

Novel method to investigate protein carbonylation by iTRAQ strategy

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Abstract This paper reports a novel methodology for relative quantitative analysis of carbonylation sites in proteins by exploiting a new isobaric tag for relative and absolute quantitation (iTRAQ) derivative, iTRAQ hydrazide (iTRAQH), and the analytical power of linear ion trap instruments (QqLIT). Because of its operational simplicity, avoiding time-consuming enrichment procedures, this new strategy seems to be well suited for quantitative large-scale proteomic profiling of carbonylation.

Keywords Protein carbonylation · iTRAQ · Quantitative proteomics

Introduction

Carbonylation of proteins is a common type of damage resulting from oxidative stress in living cells [1, 2]. It is induced by a variety of reactive oxygen species (ROS) as well as by-products of lipid oxidation [3, 4].

Several proteomic methods for the identification of carbonylated proteins have been reported. Six different classes

of reagents used to target carbonylated peptides were reported in a recent review [5]. Among these, biotin hydrazide, introduced by Regnier [6] in 2005, has been quite broadly used to study oxidized proteins in yeast [7], rats [8], and humans [9].

However, in the fast-progressing post-genomic age there is a growing interest in quantitative proteomic studies [10]. A number of methods, aimed both at the identification and quantification of oxidation sites in proteins, have recently been described. The use of light and heavy deuterium labeled Girard's Reagent P (1-(2-hydrazino-2-oxoethyl)pyridinium chloride) was introduced by Mirzaei and Regnier [11]. Differently oxidized samples were reacted with GRP- d_0 and GRP- d_5 and analyzed, after an affinity enrichment step, by reversed-phase high-performance chromatography (RP-HPLC) and tandem mass spectrometry for peptide identification. Carbonylated peptides appeared as doublet clusters of ions separated by 5 Da or multiples thereof in relation to the number of carbonyls that were present in the peptide [11].

In a similar way relative quantification studies have been suggested to be carried out by light and heavy $^{13}\text{C}_6$ -labeled-dinitrophenylhydrazine (DNPH). Carbonylated peptides appear, this time, as doublet clusters of ions separated by 6 Da or multiples thereof [12].

A carbonyl-specific reagent, O-ECAT (oxidation-dependent element coded affinity tags), which can be loaded with various rare earth metals thus allowing differential quantitation of oxidized samples, was designed by Lee et al. [13].

The widely used biotin hydrazide proteomic approach has been turned into quantitative analysis by labeling the trypsin-digested peptides, arising from affinity-enriched carbonylated proteins, with isobaric tags for relative and absolute quantitation (iTRAQ) reagent(s) [14, 15].

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All the above methods suffer from several drawbacks particularly in terms of recovery and reproducibility because of the erratic yields of affinity purification steps which are involved in most of them. Furthermore, it is well known that in the biotinylation approach, biotin tags reduce the ionization efficiency and alter the fragmentation pattern of the modified peptide causing a loss in signal intensities and in the quality of MS/MS spectra.

Our research group recently reported an innovative RIGHT (Reporter Ion Generating Tag) approach for the selective analysis of protein carbonylation, involving dansyl labeling of carbonylation sites [16]. Unfortunately no isotope coded dansyl reagents are commercially available to directly extend the above procedure into a quantitative one. We therefore sought to expand the chemistry of iTRAQ reagents by synthesizing iTRAQ hydrazide (iTRAQH) in order to set up a novel methodology for the selective labeling and relative quantitative analysis of carbonyl groups in proteins. In other words we suggest to avoid the double labeling procedure and the use of an affinity step as reported elsewhere [14, 15] and to directly label the carbonylated sites by using isobaric reagents, which are more sensitive than isotopic tags [17]. Furthermore the tremendous analytical potential of precursor ion scan (PIS) analysis in combination with the high sensitivity of linear ion trap instruments can be exploited, thus avoiding the enrichment steps.

Experimental

Materials

Trypsin, α -cyano-4-hydroxycinnamic acid, sodium hypochlorite, standard proteins (bovine ribonuclease A (RNase A), chicken egg lysozyme, horse myoglobin, horse carbonic anhydrase, bovine serum albumin), ammonium hydrogen carbonate (AMBIC), and hydrazine were from Sigma (St. Louis, MO). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Romil (Waterbeach, Cambridge, UK). iTRAQ molecules were from ABSciex (Foster City, California, USA).

Preparation of standard oxidized RNase A (OX-RNase A)

Lyophilized RNase A was dissolved in a 5 mM NaClO solution to a final concentration of 0.5 mg/ml. The reaction was carried out at 37 °C for 15 min. Carbonylated proteins were desalted by size exclusion chromatography on a Sephadex G-25 M column (GE Healthcare). Protein elution was monitored at 220 and 280 nm. Protein fractions were collected, lyophilized, and stored at -20 °C.

iTRAQH synthesis

iTRAQH was synthesized from iTRAQ 114 and 117 (1 nmol/ μ l dissolved in ethanol) and hydrazine (50 nmol/ μ l in ethanol) (molar ratio 2:1). The reaction was carried out at room temperature for 6 h. The product was purified by RP-HPLC on an Agilent Zorbax C18 column (4.6 mm \times 150 mm) (Palo Alto, California) using a 10 % to 65 % linear gradient in 20 min from water to ACN and verified by MALDI mass spectrometry.

Carbonyl labeling with iTRAQH

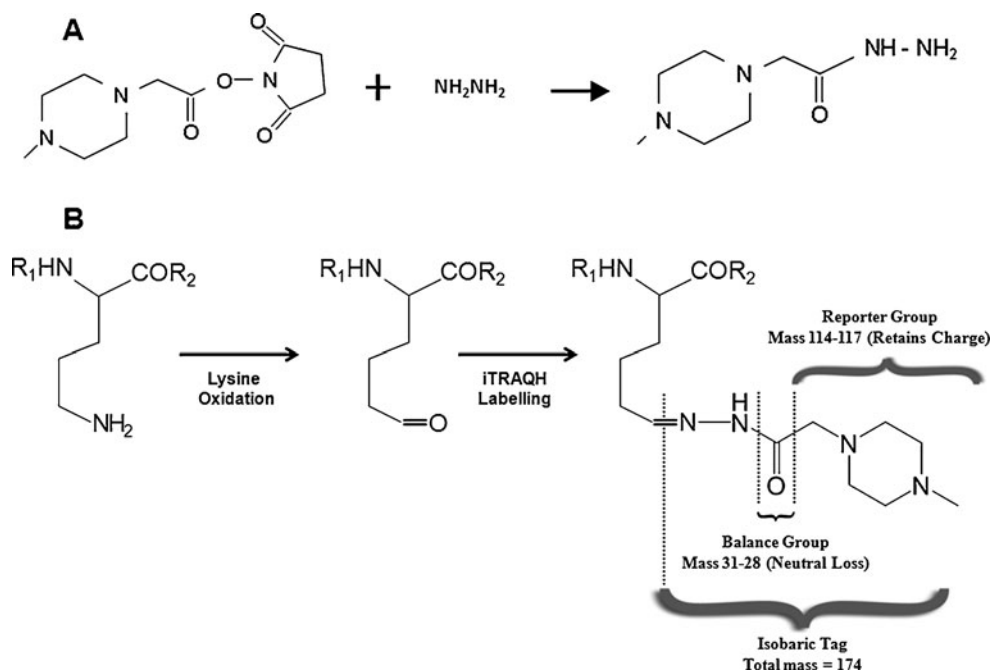
OX-RNase A was denatured and reduced as previously described [16] and then dissolved in 50 mM AMBIC, pH 8.0. Trypsin digestion was performed using an enzyme/substrate ratio of 1:50 w/w at 37 °C for 16 h. Peptide samples were lyophilized and then dissolved in 100 μ l of 10 % H₂O, 6.5 % of TFA, 83.5 % ACN. An equal volume of a 5 mM iTRAQH solution in ACN was then added and the solutions were allowed to react at 37 °C for 18 h.

Mass spectrometry

MALDI experiments were performed on a Voyager-DE STR MALDITOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (337 nm). A 1- μ l aliquot of the total mixture was mixed (1:1, v/v) with α -cyano-4-hydroxycinnamic acid 10 mg/ml in ACN/50 mM citrate buffer, 70:30 v/v.

NanoLC-MS/MS experiments were performed on a 4000QTrap mass spectrometer (Applied Biosystems), a QqLIT instrument, coupled to a 1100 nanoHPLC system (Agilent Technologies). Peptide mixtures were loaded onto an Agilent reversed-phase pre-column cartridge (Zorbax 300 SB-C18, 5 \times 75 μ m, 3.5 μ m) at 20 μ l/min with solvent A (0.1 % formic acid, 2 % ACN in water, loading time 5 min). Peptides were then separated on an Agilent reversed-phase column (Zorbax 300 SB-C18, 150 mm \times 75 μ m, 3.5 μ m) at a flow rate of 300 nl/min using 0.1 % formic acid, 2 % ACN in water as solvent A and 0.1 % formic acid, 2 % water in ACN as solvent B. The elution was accomplished by a 7–50 % linear gradient of solvent B in 50 min. A nano-ionspray source was used at 2 kV with liquid coupling, with a declustering potential of 50 eV, using an uncoated silica tip (o.d. 150 μ m, i.d. 20 μ m, tip diameter 10 μ m) from New Objectives (Ringoos, NJ). Spectra acquisition was based on a survey precursor ion scan for m/z 114/117. The Q₁ was scanned from 400 to 1,400 in 2 s with “low” resolution, and the precursor ions were fragmented in q₂ using a linear gradient of collision energy from 30 to 80 eV. Finally, Q₃ was set to transmit only ions at m/z 114/117 with “unit” resolution. This scan mode was followed by

Fig. 1 **a** Reaction scheme for the synthesis of iTRAQH. **b** Modification of α -aminoadipic semialdehyde residues by iTRAQH



an enhanced resolution experiment for the ions of interest and then by enhanced product ion (EPI) experiments for the three most abundant species. MS² spectra were acquired using the best collision energy calculated on the basis of m/z values and charge state (rolling collision energy), scanning the mass range from 100 to 1,400 m/z at 4,000 amu/s [18].

Results and discussion

A novel iTRAQ derivative, namely iTRAQ hydrazide (iTRAQH) (Fig. 1a), was designed by simply reacting iTRAQ, as its *N*-hydroxysuccinimide activated ester [17], with an excess of hydrazine. It is worth noting that the formation of the hydrazide derivative does not

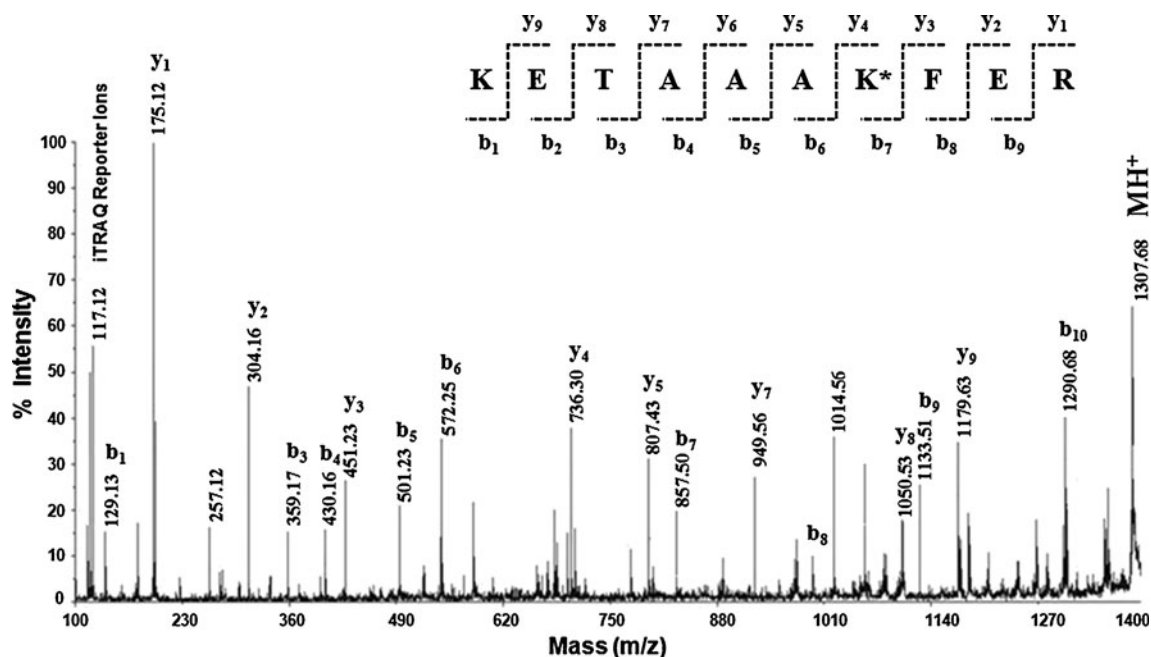
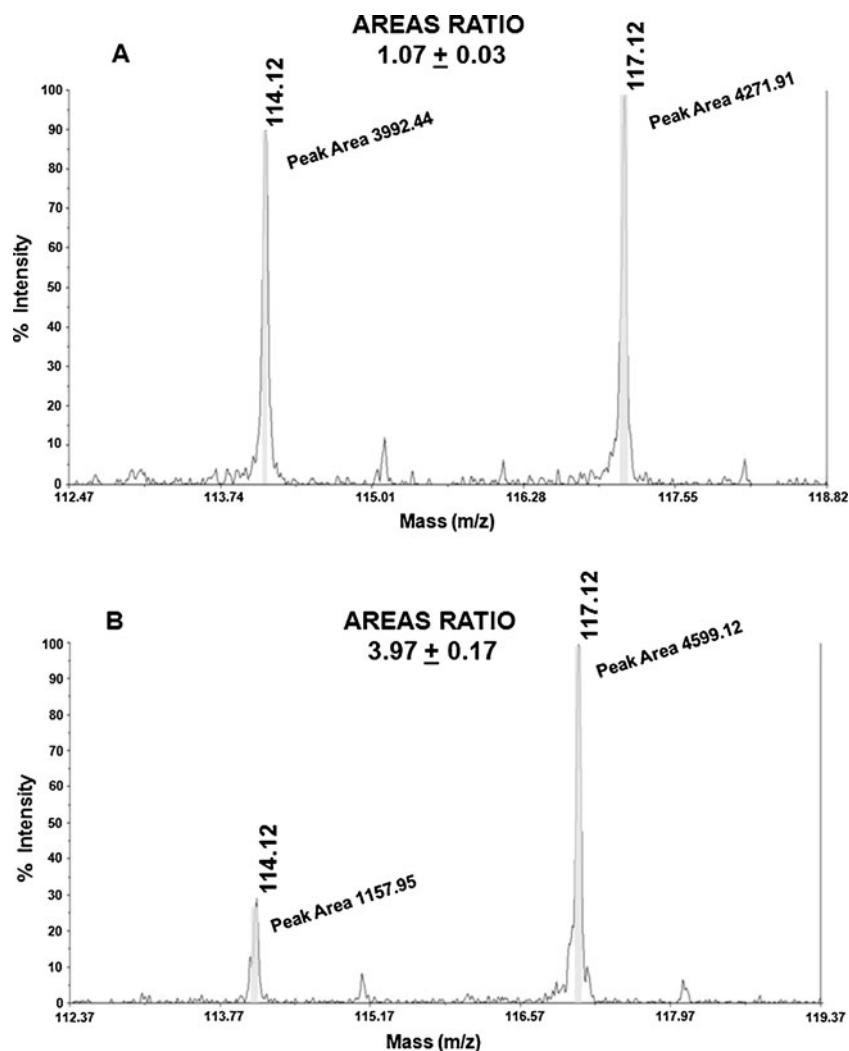


Fig. 2 MS/MS spectrum of the precursor ion at m/z 1,307.7 attributed to iTRAQ-modified oxidized peptide 1–10 of RNase A. The spectrum contains all the expected fragments of y and b series and by manual interpretation it was possible to reconstruct the entire sequence of the

peptide 1–10 of RNase A retaining the modified residue. The modification site on the lysine residue in position 7 was inferred. The low mass region of the spectrum showed the presence of the signals of iTRAQ reporter ions that could be used for quantitative analysis

Fig. 3 Magnified sections of MS/MS spectra of modified peptide 1–10 of RNase A for 1:1 (**a**) and 1:4 (**b**) mixtures. The signals of reporter ions of iTRAQ can be used for quantitative analysis of carbonylated peptides



alter the isobaric nature of iTRAQ reagents as shown in Fig. 1.

In order to set up proof-of-concept experiments we attempted to prepare standard carbonylated derivatives of five model proteins (bovine ribonuclease A, chicken egg lysozyme, horse myoglobin, horse carbonic anhydrase, bovine serum albumin) by using reported oxidation protocols [19] with different oxidants (i.e., hypochlorite and/or $\text{Fe}^{2+}/\text{EDTA}/\text{H}_2\text{O}_2$). We aimed to selectively oxidize specific residues, without undesired side reactions, and to introduce only a small number of modifications, in a controlled way, to mimic the *in vivo* sub-stoichiometric conditions. Unfortunately, MALDI analysis of the resulting oxidized species was satisfactory only for RNase A and lysozyme. However, in the latter case, the generated carbonyl moiety occurred at the N-terminus thus forming a cyclic imine [16] and rendering the modified residue unavailable to react with hydrazine-derived reagents.

In the case of myoglobin, carbonic anhydrase, and bovine serum albumin, MALDI analysis of samples, either directly

and/or after tryptic digestion, showed that the parent proteins were either oxidatively degraded and/or underwent side-chain modifications unrelated to the carbonyl formation (data not shown). This result was not surprising because of the complex pattern of reactions generated by radical species [17]. Therefore only carbonylated RNase A, prepared as described in the **Experimental** section (OX-RNase A), was used to set up the method.

MALDI mapping of OX-RNase A led to the verification of about 95 % of protein sequence and showed the occurrence of a single anomalous signal at m/z 1,149.63, thus showing that the rest of the protein remained unaffected. The m/z 1,149.63 signal was assigned to the oxidized peptide 1–10, possibly suggesting the conversion of a single lysine residue to α -aminoaldehyde. Moreover, the presence in the MALDI mapping spectrum of a signal at m/z 1,150.61, assigned to the unreacted peptide 1–10 of RNase A, showed that the oxidation reaction was sub-stoichiometric. We considered the latter result quite in

line with our intent because both the presence and absence of oxidized species in the preparation actually mimic the sub-stoichiometric nature of the oxidative modifications [3].

To test the effectiveness of the procedure for relative quantitation of carbonylated residues, fixed amounts of OX-RNase A, in the nanomolar range, were digested with trypsin and reacted with either iTRAQH 114 or iTRAQH 117; the two different peptide mixtures were then mixed in a 1:1 and 1:4 ratio, respectively, and analyzed.

MALDI mapping showed the occurrence of a signal at m/z 1,307.7 ($\Delta M=+158$ Da) assigned to the carbonylated peptide 1–10 modified with iTRAQH resulting in the formation of a hydrazone moiety, thus confirming the presence of a real carbonylation moiety in the 1–10 sequence of RNase A.

Because of the isobaric nature of iTRAQ molecules (Fig. 1b), differentially labeled peptides were indistinguishable by MS analysis. The iTRAQ reporter ions generated following fragmentation events allowed the quantitative analysis by using MS/MS analysis. The two peptide mixtures were analyzed by LC-MS/MS experiments in precursor ion scan (PIS) mode using the ions at m/z 114 or 117 (i.e., the iTRAQ reporter ions) as targets for precursor selection. By exploiting the capability of the PIS mode, one can avoid the need for any enrichment step prior to LC-MS/MS analysis.

To gain information about the carbonylation site, a second stage of mass analysis is performed. The MS/MS spectrum (Fig. 2) led to the reconstruction of the peptide sequence with y ions and b ions retaining the modified residue, clearly showing that carbonylation occurs at the level of the lysine in position 7, whereas the lysine in position 1 remains unmodified.

Moreover the presence of the iTRAQ reporter ions in the low mass region of the MS/MS spectrum allowed us to perform the relative quantitative analysis. Figure 3 shows a magnified section of an MS/MS spectrum for both mixtures, in which the signals of iTRAQ reporter ions are shown. By replicating these experiments it was possible to calculate an experimental ratio of 1.07 ± 0.03 and 3.97 ± 0.17 for the 1:1 and 1:4 mixtures, respectively. These preliminary data demonstrate the feasibility of this method for relative quantitative analysis of protein carbonylation.

Conclusions

This communication reports preliminary results aimed at setting up a new methodology for the selective qualitative and quantitative analysis of carbonylation sites in proteins by exploiting a novel iTRAQ-derived reagent. The strategy

suggested here takes substantial advantage of QqLIT instruments to select specific labeled peptides giving rise to diagnostic MS^2 product ions. Owing to its operational simplicity this new strategy seems to be well suited for the (a) direct identification of modified residues avoiding any enrichment step and (b) relative quantitation of carbonylated residues in different cellular states up to 4 or 8, given the availability of iTRAQ 4-plex and 8-plex reagent kits. The reagent described when used in combination with other isotopically labeled tags, such as TMT [20], can provide quantitative data which take into account the change of protein abundances in relation to the extent of carbonylation.

We have outlined a simpler and more effective approach for quantitative proteomics studies in the field of protein carbonylation with respect to other methods so far suggested [11–15]. The use of iTRAQH on real samples is currently under investigation by analyzing carbonylation in different protein yeast extracts under different oxidative stress conditions.

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