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A step-by-step protocol for assaying protein carbonylation in biological samples

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ABSTRACT

Protein carbonylation represents the most frequent and usually irreversible oxidative modification affecting proteins. This modification is chemically stable and this feature is particularly important for storage and detection of carbonylated proteins. Many biochemical and analytical methods have been developed during the last thirty years to assay protein carbonylation. The most successful method consists on protein carbonyl (PCO) derivatization with 2,4-dinitrophenylhydrazine (DNPH) and consequent spectrophotometric assay. This assay allows a global quantification of PCO content due to the ability of DNPH to react with carbonyl giving rise to an adduct able to absorb at 366 nm. Similar approaches were also developed employing chromatographic separation, in particular HPLC, and parallel detection of absorbing adducts. Subsequently, immunological techniques, such as Western immunoblot or ELISA, have been developed leading to an increase of sensitivity in protein carbonylation detection. Currently, they are widely employed to evaluate change in total protein carbonylation and eventually to highlight the specific proteins undergoing selective oxidation. In the last decade, many mass spectrometry (MS) approaches have been developed for the identification of the carbonylated proteins and the relative amino acid residues modified to carbonyl derivatives. Although these MS methods are much more focused and detailed due to their ability to identify the amino acid residues undergoing carbonylation, they still require too expensive equipments and, therefore, are limited in distribution. In this protocol paper, we summarise and comment on the most diffuse protocols that a standard laboratory can employ to assess protein carbonylation; in particular, we describe step-by-step the different protocols, adding suggestions coming from our on-bench experience.

1. Introduction

The introduction of reactive carbonyl groups (CO), mainly aldehydes and ketones, into a protein structure is defined "protein carbonylation" [1]. Protein carbonylation includes many chemical modifications occurring through different reaction mechanisms that can be summarised as follows:

1) Direct oxidation of several amino acid residues [2,3] induced by hydroxyl radical (HO•): this radical can be generated by Fenton reaction of metal cations with hydrogen peroxide [1,4] or by ionizing radiations [5]. HO• induces oxidation of proline, arginine, lysine, and threonine residue side chains to aldehydes or ketones. Two typical products of oxidation are glutamic semialdehyde and aminoadipic semialdehyde from arginine and lysine oxidation, respectively [6].

Abbreviations: 2D-GE, two-dimensional gel electrophoresis; 4-HNE, 4-hydroxynonenal; ARP, aldehyde-reactive probe; BHZ, biotin-hydrazide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMF, dimethyl formammide; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiotreitol; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; FTC, fluorescein-5-thiosemicarbazide; HGFs, human gingival fibroblasts; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; HSA, human serum albumin; IAM, iodoacetamide; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; MW, molecular weight; PBST, phosphate buffered saline with Tween-20; PCO, protein carbonyls; PNGase F, peptide N-glycosidase F; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; RT, room temperature; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBP, tri-butyl phosphine; TCA, trichloroacetic acid; UTC, urea-thiourea-CHAPS solution.

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2) Protein backbone hydrolysis: hydroxyl radical attack to protein backbone can induce hydrolysis through α-amidation pathway [7].
3) "Michael addition" reactions: cysteine, histidine, and lysine residues can react with carbonyl species, such as 4-hydroxynonenal (4-HNE), 2-propenal (acrolein), and malondialdehyde, generated during lipid peroxidation [8].
4) Glycation/glycoxidation reactions: the amino group of lysine residues can react with reducing sugars or their oxidative products to generate carbonyl species such as carboxymethyl lysine [9].

Detection and quantification of protein carbonyls (PCO) in biological samples is an indirect way to determine the level of oxidative stress. At the current time, protein carbonylation is a widely occurring and accepted irreversible marker of protein oxidation and hundreds of published papers reported PCO quantification.

There are many methods used nowadays for evaluation of the content of carbonylated proteins and, among them, the most employed is based on 2,4-dinitrophenylhydrazine (DNPH) and was originally developed by Levine et al. [10]. This molecule reacts with carbonyl groups leading to the formation of the stable 2,4-dinitrophenylhydrazone. The dinitrophenyl group (DNP) can be detected and quantified spectrophotometrically because it is characterized by a typical absorption spectrum with a maximum at 365–375 nm [10]. In addition, spectrophotometric measurement of DNP can also be used in association with HPLC protein separation adding the possibility to detect more precisely the proteins undergoing carbonylation [11,12].

Within a few years from the fundamental paper of Levine et al. [10], the development of good antibodies able to recognize the DNP adducts has opened the possibility to increase the sensitivity of PCO detection. The only use of the high recognition specificity of anti DNP antibodies or the combination with other standard protein separation and/or detection methods (e.g. electrophoretic techniques) allowed researchers to detect protein carbonylation with dot blot, immunochemistry, ELISA and Western blot protocols. Today, hundreds of papers investigating PCO and using these well ascertained methods are reported in literature [13–17].

A more recent and powerful approach consists on using MS techniques as investigation tools. These methods are not accessible to all laboratories due to the high cost for analysis instrumentation. Although these methods are very sensitive and can identify specific oxidized residues without, in theory, requiring protein labelling, in practice, protein labelling is often used in mass spectrometric analysis because it has the advantage to allow protein enrichment (e.g. biotin-hydrazide derivatization of proteins can be followed by affinity chromatography column enrichment with immobilized streptavidin). For instance, using a biotin-hydrazide based approach, it was possible to identify 100 carbonylated proteins, including low abundance receptors, in brain homogenates of mice of different ages [18]. Alternatively, label-free approaches have also been developed as reported by capture of carbonylated peptides by a solid-phase hydrazide reagent [19] or by immobilized oxalyldihydrazide on a microchip [20].

Carbonyl content of purified proteins is usually expressed as moles carbonyl/mole protein. Otherwise, for cell or tissue homogenates, protein carbonyl content is expressed as nmol carbonyl/mg protein. This means that both carbonyl levels and protein levels need to be accurately determined. Carbonyl content can be indirectly measured evaluating DNPH incorporation using a spectrophotometer (Section 2.2), while protein amount is usually determined by protein assay.

In case of immunowestern blot analysis (Sections 2.4 and 2.5), the carbonyl levels can be densitometrically defined using specific software, whereas the protein levels are usually evaluated after Amido Black or Coomassie blue stain of western blot membrane. The ratio between the carbonyl signal intensity and the protein signal intensity will give specific carbonyl content. In general, in this kind of experiment, many researchers consider sufficient to express protein carbonyl content in treated samples as a percentage increase/decrease respect to control one. In case a more accurate quantification is required and protein carbonyl content needs to be expressed as nmol carbonyl/mg protein, an oxidized protein standard is required to compare sample signals to well known carbonyl protein content standard.

The protein carbonyl content highly increases under pathological conditions related to oxidative stress. For example, in plasma proteins of children with different forms of juvenile chronic arthritis, the carbonyl content was significantly higher than in healthy group (1.36 ± 0.68 vs. 0.807 ± 0.16 nmol carbonyl/mg of protein) [21]. A more evident difference was observed comparing plasma protein carbonyl groups among normal volunteers (0.76 ± 0.51 μmol/l), patients with chronic renal failure (13.73 ± 4.45 μmol/l) and patients on chronic maintenance haemodialysis (16.95 ± 2.62 μmol/l) [22].

In this article, we describe in a user-friendly and step-by-step way, with detailed guidelines coming from our on-bench experience, the most used methods to detect and quantify protein carbonylation in a standard research laboratory devoted to studying protein oxidative modifications and whose equipment is composed by relatively cheap and highly diffuse instrumentation (e.g., spectrophotometer, SDS-PAGE, 2D-GE and Western blot apparatuses).

2. Protocols

2.1. PCO labelling with DNPH

The molecule DNPH, also known as Brady's reagent, is a specific probe able to react with PCO leading to the formation of protein-conjugated dinitrophenylhydrazones (DNP) (Fig. 1). These

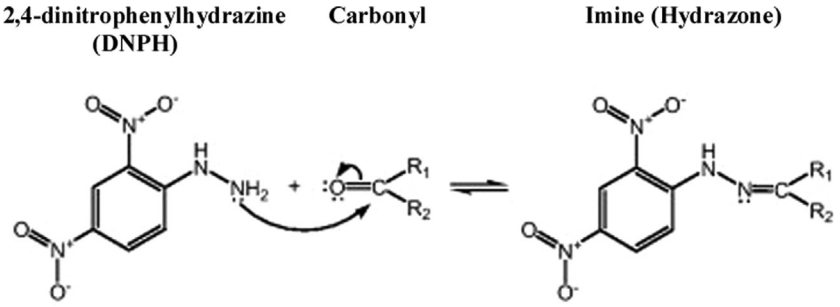

Fig. 1. DNPH reaction with carbonyl species. DNPH reacts readily with aldehydes and ketones via a condensation reaction to produce the corresponding hydrazine. Modified from [11]. Copyright© 2013 Elsevier Ireland Ltd.