Investigation of the Carbonylation Process of Protein Induced “in vitro” by Different Hydroxyl Radical Generating Systems

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The aim of this study was to assess the carbonylation process induced in vitro by different hydroxyl radical generating systems and to characterize the obtained carbonyl products using capillary electrophoresis and UV-Vis molecular absorption spectrometry methods. Experimental results revealed that the system potassium ascorbate / iron(III) chloride induced the best yield for carbonyl generation, compared with the other two hydroxyl radical generating systems. Carbonyl formation was time and concentration dependent in the range investigated. Using capillary electrophoresis more than 10 derivates were separated. Capillary electrophoresis showed superior specificity and sensitivity in the detection of carbonyl derivates compared with the classical UV-Vis analysis.

Key words: protein carbonyls; hydroxyl radical generating systems; capillary electrophoresis

The carbonylation of protein represents the introduction of carbonyl groups (as aldehyde or ketone) in the protein structure, through several mechanisms, such as direct oxidation of the residues of lysine, arginine, proline and threonine residues from the protein chain, by interaction with lipid peroxidation products with aldehyde groups, or by the interaction with the compounds with the carbonyl groups resulting from the degradation of the lipid or glycoxidation [1 - 3].

Compared with other oxidative changes (such as cysteine disulfide bond formation), the carbonylation process is irreversible, therefore the final compounds are stable and can be quantified [4]. As only certain proteins can undergo carbonylation and the protein structure determines the preferential sites of carbonylation, a specific pattern of carbonylation can be highlighted for a given protein or class of proteins. The most investigated carbonyl derivates are represented by (2S)-2-Amino-5-oxopentanoic acid which is generated from the degradation of arginine residue and 2-amino-6-oxo-hexadienoic acid, derived from lysine (fig.1) [5].

Fig. 1. Chemical structures of the main carbonyl derivates

The assessment of protein carbonyls offers some advantages over other oxidative stress markers as the process occurs in the early stages of human diseases (e.g. Parkinson’s and Alzheimer’s diseases, amyotrophic lateral sclerosis, cataractogenesis, cystic fibrosis, diabetes) and remains in circulation for a longer time, compared to other biomarkers, such as malondialdehyde or 4-hydroxy-2-nonenal. Their chemical stability (up to 3 months at -80°C) in different types of samples and its clinical accessibility makes protein carbonyls suitable for routine laboratory measurement [6].

In the last decade, various analytical methods for the assessment of protein carbonyls were developed and validated, including spectrophotometric assays [7 - 10], high-performance liquid chromatography with diode-array or fluorescence detectors [11, 12], enzyme-linked immunosorbent assays (ELISA) [13, 14], one- or two-dimensional electrophoresis and Western Blot immunoassays [10] or capillary electrophoresis with laser induced fluorescence [15].

The aim of the present study was to investigate the carbonylation process induced by different hydroxyl radical generating systems and to characterize the obtained carbonyl products using capillary electrophoresis and UV-Vis molecular absorption spectrometry methods.

Experimental part
Reagents

All the reagents used were of analytically grade and all solutions were prepared with bidistilled water. Iron(II) sulfate, iron(III) chloride, cooper(II) sulfate, DNPH(2,4-dinitrophenylhydrazine), acorbic acid, potassium ascorbate, trifluoroacetic acid (99% GC), guanidine hydrochloride (>99%), bovine serum albumin (BSA> 98% 66,000 Da) were bought from Sigma Aldrich. Ethylacetate (ULC-MS) and ethanol were purchased from Scharlau Chemie SA.

Carbonylation process

The formation of protein carbonyls was investigated on bovine serum albumin (BSA), which was subject to three different hydroxyl radical generating systems: 1 mM iron(III) chloride / 25 mM sodium ascorbate; 0.95 mM iron(II) sulfate / 1.5 mM ascorbic acid and 0.05mM cooper(II) sulfate / 2 mM hydrogen peroxide. Each of the three oxidation systems was applied to 1mg/mL or 5 mg/ml BSA solutions, using incubation at 37°C for 1 to 18 h.

Protein carbonyl evaluation was performed by derivatisation with DNPH 10 mM / HCl 2.5 mM at room temperature for 15 min, followed by precipitation with TCA 10% and then incubation for 30 min at -10°C.
The separation of hydrazone pellets from the sample was done according to Levin method [16], with two major changes in the technique: (1) centrifugation was done at 4000 g for 20 min at 4°C and (2) the free DNP was removed from the suspended pellets with only two successive rinses with ice cold ethanol–ethyl acetate mixture (50:50 v/v). The obtained pellets were redissolved in 6.0 M guanidine hydrochloride solution at 37°C, for 9 hours.

**Equipment**

For the UV-Vis analysis of protein carbonyls, a Cary 100 Bio (Varian Inc.) UV-Vis absorption spectrophotometer was used. The blank corresponds to 6 M guanidine hydrochloride.

For the assessment of the carbonylation pattern of analysis was performed on a G1600A (Agilent) capillary electrophoresis (CE) with diode-array detector (DAD) controlled by Agilent ChemStation ver. B.0 2.0x software.

**Methods**

A 64.5 cm (effective length 56 cm) x 75 μm i.d. fused-silica separation capillary column was used for electrophoretic experiments; the column was thermostated at 25°C all along the analysis.

Before the first use the capillary was conditioned by flushing with 0.1M NaOH for 60 min, then with water for 30 min, and finally with the background borate buffer 20 mM for 20 min.

Electrophoretic separation was performed using an aqueous electrolyte system (20 mM borate buffer) with pH 9.0. Before use, the electrolyte solutions and samples were filtered through a 0.45 μm microfilter and degassed for 10 min. At the beginning of each analysis the capillary was washed for 5 min with 0.1M NaOH, 5 min with water and 15 min with the 20 mM borate buffer. Injection was performed in the hydrodynamic way at 0.5 psi for 10s, and the applied voltage was 25 kV. The detection was done at 370 nm, 365 and 214 nm.

**Statistical analysis**

Carbonyl content was expressed as nmol carbonyl/mg protein. Carbonyl concentration was calculated using the Levine’s formula [10, 16], using the maximum absorbance at 360-380 nm and the molar extinction coefficient of the hydrazones (22,000 M⁻¹cm⁻¹). The protein concentration of each sample was determined using Lowry’s method [17]; the spectral method using the UV absorbance at 280 nm was discarded due to interferences (such as DNA). The concentration was expressed as mg/mL. The mean carbonyl concentration and protein concentrations were determined based on triplicate determination for each sample. Results are expressed as means ± standard deviation (SD).

**Results and discussions**

**Spectrophotometric analysis**

UV absorption spectrum of DNPH-derivatised protein carbonyl (fig. 2) revealed a broad UV-VIS absorption band (360-380 nm), suggesting that after derivatization with DNPH a mixture of DNPH-derivates is obtained, not only a single compound.

The carbonylation process is influenced by different factors, therefore in the first stages we investigated three in vitro hydroxyl radical generating systems on the carbonyl formation yield for the influence of BSA concentration and the influence of exposure time.

Carbonyl formation was dependent on the BSA concentration and for all three hydroxyl radical generating systems; the content of carbonyl proteins was significantly higher compared with physiological levels. According to Dale-Done et al. [4], the physiological levels of protein carbonyls in the plasma range within 0.5 to 4.0 nmol/mg proteins, while in the serum between 0.1 to 1.0 nmol/mg proteins, and in human brain cortex vary between 1.5 to 6.4 nmol/mg proteins.

The sensibility of the method (assessed by successive dilutions) revealed that 75 mg protein generated enough carbonyl protein to be quantified if the carbonylation is done using system potassium ascorbate/ferric chloride.

The influence of time exposure was studied on BSA with the three oxidizing systems from 1 to 18 h using 5 mg/mL BSA solution. Experimental results (fig. 3) revealed that the system potassium ascorbate/ferric chloride induced the best yield for carbonyl generation, after 9 h degradation time, compared with the other two hydroxyl radical generating systems. After 9 h of incubation the average content of carbonyl groups in the oxidized BSA with the system potassium ascorbate/ferric chloride corresponds to 30.11 nmol carbonyl/mg of protein.

Carbonyl formation was time-dependent up to 9 h, at 37°C. Experimental results (fig. 4) revealed that prolonging incubation time more than 9 h results in a decreasing tendency, which is correlated with their high reactivity.

**Preliminary investigation of the BSA carbonylation pattern**

The spectrophotometric assay, based on derivatization with DNPH, quantified the global carbonyls/mg of protein, without making any distinction between carbonyl proteins. Using CE/DAD analysis it was possible to separate the DNPH-derivates.

CE/DAD analysis at 370 nm (fig. 5) confirmed a the presence of more than 10 derivates. Our CE/DAD results are in line with other published studies on degraded BSA...
samples, where the components of the mixture were identified using MALDI and ESI-MS [18].

Two instrumental conditions were considered critical for this separation: the internal diameter of the capillary and the choice of running buffer. The capillary with internal diameter 75 μm was selected taking into account that DNPH-derivates could have a large molecular weight. A classical capillary with an internal diameter of 50 μm will not be appropriate as the DNPH-derivates could physically block the capillary. As running buffer we selected the pH 9.0 20 mM borate buffer with taking into account its low conductivity, the structure of carbonyl derivates and their global pKa. We should underline here that the 6 M guanidine hydrochloride, used for the solubilisation of DNPH-derivates, exhibited another positive effect, preventing the

![Fig. 4. Influence the time of exposure on the levels of carbonyl proteins](image)

![Fig. 5. Electrophoregram of DNPH- carbonilated peptides obtained from degraded BSA (1 mg/mL) with 1 mM iron(III) chloride/25 mM sodium ascorbate; (detection at 365 nm)](image)

![Fig. 6. Electrophoregram of DNPH- carbonilated peptides (detection at 214 nm)](image)

<table>
<thead>
<tr>
<th>Carbonyl derivates</th>
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<th>Area</th>
<th>Height</th>
<th>Width</th>
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![Table 1. CARBONYL-DNPH COMPONENTS OF DEGRADED BSA OBTAINED BY CAPILLARY ELECTROPHORESIS (PEAKS AT 370 nm)](image)
adsorption of derivates on the negative charged capillary wall.

Figure 5 revealed that after derivatization with DNPH, a complex mixture of hidrazones was obtained. Using detection at 370 nm and 365 nm, as much as twelve DNPH derivates were separated in the BSA degraded samples with the system potassium ascorbate/iron(III) chloride.

The same 12 derivates were obtained in more than 20 different samples, this indicating the reproducibility of the simulated carbonylation process.

According to table 1, all derivates migrated in less than 10 minutes proving a good velocity, which is correlated with small molecular weight.

The peptide nature of the derivates was confirmed by the signal at 214 nm (fig.6). CE/DAD method showed superior specificity and sensitivity in the detection of DNPH-derivates compared with the classical spectrophotometry.

Conclusions

The carbonylation of protein is a complex process that reflects the level of oxidative stress. Results revealed that the system potassium ascorbate/iron(III) chloride induced the best yield for carbonyl generation, compared with the other two tested hydroxyl radical generating systems (iron (II) sulfate/ascorbic acid and cooper sulfate/ hydrogen peroxide). Carbonyl formation was time-dependent in the range investigated (up to 9h), at 37°C. Prolonging incubation time more than 9 h resulted in decreased yield, attributed to their high reactivity. Carbonyl formation was dependent on the BSA concentration of the samples, a 5 mg/mL solution being found as optimum for the in vitro degradation studies. Spectrophotometric analysis of protein carbonyl using DNPH derivatisation method revealed a broad UV-Vis absorption band (360-380 nm), suggesting the formation of a mixture of DNPH derivates. The CE-DAD capillary method (64.5 cm (effective length 56 cm) x 75 μm i.d. fused-silica capillary column, 20 mM borate buffer pH 9.0, hydrodynamic injection at 0.5 psi for 10s, 25 kV running voltage, detection at 370 and 214 nm) indicated a complex mixture of twelve DNPH-derivates. The separated derivates migrated in less than 10 minutes, proving a good velocity and small molecular weight. The peptide nature was confirmed by the signal at 214 nm.

Further investigation are needed in order establish a pattern of human albumin carbonylation process.

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References


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