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Quantification of proteins using $^{13}C_7$-labeled and unlabeled iodoacetanilide by nano liquid chromatography/nanoelectrospray ionization and by selected reaction monitoring mass spectrometry

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ABSTRACT

The combination of cysteine-specific modifiers, iodoacetanilide (IAA) and $^{13}C_7$-labeled iodoacetanilide ($^{13}C_7$-IAA), has been applied to absolute quantification of proteins. The selected reaction monitoring (SRM) with the use of nano liquid chromatography/nanoelectrospray ionization ion trap mass spectrometry (nano LC/nano-ESI-IT-MS) analysis was applied to precise quantification of three commercial proteins. Good correlation was observed between the theoretical ratios and observed ratios for all these proteins both in a simple buffer solution and in a complex protein environment. Due to efficient tagging, this method does not require separate synthesis of isotope-labeled peptides for the SRM studies. Therefore, this method is expected to be a useful tool for proteomics research.

Proteomics studies a set of proteins in complex protein mixtures, and identification and quantification of sample protein constitutes an essential part of modern proteomic studies. Proteomic technologies have undergone considerable growth recently. One of the most common approaches is a so-called 'shotgun approach', in which the sample proteins are digested to sets of peptides with the use of enzymes, followed by mass spectrometric (MS) analysis. This approach has been a norm for a long time, allowing comprehensive detection of hundreds to thousands of proteins through mass spectrometric analysis in biological samples, and has a potential to discover new protein candidates for clinical studies, for example. However, drawbacks of this approach include limited sensitivity and rather poor reproducibility, resulting in only partial overlap of sets of proteins identified from significantly similar analytes. The latter shortcoming is a particular problem, for example, for systems biology, which requires consistent identification and precise quantification of sets of proteins from multiple samples.

In recent years, selected reaction monitoring (SRM) has emerged, complementing this shotgun approach.[1] In this SRM approach, a predefined precursor ion called signature ion and one of its fragments are selected, and monitored for more accurate quantification. SRM is particularly useful when predetermined sets of proteins need to be more precisely quantified across multiple samples consistently and reproducibly, as well as for absolute quantification of protein samples.

Earlier, we reported methodologies for both identification and relative quantification of proteins with the use of combinations of stable isotope-labeled and unlabeled small organic molecules that specifically react with the sulfhydryl group of cysteine residues of peptides or proteins followed by analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[2–7] or nano liquid chromatography/nanoelectrospray ionization ion trap mass spectrometry (nano LC/nano-ESI-IT-MS).[8,9] The combinations we reported are d_5-labeled and unlabeled *N*-ethyl maleimides,[2,6–9] $^{13}C_6$ or $^{13}C_7$-labeled and unlabeled iodoacetanilides,[3,4,6–9] and d_7-labeled and unlabeled *N*-β-naphthyliodoacetamides.[5] We have demonstrated their applicability to identification and quantification for proteomics research in the context of the shotgun approach with certain advantages over existing methods.

Here we report the application to absolute quantification of peptides by the SRM approach by one of the combinations of the cysteine modifiers that we previously reported. The combination we chose for this study is IAA (**1**) and $^{13}C_7$-IAA (**2**), as these

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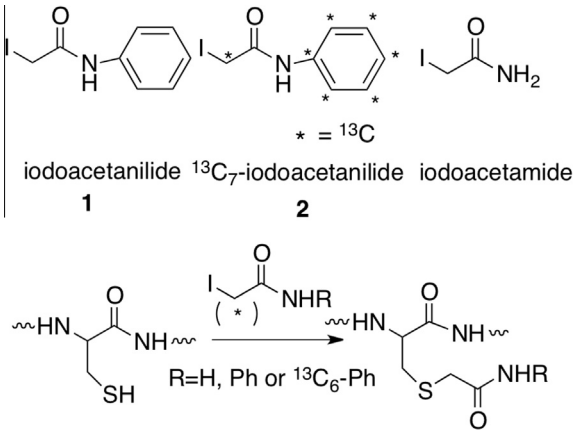
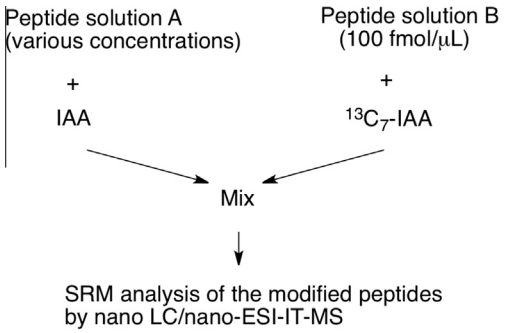
compounds were the most soluble in aqueous media. Furthermore, they were found not to be interfered by commonly observed isotope-effects under the LC conditions,[9] which are necessary for SRM. They are derivatives of the well-known commercially available cysteine modifier, iodoacetamide, with similar reaction mechanisms (Scheme 1). Precursor ions were selected, and a few of the most intense and most reliable collision-induced dissociation (CID) product ions were specifically monitored by nano LC/nano-ESI-IT-MS. Precise quantifications of three commercial proteins were attempted in this way. We used the nano LC/nano-ESI-IT-MS for this SRM study, as we had monitored many ion species in previous studies,[9] and the collision energy had been optimized to minimize the undesirable cleavage of the modifiers. IT-CID fragmentation spectra also tend to show higher signal and signal-to-noise ratios than QqQ (triple quadrupole)-CID, which is more commonly applied to SRM.[10]

In general, the first essential step for the SRM study is to select from tryptic peptides the pairs of precursor (signature) ions and their product ions, which are called 'transitions', to be monitored for quantitative analysis of the corresponding proteins. The quantification is performed by obtaining standard curves with the use of synthetic peptides, followed by the same operation with the sample tryptic peptides run against known amounts of synthetic peptides. In this study, we focused on the examination of feasibility of the quantitative analysis of synthetic peptides for three commercial proteins with the use of IAA (**1**) and $^{13}C_7$-IAA (**2**). Scheme 2 shows the workflow for the quantification of the peptides, hence the proteins, by our SRM approach. Two cluster peaks of the same peptide species modified with IAA (**1**) and $^{13}C_7$-IAA (**2**) were observed to be 7 Da apart due to the presence of seven ^{13}C atoms and were detected with the same retention time, which means that the isotope effects by the introduction of IAA and $^{13}C_7$-IAA do not exist in this system.[9] Therefore, we used



Scheme 1. Iodoacetanilide, $^{13}C_7$-iodoacetanilide, iodoacetamide and their reactions with cysteine.



Scheme 2. Procedure of quantification of proteins.

these two cluster ions of the same peptide species modified with IAA (**1**) and $^{13}C_7$-IAA (**2**) as precursor ions for SRM.

To select precursor ions and product ions for SRM, MS and MS/MS measurements were conducted using IAA (**1**)- or $^{13}C_7$-IAA (**2**)-modified tryptic peptides for three kinds of commercial proteins, bovine serum albumin (BSA), ovalbumin (OVA), and α-lactalbumin (LCA), by nano LC/nano-ESI-IT-MS and -MS/MS.[11] Each protein was confirmed as BSA, OVA, and LCA from the tryptic peptides modified with IAA (**1**) by Mascot MS/MS Ion Search. Precursor ions were chosen for the IAA (**1**)- or $^{13}C_7$-IAA (**2**)-modified tryptic peptide ions with the intense extracted ion chromatograms (EICs, data not shown) by the nano LC/nano-ESI-IT-MS among the IAA (**1**)- or $^{13}C_7$-IAA (**2**)-modified tryptic peptides identified at the stage of MS/MS. They were 487.8 and 491.3 for BSA modified with IAA (**1**) and that with $^{13}C_7$-IAA (**2**), respectively, 538.2 and 545.2 for OVA modified with IAA (**1**) and that with $^{13}C_7$-IAA (**2**), respectively, and 786.4 and 793.4 for LCA modified with IAA (**1**) and that with $^{13}C_7$-IAA (**2**), respectively, (Table 1).

Figure 1 shows MS/MS spectra of IAA (**1**)- or $^{13}C_7$-IAA (**2**)-modified tryptic peptides, which were detected from mixtures of IAA (**1**)-modified protein and $^{13}C_7$-IAA (**2**)-modified protein (1:1).[9] Both spectra of IAA (**1**) and $^{13}C_7$-IAA (**2**)-modified tryptic peptides from the same protein were quite similar, except for some peaks with a different *m*/*z* due to ^{13}C atoms as marked with '#'. Most b and y ions were detected, and the signature (precursor) ion peaks (marked by Ⓢ) did not appear intensely in any of the spectra. Moreover, these spectra indicate that the modifiers remained intact during CID in most cases, although some product ion peaks detected from LCA were characterized as peptides that include partial or whole cleavage of the modifiers.[12] According to the MS/MS spectra, we chose intense and stable product ion peaks for SRM as follows: *m*/*z* (from light/heavy precursor ions) 333.2 and 625.4/340.2 and 625.4 for BSA, 423.2/430.2 for OVA, and 465.2/472.2 for LCA (Table 1). All the product ions chosen for SRM, except *m*/*z* 625.4 ions observed from BSA, include one IAA (**1**) or one $^{13}C_7$-IAA (**2**).

As summarized in Table 1, the SRM *m*/*z* transitions were as follows: 487.8/333.2 (precursor^{2+}/b_2-NH_3) and 487.8/625.4

Table 1
Target proteins, their signature peptide sequences and MS/MS transitions in SRM

Protein name	MW (kDa)	Signature peptide[a]			SRM transition *m*/*z*
		Sequence	Charge	Theoretical monoisotopic *m*/*z*	
Bovine serum albumin	69	LC(+IAA)VLHEK	2	487.8	487.8 → 333.2, 625.4
		LC(+$^{13}C_7$-IAA)VLHEK	2	491.3	491.3 → 340.2, 625.4
Ovalbumin	43	C(+IAA)VSP	1	538.2	538.2 → 423.2
		C(+$^{13}C_7$-IAA)VSP	1	545.2	545.2 → 430.2
α-Lactalbumin	16	C(+IAA)EVFR	1	786.4	786.4 → 465.2
		C(+$^{13}C_7$-IAA)EVFR	1	793.4	793.4 → 472.2

[a] All peptides were synthesized, followed by being modified with IAA (**1**) or $^{13}C_7$-IAA (**2**).