Chapter 18

Thiol-Redox Proteomics to Study Reversible Protein Thiol Oxidations in Bacteria

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Abstract

Thiol-redox proteomics methods are rapidly developing tools in redox biology. These are applied to identify and quantify proteins with reversible thiol oxidations that are formed under normal growth and oxidative stress conditions inside cells. The proteins with reversible thiol oxidations are usually prepared by alkylation of reduced thiols, subsequent reduction of disulfide bonds followed by a second differential alkylation of newly released thiols. Here, we describe two methods for detection of protein *S*-thiolations in Gram-positive bacteria using the direct shotgun approach and the fluorescent-label thiol-redox proteomics method that have been successfully applied in our previous work.

Key words Fluorescent-based redox proteomics, Thiol trapping, Mass spectrometry, Reversible thiol oxidations, Protein *S*-thiolations

1 Introduction

Bacteria are exposed to reactive oxygen species (ROS) during aerobic respiration or under infection conditions by the oxidative burst encountered by activated neutrophils [1, 2]. ROS can damage all cellular macromolecules, but the most susceptible target is the thiol group of cysteine in proteins. The thiol group is subject to reversible oxidation by ROS to form intramolecular or intermolecular protein disulfides or mixed protein disulfides with low molecular weight (LMW) thiols (termed as *S*-thiolations). Highly reactive ROS, such as hydroxyl radicals are formed in the Fenton reaction and can lead to irreversible overoxidation of protein thiols to sulfinic or sulfonic acids leading to a loss of protein function [3] (Fig. 1).

Protein *S*-thiolations protect the thiol groups against overoxidation and function as thiol-redox switches to control protein activities. In eukaryotes and Gram-negative bacteria, the LMW

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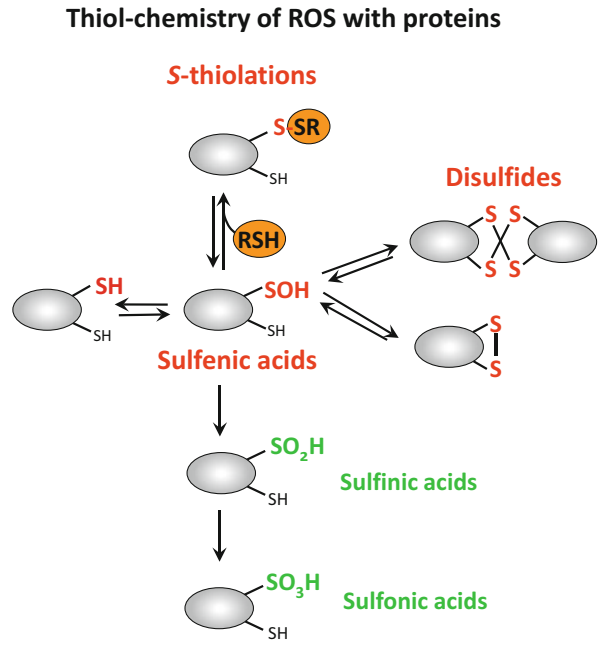

Fig. 1 Thiol chemistry of reactive oxygen species (ROS). Reversible thiol oxidation by ROS leads first to a Cys sulfenic acid intermediate (R-SOH) that is unstable and reacts further to form intramolecular and intermolecular disulfides or mixed disulfides with LMW thiols, such as glutathione, bacillithiol, cysteine, or CoASH, termed as *S*-thiolations. The Cys sulfenic acid can be also overoxidized to Cys sulfinic and sulfonic acids which are irreversible thiol oxidations

thiol glutathione (GSH) is used for protein *S*-glutathionylation. Protein *S*-glutathionylation controls numerous cellular functions and is involved in many physiological and pathophysiological processes [4]. However, most Gram-positive bacteria lack the ability to synthesize GSH and alternative redox buffers are used for protein *S*-thiolations. Actinomycetes utilize the alternative LMW thiol mycothiol (MSH, Acetyl-Cys-GlcN-myoinositol) and Firmicutes produce bacillithiol (BSH, Cys-GlcN-malate) [5]. Thus far, the knowledge about the targets and the physiological role of protein *S*-thiolations in bacteria is limited. Recent advances in redox proteomics and mass spectrometry have facilitated the detection of various forms of protein *S*-thiolations in bacteria, including *S*-cysteinylation, *S*-bacillithiolations, and *S*-mycothiolations [6–10]. Here, we provide two protocols that were applied in our lab to identify these different forms of protein *S*-thiolations in Gram-positive bacteria, such as *Staphylococcus carnosus, Bacillus subtilis*, and *Corynebacterium glutamicum*. We describe first the fluorescent-label based thiol-redox proteomics method that was developed to quantify and visualize reversibly oxidized protein thiols using the two-dimensional gel electrophoresis method (2D PAGE) [7, 9]. In this approach, all reversibly oxidized proteins are reduced and labeled with the fluorescent dye BODIPY FL C_1-IA

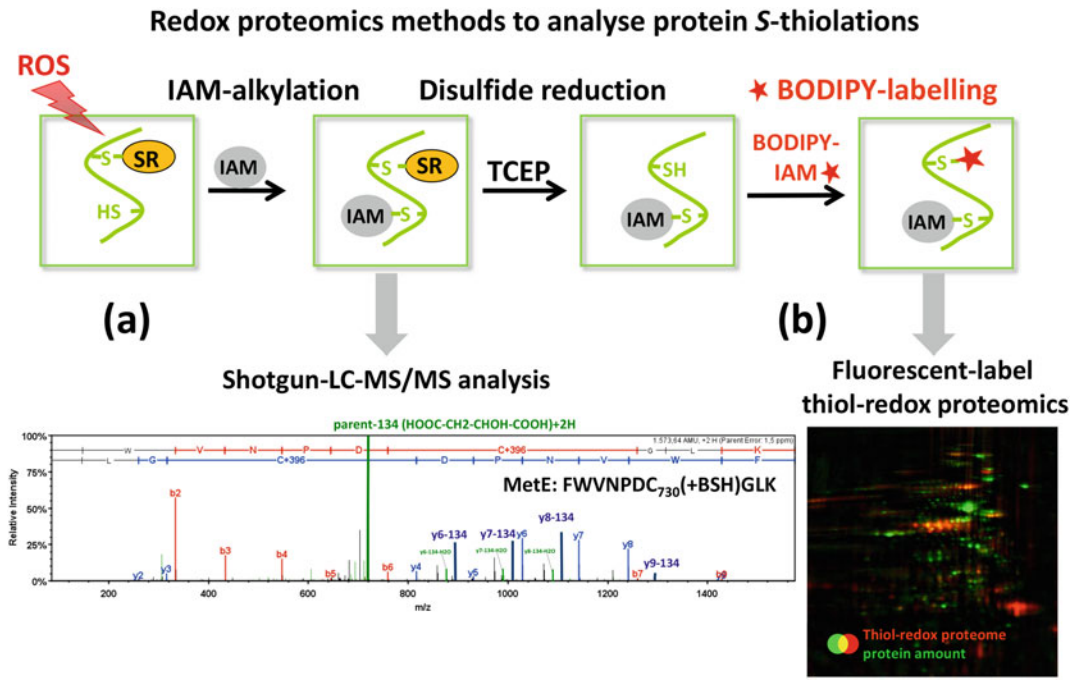

Fig. 2 Workflow for preparation of proteins for shotgun LC-MS/MS analyses of protein *S*-thiolations (**a**) and fluorescent-label thiol-redox proteomics (**b**). (**a**) Bacterial cells are harvested and reduced thiols are alkylated with IAM and subjected to non-reducing SDS-PAGE and in-gel tryptic digestion. LC-MS/MS analysis of the tryptic peptides is performed using a LTQ-Orbitrap-Velos mass spectrometer. The MS/MS spectrum shows as example the *S*-bacillithiolated MetE-Cys730 peptide. Bacillithiol (BSH) is composed of cysteine, glucosamine and malate. The abundant neutral loss precursor ion is characteristic for the loss of malate (−134 Da) and serves as indicator for the *S*-bacillithiolated peptide since malate of BSH is lost during fragmentation. (**b**) For the thiol redox proteome analysis, reduced protein thiols are alkylated with IAM and reversibly oxidized proteins (-S-SR) are reduced with TCEP and labeled with the fluorescent dye BODIPY FL C_1-IA. Proteins are separated using 2D PAGE, scanned for BODIPY fluorescence and stained with Colloidal Coomassie blue

(*N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-l) methyl)iodoacetamide). The BODIPY-labeled protein extracts are separated by 2D-PAGE and scanned for BODIPY-fluorescence. The gels are subsequently stained for total protein amounts by Coomassie blue or a compatible fluorescent protein dye. Using the Decodon Delta 2D software an overlay image is generated of the fluorescent-labeled thiol-redox proteome and the Coomassie stained protein amount image. Finally, the level of oxidation is quantified in comparison to the protein amount and the oxidized proteins can be identified using MALDI-TOF mass spectrometry (Figs. 2b and 3).

The direct identification of the different forms of protein *S*-thiolations is based on a bottom-up proteomics approach using shotgun LC-MS/MS analysis of alkylated proteins samples under non-reducing conditions [6, 7, 10]. The reduced thiols in the protein extracts are blocked using iodoacetamide (IAM) or N-ethylmaleimide (NEM) during cell disruption and the extracts

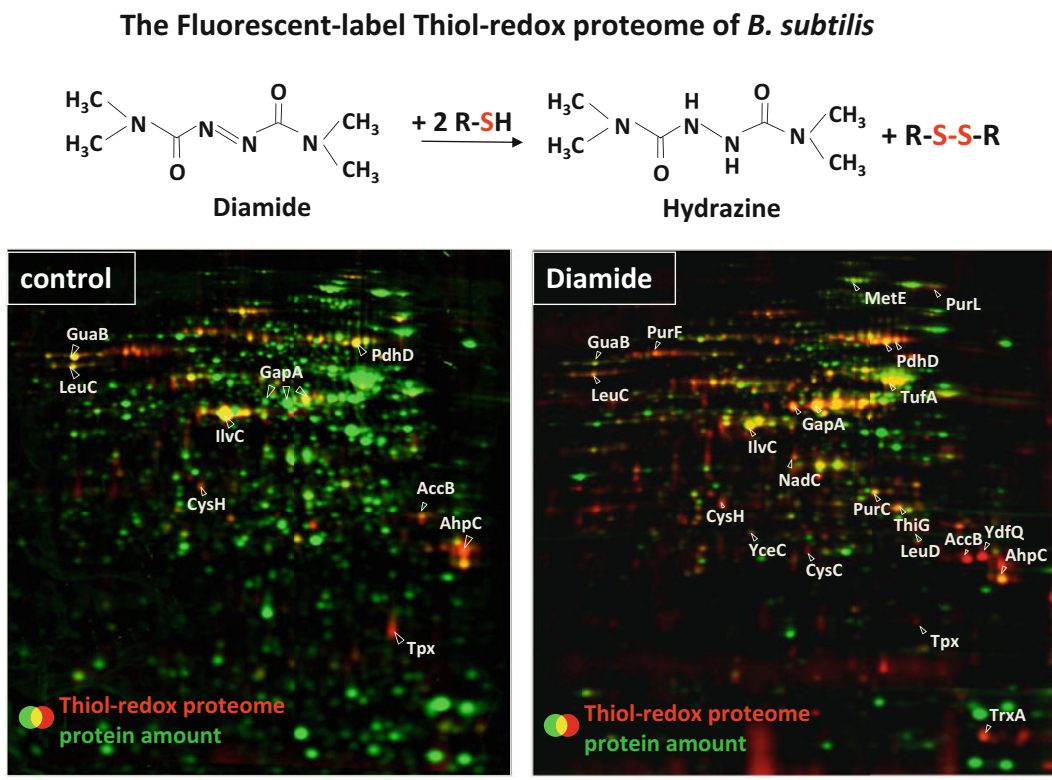

Fig. 3 Fluorescence-label thiol redox proteomics to visualize reversibly oxidized proteins in *B. subtilis* before (control) and after diamide stress. Diamide is a thiol-reactive azo compound that causes disulfide stress in cells as shown by the chemistry above. For the thiol redox proteome analysis, cells were harvested at control and diamide stress conditions and prepared as described in Fig. 2b. The overlay image shows the BODIPY fluorescence image (redox proteome, red image) compared with the Coomassie-stained protein amount image (green image). Quantitative image analysis is performed using the DECODON Delta 2D software (http://www.decodon.com) to calculate fluorescence (redox) ratios versus protein amounts. Proteins which appear in red in the overlay images are highly oxidized proteins with low protein levels, yellow spots are abundant oxidized proteins and green proteins are reduced proteins. The strongly increased reversible protein thiol oxidation after diamide stress is visualized which includes also protein *S*-thiolations

are directly applied for tryptic in-gel digestion and mass spectrometry to identify for example *S*-bacillithiolated or *S*-mycothiolated peptides (Fig. 2a). The 2D gel-based method and gel-free shotgun approach use similar protocols for thiol-trapping by IAM or NEM during sample preparation leading to a significant overlap between the identified reversibly oxidized proteins with those identified with *S*-thiolations [7–9]. However, even in combination both methods cover only a small fraction of protein *S*-thiolations and have several limitations. For example, false-positive redox-regulated proteins may be identified using the 2D gel-based approach and *S*-thiolated proteins cannot be enriched using both methods. Hence, these 2D gel-based method and shotgun-approach should be combined with more sensitive mass spectrometry-based methods of redox

proteomics, such as OxICAT or the NEM-Biotin-switch assay for comprehensive quantification of protein *S*-thiolations [11–14]. These mass spectrometry-based thiol-trapping assays could be even further developed to use purified bacilliredoxins or mycoredoxins for selective reduction of *S*-bacillithiolations and *S*-mycothiolations, respectively. This would facilitate the enrichment and more comprehensive identification and quantification of the various protein *S*-thiolation forms in bacteria. Similarly, the glutaredoxin-coupled NEM-Biotin-switch assay has been successfully applied for global identification of *S*-glutathionylations in different eukaryotic cells [11, 13]. Further advances in mass spectrometry and the design of novel chemical probes will provide leads for a more comprehensive description of the dynamics of the complete *S*-thiolome in various bacterial cells.

2 Materials

Centrifugation of protein samples in Eppendorf tubes is performed using a microfuge. For concentration of protein samples, a vacuum concentrator (SpeedVac) is used. Cells are disrupted using a Precellys-homogenizer (Peqlab). The rehydration chamber is required for rehydration of the IPG-strips. The Multiphor electrophoresis instrument including a power supply (e.g., EPS3500-XL from Pharmacia) is used for the IEF run. The 2D electrophoresis unit and a power supply are used for the subsequent second dimension (SDS-PAGE). In addition, casting trays must be provided for casting the 1D and 2D gels. Scanning of the 2D redox gels is performed using the Typhoon Scanner.

2.1 Components for Fluorescent-Label Thiol-Redox Proteomics

Prepare solutions for thiol-trapping, reduction and BODIPY-IAM-fluorescent-labeling of thiols using double distilled water.

2.1.1 Components for Preparation of Fluorescent-Labeled Protein Samples

1. TE-IAM buffer (for washing bacterial cell pellets): 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mM iodoacetamide (IAM).
2. UCE buffer (for protein denaturation): 8 M urea, 1% (w/v) CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), 1 mM EDTA, 200 mM Tris–HCl, pH 8.0. Store in Falcon tubes at −20 °C.
3. UCE-IAM buffer (for thiol-blocking): 8 M urea, 1% (w/v) CHAPS, 1 mM EDTA, 200 mM Tris–HCl, pH 8.0, 100 mM IAM. Store in Falcon tubes at −20 °C.
4. 80% acetone (v/v) in Aqua dest (for removal of excess IAM after thiol-blocking).

2.1.2 Components for 2D-PAGE and Staining Used in Fluorescent-Label Thiol-Redox Proteomics

5. Rehydration solution (RH) for IPG strips: 8 M urea, 2 M thiourea, 0.5% (v/v) CHAPS, 20 mM DTT, 0.5% (v/v) Pharmalytes pH 3–10. Add fresh DTT, CHAPS and Pharmalytes to 20 mL urea/thiourea solution before usage for isoelectric focusing (IEF).
6. SERVA IPG Blue Strips pH 4–7 NL/18 cm (SERVA Electrophoresis GmbH).
7. Electrode wicks.
8. Mineral oil for IEF.
9. TCEP stock solution: 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) should be prepared in Aqua dest.
10. 15 mM BODIPY-IAM (Invitrogen D-6003) stock solution: Dissolve one flask of BODIPY FL C1-IA in 800 µL 100% acetonitrile and store as 80 µL aliquots at −20 °C in dark tubes (*see* **Note 1**).
11. Micro Bio-Spin six Chromatography Columns (e.g., Bio-Rad 732-6221) (for removal of excess BODIPY-IAM from the protein samples).
12. Separating gel buffer: 1.5 M Tris–HCl, pH 8.8. Dissolve 181.71 g Tris base in 800 mL distilled water, adjust with HCl to pH 8.8 and fill up to 1 L.
13. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8. Dissolve 60.57 g Tris base in 800 mL distilled water, adjust with HCl to pH 6.8 and fill up to 1 L.
14. Sodium dodecyl sulfate (SDS): 10% (w/v) SDS in distilled water.
15. Ammonium persulfate (APS): 10% (w/v) Ammonium persulfate in distilled water. Store at 4 °C.
16. N, N, N, N-tetramethyl-ethylenediamine (TEMED).
17. Acrylamide solution: 40% (w/v) acrylamide solution (e.g., SERVA).
18. Bisacrylamide solution: 2% (w/v) bisacrylamide solution (e.g., SERVA).
19. Separating gel solution (12.5%): acrylamide solution, bisacrylamide solution, separating gel buffer, SDS, APS, TEMED. Mix 266 mL distilled water with 304 mL 40% (w/v) acrylamide solution, 170 mL 2% (w/v) bisacrylamide solution, 250 mL separation gel buffer, and 10 mL 10% (w/v) SDS using a magnetic stirrer. Add 2.5 mL 10% (w/v) APS and 500 µL TEMED to initiate the polymerization process of the separating gel.
20. Stacking gel solution: acrylamide solution, bisacrylamide solution, stacking gel buffer, 10% (w/v) SDS. Mix 60 mL distilled

water, 9 mL 40% (w/v) acrylamide solution, 4.5 mL 2% (w/v) bisacrylamide solution, 25 mL stacking gel buffer, and 1 mL 10% (w/v) SDS using a magnetic stirrer.

21. SDS–Tris–Glycine running buffer (tenfold): Dissolve 30 g Tris base, 144 g glycine, and 10 g SDS in distilled water and fill up to 1 L. The tenfold running buffer is stored at RT and used as a dilution of 1:10 in distilled water.
22. Equilibration solution for IPG strips: To prepare 1 L of the equilibration solution, dissolve the following components in Aqua dest and fill up to 1 L: 100 mL 0.5 M Tris–HCl pH 6.8 (final conc. 50 mM), 360 g urea (final conc. 6 M), 300 mL 99% (v/v) glycerol (final conc. 30% (v/v)), 40 g SDS (final conc. 4% (w/v)). Divide the equilibration solution into two parts (A, B). Add 1.75 g DTT to 500 mL solution (A) corresponding to a final DTT concentration of 3.5 mg/mL and store as 50 mL aliquots in Falcon tubes at −20 °C. Add 22.5 g IAM and traces of bromophenol blue to 500 mL solution (B) corresponding to a final IAM concentration of 45 mg/mL and store as 50 mL aliquots in Falcon tubes at −20 °C.
23. Gel fixation solution: 40% (v/v) ethanol, 10% (v/v) acetic acid.
24. Colloidal Coomassie staining solution: Prepare 1 L Colloidal Coomassie staining solution by dissolving 100 g ammonium sulfate, 12 mL orthophosphoric acid 85%, and 20 mL of 5% (w/v) Coomassie Brilliant Blue stock in distilled water and fill up to 1 L. Add methanol directly before the staining of the gels.
25. Methanol, purity >99.95%.

2.2 Components for Mass Spectrometry-Based Identification of Protein S-Thiolations

2.2.1 Components for Preparation of Protein Samples

1. TE-IAM buffer (for washing bacterial cell pellets) is described in Subheading 2.1.1, **item 1**.
2. UCE-buffer (for denaturation of proteins) is described in Subheading 2.1.1, **item 2**.
3. Bradford reagent (e.g., Roti-Nanoquant, Fa Roth) for protein determination.

2.2.2 Components for SDS-PAGE

4. Separating and stacking gel buffer, SDS 10% (w/v), APS 10% (w/v) and TEMED are described in Subheading 2.1.2, **items 12–16**.
5. Nonreducing SDS-PAGE sample buffer: Stacking gel buffer, SDS, glycerol, Bromophenol Blue, Aqua dest. To 3 mL Aqua dest add 1.2 mL stacking gel buffer, 4.0 mL 10% (w/v) SDS, 1.0 mL glycerol, and a small amount (tip of the spatula) Bromophenol Blue and fill up to 10 mL with distilled water. This buffer is used as sample buffer to dissolve proteins with *S*-thiolations before non-reducing SDS-PAGE.

6. Acrylamide–bisacrylamide solution: 30:0.8% (w/v) acrylamide–bisacrylamide solution (e.g., Applichem).
7. 1D Separating gel solution: Separating gel buffer, acrylamide–-bisacrylamide solution, SDS, APS, TEMED, Aqua dest. For preparation of the separation gel solution mix 4.7 mL distilled water, 5 mL separating gel buffer, 10 mL acrylamide–bisacrylamide solution, and 0.2 mL 10% (w/v) SDS. Add 100 μL APS and 10 μL TEMED to initiate the polymerization process.
8. 1D Stacking gel solution: Stacking gel buffer, acrylamide–bisacrylamide solution, SDS, APS, TEMED, Aqua dest. For preparation of stacking gel solution mix 6.1 mL distilled water, 2.5 mL Stacking gel buffer, 1.3 mL 30:0.8% (w/v) acrylamide–bis, and 100 μL 10% (w/v) SDS. Add 50 μL 10% (w/v) APS and 5 μL TEMED to initiate the polymerization process.
9. SDS–Tris–Glycine running buffer: is described in Subheading 2.1.2, **item 21**.
10. Colloidal Coomassie staining solution: is described in Subheading 2.1.2, **item 24**.
11. Gel fixation solution: 40% (v/v) ethanol, 10% (v/v) acetic acid.
12. Digester solutions for in-gel tryptic digestion: Digester-A: 50 mM NH_4HCO_3, pH 8; 50% (v/v) acetonitrile. Digester-B: 75% (v/v) acetonitrile. Spotter solution: 0.5% (v/v) TFA; 50% (v/v) acetonitrile.
13. Trypsin solution: 20 μg trypsin (e.g., Promega) is dissolved in 1 mL distilled water.

3 Methods

Fluorescent-label thiol-redox proteomics to visualize reversibly oxidized proteins in bacteria in response to oxidative stress.

3.1 Fluorescent-Label Thiol-Redox Proteomics: Growth, Harvesting of Cells, and Blocking of Reduced Thiols by IAM

Fluorescent-label thiol-redox proteomics is carried out to visualize reversibly oxidized proteins in bacteria in response to oxidative stress.

1. Inoculate an overnight culture of bacterial cells into two flasks each containing 100 mL defined minimal medium to an optical density at 500 nm (OD_{500}) of 0.05.
2. Grow bacterial cells until the mid-exponential phase (e.g., OD_{500} of 0.4). Flask 1 is used as control and flask 2 is exposed to sublethal NaOCl concentration which allows the cells to recover from growth arrest. The sublethal NaOCl concentration has to be determined for the specific bacterium before the stress experiment.

3. Harvest bacterial cultures in flask 1 before the stress (control) and that in flask 2 after 30 min of NaOCl stress exposure in 2 × 50 mL Falcon-like tubes on ice by rapid centrifugation (ca 9500 × *g*, 5 min, 4 °C).
4. Wash cell pellets immediately in 1–2 mL TE-IAM buffer to remove the oxidant, transfer the cells to 2 mL Eppendorf-like tubes, repeat centrifugation using a microfuge (19,400 × *g*, 5 min, 4 °C).
5. Resuspend washed cell pellet on ice immediately in 400 µL UCE buffer with 100 mM IAM. Do not vortex to avoid air bubbles and resuspend the cell pellet slowly using a pipette tip (*see* **Note 2**).
6. Break cells in UCE-IAM buffer immediately using a homogenizer in the presence of glass beads and remove glass beads by short centrifugation in a microfuge (19,400 × *g*, 5 min, 4 °C). Thiol alkylation is performed for 15 min in the dark (*see* **Note 2**).
7. Add four parts pure acetone to one part of protein extract in 2 mL Eppendorf-like tubes (e.g., add 0.4 mL protein extract to 1.6 mL acetone), precipitate proteins for 1 h on ice and centrifuge protein pellet in a microfuge (19,400 × *g*, 20 min, room temperature (RT)).
8. Wash protein pellet with 80% (v/v) acetone by mixing up the pellet mechanically with a pipette tip to ensure the proteins are suspended and any trace of IAM is removed. Remove supernatant after centrifugation using a microfuge (19,400 × *g*, 20 min, 20 °C) and repeat washing and centrifugation with 80% (v/v) acetone four times. Dry the washed protein pellet in a vacuum centrifuge and store proteins frozen at −20 °C until reduction and BODIPY labeling **(***see* **Note 3).**

3.2 Fluorescent Labeling of Reversibly Oxidized Proteins by BODIPY-IAM and Isoelectric Focusing of the Labeled Protein Extracts

1. Dissolve dried protein pellet from a 50 mL cell culture in 150 µL UCE buffer (without IAM) by shaking for 30–60 min. Remove cell waste by centrifugation in a microfuge (20 min, 17,900 × *g*, RT), transfer the supernatant into a new Eppendorf tube and determine protein concentration using Bradford reagent according to manufacturer instructions.
2. Transfer 250 µg protein extract into a new Eppendorf tube and fill up to 50 µL with UCE buffer. Reduce protein disulfides with 1 mM TCEP for 30 min at RT in the dark (add 5 µL 10 mM TCEP to 50 µL protein extract). Perform fluorescence labeling of the protein extract by addition of 6 µL BODIPY-IAM stock solution and incubate for 15 min at RT in the dark (*see* **Note 4**).
3. Equilibrate spin columns with 500 µL RH solution, centrifuge twice (1 min, 1020 × *g*, RT) and discard the flow-through.

Add BODIPY-IAM-labeled protein extract to the spin columns, centrifuge (4 min, 1020 × *g*, RT) and collect the protein containing flow-through into a new Eppendorf-like tube. The excess BODIPY-IAM will be retained in the spin columns. Add 280 μL RH solution to the BODIPY-IAM-labeled proteins, centrifuge (15 min, 17,900 × *g*, RT) and use the supernatant for rehydration of IPG strips in the dark overnight (*see* **Note 5**).

4. For rehydration of IPG strips use SERVA IPG Blue Strips pH 4–7 NL/18 cm. Add 360 μL of RH solution containing the BODIPY-IAM-labeled proteins to the rehydration chamber. Place IPG strips into the rehydration solution within the chamber and remove air bubbles below the IPG strips. Cover the rehydration chamber with a parafilm to avoid drying out of IPG strips. Protect rehydration chamber from light. Reswelling of the IPG strips is performed overnight at RT (*see* **Note 6**).
5. Place the IPG strips in the isoelectric focusing chamber. On top at both ends of the IPG strips place electrode strips that are soaked with distilled water. Place the electrodes on top of these electrode strips and connect these with the cathode and anode. Overlay the IPG strips with mineral oil, start running the IEF using the power supply EPS3500-XL according to the IEF following parameters (*see* **Note 7**):

1.	Step: 150 V	1 mA	5 W	150 Vh	1 h
2.	Step: 300 V	1 mA	5 W	300 Vh	1 h
3.	Step: 600 V	1 mA	5 W	600 Vh	1 h
4.	Step: 1500 V	1 mA	5 W	1500 Vh	1 h
5.	Step: 3000 V	1 mA	5 W	57,000 Vh	19 h

6. Remove IPG strips from the chamber and soak the mineral oil from the surface of the strips by a filter paper. Store IPG strips at −20 °C until separation by SDS-PAGE.

3.3 Second Dimension of 2D SDS-PAGE of Fluorescent-Labeled Proteins

1. For 12.5% acrylamide–bisacrylamide 2D gels prepare the separating gel solution as described in Subheading 2.1.2, **item 19**. Immediately after initiating the polymerization process cast the separating gel solution into the funnel of the multicasting chamber. Remove bubbles by pressing the flexible tube which connects the funnel and the multicasting chamber. Open the valve to cast slowly the gel solution through the flexible tube into the multicasting chamber. Make sure to avoid air bubbles in the chamber. Cover the separating gels with butanol to ensure a homogeneous polymerization of the gel. Acrylamide polymerization is finished within 2 h.

2. Prepare 100 mL stacking gel solution as described in Subheading 2.1.2, **item 20**. Keep 30 mL of the stacking gel solution for embedding the IPG strips at 4 °C. Rinse the surface of the gels with distilled water to remove the butanol. Add 380 μL 10% (w/v) APS and 62.5 μL TEMED to the stacking gel solution to initiate the polymerization process and mix gently. Load stacking gel solution with APS and TEMED immediately on top of the separating gel using a pipette. Cover the stacking gel with butanol to ensure a homogeneous polymerization of the gel. Polymerization is finished within 1 h.
3. After stacking gel polymerization, remove the 2D gels from the multicasting chamber and insert it into the running gel chamber. Fill the running gel chamber with 1× SDS–Tris–glycine running buffer. Equilibrate each IPG strip with 4.5 mL equilibration solution (A) for 15 min. Remove solution (A) and equilibrate each IPG strip with 4.5 mL solution (B) for 15 min. Remove solution (B), and load the IPG strips onto the 2D gels. Mix 30 mL stacking gel solution (from **step 2** in this section) with 460 μL 10% (w/v) APS and 75 μL TEMED using a magnetic stirrer and cover IPG with this stacking gel solution. Finally, fill the upper gel chamber with 1× SDS–Tris–glycine running buffer. Perform the SDS-PAGE run according to the following parameters: 300 V and 300 mA; 10 min 20 W per gel and overnight 2 W per gel. Stop the run when the bromphenol blue front has reached the bottom of the gel. After the run is finished, remove the gels from the glass plates. Scan the gels to observe fluorescence images immediately using a Typhoon scanner according to the following parameters: Fluorescence setup: 520 nm, BP40, 488 nm; Filter: Blue FAM; Mode: platen.
4. Fix 2D-gels in gel fixation solution for at least 1 h. Mix 200 mL Colloidal Coomassie stock solution with 50 mL methanol and use this solution for staining of the gels overnight. Next day, rinse the gels with distilled water twice and scan the Coomassie image. 2D gel images are quantified using the Decodon Delta 2D software by calculating ratios of fluorescence/protein amounts (*see* **Note 8**) (Figs. 2b and 3).

3.4 Bacterial Cultivation, Harvesting of Cells, and Preparation of Protein Extract for Identification of Protein S*-Thiolation Using ESI-LC-MS/MS Analysis*

1. Inoculate three flasks each containing 100 mL minimal medium with overnight cultures to an optical density of OD_{500} of 0.07 and grow cells until the mid-exponential growth phase (OD_{500} of 0.5–1.0). Flask 1 is used as untreated control. Flask 2 is exposed to diamide, and flask 3 is treated with NaOCl at sublethal concentrations each for 30 min. These sublethal NaOCl and diamide concentrations should reduce the growth rate and have to be determined before in detailed growth analyses.

2. Harvest the three bacterial cultures from untreated cells (control) and cells exposed for 30 min to diamide and NaOCl stress in 50 mL Falcon tubes on ice by rapid centrifugation (9500 × *g*, 10 min, 4 °C).
3. Wash bacterial cell pellets in TE-IAM buffer, transfer to Eppendorf tubes, centrifuge again (17,900 × *g*, 5 min, 4 °C) and resuspend the cell pellets in 1 mL UCE-IAM buffer on ice.
4. Disrupt cells using the homogenizer on ice and centrifuge cell extracts twice for 30 min at 4 °C to remove the cell debris. Alkylate the proteins containing free thiol-groups by incubation of the protein extract for 15 min at RT in the dark.
5. Determine the protein concentration by using Bradford reagent according to manufacturer instructions. Concentrate aliquots of 200 μg protein extract in the vacuum centrifuge to 15 μL and dissolve proteins in 15 μL non-reducing SDS-sample buffer (without DTT). Continue with protein separation using SDS gels.

3.5 Protein Fractionation by Separation on 15% SDS-PAGE for Identification of Protein S-Thiolation Using ESI-LC-MS/MS Analysis

1. For preparation of two 15% non-reducing SDS-PAGE separating gels, prepare 1D separating gel solution freshly (Subheading 2.2.2, **item 7**) and immediately cast the separating gels between two glass plates, cover the gel surface with 1 mL butanol and allow polymerization for 30–45 min.
2. For preparation of 1D SDS-PAGE stacking gel, first remove butanol and rinse the gel surface with Aqua dest. Prepare 1 D stacking gel solution (Subheading 2.2.2, **item 8**) and cast the stacking gel solution on top of the separation gel. Place the comb into the stacking gels and allow polymerization for 40 min.
3. Place the gels into the running chamber and fill up with running buffer. For protein fractionation, load 2 wells each with 200 μg protein extract in non-reducing SDS-sample buffer for control, diamide, and NaOCl extract, respectively. Separate proteins in 15% non-reducing SDS-PAGE according to manufacturer's description. Fix the SDS gels in gel fixation solution for 1 h. Mix 200 mL of Colloidal Coomassie stock with 50 mL methanol and stain the gels overnight. Next day, rinse the gels with distilled water twice. At this point gel can be stored at 4 °C covered in for example transparent film to avoid drying till in-gel digestion. Each gel lane is cut into ten slices and used for in-gel tryptic digestion and subsequent LC-MS/MS-analysis for identification of protein *S*-thiolations (*see* **Note 9**).

3.6 In-Gel Tryptic Digestion and Peptide Preparation for Mass Spectrometry

1. Destain the gel pieces using Digester-A solution under vigorous agitation for 20 min at 37 °C. Discard the supernatant and repeat destaining several times until the gel pieces are completely destained.
2. Shrink the destained gel pieces in Digester-B solution under vigorous agitation for 30 min at RT. Discard the supernatant and dry the gel pieces in a vacuum centrifuge for 10–20 min.
3. For the tryptic in-gel digestion add 5–20 μL trypsin solution to the dried gel pieces until the gel is completely reswollen. Perform tryptic digestion overnight at 37 °C.
4. Extract tryptic peptides with 100 μL spotter solution under vigorous agitation for 60 min at RT. Concentrate the peptides in the vacuum centrifuge and store at −20 °C until LC-MS/MS analysis.

4 Notes

1. The BODIPY FL C1-IA stock solution can be stored as frozen aliquots for 6 month and should be discarded when precipitates appear in the stock solution.
2. Cell sampling, washing, and blocking of reduced thiols by IAM for the fluorescent-label Thiol-Redox proteomics method needs to be done at the same day until the acetone-precipitation step is completed and the IAM-alkylated protein extracts is dried (**step 8**). If the cell pellet is not quickly dissolved and broken in UCE-IAM buffer, artificial oxidation can occur since reduced thiols are not blocked by IAM. The acetone precipitated protein pellet can be stored for several days at −20 °C.
3. Washing of the IAM-alkylated protein extract in acetone must be done carefully by using a pipette tip to mechanically disturb the protein pellet. Traces of remaining IAM must be avoided since these prevent the BODIPY-IAM labeling.
4. For BODIPY-IAM labeling, we recommend to spin down the BODIPY-IAM stock solution for removal of precipitated fluorescent dye which affects the thiol labeling.
5. Equilibrate the Biorad spin columns directly before the removal of excess BODIPY-IAM dye from the samples.
6. Reswelling of the IPG strips, IEF and the SDS-PAGE should be performed in the dark. The IPG strips should be subjected to SDS-PAGE directly after the IEF run is finished to avoid loss of the fluorescence label.

7. The IEF running parameters are used according to the instructions as recommended for IPG BlueStrips from SERVA (http://www.scie-plas.com/documents/SCIEPLAS/distributors/445.pdf.)
8. The scanning of the fluorescence images is performed using a Typhoon scanner and requires to wet the surface of the scanner with distilled water. After the gel run, the 2D gel is removed from the glass plates and placed immediately to the scanner surface in the water by avoiding air bubbles under the gel. For gel scanning, the PMT voltage needs to be adjusted according to the fluorescence intensity of the spots. We further recommend for the fluorescence quantification to run all gels of one experiment (e.g., gels of controls and stress samples) at the same day in one 2D gel run.
9. For preparation of protein extracts for direct identifications of *S*-thiolations using Orbitrap LC-MS/MS analysis, the reduced thiols need to be IAM-alkylated directly after cell harvesting and alkylated proteins are subsequently used for fractionation by non-reducing SDS-PAGE. Protein sample processing at the same day avoids precipitation of IAM-alkylated proteins which often occurs after freezing the IAM-alkylated protein extracts. To maintain the thiol-modifications, it is required that cells need to be broken, alkylated, and processed by SDS-PAGE at the same day. However, it is possible to store cells frozen at −70 °C before preparation of protein extracts for SDS-PAGE and mass spectrometry.

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