CHAPTER FIFTEEN

Detection of protein persulfidation in plants by the dimedone switch method

Angeles Aroca[a], Ana Jurado-Flores[a], Milos R. Filipovic[b], Cecilia Gotor[a,*], and Luis C. Romero[a,*]

[a]Institute of Plant Biochemistry and Photosynthesis, CSIC-University of Seville, Seville, Spain
[b]Leibniz Institute for Analytical Sciences, ISAS e.V., Dortmund, Germany
*Corresponding authors: e-mail address: gotor@ibvf.csic.es; lromero@ibvf.csic.es

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Abstract

Hydrogen sulfide (H_2S) is a well-known signaling molecule in both animals and plants, endogenously produced by cells, and involved in a wide variety of biological functions. In plants, H_2S regulates a wide range of essential aspects of plant life, including plant responses to numerous stresses and physiological processes as important as abscisic acid (ABA)-dependent stomatal movement, photosynthesis, and autophagy. The best studied molecular mechanism responsible of sulfide signaling is protein persulfidation, a post-translational modification of cysteine residues, where a thiol group (P-SH) is transformed into a persulfide group (P-SSH). In this way, persulfidation has emerged as a new type of cellular redox mechanism that can regulate protein structure and function and interest in this modification has increased exponentially. However, the identification and the development of detection methods have been challenging. Nevertheless, on the basis of the chemical differences between the thiol and the persulfide groups, different methods have been implemented. In plants, different high-throughput proteomic

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analyzes have been performed using a tag-switch method where in the first step all thiols and persulfides are blocked and then in the second step persulfides are selectively labeled using a specific nucleophile. This chapter outlines a new method, previously described in mammals, that has been applied to detect persulfidation in plants and is based on the same chemical premise but consists of chemoselective persulfide labeling with dimedone-based probes. Here, we provide a detailed workflow of this method that includes procedures for the determination of the persulfidation level of a protein extract visualized and quantified by fluorescence on the gel on one side, and on the other, the labeling and purification of persulfidated proteins for identification by mass spectrometry.

1. Introduction

Hydrogen sulfide (H_2S) is a colorless and flammable gas with a characteristic scent of rotten eggs. It is naturally produced in nature found in petroleum, volcanic emissions, and natural gas. It has been always considered as a pollutant in the environment and a hazardous toxic gas to living organisms because it inhibits complex IV of the electron transport chain in mitochondria. Nevertheless, life on Earth emerged in an atmosphere rich in H_2S and, still, H_2S is necessary for some organisms such as sulfur-oxidizing bacteria, which use H_2S as an electron donor in anoxygenic photosynthesis to produce oxidized sulfur compounds (Johnston, Wolfe-Simon, Pearson, & Knoll, 2009). Moreover, it is known that H_2S is endogenously produced enzymatically by cells throughout the regna, from bacteria to plants and mammals (Aroca & Gotor, 2022). Therefore, all these data were the first premises to think about H_2S as a molecule with additional functions different from toxic.

In mammals, H_2S was found to act as a neuromodulator (Abe & Kimura, 1996), to induce hibernation states in mice (Blackstone, Morrison, & Roth, 2005), and a regulator of inflammation (Szabo, 2007) and aging (Zivanovic et al., 2019). In plants, it is now well known that H_2S regulates a wide range of vital processes. H_2S protects plants against several stresses such as drought, heat, oxidative, and metal stresses (Aroca, Gotor, & Romero, 2018; Shen et al., 2013; Wang, Shi, Li, & Zhang, 2010; Zhang et al., 2008) and regulates important physiological processes, including abscisic acid (ABA)-dependent stomatal movement (Garcia-Mata & Lamattina, 2010; Scuffi et al., 2014), photosynthesis (Chen et al., 2011), and autophagy (Alvarez et al., 2012; Gotor, Garcia, Crespo, & Romero, 2013; Laureano-Marín et al., 2016).

The production of H_2S in plants occurs mainly during the photosynthetic sulfate-assimilation pathway in chloroplast (Gotor et al., 2019). H_2S is also produced in other cell compartments through different enzymes involved in cysteine metabolism, such as L-cysteine desulfhydrase 1 (DES1), which is the main producer of endogenous cytosolic H_2S (Álvarez, Calo, Romero, García, & Gotor, 2010). Several publications in the last decade reveal the interplay of sulfide with plant hormones and other signaling molecules, such as hydrogen peroxide (H_2O_2) and nitric oxide (NO), and how this interaction can regulate differently several physiological processes (Aroca, Gotor, Bassham, & Romero, 2020; Lisjak et al., 2010; Liu, Hou, Liu, Liu, & Wang, 2011; Scuffi, Lamattina, & García-Mata, 2016; Xie et al., 2014; Zhang et al., 2010).

The molecular mechanisms by which sulfide regulates a huge variety of processes were unknown until the post-translational modification of protein thiols (persulfidation, formerly called *S*-sulfhydration) was identified in mice (Mustafa et al., 2009) and Arabidopsis (Aroca, Serna, Gotor, & Romero, 2015). Since then, multiple papers have identified this process in animals and in plants with numerous proteins described as targets for persulfidation (Aroca & Gotor, 2022).

Persulfidation is a post-translational modification of protein cysteine residues where a thiol group (P-SH) is modified to a persulfide group (P-SSH) (Fig. 1). Persulfidation has emerged as a new type of redox regulation of protein structure and function in diverse biological processes, in a similar way to how nitric oxide (NO) regulates cellular processes through S-nitrosylation (P-SNO) or hydrogen peroxide (H_2O_2) through S-sulfenylation (P-SOH).

Persulfidation affects the structure of proteins, the enzymatic activity, and the subcellular localization of proteins (Aroca, Schneider, Scheibe, Gotor, & Romero, 2017; Aroca et al., 2015; Kimura, 2015; Mustafa et al., 2009). In plants, the role of persulfidation was first demonstrated in the activity of several enzymes, glutamine synthetase (GS2), ascorbate peroxidase (APX1), cytosolic glyceraldehyde 3-phosphate dehydrogenase

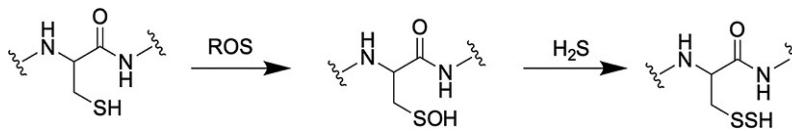

Fig. 1 Proposed mechanism of the reaction of H_2S with protein sulfenic acid to form protein persulfide.

(GapC1) (Aroca et al., 2015). Persulfidation was shown to activate APX1 and GapC1 in Arabidopsis protein extracts and recombinant proteins, while persulfidation inactivated GS2. Furthermore, other studies also confirm the regulation of different enzymes by persulfidation, demonstrate that H_2S-mediated persulfidation regulates the activities of catalase (CAT), APX, and peroxidase (POD) and enhances the antioxidant response of plants to oxidative stress (Li et al., 2020). Persulfidation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidases 1 and 2 also negatively regulates ethylene biosynthesis in tomato, which identifies another role for persulfidation in plants (Jia et al., 2018). Likewise, persulfidation affects the cytosolic/nuclear subcellular location of GapC1 (Aroca, Schneider, et al., 2017), which could alter its function from its metabolic role in the cytosol to its transcriptional role in mRNA regulation.

More recently, several proteins involved in the autophagy process have been described to be targets of persulfidation, such as autophagy-related protein 2 (ATG2), ATG3, ATG4, ATG5, ATG7, ATG11, ATG13, and ATG18a, as well as other 10 related proteins to autophagy, such as the serine/threonine kinase target of rapamycin (TOR), the regulatory-associated protein of TOR1 (RAPTOR1), the lethal with *sec* thirteen protein 8 (LST8), five subunits of protein phosphatase 2A (PP2A), the regulatory subunit of PP2A (TAP46) and the serine/threonine-protein kinase (VPS15) (Aroca, Benito, Gotor, & Romero, 2017; Jurado-Flores, Romero, & Gotor, 2021; Laureano-Marín et al., 2020). Currently, the role of persulfidation as the underlying mechanism to regulate autophagy has been demonstrated in ATG4 and ATG18a. In both cases, persulfidation of the ATG protein had a direct impact on autophagosome progression to negatively regulate the autophagy process (Aroca, Yruela, Gotor, & Bassham, 2021; Gotor, Aroca, & Romero, 2022; Laureano-Marín et al., 2020).

Various proteins involved in ABA signaling have been also revealed to be susceptible to persulfidation and the role of H_2S in guard cell ABA signaling was demonstrated through persulfidation of specific targets. In this way, several studies showed that ABA triggers the DES1 activity to induce the production of H_2S for persulfidation of DES1 itself, open stomata 1 (OST1), NADPH oxidase RBOHD and the transcription factor ABA insensitive 4 (ABI4), highlighting the complexity of the H_2S mechanism of action to regulate the ABA-dependent stomatal closure process (Chen et al., 2020, 2021; Shen et al., 2020; Zhou et al., 2021).

Furthermore, there is evidence of a crosstalk between reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species

(RSS) regulating biological processes. This provides a link between persulfidation, S-nitrosylation, and S-sulfenylation that allow plants to cope with stress stimuli. More than 600 proteins have been found to be modified by both persulfidation and S-nitrosylation (Aroca et al., 2018), and more than 1700 proteins were modified by persulfidation or S-sulfenylation, which makes 82% of the sulfenylated proteome described in Arabidopsis also undergo persulfidation (Aroca, Zhang, Xie, Romero, & Gotor, 2021). Therefore, numerous studies in plants have demonstrated that persulfidation is a ubiquitous modification of proteins that regulates a wide variety of biological processes and interacts with other redox modifications that might change the outcome of a certain protein, which explains the need for the scientific community to understand the molecular and cellular mechanisms of this modification.

During the last decade, the interest in persulfidation has increased exponentially, but researchers have coped with the intrinsically unstable chemistry of this modification and its similarity to both thiols and disulfides, which made its identification and the development of detection methods very challenging. Due to the asymmetry of the persulfide group, which contains an inner sulfane sulfur susceptible to nucleophilic attack (oxidation state: 0) and an outer sulfur susceptible to electrophilic attack (oxidation state: −1), and its anionic form at physiological pH 7.4 (P-SS-), persulfide groups have a more nucleophilic nature than the thiol groups (Cuevasanta et al., 2015; Filipovic, 2015; Filipovic, Zivanovic, Alvarez, & Banerjee, 2018). Based on these chemical differences, new methods have been described to increase the number of identifications of protein targets.

In plants, three high-throughput proteomic analyses using different labeling methods have been published to reveal more than 3400 proteins in leaves and more than 5200 in roots as modified by persulfidation (Aroca, Benito, et al., 2017; Aroca et al., 2015; Jurado-Flores et al., 2021). Most of these methods were based on chemical differences among the thiol and persulfide groups using a first step to block all thiols and persulfides followed by a second step to specifically label persulfide groups with a reactive nucleophilic compound. In addition to these, a new method has been described in the search for higher specificity and easier detection of persulfidation. Here we report a dimedone switch method, previously described in mammals (Zivanovic et al., 2019), that we now use to detect persulfidation in plants. This method is a chemoselective persulfide labeling approach using dimedone-based probes, which allow visualization of the persulfidation level in the gel or the labeling and purification of persulfidated proteins to be further analyzed by other

techniques, such as mass spectrometry. We describe a set of protocols to determine: (a) the level of persulfidation within a protein extract which can be visualized and quantified by fluorescence in the gel (Fig. 2) and (b) to label and purify the persulfidated proteins from a protein extract and identify them by mass spectrometry. The procedures detailed here have been optimized for protein extracts from *Arabidopsis thaliana*, although they can be used for any other species.

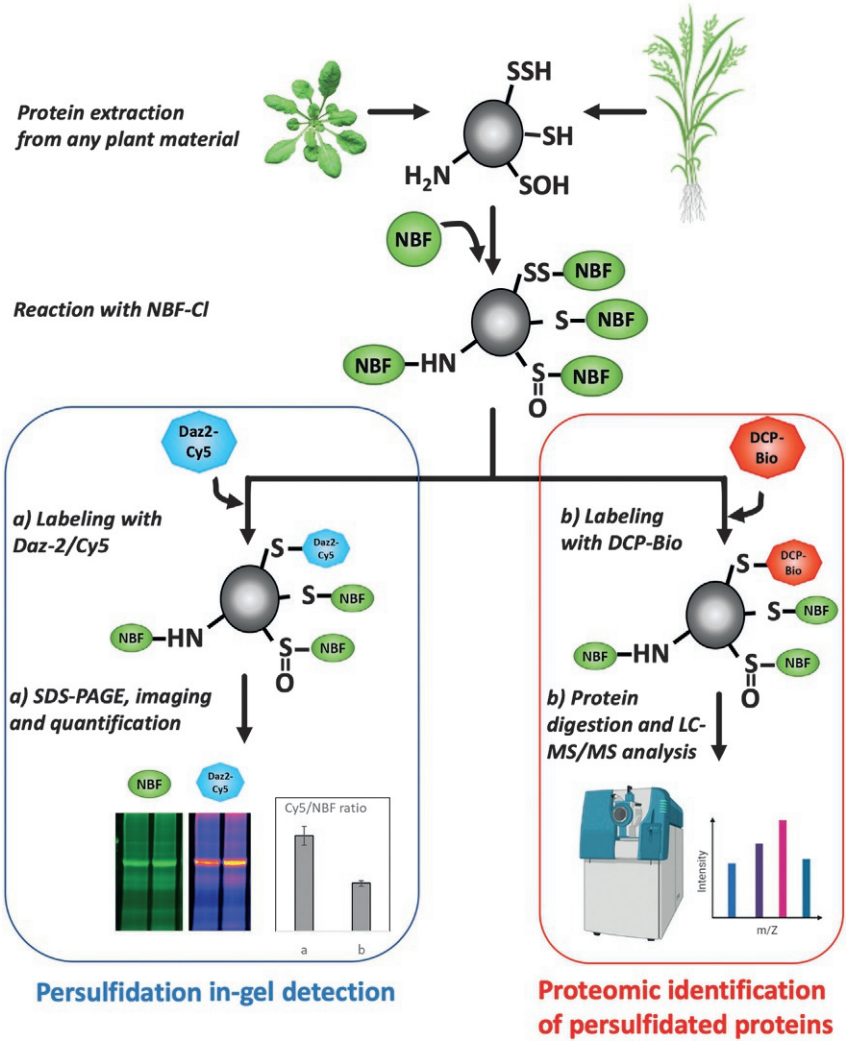

Fig. 2 Graphical scheme of persulfidation in-gel detection protocol, and purification and identification of persulfidated proteins by mass spectrometry.

2. In-gel detection of protein persulfidation of plant proteins

Here we describe the assay to detect the protein persulfidation pattern in a particular plant tissue or under certain conditions, so that the persulfidation levels in different samples can be compared in a semiquantitative way. The dimedone switch detection method consists of two main steps that are performed in protein extracts (Zivanovic et al., 2019). In the first step, all cysteines and amino groups in proteins react with 4-chloro-7-nitrobenzofurazan (NBF-Cl) to yield a variety of adducts with a characteristic green fluorescence signal that could be used to measure the total protein load. In the second step, only the persulfidated cysteine adducts are selectively labeled with a mixture of dimedone azide-2 (Daz2) and Cy5-alkyne, which contains a Cy5-fluorescence moiety for generating a red fluorescence signal. The total labeled protein extracts are separated on SDS-PAGE gels and imaged at 640 nm for the Cy5 signal and 488 nm for the NBF-Cl signal with the fluorescence intensity signal ratio of Cy5/NBF-Cl constituting the total protein persulfidation level (Fig. 2).

2.1 Reaction with NBF-Cl

Materials and equipment

- Liquid nitrogen
- PBS 1×, pH 7.4, Gibco™
- EDTA-Na_2-$2H_2O$ Ultrapure, Invitrogen™. Stock solution 0.5 M dissolved in distilled water
- SDS BioChemica, AppliChem. 10% stock solution
- Pierce™ Protease Inhibitor Tablets, EDTA-Free, Thermo Scientific. Stock solution 25× in distilled water
- 4-Chloro-7-nitrobenzofurazan (NBF-Cl), SigmaAldrich. Stock solution 500 mM in DMSO
- Methanol 99.9%, Fisher Chemical
- Chloroform, Merck
- DC (detergent compatible) Protein Assay, Bio-Rad
- Mortar and pestle
- Eppendorf ThermoMixer™ C
- Eppendorf™ Centrifuge 5424 R
- Vortex VWR® International
- Flowtronic Fume hood
- Bioruptor® Pico minichiller 300, Diagedone SA

Procedure

1. Ground plant tissues (100 mg) in liquid nitrogen using mortar and pestle until a fine powder and resuspend it in 200 μL of cold PBS lysis buffer (PBS 1 × pH 7.4, 1 mM EDTA, 2% SDS) supplemented with 1 × protease inhibitor and 5 mM NBF-Cl
2. Immediately incubate the mixtures at 37 °C for 30 min, protected from light
3. Centrifuge the samples at 15,000 rpm (21,130 × *g*) in a microfuge for 2 min at 4 °C to remove debris, collect the supernatants in a new fresh microtube. Discard the pellets
4. Next, proteins are precipitated by adding to the supernatants (volume should be determined), equal volume of methanol and a quarter of a volume of chloroform and then vortexed (1:1:0.25, protein extract: MeOH:chloroform)
5. Centrifuge the mixtures at 15,000 rpm (21,130 × *g*) for 15 min at 4 °C. The obtained pellet is located between the two layers. Carefully remove the aqueous phase, twist the tube to locate the pellet in the wall of the tube, and carefully remove the chloroform phase (Fig. 3)
6. Wash the pellet with 200 μL methanol, 200 μL distilled water, and 50 μL chloroform, and vortex vigorously
7. Centrifuge again at 15,000 rpm (21,130 × *g*) for 15 min at 4 °C, and remove the liquid as explained before in step 5
8. Perform a last washing adding 500 μL of methanol to the obtained pellet, vortex vigorously and centrifuge at 15,000 rpm (21,130 × *g*) for 15 min at 4 °C

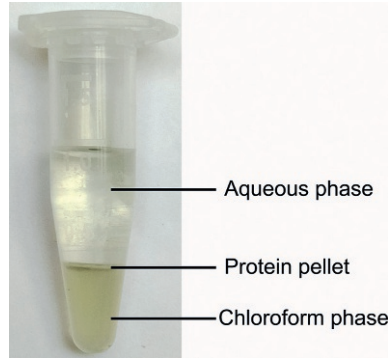

Fig. 3 Separation of phases after centrifugation during methanol/chloroform precipitation.

9. Remove the methanol and air-dry the pellet protected from the light for 10 min at room temperature. At this point, the samples can be stored at −20 °C, and continue the protocol at your convenience
10. Resuspend the samples by first adding 30 μL of 10% SDS directly to the pellet. Add then 120 μL PBS 1 × supplemented with 1 × protease inhibitor. Vortex and made use of the Bioruptor (or sonication bath) if necessary until it is fully dissolved. Program Bioruptor with 10 cycles of 60 s ON and 30 s OFF
11. Determine protein concentration spectrophotometrically using the DC Protein Assay, Bio-Rad
12. Adjust the final protein concentration to 2–3 mg/mL, to continue the second step of labeling

2.2 Labeling with Daz2-Cy5

Materials and equipment

- Acetonitrile, Merck
- PBS 1 ×, pH 7.4, Gibco™
- Dimedone azide-2 (Daz2), Cayman Chemical. Stock solution 50 mM in DMSO
- Cy5-alkyne, Lumiprobe. Stock solution 20 mM in DMSO
- TBTA-Cu(II) complex 10 mM, Lumiprobe
- Dimethyl sulfoxide 99.9% (DMSO), Sigma Aldrich
- L-Ascorbic acid, Sigma Aldrich. Stock solution 100 mM in distilled water (fresh prepared)
- EDTA-Na_2-$2H_2O$ Ultrapure, Invitrogen™. Stock solution 0.5 M dissolved in distilled water
- Methanol 99.9%, Fisher Chemical
- Chloroform, Merck
- SDS BioChemica, AppliChem. 10% (*w*/*v*) stock solution
- Pierce™ Protease Inhibitor Tablets, EDTA-Free, Thermo Scientific. Stock solution 25 × in distilled water
- Eppendorf ThermoMixer™ C
- Eppendorf™ Centrifuge 5424 R
- Vortex VWR® International
- Flowtronic Fume hood

Procedure

1. Prepare the Daz2-Cy5 mix by combining, PBS 1 ×, pH 7.4, 30% (v/v) acetonitrile, 1 mM DAz2, 1 mM Cy5-alkyne, 2 mM TBTA-Cu

complex and 4 mM ascorbic acid, in a final volume of 150 μL. Keep the mixture in a rotator shaker overnight at room temperature to obtain a homogeneous mixture

2. The next day, the reaction is quenched with the final concentration of 20 mM EDTA for 1 h and the final mixture can be aliquoted and stored at −20 °C. Small volume aliquots must be prepared to avoid freezing and thawing (~20 μL). Do not store aliquots for more than 2 months
3. Incubate the protein samples obtained in Section 2.1 (Reaction with NBF-Cl) with 25 μM Daz2-Cy5 mix for 30 min at 37 °C and protected from light
4. Precipitate the proteins following the same procedure described in Section 2.1 (steps 4–9). At this point, the samples can be stored at −20 °C and continue the protocol as desired

2.3 SDS-PAGE of labeled proteins and imaging

Materials and equipment

- PBS 1 ×, pH 7.4, Gibco™
- SDS BioChemica, AppliChem. 10% stock solution
- Pierce™ Protease Inhibitor Tablets, EDTA-Free, Thermo Scientific. Stock solution 25 × in distilled water
- DC (detergent compatible) Protein Assay, Bio-Rad
- 4 × Laemmli buffer, Bio-Rad
- β-Mercaptoethanol, Merck
- Methanol 99.9%, Fisher Chemical
- Acetic acid 99.5% pure, Acros Organics
- Eppendorf ThermoMixer™ C
- Mini-PROTEAN® Tetra Vertical Electrophoresis Cell for Mini Precast Gels and PowerPac™ Basic Power Supply, Bio-Rad
- Precast gels of 10% (w/v) polyacrylamide
- Heidolph Unimax 1010 Orbital Shaker
- Typhoon FLA 9500, GE healthcare
- Varioskan Lux, Thermo scientific
- Multi-RS, Programmable rotator, Biosan
- DC (detergent compatible) Protein Assay, Bio-Rad

Procedure

1. Dissolve the samples obtained in Section 2.2 (Labeling with Daz2-Cy5) in 30 μL of 10% (w/v) SDS and 120 μL of PBS 1 ×, pH 7.4, supplemented with 1 × protease inhibitor

2. Determine protein concentration spectrophotometrically using the DC Protein Assay, Bio-Rad
3. Mix 3 parts of the dissolved sample containing 40–50 µg of protein, with 1 part of 4× Laemmli buffer and supplement with 10% (v/v) β-mercaptoethanol. Boil at 95 °C for 5 min
4. Place the 10% polyacrylamide gel pre-cast on the electrophoresis module
5. Load the mixtures into the wells and run the electrophoresis, first 80 V until the samples go into the separating phase and then 120 V for about 2 h. Cover the electrophoresis module to protect samples from light during the running
6. Prepare the gel fixation solution containing 12.5% (v/v) methanol and 4% (v/v) acetic acid in water
7. Cover the gels with the fixation solution and incubate for 30 min with gentle agitation and protected from light
8. Record the gel at 640 nm for the Cy5 signal and 488 nm for the NBF-Cl signal on the Typhoon 9500 FLA Imager
9. Quantify the persulfidation levels by measuring the Cy5/NBF-Cl fluorescence signal ratio (Fig. 4)

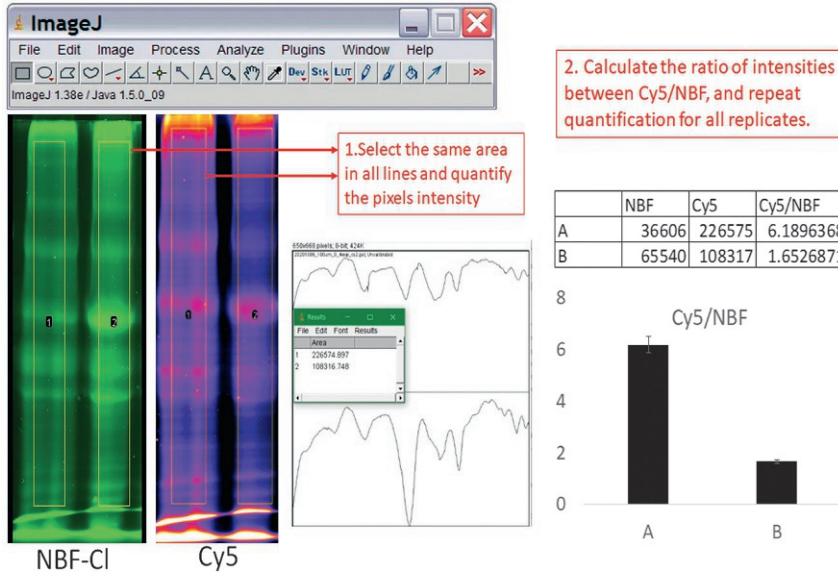

Fig. 4 Example of the persulfidation level quantification in protein extracts from Arabidopsis leaves using ImageJ software. The fluorescent signal for NBF-Cl is in green and the Cy5 signal in red color.

2.4 Notes

1. The details of the Daz2-Cy5 mix preparation are as follows: Bring all compounds to room temperature before to start and in an Eppendorf tube
   - First add 60.5 μL of PBS 1 ×, pH 7.4
   - Add 43 μL of acetonitrile and vortex
   - Add 3 μL of 50 mM DAz2 and vortex
   - Add 7.5 μL of 20 mM Cy5-alkyne and vortex
   - Add 30 μL of 10 mM Copper-TBTA complex and vortex
   - Add H_2O to solid ascorbic acid (100 mM) and vortex until well dissolved
   - Add 6 μL of 100 mM ascorbic acid
   - Cover with aluminum foil and leave in a rotator shaker overnight at room temperature
   - Next day add 10 μL of 300 mM EDTA, cover again and leave in a rotator shaker for 1 h
2. Special caution should be taken against the use of reducing agents in the composition of the PBS lysis buffer to avoid reduction of persulfide groups to thiols
3. To have quantitative data with reduced SD, perform the experiment at least with four biological replicates and two experimental replicates for each biological one. When plant protein extracts are used, the variability of the samples is higher than that observed in cell cultures
4. Due to the method detection is based on fluorescent signals, it is very sensitive to small changes during the procedure and the variability can be increased if the replicas are run on SDS-PAGE gels at very different time period. It is highly recommended to run replicates on the same gel and at near time. Furthermore, it is essential to protect the reagents/samples from light when this is indicated in the procedure
5. To avoid erroneous results, it is imperative that the fluorescent signals are not over-exposed. If this is the case, it is necessary to reduce the amount of protein loaded onto the gels
6. Avoid long storage of either the Daz2-Cy5 mixture or labeled proteins because the fluorescence signal can be decreased
7. Instead of a Bioruptor, a sonication bath might be used at 4 °C

3. Identification of persulfidated proteins by mass spectrometry analysis

A similar method based on dimedone probes is also described here to identify persulfidated proteins by proteomic analysis. The method consists

first of full blocking with the NBF-Cl reagent as described in the previous procedure and then reaction of the modified persulfidated proteins to specifically tag-switch with DCP-Bio1, a biotinylated form of the dimedone. Finally, biotin-labeled proteins are purified with streptavidin and subjected to trypsin digestion for further mass spectrometry analysis (Fig. 2).

3.1 Labeling with DCP-Bio1

Materials and equipment

- PBS 1 ×, pH 7.4, Gibco™
- SDS BioChemica, AppliChem. 10% (w/v) stock solution
- Pierce™ Protease Inhibitor Tablets, EDTA-Free, Thermo Scientific. Stock solution 25 × in distilled water
- DCP-Bio1, Merck. Stock solution 25 mM in DMSO
- Sera-Mag Magnetic Streptavidin, Cytiva
- Tween® 20, Fisher Chemical
- H_2O Optima® for LC/MS, Fisher Chemical
- Ammonium hydroxide solution 5 M, Fluka Analytical
- Formic acid for LC/MS, Fisher Chemical
- DC (detergent compatible) Protein Assay, Bio-Rad
- Eppendorf ThermoMixer™ C
- Eppendorf™ Centrifuge 5424 R
- Flowtronic Fume hood
- Vortex VWR® International
- Bioruptor® Pico minichiller 300, Diagedone SA
- Varioskan Lux, Thermo scientific
- Multi-RS, Programmable rotator, Biosan
- Heidolph Unimax 1010 Orbital Shaker
- Invitrogen™DynaMag™-Spin Magnet
- Qubit ™ Protein Assay Kit, Invitrogen
- Qubit 4 Fluorometer, Invitrogen

Procedure

1. In this procedure, grind 500 mg of plant tissue and resuspend in 500 µL of lysis buffer PBS 1 × supplemented with 2% (w/v) SDS, 1 × protease inhibitor, and 5 mM NBF-Cl. Perform the reaction with NBF-Cl previously described in Section 2.1 (Reaction with NBF-Cl), from step 1 to 9
2. Add 200 µM DCP-Bio1 and incubate the mixture at 37 °C for 2 h (Fig. 2)
3. Precipitate the proteins following the same procedure described in Section 2.1 (steps 4–9) (Fig. 3)

4. Resuspend protein samples in 500 μL of PBS 1 × supplemented with 0.1% SDS and 1 × protease inhibitor
5. Add 150 μL of Sera-Mag Magnetic Streptavidin beads and incubate the mixture at 4 °C overnight with gentle agitation on the rotator shaker
6. The microtubes containing the magnetic beads are located in the Invitrogen™DynaMag™-Spin Magnet and after several seconds, the beads are separated from the supernatant, which is discarded
7. The microtubes are removed from the magnet and the magnetic beads are washed by resuspending them in 1 mL of PBS 1 × supplemented with Tween 0.001% (v/v) at room temperature. Afterwards, the microtubes are located again into the Magnet and the supernatant is discarded. This wash is repeated three times
8. The magnetic beads are then washed twice with 1 mL of PBS 1 × as previously described and once with 1 mL of pure H_2O for LC/MS
9. After the six washes, the beads are incubated with 2.25 M ammonium hydroxide at room temperature overnight, with gentle agitation
10. Then, samples are located again in the Invitrogen™DynaMag™-Spin Magnet and supernatants are transferred to fresh microtubes. Magnetic beads are discarded
11. Samples are neutralized with pure formic acid for LC/MS
12. Determine protein concentration spectrophotometrically using the Qubit™ Protein Assay kit

3.2 Liquid chromatography and mass spectrometry analysis (LC-MS/MS)

Protein persulfide labeled proteins with DCP-Bio 1 can be analyzed by a variety of high-resolution LC-MS/MS mass spectrometers after peptide digestion with either trypsin or chymotrypsin following standard protocols. A total of 50 μg of proteins are digested and analyzed by LC-MS/MS. The chromatographic separation and fragmentation of the peptides will depend on the optimal conditions of each commercial brand, recommending analysis in equipment with high sensitivity and resolution.

Regardless of the spectrometer, peptide identification and search setting are following:

- precursor Δm tolerance = 10 ppm
- -fragment Δm tolerance = 0.2 Da
- missed cleavages = 2
- modifications of lysine and arginine: NBF (163.0012)
- modifications of cysteine: NBF (163.0012), hydrolyzed DCP-Bio1 (168.0786) and/or DCP-Bio1 (394.1557)

3.3 Notes

1. As a general rule, all the plasticware used during the treatments of the samples to be analyzed by mass spectrometry is imperative to be of very high quality (Eppendorf-type) to avoid contamination of samples and the obtaining of unusable results
2. The solvents and water used to prepare the buffers should be of HPLC-MS quality
3. It is mandatory to use streptavidin beads with high nominal biotin binding capacities (4500–5500 pmol/mg) to optimize the enrichment of the persulfidated proteins (DCP-Bio1 labeled) and to avoid as much as possible the limitation of this step
4. When it is expected that the protein extract could contain a significant amount of endogenous biotinylated proteins, a previous step prior to incubation with DCP-Bio1 should be performed. The biotinylated proteins are precleared using the streptavidin magnetic beads (low-medium biotin binding capacity can be used) as described before
5. Step 12 for protein quantification might be performed with other protein assay methods, but Qubit is recommended because it is a fluorescent method with high sensitivity

Acknowledgments

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