Research article

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Peptide peak intensities enhanced by cysteine modifiers and MALDI TOF MS

Masoud Zabet-Moghaddam,[a,b] Aarif L. Shaikh[a] and Satomi Niwayama[a]*

Two cysteine-specific modifiers we reported previously, *N*-ethyl maleimide (NEM) and iodoacetanilide (IAA), have been applied to the labeling of cysteine residues of peptides for the purpose of examining the enhancement of ionization efficiencies in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS). The peak intensities of the peptides as a result of modification with these modifiers were compared with the peak intensities of peptides modified with a commercially available cysteine-specific modifier, iodoacetamide (IA). Our experiments show significant enhancement in the peak intensities of three cysteine-containing synthetic peptides modified with IAA compared to those modified with IA. The results showed a 4.5–6-fold increase as a result of modification with IAA compared to modification with IA. Furthermore, it was found that IAA modification also significantly enhanced the peak intensities of many peptides of a commercially available proteins, bovine serum albumin (BSA), compared to those modified with IA. This significant enhancement helped identify a greater number of peptides of these proteins, leading to a higher sequence coverage with greater confidence scores in identification of proteins with the use of IAA. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: ionization efficiencies; peak enhancement; cysteine modifier; MALDI TOF; alkylation

Introduction

Structural modification of peptides by tagging reagents plays an important role in combination with mass spectrometry in various biochemical and biological applications. Some examples include selective separation of the tagged peptides by selective adsorption through affinity tag technology[1] and identification of spatial relationships between amino acids in proteins or protein complexes by cross-linking.[2] One of the most beneficial aspects of chemical modification of peptides for observation of the mass spectrum is improvement of the ionization efficiencies, allowing detection of the peptides at a lower concentration.[3] This improvement also assists in identification of proteins. More recently, various tagging reagents have been invented for the purpose of identification and quantitative analysis of proteins or peptides in proteomics research. The combination of stable isotope-labeled and unlabeled tagging of peptides and proteins allows quantitative analysis of peptide or protein samples obtained from two different external stimuli. Some of the most well-known examples include the ICAT[4] and iTRAQ reagents,[5] and many other researchers reported other tagging reagents.[6] In this context, earlier we reported several sets of isotope-labeled and unlabeled small organic molecules that specifically react with cysteine residues for the purpose of identification and quantitative analysis of peptides and proteins in combination with 1D and 2D electrophoresis and soft ionization mass spectrometry, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).[7–11] We demonstrated that these sets of reagents enable quantitative analysis of proteins and peptides as well as identification of proteins, and hence they are expected to be a useful tool for proteomics research. In particular, we reported that *N*-ethyl maleimide (NEM), **1**, and iodoacetanilide (IAA), **2**, exhibited significant enhancement of ionization efficiencies of peptides in combination with ESI. However, since MALDI is as commonly applied as ESI, it is also important to assess the effects by these modifiers in combination with MALDI.

Here, we examined the effects on enhancement of ionization efficiencies as a result of modification with two cysteine-specific modifiers, NEM, **1**, and IAA, **2**, in MALDI mass spectrometry (Scheme 1).[12,13] In particular, we compared the effects of these modifiers with the effects of commercially available iodoacetamide (IA), **3**, which is commonly applied.

Results and discussion

Peak enhancement of peptides

We first examined the effects of enhancement of peak intensities of three model peptides with different amino acid sequences and molecular weights after the modification of cysteine residues. The model peptides were PEP 60, PEP 13 and PEP 31 with the amino acid sequences, molecular weight and pI values of ALVCEQEAR, 1017.49 Da, 4.4; SDTCSSQKTEVSTVSSTQK, 2001.92 Da, 6.2 and KEEPPHHEVPESETC, 1746.75 Da, 4.5, respectively, and each model peptide contained a cysteine residue. Each peptide was modified with IAA, NEM or IA, and the signal-to-noise (S/N) ratios were compared. As each peptide was reacted with an excess of each modifier, no unmodified peptide was observed in each case. The relative S/N ratios which were calculated by the S/N ratios of IAA- or NEM-modified peptides divided by those of IA-modified

* *Correspondence to: Satomi Niwayama, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, USA. E-mail: satomi.niwayama@ttu.edu*

a *Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061, USA*

b *Current address: Center for Biotechnology and Genomics, Texas Tech University, Lubbock, Texas 79409-3132, USA*

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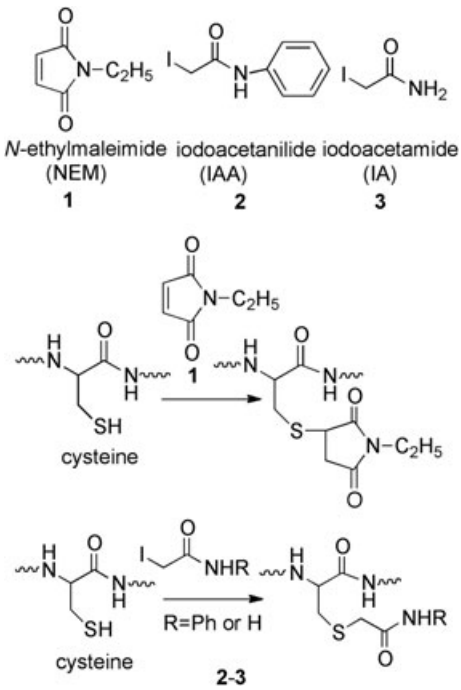

Scheme 1. *N*-ethylmaleimide, iodoacetanilide, iodoacetamide and their reactions with cysteine.

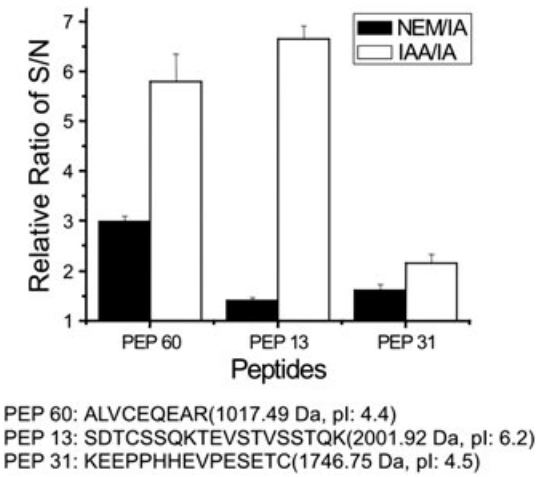

Figure 1. The average relative S/N ratios of NEM-peptides/IA-peptides and IAA-peptides/IA-peptides. The results show a nearly sixfold enhancement by IAA over IA for PEP 60 and PEP 13 and a nearly twofold enhancement for PEP 31. Error bars show the error ranges in five independent measurements.

peptides (IAA or NEM-modified peptide/IA-modified peptide) are summarized in Fig. 1.

As can be seen from Fig. 1, significant enhancement was observed as a result of IAA modification compared to IA modification. The S/N ratios were increased about six times for PEP 60 and PEP 13 and more than two times for PEP 31 as a result of IAA modification over IA modification. The modification with NEM also enhanced the S/N ratios for all these peptides over the modification with IA, but the effect was not as remarkable. Only the smallest peptide, PEP 60, showed a threefold enhancement with NEM over IA.

The enhancement observed above also resulted in improvement of the limit of detection (LOD). Each peptide modified with IAA, NEM or IA was mixed together at the same concentration, and measurement of the S/N ratios was performed to find out the LOD of the peptide modified with each modifier. The results obtained are summarized in Table 1. As can be seen, the LOD was reduced fourfold particularly as a result of IAA modification over IA modification for PEP 60 and PEP 13, and twofold for PEP 31, while NEM modification improved the S/N ratio only for PEP 60.

Figure 2 shows the spectra of PEP 60 modified by IAA, IA and NEM in four successive dilutions. The starting amount of each modified peptide on the MALDI-plate was 1.6 fmol followed by dilution to 0.8, 0.4 and 0.2 fmol. In accordance with the above result, the S/N ratio of the IAA-peptide (252) was far higher than the two other modified peptides, IA (46) or NEM-peptide (37), at the same amount of 1.6 fmol on the plate. As the sample was diluted by half, 0.8 fmol for each, the S/N ratios of all three peptides were reduced but still identifiable. At the concentration of 0.4 fmol, the signals of IA- and NEM-peptides were no longer identifiable, whereas a clear signal with the S/N ratio of 49 was observed for the IAA-peptide. The signal corresponding to the IAA-modified peptide was identified even after dilution to 0.2 fmol on the plate with the S/N ratio of 18, demonstrating a fourfold improvement of LOD for PEP 60 in comparison with the other two modifiers, IA and NEM.

Protein identification

Next, we examined the effects of these modifiers in comparison to IA on alkylation of a commercial protein, bovine serum albumin (BSA). BSA was alkylated with an excess of IA, IAA or NEM, and each sample was spotted six times to overcome the problem caused by non-homogeneity of samples in matrix crystals on the MALDI plate. All the spectra were checked carefully, to ensure in each sample that the masses of alkylated peptides (m/z) were truly derived from the peptides where all the cysteine residues were alkylated rather than non-alkylated peptides with the same m/z or those where another amino acid residue was alkylated, as each alkylation reagent adds a different mass to the respective peptides. The masses of 57, 133 and 125 Da for IA, IAA and NEM, respectively, were added to the masses of peptides for calculation of the alkylated peptide masses in the spectra. These extra masses were subjected to a MASCOT database search. Figure 3 shows examples of the spectra obtained as a result of modification by each alkylation reagent investigated in this study.

The alkylated peptides with their masses for each alkylation reagent are listed in Table 2. The alkylated peptides were taken into account only if they were identified in at least four out of six identically measured spots. Therefore, although the IA-peptide No. 12 was identified in three spots, it was not counted in Table 2. As can be seen, a greater number of alkylated peptides and alkylated cysteine residues was identified as a result of IAA modification than IA modification. The NEM modification indicated the smallest number of identified alkylated peptides. A total of 23 alkylated peptides were identified using IA, whereas this number was 28 for IAA-peptides, which indicates an 8% improvement in identification of cysteine-containing peptides, as the total theoretical number of cysteine-containing peptides is 71. The total number of identified peptides alkylated with NEM was only 14. In addition, the number of alkylated cysteine residues was greater with the use of IAA (30) compared to IA (28). Only 17 alkylated cysteines were identified with NEM.

The influence on ionization efficiencies of alkylated peptides by IA, IAA and NEM was more closely examined on the identical peptides modified with IA, IAA or NEM. The comparison was made between IAA-peptides/IA-peptides and NEM-peptides/IA-

Table 1. Limit of detections (LOD) and the S/N ratios for three model peptides modified by IA, IAA and NEM. The amounts on the plate and corresponding S/N value are given for each peptide. The LOD was calculated based on the ratios of amounts of peptides in which the signal was observed to the amount of peptide detected only by IAA for all three model peptides modified by IA, IAA and NEM

Peptides	**IA**-Peptide amount on the plate/S/N value	**NEM**-Peptide amount on the plate/S/N value	**IAA**-Peptide amount on the plate/S/N value	**Improved LOD by IAA**
PEP 60	800 amol/19 not detected	800 amol/33 not detected	800 amol/117 200 amol/18	fourfold
PEP 31	800 amol/21 not detected	800 amol/26 not detected	800 amol/45 300 amol/18	2.6-fold
PEP13	10 fmol/22 not detected	10 fmol/23 not detected	10 fmol/143 2.5 fmol/34	fourfold

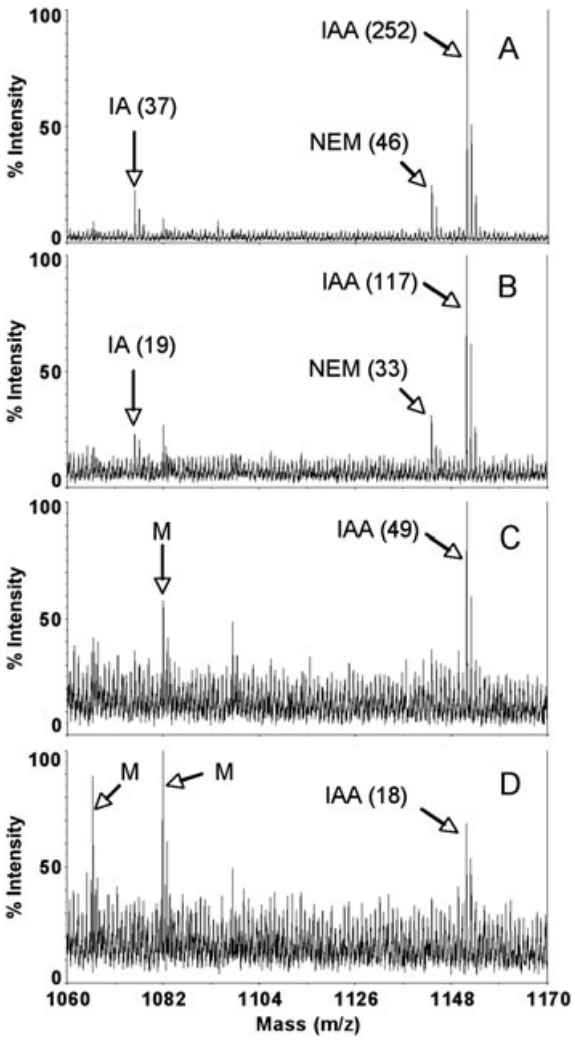

Figure 2. The spectra of PEP 60 modified with IAA, NEM or IA. The amounts of each peptide on the plate for each spectrum are A) 1.6 fmol, B) 0.8 fmol, C) 0.4 fmol and D) 0.2 fmol. M: matrix cluster.

peptides. Figure 4 shows the logarithmic scale of the ratio of S/N values of IAA/IA-peptides and NEM/IA-peptides.

Ionization efficiencies of the peptides with respect to the alkylation reagent used were observed to be different. The S/N values of most of the peptides modified by IAA were enhanced compared to those modified by IA and NEM. Some of the peptides were uniquely identified either by IAA modification or IA modification, as shown also in Table 2. NEM not only delivered the lowest number of peptides, but also showed a negative influence of ionization efficiencies on most of the peptides in comparison to IAA or IA. Previously, we found that NEM is less reactive than IAA or IA under certain conditions,[10] but the tendency of the enhanced ionization efficiencies by IAA is consonant with the synthetic peptides described above. Depending on the sequence of amino acids of the peptides and the length of the peptides, the effects of IAA, IA and NEM on ionization of the peptides were different. In a certain case, a nearly 50-fold enhancement was observed with the use of IAA over IA (peptide with m/z of 1024.521). In some cases, the S/N values of IA-peptides were greater than those of IAA-peptides. For example, the two peptides with the highest enhancements with the use of IA over IAA were observed in the peptide with m/z 2441.251 with a fivefold enhancement and the peptide with m/z 2472.286 with a 3.5-fold enhancement. However, this enhancement is far lower compared to those achieved by IAA (up to 50-fold). Overall, the alkylated peptides showed far higher S/N values when IAA was applied in comparison to IA. Interestingly, the influence of IAA on ionization efficiencies of the peptides was more pronounced for peptides with smaller molecular weights (m/z up to about 2000 Da), whereas IA showed greater enhancement of ionization efficiencies for peptides with greater molecular weights (m/z $>$ about 2000 Da) (Fig. 4) for BSA.

Due to the enhancement of peak intensities by use of IAA, some of the peptides were identified uniquely by IAA. Although some peptides were uniquely identified by IA modification, the number uniquely identified by IAA modification (9) increased by more than 50% in comparison to those uniquely identified by IA modification (4). In accordance with the earlier results mentioned above, all the peptides uniquely identified by IA are peptides with greater molecular weights, with m/z greater than 2000 Da, and those uniquely identified by IAA modification are peptides with smaller molecular weights, with m/z less than 2000 Da.

We further confirmed that the S/N values of the non-alkylated peptides are in a similar error bar range in all the samples we attempted to react with IA, IAA or NEM, indicating that the changes in peak intensities of cysteine-containing peptides are mainly the result of modification by IA, IAA or NEM (data not shown).

Protein identification at the LOD

Identification of proteins in a small amount is still challenging in proteomics. Therefore, peak enhancement of the peptides obtained from digestion of a protein is expected to make a significant contribution to detection of small quantities of proteins. In order to

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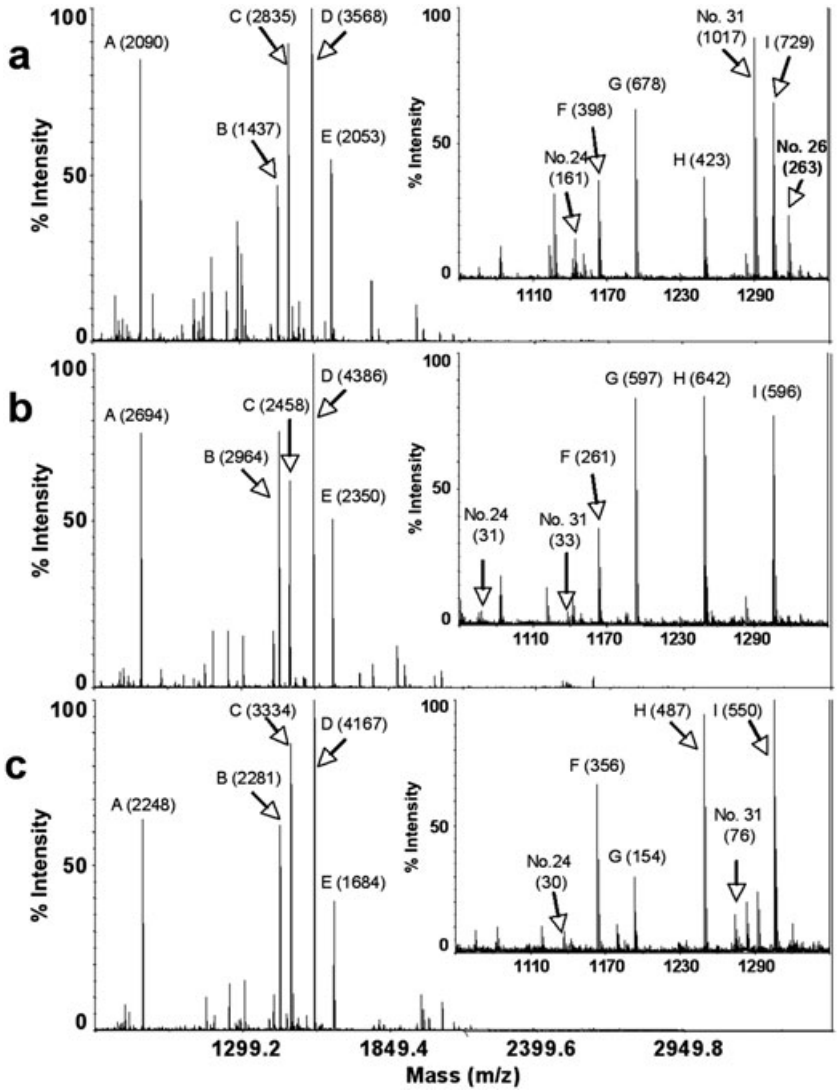

Figure 3. The spectra of BSA digest alkylated with a) IAA b) IA and c) NEM. Some S/N values are shown in parentheses. The peptides assigned letters are non-alkylated. The inserted spectra show enlarged views of the m/z range of 1050–1350 Da. The numbered peptides shown in the inserted spectra are referred to in Table 2.

take advantage of peak enhancement using IAA, we compared the number of identified peptides from a small amount of the BSA digest solutions (1.5 fmol) modified with IA or IAA on the MALDI plate. NEM was not tried since it did not show any significant improvement in the ionization efficiencies in comparison to the use of IA.

Table 3 lists all the peptides obtained from the in-gel digestion of gel bands containing BSA. As can be seen, the total number of peptides identified (alkylated or non-alkylated) was 14 with the use of IA, and 19 with the use of IAA. The five additional peptides identified with the use of IAA were all alkylated peptides. It should be mentioned here that the number of non-alkylated peptides identified was the same with the use of IA and IAA, and hence the alkylated peptides constitute the main difference in this comparison. The identified number of cysteine residues alkylated with IAA was 12, and therefore far greater than that with IA (3), again demonstrating enhanced ionization efficiencies by IAA. Six peptides were uniquely identified with the use of IAA, while only one peptide was identified with the use of IA. The only peptide uniquely identified by IA modification was peptide No. 17 (Table 3) with a high m/z of 2529.03 Da. All the peptides uniquely identified by IAA modification were those with m/z less than 2000 Da. These results are consistent with the earlier results shown above, wherein detection was more effective by IAA for smaller peptides ($m/z < 2000$ Da).

As a result of peak enhancement with the use of IAA, the sequence coverage of the protein improved from 24% with the use of IA to 33% with the use of IAA (Table 3). This improvement in the sequence coverage was entirely due to the uniquely alkylated peptides identified by IAA. Similarly, the score of the identified protein was also increased using IAA (255) compared to IA (203), indicating higher confidence for identification of protein as a result of IAA modification over IA modification.

Conclusions

Both the NEM and IAA we reported before showed enhancement of MALDI ionization efficiencies over IA in model peptides and a commercial protein. In particular, IAA modification showed an up to 50-fold enhancement in ionization efficiencies compared to IA modification. This enhancement with the use of IAA led to

Table 2. The identified peptides from BSA modified with the three different alkylation reagents IA, IAA or NEM. The numbers represent the obtained masses of the respective alkylated peptide. MC: missed cleavage, C: no. of cysteines

No	Position	Peptide	MC	C	IA	IAA	NEM
1	45 – 65	K.GLVLIAFSQYLQQCPFDEHVK.L	0	1	2492.314(1)	–	–
2	76 – 88	K.TCVADESHAGCEK.S	0	2	1463.633(2)	1615.663(2)	–
3	89 – 100	K.SLHTLFGDELCK.V	0	1	1419.717(1)	1495.736(1)	1487.718(1)
4	101 – 117	K.VASLRETYGDMADCCEK.Q*	1	2	–	2172.918(2)	–
5	106 – 117	R.ETYGDMADCCEK.Q*	0	2	–	1646.586(2)	–
6	123 – 138	R.NECFLSHKDDSPDLPK.L	1	1	–	1977.920(1)	–
7	139 – 151	K.LKPDPNTLCDEFK.A	0	1	1576.798(1)	1652.819(1)	1644.790(1)
8	139 – 155	K.LKPDPNTLCDEFKADEK.K	1	1	2020.012(1)	2096.025(1)	2087.997(1)
9	184 – 197	K.YNGVFQECCQAEDK.G	0	2	1747.745(2)	1899.793(2)	–
10	184 – 204	K.YNGVFQECCQAEDKGACLLPK.I	1	3	2430.202(2)	–	–
11	198 – 204	K.GACLLPK.I	0	1	758.441(1)	834.464(1)	–
12	223 – 228	R.CASIQK.F	0	1	–	782.391(1)	774.375(1)
13	267 – 285	K.ECCHGDLLECADDRADLAK.Y	1	3	2247.996(3)	–	–
14	286 – 297	K.YICDNQDTISSK.L	0	1	1443.725(1)	1519.692(1)	1511.671(1)
15	298 – 309	K.LKECCDKPLLEK.S	1	2	1532.813(2)	1684.863(2)	–
16	310 – 318	K.SHCIAEVEK.D	0	1	–	1148.566(1)	–
17	372 – 386	R.LAKEYEATLEECCAK.D	1	2	1814.867(2)	1966.937(2)	–
18	375 – 386	K.EYEATLEECCAK.D	0	2	–	1654.731(2)	1638.857(2)
19	387 – 399	K.DDPHACYSTVFDK.L	0	1	–	1630.677(1)	–
20	387 – 401	K.DDPHACYSTVFDKLK.H	1	1	1795.858(1)	1871.897(1)	1863.870(1)
21	402 – 420	K.HLVDEPQNLIKQNCDQFEK.L	1	1	2355.167(1)	–	–
22	413 – 420	K.QNCDQFEK.L	0	1	1068.456 (1)	1144.477(1)	1136.463(1)
23	413 – 433	K.QNCDQFEKLGEYGFQNALIVR.Y	1	1	2529.286(1)	2605.262(1)	–
24	460 – 468	R.CCTKPESER.M	0	2	–	1318.569(2)	1302.552(2)
25	469 – 482	R.MPCTEDYLSLILNR.L*	0	1	1740.864(1)	1816.878(1)	1808.859(1)
26	483 – 489	R.LCVLHEK.T	0	1	898.504(1)	974.516(1)	–
27	499 – 507	K.CCTESLVNR.R	0	2	–	1157.555(1)	–
28	499 – 507	K.CCTESLVNR.R	0	2	1138.521(2)	1290.570(2)	1274.542(2)
29	508 – 523	R.RPCFSALTPDETYVPK.A	0	1	1880.958(1)	1956.971(1)	1948.955(1)
30	524 – 544	K.AFDEKLFTFHADICTLPDTEK.Q	1	1	2498.254 (1)	2574.219(1)	2566.248(1)
31	529 – 544	K.LFTFHADICTLPDTEK.Q	0	1	1907.963(1)	1983.977(1)	1975.950(1)
32	581 – 597	K.CCAADDKEACFAVEGPK.L	1	3	1927.848 (3)	2155.926(3)	2131.899(3)
No. of alkylated Cysteines identified (theoretical 35)					28 (80%)	30 (85.7%)	17 (48.5%)
No. of alkylated peptides identified (theoretical 71)					23 (32%)	28 (39.5%)	14 (20%)
No. of uniquely alkylated peptides identified					4	9	

improvement of identification of proteins in a smaller amount over IA, and a higher sequence coverage and a higher score in the database search. Therefore, we found that IAA has considerable advantages over IA in combination with MALDI.

Earlier, we observed that NEM modification showed ionization efficiencies more enhanced than IAA modification in combination with ESI, which significantly contrasts with the present results with MALDI. While the reason is not clear, it may be speculated that the existence of the phenyl ring might increase absorption at the laser wavelength applied in MALDI, contributing to more desorption of a greater amount of peptides modified with IAA. These observations are anticipated to be useful in designing new modifiers according to the types of mass spectrometer to be applied, and in more confident identification of proteins in the future.

Experimental

Alkylation of peptides

The alkylation of model peptides was performed as described earlier.[1] Briefly, the stock solutions of peptides were prepared at a concentration of 0.6 mM (Tris-buffer, 50 mM, pH = 8.5). The stock solutions of IAA, IA and NEM were, respectively, prepared in DMSO at a concentration of 20 mM, in a Tris-buffer at a concentration of 50 mM at pH = 8.5 and in DMSO at a concentration of 10 mM. Each peptide stock solution (2 μL) was mixed with 2 μL of the stock solution of IA, IAA or NEM and left for 0.5–1 h in a dark place. A large excess of β-mercaptoethanol was added to each solution to terminate the alkylation reaction. Samples were diluted with 50% acetonitrile containing 0.1% TFA prior to MS measurement to obtain a sufficient amount on the MALDI-plate. The comparison of the peak intensities was performed against IA-modified peptides. The alkylated peptides (IAA or NEM) were mixed with IA-peptides at the same amount of 8 fmol each (IAA-peptides with IA-peptides or NEM-peptides with IA-peptides) for PEP 61 and PEP 31, and 50 fmol for PEP 13 on the plate. For experiments of the LOD, peptides alkylated with IA, IAA or NEM were mixed together in an equal mole, and the MALDI measurement of successive dilution of the mixture was performed to determine the LOD for each alkylating reagent used in this study.

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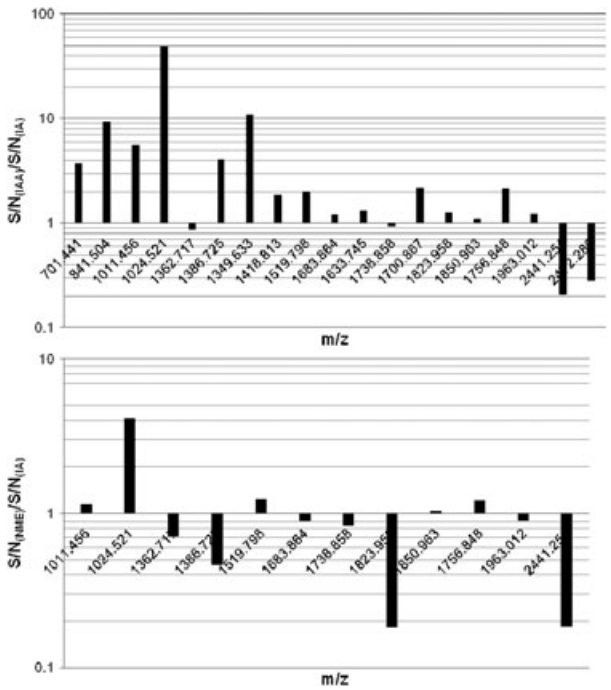

Figure 4. The logarithmic scale of the S/N values of IAA-peptides/IA-peptides (top) and NEM-peptides/IA-peptides (bottom). The m/z values are the masses of peptides without modification by alkylation reagent IA, IAA or NEM.

Alkylation of a protein

The procedure for cysteine alkylation of a model protein, BSA, was performed before the sample was subjected to SDS-PAGE as described previously.[10] The stock solution of protein was prepared in a buffer containing Tris-HCl (100 mM), 3% SDS, 20 mM tributylphosphine at a concentration of 0.1 mg/mL and pH = 8.5, and left for 1 h at room temperature for completion of the reduction. The protein solution (10 μL) was incubated with 1 μL of the solution of alkylation reagent, IAA (200 mM in DMSO), IA (200 mM in the above Tris-buffer) or NEM (100 mM in DMSO). The mixture was shaken and kept in a dark place for 2 additional hours.

1D SDS-PAGE

To the protein reaction mixture prepared above was added 2 μL of a solution containing 20% (v/v) glycerol and 0.1% (w/v) bromophenol blue. The amount of protein loaded on the gel was 1 μg. The mixture was directly subjected to SDS-PAGE, and separation was performed at 120 V for 90 min. The gel was stained by Coomassie Brilliant Blue for overnight.

In-gel digestion

The protein bands from the 1D electrophoresis above were washed with 100 μL H_2O for 5 min and with 100 μL of a solution H_2O/acetonitrile (50/50) at 37°C and 600 rpm on a Thermomixer comfort (Eppendorf). The latter step was repeated until the

Table 3. The identified peptides (alkylated and non-alkylated) obtained from the digestion of BSA alkylated either by IAA or IA. The numbers represent the obtained masses of the respective alkylated peptide. The numbers given in parentheses are the S/N values of the respective peptides. MC: missed cleavage, C: no. of cysteines

No.	Position	Peptide	MC	C	IA	IAA
1	25–34	R.DTHKSEIAHR.F	1	0	1193.520 (30)	1193.505 (20)
2	35–44	R.FKDLGEEHFK.G	1	0	1249.517 (38)	1249.522 (13)
2	66–75	K.LVNELTEFAK.T	0	0	1163.534 (17)	1163.541 (13)
3	76–88	K.TCVADESHAGCEK.S	0	2	---------	1615.549 (26)
4	89–100	K.SLHTLFGDELCK.V	0	1	1419.581 (45)	1495.606 (67)
5	161–167	K.YLYEIAR.R	0	0	927.418 (237)	927.416 (137)
6	161–168	K.YLYEIARR.H	1	0	1083.513 (15)	1083.506 (18)
9	233–241	R.ALKAWSVAR.L	1	0	1001.506 (36)	1001.500 (48)
11	286–297	K.YICDNQDTISSK.L	0	1	---------	1519.550 (29)
12	298–309	K.LKECCDKPLLEK.S	1	2	---------	1684.716 (28)
13	347–359	K.DAFLGSFLYEYSR.R	0	0	1567.632 (131)	1567.626 (93)
14	360–371	R.RHPEYAVSVLLR.L	1	0	1439.704 (219)	1439.693 (192)
16	402–412	K.HLVDEPQNLIK.Q	0	0	1305.607 (16)	1305.600 (24)
17	413–433	K.QNCDQFEKLGEYGFQNALIVR.Y	1	1	2529.030 (15)	----------
18	421–433	K.LGEYGFQNALIVR.Y	0	0	1479.684 (48)	1479.673 (46)
19	437–451	R.KVPQVSTPTLVEVSR.S	1	0	1639.821 (111)	1639.808 (128)
20	460–468	R.CCTKPESER.M	0	2	----------	1318.450 (29)
21	469–482	R.MPCTEDYLSLILNR.L	0	1	---------	1816.718 (11)
22	499–507	K.CCTESLVNR.R	0	2	----------	1290.467 (18)
23	508–523	R.RPCFSALTPDETYVPK.A	0	1	1880.785 (15)	1956.901 (11)
No. of peptides identified					14	19
No. of unique peptides identified					1	6
No. of Cysteine containing peptides identified					3	8
No. of cysteine alkylated					3	12
Sequence coverage					24%	33%
Score					203	255

Coomassie Brilliant Blue dye was completely removed, and the protein bands were incubated for 1 min 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 300 ng of trypsin (Promega Sequencing Grade Modified Trypsin, Promega Corporation, Madison, USA) at 37°C overnight, and peptide extraction was performed as described previously.[10] The extracted peptide solution was dried by Speed Vacuum and kept at −20°C for further analysis by the MALDI mass spectrometer.

Mass spectrometric analysis

All the mass spectrometric analysis described here with peptides and proteins was performed with a MALDI- time-of-flight (TOF)/TOF 4800 mass spectrometer (Applied Biosystems). The dried protein samples were mixed with 100 μL of the solution consisting of 50% CH_3CN, 0.1% trifluoroacetic acid (TFA). The protein digest sample or peptide sample solution (0.5 μL) was spotted on the MALDI plate followed by the addition of 0.5 μL of a matrix consisting of 5 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA. The mass spectra were acquired automatically in the positive mode, and a total of 1000 shots were accumulated per spectrum. The mass range was selected to be between 600 and 4000 m/z.

Identification of proteins

For identification of the protein, the MASCOT search engine (Matrix Science, V2.1) was utilized to perform the search for the obtained MS data. The following parameters were used for the search: enzyme, trypsin; allowed missed cleavages, 1; variable modification, oxidation of methionine. The mass tolerance for precursors was set to ±50 ppm for MS. The fixed modification was introduced for IAA-modified peptides (+133 Da) and NEM-modified peptides (+125 Da).

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References

[1] D. Wang, M. Douma, B. Swift, R. D. Oleschuk, J. H. Horton. The adsorption of globular proteins onto a fluorinated PDMS surface. J. Colloid Interface Sci. **2009**, *331*, 90.

[2] J. Toews, J. C. Rogalski, T. J. Clark, J. Kast. Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. Anal. Chim. Acta **2008**, *618*, 168.

[3] For example, (a) F. L. Brancia, S. G. Oliver, S. J. Gaskell. Improved matrix-assisted laser desorption/ionization mass spectrometric analysis of tryptic hydrolysates of proteins following guanidination of lysine-containing peptides. Rapid Commun. Mass Spectrom. **2000**, *14*, 2070; (b) S. Sechi, B. T. Chait. Modification of cysteine residues by alkylation: A tool in peptide mapping and protein identification. Anal. Chem. **1998**, *70*, 5150; (c) Z. Yang, A. B. Attygalle. LC/MS characterization of undesired products formed during iodoacetamide derivatization of sulfhydryl groups of peptides. J. Mass Spectrom. **2007**, *42*, 233; (d) J. J. Hedberg, E. J. Bjerneld, S. Cetinkaya, J. Goscinski, I. Grigorescu, D. Haid, Y. Laurin, B. Bjellqvist. A simplified 2-D electrophoresis protocol with the aid of an organic disulfide. Proteomics **2005**, *5*, 3088; (e) T. Kinumi, Y. Shimomae, R. Arakawa, Y. Tatsu, Y. Shigeri, N. Yumoto, E. Niki. Effective detection of peptides containing cysteine sulfonic acid using matrix-assisted laser desorption/ionization and laser desorption/ionization on porous silicon mass spectrometry. J. Mass Spectrom. **2006**, *41*, 103; (f) C. Hagman, M. Ramstrom, P. Hakansson, J. Bergquist. Quantitative analysis of tryptic protein mixtures using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. J. Proteome Res. **2004**, *3*, 587; (g) V. A. Gorshkov, K. A. Artemenko, T. Y. Samgina, A. T. Lebedev. Signal enhancement for protonated peptides in matrix-assisted laser desorption/ionization by doping with organic molecules. Mass-Spektrometriya **2007**, *4*, 5; (h) J. L. Frahm, I. D. Bori, D. L. Comins, A. M. Hawkridge, D. C. Muddiman, Anal. Chem. **2007**, *79*, 3989; (i) D. K. William, C. W. Meadows, I. D. Bori, A. M. Hawkridge, D. L. Comins, D. C. Muddiman. J. Am. Chem. Soc. **2008**, *130*, 2122.

[4] S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb, R. Aebersold. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat. Biotechnol. **1999**, *17*, 994.

[5] P. L. Ross, Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniel, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, D. J. Pappin. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics **2004**, *3*, 1154.

[6] For example, (a) M. B. Goshe, R. D. Smith. Stable isotope-coded proteomics mass spectrometry. Curr. Opin. Biotechnol. **2003**, *14*, 101; (b) W. A. Tao, R. Aebersold. Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. Curr. Opin. Biotechnol. **2003**, *14*, 110; (c) M. R. Flory, T. J. Griffin, D. Martin, R. Aebersold, Advances in quantitative proteomics using stable isotope tags. Trends Biotechnol. **2002**, *20*, S23; (d) S. D. Patterson. Proteomics: The industrialization of protein chemistry. Curr. Opin. Biotechnol. **2000**, *11*, 413–418; (e) T. J. Griffin, D. R. Goodlett, R. Aebersold. Advances in proteome analysis by mass spectrometry. Curr. Opin. Biotechnol. **2001**, *12*, 607; (f) R. Aebersold, D. R. Goodlett. Mass spectrometry in proteomics. Chem. Rev. **2001**, *101*, 269; (g) M. Hamdan, P. G. Righetti. Modern strategies for protein quantification in proteome analysis: advantages and limitations. Mass Spectrom. Rev. **2002**, *21*, 287; (h) R. Aebersold, M. Mann. Mass spectrometry-based proteomics. Nature **2003**, *422*, 198; (i) S. Julka, F. Regnier. Quantification in proteomics through stable isotope coding: a review. J. Proteome Res. **2004**, *3*, 350; (j) M. Broenstrup. Absolute quantification strategies in proteomics based on mass spectrometry. Expert Rev. Proteomics **2004**, *1*, 503; (k) P. G. Righetti, N. Campostrini, J. Pascali, M. Hamdan, H. Astner. Quantitative proteomics: a review of different methodologies. Eur. J. Mass Spectrom. **2004**, *10*, 335; (l) A. Panchaud, J. Hansson, M. Affolter, R. B. Rhlid, S. Piu, P. Moreillon, M. Kussmann. ANIBAL, stable isotope-based quantitative proteomics by aniline and benzoic acid labeling of amino and carboxylic groups. Mol. Cell. Proteomics **2008**, *7*, 800; (m) J. Zhang, L. Zhang, Y. Zhou, Y.-L. Guo. A novel pyrimidine-based stable-isotope labeling reagent and its application to quantitative analysis using matrix-assisted laser desorption/ionization mass spectrometry. J. Mass Spectrom. **2007**, *42*, 1514; (n) J. Rivers, D. M. Simpson, D. H. L. Robertson, S. J. Gaskell, R. J. Beynon. Absolute multiplexed quantitative analysis of protein expression during muscle development using QconCAT. Mol. Cell. Proteomics **2007**, *6*, 1416; (o) C. Guerrero, C. Tagwerker, P. Kaiser, L. Huang. An integrated mass spectrometry-based proteomic approach. Quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. Mol. Cell. Proteomics **2006**, *5*, 366.

[7] S. Niwayama, S. Kurono, H. Matsumoto. Synthesis of d-labeled N-alkylmaleimides and application to quantitative peptide analysis by isotope differential mass spectrometry. Bioorg. Med. Chem. Lett. **2001**, *11*, 2257.

[8] S. Niwayama, S. Kurono, H. Matsumoto. Synthesis of ^{13}C-labeled iodoacetanilide and application to quantitative peptide analysis by isotope differential mass spectrometry. Bioorg. Med. Chem. Lett. **2003**, *13*, 2913.

[9] S. Niwayama, S. Kurono, H. Cho, H. Matsumoto. Synthesis of d-labeled naphthyliodoacetamide and application to quantitative peptide analysis by isotope differential mass spectrometry. Bioorg. Med. Chem. Lett. **2006**, *16*, 5054.

[10] S. Kurono, T. Kurono, N. Komori, S. Niwayama, H. Matsumoto. Quantitative proteome analysis using d-labeled *N*-ethylmaleimide and ^{13}C-labeled iodoacetanilide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Bioorg. Med. Chem. **2006**, *14*, 8197.

[11] M. Zabet-Moghaddam, T. Kawamura, E. Yatagai, S. Niwayama. Electrospray ionization mass spectroscopic analysis of peptides modified with *N*-ethylmaleimide or iodoacetanilide. Bioorg. Med. Chem. Lett. **2008**, *18*, 4891.
[12] One of the cysteine-modifiers we reported previously, *N*-β-naphthyliodoacetamide, has a limited solubility in buffer solutions and therefore it was found unsuitable for studies with proteins, although it showed enhancement of ionization efficiencies of peptides, which were comparable to modification with IAA.
[13] We also prepared pyridinyl derivative, 2-iodo-*N*-(pyridin-3-yl) acetamide and examined its effects on enhancement of ionization efficiencies. However, this compound turned out to be rather unstable and showed only comparable or slightly lower enhancement of the ionization efficiencies of synthetic peptides or peptides from BSA.

