

Detergent-free biotin switch combined with liquid chromatography/tandem mass spectrometry in the analysis of S-nitrosylated proteins

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High-throughput proteomic analysis based on a biotin switch combined with liquid chromatography/tandem mass spectrometry (LC/MS/MS) enables simultaneous identification of S-nitrosylated sites and their cognate proteins in complex biological mixtures, which is a great help in elucidating the functions and mechanisms of this redox-based post-translational modification. However, detergents such as sodium dodecyl sulfate (SDS) and Triton X-100 adopted in these systems, which are hard to fully remove in the subsequent MS-based analyses, can suppress the peptide signals and influence the SNO-Cys site identification and the reproducibility of the experiments. Here we developed a detergent-free biotin-switch method, which applied urea to replace detergents, and successfully combined it with LC/MS/MS in the analysis of S-nitrosylated proteins. With this approach, 44 SNO-Cys sites were specified on 35 distinct proteins in S-nitrosoglutathione (GSNO)-treated HeLa cell extracts of proteins with good reproducibility. The LC/MS performance was greatly improved as analyzed with Pep3D and the amount of samples for analysis reduced from 40 mg used in the literature to 3–5 mg. For S-nitrosylated targets detected both in the control sample and in the GSNO-treated sample, extracted ion chromatography (XIC) was employed to estimate the quantitative change of S-nitrosylation (S-nitrosation), which facilitates the judgment on 'accept or reject' of the identified targets. Copyright © 2008 John Wiley & Sons, Ltd.

Accumulated evidence has shown that reversible addition of nitric oxide (NO) to Cys-sulfur in proteins, a modification termed S-nitrosylation, has emerged as a ubiquitous signaling mechanism for regulating diverse cellular processes.^{1,2} The high-throughput proteomic approach shows significant advantage in this investigation: On one hand, it enables simultaneous identification of S-nitrosylated sites and their cognate proteins in complex biological mixtures, on the other hand, it facilitates the screening of new S-nitrosylated targets and acquiring the panorama of S-nitrosylation from a systematic view.³ A biotin-switch method⁴ combined with liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been developed, in which peptides possessing an SNO moiety were converted into biotin-tagged peptides, purified by (strept)avidin and analyzed by LC/MS/MS.^{3,5} With this method, much

information on protein identification as well as their modified cysteines could be acquired in a single experiment.

However, in purifying the S-nitrosated proteins, a high concentration of sodium dodecyl sulfate (SDS) was applied in steps of thiol blocking (2.5% SDS) and labeling (1% SDS) in the biotin switch and 0.4% of Triton X-100 was used in the washing buffer in the streptavidin-based purification step. The ionic detergent SDS and the non-ionic detergent TritonX-100 are notoriously detrimental in electrospray ionization (ESI) LC/MS research, because they can cause distortion of the shape of protein signals, suppress protein signals, cause adduct formation, and shift the mass-to-charge signals.⁶ SDS shows significant signal suppression even at 0.01%. Besides, SDS and Triton X-100 have a low critical micelle concentration (CMC) and they are prone to form micelles in the sample preparation step, which are difficult to remove by dialysis, ultrafiltration, etc. In our practice of using the biotin-switch method (where detergents were used as described above) combined with LC/MS/MS in the analysis of S-nitrosylated targets, we found that detergents were hard to fully remove from samples and the reversed-phase C18 columns, causing a typical and very strong –44 Da series (or –22 Da series in doubly charged form) of the oxyethylene ions (clusters) and severe peptide signal suppression and the experiments were difficult to reproduce. Thus, in LC/MS-based experiments, detergents should be

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completely avoided or reduced below its maximum tolerable detergent concentration (MTDC).⁷

In this study, we developed a detergent-free biotin-switch method, in which urea rather than SDS was used as a denaturing environment to ensure thiol blockage and biotinylation. Urea is often applied to dissolve and unfold proteins in denaturing studies and it is completely compatible with tryptic digestion and subsequent MS analysis. Urea does not form micelles and is convenient to remove, which makes it easy to obtain reproducible ESI-MS results. With such a method, we successfully identified 35 S-nitrosylated proteins and their modified cysteines from an S-nitrosoglutathione (GSNO)-treated HeLa cell extract of proteins with good repeatability. The performance of the LC/MS/MS experiment was greatly ameliorated as analyzed by Pep3D. All MS/MS data were validated by Trans-Proteomics Pipeline, a probability score-based analysis system and extracted ion chromatography (XIC) was employed to estimate the quantitative change of S-nitrosylation, which facilitates the judgment on 'accept or reject' of the identified targets.

EXPERIMENTAL

Materials

Methyl methanethiosulfonate (MMTS), biotin-HPDP (HPDP = *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamido), the BCATM protein assay kit and the *Slide-A-Lyzer* dialysis cassette (0.5 mL to 3 mL, 7K, molecular-weight cutoff (MWCO)) were from Pierce (Rockford, IL, USA). PlusOneTM urea was from GE Healthcare (Piscataway, NJ, USA). Protease inhibitor cocktail tablets (Complete, Mini, EDTA-free) were from Roche Applied Sciences (Indianapolis, IN, USA). Sequencing-grade modified trypsin (V5111) was from Promega (Madison, WI, USA). Solvents used in LC/MS analysis, including water, formic acid (FA), acetonitrile (ACN) and methanol, were from J. T. Baker Chemicals (Philipsburg, NJ, USA). S-Nitrosoglutathione (GSNO) was synthesized as described.⁸ The concentration was determined by its optical density (O.D.) value at 334 nm (with extinction coefficient 800 M⁻¹ · cm⁻¹). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture and extract preparation

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin, maintained in a 5% CO₂ incubator at 37°C in T75 flasks (Corning). Cells approaching 80% of confluence were gently washed twice with ice-cold phosphate-buffered saline (PBS), trypsinized and collected with a cell scraper. Cell pellets were collected by centrifugation at 300 g for 5 min at 4°C and washed twice with ice-cold PBS, then re-suspended in the lysis buffer (20 mM Tris · Cl, pH 7.7/1 mM EDTA/0.4% Nonidet P-40/0.1 mM neocuproine/protease inhibitor cocktail tablets) and lysed on ice for 15 min. Supernatant was collected after centrifugation at 13200 g for 30 min at 4°C. Protein concentration was determined using the BCATM protein assay kit and adjusted to 1 mg/mL. The protein

sample was aliquoted into three groups, each containing 3 mg protein.

Detergent-free biotin-switch method for SNO peptide isolation

All steps were performed in the dark unless noted otherwise. The three aliquots were treated with 5 mM dithiothreitol (DTT) (37°C, 30 min), 100 µM glutathione (GSH) (room temperature (R.T.), 1 h) and 100 µM GSNO (R.T., 1 h), designated as the Negative CTRL group, the CTRL group and the GSNO group, respectively. After treatment, the three groups were precipitated twice with ice-cold acetone for 30 min and the protein pellets were collected after centrifugation at 2000 g for 10 min. The pellets were then dissolved in 3 mL blocking buffer (20 mM Tris · Cl, pH 7.7/1 mM EDTA/0.1 mM neocuproine/8 M urea/20 mM MMTS) to give a final protein concentration of 1.0 mg/mL and incubated for 60 min at R.T. The excess MMTS was removed by fast dialysis into 1 L Labeling Buffer (20 mM Tris · Cl, pH 7.7/1 mM EDTA/0.01 mM neocuproine/4 M urea) within the *Slide-A-Lyzer* dialysis cassette three times (about 1 h per one dialysis) in the dark according to the manufacturer's guide. A final concentration of 200 µM of biotin-HPDP and 5 mM sodium ascorbate were sequentially injected into the cassette, mixed well and the cassette was kept in a self-sealing bag and left to react on a rotator for 120 min at R.T. When labeling was over, the samples were exchanged to digestion buffer (100 mM NH₄HCO₃, 1 M urea) in a stepwise mode followed by digestion with 1:50 (w/w) sequencing-grade modified trypsin at 37°C for 18–20 h. Digestion was terminated by the addition of an appropriate amount of protease inhibitor tablets. The peptide mixture was diluted two-fold with neutralization buffer (20 mM Tris · Cl, pH 7.7/1 mM EDTA) and incubated with 200 µL streptavidin-agarose slurry for 1 h with intermittent rotating at R.T. The beads were washed five times with 1 mL washing buffer A (20 mM Tris · Cl, pH 7.7/1 mM EDTA plus 1 M NaCl), followed by five washes with washing buffer B (5 mM NH₄HCO₃/20% ACN). Streptavidin-bound peptides were eluted with 500 µL 10 mM tris(2-carboxylethyl)phosphine hydrochloride (TCEP) at 37°C for 15 min and alkylated with 5 mM iodoacetamide (IAM) at R.T. for 1 h in the dark. The purified peptides were lyophilized to near dryness in a Speed Vac (ThermoFisher) to reach a volume of approximate 10 µL. The sample was diluted with water and FA was added to give a final concentration of 0.1%, and the samples were analyzed by LC/MS/MS or kept under -80°C until analysis. For comparison, the detergent-based biotin-switch method for SNO peptide isolation was carried out as described previously³ with an initial amount of protein of 3 mg.

LC/MS/MS analysis

Acidified samples were analyzed on a Thermo LTQ linear trap instrument equipped with a Thermo nano-electrospray source, a Thermo Surveyor pump and an autosampler (Thermo Electron Corporation, San Jose, CA, USA). LC/MS/MS analysis was performed with a fused-silica capillary (75 µm i.d. × 12 cm) packed with Monitor C18 (3 µm) with the flow rate set at 150 µL/min. Buffer A consisted of 0.1% FA in 5% ACN and buffer B consisted of 0.1% FA in 80%

ACN. Peptides were eluted initially with 100% A for 1 min, then to 90% A at 5 min, then a linear gradient to 55% A by 60 min, then to 0% A at 70 min and held to 80 min, then to 100% A at 80.01 min and held until 90 min. MS/MS spectra were acquired by using a full scan followed by five MS/MS scans on the five most intense precursor ions in data-dependent mode. Precursors that were detected twice within 15 s were put into a dynamic exclusion list for a period of 60 s.

Database search of MS/MS data and the data validation

MS/MS spectra were extracted from the raw data files by using TurboSEQUENT™ (licensed to Bioworks 3.2) with the following parameters: molecular weight (MW) range, 400–3500; threshold, 1000; precursor mass tolerance, 2.0; fragment ion tolerance, 0.5. Raw data were searched against the human NCBI RefSeq database (version 2007.9.18). Cysteine alkylation (+57.02) and methionine oxidation (+16) were specified as static and differential modification, respectively. The search results were validated through open source software Trans-Proteomics Pipeline (ISB, Seattle, WA) using the PeptideProphet algorithm,⁹ which assigns a comprehensive probability score from 0 to 1 to each peptide sequence match based on SEQUEST scores (X_{corr} , ΔCn , Sp , RSp , etc.). Only peptide sequence matches fully tryptic ($NTT = 2$) and with assigned scores greater than the minimal probability threshold (MPT) were accepted as the true matches. The web-based INTERACT program was used for data organization and the ProteinProphet algorithm^{10,11} was used to validate the presence of target proteins. Single-hit matches were further validated by manual inspection upon the above criteria, ensuring that major peaks detected matched the expected forms of b and y ions. Pep3D, a graphic tool for the analysis of performance of LC/MS/MS,¹² was inspected. The MS intensity of XIC data from the Negative CTRL group, the CTRL group and the GSNO group was first normalized and compared.

RESULTS

Comparison of the LC/MS performance between the urea-based method (detergent-free) and the detergent-based method

To elucidate the crucial influence of the detergents on LC/MS analysis, the LC/MS performance was compared between the detergent-based method and the urea-based method by analysis with Pep3D, an effective visualization and evaluation software developed by Li *et al.*¹² The software displays ESI-MS raw data in a two-dimensional array of density spots (retention time vs. m/z), in which the darkness of each spot reflects the relative intensity of the detected signals, indicated by the gray-gradient bar on the right-hand side. As shown in Fig. 1, Pep3D images were generated from raw data with the detergent-based method (Figs. 1(A) and 1(B)) and with the urea-based method (Figs. 1(C) and 1(D)), respectively, with the time range set from 0 to 65 min and the m/z range set from 400–1300. In the Pep3D images derived from the detergent-based method (Figs. 1(A) and 1(B)), data spots were distributed in an obviously recognizable 'slash-like' pattern as annotated by the arrows.

These 'slash-like' patterns distribute across the whole m/z range and mainly exist in the low m/z range (400–700) when the concentration of ACN goes higher. The data spots with the pattern featured an equal m/z difference of 44 or 22 Da and an extremely high intensity compared to other spots. Furthermore, a trailing effect was clearly seen behind each spot with this pattern, indicating something was eluted constantly along the timeline and was hard to be fully removed from the reversed-phase C18 column, whereas, in the Pep3D images generated from the detergent-free biotin-switch method (Figs. 1(C) and 1(D)), data spots were evenly scattered across the m/z range of 500–1000 from about 12 to 40 min and a significant increase in relative intensity was observed in the GSNO-treated sample. The substitution of detergent with urea greatly improves the performance of LC/MS/MS analysis. At the bottom of all the Pep3D images, several long horizontal streaks of fixed m/z in the low m/z range (400–600) across the whole timescale were also observed. These streaks represent some background signals, which could be easily put into the dynamic exclusion list without interfering with the peptide identification.

Identification of S-nitrosylated proteins in the GSNO-treated HeLa cell extract with the detergent-free biotin-switch method combined with LC/MS/MS

To validate this method, we examined S-nitrosylated proteins in HeLa extracts upon (*in vitro*) treatment with 100 μ M GSNO based on the detergent-free biotin-switch method. In this work, three groups of samples were prepared in each experiment. One aliquot of sample was treated with 5 mM DTT at 37°C for 30 min in order to fully reduce all the sulfhydryls in a variety of oxidized states such as S-nitrosylation, disulfide bonds, etc. This group of samples was used as a negative control to monitor the false-positive effect resulting from the incomplete blockage of free thiols with MMTS. Another two aliquots of sample were separately treated with 100 μ M GSH and 100 μ M GSNO. The MS/MS spectra were searched using TurboSequest (Bioworks 3.2) as described above. Rather than filtering searched results by merely setting a series of filters such as X_{corr} , ΔCn , Sp , RSp , etc., Trans-Proteomics Pipeline (a probability-based algorithm system developed by the Institute for Systems Biology, Seattle, WA, USA) was applied for data validation. The minimal probability threshold (MPT) for identified peptides to accept was set to 0.99^{13,14} and the estimated false-positive rate was less than 0.4%, the minimal protein probability (MPP) set for the Negative CTRL sample, the CTRL sample and the GSNO sample was 0.99 and the estimated false-positive rate was less than 0.3% (see S-Table 1, Supplementary Material). Following the above criteria, 35 proteins in the GSNO group were identified as S-nitrosylated targets, including metabolic enzymes such as enolase-3, GAPDH, creatine kinase, structural proteins such as tubulin α and other previously reported proteins such as chaperones, eukaryotic translation initiation factor 1. A detailed list of all identified S-nitrosylated proteins and their modified cysteines, X_{corr} , Cn , $PeptideProphet$ and $ProteinProphet$ values is shown in Table 1. As no detergents were applied in the whole experimental process, there is no MS signal suppres-

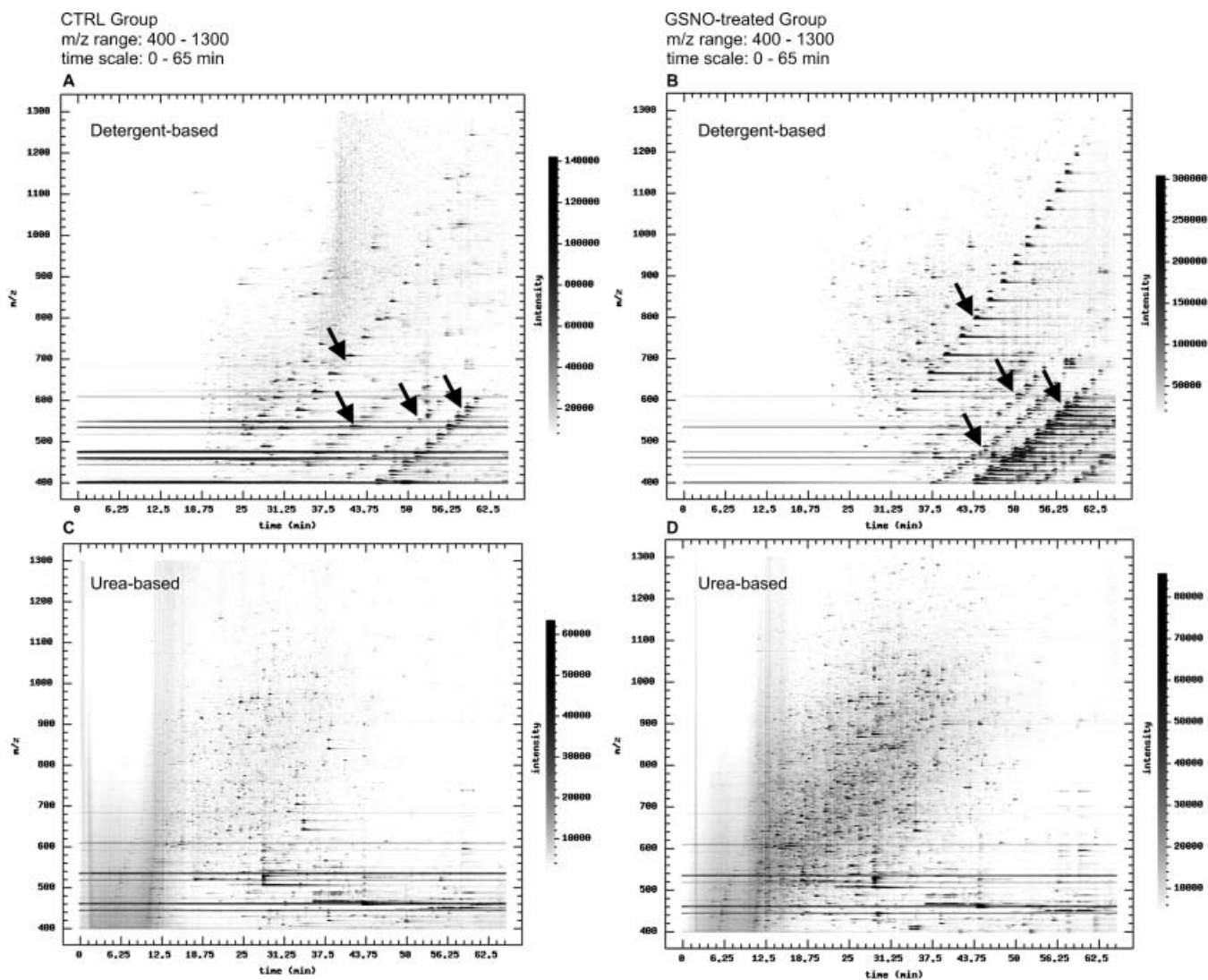


Figure 1. Comparison of LC/MS performance with Pep3D images generated from raw LC/ESI-MS data in the detergent-based method and in the urea-based biotin-switch method. The two Pep3D images at the top (A, B) were derived from raw LC/ESI-MS data performed with the detergent-based biotin switch method, while the other two images at the bottom (C, D) were generated from raw LC/ESI-MS data with the urea-based detergent free biotin switch method. As shown in (A) and (B), the 'slash-like' pattern has an extremely strong MS response as compared with other evenly scattered spots, characterized as equal mass-to-charge difference of 44 or 22 Da from 400–1300, with a trailing effect after each spot along the retention time (RT) scale. Such a pattern represents a common chemical contaminant derived from detergents with a polyoxyethylene side chain like Triton X-100. In images (C) and (D), the MS response of each spot was evenly distributed along the RT and m/z scales, with comparable signal intensity as indicated by the gray-gradient bar on the side. In addition, more signal spots were observed with increased pixel intensity in the GSNO-treated group (D) than in the CTRL group (C). These images are representative of at least three independent experiments.

sion, which greatly reduced the initial amount of sample needed for each experiment from that commonly used in the literature of 40 mg (1.0 mg/mL, 40 mL)¹⁵ to about 3–5 mg (1.0 mg/mL, 3–5 mL).

Utility of XIC in the analysis of the S-nitrosylated targets both in the control and GSNO-treated groups

As shown in Table 1, some peptides such as peptide VNQIGSVTESLQACK from enolase-3 and peptide SIQFVDWCPTGFK from tubulin α were detected in all three groups of samples (Negative CTRL group, CTRL group or GSNO group). As no methods to quantify the amount of

S-nitrosylation by LC/MS/MS is available so far, such protein targets were often discarded from the results list. As identical experimental conditions were used during sample manipulation, a rough estimation of the level of S-nitrosylation could be achieved by comparison of the XIC data among these three groups.^{16,17} As exemplified by peptide VNQIGSVTESLQACK from enolase-3 (Figs. 2(A)–2(C)) and peptide SIQFVDWCPTGFK from tubulin α in three samples (Figs. 2(D)–2(F)), each XIC profile extracted from the Negative CTRL group, the CTRL group or the GSNO group was put together and compared after normalization. As shown in Fig. 2(A), little signal was observed in the Negative CTRL group, indicating that almost all the free

Table 1. Identification of S-nitrosylated proteins in GSNO-treated HeLa cell extract with detergent-free biotin-switch method combined with LC/MS/MS

Sample group	Protein description	ProteinProphet™	Peptide	PeptideProphet™	Matched ions	Assumed charge	Xcorr	ΔCn	
HeLa (+5 mM DTT), Negative CTRL Group	glyceraldehyde-3-phosphate dehydrogenase	1.00	IISVASC ⁺ TTNC ⁺ LAPLAK	1.0000	21/32	2	3.820	0.616	
		1.00	VPTANVSVDLTC ⁺ R	1.0000	18/26	2	3.780	0.568	
		1.00	AC ⁺ ANPAAGSVILLENLR	0.9966	24/32	2	3.968	0.350	
		1.00	VNQIGSVTESLQAC ⁺ K	1.0000	22/28	2	4.782	0.554	
	HeLa (+100 μM GSH), CTRL Group	glyceraldehyde-3-phosphate dehydrogenase	1.00	IISVASC ⁺ TTNC ⁺ LAPLAK	1.0000	24/32	2	3.955	0.623
			1.00	VPTANVSVDLTC ⁺ R	1.0000	19/26	2	4.220	0.623
		phosphoglycerate kinase 1	1.00	AC ⁺ ANPAAGSVILLENLR	1.0000	24/32	2	5.079	0.511
			1.00	AAVEEGIVLGGC ⁺ ALLR	1.0000	24/32	2	4.831	0.566
		pyruvate kinase 3 isoform 1	1.00	NTGIIIC ⁺ TIGPASR	1.0000	20/24	2	4.242	0.554
			1.00	NC ⁺ AVEFNFGQR	1.0000	17/20	2	3.215	0.525
HeLa (+100 μM GSNO) GSNO Group	ribosomal protein L4	1.00	FC ⁺ IWTESAFR	1.0000	16/18	2	3.454	0.503	
		1.00	YWLC ⁺ AATGPSIK	0.9923	18/22	2	2.977	0.491	
	beta polypeptide 2-like 1	1.00	VNQIGSVTESLQAC ⁺ K	1.0000	24/28	2	5.101	0.567	
		1.00	SIQFVDC ⁺ PTGFK	1.0000	19/24	2	4.384	0.559	
	brain creatine kinase	1.00	FC ⁺ TGLTQIETLFK	0.9998	20/24	2	3.914	0.508	
		1.00	VIVVGNPANTNC ⁺ LTASK	1.0000	27/32	2	4.396	0.633	
	cytosolic malate dehydrogenase	1.00	AFQVETHGEVC ⁺ PANWTPDSTPIKPSFAASK	1.0000	37/120	3	5.955	0.585	
		1.00	C ⁺ VLLSNLSSTSHVPEVDPCSALQLK	1.0000	34/96	3	4.778	0.373	
	fatty acid synthase	1.00	C ⁺ QLEINFNTLQTK	1.0000	20/24	2	4.515	0.501	
		1.00	GHPYPLPNNYSYGLC ⁺ PGNGTTK	1.0000	34/88	3	5.242	0.542	
actinin, alpha	1.00	WFLTC ⁺ INQPQFR	0.9998	15/22	2	3.883	0.433		
	1.00	TREEC ⁺ HFYAGGQVYPGEASR	1.0000	32/80	3	4.645	0.552		
soluble carrier family 43, member 3	1.00	GLC ⁺ ALQAESLR	0.9979	15/22	2	2.825	0.357		
	1.00	NC ⁺ VILDSTPYR	1.0000	19/22	2	4.072	0.540		
eukaryotic translation elongation factor 1-γ	1.00	LC ⁺ AAAASILGKPADR	0.9999	22/28	2	4.014	0.422		
	1.00	GGDISVC ⁺ EWYQR	0.9998	15/22	2	3.444	0.483		
thioredoxin peroxidase	1.00	IISVASC ⁺ TTNC ⁺ LAPLAK	1.0000	24/32	2	4.700	0.633		
	1.00	VPTANVSVDLTC ⁺ R	1.0000	20/26	2	4.275	0.655		
ribosomal protein S3	1.00	AC ⁺ ANPAAGSVILLENLR	1.0000	26/32	2	5.516	0.544		
	1.00	AAVEEGIVLGGC ⁺ ALLR	1.0000	24/32	2	5.121	0.468		
ribosomal protein S8	1.00	C ⁺ EFQDAYVLISEK	1.0000	20/24	2	4.802	0.541		
	1.00	C ⁺ IPALDSLTPANEDQK	1.0000	21/30	2	4.173	0.526		
D-dopachrome tautomerase	1.00	NTGIIIC ⁺ TIGPASR	1.0000	19/24	2	4.087	0.554		
	1.00	pyruvate kinase 3 isoform 1/2							

(Continues)

Table 1. (Continued)

Sample group	Protein description	ProteinProphet™	Peptide	PeptideProphet™	Matched ions	Assumed charge	Xcorr	ΔCn
	E1B-55kDa-associated protein 5 isoform d	1.00	NC*AVEFNFGQR	1.0000	16/20	2	2.988	0.408
	ribosomal protein L4	1.00	WDVLIQQATQC*LNR	1.0000	22/26	2	4.390	0.499
		1.00	FC*TWTESAFR	1.0000	16/18	2	3.460	0.487
		1.00	RGPC*INVEDNGHIK	1.0000	24/56	3	3.568	0.439
	enolase-3	1.00	VNQGVSFTESLQAC*K	1.0000	23/28	2	5.467	0.518
	tubulin, alpha 1	1.00	SIQFVDWC*PTGFK	1.0000	19/24	2	4.570	0.594
	brain creatine kinase	1.00	FC*TGLTQJELFK	1.0000	20/24	2	3.898	0.487
	peroxiredoxin 1	1.00	HGEVC*PAGWKPGSDTIKPDVQK	1.0000	31/84	3	4.617	0.499
	cytosolic malate dehydrogenase	1.00	VIVVGNPANTNC*LTASK	1.0000	26/32	2	4.545	0.639
	clathrin heavy chain 1	1.00	HSSLAGC*QIINYR	1.0000	16/24	2	3.908	0.629
	ribosomal protein L30	1.00	LVILANNC*PALR	0.9999	19/22	2	3.616	0.465
		1.00	VC*TLAIIDPFGDSDIIR	0.9995	20/30	2	3.864	0.393
	ribosomal protein S5	1.00	TIAEC*LADELINAAK	1.0000	22/28	2	4.818	0.573
		1.00	VNQAIIWLLC*TGAR	1.0000	19/24	2	3.870	0.626
	ribosomal protein L12	1.00	EILGTAQSVGC*NVDGR	1.0000	24/30	2	4.726	0.659
		1.00	LGEWVGLC*K	1.0000	14/16	2	3.128	0.520
	ribosomal protein P0	1.00	AGAIAPC*EVTVP AQNTGLGPEK	1.0000	27/42	2	4.067	0.528
	filamin 1 (actin-binding protein-280)	1.00	VQVDNEGC*PVEALVK	1.0000	22/30	2	4.970	0.530
	phosphoglycerate dehydrogenase	1.00	ALVDHENVISC*PHLGASTK	1.0000	20/36	2	4.638	0.594
		1.00	NAGNC*LSPAVIVGLLK	0.9999	21/30	2	4.013	0.427
	GDP dissociation inhibitor 2	1.00	TDDYLDQPC*YETINR	1.0000	20/28	2	4.285	0.484
	beta-galactoside-binding lectin precursor	1.00	FNAHGDAITVC*NSK	1.0000	17/28	2	4.093	0.590
	eukaryotic translation initiation factor 1	1.00	FAC*NGTVIEHPPEYGEVIQLQGDQQR	1.0000	29/92	3	4.645	0.531
	cofilin 1 (non-muscle)	1.00	HELQANC*YBEVKDR	1.0000	25/52	3	4.083	0.534
	S100 calcium binding protein A11	1.00	C*TESLIAVFQK	0.9999	17/20	2	3.626	0.433
	ATP citrate lyase isoform 1/2	1.00	FIC*TTSAIQNR	1.0000	17/20	2	3.614	0.374
	matrin 3	1.00	LC*SLFYTNEEVAK	0.9996	17/24	2	3.476	0.432
	carbamoyl-phosphate synthetase 1, mitochondrial	1.00	SAYALGGLGSGIC*PNR	1.0000	24/30	2	4.461	0.472
	ras-related nuclear protein	1.00	VC*ENIPVLC*GNK	0.9997	21/24	2	3.903	0.406
	HLA-B associated transcript 1	1.00	NC*PHIVVGTGPR	0.9997	17/22	2	3.138	0.483
	ribosomal protein S11	1.00	DVQIGDIVTGGC*RPLSK	1.0000	21/34	2	4.367	0.524
	ribosomal protein S3a	1.00	AC*QSYPLHDVTVR	1.0000	20/26	2	4.250	0.555
	ribosomal protein L18a	1.00	SSGEIVYC*GOVFEK	1.0000	19/26	2	4.349	0.489
	ribosomal protein S8	0.99	NC*IVLIDSTPYR	0.9963	16/22	2	2.746	0.356
	acyl-CoA synthetase long-chain family member 3	1.00	LLLL*GGAPLSAITQR	0.9997	17/28	2	3.596	0.414
	T-complex protein 1 isoform a/b	0.99	IC*IDDELILIK	0.9952	15/18	2	3.103	0.264
	voltage-dependent anion channel 2	1.00	WC*EYGLTFTEK	0.9993	17/20	2	3.047	0.434

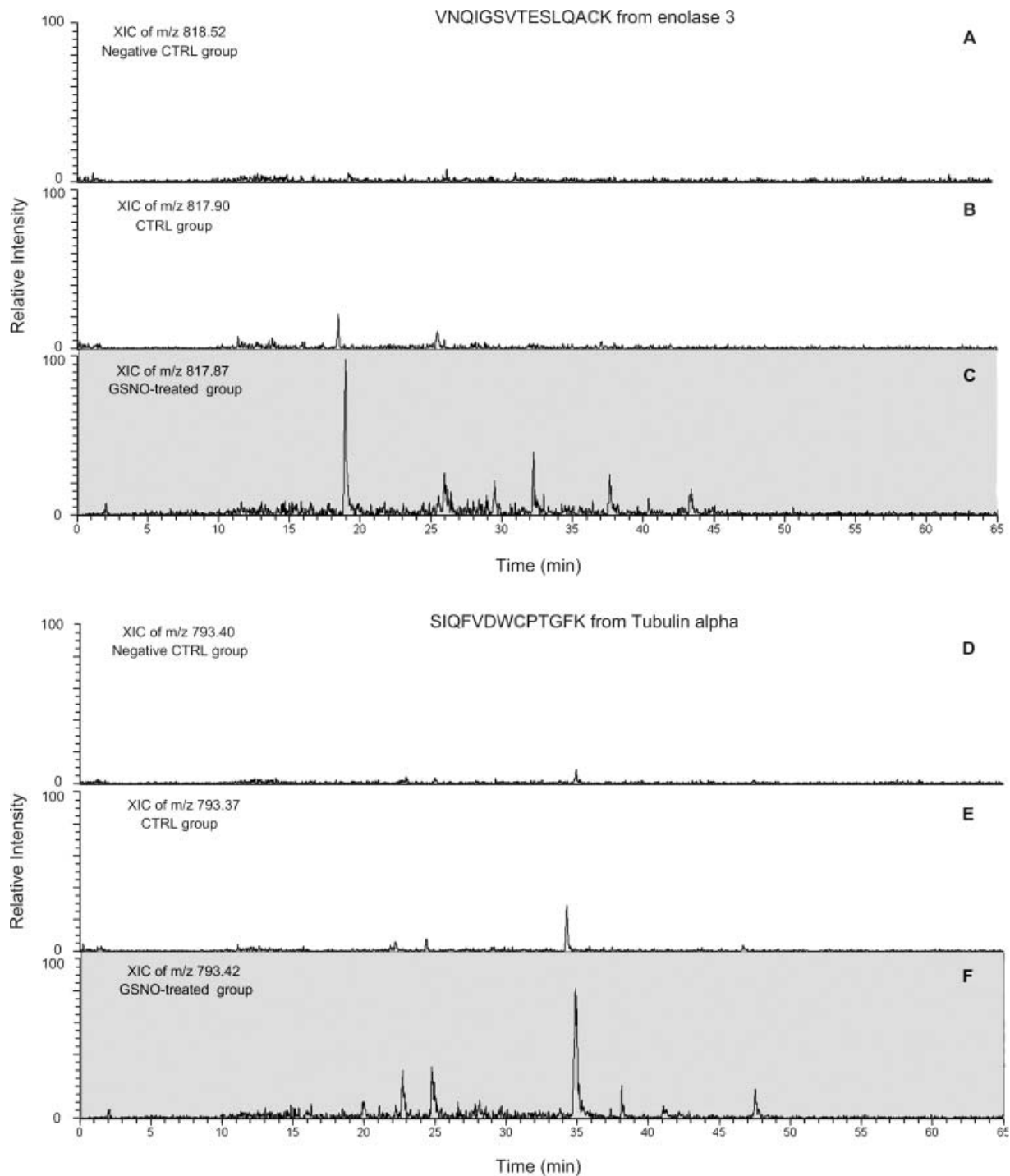


Figure 2. Comparison of the normalized extracted ion chromatograms of S-nitrosylated peptides identified from enolase-3 and tubulin α . The top images (A–C) represent the XIC traces of peptides **VnQIGSVTESLQACK** from enolase-3 identified in the Negative CTRL group (m/z 818.52), the CTRL group (m/z 817.90) and the GSNO-treated group (m/z 817.87). The extracted spectra were smoothed and background noises were subtracted. Almost no signal was observed in the chromatogram of the Negative CTRL group (A), while a tiny peak was detected in the chromatogram of the CTRL group (B). In contrast, a large rise in MS response was found in the GSNO-treated group, indicating that the peptide was actually S-nitrosylated by GSNO. Similar results were observed in the XIC trace of peptide **SIQFVDWCPTGFK** identified from tubulin α in the Negative CTRL group (m/z 793.40), the CTRL group (m/z 793.37) and the GSNO-treated group (m/z 793.42).

sulfhydryls have been blocked during the urea-based biotin switch. Nevertheless, there was a small increase in the signal intensity in the GSH-treated group (Fig. 2(B)) and a large increase in the GSNO-treated group (Fig. 2(C)) compared

with the negative control group. A similar result was observed in tubulin α (Figs. 2(D)–2(F)). Comparison of the XIC traces from identical peptides among all three groups together could help to reveal valuable

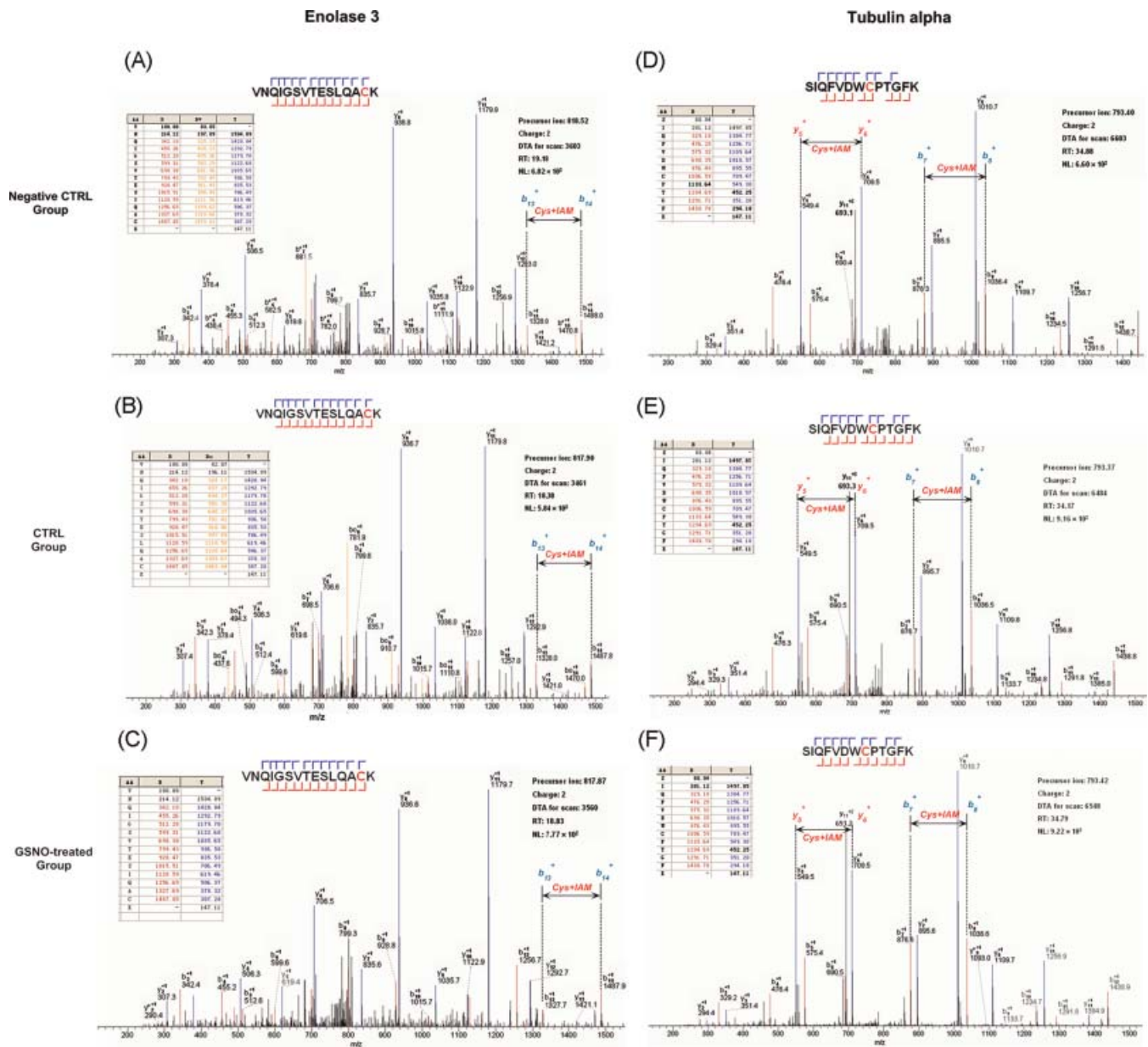


Figure 3. The MS/MS spectra of peptide VNQIGSVTESLQACK from enolase-3 (A–C) and peptide SIQFVDWCPTGFK from tubulin α (D–F) identified in the Negative CTRL group, the CTRL group and the GSNO-treated group, respectively. Doubly charged precursors were fragmented by the CID process. Matched y -ion series are colored in blue, while matched b -ion series are colored in red. A nearly identical distribution pattern of b - and y -ion series of the same peptide in different groups was clearly seen. As shown in the table in the top left-hand corner within each MS/MS spectrum, a continuous match of at least five b ions and y ions was achieved. All major ion fragment peaks were matched to either b or y ions or other adducts. In addition, the validation of the m/z difference in modified cysteine has been highlighted by dotted lines in each spectrum. All peptides identified were validated for acceptance by the PeptideProphetTM algorithm with a computed peptide probability (p_{comp}) of at least 0.99. Other parameters such as X_{corr} , Δeltn , Cn , etc., are listed in detail in Table 1. This figure is available in colour online at www.interscience.wiley.com/journal/rcm.

information on false-positive interference as well as endogenous S-nitrosylation during sample preparation. Figure 3 presents the MS/MS spectra of peptide VNQIGSVTESLQACK from enolase-3 (A–C) and peptide SIQFVDWCPTGFK from tubulin α (D–F) identified in the Negative CTRL group, the CTRL group and the GSNO-treated group, respectively. As shown in the table on the top left within each MS/MS spectrum, a continuous match of at least five b and y ions was achieved. In Fig. 3, b -ion series and y -ion series of

the modified cysteine (the original S-nitrosylated cysteine has been modified to alkylated cysteine, designated as Cys+IAM) have been highlighted by dotted lines. For example, in the MS/MS spectra of tubulin α , the cysteine residue in peptide SIQFVDWCPTGFK was identified as the S-nitrosylated one. The m/z difference between y_5^+ and y_6^+ , b_7^+ and b_8^+ is equal to the m/z of the alkylated cysteine. Based on this analysis, enolase-3 and tubulin α should be kept as potential S-nitrosylated targets.

DISCUSSION

In the study reported in this paper, we developed a detergent-free biotin-switch method and combined it with LC/MS/MS to identify the S-nitrosylated targets. 8 M urea was chosen as the denaturing environment to ensure the complete opening up of the protein structure and to ensure that MMTS reached the buried cysteine residues of the proteins. In the labeling step, 2 M to 4 M urea was tested for biotin-HPDP labeling, and 4 M urea was finally chosen for the maintenance of the denaturing state of the protein as well as labeling efficiency (data not shown). Besides, in the streptavidin-based purification step, the detergent Triton X-100 was omitted from washing buffer A. Instead, the salt concentration (NaCl) was changed from 600 mM to 1 M to increase the capacity of removing the non-specific binding. The modified method was successfully used in the analysis of a GSNO-treated HeLa cell extract of proteins.

By switching from the detergents SDS and Triton X-100 to urea in our developed method, the LC/MS performance for the proteomic analysis of S-nitrosylated proteins was greatly ameliorated, as evaluated by Pep3D. Meanwhile, the contamination of the reversed-phase column caused by detergents was completely avoided and the repeatability of experiments was greatly improved. Furthermore, as the MS signal suppression resulting from the detergents was eliminated, the amount of sample needed for each experiment was also significantly reduced, which is particularly helpful in the analysis of small amounts of rare samples.

For data processing, all MS/MS data from our experiments were validated by probability-based algorithms developed by the Institute for Systems Biology, Seattle, WA, USA. Above the designated minimal probability cutoff for peptides and proteins, the sensitivity and false error rate are accurately estimated, providing an object and transparent platform for the validation of identified targets within a dataset.¹⁸ In addition, for the peptides detected in both the CTRL group and the GSNO-treated group, they should not be simply discarded. XIC may be used to supply some quantitative information to facilitate the judgment on 'accept or reject' of the identified S-nitrosylated targets. Although more accurate methods for quantitation of S-nitrosylation by LC/MS/MS are expected, such preliminary estimation is

also very helpful to obtain information on the change of S-nitrosylation.

In conclusion, the urea-based detergent-free biotin-switch method combined with LC/MS/MS showed great advantage in proteomic identification of S-nitrosylated proteins. Proper analysis of S-nitrosylation results based on MS is important for further investigation.

SUPPLEMENTARY MATERIAL

The supplementary electronic material for this paper is available in Wiley InterScience at: <http://www.interscience.wiley.com/jpages/0951-4198/suppmat/>.

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