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APPLICATION NOTE

A New Strategy of Using O^{18}-Labeled Iodoacetic Acid for Mass Spectrometry-Based Protein Quantitation

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Abstract

A new O^{18} labeling protocol is designed to assist quantitation of cysteine-containing proteins using LC/MS. Unlike other O^{18} labeling strategies, the labeling is carried out at the intact protein level (prior to its digestion) during reduction/alkylation of cysteine side chains using O^{18}-labeled iodoacetic acid (IAA). The latter can be easily prepared by exchanging carboxylic oxygen atoms of commercially available IAA in O^{18}-enriched water at low pH. Since incorporation of the O^{18} label in the protein occurs at the whole protein, rather than peptide level, the quantitation results are not peptide-dependent. The excellent stability of the label in mild pH conditions provides flexibility and robustness needed of sample processing steps following the labeling. In contrast to generally costly isotope labeling reagents, this approach uses only two relatively inexpensive commercially available reagents (IAA and H_2O^{18}). The feasibility of the new method is demonstrated using an 80 kDa human serum transferrin (hTf) as a model, where linear quantitation is achieved across a dynamic range spanning three orders of magnitude. The new approach can be used in quantitative proteomics applications and is particularly suitable for a variety of tasks in the biopharmaceutical sector, ranging from pharmacokinetic studies to quality control of protein therapeutics.

Key words: Pharmacokinetics, Stable isotope labeling, Protein quantitation

Introduction

Mass spectrometry (MS) has already become an indispensable tool in quantitative proteomics and is beginning to enjoy popularity in other areas where reliable protein quantitation is critical, such as pharmacokinetic studies of protein drugs [1, 2]. To achieve accurate quantitation, most of the MS-based methods apply differential stable isotope labeling to introduce the internal standard with a specific isotope label. Although the isotope label can be introduced with relative ease in prokaryotic systems metabolically by using isotope-enriched (e.g., N^{15} or C^{13}) cell culture medium, production of labeled proteins in eukaryotic expression systems is significantly more costly and labor-intensive [3, 4]. Alternative methods of placing isotopic label on internal standards include production of surrogate standards [5], or chemical derivatization of specific amino acid residues using isotope labeling reagents (such as ICAT [6], iTRAQ [7]). Enzyme-catalyzed O^{18} labeling [8] (as well as its modifications that use acid catalysis [9–11]) is another elegant way to introduce isotope labeling. All those approaches have gained wide acceptance in the field of quantitative proteomics, and also provide powerful tools for targeted protein quantitation, especially in the pharmacokinetic studies of protein drugs. Currently, the most commonly applied method for targeted protein quantitation is known as absolute quantification of proteins (AQUA) [12], in which synthesized stable isotope-labeled peptides are used as internal standards. However, an intrinsic disadvantage of using synthesized peptide standards for protein quantitation is the combination of the standards and endogenous peptides at the very end of the process, which inevitably introduces more sources of quantitation error [13]. Although the use of isotope-labeled protein standards [14] can

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eliminate this problem, its application is limited due to high costs and difficulties of producing eukaryotic protein standards.

We propose a new O^{18} labeling strategy where the labeling is carried out at the intact protein level by using O^{18}-labeled iodoacetic acid (IAA) to cap the cysteine residues. Since the labeling is done at the intact protein level, the sample and the internal standard can be combined at the very beginning of the process, which eliminates possible discriminatory effects and quantitation errors caused by differential processing of the protein within the sample and the internal standard prior to MS analysis. Feasibility of the new method is demonstrated using as a model an 80 kDa protein non-glycosylated human serum transferrin (hTf), which is a part of a number of biopharmaceutical products that are currently under development [15].

Experimental

Non-glycosylated human serum transferrin (hTf) was provided by Professor Anne B. Mason (University of Vermont College of Medicine, Burlington, VT, USA); H_2O^{18} (97 % purity), iodoacetic acid (IAA), and proteomic-grade trypsin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used in this work were of analytical grade or higher.

The O^{18}-labeled IAA solution was prepared by dissolving 80.0 mg of IAA in 495 μL of O^{18} enriched water and incubating in the presence of 1 % trifluoroacetic acid at 50 °*C* for 1 day. Progress of O^{18} incorporation into IAA was monitored by an API-2000 triple quadrupole mass spectrometer (AB SCIEX, Toronto, Canada) in negative ion mode. Following pH adjustment to near-neutral level, the O^{18}-labeled IAA solution was sealed to prevent the oxidation from air and covered by aluminum foil to avoid light, and kept at −20 °*C* for future use.

The O^{18} labeling of hTf was introduced by alkylation of free cysteines. Briefly, 20 μg of hTf was prepared in 200 μL of 100 μM ammonium bicarbonate (pH 8.0), and then reduced with 10 mM of DTT at 50 °*C* for 45 min. The alkylation of cysteine residues was followed by addition of O^{18}-labeled IAA stock solution to a concentration of 30 mM, and incubation at 50 °*C* for 45 min in the dark. The digestion was initiated by addition of trypsin at a 20:1 substrate to enzyme ratio and followed by incubation at 37 °*C* for overnight.

To investigate the back exchange of O^{18}-labeled peptides under typical RP-HPLC conditions and near neutral pH, a 20 μL aliquot of the tryptic digests was diluted into 100 μL of H_2O^{16} with 0.1 % formic acid or 100 μL of 100 mM ammonium bicarbonate, respectively, and sampled hourly or daily for LC/MS/MS analysis.

To use this strategy for protein quantitation, two batches of hTf were alkylated by O^{18}-labeled and unlabeled IAA, respectively. Right after the alkylation, two samples were mixed in ratios of 1:50, 1:20, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, 20:1, 50:1 and digested by trypsin using the procedure outlined above.

The digested sample was analyzed with LC/MS/MS using an LC Packings Ultimate (Dionex/Thermo Fisher Scientific, Sunnyvale, CA, USA) nano-HPLC system coupled with a QStar-XL (AB SCIEX, Toronto, Canada) hybrid quadrupole/TOF MS. A previously reported setup and method were used for the nano-LC/MS/MS analysis [9].

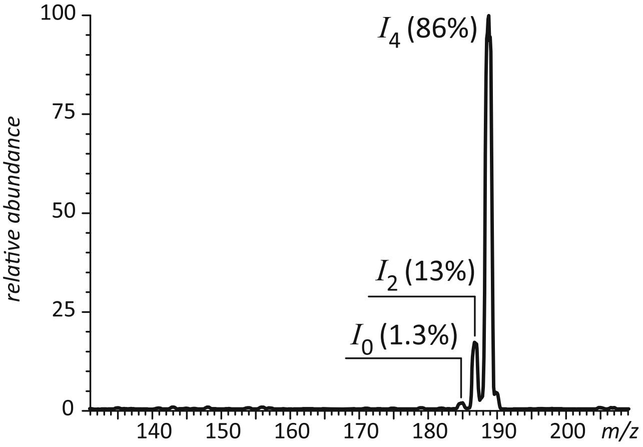

Figure 1. ESI MS O^{18}-labeled IAA in the negative ion mode showing the isotopic distribution of IAA anion

Results and Discussion

The O^{18}-labeled IAA acid was prepared by exchanging oxygen atoms in its carboxylic group with H_2O^{18} at low pH. The labeling results are shown in Figure 1, where the isotopic distribution of IAA after 1 day's incubation at 50 °*C*, in the presence of 1 % TFA, shows >85 % exchange of both carboxylic oxygen atoms and >98 % exchange of at least one oxygen atom. These numbers yield the overall extent of O^{18} incorporation being 93 %, whereas the theoretical value is calculated to be 93.9 % after taking into account the presence of residual O^{16} in the reaction mixture. The consistent results suggested that the O^{18} exchange between IAA and water had already reached the equilibrium. The presence of iodide ions were also observed in the mass spectra, which was quite common even for freshly prepared IAA solution.

The alkylation of cysteine residues with the O^{18}-labeled IAA was further investigated using hTf as the model protein, which has 38 cysteine residues. Figure 2 presents a nano-LC/MS/MS analysis of the mixture of tryptic digests from two batches of hTf capped by unlabeled and O^{18}-labeled IAA, respectively. The mass spectrum (Figure 2b) of a tryptic peptide KPVEEYANC*HLAR indicates a maximum mass shift of 4 Da between the unlabeled (black trace) and the labeled (grey trace) peptides. Extracted ion chromatograms plotted for their most abundant isotopic peaks, I_0 and I_4 (Figure 2a), clearly show identical elution time for the O^{18}-labeled peptide and its unlabeled counterpart, which is critically important for reliable quantitation. The tandem MS data (Figure 2c and d) suggest that the presence of IAA moiety on a peptide ion does not interfere with its identification using CID (contrary to ICAT labeling [16]). Furthermore, the isotopic distributions of y_4^+ and y_5^+ ions provide clear indication as to the localization of O^{18} atoms within the peptide (cysteine residue).

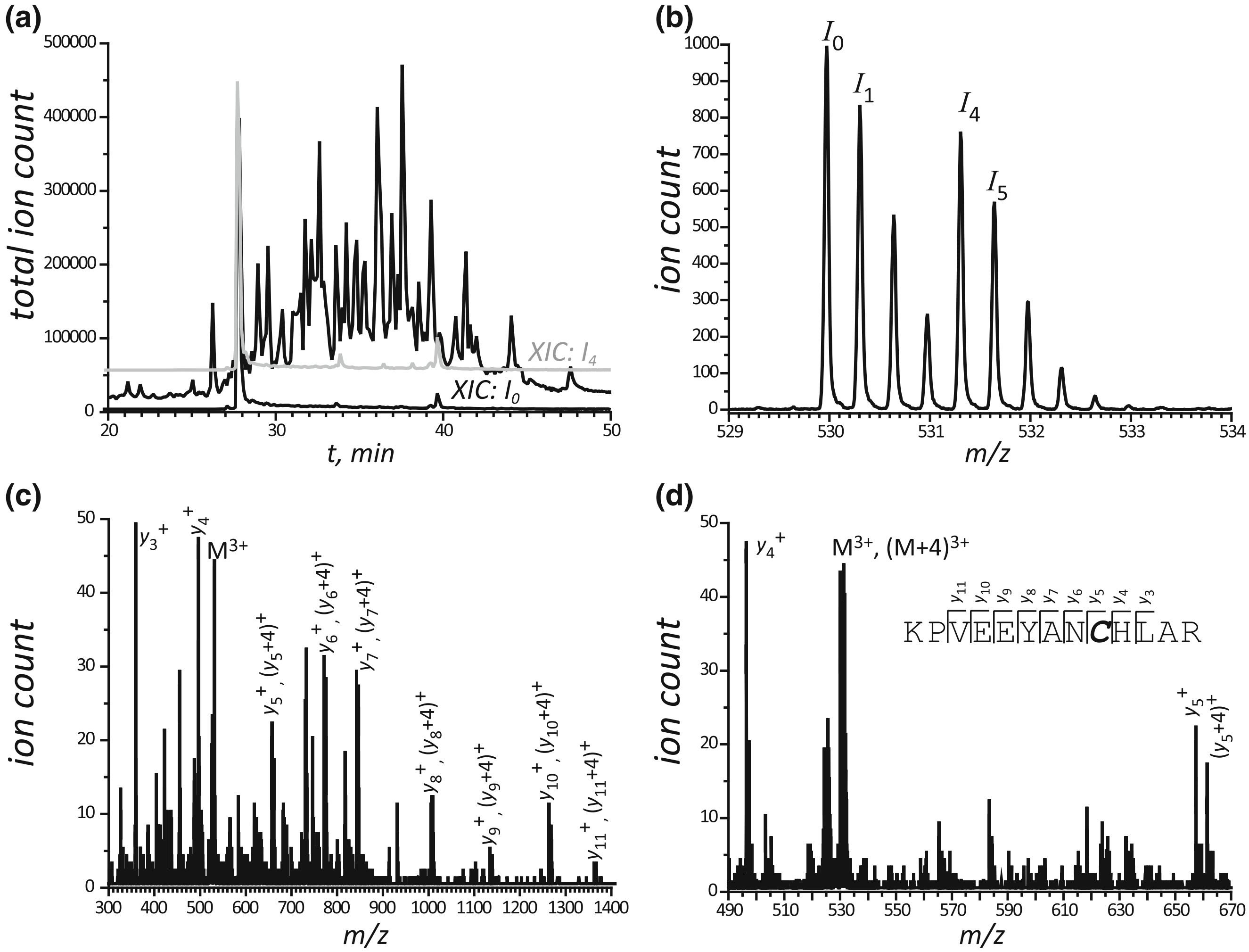

Figure 2. Nano-LC/MS/MS analysis of tryptic peptides derived from a mixture of hTf capped with unlabeled and O^{18}-labeled IAA. **(a)** Total ion chromatogram of the digest and extracted ion chromatograms of peak I_0 and I_4 representing unlabeled (black trace) and O^{18}-labeled (gray trace) tryptic fragment KPVEEYANC*HLAR. **(b)** Isotopic distribution of a triply charged peptide ion KPVEEYANC*HLAR. **(c)** MS/MS (CID) of peptide ion KPVEEYANC*HLAR shown in **(b)**. **(d)** A zoom view of the MS/MS spectrum **(c)** showing y_4^+ and y_5^+ fragment ions

The O^{18} labeling of other cysteine-containing tryptic peptides is summarized in Table 1. For peptides containing one cysteine, the full labeling products (with both two O^{18} atoms incorporated) made up to 87 %–90 %, consistent with the labeling results of IAA (vide supra). Unlike enzyme-catalyzed O^{18} labeling which has peptide-specific O^{18} incorporation rate [17], the use of O^{18}-labeled IAA generates almost the same labeling efficiency for all the cysteine-containing

Table 1. Efficiency of O^{18} Incorporation into Cysteine-Containing Tryptic Fragments of Human Serum Transferrin (hTf)

Tryptic Peptides	Percentage of differentially O^{18}-incorporated peptides				
	0×O^{18}	1×O^{18}	2×O^{18}	3×O^{18}	4×O^{18}
KPVEEYANC*HLAR	<1 %	11 %	89 %	N/A	N/A
WC*ALSHHER	<1 %	12 %	88 %	N/A	N/A
DYELLC*LDGTR	<1 %	13 %	87 %	N/A	N/A
C*LKDGAGDVAFVK	<1 %	10 %	90 %	N/A	N/A
SVIPSDGPSVAC*VKK	<1 %	11 %	89 %	N/A	N/A
DLLFRDDTVC*LAK	<1 %	10 %	90 %	N/A	N/A
FDEFFSEGC*APGSK	<1 %	10 %	90 %	N/A	N/A
WC*AVSEHEATK	<1 %	13 %	87 %	N/A	N/A
C*STSSLLEAC*TFR	<1 %	<1 %	1.8 %	17 %	81 %
EGTC*PEAPTDEC*KPVK	<1 %	<1 %	2.0 %	18 %	80 %
IEC*VSAETTEDC*IAK	<1 %	<1 %	2.0 %	17 %	81 %

peptides. This is particularly helpful in quantitative measurements when the mass shift is not sufficient to completely separate the isotopic clusters of the labeled and unlabeled peptides [18]. The ratio between unlabeled and labeled peptides can be easily estimated by the following equation:

$$peptide\ ratio = \frac{I_1}{I_5 \cdot (1+\alpha)},$$

where α (α=0.14 in this work) is a fraction of partially labeled peptides (only one O^{18} atom incorporated); and I_1 and I_5 represent the observed relative intensities of the second isotopic peaks for the unlabeled peptides and fully O^{18}-labeled peptides, respectively (see Figure 2b).

Since the O^{18} labeling is introduced at the protein level, any consecutive steps, including purification, digestion, and LC/MS analysis will become sources of back exchange by exposing the O^{18} labeled carboxylic acid group in O^{16} water. Thus, the stability of the O^{18} labeling was investigated at the RP-HPLC conditions (0.1 % FA) as well as at pH 8 for tryptic digestion (see Supplementary Material). The relative abundance of the isotopic peak I_2 (as percentage of the total ionic signal) was calculated and monitored over time. No noticeable back exchange was observed in RP-HPLC conditions for at least 8 h, or under near neutral pH for at least 6 d. The good stability of O^{18} labeling under near neutral pH allows overnight tryptic digestion as well as any desired purification process as long as it does not require a long period of time in extreme pH conditions. Furthermore, the good stability of O^{18} labeling in RP-HPLC conditions allows the feasibility of applying long LC gradient separation for complex samples.

To demonstrate the feasibility of using O^{18}-labeled IAA for protein quantitation, we generated hTf capped by unlabeled and O^{18}-labeled IAA at various ratios covering three orders of magnitude, and performed nano-LC/MS analysis. The quantitation was achieved using two cysteine-containing tryptic peptides from hTf respectively (see Supplementary Material). High correlation of the theoretical and experimentally observed protein ratios was achieved (R^2>0.99). Two reference peptides both showed good linearity in up to three orders of magnitude. Furthermore, the quantitation results generated by these two peptides were highly consistent, highlighting the reliability of the quantitation.

In contrast to enzyme-catalyzed O^{18} labeling, which universally incorporates O^{18} into carboxylic terminus of each peptide, the O^{18}-labeled IAA only targets the cysteine-containing tryptic peptides and, therefore, cannot be applied to quantitate proteins containing no cysteine residues. Nevertheless, even the most conservative estimates of the fraction of completely cysteine-free proteins in the proteomes of higher organisms put this number as low as 8 % [19], while the SWISS-Prot database puts the number of human proteins that have at least one cysteine residue in the sequence as high as 97 %. Obviously, the frequency of occurrence of cysteine residues in prokaryotic organisms is significantly lower, but quantitation of prokaryotic proteins can be easily achieved by generating isotopically labeled internal standards at the protein expression step [4].

Conclusion

O^{18}-labeled IAA was prepared in O^{18}-enriched water at low pH, and then used for MS-based protein quantitation. This approach offers several advantages over enzyme catalyzed O^{18} labeling. First, full labeling can be easily achieved at the equilibrium of the oxygen exchange reaction. Furthermore, the O^{18} incorporation efficiency for peptides is consistent rather than peptide-specific, which makes the 'unlabeled/labeled' calculation much easier. Finally, the combination of unlabeled and labeled samples at protein level excludes all sources of quantitation error introduced afterwards. The feasibility of this method was demonstrated by using hTf as the model protein; linear quantitation with dynamic range spanning three orders of magnitude was achieved. Due to the thiol-specific feature, its utility is limited to cysteine-containing proteins. However, since only a very small percentage of eukaryotic proteins contain no cysteine residues, this approach can still be generally applied to most of the protein quantitation works. Furthermore, virtually all of the existing protein therapeutics contain cysteine residues, making this new technique particularly suitable for tasks ranging from pharmacokinetic studies to quality control of biopharmaceutical products, a vast emerging field in protein quantitation [1].

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