Chapter 22

Thioredoxin-Dependent Decomposition of Protein *S*-Nitrosothiols

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Abstract

The addition of nitric oxide to cysteine moieties of proteins results in the formation of *S*-nitrosothiols (SNO) that have emerged as important posttranslational signaling cues in a wide variety of eukaryotic processes. While formation of protein-SNO is largely nonenzymatic, the conserved family of Thioredoxin (TRX) enzymes are capable of selectively reducing protein-SNO. Consequently, TRX enzymes are thought to provide reversibility and specificity to protein-SNO signaling networks. Here, we describe an in vitro methodology based on enzymatic oxidoreductase and biotin-switch techniques, allowing for the detection of protein-SNO targets of TRX enzymes. We show that this methodology identifies both global and specific protein-SNO targets of TRX in plant cell extracts.

Key words Biotin switch technique, *S*-nitrosylation, Denitrosylation, Thioredoxin, Protein-SNO reductase, Nitric oxide

1 Introduction

Cysteine reactivity toward nitric oxide (NO) leads to the formation of *S*-nitrosothiols (SNO) in a process termed *S*-nitrosylation [1]. Protein *S*-nitrosylation has been shown to be a critical post-translational control mechanism that alters the function, activity, localization, and stability of signaling proteins in a wide variety of developmental and stress-related processes [1–5]. Although protein-SNO have been studied intensively in the past few years, the labile nature of the relatively weakly oxidized SNO group makes the detection and identification of protein-SNO experimentally challenging [6]. However, an effective methodology first proposed by Jaffrey and Snyder [7, 8] and optimized by others [9, 10], the biotin switch technique (BST), has become increasingly popular in studies on protein *S*-nitrosylation largely due to its compatibility with established downstream protein analysis procedures such as western blotting, silver staining and mass spectrometry. The basic concept of the BST involves selectively reducing the -SNO group and labeling the resulting free thiol (–SH)

Alexander Mengel and Christian Lindermayr (eds.), *Nitric Oxide: Methods and Protocols*, Methods in Molecular Biology, vol. 1747,
https://doi.org/10.1007/978-1-4939-7695-9_22, © Springer Science+Business Media, LLC, part of Springer Nature 2018

with a biotin tag that can readily be purified or detected by a variety of methods [7]. The process can be broken down into three main steps, the first of which requires a methylthiolating or alkylating agent to “block” free thiols under denaturing conditions that expose poorly accessible and buried cysteine residues in proteins. Subsequently, the covalent S-NO bond is reduced by ascorbate—an antioxidant that exhibits concentration-dependent specific reactivity toward SNOs. Finally, the free thiol groups resulting from ascorbate-mediated reduction are labeled with sulfhydryl-targeting pyridyldithiol-biotin (biotin-HPDP) [7, 9]. From here, there are several options for protein analysis, including immediate global detection of biotinylated proteins by immunoblotting against anti-biotin as well as specific detection by first purifying biotin-labeled proteins using a streptavidin-linked solid support prior to immunoblotting with protein-specific antibodies or mass spectrometry. Indeed, since its inception, the BST strategy has been adapted by many groups to improve its suitability for protein analysis by mass spectrometry [11–14], and has enabled the identification of many protein-SNO.

In recent years, it has been demonstrated that in addition to their well-established role in disulfide reduction [15], Thioredoxin (TRX) enzymes are potent protein-SNO reductases that are major contributors to the signaling roles of protein-SNO [16, 17]. TRX-mediated decomposition of protein-SNO has been shown to be involved in a wide variety of developmental processes and pathologies [18]. Moreover, it was shown in plants that at least one TRX enzyme exhibited selectivity toward specific protein-SNO substrates [16]. As TRX enzymes are typically part of large gene families, especially in higher plants [19], these findings suggests that each TRX family member may target distinct subsets of protein-SNO. To dissect different TRX-mediated denitrosylation pathways, it is therefore important to identify and characterize the protein-SNO substrate ranges of each TRX. To this end substrate trapping approaches have been designed that are based on the substrate reduction mechanism utilized by TRX. Mammalian TRX catalyzes the reduction of protein-SNO by utilizing two active site cysteine residues [17]. The more N-terminal active site cysteine is thought to displace NO from the target cysteine by heterolytic cleavage, resulting in formation of a mixed disulfide between TRX and its target substrate. Subsequently, the second active site cysteine of TRX intramolecularly attacks and resolves this mixed disulfide, thereby releasing the now reduced substrate and rendering the TRX active site oxidized. Mutation of the second site cysteine stabilizes the normally transient mixed disulfide intermediate and thus traps protein-SNO substrates onto TRX. This substrate trapping strategy has been successfully applied to identify protein-SNO targets of mammalian TRX [20, 21]. In plants, however, TRX enzymes are thought to denitrosylate protein-SNO via a distinct mechanism

that does not involve the formation of a mixed disulfide intermediate. The immune-inducible TRX*h*5 enzyme was found to selectively reduce protein-SNO via a trans-denitrosylation mechanism in which the NO group is transferred from the substrate onto the TRX*h*5 active site [16]. Consequently, mutation of the second active site cysteine of TRX*h*5 (i.e., C42S) can separate its disulfide reduction activity from its denitrosylation activity. Indeed, mutation of this cysteine blocked disulfide reduction activity of TRX*h*5 (Fig. 1a), but did not block its protein-SNO reductase activity (Fig. 1b). By contrast, mutation of both active site cysteines (i.e., C39/42S) resulted in loss of both reductase activities (Fig. 1). Because these data indicate that a substrate trapping approach based

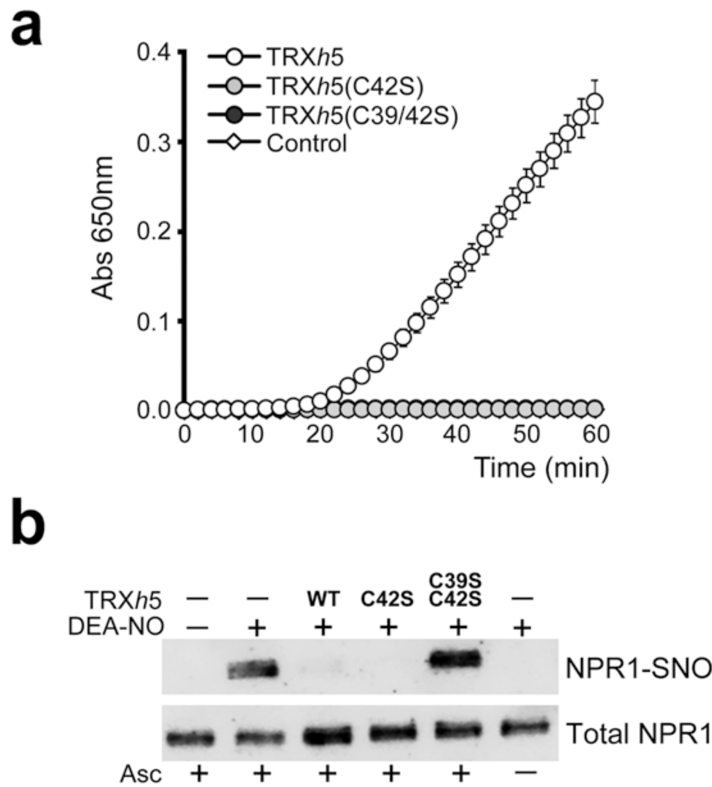

Fig. 1 Plant TRX*h*5 utilizes different mechanisms for disulfide reduction and protein-SNO decomposition. (**a**) Whilst TRX*h*5 can reduce insulin disulfides, both active site cysteine mutants are unable to do so. Insulin assay was performed as described by Holmgren [22] to measure the disulfide reduction activities of wild-type recombinant TRX*h*5 and the cysteine mutants TRX*h*5(C42S) and TRX*h*5(C39/42S). Reactions contained the complete TRX system consisting of TRX*h*5, NTRA and NADPH. Measurements at 650 nm were taken at 2 min intervals using an Infinite® 200 NanoQuant (TECAN). Samples were tested in triplicate. (**b**) TRX*h*5 denitrosylates protein-SNO by a monocysteinic mechanism. NPR1-FLAG protein treated with or without (lane 1) DEA-NO was subjected to the BST in the presence or absence (lane 6) of ascorbate. Wild-type recombinant TRX*h*5 or the cysteine mutants TRX*h*5(C42S) and TRX*h*5(C39/42S) were used in molar excess (40 μM) to denitrosylate *S*-nitrosylated NPR1-FLAG (NPR1-SNO). Total and *S*-nitrosylated NPR1-FLAG were detected by immunoblotting with an anti-FLAG antibody

on mixed disulfides will not suffice for plant TRX enzymes, we describe here a distinct in vitro strategy for visualization and identification of protein-SNO substrates of TRX enzymes. This protocol is applicable to both total plant cell extracts and recombinant proteins and detects protein-SNO targets of TRX as demonstrated by silver staining (Fig. 2) and immunoblotting (Fig. 1b).

This protocol utilizes a well-established procedure, the BST, in combination with in vitro TRX-mediated decomposition of protein-SNO to detect and identify targets. Provided they can be produced recombinantly, the protocol is adaptable to many different TRX superfamily members as well as recombinant protein targets. In addition, the ability to produce cysteine mutants of TRX allows a great opportunity for mechanistic studies into the SNO reductase activities of various TRX enzymes. Particularly advantageous to this methodology is its compatibility with downstream protein analysis techniques, as demonstrated here by silver staining (Fig. 2) and immunoblotting (Fig. 1b), providing the ability to easily detect and identify proteins-SNO targets of TRX. It is also compatible with downstream mass spectrometry to reveal the identity of unknown protein-SNO targets. Like most adaptations of the BST, this methodology does carry the disadvantage of being time-consuming and technically difficult. Care should be taken in optimizing each procedural step as appropriate and inclusion of negative controls is essential to

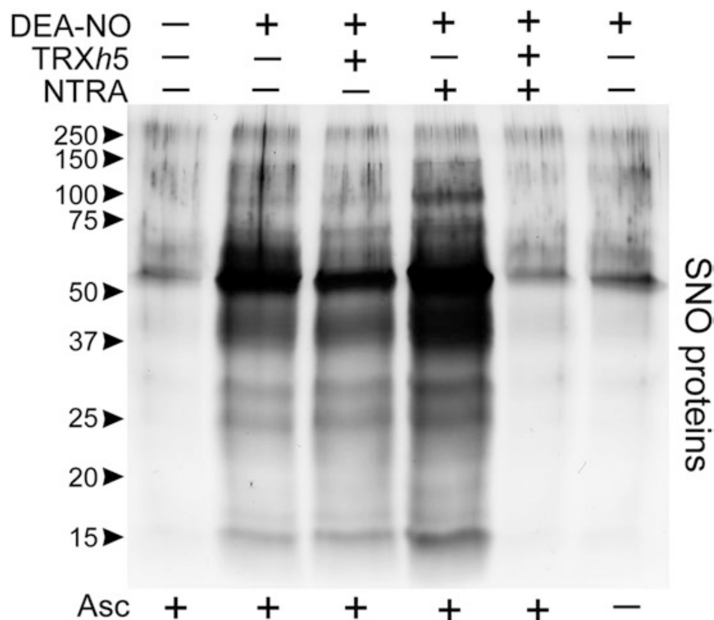

Fig. 2 Silver stain of protein-SNO targets of TRX*h*5 in total plant extracts. Total plant extracts were treated with or without DEA-NO (3 mM), proteins denitrosylated with the NADPH-dependent TRX*h*5/NTRA system, and purified with the BST in the presence or absence of ascorbate before visualizing by silver staining

correct interpretation of the results. Consequently, the number of methodological steps, negative controls and sample replicates that may be required to produce enough protein for silver staining or mass spectrometry makes this assay labor-intensive. A major consideration should be the potentially altered selectivity of TRX enzymes in vitro and the generation of false-positives (i.e., protein-SNO that can be reduced by TRX in vitro but are not physiological targets of this enzyme). For this reason, results obtained using this methodology should be used to provide guidance and support for further in vivo studies.

2 Materials

Prepare all solutions as stock solutions and store at room temperature unless otherwise stated. Solutions to be prepared fresh are indicated and were prepared in double distilled water unless indicated otherwise.

2.1 Expression and Purification of Recombinant TRX Protein

1. BL21(DE3) competent *E. coli* carrying a TRX protein expression plasmid. This protocol utilized the pET28a/His_6-TRX*h*5 plasmid that confers kanamycin resistance in bacteria. Store at −80 °C in 15% glycerol.
2. Sterile petri dishes.
3. Kanamycin: 50 mg/mL. Store at – 20 °C.
4. LB medium: Dissolve 10 g tryptone, 5 g yeast extract and 10 g NaCl in 950 mL water. Adjust pH to 7.0 with 5 N NaOH and make up volume to 1 L. Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.
5. LB agar selection plates: As LB medium but additionally add 15 g agar and autoclave. When media is still warm but not piping hot, add kanamycin to a final concentration of 50 μg/mL (from 50 mg/mL stock). Pour into petri dishes, allow to air-dry and store at 4 °C.
6. 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Sterilize by filtration through a 0.22 μm filter and store at 4 °C in the dark.
7. 200× Protease inhibitor cocktail: 50 mg/mL N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 50 mg/mL Nα-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), and 0.6 mM phenylmethanesulfonyl fluoride (PMSF) in methanol. Store at −20 °C.
8. Extraction buffer: 1× Bugbuster reagent (Novagen), 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, 10 mM imidazole. Store at 4 °C. Just prior to use add 10 mM 2-mercaptoethanol and 1× protease inhibitor cocktail (from 200× stock).

9. Benzonase nuclease (25 U/μL). Store at −20 °C.
10. Wash buffer: 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, 10 mM imidazole. Store at 4 °C. Prepare fresh by adding 1× protease inhibitor cocktail (from 200× stock).
11. HisPur Cobalt resin (Thermo Scientific) or equivalent. Store at 4 °C.
12. Elution buffer: 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, 500 mM imidazole. Store at 4 °C.
13. TRX storage buffer: 100 mM potassium phosphate (pH 7.0), 10% glycerol.
14. TRX dialysis buffer: 100 mM potassium phosphate (pH 7.0).
15. 500 mL sterile flasks.
16. 15 mL falcon tubes.
17. 37 °C incubator (with shaking).
18. 2 mL centrifuge columns (Pierce).
19. Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific): 3500 MWCO, 3 mL.
20. 5 mL syringe and 50 mm needle.
21. Bio-Rad Protein Assay Dye Reagent Concentrate. Store at 4 °C.
22. 10 mg/mL bovine serum albumin (BSA). Store at −20 °C.
23. Spectrophotometer.

2.2 S-Nitrosylation of Target Proteins

1. DEA-NO: 50 mM in 0.001 M NaOH. Store at −80 °C. Other NO donors may also be used.
2. Large (8.3 mL) Sephadex G-25 desalting columns (e.g., PD-10 columns).
3. 50 mL Collection tubes with column adaptors.

2.3 TRX-Mediated Denitrosylation of Protein-SNO

1. Recombinant proteins: epitope-tagged TRX (e.g., Arabidopsis His_6-TRX*h*5; *see* Subheadings 2.1, 3.1 and 3.2) and NADPH-dependent TRX reductase, (e.g., Arabidopsis His_6-NTRA). Store at −20 °C in TRX storage buffer.
2. Nicotinamide adenine dinucleotide phosphate (NADPH): 10 mM in 1× HEN (*see* Subheading 2.4). Store at −20 °C.
3. 1 M dithiothreitol (DTT): Store at −20 °C.
4. Small 0.5 mL Sephadex G-25 desalting columns (e.g., Zeba Spin Desalting columns, Thermo Scientific).

2.4 Biotin-Switch Technique

1. Plant cell extract: Plant tissue frozen in liquid nitrogen (~200 mg will produce the required 0.5–2 mg total protein per sample). Store at −80 °C.
2. Neocuproine: 100 mM in methanol. Store at −20 °C.

3. 10% Sodium dodecyl sulfate (SDS).
4. 4× HEN buffer: 1 M HEPES–NaOH (pH 7.7), 4 mM EDTA. Prepare fresh 1× buffer by diluting in water, adding 0.1 mM neocuproine (from 100 mM stock) and 1× protease inhibitor cocktail (from 200× stock).
5. 4× $H_{25}EN$ buffer: 100 mM HEPES–NaOH (pH 7.7), 4 mM EDTA. Prepare fresh 1× buffer by diluting in water, adding 0.1 mM neocuproine (from 100 mM stock) and 1× protease inhibitor cocktail (from 200× stock).
6. N-ethylmaleimide: 2.5 M in DMSO. Store at −20 °C.
7. Sodium ascorbate: 500 mM in 1× HEN buffer. Prepare fresh.
8. Biotin-HPDP: 4 mM in DMSO. Store at −20 °C
9. Neutralization buffer: 10 mM HEPES–NaOH (pH 7.7), 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100. Just before use add 1× protease inhibitor cocktail.
10. Wash buffer: 10 mM HEPES–NaOH (pH 7.7), 1 mM EDTA, 600 mM NaCl, 0.5% Triton X-100. Just before use add 1× protease inhibitor cocktail.
11. Elution buffer: 10 mM HEPES–NaOH (pH 7.7), 1 mM EDTA, 1% 2-mercaptoethanol, 1× protease inhibitor cocktail. Prepare this buffer immediately prior to use.
12. Streptavidin agarose. Store at 4 °C.
13. 100% acetone: Store at −20 °C.
14. 70% acetone: Store at −20 °C.
15. 4× Sample buffer: 8% SDS, 200 mM Tris–HCl (pH 6.8), 40% glycerol, 0.4% Bromophenol Blue.

2.5 SDS-PAGE

1. Precast polyacrylamide gel: 4–20%. Store at 4 °C.
2. Electrophoresis container and associated power pack.
3. Prestained Protein ladder (Precision Plus Protein™ Dual Color Standards, Bio-Rad).
4. 10× Electrophoresis buffer: 250 mM Tris, 1.92 M glycine, 1% SDS. Prepare fresh 1× buffer by dilution in water.

2.6 Silver Staining

1. Sensitizer solution: 0.02% sodium thiosulfate ($Na_2S_2O_3$). Prepare fresh.
2. Staining solution: 0.1% silver nitrate ($AgNO_3$). Prepare fresh and chill to 4 °C before use.
3. Developer solution: 2% Na_2CO_3, 0.04% formaldehyde. Prepare fresh.
4. Fixing solution: 50% methanol, 5% acetic acid.
5. 50% methanol.

6. Stop solution: 5% acetic acid.
7. Clean glass or plastic container.

2.7 Immunoblotting

1. Nitrocellulose membrane.
2. Whatman 3 mm chromatography paper.
3. Mini Trans-Blot® Cell (Bio-Rad) or equivalent.
4. Methanol.
5. 10× Transfer buffer: 250 mM Tris, 19.2 M glycine. To prepare 1× transfer buffer add 100 mL of 10× transfer buffer to 100 mL methanol and 800 mL water and store at 4 °C prior to use.
6. 10× Phosphate buffered saline (PBS): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2PO_4, 18 mM KH_2PO_4 [pH 7.4]. Prepare 1× solution in water.
7. Blocking buffer: 1× PBS containing 5% fat-free milk powder and 0.05% Tween-20. Prepare fresh.
8. Sealable plastic bag.
9. Appropriate primary antibody. Store at −20 °C.
10. Appropriate HRP-linked secondary antibody. Store at −20 °C.
11. HRP substrate for chemiluminescent detection (e.g., SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific)).

3 Methods

3.1 Expression of Recombinant TRX Protein

1. From a −80 °C glycerol stock spread BL21(DE3) cells carrying pET28a/His_6-TRX*h*5 (or equivalent) on LB agar selection plates. Grow overnight at 37 °C. This plate can be used as stock for up to 2 weeks by storing at 4 °C.
2. Pick a single colony and inoculate into 5 mL LB medium supplemented with 50 μg/mL kanamycin. Grow overnight at 37 °C in shaking incubator.
3. Add 3 mL of the preculture (from **step 2**) to 100 mL LB medium supplemented with 50 μg/mL kanamycin in a 500 mL sterile flask (*see* **Note 1**). Grow to OD_{600}: 0.6–0.8 (around 1.5 h) at 37 °C with shaking.
4. Add 1 mL sterile-filtered 100 mM IPTG (final concentration will be 1 mM) and continue growing at 37 °C with shaking for ~5 h.
5. Harvest cells by centrifugation in a Sorvall fixed-angle rotor centrifuge at 10,000 × *g* for 15 min.
6. Pour off liquid LB medium and remove excess liquid by blotting on paper. Place pellet at −20 °C for short storage or proceed directly to Subheading 3.2.

3.2 Purification of Recombinant TRX Protein

1. Resuspend bacterial pellet in 5 mL of Extraction buffer and add 1 μL/mL benzonase nuclease. Transfer into Eppendorf tubes.
2. Incubate cell suspensions on a rotating mixer at a slow setting for 20 min.
3. Remove insoluble cell debris by centrifugation in a microcentrifuge at 16,000 × *g* for 20 min at 4 °C.
4. Combine supernatants in a fresh 15 mL falcon tube and keep on ice.
5. Pack a column with 0.5 mL of HisPur cobalt resin or equivalent (*see* **Note 2**). Allow storage buffer to drain by gravity flow (*see* **Note 3**).
6. Equilibrate column with 2 resin-bed volumes (1 mL) of Extraction buffer.
7. Apply protein extract from **step 4** to the column and collect the flow-through in a 15 mL falcon tube (*see* **Note 4**). Reapply the flow-through to the column.
8. Wash column three times with 2 resin-bed volumes (1 mL) of Wash buffer.
9. When the Wash buffer is completely drained, immediately elute three times with 2 resin-bed volumes (1 mL) of Elution buffer (*see* **Note 5**). Collect each elution in a 1.5 mL Eppendorf and place eluates on ice.
10. To dialyze, first combine all protein-containing eluates into a single sample (*see* **Note 6**). Attach a needle to a 5 mL syringe and draw up ~1 mL of air, followed by the sample. Insert the needle into the top left hand corner of a dialysis cassette, until it is just inside the dialysis chamber. Being careful not to pierce the chamber, slowly expel the sample (*see* **Note 7**). Next, remove the air from the dialysis chamber by drawing up the air into the syringe. Attach the cassette to a float and incubate in 1 L of TRX dialysis buffer with stirring for 1 h. Exchange the dialysis buffer three times and after the third exchange, incubate at 4 °C overnight with stirring.
11. To remove the sample from the dialysis cassettes, first attach a needle to a 5 mL syringe and draw up ~1 mL of air. Insert the needle into the top right hand corner of the dialysis cassette until it just enters the edge of the dialysis chamber. Angle the cassette so that the needle is going vertically down. Gently expel the air into the chamber and then rotate the cassette 180°. Pull the syringe plunger downward slowly to take in the sample (*see* **Note 7**). Once the sample is completely drawn into the syringe, remove the needle from the cassette and expel the sample into a fresh 1.5 mL Eppendorf tube on ice.

12. Measure the protein concentration using a Bradford assay. Prepare a standard curve ranging from 0 to 1 mg/mL BSA in TRX dialysis buffer. Add 10 μL of standard or sample to 790 μL water and 200 μL Bio-Rad Protein Assay Dye Reagent Concentrate. Incubate for 5 min and then measure the A_{600}. It may be necessary to dilute the sample in TRX dialysis buffer if it falls outside the range of the standard curve.

3.3 Preparation of Reduced Recombinant TRX

1. Incubate 50 μL of 200 μM recombinant TRX protein with 0.33 mM DTT per sample for 30–60 min (*see* **Note 8**).
2. To desalt the TRX recombinant protein, first place a small Sephadex G-25 desalting column in an open eppendorf tube and centrifuge at 1500 × *g* for 1 min in a microcentrifuge. Discard the flow through and place column into a fresh Eppendorf tube.
3. Gently add TRX recombinant protein solution to the top of the desalting column, ensuring not to disturb the Sephadex G-25, and centrifuge at 1500 × *g* for 2 min in a microcentrifuge (*see* **Note 9**). This step will remove the DTT to prevent it from interfering with TRX-mediated decomposition of SNO groups.

3.4 S-Nitrosylation and TRX-Mediated Denitrosylation of Cellular Protein-SNO

1. Grind plant tissue to a very fine powder in liquid nitrogen (*see* **Note 10**). Use ~200 mg of plant tissue per sample.
2. Resuspend finely ground plant tissue in 1× H_{25}EN buffer. Adding 1.5 mL buffer for every 1 g of tissue will give a protein concentration of ~5 mg/mL (*see* **Note 11**). Transfer homogenate into precooled 1.5 mL reaction tubes.
3. Centrifuge samples for 15 min at 16,000 × *g* at 4 °C in a microcentrifuge (*see* **Note 12**), combine supernatants in a prechilled 15 mL reaction tube and keep on ice.
4. Equilibrate a large Sephadex G-25 column by adding 1× H_{25}EN buffer to the rim of the column and allowing the buffer to enter the resin while discarding the flow through. Refill and drain the column three more times. Finally, fill the column a fifth time with 1× H_{25}EN buffer (a total of ~25 mL of buffer should be used for an 8.3 mL Sephadex G-25 column), place in a 50 mL collection tube with column adaptor and centrifuge at 1000 × *g* for 2 min in a benchtop centrifuge with swing-out rotor.
5. Desalt the supernatant from **step 3** by adding 1.75–2.5 mL to the center of the Sephadex G-25 column. Place the column in a 50 mL collection tube with column adaptor and centrifuge at 1000 × *g* for 2 min in a benchtop centrifuge with swing-out rotor. Place the desalted supernatant on ice. This step removes a large proportion (>95%) of naturally occurring antioxidants found in plant cells, and prevents unwanted decomposition of SNO groups generated in the next step.

6. In the dark, add DEA-NO (+ NO samples) to a final concentration of 3 mM. For control samples (− NO samples) add an equivalent amount of 0.001 M NaOH (*see* **Notes 13–15**). Incubate the samples at room temperature in the dark for 20 min.
7. In the dark, desalt 125 μL extract per sample as in **steps 2** and **3** of Subheading 3.3, keeping (− NO) and (+ NO) samples separate.
8. To 100 μL DEA-NO or control-treated extract from **step 7**, add 10 μL of 10 mM NADPH, 0.2 μM NTRA, 5 μM TRX and 1× H_{25}EN to a final volume of 125 μL. Incubate the samples at room temperature in the dark for 45 min (*see* **Notes 16** and **17**).

3.5 Blocking Free Thiols

1. Add 250 μL of 2× HEN buffer supplemented with 5% SDS, and 20 mM N-ethylmaleimide. Subsequently adjust volume to 500 μL by adding 125 μL H_2O. Mix well and incubate at 50 °C in the dark for 20 min with continuous or frequent vortexing (*see* **Note 18**).
2. Add 2 volumes (1 mL) of ice-cold acetone (100%) and incubate in the dark at −20 °C for 20 min.
3. Centrifuge samples at 4 °C for 10 min at 6500 × *g* and wash pellet three times with 1 mL of ice-cold acetone (70%) (*see* **Note 19**).

3.6 Biotin Switch and Immunoprecipitation

1. Dissolve pellet completely by vigorous pipetting in 425 μL of 1× HEN buffer supplemented with 1% SDS. Add 50 μL of 4 mM Biotin-HPDP and 25 μL of either 500 mM sodium ascorbate or 1× HEN as a negative control (*see* **Note 20**). This step is extremely light sensitive so perform in darkness. Incubate at room temperature in complete darkness for 1 h with gentle rotation (*see* **Note 21**).
2. Add 2 volumes (1 mL) of ice-cold acetone (100%) and incubate in the dark at −20 °C for 20 min.
3. Centrifuge samples at 4 °C for 10 min at 6500 × *g* and wash pellet 3 times with ice-cold acetone (70%) (*see* **Note 19**).
4. Dissolve pellet completely by vigorous pipetting in 250 μL 1× HEN buffer supplemented with 1% SDS. Add 750 μL Neutralization buffer containing 10 μL packed streptavidin agarose (*see* **Note 22**). Incubate at 4 °C overnight with rotation. At this stage samples may be exposed to laboratory light again.
5. Wash beads three to five times with Wash buffer. Incubate every wash at room temperature for 5 min with rotation followed by centrifugation for 1 min at 2400 × *g* and 1 min resting on ice.

6. Add 40 μL Elution buffer. Incubate at room temperature for 30 min with frequent agitation (*see* **Note 23**).
7. Centrifuge for 1 min at 16,000 × *g* and let sit on ice for 1 min.
8. Take off supernatant and add 10 μL of 4× SDS-PAGE sample buffer.
9. Incubate at 70 °C for 10 min. Centrifuge at 16,000 × *g*.

3.7 SDS-PAGE Gel Electrophoresis

1. Place the 4–20% precast polyacrylamide gel in a vertical electrophoresis container and fill both the inner and outer chambers with 1× Electrophoresis buffer, ensuring all electrodes are submerged.
2. Load samples from **step 9** of Subheading 3.6 into each well of the gel and include one well with a prestained protein ladder (*see* **Note 24**).
3. Connect electrophoresis chamber to a power pack and run gel at 100–150 V until the dye front reaches the bottom of the gel.
4. Remove the gel from the electrophoresis container and gently separate the gel sandwich glass or plastic plates. Continue to silver staining (*see* Subheading 3.8) or immunoblotting (*see* Subheading 3.9).

3.8 Silver Staining (See Note 25)

1. Wash gel in water for 5 min. Replace water and repeat once (*see* **Note 25**).
2. Incubate gel in 100 mL Fixing solution for 15 min. Replace solution and repeat once. Fixing step can be extended overnight without disrupting staining performance.
3. Incubate gel in 100 mL 50% methanol for 5 min. Replace solution and incubate a further 5 min.
4. Wash gel in 100 mL water for 5 min. Replace water and incubate a further 5 min.
5. Incubate gel in 100 mL Sensitizer solution for 1 min.
6. Wash gel in 100 mL water for 1 min. Replace water and wash a further 1 min.
7. Incubate gel in 100 mL chilled Staining solution for 30 min. This step must be performed in the dark.
8. Wash gel with 100 mL water for 1 min. Replace water and wash a further 1 min.
9. Add 100 mL Developer solution and incubate until bands appear (2–3 min) (*see* **Note 26**).
10. When desired band intensity is achieved, immediately replace the Developer solution with Stop solution. Incubate for 1 min and then replace with fresh Stop solution and incubate for 10 min.

3.9 Electrophoretic Transfer and Immunoblotting

1. Cut a nitrocellulose membrane and 4 sheets of Whatman 3 mm chromatography paper to the size of the gel and soak in 1× Transfer buffer.
2. Rinse gel in 1× Transfer buffer.
3. Assemble transfer cassette with the cathode side down while keeping submerged in 1× Transfer buffer. First add a foam pad, then 2 sheets of Whatman 3 mm chromatography paper followed by the nitrocellulose membrane. Add your gel on top followed by two more sheets of Whatman 3 mm chromatography paper and a second foam pad. When closing the cassette, avoid bubbles (*see* **Note 27**).
4. Place assembled cassette into transfer chamber filled with 1× Transfer buffer and transfer at 25 V overnight at 4 °C. This step can also be performed for 1 h at 90–100 V for protein products of ~70 kDa or less.
5. Remove membrane from cassette carefully, place in a plastic container and wash for 5 min in 50 mL of 1× PBS (*see* **Note 28**).
6. Incubate membrane with 100 mL Blocking buffer for 1 h.
7. Incubate membrane in a sealed plastic bag with an appropriate concentration of primary antibody (1:5000 anti-Flag mouse antibody was used in Fig. 1b) diluted in 5 mL Blocking buffer for 1.5–3 h. This step can also be performed overnight at 4 °C.
8. Wash membrane three times for 10 min in 50 mL Blocking buffer.
9. Incubate membrane in a sealed plastic bag with an appropriate concentration of secondary antibody (1:1000 HRP-linked anti-mouse antibody was used in Fig. 1b) in 5 mL Blocking buffer for 1–2 h.
10. Wash membrane three times for 10 min in 50 mL Blocking buffer.
11. Wash membrane for 10 min in 50 mL 1× PBS.
12. Remove 1× PBS and cover membrane in 1 mL SuperSignal West Pico Chemiluminescent Substrate or equivalent. Incubate membrane for 1–5 min and then tip off substrate.
13. Use luminescent detection by light-sensitive film or imaging equipment such as LICOR Odyssey to view protein bands.

4 Notes

1. Do not use a smaller flask, as this will prevent aeration during bacterial growth.

2. HisPur cobalt resin is provided as 50% slurry. Therefore, use twice the amount needed for a packed volume (e.g., for 0.5 mL packed volume, pipette 1 mL into the column). Mix resin well before use and pipette with tips from which the ends are cut off.
3. After the storage buffer has drained out of the column, do not allow the resin to dry at any time.
4. If protein extracts appear cloudy, filter through a 0.22 μm filter before application to the column.
5. As soon as the Wash buffer has completely drained through the column, add the Elution buffer. Immediate application of the Elution buffer creates a sharp elution peak resulting in the highest concentration of sample possible. The most concentrated eluate will be in the first and second collection tube.
6. We recommend performing a quick Bradford assay directly after elution in which 10 μL of each sample is added to 790 μL of water and 200 μL of Bio-Rad Protein Assay Dye Reagent Concentrate and compared to 10 μL of Elution buffer in the same reaction mix. Those samples that turn blue contain protein and can be combined for dialysis.
7. It is essential that the dialysis chamber is not pierced. When inserting the needle, do it slowly and stop as soon as the tip of the needle enters the dialysis chamber at the corner. When drawing up or expelling liquid, be careful not to push the needle further into the chamber.
8. This step should be performed just prior to the use of TRX enzymes to ensure that upon addition to the protein-SNO containing sample, the TRX cysteines are in reduced state. It is only necessary to prereduce TRX when using a molar excess (40 μM) of the enzyme in absence of a NADPH-dependent TRX Reductase rather than using the fully constituted system (i.e., TRX, NTRA, and NADPH combined) (*see* also **Notes 16** and **17**). Note that only wild-type TRX enzymes but not active site mutants show denitrosylation activity using the fully constituted system.
9. Protein concentration may be slightly reduced during the desalting procedure, so it is advised to measure the concentration of recombinant TRX protein (e.g., Bradford or BCA assay) after desalting to ensure the use of the correct final concentration in subsequent steps (40 μM in a 125 μL sample).
10. Prechill one mortar and pestle with liquid nitrogen before grinding tissue to a fine powder. Transfer powder to a second mortar prechilled on ice and resuspend in buffer. Wear goggles during this step.
11. Use enough starting tissue to generate more protein than needed because each desalting step on Sephadex G-25 columns

will marginally reduce the volume and protein concentration of the extract. A minimum of 0.5 mg protein per sample (in 125 μL) is required at the beginning of the blocking procedure (*see* Subheading 3.5).

12. After this step protein concentration should be measured (e.g., Bradford or BCA assay). A single sample requires a minimum of ~0.5 mg protein when immunoblotting. For optimum results in a silver stain assay, ~1.5 mg protein should be used. To achieve this, it is necessary to duplicate or triplicate samples (e.g., results obtained in Fig. 2 utilized triplicates of each sample and were only combined into a 15 mL round-bottomed tube at **step 4** in Subheading 3.6). We do not recommend scaling up the suggested volumes as this reduces experimental efficiency and increases background.
13. For all steps to be performed in the dark, be aware of the indirect light from adjacent windows, which can affect experimental performance. Where possible shut window blinds and turn of all lights.
14. Alternate sources of NO that can be used include *S*-nitrosylated cysteine (Cys-NO) and *S*-nitrosoglutathione (GSNO). These may be used at a concentration of 1–3 mM.
15. This protocol can also be adapted for use with recombinant target protein at an experimentally determined appropriate concentration. When using recombinant proteins, **steps 1–5** of Subheading 3.4 can be eliminated.
16. When using the complete TRX system (i.e., TRX, NTRA, and NADPH combined), it is recommended to include controls that lack TRX and NTRA by replacing these with equivalent amounts of 1× H_{25}EN. Because NTRA and NADPH will recycle the redox activity of TRX, Subheading 3.3 can be skipped when using the fully constituted TRX system.
17. It is possible to perform denitrosylation assays in absence of NTRA and NADPH by using a molar excess of TRX (i.e., at least 2× compared to substrate), which enables the investigation of TRX cysteine mutants whose redox activity cannot be recycled by NTRA (Fig. 1). A good control to use here is an oxidoreductase dead mutant of TRX in which both active site cysteines are mutated (Fig. 1). When using a molar excess of TRX it is essential to prereduce TRX enzymes as described in Subheading 3.3.
18. Wrap each Eppendorf in tin foil to avoid any light from reaching the samples. Either vortex samples every 4 min or use a thermomixer to continuously shake samples.
19. Avoid exposure to day light and instead work under dimmed artificial light. Acetone pellets will be visible at the bottom of

the Eppendorf tube but are likely also spread along the wall of the tube. Therefore, when washing, tilt tube to ensure a complete wash. Add and remove wash solution gently and carefully so as not to lose any of the pellet. It may be necessary to centrifuge the samples for 1 min at 6500 × *g* to remove residual acetone after the final wash. Note that centrifugation at higher speeds is not recommended as this yields a dense pellet that is difficult to resuspend in subsequent steps.

20. Prepare sodium ascorbate fresh every time and use immediately. Inclusion of a negative control sample in which sodium ascorbate is omitted, is essential to determining background detection of false positives.
21. Do not put tubes on a rotator but instead place tubes on their sides on a rocking table to prevent SDS from foaming.
22. Mix streptavidin agarose well before use and pipette with tips from which the ends are cut off. Streptavidin agarose is usually provided as a 50% slurry, so for 10 μL packed volume use 20 μL per sample. Spin down streptavidin in an Eppendorf tube at 2400 × *g* for 1 min followed by resting on ice for 1 min. Remove storage buffer from the top of the agarose resin and wash in 500 μL Neutralization buffer. Centrifuge and rest on ice as above, remove Neutralization buffer and resuspend in fresh 750 μL Neutralization buffer. Prior to dispensing, pipette up and down slowly to mix streptavidin solution to avoid settling to the bottom.
23. Do not vortex samples during elution as the agarose will splash up and stick to the sides of the tube. Instead, gently flick the bottom of the tube every ~4 min to mix.
24. Use 2 μL and 6 μL protein marker for silver staining and immunoblotting respectively. For silver staining, leave a blank well between the protein marker and the first sample.
25. Every step of Subheading 3.8 requires gentle shaking. Wear clean gloves when handling all materials. Take special care to touch only the same corner of the gel to prevent staining artifacts. Clean plastic or glass container carefully before staining.
26. When developing, hold container over a blank white sheet of paper to see bands more clearly. If signals are weak the Developer solution can be replaced after 2–3 min to allow further incubation.
27. To avoid bubbles, keep all materials soaked in 1× Transfer buffer while assembling the gel cassette. It is possible to use a roller to gently push out bubbles that form whilst stacking the materials.
28. All washing, blocking and antibody incubation steps from this point on should be performed with gentle rocking.

Acknowledgments

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement No 678511), a Royal Society University Research Fellowship (UF090321), and a Wellcome Trust-University of Edinburgh Institutional Strategic Support Fund (ISSF).

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