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Review Article

Determination of protein carbonyls in plasma, cell extracts, tissue homogenates, isolated proteins: Focus on sample preparation and derivatization conditions

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ABSTRACT

Protein oxidation is involved in regulatory physiological events as well as in damage to tissues and is thought to play a key role in the pathophysiology of diseases and in the aging process. Protein-bound carbonyls represent a marker of global protein oxidation, as they are generated by multiple different reactive oxygen species in blood, tissues and cells. Sample preparation and stabilization are key steps in the accurate quantification of oxidation-related products and examination of physiological/pathological processes. This review therefore focuses on the sample preparation processes used in the most relevant methods to detect protein carbonyls after derivatization with 2,4-dinitrophenylhydrazine with an emphasis on measurement in plasma, cells, organ homogenates, isolated proteins and organelles. Sample preparation, derivatization conditions and protein handling are presented for the spectrophotometric and HPLC method as well as for immunoblotting and ELISA. An extensive overview covering these methods in previously published articles is given for researchers who plan to measure protein carbonyls in different samples.

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Introduction

In recent years, more and more evidence has arisen that oxidative processes play a key role in the pathophysiology of many diseases and in the aging process. Besides regulatory events, a plethora of damaging effects is induced by oxidative processes, one of these damaging processes is protein oxidation [1]. Oxidative damage to proteins results in a multitude of products (for reviews see [2–6]), arising from modification of a wide range of amino acids. These include damage to sulfur-containing, aromatic, and aliphatic amino acids [7–10] (Table 1). Protein carbonyls

Abbreviations: AP, alkaline phosphatase; BAL, bronchoalveolar; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BHT, butylated hydroxytoluene; DMSO, dimethyl sulfoxide; DNP, 2,4-dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HCl, hydrochloric acid; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; Hsf1, heat shock factor 1; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; n.a., not available; NBT, nitro blue tetrazolium; PVDF, polyvinylidene difluoride; RT, room temperature; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoracetic acid

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Table 1
Possible oxidation products of amino acid residues resulting in protein carbonyl formation.

Amino acid	Oxidation products
Proline	Glutamic semialdehyde and other ring opened products
Arginine	Glutamic semialdehyde and other side-chain products
Lysine	Aminoadipic semialdehyde and other side-chain products
Threonine	Carbonyls formed at side chain sites
Methionine	Methional
Tryptophan	N-formylkynurenine, kynurenine
Histidine	2-Oxo-histidine and ring-opened species
Alanine	Formaldehyde and carbonyls from methyl group
Valine	Acetone, formaldehyde and carbonyls on side-chain methyl groups
Leucine	Isobutyraldehyde, acetone, formaldehyde, and carbonyls on side-chain
Aspartate	Glyoxylic acid
Isoleucine	Formaldehyde, carbonyls on side-chain

represent an irreversible form of protein modification and have been demonstrated to be relatively stable (degradation/clearance in hours/days) in contrast to lipid peroxidation products that are removed within minutes [11,12]. In addition, protein carbonyls are formed early during oxidative stress conditions and are not a result of one specific oxidant, thus they can be called a marker of overall protein oxidation. Due to the great variety of different modifications [13], one obstacle in the detection of protein-related oxidative stress biomarkers is the requirement of complex procedures for their determination. Furthermore, the instability of some of these products as a result of repair processes (in the case of methionine sulfoxides) [14] and by peroxiredoxins and disulfide reductases [15] can contribute to difficulties in assessing and quantifying oxidation status. It is worth pointing out that some of the formed species resulting from protein oxidation (formaldehyde, acetaldehyde, acetone, etc.) do not remain protein bound but are released. Hence they are not detected by any assays that involve protein separation or precipitation. Depending on the radical treatment used, released carbonyls can however be major products [16].

Various oxidants may attack several amino acids and are thus able to produce both protein-bound and released carbonyl groups (Table 1 and Fig. 1). The yield of these species is however oxidant dependent. Due to the structure of normal amino acids it is expected, that no carbonyl groups are part of a native protein. However, this seems to be a simplifying assumption since many proteins undergo (enzymatic) post-translational modifications where carbonyl groups might be introduced into the native, functional protein. The presence of such structures could be the reason for the high basal (not stress-induced) level of protein carbonyls found in some proteins. Furthermore, inappropriate sample handling might contribute to elevated concentrations observed in some studies.

The most commonly used marker to assess protein oxidation is via the determination of protein-bound carbonyls. Protein carbonyls can be detected by various methods, all relying on the derivatization of the carbonyl group. The reduction with radiolabeled borohydride introduces a measurable radiolabel into the protein, whereas several hydrazine derivatives, most commonly 2,4-dinitrophenylhydrazine (DNPH, Fig. 2) or biotin hydrazine, introduce detectable functional groups into the oxidized protein. So, the most often used procedure to detect protein carbonyls is after their derivatization with DNPH. During the last three decades most of these methods have referred to the basic methods described by Levine et al. [17,18] using the highly-sensitive DNP-modification of protein carbonyls followed by a detection either by spectrophotometric methods, by an HPLC-based technique or using anti-DNP antibodies in immunoblotting [19] or ELISA [20] (see Fig. 3). In addition to this, proteomic techniques have been applied to get a more detailed insight into the mechanism of protein damage, e.g. in blood [21].

In the following we will concentrate on the determination of protein carbonyls in plasma, cell culture, organ homogenate and isolated protein/organelle samples by the methods of Levine et al. [17,18], Shacter et al. [19], Keller et al. [22] and Buss et al. [20].

As described above, DNP-derivatized proteins can be detected by different methods; hence every laboratory should be able to detect carbonyl groups either by the simple spectrophotometric assay or by more complex procedures. The fact that no special equipment is needed for the analysis of DNP-derivatized proteins has led to the application of these methods in numerous publications. The search term “protein carbonyl” leads to more than 15,000 publications on PubMed and “protein carbonyl assay” still leads to around 6000 results (accessed in April 2015).

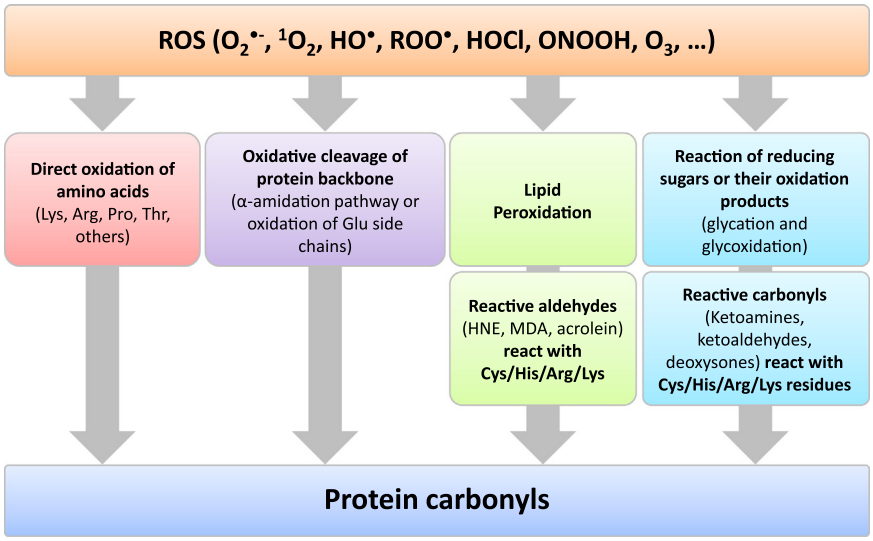

Fig. 1. Protein oxidation resulting in protein carbonyl formation. Reactive oxygen species (ROS) may either react directly with some amino acid residues or lead to oxidative cleavage of the protein backbone. Other possible formation routes of protein carbonyls are via the oxidation of lipids resulting in reactive aldehydes which react with cysteine (Cys), histidine (His), arginine (Arg) and lysine (Lys) residues and thus introduce carbonyl groups and furthermore via the reaction of reducing sugars or their oxidation products with the same residues.