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Synthesis of d-Labeled *N*-Alkylmaleimides and Application to Quantitative Peptide Analysis by Isotope Differential Mass Spectrometry

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Abstract—d-Labeled *N*-alkylmaleimides have been prepared for specific modification of the terminal SH groups of cysteine residues in proteins or peptides. These reagents are useful tools for quantitative analysis of peptides by stable isotope differential mass spectrometry.

Quantitative measurement of a specific protein within a crude protein mixture is important for proteome analysis. Most methods hitherto reported for proteome analysis are performed by protein separation, usually by high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by peptide mass fingerprinting,[1] sometimes combined with peptide fragmentation analysis by MS/MS and/or with other supplementary data such as Edman degradation.[2] Quantification of proteins in such analysis can be done, for example, by two-dimensional densitometry of the gel,[3] by extracting the Coomassie blue-stained gel spots followed by spectrophotometry,[4] or by metabolic radioisotope labeling of the total proteins followed by counting the incorporated radioactivity.[5] These methods are laborious and apt to induce experimental errors.

Quantification of proteomes by mass spectrum analysis eliminates these laborious procedures due to its high sensitivity. Some successful examples have been reported, for example, for quantification of phosphopeptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) by Matsumoto et al.[6] More sophisticated methods of peptide quantification have been reported using stable isotopes.[7,8] Since isotopically-labeled specific residue modifiers have identical chemical properties, a set of isotopically labeled and unlabeled peptides provides an accurate measure of the relative abundance of the peptides (and hence proteins). Those methods utilize metabolic labeling with ^{15}N-enriched media[7] and deuterium-labeled isotope-coded affinity tags (ICATs).[8] Both of these methods produced encouraging results for quantitative analysis of proteins and peptides by mass spectrometry. The former method[7] involves a metabolic labeling which may not be suitable for the investigation of proteomes in higher mammals. The latter[8] utilizes chemical modification of extracted proteins, making it more widely applicable than the former method. Moreover, the use of affinity tags makes the analysis of a protein mixture possible, eliminating the use of 2D-PAGE. The disadvantage of the ICAT method, however, is that the ICAT reagents are not available commercially. Since our group has been routinely using 2D-PAGE for the initial display of proteins extracted from animal tissues, in our case, retinas from *Drosophila*,[9] bovines,[10,11] and rodents,[12,13] affinity tags are unessential.

Here, we describe a methodology for accurate quantification of a peptide as a first step toward quantitative proteome analysis by introducing a d-labeled SH group modifier followed by MALDI-TOF MS. In this method, both isotopically heavy and light peptides are prepared using d-labeled and unlabeled *N*-alkylmaleimides, and the relative quantities are measured by MALDI-TOF

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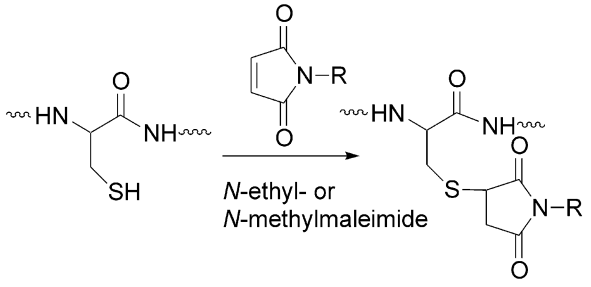
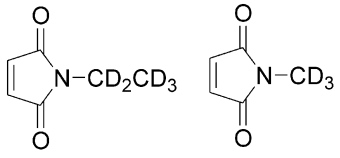
MS. Under optimized conditions, this method is expected to be less susceptible to interference caused by overlapping 2D-gel spots or by highly abundant proteins, as in most existing methods. In the cases where multiple cysteinyl residues are present in the same protein, this method will give multiple data points in a single run. Therefore, quantification is expected to be more accurate and efficient than with methods that require separation. Moreover, these d-labeled alkylmaleimides are small molecules and therefore react with SH residues efficiently, and the resultant peptides are expected not to change their physical properties such as solubility, hydrophilicity and hydrophobicity, and their synthesis is quite straightforward.

N-Alkylmaleimides are known to be useful reagents for specifically modifying terminal SH groups of cysteine residues in proteins (Scheme 1). In fact, *N*-ethyl- and *N*-methylmaleimide are commercially available, but no d-labeled *N*-alkylmaleimide is commercially available to date.



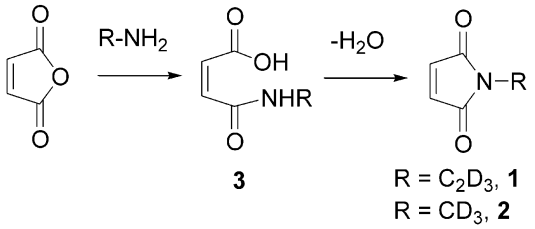
Scheme 1.

Therefore, we synthesized d-labeled *N*-ethylmaleimide (**1**) and *N*-methylmaleimide (**2**) starting from maleic anhydride and HCl salts of $C_2D_5NH_2$ and CD_3NH_2, which are commercially available, and applied them as a model system for quantification of a peptide. In particular, *N*-ethylmaleimide (**1**) is expected to be a useful reagent, since the d_5-*N*-ethylmaleimide-modified cysteinyl residue will give mass peaks that are 5 Da higher than unlabelled *N*-ethylmaleimide-modified cysteinyl residue peaks, which can be detected by widely available mass spectrometers such as ESI quadrupole MS or MALDI-TOF MS, while *N*-methylmaleimide (**2**) is still expected to be useful with high resolution mass spectrometry such as FT-ICR MS (Scheme 2).



Scheme 2.

N-Alkylmaleimides are generally prepared by dehydration of the corresponding maleamic acid (**3**) as in Scheme 3. The yields are usually not very high due to the high reactivity of the *N*-alkylmaleimide and potential rearrangements, while the maleamic acids (**3**) are often formed in reasonably high yields.



Scheme 3.

Several efficient methods are reported for dehydration of maleamic acids, such as applying thionyl chloride[14] or polyphosphoric acid.[15] However, the former method is accompanied by formation of α-chloro-*N*-alkylsuccinimide, and the latter method is laborious because the reaction mixture must be treated with extremely viscous polyphosphoric acid, which is difficult to weigh accurately. In our dehydrating method, we heated the reaction mixture in anhydrous acetic anhydride at 110 °C in the presence of anhydrous NaOAc, which is quite straightforward.

The d-labeled methyl- and ethylamines are commercially available as easily manageable HCl salts, which become free amines by mixing with triethylamine.[16] The maleamic acids were heated in acetic anhydride in the presence of NaOAc for about 15 min at 110 °C.[17] The corresponding *N*-alkylmaleimide was clearly observed as the main product. After purification by routine silica gel column chromatography and recrystallization, *N*-alkylmaleimides were obtained.[18] The procedure is quite mild and straightforward and does not require any special techniques.

In order to test the reactivity of these reagents, a synthetic peptide, MAT31, with a sequence and estimated pI value of KEEPPHHEVPESETC and 4.5, respectively, was prepared and solutions of this peptide were treated with these labeled or unlabeled *N*-alkylmaleimides.[19] There is a possibility that the reactivity of a thiol group against *N*-alkylmaleimides varies depending on the adjacent amino acid side chains. In order to avoid ambiguity that might come from the difference in reactivity, we added the maleimides three times in excess. Under this condition more than 95% of peptides were converted into their corresponding maleimide adducts. After adding a matrix to the sample solutions, the ion peaks of these solutions were analyzed by MALDI-TOF MS.[20]

Figure 1 shows the MALDI-TOF spectra of MAT31, for which the monoisotopic mass is 1746.75 Da, and *N*-methyl- or *N*-ethylmaleimide-modified MAT31. As can be seen in Figure 1, unlabeled *N*-methylmaleimide (NMM) added 111 Da to MAT31, while d-labeled *N*-methylmaleimide (d_3-NMM) (**2**) added 114 Da to MAT31 as a result of their reaction with this peptide. Similarly, unlabeled *N*-ethylmaleimide (NEM) added 125 Da to MAT31, while d-labeled *N*-ethylmaleimide

(d_5-NEM) (**1**) added 130 Da to MAT31. Due to the presence of ^{13}C and other natural isotopes, each ion peak shows a series of isotope peaks that are 1–5 Da higher than its monoisotopic ion peak.

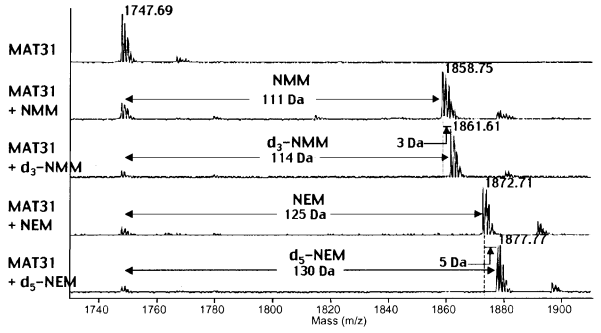


Figure 1. MALDI spectra of MAT31 and *N*-alkylmaleimide modified MAT31.

In the next step, in order to examine the applicability of these reagents to quantification of peptides, several MAT31 solutions were quantitatively analyzed using d_5-*N*-ethylmaleimide (**1**) and *N*-ethylmaleimide, as a model system, since the combination of these reagents gives 5 Da difference in the molecular weight, providing clearer separation than with the combination of d_3-*N*-methylmaleimide (**2**) and *N*-methylmaleimide.[21] The experiments were performed in the following manner. MAT31 was individually treated with d-labeled (heavy) or unlabeled (light) *N*-ethylmaleimide in excess. Then the differentially labeled MAT31 solutions were mixed in such a way that ratios of NEM-MAT31 to d_5-NEM-MAT31 in the final solutions were 9:1, 3:1, 1:1 and 1:3, respectively. Finally, the quantities of MAT31 in each solution were measured from the relative signal intensities for pairs of peptide ions that were modified with the heavy or light *N*-ethylmaleimides.

As can be seen in Figure 2, these spectra revealed isotope peaks due to the presence of ^{13}C and other stable isotopes within the peptide sequence. The relative signal intensities of each ion peak area were measured and the totals of the areas were summed. In this way, the relative ratios of MAT31 in the four sets of the two solutions obtained in these experiments were plotted against their theoretical (calculated) ratios in Figure 3a.

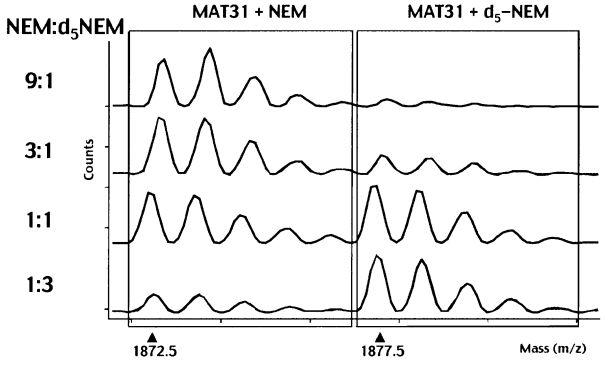


Figure 2. MALDI spectra of the sets of MAT31 with varied concentrations.

As seen in Figure 3a, the observed relative ratios and the theoretical relative ratios for d-labeled and unlabeled *N*-ethylmaleimide-modified MAT31 are well correlated ($r^2 = 0.9931$, inclination = 0.9565). Thus we conclude that the ionization efficiencies of the d-labeled MAT31 and those of the unlabeled MAT31 are the same within the experimental error. They also indicate that the overlap between the light and heavy *N*-ethylmaleimide-modified peptides below the *m*/*z* 1877 signal

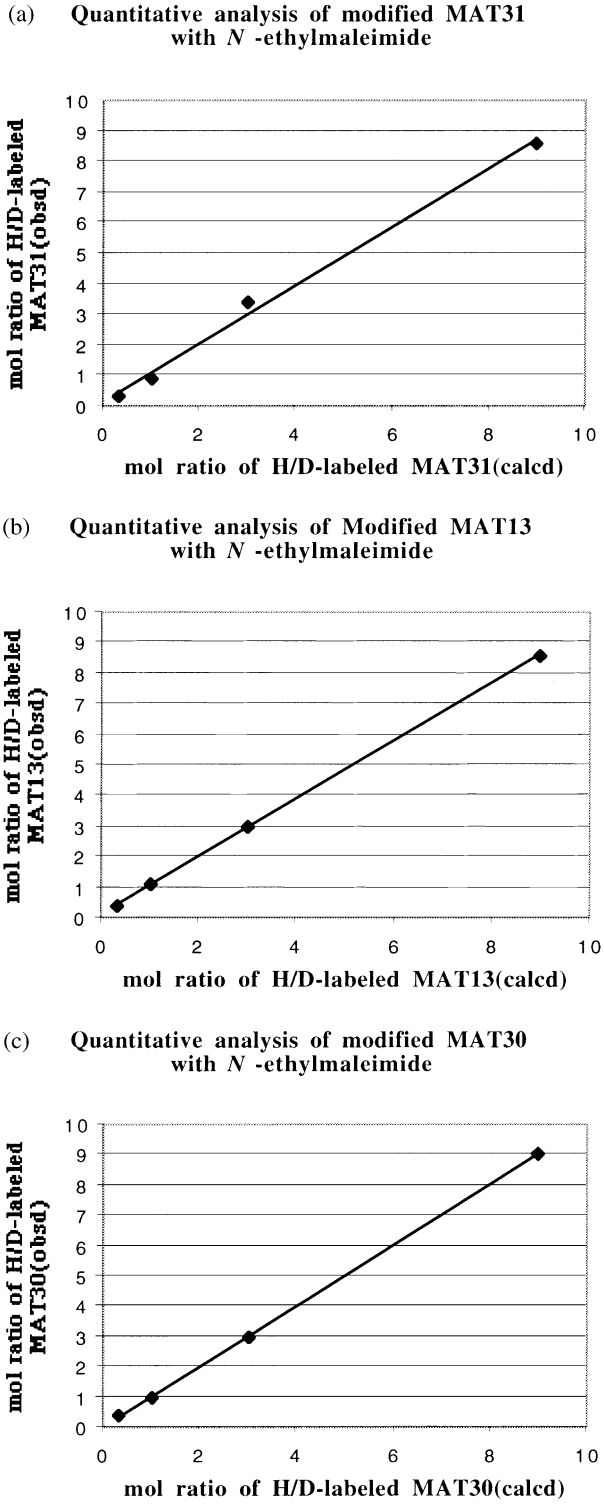


Figure 3.

is practically negligible. Therefore, the molar ratio of the peptide can be determined at a high accuracy with this method. In order to investigate the generality of this method, we performed further analyses using two additional synthetic peptides, MAT13 and MAT30. The sequences, monoisotopic masses, and estimated pIs are SDTCSSQKTEVSTVSSTQK, 2001.92 Da and 6.2 for MAT13, and Ac-HRSTVASMHRQEAVDCLKKF-NARRKLKGA-NH_2, 3377.82 Da and 11.6 for MAT30, respectively.

The results for MAT13 and MAT30 are shown in Figure 3b and c, respectively, indicating that the theoretical and observed relative ratios for d-labeled and unlabeled *N*-ethylmaleimide-modified MAT13 and MAT30 are also well correlated ($r^2 = 0.9998$ and 1.000, inclination = 0.9441 and 0.9968, respectively), and practically the same as those of MAT31. These results suggest that the ionization efficiencies of the d-labeled and unlabeled *N*-ethylmaleimide-modified synthetic peptides are the same within the experimental error.

In summary, we developed a new method for quantitative analysis of a variety of peptides having about 1.5–3.5 kDa molecular weight and a pI range of about 4–12 as a model system to quantify a protein in a complex mixture. The application of this method toward a complex mixture of proteins will be reported in due course.

Acknowledgements

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16. Labeled $C_2D_5NH_2$·HCl was purchased from Isotech Inc., Miamisburg, OH, USA, and CD_3NH_2·HCl was purchased from Aldrich, Milwaukee, WI, USA. For the solvents for reactions, commercially available anhydrous DMF and reagent grade acetic anhydride were used without further purification.
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18. The synthetic procedure for d-labeled *N*-ethylmaleimide, **1**, is as follows. Under N_2 atmosphere, $C_2D_5NH_2$·HCl (716 mg, 8.3 mmol) was dissolved in anhydrous DMF (4 mL) at room temperature, and triethylamine (1.15 g, 8.3 mmol) was added dropwise. The mixture was cooled to 0 °C, maleic anhydride (811 mg, 8.3 mmol) was added, and the mixture was stirred at room temperature. After about an hour, approximately 40 mL of chloroform and 30 mL of water were added. The reaction mixture was extracted with chloroform (×3) and washed with saturated sodium chloride (×1), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the mixture was purified by silica gel column chromatography (ethyl acetate) to afford *N*-(ethyl)-maleamic acid (1.16 g, 95%).

The *N*-(ethyl)maleamic acid (432 mg, 2.9 mmol) was heated at 110 °C in acetic anhydride (3 mL) in the presence of anhydrous NaOAc (150 mg) for 15 min. The reaction mixture was cooled and poured into an iced, saturated aqueous $NaHCO_3$ solution. The mixture was extracted with ether (30 mL, ×4), washed with saturated aqueous NaCl solution, and dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by silica gel column chromatography (hexane/ethyl acetate = 5:1) to afford pure d_5-*N*-ethylmaleimide, **1** (188 mg, 50%) as oil, which was triturated with cold hexane to form a white solid. *N*-ethylmaleimide, **2**, was synthesized in the same way. However, the yield was diminished (33%) due to instability of **2** as well as low solubility of d_3-methylamine HCl salt in DMF even in the presence of triethylamine. Spectrum data for **1** and **2** are as follows. **1**: white solid, mp 43–44 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.69 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 133.8, 31.6, 12.4, IR (neat, cm^{-1}) 3099, 1641, 1504 HRMS *m/e* calcd for $C_6H_6D_5N_2O_2$ $(M+NH_4)^+$: 148.1212, found: 148.1211.

2: White solid, mp 94 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.73 (s, 2H); ^{13}CNMR (75 MHz, $CDCl_3$) δ170.7, 134.1, 22.9. IR (neat, cm^{-1}) 3099, 1700, 1664, HRMS *m/e* calcd for $C_5H_6D_3N_2O_2$ $(M+NH_4)^+$: 132.0849, found: 132.0838.
19. The typical procedures are as follows: the 0.6 mM aqueous solution of MAT31(5 μL), 10 mM Tris–HCl buffer solution (pH 7.0, 5 μL), and 1 μL of 10 mM *N*-methylmaleimide in ethanol were mixed as a sample solution and incubated at room temperature for 10 min. Then, 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution prepared as below was added to 1 μL of the sample solution, and 1 μL of this mixture was analyzed by MALDI-TOF MS.
20. The spectra were obtained using MALDI-TOF MS, Voyager Elite BioSpectrometry Research Station (Serial No. 130), equipped with a delayed extraction option (PerSeptive Biosystems, Framingham, MA, USA) operated at the accelerating voltage, 20 kV; grid voltage, 75%; guide wire voltage, 0.1%; and pulse delay time, 250 ns. A pulsed nitrogen laser

emitting at 337 nm was used as a desorption/ionization source. Mass spectrometry was performed in a reflector with positive ion detection. The ion signal was recorded using a 500-MHz transient digitizer. The data were analyzed using GRAMS/386 (Galactic Industries Corp., Salem, NH, USA). The measurement was externally calibrated by angiotensin I ($[M+H]^+ = 1296.69$, monoisotopic mass) and a synthetic peptide ($[M+H]^+ = 2110.94$, monoisotopic mass). The matrix solution was α-cyano-4-hydroxycinnamic acid (CHCA) at 10 mg/mL dissolved in 50% CH_3CN/0.1% trifluoroacetic acid.

21. We plan to quantitatively analyze peptide solutions using the set of d-labeled and unlabeled *N*-methylmaleimides by high resolution mass spectrometry in the near future.