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► **To cite this version:**

Corentin Moreau, Emmanuelle Issakidis-Bourguet. A Simplified Method to Assay Protein Carbonylation by Spectrophotometry. Amna Mhamdi. Reactive Oxygen Species in Plants. Methods and Protocols, 2526, Springer US, pp.135-141, 2022, Methods in Molecular Biology, 978-1-0716-2468-5. <10.1007/978-1-0716-2469-2_10>. <hal-03866183>

HAL Id: hal-03866183

<https://hal.science/hal-03866183v1>

Submitted on 28 Nov 2023

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HAL Authorization

Methods in
Molecular Biology 2526

Springer Protocols

Amna Mhamdi *Editor*

Reactive Oxygen Species in Plants

Methods and Protocols

MOREMEDIA



Humana Press



A Simplified Method to Assay Protein Carbonylation by Spectrophotometry

Corentin Moreau and Emmanuelle Issakidis-Bourguet

Abstract

Protein carbonylation is an irreversible oxidation process leading to a loss of function of carbonylated proteins. Carbonylation is largely considered as a hallmark of oxidative stress, the level of protein carbonylation being an indicator of the oxidative cellular status. The method described herein represents an adaptation to the commonly used 2,4-dinitrophenylhydrazine (DNPH)-based spectrophotometric method to monitor protein carbonylation level. The classical final sample precipitation was replaced by a gel filtration step avoiding the tedious and repetitive washings of the protein pellet to remove free DNPH while allowing optimal protein recovery.

This improved protocol here implemented to assay protein carbonylation in plant leaves can potentially be used with any cellular extract.

Key words Protein oxidation, Carbonylation, Spectrophotometry

1 Introduction

Carbonylation is the most common protein modification induced by Reactive Oxygen Species (ROS) [1], and carbonylated proteins have been identified in many plant species at different stages of growth and development (reviewed in Ref. [2]).

Carbonylation is induced by direct ROS attack of exposed side chains of amino acid residues such as Pro, Arg, Lys, and Thr, but protein carbonylation can also be indirectly induced by ROS through lipid peroxides (for Cys, His, and Lys) and even by glycation/glycooxidation (for Lys) [3].

Because the formation of carbonylated proteins is a major product of protein oxidation and protein carbonylation is considered to be a stable and irreversible modification, it is often used as a marker to evaluate cellular oxidation.

In plants, oxidative stress (carbonylation) was shown to accompany senescence/aging in plant leaves as well as in seeds where it is associated to loss of seed viability [2, 4–6]. Extensive carbonyl

formation has been also shown in protein extracts from leaves exposed to abiotic stresses such as low and high temperatures, salinity, or drought [7–11]. It is postulated that carbonylation of proteins induces a loss of their functional properties and/or may serve as a signal for degradation by increasing their susceptibility towards proteolysis by the proteasome and organellar proteolytic systems [12]. Also, it was shown that carbonylated proteins can escape degradation and form high-molecular-weight aggregates that accumulate with age [3].

Protein carbonylation can be detected and quantified at the global level in proteins and protein mixtures using derivatization of carbonyl groups by 2,4-dinitrophenylhydrazine (DNPH) which reacts with the ketone and aldehyde functional groups and produces a stable 2,4-dinitrophenyl (DNP) hydrazone product. The specific UV absorption of DNP-hydrazone at 370 nm allows detection and quantification of carbonyls with a spectrophotometer.

Here, we have adapted the DNPH-based spectrophotometric method [13]. It classically includes a final precipitation step of the sample yielding a protein pellet that needs to be carefully washed to ensure reliable and reproducible data. Complete removal of free DNPH is conditional to optimal pellet dispersion. This is achieved by vigorous vortexing (sonication of the pellet is often recommended in commercial kits) to dislodge the pellet and by repeating washing/centrifugation cycles several times (usually 3–5). To overcome this problem, Xia et al. [14] soundly proposed to perform the final step centrifugation at slow speed to form a loose easily dispersible pellet facilitating re-dissolving/washing. But protein loss at washing steps is a major drawback of the DNPH-based method as it results in a relatively low reproducibility and in high standard deviation of the data [15].

We have conveniently modified this commonly used method by replacing the final precipitation by a gel filtration allowing a simple, fast, and efficient removal of free DNPH in a single step combined with optimal protein recovery.

Herein, this improved protocol was used to measure protein oxidation in plant extracts, but it can potentially be used with any cellular extract.

2 Materials

Prepare all buffers using ultrapure water (double distilled or MilliQ grade).

1. Extraction buffer: 100 mM Tris-HCl pH 6.8.
2. 10% (w/v) streptomycin sulfate prepared in water.
3. Mini clarification spin columns (*see Note 1*).

4. Carbonyl labelling solution: 30 mM DNPH prepared in DMSO (*see Note 2*).
5. 1 mL G-25 spin column prepared in TE (Tris-HCl 100 mM pH 7.9, 1 mM EDTA) buffer at 20% (w/v) (*see Notes 3 and 4*).
6. Qubit protein assay kit from Invitrogen (protein dye reagent and dedicated fluorometer) (*see Note 5*).

3 Methods

3.1 Sample Extraction and Clarification

1. Grind around 100 mg of tissue per sample in liquid nitrogen (*see Note 6*).
2. Add 400 μ L of extraction buffer supplemented with a cocktail of protease inhibitors, and homogenize by vigorously vortexing the sample for 5 s (2 times with a 1–2 min stand on ice in the interval) (*see Note 7*).
3. Centrifuge for 15 min at 4 °C and 18,000 $\times g$.
4. Further clarify the extract by filtrating the supernatant (380 μ L) through a clarification unit (*see Note 1*).
5. Centrifuge at RT for 2 min at 12,000 $\times g$ (*see Note 8*).

3.2 Removal of Nucleic Acids

1. Transfer 360 μ L of clarified extract to a new tube, and add 40 μ L of 10% streptomycin sulfate to precipitate nucleic acids (*see Note 9*).
2. Mix by a brief vortexing.
3. Incubate at RT for 20 min with mixing (*see Note 10*).
4. Centrifuge at RT for 10 min at 12,000 $\times g$.

3.3 Derivatization with DNPH

1. Transfer 380 μ L of supernatant to a new tube, and add 90 μ L of 30 mM DNPH (*see Note 11*).
2. Mix by a brief vortexing.
3. Incubate at RT, in the dark, for 20 min with mixing (*see Note 10*).

3.4 Removal of Unincorporated DNPH by Gel Filtration

1. Build up gel filtration column (all steps performed at RT) by adding 1.5 mL of swollen G-25 slurry to a 2 mL centrifuge empty column (*see Note 3*).
2. Remove twist-off bottom closure and let the column set for 10 min. Place column onto a 15 mL collection tube and centrifuge at 800 $\times g$ for 30 s. Check that the resin has packed to a 1 mL volume (graduation on the centrifuge column tube) (*see Note 4*).
3. Load 210 μ L of the sample onto a 1 mL G-25 spin column placed on a new 15 mL collection tube.

4. Centrifuge at RT for 15 s at $800 \times g$ (see **Note 12**).
5. Transfer the 210 μL eluate into a new tube. Keep the sample on ice.

3.5 Quantification of Carbonyls

1. Determine carbonyl concentration by reading the sample absorbance at 370 nm using TE buffer or the related control sample (without DNPH) as blank (see **Note 13**).
2. Determine protein concentration using 2–5 μL sample using a protein assay (see **Note 5**).
3. Calculate carbonyl content using a molar absorption coefficient for aliphatic hydrazones of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed in nmol carbonyl mg^{-1} protein, using the following equation:

$$\text{Carbonyl (M)} = \frac{A_{370}}{22,000}$$

$$\text{Carbonyl (nmol/mg protein)} = \frac{A_{370}}{22,000}$$

$$\times 10^6 \text{ (nmol/mL) / protein concentration (mg/mL)}$$

Using the present method, we quantified carbonyls in *Arabidopsis* and wheat leaves and found an overall variation of 2–8% of results within experiments. We measured a carbonyl content of 26.46 nmol/mg protein in the leaves of 3-week-old *Arabidopsis* plants grown on MS medium under short day conditions. We also measured protein oxidation in wheat in the context of heat stress. The results obtained are presented in Fig. 1. Upon heat stress, a marked increase (+ 42–62% in 3 independent experiments) in the carbonyl content of the flag leaf was found, signifying that protein oxidation has massively occurred. An increase in the protein carbonylation level was also found in dehydrated wheat seedlings [10] suggesting a possible link between protein carbonylation and wheat sensitivity to unfavorable environmental conditions. Thus, experimental work points to the idea that protein oxidation level assessed as protein carbonylation may be taken as a biochemical marker for screening stress-tolerant wheat germplasm in breeding programs.

4 Notes

1. We used Proteus Mini Clarification Spin Columns from GENERON. These filter units (hydrophilic PVDF) are designed to remove microorganisms, particles, and precipitates larger than 0.2 μm in size from aqueous solutions. Any equivalent filters may be potentially used. Alternatively, the homogenate can be filtered through Miracloth (cellulosic filtration medium).

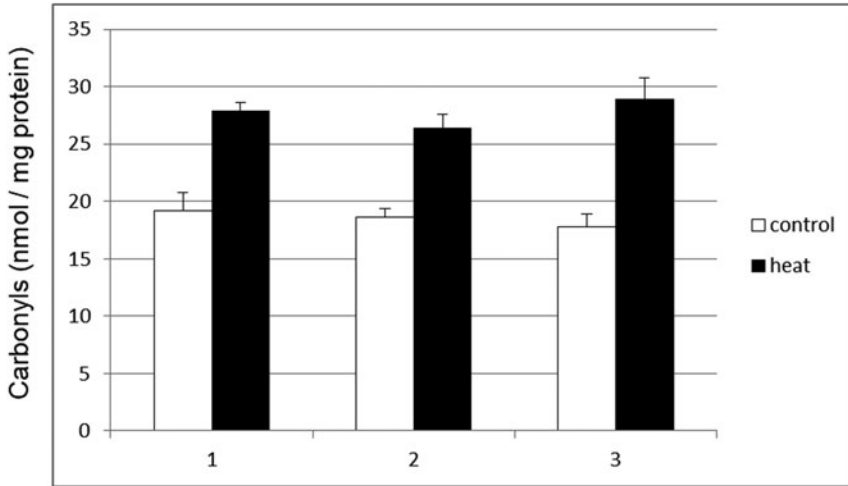


Fig. 1 Effect of heat stress on protein oxidation in wheat. Winter wheat (*Triticum aestivum* L.) was cultivated under a 16 h photoperiod ($120\text{--}250\ \mu\text{E}/\text{m}^2/\text{s}$) at $24\ \text{°C}/18\ \text{°C}$ day/night temperatures with fertilized watering supplied 3 times per week. Heat stress ($30\text{--}32\ \text{°C}$, under a light of $650\text{--}700\ \mu\text{E}/\text{m}^2/\text{s}$, for 4 h at midday) was conducted during grain filling at the kernel milk stage corresponding to $250\text{--}300$ degree days after anthesis. Flag leaf samples (2/3 median part of the leaf) were taken at the end of the heat treatment and immediately frozen in liquid N_2 and stored at $-80\ \text{°C}$ until analysis. Bars represent the mean \pm SD ($n = 8\text{--}12$) for 3 independent experiments

2. When preparing the DNPH solution, the actual content of DNPH in the reagent (the powder contains $> 30\%$ water) should be taken into account. The DNPH stock solution (protected from light) is stable at RT for several days.
3. We used G-25 gel filtration resin to remove free DNPH from labelled protein samples. Proteins and peptides larger than Mr 5000 are separated from molecules with Mr of less than 1000 (DNPH MW $198.14\ \text{g}/\text{mol}$). We found optimal DNPH removal and protein recovery with a minimum sample dilution using Sephadex G-25 superfine resin. We used 2 mL centrifuge empty column tubes from Pierce.
4. The G-25 resin may be cleaned and re-used according to the supplier's recommendations. Alternatively, pre-packed commercially available columns may be used with protocol adaptation.
5. We used the Qubit protein assay kit from Invitrogen, but alternatively, protein quantitation can be performed using the bicinchoninic acid (BCA) assay [16], or any other protein assay with a suitable range of sensitivity, and a standard spectrophotometer.
6. Freshly harvested tissues or tissues stored at $-80\ \text{°C}$ are suitable. Extraction can be performed using a bead mill or

manually using a mortar and a pestle. Preliminary cooling is needed to avoid protein degradation.

7. Protease inhibitor cocktails are commercially available and usually provided as ready-to-use 100 times concentrated. We used a protease inhibitor cocktail optimized for plant cell extracts.
8. Filters can be re-used after a careful washing and storage in 20% ethanol. Drain (centrifuge at RT for 2 min at $12,000 \times g$) and rinse the unit with 400 μL water (centrifuge in the same conditions).
9. Nucleic acids also contain carbonyl groups and will react with DNPH. To avoid an erroneously high estimate of protein-bound carbonyls, cellular extracts are treated with 1% streptomycin sulfate to decrease nucleic acid contamination. 1% streptomycin sulfate is usually effective in minimizing interference, but this should be confirmed for specific samples for which higher concentrations of streptomycin sulfate may be required. Interference by nucleic acids can be checked by the ratio of absorbance at 280 nm to that at 260 nm that must be greater than 1.
10. Samples were continuously mixed using a Thermomixer set at 1100 rpm, or alternatively a brief vortex of the samples was performed occasionally during incubation.
11. At this step, a reagent blank can be prepared for each sample by mixing 190 μL of clarified extract with 45 μL DMSO. The carbonyl labelling sample is prepared by mixing 190 μL of clarified extract with 45 μL 30 mM DNPH. We have found that absorbance of reagent blank samples at 370 nm was null using the method described here for *Arabidopsis* and wheat leaf extracts.
12. We used an Eppendorf 5810R centrifuge equipped with a swinging-bucket rotor for 15 mL conical tubes.
13. Sample preparation (*see Note 11*) allows performing, for each sample, either a blank or a technical duplicate of the protein carbonyl assay. We used plastic UV-compatible micro cuvettes allowing reads in triplicate with 70 μL of assay samples and a standard spectrophotometer compatible with micro cuvettes.

Acknowledgments

This work was financially supported, thanks to the Poc in Labs initiative of the University Paris-Saclay and the Saclay Plant Sciences network, by the program “Investing for the Future” launched by the French State and implemented by the ANR (French National Research Agency). Former undergraduate students of the laboratory, Mai Pham Thy, Albert Kwarteng, and Oscar Freyschlag, are acknowledged for their participation to this work.

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