# Optimization of the isolation and enrichment of *S*-nitrosylated proteins from brain tissues of rodents and humans with various prion diseases for iTRAQ-based proteomics

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**Abstract.** Accumulating evidence indicates that *S*-nitrosylation plays an important role in protein function either physiologically or pathologically. In the present study, we describe a modified method for the isolation and enrichment of *S*-nitrosylated (SNO) proteins from brain tissue based on a biotin labeling system using the biotin switch technique (BST). Various working conditions for the incubation of biotin-labeled samples with streptavidin beads and for the elution of SNO proteins from streptavidin beads were comparatively evaluated. The working conditions were optimized with incubation at a ratio of 1:3 (streptavidin beads/brain homogenates) at 25˚C for 120 min, and the elution conditions were optimized using buffer containing 0.5% sodium dodecyl sulfate. Under these conditions, we found that at least 12 rounds of successive incubation were required in order to recover all the SNO proteins in the human and rodent brain homogenates. Western blot analyses of some of the eluted products confirmed the reliable immunoreactivity of the isolated SNO proteins. iTRAQ-based mass spectrometric (MS) analysis of the eluted products from the brain tissues of a normal healthy subject and patients with various prion diseases identified 1,509 SNO proteins with high confidence [false discovery rate (FDR) <1%]. These data indicate that with this optimized method, the endogenous SNO proteins from the brain tissue of humans and rodents can be sufficiently isolated, which can then be used directly in further assays, such as large-scale analysis of the *S*-nitrosoproteome in complex backgrounds.

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## Introduction

Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of fatal and infectious neurodegenerative diseases that can affect humans and animals, including Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, transmissible mink encephalopathy (TME) in mink and chronic wasting disease (CWD) in elk and deer (1). The pathogen for these diseases is believed to be prion, whose infectious isoform termed $PrP^{Sc}$ is derived from the cellular isoform termed $PrP^{C}$ (2,3). The conversion from $PrP^{C}$ to $PrP^{Sc}$ is the central event in prion diseases which may occur spontaneously and can be acquired or induced by autosomal dominant mutations of the *PRNP* gene (4).

Nitric oxide (NO) and other reactive nitrogen species (RNS) are biologically active small molecules involved in the pathogeneses of a series of neurodegenerative diseases (5). The transfer of a NO group to cysteine sulfhydryls on proteins is known as *S*-nitrosylation. These *S*-nitrosylated proteins are thus referred to as SNO proteins (6-8). Similar to other post-translational modifications, *S*-nitrosylation can activate or inhibit the activity of the target protein, alter protein interactions with other molecules and affect protein aggregation or localization under physiological conditions, thus influencing or balancing cell signal transduction pathways and cellular functions in a number of biological processes (9). Under pathological conditions, aberrant *S*-nitrosylation can occur in response to nitrosative stress and may stimulate cell destructive processes,

contributing to neurodegeneration through the disruption of a number of pathways (5). Furthermore, the temporal and spatial dynamics of SNO proteins may critically affect their modulatory role in response to nitrosative stress. For instance, in the brains of patients with Alzheimer's disease (AD), *S*-nitrosylated dynamin-related protein (SNO-Drp1) is related to the formation of β-amyloid and subsequent mitochondrial fission activation (10,11). *S*-nitrosylated Parkin (SNO-Parkin) and *S*-nitrosylated peroxiredoxin 2 (SNO-Prx2) are associated with the pathogenesis of sporadic Parkinson's disease (PD), in which SNO-Parkin causes proteasomal dysfunction (12), and SNO-Prx2 promotes oxidative stress-induced neuronal cell death (13). In addition, a number of biological proteins involved in cellular apoptosis [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-Siah, X-linked inhibitor of apoptosis (XIAP) and caspase-3], phosphorylation [phosphatase with sequence homology to tensin (PTEN)], neuroinflammation [cyclooxygenase-2 (COX-2)] and autophagy [c-Jun N-terminal kinase (JNK)1 and IκB kinase (IKKβ)] are also *S*-nitrosylated (14). In addition to AD and PD (10,15-17), we have also reported that the expression levels of some SNO proteins, such as *S*-nitrosylated protein disulphide isomerase (SNO-PDI) (18) and 14-3-3 (19), are abnormally increased in the brains of scrapie-infected rodents at the terminal stage, highlighting the significance of SNO proteins in prion diseases.

Greatly improved proteomics technologies coupled with bioinformatics provide the potential for hundreds of proteins to be discovered and verified experimentally (20-23), which makes it possible to analyze the *S*-nitrosoproteome for prion diseases. Since the *S*-nitrosylated isoform may usually occupy only a small fraction of the relevant protein at a given time, methods to effectively enrich SNO proteins are in great need. Although studies have dealt with SNO protein isolation and enrichment through multiple methods (22,24-26), direct usage in mass spectrometry (MS)-based proteomics remains quite problematic. In this study, we describe an optimized protocol for the isolation and enrichment of SNO proteins from brain tissue, based on a commercial SNO protein detection assay kit. Several essential parameters were carefully evaluated and optimized. We also provide evidence that SNO proteins isolated using such a protocol from the brain tissues of humans with various prion diseases can be used in further assays, such as Western blot analysis and iTRAQ-based proteomics.

## Materials and methods

*Antibodies*. The following antibodies were used in this study, including anti-actin monoclonal antibody (mAb) (Thermo Fisher Scientific, Rockford, IL, USA), anti-14-3-3 polyclonal antibody (pAb), anti-glycogen synthase kinase (GSK)-3β pAb, anti-heat shock protein 27 (Hsp27) mAb and anti-sirtuin 1 (Sirt1) pAb (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-GAPDH and horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse immunoglobulin G (Thermo Fisher Scientific).

*Preparation of brain tissue samples*. The stored frozen brain tissue from normal healthy hamsters (n=3) and hamsters inoculated intracerebrally with hamster-adapted scrapie agent 263K (n=3), as well as the post-mortem cortex and cerebellum of patients with sporadic CJD (sCJD, n=1), FFI (n=3), G114V genetic CJD (G114V gCJD, n=1) and a healthy subject (n=1) were enrolled in this study. Brain homogenates (10%, w/v) were prepared based on a previously described protocol (27). Briefly, brain tissues were homogenized in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris, pH 7.5) containing 1% protease inhibitor cocktail (Abcam, Cambridge, MA, USA). The tissue debris was removed by low-speed centrifugation at 2,000 x g for 10 min and the supernatants were collected for further analysis.

*Use of the biotin-switch technique (BST) for the analysis of the S-nitrosylation of proteins*. SNO proteins in the brain tissue were determined using a commercial SNO protein detection assay kit (Cayman Chemical, Inc., Ann Arbor, MI, USA) following the manufacturer's instructions. Briefly, all tested samples were normalized to 100 mg (approximately 1 ml 10% brain homogenate/sample) total brain proteins and transferred to 1.5-ml Eppendorf tubes. The brain samples were homogenized in 0.5 ml buffer A containing blocking reagent (thiol-blocking reagent at 2 mg/ml). Following incubation at 4˚C for 30 min in the dark, the samples were clarified by centrifugation at 4˚C for 10 min. The supernatants were transferred to 2-ml tubes and mixed with pre-cooling acetone (1:4, v/v), and total proteins were precipitated at -20˚C for 2 h. Following centrifugation at 4˚C for 15 min, the pellet was resuspended in 0.5 ml buffer B containing reducing and labeling reagents, and further incubated at room temperature for 2 h on a rocker with gentle agitation. Acetone precipitation was conducted again as described above. The protein pellet was thoroughly dissolved in dissolving buffer (8 M urea, 30 mM HEPES, 10 mM DTT, 2 mM EDTA and 1 mM PMSF, pH 8.2) at the same volume as the brain homogenates initially used. The products were stored for further analysis.

*Binding to streptavidin beads*. To pull-down SNO proteins, streptavidin-conjugated magnetic beads (2.8 $\mu$m, Dynal magnetic beads; Invitrogen Life Technologies Corporation, Carlsbad, CA, USA) were used according to the manufacturer's instructions. Briefly, the beads were washed 3 times with phosphate-buffered saline (PBS, pH 7.4) containing 0.01% (v/v) Tween-20 and added to the samples mentioned above. The mixture was rotated at room temperature for 1 h. Bound beads were washed 5 times in PBS containing 0.1% bovine serum albumin (BSA) (w/v) and then resuspended in the same volume of PBS as the initial volume of beads used.

*Optimization of the working conditions for elution*. Four different elution buffers with different elution conditions were tested for releasing SNO proteins from streptavidin beads: i) incubation in non-ionic water at 75˚C for 5 min as previously described by Holmberg *et al* (28); ii) incubation in 10 mM EDTA pH 8.2 with 95% formamide at 90˚C for 2 min as described in the instructions provided with the Dynabeads® M-280 (Life Technologies); iii) incubation in HEPES elution buffer (20 mM HEPES-NaOH, 100 mM NaCl, 1 mM EDTA, 100 mM 2-ME, pH 7.7) at room temperature for 20 min as previously described by Xu *et al* (29); iv) incubation with various concentrations of sodium dodecyl sulfate (SDS; 0.1, 0.2, 0.5 and 1% SDS) at 100˚C for 5 min. SNO proteins eluted with 1.0 ml of various elution buffers were precipitated with acetone at -20˚C for 2 h.

Following centrifugation at 10,000 x g at 4˚C for 15 min, the pellets were resuspended with dissolving buffer at the same volume as the brain homogenates initially used.

*Optimization of the working conditions for incubation*. Three different bead/homogenate ratios (v/v) were tested, including 1:6, 1:3 and 1:1. In addition, 3 different incubation times at 2 incubation temperatures were evaluated: 4˚C for 30, 60 or 120 min; and 25˚C for 30, 60 or 120 min. All other experimental conditions were maintained invariable.

*Determination of protein concentration*. The protein concentration of the redissolved products was determined in triplicate using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

*Western blot analysis*. SNO protein samples were mixed with 5X loading buffer and boiled for 8 min. Proteins were separated in 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose (NC) membranes (Whatman, Pittsburgh, PA, USA) by the semi-dry method in transfer buffer and immunoblotted with anti-biotin antibody (Cayman Chemical, Inc.). Individual SNO proteins were measured by specific antibodies, including anti-14-3-3 used at 1:1,000, anti-GSK-3β used at 1:1,500, anti-Hsp27 used at 1:2,000, anti-Sirt1 used at 1:800, anti-β-actin used at 1:5,000 and anti-GAPDH antibodies used at 1:2,000. Reactive signals were visualized using an enhanced chemiluminescence (ECL) kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA).

*Dot blot for the detection of SNO proteins*. One microliter of the isolated SNO proteins was dotted onto NC strips, which were allowed to dry at room temperature. The strips were blocked with 2% BSA (w/v) in Tris-buffered saline containing 0.5% Tween-20 (TBST, w/v) and incubated overnight at 4˚C to block the residual binding sites on the paper. Subsequently, the strips were further incubated for 1 h at 37˚C with anti-biotin antibody diluted 1:1,000 in 2% BSA. The strips were washed 3 times with TBST and reactive signals were visualized using an ECL kit.

*iTRAQ labeling and MS analysis*. iTRAQ labeling for human brain specimens was carried out using the iTRAQ® Reagent-8Plex kit (AB Sciex, Framingham, MA, USA) according to the manufacturer's instructions. The purified SNO proteins of the cerebellum and cortex from the normal control were labeled with iTRAQ labeling reagent 113 and 117, while those of the cerebellum and cortex from the patients with sCJD, FFI and gCJD were labeled with iTRAQ labeling reagents 114 and 118, 115 and 119, 116 and 121. The amounts of the purified SNO proteins were 100 μg/label. The labeled products were digested with trypsin at a ratio of 1:20 (w/w, trypsin/protein) at 37˚C for 36 h. The digested peptides were subsequently dried and reconstituted with 0.5 M triethylammonium bicarbonate (TEAB; Sigma, St. Louis, MO, USA) and 0.1% SDS. The dried peptides were then labeled with respective isobaric tags, and incubated at room temperature for 1 h before being combined.

To remove all interfering substances, such as dissolving buffer, ethanol, acetonitrile, SDS, excess iTRAQ reagents, strong cation exchange chromatography (SCX) was carried out for the combined iTRAQ-labeled peptides using the cation exchange system provided in the iTRAQ method development kit (AB Sciex). The eluted fraction was desalted using Sep-Pak C18 cartridges, dried and then reconstituted with 0.1% formic acid (FA) for nano-flow liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis coupled with the ultimate LC system using Q-Exactive Mass Spectrometer (Thermo Fisher Scientific). Peptide separations were performed in a 100x75 mm column (BEH130 C18) using mobile phase A (0.1% FA in LC-MS grade water) and mobile phase B (0.1% FA in LC-MS grade ACN). The flow rate was set at 400 nl/min. The LC fluent was directed to the electrospray ionization (ESI) source for quadrupole mass spectrometry (Q-MS) analysis, using precursor ions that were selected across the mass range of 350-2,000 m/z with 250 msec accumulation time/spectrum. A maximum of 20 precursors per cycle from each MS spectra was selected for MS/MS analyses with 100 msec minimum accumulation time for each precursor and dynamic exclusion for 15 sec.

The software used for data interpretation was proteome discoverer version 1.3 (Thermo Fisher Scientific) and Mascot version 2.3.0. The database searched was the peptide sequence library in the Swissport database restricted to the human protein sequence data set.

*Ethics statement*. Usage of the stored human and animal brain specimens in this study was approved by the Ethics Committee of the National Institute for Viral Disease Prevention and Control, China CDC under protocol 2009ZX10004-101. All Chinese golden hamsters were maintained under clean grade. Housing and experimental protocols were in accordance with the Chinese Regulations for the Administration of Affairs Concerning Experimental Animals.

## Results

*Optimization of the eluting conditions for SNO proteins from the beads*. Several methods have been described for eluting biotin-labeled biological macromolecules from streptavidin beads, such as $ddH_2O$, EDTA, HEPES or SDS. To evaluate the eluting abilities of the different methods, 30 μl of brain homogenate of normal healthy hamsters (roughly 30 μg total proteins) were subjected to BST for biotin-labeling and bound with streptavidin-conjugated beads. The biotin-labeled SNO proteins bound on the beads were separately eluted with various eluting conditions and the biotin signals in the eluting fractions and beads were examined by western blot analysis for biotin. After being eluted with $ddH_2O$, only a small portion of biotin-positive signals was detected in the fraction of elution (Fig. 1A, left panel). More biotin signals were observed in the elution fraction following treatment with 10 mM EDTA with 95% formamide (pH 8.2), but there were still biotin signals left in the beads (Fig. 1A, middle panel). In the preparation of HEPES, almost all biotin signals were observed in the fraction of beads (Fig. 1A, right panel). These results indicate that it is not possible to completely elute the bound biotin-SNO protein from streptavidin beads with these 3 buffers.

Subsequently, the eluting capacity of the SDS buffer was evaluated based on the same experimental conditions.

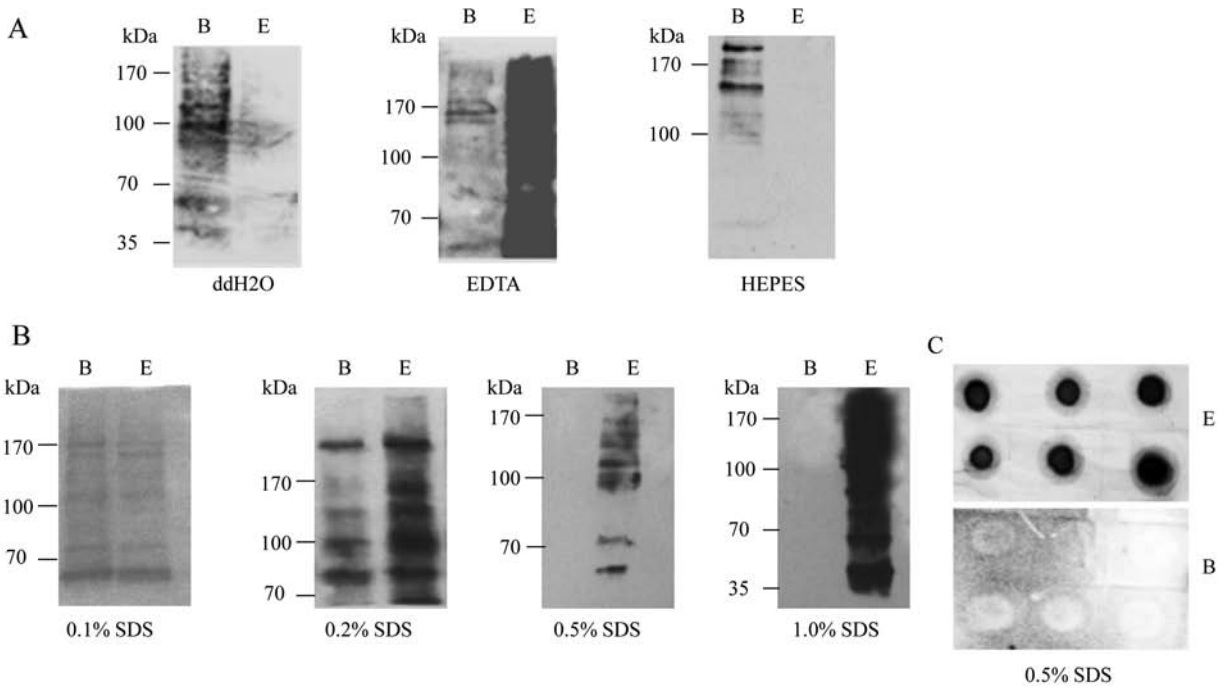


Figure 1. Optimization of the eluting conditions for *S*-nitrosylated (SNO) proteins in the brain tissues of normal healthy hamsters from the streptavidin beads. (A) Biotin-specific western blots of SNO proteins eluted by the buffers, $ddH_2O$, EDTA and HEPES. (B) Western blots of SNO proteins eluted by the buffer of 0.1, 0.2, 0.5 and 1% sodium dodecyl sulfate (SDS). (C) Dot blots of SNO proteins eluted by 0.5% SDS. B, streptavidin beads after elution; E, eluting products. The molecular markers are indicated on the left.

Following treatment with various concentrations of SDS buffer, the SNO proteins in the elution fraction and beads were evaluated by biotin-specific western blot analysis. The results revealed that the biotin signals were distributed almost equally in the fractions of elution and beads in the reaction of 0.1% SDS, but increased significantly in the fraction of elution in the reaction of 0.2% SDS. In the reactions of 0.5 and 1.0% SDS, all biotin signals were observed in the fraction of elution (Fig. 1B). Considering that a high concentration of SDS may affect the subsequent MS analysis (30) and other assays, we used 0.5% SDS as the elution buffer to break biotin-streptavidin interaction for further experiments.

To simplify the detection of SNO proteins, we used the biotin-specific dot blot technique instead of western blot analysis. In the reaction of 0.5% SDS, biotin signals were merely observed in the eluting products, but not in the beads (Fig. 1C); these results were consistent with those of western blot analysis.

*Optimization of the incubation ratio of streptavidin beads and biotin-labeled brain homogenate*. To optimize the volume ratio of the streptavidin beads to the brain homogenate (10% w/v), the protein capturing abilities of the different working volume ratios (bead/homogenate, 1:6, 1:3 and 1:1) were examined. Different amounts of bead were mixed with 30 $\mu$l brain homogenates prepared from normal or 263K scrapie-infected hamsters after being labeled with biotin by BST. After being eluted with 0.5% SDS buffer, the protein concentrations in the eluted products were measured. As shown in Fig. 2A, only very small amounts of protein were detected in the eluted products at a ratio of 1:6, whereas a markedly larger amount of protein was eluted in the reactions at a ratio of 1:3 and 1:1. The protein-capturing abilities were approximately the same between the reactions of ratios 1:3 and 1:1 in either the normal or infected brain homogenates. Based on higher cost and effective ratio, we selected the ratio of 1:3 (bead/homogenate) as the optimal working volume ratio.

*Optimization of the incubation temperature and incubation time of streptavidin beads and biotin-labeled brain homogenate*. To optimize the incubation temperature and incubation time for the streptavidin beads with biotin-labeled brain homogenate, 10 $\mu$l of beads were mixed with 30 $\mu$l normal or 263K-infected hamster brain homogenates at the volume ratio of 1:3 (bead/homogenate). Following incubation at 2 different temperatures (4 and 25˚C) for 3 different periods of time (30, 60 and 120 min), the biotin-labeled proteins were eluted from the beads with 0.5% SDS buffer and the protein amounts were evaluated using the BCA method. The results revealed similar alternative curves of the eluted protein contents in both the normal and infected homogenates, along with the changes in incubation temperature and duration (Fig. 2B). It was apparent that incubation at 25˚C yielded higher protein concentrations in the eluted products than at 4˚C. The increase in the incubation time, particularly with the incubation temperature of 25˚C, yielded more proteins in the elution products. Based on these data, incubation at 25˚C for 120 min was regarded as the optimal working condition.

*Optimization of the number of incubations for recovery of the whole SNO proteins in brain homogenates*. To optimize the number of incubations in order to recover all SNO proteins from the brain homogenates, 15 $\mu$l of BST-treated brain homogenates of normal hamsters (roughly 15 $\mu$g total proteins)

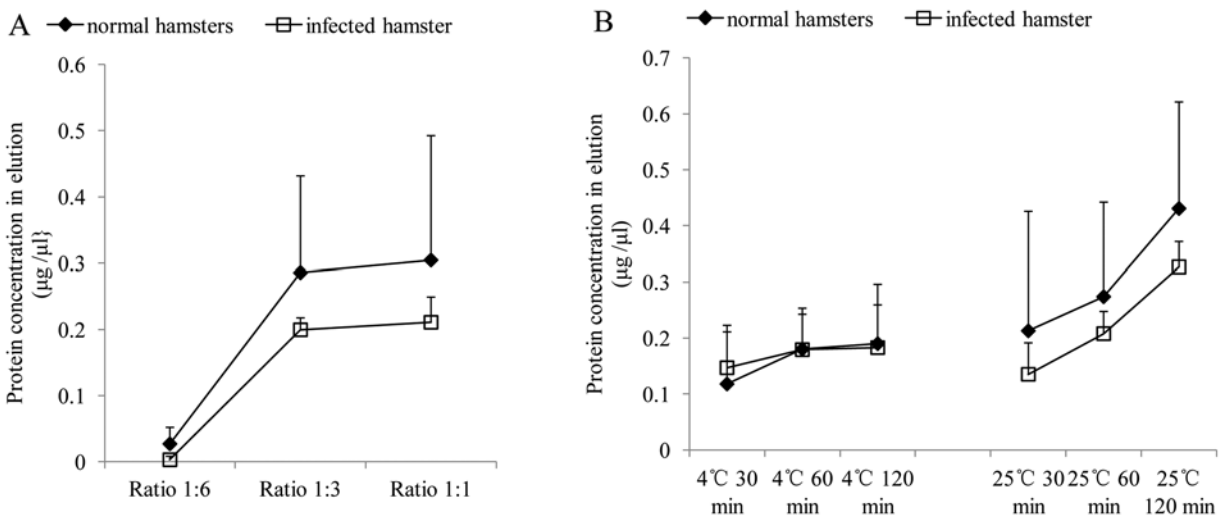


Figure 2. Optimization of the working conditions for the incubation of biotin-labeled brain homogenates of normal (healthy) and scrapie 263K-infected hamsters with streptavidin beads. Protein concentrations in the eluting products were measured using the bicinchoninic acid (BCA) kit. (A) Protein concentrations in the eluting products following incubation with different ratios of bead/homogenate (1:6, 1:3 and 1:1). (B) Protein concentrations in the eluting products following incubation under various conditions (4˚C 30 min, 4˚C 60 min, 4˚C 120 min, 25˚C 30 min, 25˚C 60 min and 25˚C 120 min). Data are presented as average ± standard error (SE) of 3 replicates.

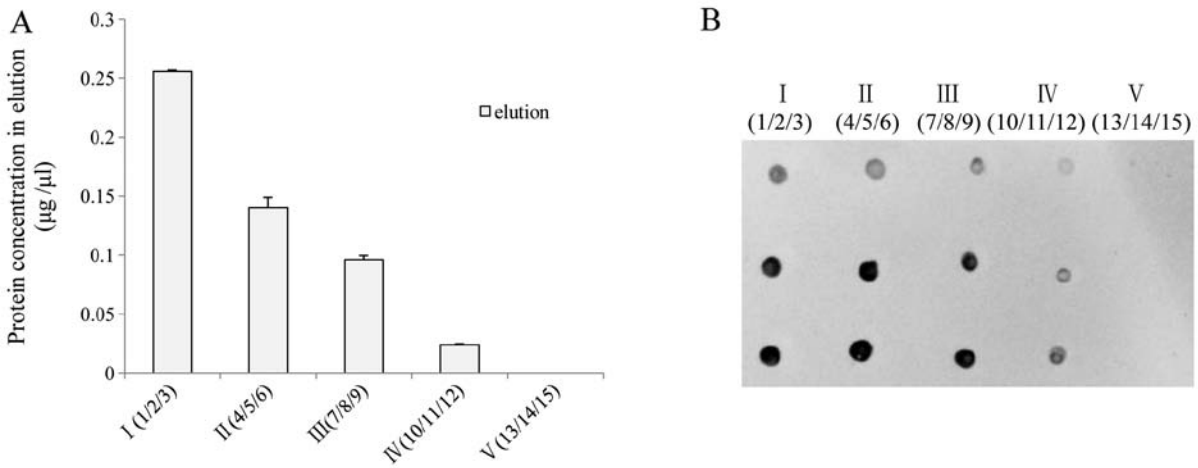


Figure 3. Recovery of whole *S*-nitrosylated (SNO) proteins from rodent brain homogenates by successive incubation with streptavidin beads. Fifteen microliters of biotin switch technique (BST)-treated brain homogenates from normal healthy hamsters were incubated with 5 $\mu$l of streptavidin beads. Following elution and discarding of used beads, the brain homogenates were continually incubated with newly input beads. This process was repeated for at least 15 rounds and 3 adjacent elution products were pooled for further assays. (A) Measurement of protein concentrations. (B) Biotin-specific dot blots; 0.5 $\mu$l (upper panel) and 1 $\mu$l (middle and bottom panels) of eluted products were separately dotted on nitrocellulose (NC) membrane. I (1/2/3), the eluting products pooled from the 1st, 2nd and 3rd round of incubation; II (4/5/6), the eluting products pooled from the 4th, 5th and 6th round of incubation; III (7/8/9), the eluting products pooled from the 7th, 8th and 9th round of incubation; IV (10/11/12), the eluting products pooled from the 10th, 11th and 12th round of incubation; V (13/14/15), the eluting products pooled from the 13th, 14th and 15th round of incubation.

were incubated with 5 $\mu$l of streptavidin beads at the optimized working conditions. Following elution and the discarding of used beads, the brain homogenates were continually incubated with newly input beads. This process was repeated for at least 15 rounds and 3 adjacent elution products were pooled for further assays. Measurement of the protein concentration in the pooled elution products revealed that the protein contents gradually decreased along with the number of incubations, in which almost no protein was detected in the last pooled elution sample (incubation number 13-15) (Fig. 3A). The biotin-labeled proteins in various pooled samples were assayed with biotin-specific dot blots. In line with the results of the protein measurement, biotin-specific signals were observed in the first 4 pooled elution samples from 3 individual brain homogenates, but not in the last one (Fig. 3B). This indicates that at least 11 rounds of continual incubations at this experimental condition are required in order to recover all SNO proteins from the rear brain extracts of the hamsters.

Furthermore, the number of incubations for the recovery of the whole SNO proteins from human brain homogenates were assessed with the same protocol, including the brain homogenates prepared from the cortex and cerebellum of the

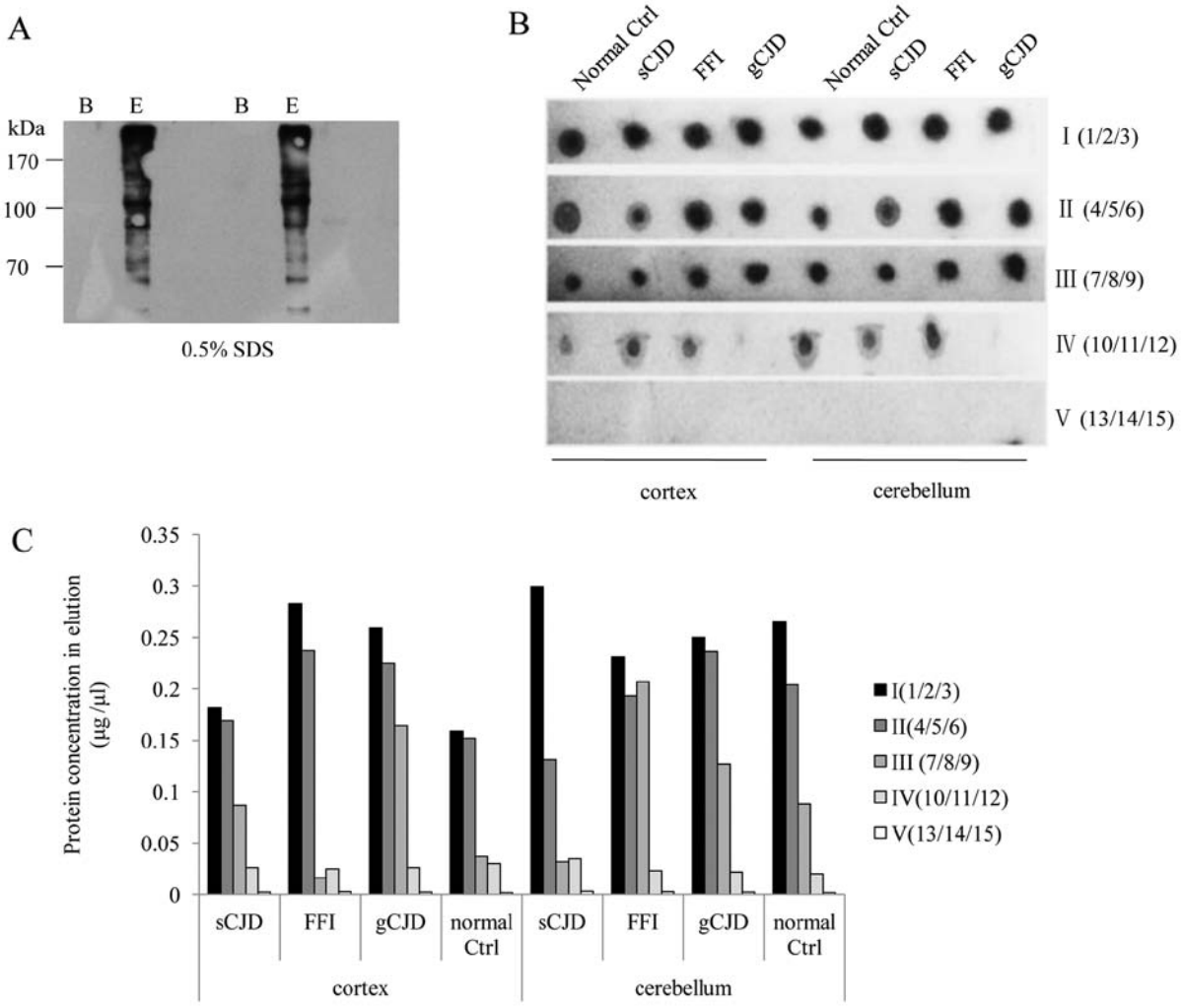


Figure 4. Recovery of *S*-nitrosylated (SNO) proteins from human brain homogenates through successive incubations with streptavidin beads. (A) Western blots of SNO proteins eluted by 0.5% sodium dodecyl sulfate (SDS). B, streptavidin beads following elution; E, eluting products. (B) Dot blots of SNO proteins in the eluting products after being successively incubated with streptavidin beads. (C) Measurement of protein concentrations in the eluting products after being successively incubated with streptavidin beads. Normal ctrl; healthy control subject; sCJD, sporadic Creutzfeldt-Jakob disease; FFI, fatal familial insomnia; gCJD, genetic Creutzfeldt-Jakob disease; I (1/2/3), the eluting products pooled from the 1st, 2nd and 3rd round of incubation; II (4/5/6), the eluting products pooled from the 4th, 5th and 6th round of incubation; III (7/8/9), the eluting products pooled from the 7th, 8th and 9th round of incubation; IV (10/11/12), the eluting products pooled from the 10th, 11th and 12th round of incubation; V (13/14/15), the eluting products pooled from the 13th, 14th and 15th round of incubation.

healthy subject, and patients with sCJD, FFI and G114V gCJD. In agreement with the results obtained with the hamster brain homogenates, biotin-specific western blot analysis confirmed that 0.5% SDS was able to elute almost all biotin-labeled proteins from the bound beads in human brain extracts (Fig. 4A). A total of 15 rounds of successive incubations of the individual BST-treated brain homogenates with beads were conducted under optimized working conditions. In agreement with the observations of the hamster brain homogenates, almost no protein was detectable in the elution sample pooled from 13 to 15 rounds in all tested extracts (Fig. 4B). Dot blots identified similar patterns, according to which the intensities of the biotin signals gradually decreased in the first 4 pooled samples and diminished in the last of all tested samples (Fig. 4C). This suggests that the optimized working conditions based on brain homogenates of hamsters are suitable for the purification of SNO proteins from human brain extracts, from either healthy subjects or patients with various prion diseases.

*Evaluation of the usage of the purified SNO proteins from human brain homogenates*. To address whether the isolated SNO proteins with the newly optimized working conditions can be used for further experiments, the immunoreactivities of the purified SNO proteins from human cortex regions were subjected to several specific western blot analyses, including for 14-3-3, GSK-3β, Hsp27, Sirt-1, actin and GAPDH. Specific bands were observed at the expected positions in all tested blots (Fig. 5), strongly indicating that the SNO proteins extracted under the newly optimized working conditions possess reliable immunoreactivities. The signal intensities of all tested proteins in the samples of prion diseases were significantly stronger than those of the normal control.

Subsequently, the isolated SNO proteins from the human cortex and cerebellum under the newly optimized working conditions were subjected to iTRAQ-based quantitative proteomics analysis. A total of 69,896 spectra was matched from a total of 448,298 spectra. After searching with Mascot software, a total

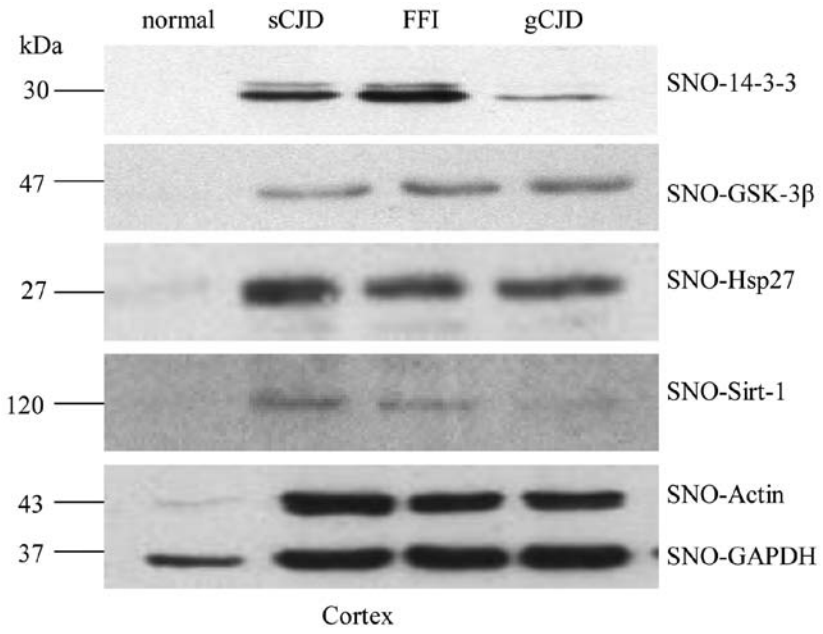


Figure 5. Western blot analysis of the purified *S*-nitrosylated (SNO) proteins in the cortex homogenates from, the healthy subject, as well as patients with sporadic Creutzfeldt-Jakob disease (sCJD), FFI and G114V genetic Creutzfeldt-Jakob disease (gCJD) with specific antibodies against 14-3-3, GSK-3β, Hsp27, actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The molecular markers are indicated on the left.

of 1,509 proteins was identified from 9,265 unique peptides with high confidence (FDR <1%). Fig. 6 illustrates the MS/MS spectra of the peptides of 3 different proteins, representing neuroblast differentiation-associated protein AHNAK isoform 1 (peptide sequence, VDIDAPDVDVHGPDWHLK) with a molecular weight of 628.7 kDa (Fig. 6A), heat shock protein gp96 precursor (peptide sequence, GVVDSDDLPLNVSR) with a molecular weight of 90.1 kDa (Fig. 6B) and S100-B (peptide sequence, AMVALIDVFHQYSGR) with a molecular weight of 10.7 kDa (Fig. 6C). The reporter region of each MS/MS spectrum illustrated the signals of the peptides labeled with the different iTRAQ tags in various brain extracts at the expected positions (m/z), including 113 and 117 for the normal control, 114 and 118 for sCJD, 115 and 119 for FFI, and 116 and 121 for G114V gCJD. This indicates that the SNO proteins purified with the present modified method are suitable for proteomics analysis.

## Discussion

As a regular process of post-translational modification, the *S*-nitrosylation of proteins occurs biologically and pathologically in different tissues. Usually, SNO proteins are in low abundance in the context of whole proteins; thereby, the isolation and enrichment of SNO proteins from tissue extracts are required prior to teh further evaluation of SNO proteins. In this study, we describe a modified protocol for the isolation and enrichment of SNO proteins from brain tissues based on a commercial SNO protein detection assay kit. Using brain homogenates, we optimized the elution reagents and the working conditions for incubation. The incubation ratio, incubation time and temperature of biotin-labeled brain homogenates with streptavidin beads were also optimized in order to obtain the opitmal efficacy/cost ratio. The workflow of such an efficient protocol was set up for SNO protein isolation and enrichment from brain extracts (Fig. 7). Briefly, aliquots of 10% brain homogenates were subjected into a biotin-switch technique kit. After being labeled with biotin, the specimens were mixed with streptavidin beads at a ratio of 3:1 (v/v) and incubated at 25˚C for 120 min. The SNO proteins are finally eluted from beads with buffer containing 0.5% SDS.

We also observed that SNO proteins in brain extracts are not able to be thoroughly isolated by one round of purification. Instead, the signals of SNO proteins in the elution products are totally undetectable after 10 to 12 rounds of purification both in rodent and human brain homogenates. Both biotin-specific dot blots and protein assays revealed large amounts of SNO proteins in the elution products of the first 6 rounds of purification. Although the distributions of SNO proteins in various eluting fractions may differ based on the molecular weights, isoelectric points or other biochemical characteristics, the present data indicate that the complete recovery of SNO proteins in 10% brain homogenates requires at least 6 rounds of purification under our experimental conditions.

Our data also highlighted that the combined usage of protein-content determination and biotin-specific dot blot may precisely reflect the alterations in SNO proteins in eluting products. Although these 2 assays do not directly represent the contents of SNO proteins and the possibility of the presence of non-SNO proteins and unlabeled free biotin in eluting products cannot be completely excluded, a combination of those 2 assays in detecting the protein contents and biotin signals in eluting products strongly suggests the reliability and feasibility of the combined use of these simple assays in monitoring the recovery of SNO proteins.

Compared with the protocols described in previous studies (28,29) or suggested by the manufacturer of Dynabeads® M-280 (Life Technologies), the denaturing

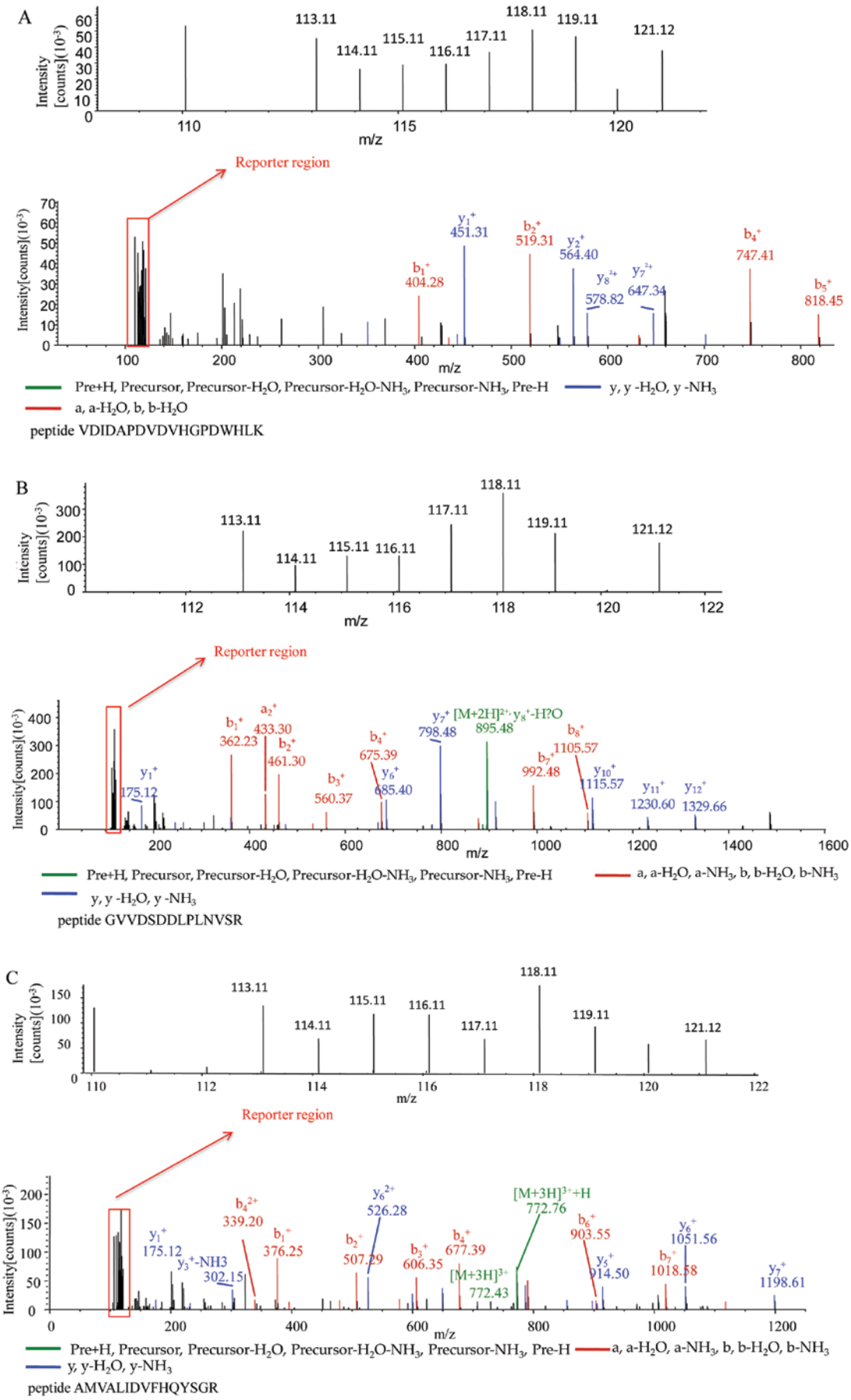


Figure 6. Mass spectrometry (MS)/MS spectra of 3 identified peptides representing (A) neuroblast differentiation-associated protein AHNAK isoform 1 (628.7 kDa), (B) heat shock protein gp96 precursor (90.1 kDa) and (C) S100-B (10.7 kDa). The spectra of reporter ions at 113, 114, 115, 116, 117, 118, 119 and 121 m/z are blown up on the top. The peptide sequences are shown at the bottom.

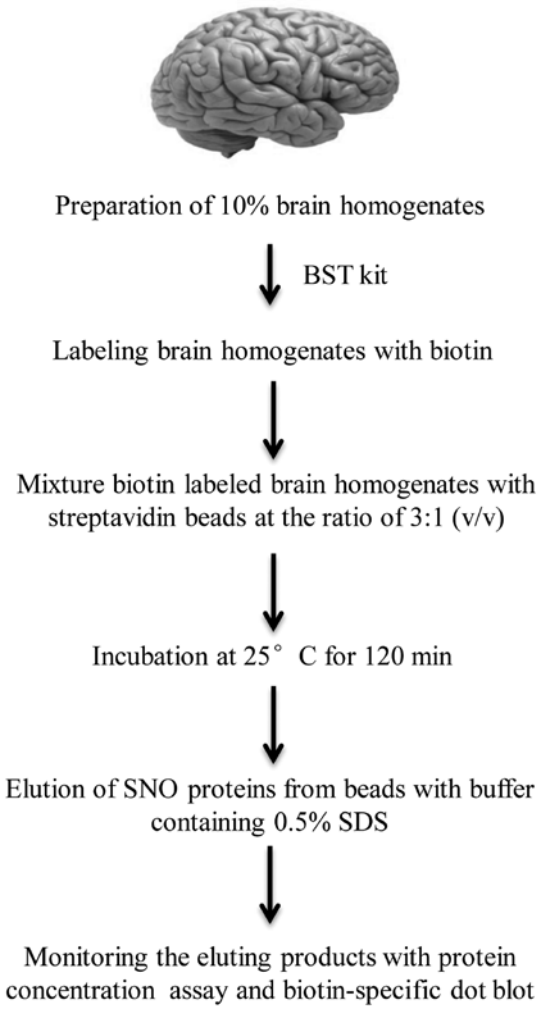


Figure 7. Summarized operation flowchart of the isolation of *S*-nitrosylated (SNO) proteins from brain tissues.

conditions with 0.5% SDS were effective on the release of biotin-labeled SNO proteins from streptavidin beads, which allowed us to obtain almost all biotin-labeled proteins from bound beads with one elution time. Further biotin-specific western blot analysis of the elution products verified a number of positive protein bands with various molecular weights. This suggests that protein free thiols covalently labeled with maleimide-biotin are not damaged under 0.5% SDS buffer. In fact, such a concentration of SDS (0.5%) is widely used for tissue and cell lysis, nucleotide acid and protein extraction, which usually does not affect further protein assays. Good immunoreactivities of the final elution products from human brain homogenates in western blot analysis with several specific antibodies confirm that the purified SNO proteins from brain tissues obtained by the optimized method in this study are suitable for further experiments.

iTRAQ-based proteomics consists of a series of complex processes, such as iTRAQ labeling, trypsin digestion, multiple peptide purifications and peptide identification. The data of iTRAQ-based proteomics demonstrate that the SNO proteins purified from various human brain homogenates with our optimized protocol can directly undergo the aforementioned processes for proteomics analysis. It should be noted that the identified peptides with high confidence by the iTRAQ-based proteomics cover large amounts of protein with a wide range of molecular weights. This highlights a broad applicability on the *S*-nitrosoproteome for a wide array of biological samples, including those derived from clinical materials.

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