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Electrospray ionization mass spectroscopic analysis of peptides modified with *N*-ethylmaleimide or iodoacetanilide

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ABSTRACT

The cysteine-specific modifiers we reported previously, *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA), have been applied to label cysteine residues of peptides in combination with electrospray ionization mass spectrometry (ESI-MS/MS), and their scope in proteomic studies was examined. Peptides modified with *N*-ethylmaleimide (NEM) or iodoacetanilide (IAA) showed significant enhancement in ionization efficiencies. These modifiers were also found to remain intact in tandem mass spectrometry. Both combinations of *N*-ethylmaleimide (NEM) and d_5-*N*-ethylmaleimide (d_5-NEM), and iodoacetanilide (IAA) and $^{13}C_6$-iodoacetanilide ($^{13}C_6$-IAA) were also shown to be applicable to quantitative analysis of a peptide.

Proteomics, which studies sets of proteins expressed under certain physiological conditions, is becoming important for solving problems in biology that involve the malfunction of complex protein networks. In particular, identification and quantitative analysis of proteins found under different external stimuli are essential parts of proteomics research. Classical methods of quantitative analysis include densitometric analysis[1] of 2D gels or radioisotope labeling.[2] More recent methods that have been proven to be effective include application of stable-isotope labeling and subsequent mass spectrometric analysis for identification and concurrent quantitative analysis of proteins.[3] To this end, we have reported several sets of isotope-labeled and unlabeled small organic molecules that specifically react with cysteine residues.[4–7] In combination with a soft ionization mass spectroscopy, MALDI TOF, we demonstrated that these sets of reagents allow quantitative analysis of proteins and peptides as well as identification of proteins, and therefore are expected to be a useful tool for proteomics research.

While MALDI is a very common type of soft ionization mass spectrometry, ESI is also an equally common soft ionization method for biochemical studies. While MALDI has certain advantages over ESI, such as ease of operation and tolerance to buffers and other additives, ESI also has certain advantages over MALDI, such as applicability to liquid samples, allowing direct connection to liquid chromatography (LC), and non-interference from matrices. In addition, ESI is more commonly applied than MALDI to the studies of ligand–protein or protein–protein interaction.[8]

Therefore, among the above cysteine-specific modifiers we previously reported, we applied *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA)[9] to modification of model peptides and examined their effects by ESI-MS and ESI-MS/MS. In addition, we performed quantitative analysis of a model peptide using *N*-ethylmaleimide (NEM) and d_5-*N*-ethylmaleimide (d_5-NEM), **1** and **2**, as well as iodoacetanilide (IAA) and $^{13}C_6$-iodoacetanilide ($^{13}C_6$-IAA), **3** and **4**, in combination with ESI-MS in order to examine their applicability to proteomics studies. In particular, we compared the results with a well-known cysteine-specific modifier, iodoacetamide (IA), **5**, which has been commercially available for a long time. These modifiers, **1**–**5**, are known to react with the sulfhydryl group of the cysteine residues as shown in Figure 1. Herein we report the results.

First we examined the ionization efficiencies of model peptides with *N*-ethylmaleimide (NEM), **1**, iodoacetanilide (IAA), **3**, and iodoacetamide (IA), **5**. Structural modifications of peptides can often cause increase or decrease in the intensities of ionization efficiencies.[10] The enhancement of ionization efficiencies would allow detection of the peptides in smaller amounts, and therefore would be a great asset for proteomics research. With the use of ESI, we noticed significant reproducible enhancement by these modifiers. We therefore compared the enhancement with another well-known commercial cysteine modifier, iodoacetamide (IA).[10b,10c]

The three model peptides used were PEP 60, PEP 13, and PEP 31, of which the amino acid sequences, molecular weight, and p*I* values are ALVCEQEAR, 1017.49 Da, 4.4; SDTCSSQKTEVSTVSSTQK, 2001.92 Da, 6.2; and KEEPPHHEVPESETC, 1746.75 Da, 4.5, respectively. Each peptide has one cysteine residue. The alkylation

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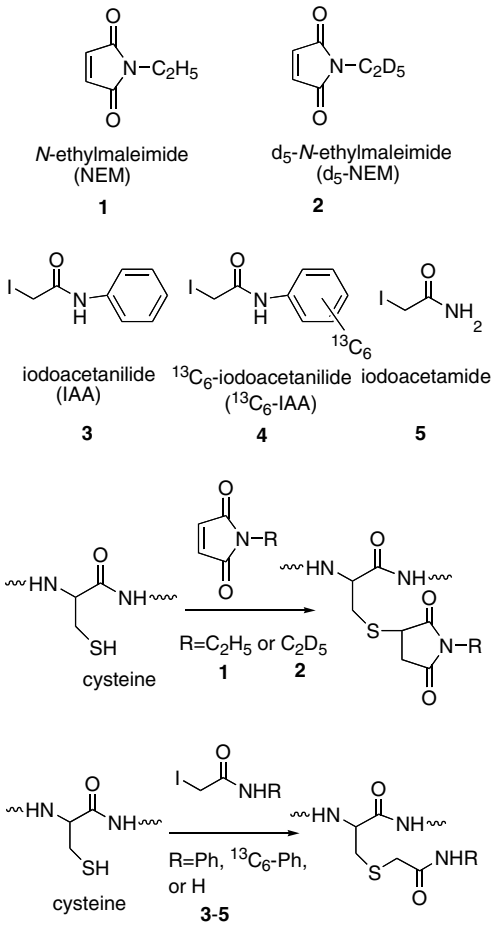

Figure. 1. *N*-Ethylmaleimide, d_5-*N*-ethylmaleimide, iodoacetanilide, $^{13}C_6$-iodoacetanilide, iodoacetamide, and their reactions with cysteine.

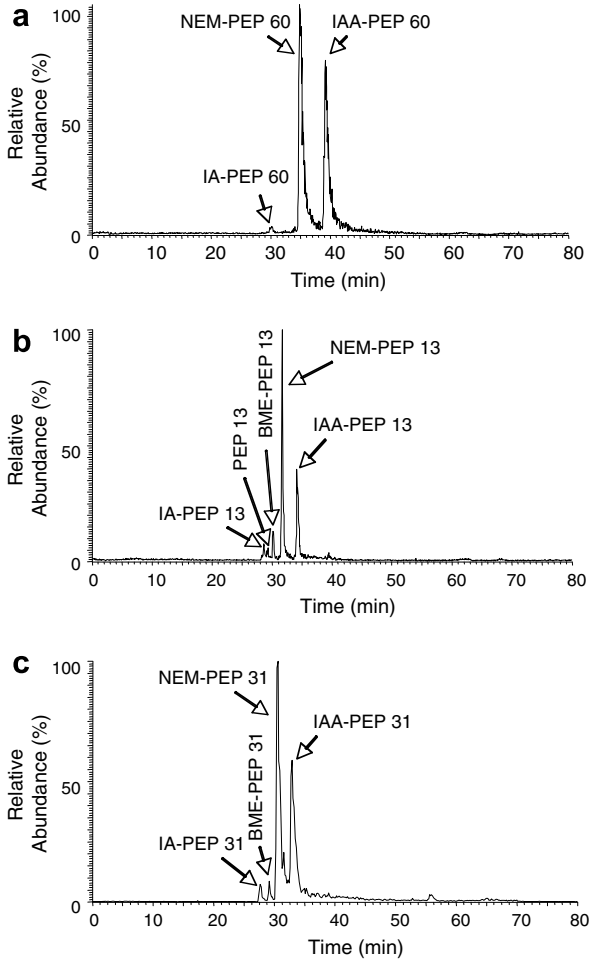

Figure. 2. The ion chromatograms obtained for the PEP 60 (a), PEP 13 (b), and PEP 31 (c), modified with iodoacetamide (IA), *N*-ethylmaleimide (NEM), or iodoacetanilide (IAA).

reaction was carried out by the mixing of each peptide solution and each modifier solution at room temperature followed by incubation for 1 h for completion of the reaction, and the reaction was terminated by the addition of β-mercaptoethanol (BME).[11] Alkylation of each peptide with iodoacetamide (IA), *N*-ethylmaleimide (NEM), or iodoacetanilide (IAA) added 57, 125, or 133 Da, respectively, to the peptide.

Figure 2 shows the ion chromatograms for the three model peptides, PEP 60 (a), PEP 13 (b), PEP 31 (c) labeled with above three modifiers. Each ion chromatogram was obtained from exactly the same amount of each modified peptide (1 pmol).

Table 1 summarizes the masses observed for each modified peptide obtained from the peaks in the ion chromatograms. The modified peptides were identified with different charge statuses. PEP 31 and PEP 13 were identified from the triply charged ($[M+3H]^{3+}$), and PEP 60 was identified from the doubly charged ion ($[M+2H]^{2+}$).

As can be seen in the ion chromatograms, all the peptides underwent the alkylation reactions to completion or near completion under these reaction conditions. In particular, PEP 60 was observed to be the most reactive, as only the peaks corresponding to IA-PEP 60, NEM-PEP 60, and IAA-PEP 60 were observed, showing that the alkylation reactions were complete. Under the same reaction conditions, however, the reaction mixture of PEP 13 and PEP 31 showed small peaks corresponding to the mass of unmodified PEP 13 and/or those likely to correspond to the starting peptides that reacted with β-mercaptoethanol (BME), although these ion chromatograms still indicate that the reactions were near complete.

Table 1
The observed masses for the model peptides modified with iodoacetamide (IA), *N*-ethylmaleimide (NEM), or iodoacetanilide (IAA)

Peptides	Modified with IA	Modified with NEM	Modified with IAA
PEP 31	602.3	625	627.7
PEP 13	687.3	710	712.8
PEP 60	538.4	572.3	576.3

Figure 3(a) and (b) shows the extent of the ionization efficiencies of the modified peptides (a) and the ratios of peak areas for NEM-modified peptides/IA-modified peptides and IAA-modified peptides/IA-modified peptides (b), which were obtained from the above ion chromatograms.

As can be seen from Figure 3, remarkable enhancement in peak intensity of the peptides modified with *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA) over those modified with iodoacetamide (IA) was observed for all three peptides. In particular, PEP 60 showed the greatest enhancement when modified with *N*-ethylmaleimide (NEM) or iodoacetanilide (IAA) on the peak area in comparison to the same peptide modified with iodoacetamide (IA). The ionization efficiency was 20 times greater with NEM-PEP 60 than with IA-PEP 60, and 15 times greater with IAA-PEP 60 than with IA-PEP 60 (Fig. 3). In the case of PEP 13, the enhancement for NEM-PEP 13 was about eightfold, and that for IAA-PEP 13 was fourfold compared to IA-PEP 13. Similar results were also observed for PEP 31.

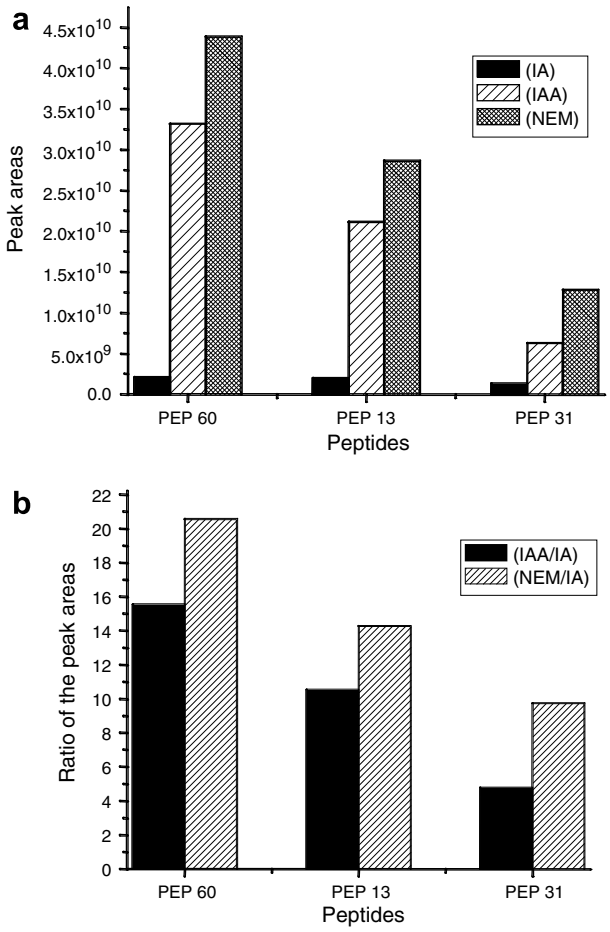

Figure. 3. (a) Observed peak areas for PEP 60, PEP 13, and PEP 31 modified with *N*-ethylmaleimide (NEM), iodoacetanilide (IAA), or iodoacetamide (IA). (b) Calculated ratios of peak areas for NEM peptide IA-peptide and IAA-peptide/IA-peptide.

The reasons for such significant enhancement observed for NEM-modified and IAA-modified peptides are not certain. However, in ESI ionization, charged droplets will form ionized analytes. More precisely, the charged droplets will be evaporated as a result of desolvation to produce smaller droplets.[12,13] As smaller droplets are produced, the surface charge density of the droplets increases, leading to enhancement of Coulombic repulsion and further explosion of the droplets into less charged smaller droplets. It has been proposed that the hydrophobicity of analytes is important in desolvation, as hydrophobicity may help a greater number of analytes to desorb more readily during the explosion of the droplets to smaller droplets by concentrating the hydrophobic group of the analytes on the surface of the droplets.[14] Therefore, it may be speculated that the presence of more hydrophobic groups in *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA) than in iodoacetamide (IA) helped a greater number of NEM-peptide or IAA-peptide ions to desorb in the desolvation process.

Next, in order to see the influence of these modifiers on collision-induced dissociation (CID)-induced fragmentation, the MS/MS spectrum of a model peptide modified with *N*-ethylmaleimide (NEM) or iodoacetanilide (IAA) was compared with that of the same peptide alkylated with a well-known modifier, iodoacetamide (IA). Tandem mass spectrometry is a powerful tool for identification of amino acid sequences of peptides, and has become increasingly popular among proteomics researchers in the last few years.[15] In particular, tandem mass spectrometry is more indispensable for identification of peptides and proteins when ESI instead of MALDI is utilized, as peptide mass fingerprinting is more complex due to formation of multiple charges. In addition, although our method for proteome analysis, in combination with IAA/$^{13}C_6$-IAA or NEM/d_5-NEM and MALDI as well as electrophoresis, allows quantitative analysis and identification of proteins by peptide mass fingerprinting without the need for tandem mass spectrometry, for many applications tandem mass spectrometry is still advantageous for analyzing more accurately the amino acid sequences initially identified by peptide mass fingerprinting.

Figure 4 shows the MS/MS spectra of the model peptide, PEP 60, which was observed to be the most reactive in the above experi-

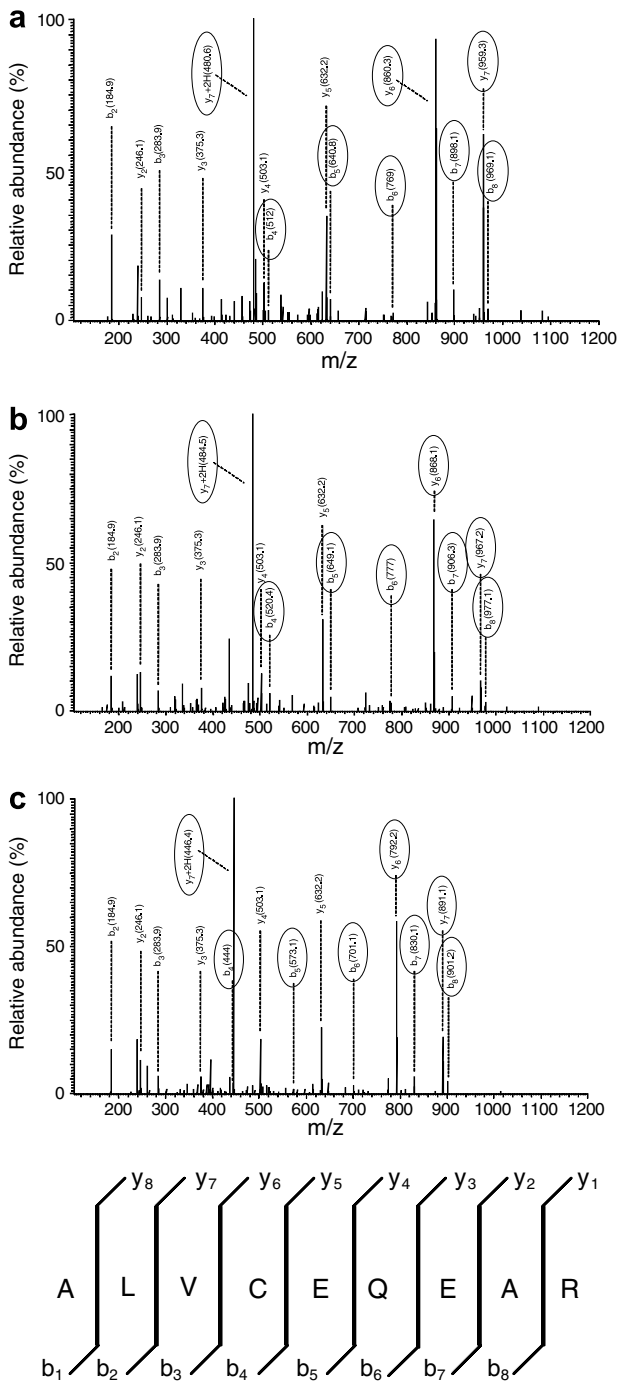

Figure. 4. The MS/MS spectra of PEP 60 (ALVCEQEAR) modified with *N*-ethylmaleimide (NEM) (a), iodoacetanilide (IAA) (b), and iodoacetamide (IA) (c). Fragments containing NEM, IAA, or IA are circled.

ments, modified with *N*-ethylmaleimide (NEM) (a), iodoacetanilide (IAA) (b), and iodoacetamide (IA) (c), respectively. Most of the b and y fragments (except for b_1, y_1, and y_8) were identified for all three modified peptides here, and it was observed that the fragmentation patterns for the NEM-modified peptide as well as the IAA-modified peptide were similar to those found in the IA-modified peptide. The fragments obtained for IA, NEM, and IAA-peptides were similar even with regard to their charge status. For example, the y_7 fragments which contain cysteine residue modified with *N*-ethylmaleimide (NEM), iodoacetanilide (IAA), or iodoacetamide (IA) were identified as $[M+H]^+$ and $[M+2H]^{2+}$, and the doubly charged fragment was the most intensive fragment in all the spectra. Therefore, both modifiers, iodoacetanilide (IAA) and *N*-ethylmaleimide (NEM), work in the same manner as the well-known modifier, iodoacetamide (IA).

It is reported that many other relatively large modifiers often undergo fragmentation by themselves during the CID-induced fragmentation, which often complicates interpretation of the spectra.[3a] Therefore, it is a special advantage that both *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA) remained intact during the CID-induced fragmentation. These results demonstrate the special expedience of our modifiers. We did not observe additional unexpected fragmentation which often complicates interpretation of the spectra.

We next performed quantitative analysis of a peptide using the above modifiers we synthesized. Quantitative analysis of proteins and peptides is also essential to proteomics research. Earlier we demonstrated that quantitative analysis of peptides and proteins using iodoacetanilide (IAA) or *N*-ethylmaleimide (NEM), and their $^{13}C_6$- or d_5-labeled derivatives in combination with MADLI-TOF-MS is possible.[4,5,7] Here, we report a similar approach using the model peptide PEP 60 to investigate the applicability of these modifiers with ESI-MS to quantitative analysis.

The quantitative analysis of the peptide was performed with different molar ratios of IAA-modified peptides/$^{13}C_6$-IAA-modified peptides or NEM-modified peptides/d_5-NEM-modified peptides,[16] as previously reported.[4–7] The results are shown in Figure 5. Good correlation was observed between the molar ratios applied for the experiments and the peak areas of IAA-modified and $^{13}C_6$-IAA-modified peptides as well as NEM-modified and d_5-NEM-modified peptides. As can be seen in Figure 5, the observed ratios and the theoretical ratios for the isotope-labeled and unlabeled IAA-modified peptide are well correlated, expressed by $R^2 = 0.997$ and inclination = 1.1. Similar results were also observed for the quantitative analysis of the same peptide modified with NEM or d_5-NEM, showing $R^2 = 0.998$ and inclination = 1.06. These correlation parameters and inclinations indicate that the ionization efficiencies of the isotope-labeled and unlabeled NEM-modified PEP 60 or IAA-modified PEP 60 are the same within experimental errors, and hence molar ratios of the peptides in two sample solutions can be measured at a high accuracy in combination with ESI-MS. In addition, the samples for ESI-MS measurement are solutions and thus tend to be more homogeneous than the samples for MALDI, and therefore smaller error ranges were observed with ESI than with MALDI. Although primary isotope effects have sometimes been reported for a combination of isotope-labeled and unlabeled peptides in other LC-based quantification methods,[3a] we observed no isotope effect during the quantitative analysis of peptides in this study, as no differential elution was observed in the ion chromatograms. The disadvantage is, however, that LC analysis is essential in general for analysis of proteins in combination with ESI mass spectrometry and therefore this LC-linked ESI-MS often requires several hours until detection of the peaks becomes possible after the injection of the sample, and thus requires a significantly greater amount of time compared to the same quantitative analysis with MALDI.

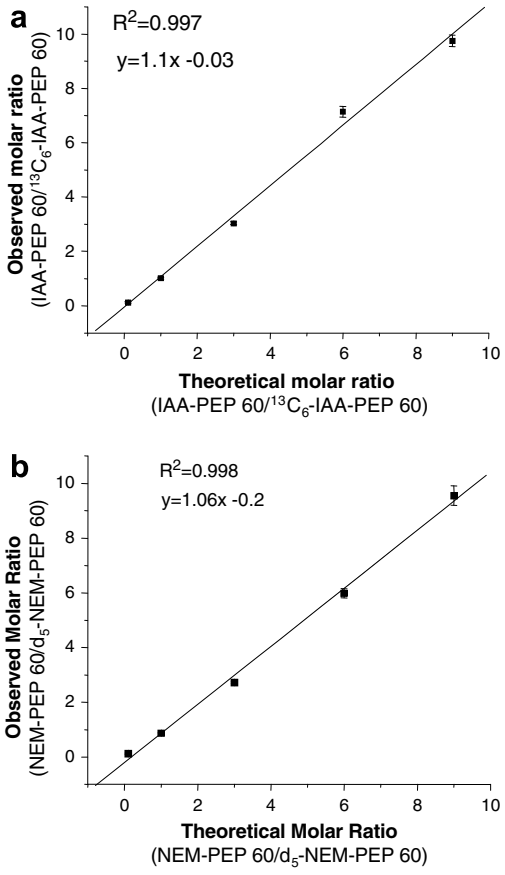

Figure. 5. Quantitative analysis of PEP 60 with IAA-PEP 60/$^{13}C_6$-IAA-PEP 60 (a) and NEM-PEP 60/d_5-NEM-PEP 60 (b).

In summary, we found that both iodoacetanilide (IAA) and *N*-ethylmaleimide (NEM) have great advantages over iodoacetamide (IA) for proteome analysis in combination with the ESI mass spectrometry. Significant enhancement of the ionization efficiencies of the IAA- or NEM-modified peptides was observed. Among the three modifiers, *N*-ethylmaleimide (NEM) showed the greatest enhancement. By MALDI we find that iodoacetanilide (IAA)-modified peptides tend to show greater enhancement in the ionization efficiencies than *N*-ethylmaleimide (NEM)-modified peptides, although the extent of the enhancement of the iodoacetanilide (IAA)-modified peptides is rather smaller, which is around 2–6 times compared to that of iodoacetamide (IA)-modified peptides.[17] These findings make interesting contrast.

In the quantitative analysis of a peptide, these modifiers did not show any isotope effects, although differential elution of isotope-labeled and unlabeled peptides during liquid chromatography is commonly reported due to primary isotope effects.[3a] We also reported earlier that no isotope effects was observed in the quantitative analysis of peptides or proteins with the use of these modifiers in combination with MALDI.[4–7] As we discussed before, this non-existence of isotope effects may be due to the use of small organic molecules,[7] which possibly increase hydrophilicity, potentially increasing the homogeneity of the aqueous reaction mixtures unlike other larger modifiers such as the isotope-coded affinity tag (ICAT),[3h] which consists of more than 20 carbon units. These modifiers, *N*-ethylmaleimide (NEM) and iodoacetanilide (IAA), allow introduction of 5 or 6 isotope atoms unlike iodoacetamide (IA), hence enabling more accurate quantitative analysis than iodoacetamide (IA) due to less overlap of the monoisotopic peak of the $^{13}C_6$ or d_5-labeled peptide and isotope peaks of ^{13}C or d-unlabeled pep-

tides. In addition, the homogeneous nature of the sample for ESI measurement is also a helpful asset. The small size of the modifiers also demonstrated their advantages in the tandem mass spectra, as they remained intact during the CID-induced fragmentation and hence showing no unexpected fragmentation. Therefore, we expect that identification of peptide or protein samples labeled with these modifiers is relatively simple compared to other modifiers with greater sizes. Although there are some disadvantages for ESI, such as the fact that the measurement requires more time in combination with LC ESI, depending on the type of project and resources available in the laboratories, the combination of iodoacetanilide (IAA) or *N*-ethylmaleimide (NEM) and ESI is expected to serve as a useful tool for analysis of proteomes in proteomics research. In particular, the significant enhancement of peptide peak intensities using ethylmaleimide (NEM) or iodoacetanilide (IAA) may lead to identification and quantification of a greater number of proteins in proteome mixture.

Acknowledgment

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11. Stock solutions of the peptides were prepared in 50 mM of Tris-buffer (pH 9) with a concentration of 0.6 mM. Stock solutions of iodoacetanilide (IAA), $^{13}C_6$-iodoacetanilide ($^{13}C_6$-IAA), *N*-ethylmaleimide (NEM), d_5-*N*-ethylmaleimide (NEM), and iodoacetamide (IA) were prepared in DMSO with a concentration of 20 mM. The alkylation reaction was started by the mixing of 2 μL of each peptide solution with 2 μL of the iodoacetanilide (IAA), $^{13}C_6$-iodoacetanilide ($^{13}C_6$-IAA), or iodoacetamide (IA) solution, or 0.8 μL of the *N*-ethylmaleimide (NEM) or d_5-*N*-ethylmaleimide (NEM) solution. The mixture was left at room temperature for 1 h in the dark, and 25 mM of β-mercaptoethanol (BME) (2 μL for IA and IAA, and 0.8 μL for NEM) was added to stop the alkylation reaction. For all the ESI-MS measurements, after dilution with 0.1% formic acid solution, 20 μL of the sample solution containing 1 pmol of each modified peptide was injected into the mass spectrometer. The analysis was performed with a nano-electrospray mass spectrometer (Thermoelectron LCQ DECA XP ^{Plus}, USA) with an ion trap mass analyzer. Peptides were separated on a C_{18} column (PicoFrit™ COLUMN, BioBasic®C18, 75 μm internal diameter) with eluents A and B. Eluent A was 0.1% formic acid in water and eluent B was 0.1% formic acid in acetonitrile, and separation of the peptides was performed for 63 min (by application of the gradient from 100% eluent A to 80% eluent B).
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16. The quantitative analysis of model peptide PEP 60 was performed as follows: The individual PEP 60 solutions were modified with IAA or $^{13}C_6$-IAA, or *N*-NEM or d_5-NEM, as described in Ref. 11, and were left for 1 h at room temperature in the dark. The reaction was terminated by addition of 25 mM of β-mercaptoethanol (BME) as described above (2 μL for IA and IAA, and 0.8 μL for NEM). The modified (isotope-labeled and unlabeled) PEP 60 solutions were mixed at a molar ratio of 0.1, 1, 3, 6, and 9 (isotope-unlabeled/labeled). Prior to MS analysis, 0.1% formic acid was added to the mixture in such a way as to include a total of 1 pmol of isotope-labeled and unlabeled modified peptides. The ratios of the IAA/$^{13}C_6$-IAA-labeled peptides and NEM/d_5-NEM peptides were calculated from the peak areas corresponding to the modified peptides. Each sample mixture was injected into the mass spectrometer twice, and the average values were plotted on the graph. For calculation of the peak areas in the ion chromatograms, Xcalibur™Revision A (ThermoFisher) was utilized after specifying the mass range of the interested peaks.
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