in this function is dansyl chloride, recently introduced by us to selectively label phospho-Ser/Thr residues. Following MS/MS fragmentation, in fact, dansyl-derivatized peptides produce a stable fragment ion at m/z 170 very useful for a precursor ion search scan. Moreover, in the MS^3 mode, these species undergo a specific and diagnostic transition from m/z 234 to 170, providing an effective second selectivity criterion.[14]

The proposed strategy consists in two simple chemical manipulation steps, namely, reduction of *o*-nitrotyrosine residues to the corresponding amino-Tyr derivatives and chemoselective labeling with DNS-Cl at pH 5.0, exploiting the lower basicity of aromatic amines compared to the aliphatic ones.

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. This experiment underlined the usefulness of the two selectivity criteria in that the precursor ion scan still showed the presence of non-nitrated peptide ions that were completely ruled out in the MS^3 scan mode. Moreover, since this combined MS approach provides only the daughter ion spectra of the nitrated peptides, this procedure always leads to the unambiguous localization of the nitrated Tyr residues.

The newly developed procedure was tested on complex systems, including the entire *E. coli* proteome and the whole milk proteins, to assess its feasibility for proteomic investigation of nitroproteins. In both cases, the results underscored the ability of the instrumental bidimensional selection to discriminate nitropeptides from their unmodified counterparts in a complex matrix. The three nitrated peptides in BSA could selectively be identified even in a mixture with the tryptic digest of the entire *E. coli* proteome, producing the same results obtained on pure BSA. Investigation of nitrated milk proteins led to the identification of several different proteins including integrin, a strongly associated membrane protein. 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. Moreover, the general rationale of the method can in principle be extended to the detection of other PTMs.

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