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Journal of Chromatography A, 1134 (2006) 122–133
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Identification and quantification of protein carbonylation using light and heavy isotope labeled Girard's P reagent

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Received 15 July 2006; received in revised form 21 August 2006; accepted 30 August 2006
Available online 22 September 2006

Abstract

Protein carbonyls are one of the most widely studied markers of oxidative stress. Determining increases in the concentration of protein carbonyls known to be associated with neurodegenerative diseases, heart disease, cancer and ageing. Identification of carbonylation sites in oxidized proteins has been a challenge. Even though recent advances in proteomics has facilitate the identification of carbonylation sites in oxidized proteins, confident identification remains a challenge due to the complicated nature of oxidative damage and the wide range of oxidative modifications. Here, we report the development of a multiplexing strategy that facilitates confident carbonylated peptide identification through a combination of heavy and light isotope coding and a multi-step filtering process. This procedure involves (1) labeling aliquots of oxidized proteins with heavy and light forms of Girard's reagent P (GPR) and combining them in a 1:1 ratio along with (2) LC/MS and MALDI-MS/MS analysis. The filtering process uses LC/MS and MALDI-MS/MS data to rule out false positives by rejecting peptide doublets that do not appear with the correct concentration ratio, retention time, tag number, or resolution. This strategy was used for the identification of heavily oxidized transferrin peptides and resulted in identification 13 distinct peptides. The competency of the method was validated in a complex mixture using oxidized transferrin in a yeast lysate as well as oxidized yeast. Twenty-five percent of the peptides identified in a pure oxidized sample of transferrin were successfully identified from the complex mixture. Analysis of yeast proteome stressed with hydrogen peroxide using this multiplexing strategy resulted in identification of 41 carbonylated peptides from 36 distinct proteins. Differential isotope coding of model peptides at different concentrations followed by mixing at different ratios was used to establish the linear dynamic range for quantification of carbonylated peptides using light and heavy forms of GPR.

Keywords: Proteomics; Protein carbonyls; Oxidized protein identification; Oxidized protein quantification; Heavy isotope labeled; Multiplexing; Strong cation exchange chromatography; Girard P reagent; Oxidized protein; Peptide sequencing, and transferrin oxidation; Yeast oxidized proteins

1. Introduction

There is accumulating evidence that oxidative stress and the concomitant production of reactive oxygen species (ROS) plays an integral role in a number of human diseases (e.g. Alzheimer disease, Parkinson's disease, arteriosclerosis, cataracts) along with ageing [1–3]. ROS generated as a result of electron leakage from the mitochondrial respiratory chain or other physiological and non-physiological processes can oxidatively modify all major groups of biomaterials including nucleic acids, proteins, lipids, and carbohydrates [4,5].

Proteins are one of the most important targets based on their high concentration in cells and complications associated with their loss of function. Among the many possible oxidative modification of proteins, irreversible introduction of carbonyl groups is one of the more common. Protein carbonyls are generated directly as a result of amino acids side chain oxidation and protein backbone cleavage or indirectly by Michael addition of 4-hydroxy-2-nonenal to protein lysine, histidine or cysteine residues or reaction of protein ammonium substituents with lipid peroxidation products.

Since reactive oxygen species are continuously generated both intracellularly and exogenously, a regulated level of oxidized proteins exists in a cell that precludes interference with cell function. However, elevation of oxidized protein levels initiates a series of cellar malfunctions that can lead to a disease state. Hence, quantification of protein oxidation to distinguish

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doi:10.1016/j.chroma.2006.08.096

healthy and disease states is of major importance. As one of the most common features of oxidative damage, protein carbonyls have been used as a marker for identification and quantification of protein damage.

Carbonylated peptides have been a major target for identification and quantification of oxidized proteins. Protein carbonyl groups have been quantified in several ways. One is by derivatization with 2,4-dinitrophenyhydrazine or tritiated borohydride followed by quantification with UV spectroscopy or radiography, respectively [6,7]. Protein carbonyl groups also react with hydrazine to from a Schiff base which can be reduced to stable secondary amines that are easily quantified. Another way is through derivatization with a fluorophore such as fluorescamine. The resulting secondary amine is fluorescent and has high molar absorptivity at 489 nm [8]. Immunological detection and quantification of protein carbonyl groups is another route. Protein carbonyl groups have been labeled with digoxigenin-hydrazide and detected by dot-blotting with an anti-digoxigenin antibody [9]. Use of anti-2,4-dinitrophenol antibodies and polyvinylidene difluoride membrane slot-blotting to capture 2,4-dinitrophenyhydrazine derivatized proteins that are detected with a peroxidase-labeled second antibody has also been reported [10]. Other methods developed for identification and quantification of oxidized proteins utilizes biotin hydrazide as a labeling reagent to react with protein carbonyls [11,12]. Biotinylated proteins were then recognized with 2-D gel electrophoresis followed by avidin–fluorescein isothiocyanate (avidin-FITC) as a staining agent or multi-dimensional liquid chromatography and mass spectrometry [11,12].

All of these methods are limited by the fact that they do not readily allow identification and quantification of oxidation sites in proteins. This is of critical importance in the study of disease associated protein oxidation. Recently, we developed a new method for labeling and enrichment of carbonylated peptides using GPR [13]. This reagent was originally developed to solubilize steroids [14] and later found to enhance MALDI ionization efficiency in mass spectral analyses [15]. Structural features of GPR critical to its analytical efficacy are the presence of a quaternary amine group that enhances ionization and a hydrazide group that reacts with carbonyl groups in proteins. The solubilizing properties of this reagent becomes especially important for oxidized proteins since they can be of reduced solubility due to cross-linking, denaturation and backbone cleavage [16]. Presence of the quaternary amine also allows enrichment of tagged peptides by strong cation exchange chromatography (SCX) [17].

Even though GPR has several important advantages over other labeling strategies for carbonylated proteins, it still suffers from inherent problems associated with complex post translational modifications such as oxidative damage. Oxidative modification targets more than 13 amino acid side chains to produce more than 30 new structures. This multiplicity problem increases the probability of false positives enormously during databse searches of the MSMS spectra. Here we report a new strategy for labeling protein carbonyls using light and heavy isotope coded GPR that allows confident identification of carbonylated proteins via multi-step filtering process [18]. Relative quantification of carbonylated peptides was also accomplished for the first time using isotopically labeled GRP.

2. Experimental procedures

2.1. Materials

Synthetic peptides Ac-Leu-Leu-Met-Aldehyde and Ac-Ile-Glu-Pro-Asp-aldehyde were purchased from BACHEM Biosience Inc. (King of Prussia, PA). Sodium cyanoborohydride ($NaBH_3CN$) and trifluoroacetic acid (TFA) were purchased from Pierce Co. (Rockford, IL). 1-(2-Hydrazino-2-oxoethyl) pyridinium chloride (Girard's P reagent), absolute ethanol (Abs. EtOH), anhydrous deuterated pyridine (C_5D_5N), ethyl chloroacetate ($ClCH_2CO_2Et$), hydrazine (NH_2NH_2), dithiothreitol (DTT), trypsin, N-alpha-tosyl-L-lysine chloromethyl ketone (TLCK), IGEPAL CA-630 nonioninc detergent, YPD-media and apo-transferrin (human) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile (ACN), Iron(III) chloride, potassium chloride, magnesium chloride, ascorbic acid, Sodium phosphate, urea, calcium chloride were purchased from Mallinkrodt. (St. Louis, MO). 218TP54 reversed-phase C_{18} column was purchased from VydacTM (W. R. Grace & Co.-Conn. Columbia, MD). DE44H10426 Zorbax reversed-phase C_{18} column (0.5 mm × 150 mm) was purchased from Agilent Technologies Inc. (Palo Alto, CA). The TSK Chelate-5PW column (7.5 mm × 7.5 mm) was purchased from TosoHaas (Montgomeryville, PA). The reversed-phase chromatography (RPC) analyses were done on a BioCAD 20 Micro-analytical Workstation (PE Biosystems, Framingham, MA). The LC system used in conjunction with mass spectrometer was an Agilent 1100 series instrument. LC/MS mass spectral analyses were done using a Sciex QSTAR^{TM} hybrid LC/MS/MS Quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). MALDI-TOF-TOF mass spectra were acquired on a 4700 Proteomics Discovery System (Applied Biosystems, Foster City, CA). All spectra were obtained in the positive ion mode.

2.2. Methods

2.2.1. Synthesis of deuteriated Girard's reagent P

1-(2-Hydrazino-2-oxoethyl)pyridinium chloride (Girard's P reagent) was synthesized according to the protocol by Girard [14]. Abs. EtOH (20 mL), $ClCH_2CO_2Et$ (9.84 g, 80 mmol) and anhydrous C_5D_5N (d_5-pyridine) (3.97 g, 47 mmol) were mixed and the mixture was refluxed for 12 h. After that an additional 2.83 g of d_5-pyridine was added and the mixture was allowed to stand until a test on acidification confirmed the complete conversion of $ClCH_2CO_2Et$. $NH_2NH_2{\cdot}H_2O$ (4 g, 80 mmol) was then added with shaking, and, after the violent reaction had subsided, the mixture set to a mass of crystaline 1-(2-Hydrazino-2-oxoethyl)pyridinium chloride (14.63 g).