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Synthesis of $^{13}C_7$-labeled iodoacetanilide and application to quantitative analysis of peptides and a protein by isotope differential mass spectrometry

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ABSTRACT

$^{13}C_7$-Labeled iodoacetanilide has been synthesized for specific labeling of sulfhydryl groups of cysteine residues and has been successfully applied to quantitative analysis of peptides and a commercial protein in combination with ^{13}C-unlabeled iodoacetanilide and a MALDI mass spectrometer. Subsequent tandem mass spectrum analysis revealed that $^{13}C_7$-labeled iodoacetanilide remained intact during the collision-induced dissociation (CID) conditions.

Development of methodologies for quantitative analysis of proteins or peptides has been an important part of proteomics research, which studies proteome, a set of proteins expressed under certain external stimuli. In particular, recently, stable isotope-labeling of proteins and subsequent mass spectrometry analysis has been emerging as a powerful tool for quantitative analysis and identification of a set of proteins.[1] Classical examples include metabolic stable isotope-labeling[2] or stable isotope-labeling during proteolytic digestion of proteins.[3] More recently, stable isotope-tagging of specific amino acid residues by chemical reactions is becoming a versatile method applicable to a wide variety of protein samples.[4] Perhaps the most pioneering work is exemplified by the isotope-coded affinity tags (ICAT)[4f] and various other stable isotope-tagging methods developed subsequently, such as the iTRAQ method.[4g] These methods typically apply liquid chromatography (LC) for separation and purification of labeled tryptic peptides from digestion of the sample protein mixture, and the identification of proteins relies on tandem mass spectrometry analysis of these tryptic peptides.[5] However, several fundamental problems have been reported, such as primary isotope-effects leading to differential elution during the LC and cleavage of the labels during the measurement of tandem mass spectrometry. Therefore, we have been developing our methodology with the use of gel electrophoresis instead of LC and stable isotope-labeled and unlabeled small organic molecule tagging.

In the past we demonstrated that several combinations of cysteine-specific modifiers can be successfully applied to quantitative analysis of peptides and proteins by soft ionization mass spectrometry, such as MALDI TOF mass spectrometry[6–9] and ESI mass spectrometry.[10] These combinations include D_5-labeled and unlabeled *N*-ethyl maleimides,[6,9,10] $^{13}C_6$-labeled and unlabeled iodoacetanilides,[7,9,10] and D_7-labeled and unlabeled *N*-β-naphthyliodoacetamides.[8] Among them, we are finding that the iodoacetanilides are the most useful due to their reactivity and hydrophilicity.

Here we report synthesis of $^{13}C_7$-iodoacetanilide, **1**, and its application to quantitative analysis of peptides and a protein in combination with the corresponding ^{13}C-unlabeled iodoacetanilide, **2**, and a MALDI mass spectrometer. The combination of these iodoacetanilides allows 7 Da difference instead of 6 Da which we reported earlier, and therefore, we expected that the combination of $^{13}C_7$-labeled and unlabeled iodoacetanilides, **1** and **2**, would provide sharper separation and serves as an even more useful tool for proteomics research. We also demonstrate that an advanced mass spectrometer with high sensitivities, MALDI TOF/TOF, provides more accurate quantitative analysis of a protein and peptides as well as enables tandem mass spectrometry analysis of amino acid sequences of peptides, thus offering more confidence in protein identification.

Iodoacetanilide is a derivative of a well-known cysteine modifier, iodoacetamide, which specifically reacts with the sulfhydryl group of the cysteine residue (Scheme 1).

The $^{13}C_7$-labeled iodoacetanilide, **1**, was synthesized from $^{13}C_6$-labeled aniline and ^{13}C-labeled iodoacetic acid (Scheme 2),

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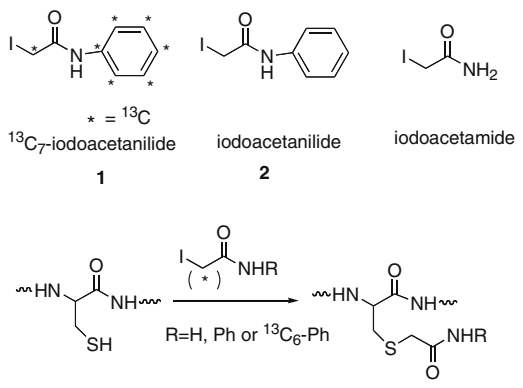
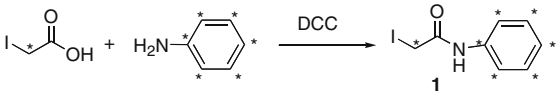


Scheme 1. $^{13}C_7$-Iodoacetanilide, iodoacetanilide, iodacetamide and their reactions with cysteine.



Scheme 2. Synthesis of $^{13}C_7$-iodoacetanilide.

and synthesis of ^{13}C-unlabeled iodoacetanilide, **2**, has been reported.[11,12]

We first tested the reactivity of this reagent with three synthetic cysteine-containing peptides with different amino acid sequences and molecular weights. These peptides are PEP 31, PEP 13, and PEP 60, and the sequences, monoisotopic masses, and estimated pIs are KEEPPHHEVPESETC, 1746.75 Da, 4.5 for PEP 31; SDTCSSQKTEVSTVSSTQK, 2001.92 Da and 6.2 for PEP 13; and ALVCEQEAR, 1017.49 and 4.4 for PEP 60.[13] Aqueous solutions of these peptides with pH values 9.0 were treated with these $^{13}C_7$-labeled or unlabeled iodoacetanilides, and the ion peaks of these solutions were analyzed by MALDI TOF MS after the addition of a matrix.

Figure 1 shows MALDI TOF mass spectra of PEP 31 itself (top) and a mixture of PEP 31 reacted with ^{13}C-unlabeled iodoacetanilide and PEP 31 reacted with $^{13}C_7$-labeled iodoacetanilide (bottom). The ion peaks show the monoisotopic mass for PEP 31 (1746.77 Da) and a series of isotope peaks that are one to several Da higher than the monoisotopic peak due to natural isotopes existing in PEP 31. The charts also demonstrate that after this peptide reacted with $^{13}C_7$-labeled or unlabeled iodoacetanilide, it increased 140 Da and 133 Da, respectively, showing that the combination of these modifications will lead to a difference of 7 Da. This difference provides sharper separation than we had reported before and is expected to be particularly useful for quantitative analysis of peptides with relatively high molecular weight.

Next, we applied these modifiers to quantitative analysis of the above three peptides. Several sets of aqueous peptide solutions

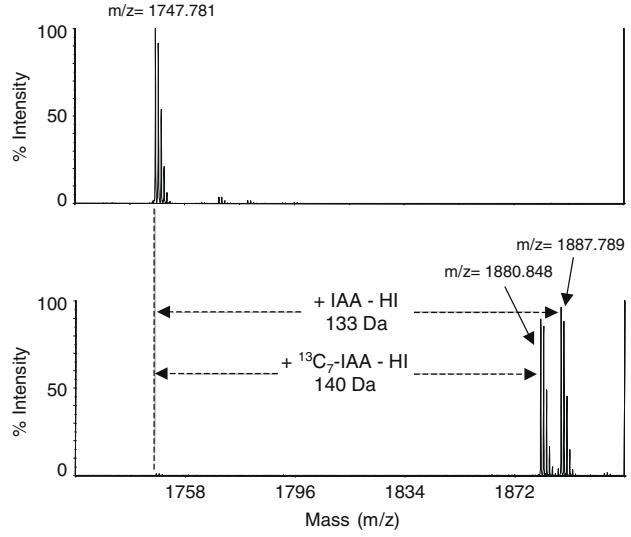


Figure 1. MALDI TOF mass spectra of PEP 31 (top) and a mixture of IAA–PEP 31 and $^{13}C_7$-IAA–PEP31 (bottom).

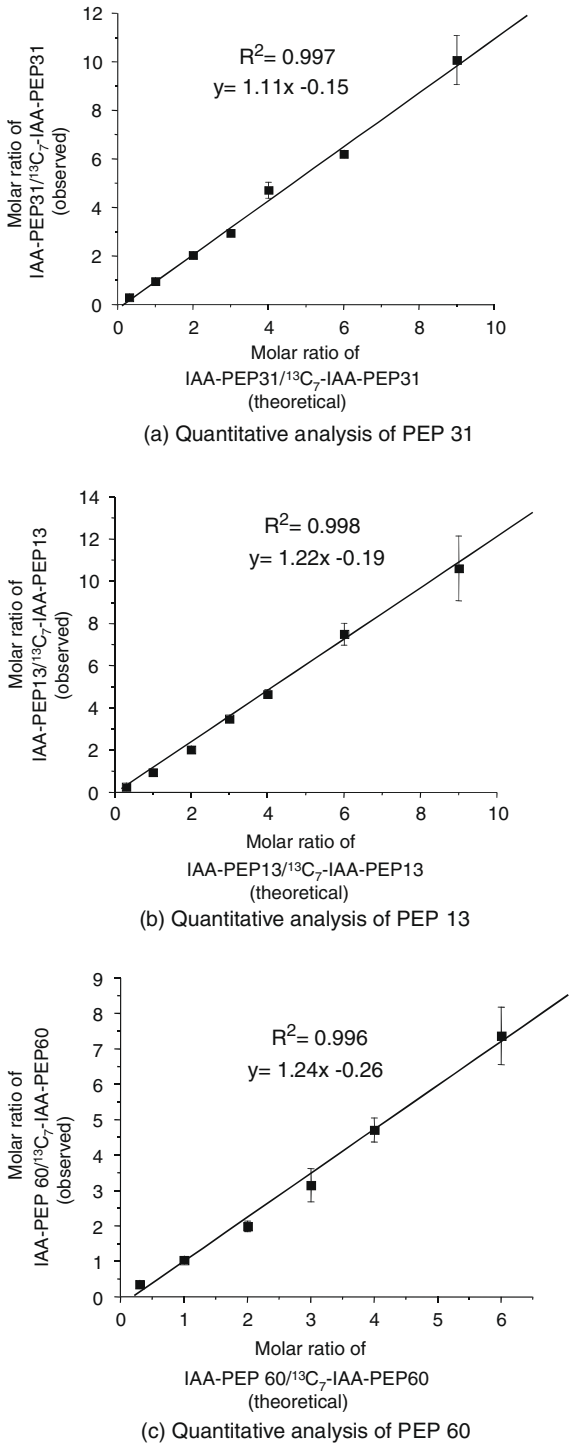


Figure 2. Quantitative analysis of three model peptides.

were prepared with pH 9, and these solutions were individually reacted with $^{13}C_7$-labeled or unlabeled iodoacetanilide. The differentially modified peptide solutions were mixed, and from the MALDI mass spectra of the mixture, the ratios of the S/N values of the monoisotopic peaks were calculated. In this way, the observed ratios of IAA-modified peptide to $^{13}C_7$-IAA-modified peptide were plotted against the theoretical ratios.[14] The graphs obtained in this way showed an excellent correlation between the theoretical ratios and observed ratios for all three peptides above (Fig. 2). Therefore the ionization efficiencies of the $^{13}C_7$-IAA-modified peptides and IAA-modified peptides are the same within experimental errors, and quantitative measurement of the relative molar ratios of the peptides in two different solutions is possible with high accuracy.

We next applied this set of modifiers to quantitative analysis of a commercial protein, bovine serum albumin (BSA).[15] The BSA solution was prepared with a concentration of 0.1 mg/mL, and modification of this protein solution was performed after the reduction with tributylphosphine (TBP). The IAA–modified BSA solution and $^{13}C_7$-IAA–modified BSA solution were mixed at various ratios, and they were purified by 1D SDS–PAGE. After in-gel digestion of the excised protein, the tryptic peptides were quantitatively analyzed.

Table 1 shows the number of the modified peptides that were detected easily for each theoretical ratio (IAA-modified/$^{13}C_7$-IAA-modified), observed ratio, and the standard deviation. The graph was obtained from the averages of these data (Fig. 3).

The correlation parameters and inclinations for all these peptides are close to 1.0. From these results, we can safely conclude that quantitative analysis of this protein is possible with high accuracy. It should also be noted that as in other sets of modifiers we reported earlier,[6–10] no isotope effect was observed for this quantitative analysis, although isotope effect is commonly reported with other sets of isotope-labeled and unlabeled modifiers.[16] Moreover, this upgraded MALDI mass spectrometer used for this study has greater sensitivity, allowing detection of various peptides at the attomol level;[17] therefore, we were able to detect a greater number of modified peptides than we reported before. When we previously tried quantitative analysis of the same BSA with the combination of $^{13}C_6$-IAA and IAA with a MALDI TOF, only a few alkylated peptides viable for quantitative analysis were observed, although BSA has 35 cysteine residues. Therefore, it appears that this upgraded MALDI mass spectrometer enables more accurate quantitative analysis of proteins.

Although our method allows identification of proteins by peptide mass fingerprinting, tandem mass spectrum analysis often provides more confident identification, especially for unknown proteins. Therefore with the use of MALDI TOF/TOF mass spectrometer, we subjected several peptides of BSA to the collision-induced dissociation (CID) conditions to examine the influence of $^{13}C_7$-IAA. The following MS/MS spectrum is an example of a cysteine-containing peptide modified with $^{13}C_7$-IAA (Fig. 4).[18] Most b and y fragments were found in the spectrum as expected, demonstrating that modification by this reagent does not affect the identification of fragments.[19] It is also a special advantage that the $^{13}C_7$-IAA remained intact during the CID-induced fragmentation,

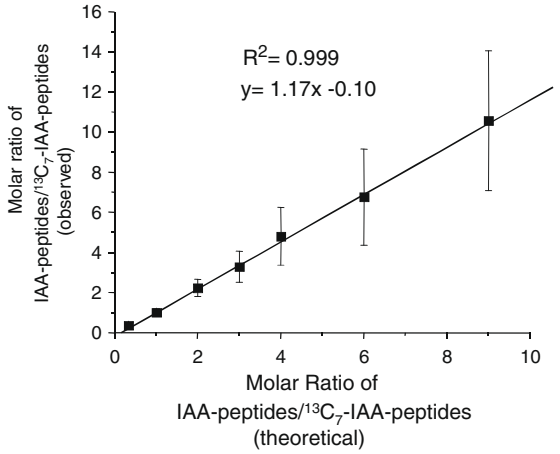


Figure 3. Quantitative analysis of bovine serum albumin (BSA).

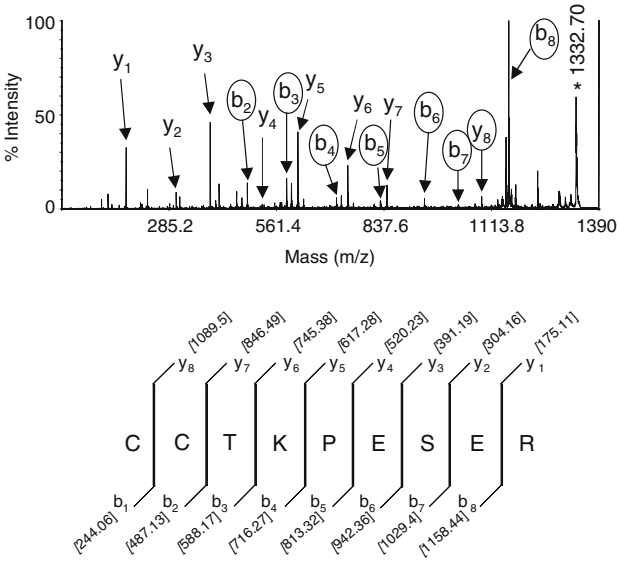


Figure 4. MS/MS spectra of a peptide from BSA. The asterisked peak indicates the precursor ion. Fragments containing $^{13}C_7$-IAA are circled.

as many other relatively large modifiers are reported to undergo fragmentation under the CID conditions.[20]

In summary, we have demonstrated that the combination of $^{13}C_7$-labeled and unlabeled iodoacetanilides as well as a MALDI mass spectrometer is a useful tool for quantitative analysis of peptides and a protein with high accuracy. We did not observe any isotope effects for quantitative analysis of a protein or peptides nor did we observe fragmentation of the labels during the collision-induced dissociation conditions for the tandem mass spectrometry. This new set of modifiers is expected to be useful for proteomics research including identification of proteins and quantitative analysis of peptides or proteins especially for those with

Table 1
The theoretical and observed ratios of IAA–peptide/$^{13}C_7$-IAA–peptide, the standard deviation, and the number of modified peptides detected.

Theoretical ratio IAA–peptide/$^{13}C_7$-IAA–peptide	0.35	1.00	2.00	3.00	4.00	6.00	9.00
Average of observed ratio IAA–peptide/$^{13}C_7$-IAA–peptide	0.38	1.02	2.23	3.29	4.81	6.77	10.57
Standard deviation	0.05	0.18	0.43	0.78	1.44	2.39	3.48
Detected modified peptides	18	20	20	19	19	16	14

relatively higher molecular weight, which typically show a greater number of isotopic peaks.[21]

Acknowledgements

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12. *The procedure for synthesis of $^{13}C_7$-iodoacetanilide is as follows*: ^{13}C-labeled iodoacetic acid (475 mg, 2.5 mmol, purchased from Cambridge Isotope Laboratories, Inc.) was dissolved in ethyl acetate (5 mL), and the mixture was cooled to 0 °C in an ice bath. To this solution, $^{13}C_6$-labeled aniline (250 mg, 2.5 mmol, purchased from Cambridge Isotope Laboratories, Inc.) was added, and dicyclohexylcarbodiimide (520 mg, 2.5 mmol) in ethyl acetate (5 mL) was added slowly by stirring. A white precipitate formed immediately after addition of the dicyclohexylcarbodiimide. The mixture was stirred at 0 °C for 30 min, and then at room temperature for an hour. The dicyclohexylurea was removed by celite filtration, and the filtrate was evaporated to dryness and purified by silica gel column chromatography (CH_2Cl_2–$CHCl_3$ = 1/1 then CH_2Cl_2) to afford the product (645 mg, 96% yield). The product was recrystallized from $CHCl_3$ (a yellowish solid, mp 143–144 °C). ^{1}H NMR (300 MHz, acetone-d_6) δ 9.54 (1H, br s), 6.82–7.97 (5H, m), 3.93 (2H, d, $^1J^*_{CH}$ = 151.8); ^{13}C NMR (75 MHz, acetone-d_6) 167.0, 140.1, 129.9, 124.9, 120.2, 0.9, HRMS *m/z* calcd for $^{12}C^{13}C_7H_8INO$ $(M+H)^+$: 268.9967, found: 268.9966.
13. PEP 13, 31 and 60 were purchased from Sigma–Aldrich, Japan, and Biomer Technology, CA, USA. The sequences of amino acids for these peptides are identical to those of peptides we reported earlier and referred to as MAT 13, 31, and 60, respectively.
14. *The typical procedures are as follows*: The 0.6 mM stock solutions of the peptides were prepared in Tris–HCl buffer (pH 9, concentration of Tris–HCl = 50 mM), and the 20 mM stock solutions of IAA and $^{13}C_7$-IAA were also prepared in DMSO. For the alkylation reaction, the peptide stock solution (2 μL) and IAA or $^{13}C_7$-IAA stock solution (2 μL) were mixed and left for one hour at room temperature. To this mixture, 2 μL of β-mercaptoethanol (BME, 25 mM) was added to stop the reaction. The IAA–peptide and $^{13}C_7$-IAA–peptide solutions prepared in this way were mixed in the molar ratios of IAA/$^{13}C_7$-IAA = 0.3, 1, 2, 3, 4, 6, and 9. These mixtures were diluted with 50% acetonitrile, 0.1% TFA and subjected to MALDI MS analysis by MALDI TOF/TOF 4800 plus™ (Applied Biosystems). The MS spectra were acquired automatically in the positive mode and a total of 1000 shots were accumulated per spectrum. The mass range was selected between 600 and 4000 *m/z*. Five data points were collected for each ratio, and the S/N values of the monoisotopic peaks of IAA or $^{13}C_7$-IAA modified peptides were used for calculation of the relative ratios. The averages of these data were plotted on the graph.
15. The modification of BSA was performed as reported previously.[9] The stock solution of BSA was prepared at a concentration of 0.1 mg/mL in a buffer containing 100 mM Tris–HCl, 3% SDS and 20 mM tributylphosphine (TBP), and the pH was adjusted to 8.5. To complete the reduction, this mixture was left for one hour at room temperature. The BSA solution (10 μL) was incubated with 1 μL of IAA (200 mM in DMSO) or 1 μL of $^{13}C_7$-IAA (200 mM in DMSO) and was shaken and kept in a dark place for two additional hours. The IAA–BSA and $^{13}C_7$-IAA–BSA solutions prepared accordingly were mixed in the molar ratios of IAA/$^{13}C_7$-IAA = 0.35, 1, 2, 3, 4, 6, and 9. The solution was mixed with 2 μL of a solution containing 20% (v/v) glycerol and 0.1% (w/v) bromophenol blue, and was directly subjected to 1D SDS–PAGE, and the separation was performed at 120 V for 90 min. The gel was stained with Coomassie Brilliant Blue overnight.The purified BSA spot was excised and subjected to in-gel digestion in the following manner. The Eppendorf tubes containing the protein bands were placed in a Thermomixer Comfort (Eppendorf). The protein bands were washed with 100 μL H_2O for 5 min at 37 °C and at 600 rpm. Next, they were washed with 100 μL of a solution H_2O/acetonitrile (50/50) for 5 min at 37 °C and at 600 rpm. The latter step was repeated until the dye was completely removed, and then the spots were incubated for one minute in 100 μL of 100% acetonitrile. After removal of the acetonitrile, the protein bands were dried for 15 min. Digestion was performed by the addition of 10 μL of an aqueous ammonium hydrogen carbonate (30 mM) containing 100 ng of trypsin (Promega Sequencing Grade Modified Trypsin, Promega Corporation, Madison, USA) at 37 °C overnight, and peptide extraction was performed as described previously.[9] The extracted peptide solution was dried by Speed Vacuum and kept at −20 °C for further analysis by the MALDI mass spectrometer.The dried samples were mixed with 10 μL of the solution containing 50% acetonitrile and 0.1% TFA. The digested sample (0.5 μL) was deposited on a MALDI-plate followed by the addition of 0.5 μL of a matrix consisting of 5 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% trifluoroacetic acid (TFA).
16. We discussed potential reasons for not observing isotope effects previously.[9,10]
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18. For the MS/MS analysis, the five strongest precursors with the S/N values higher than 50 in the MS spectrum were selected for further MS/MS analysis. The MS/MS was performed with the 1 kV positive mode under the collision-induced dissociation conditions. A maximum of 2000 laser shots were accumulated per MS/MS spectrum. A combination of MS and MS/MS data was used for a database search utilizing MASCOT (Matrix Science, V2.1). The following parameters were used for the MASCOT database search: enzyme, trypsin; allowed missed cleavages, 1; variable modification, oxidation of methionine. The mass tolerance for precursors was set to ±50 ppm and for MS/MS fragment ions to ±0.25 Da. The fixed modification was introduced for IAA-modified peptides (+133 Da) and also $^{13}C_7$-IAA modified peptides (+140).
19. Similar observations have been reported with the use of isotope-unlabeled iodoacetanilide. See Ref. 5a.

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21. We conducted quantitative analysis of the same peptides by nanoLC/ESI Ion Trap mass spectrometer (Brucker HCTultra) as well and obtained a similarly good correlation between the theoretical ratios and observed ratios as follows: PEP 31 (doubly charged) inclination = 1.05, R^2 = 0.996; PEP 31 (triply charged) inclination = 0.95, R^2 = 0.997; PEP 13 (doubly charged) inclination = 1.15, R^2 = 0.999; PEP 13 (triply charged) inclination = 1.03, R^2 = 0.946; PEP 60 (doubly charged) inclination = 1.12, R^2 = 0.996.