Mass Spectrometry
DOI: 10.5702/massspectrometry.A0059

Vol. 6 (2017), A0059

Original Article

A Facile Method for Preferential Modification of the N-Terminal Amino Group of Peptides Using Triazine-Based Coupling Reagents

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It has been shown that chemical modification of the peptide N-terminus with a charged tag greatly affects the fragmentation process caused by collision-induced dissociation to obtain more interpretable product ion spectra. In this study, we examined the selective introduction of a charged tag, 4-(guanidinomethyl)-benzoic acid (Gmb), into the peptide N-terminus. After optimization of the reaction conditions, we found that the most effective conversion in terms of the reaction rate and selectivity was achieved by reacting the peptide with the active ester of Gmb, prepared using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) at pH 7. This method is applicable to the introduction of various carboxylic acid-containing compounds into the N-terminus of peptides, which will be useful not only for improvement of MS/MS fragmentation but also for various biochemical studies of peptides and proteins.

Please cite this article as: Mass Spectrom (Tokyo) 2017; 6(1): A0059

Keywords: chemical tag, derivatization, MS/MS, fragmentation, active ester

(Received March 23, 2017; Accepted June 10, 2017)

INTRODUCTION

Chemical modification of peptides and proteins has been utilized for the modulation of their function and development of biotherapeutics.[1)] Introduction of various chemical tags also provides many useful applications in the field of analytical sciences including proteomics. The isotope-coded tags, for example, are essential for the precise quantitation of protein expression by mass spectrometry.[2)] We have been interested in the charged tags that affect the fragmentation process caused by collision-induced dissociation (CID) in tandem mass spectrometers, because it provides more easily interpretable product ion spectra to facilitate the *de novo* sequencing of peptides.[3)]

These modifications are usually performed at the N-terminal amino group of peptides, because it readily forms an amide bond with carboxyl group-containing compounds. However, due to the slight difference in reactivity between N-terminal α- and Lys ε-amino groups, both types of amino groups are modified unless special attention is paid for the reaction conditions. This is particularly disadvantageous in the case of MS-based *de novo* sequencing, where N-terminal-specific modification is essential to facilitate peptide fragmentation in a more interpretable fashion.[3)]

To achieve N-terminal-specific modification, various methods have been developed. One of the most commonly employed approaches is the precise control of pH values of the reaction medium, which takes advantage of the slight difference in pK_a values between the α- and ε-amino groups.[4)] To date, *N*-hydroxysuccinimide (NHS) esters of carboxylic acids have been widely used for this purpose, because of its ease of preparation and relatively high stability. However, even under judiciously controlled pH conditions, the selectivity is often unsatisfactory, accompanying a considerable level of modification at the Lys side chains.[5)] As an alternative approach, utilization of transamination reaction was developed,[6)] in which the N-terminal amino acid residue is specifically converted into α-ketoacid using aldehyde reagents for the subsequent introduction of various components having a hydroxylamine moiety. While this method is demonstrated to be effective in terms of selectivity, it is incompatible with peptides having the particular amino acid residues, such as Ser, Thr, Cys, and Trp, at the N-terminus because of their side reactions with aldehydes, limiting the range of applications. Thus, to overcome this problem, the same research group revised the method by utilizing 2-pyridinecarboxyaldehyde to form an imidazolizinone structure specifically at the N-terminus through an imine intermedi-

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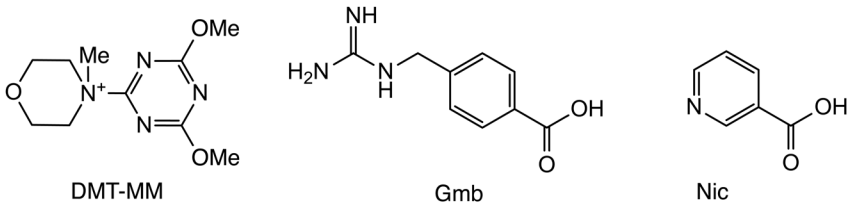

Fig. 1. Structure of compounds used in this study.

ate.[7] Likewise, Chan *et al.* also reported a method for selective N-terminal modification using ketene-containing compounds.[5] Although above-cited methods allow highly selective modifications of the peptide N-terminus regardless of the amino acid structure, they are disadvantageous in that they require laborious and time-consuming preparation of reagents containing a 2-pyridinecarboxyaldehyde or ketene moiety. Moreover, since ketene is highly reactive, care must be taken to avoid degradation of the reagents during storage. Thus, a simple and facile method for the N-terminal selective modification using commercially available reagents would be of great value.

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM, Fig. 1) is known to be a powerful condensation reagent for formation of amide bonds even in water as a solvent.[8] This property is suitable for chemical modification of peptides and proteins, because water is the best reaction solvent for these molecules in most cases. Although DMT-MM has been widely employed for peptide and protein modifications, its capability for selective modification at the N-terminal amino group of peptides has never been investigated. In this study, we examined the effectiveness of DMT-MM on N-terminal-specific introduction of 4-(guanidinomethyl)benzoic acid (Gmb, Fig. 1) as a charged tag, which is known to improve the MS/MS fragmentation of peptides.[9]

EXPERIMENTAL

Chemicals

Gmb and nicotinoyloxysuccinimide (Nic-NHS) were synthesized as previously described.[9] DMT-MM was obtained from Kokusan Chemical (Tokyo, Japan). The peptides used in this study were synthesized using Fmoc solid-phase chemistry in our laboratory. Endoproteinase Lys-C (mass spectrometry grade) was purchased from Wako Pure Chemical Industries, Ltd.

Derivatization of model peptides

The Gmb (10 nmol) was mixed with the DMT-MM (10 nmol) in H_2O (11 μL) for 20 h to obtain the active ester of Gmb (Gmb-DMT). After lyophilizing the reaction mixture, the peptide solution (1 nmol) in 0.2 M sodium phosphate buffer (pH 7.0, 14 μL) was added to Gmb-DMT (10 nmol). After reaction for 30 min at room temperature, the progress of the reaction was monitored by HPLC and LC/MS. Conversion rates were calculated by dividing the UV peak area (absorbance at 215 nm) of both mono- and di-modified peptides by the total area of unmodified, mono- and di-modified peptides. The N-terminal selectivity was determined based on the UV peak area of the peptides modified at the N-terminus, Lys side chains or both.

Derivatization of enzymatic digests of LaIT1

LaIT1 (40 nmol) was treated with 45 mM DTT in 0.2 M sodium phosphate buffer (pH 7.8, 80 μL) for 30 min at 50°C. Then, 100 mM iodoacetic acid in 0.2 M sodium phosphate buffer (pH 7.8, 80 μL) was added to this reaction mixture. After reaction for 30 min at room temperature, the reagents were removed by a C18 spin column (MonoSpin C18, GL Sciences, Tokyo, Japan). The resultant peptide was subjected to enzymatic digestion with endoproteinase Lys-C at a peptide/enzyme ratio of 50:1 (w/w) in 0.2 M sodium phosphate buffer (pH 7.8) for 18 h at 37°C. The peptides were partially purified by a C18 spin column. The digested peptides (0.3 nmol each) in 0.2 M phosphate buffer (pH 7.0, 10 μL) were mixed with Gmb-DMT (20 nmol) prepared as described above. After reaction for 30 min at room temperature, the progress of the reaction was monitored by LC/MS.

HPLC analysis

HPLC analysis was performed using a Vydac Everest C18 column (1×250 mm, Grace, Columbia, MD). The column was eluted at a flow rate of 0.05 mL/min using a mixture of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) with a 20-min linear gradient from 5 to 40% solvent B. The amount of each peptide was estimated based on peak areas of UV chromatograms monitored at 215 nm absorbance.

Mass spectrometric analysis

LC/MS/MS analysis was performed on an LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization probe. The HPLC separations were performed with a TSK-ODS V100 column (1×150 mm, TOSOH, Tokyo, Japan). The column was eluted at a flow rate of 0.05 mL/min using a mixture of 0.1% formic acid in water (solvent C) and 0.1% formic acid in acetonitrile (solvent D) with a 30-min linear gradient from 10 to 50% solvent D. All mass spectra were obtained in the positive mode, and precursor ions for MS/MS analysis were automatically selected based on the ion intensity. The amount of each peptide was estimated based on peak areas in total ion current (TIC) chromatograms.

RESULTS AND DISCUSSION

We previously demonstrated that modification of the peptide N-terminus with Gmb is effective for improving the backbone fragmentation by low-energy CID,[9] as well as for enhancing the side chain fragmentation by high-energy CID due to its high proton affinity.[10] Since Gmb contains a carboxyl group, its introduction can be achieved by formation of an amide bond with the amino group of peptides using condensation reagents. DMT-MM has been widely used for amide bond formation between various compounds, due

to its high reactivity superior to other reagents such as carbodiimide or NHS.[11] Therefore, we investigated the capability of DMT-MM for N-terminal-specific introduction of Gmb into peptides.

First, introduction of Gmb into the peptide N-terminus was evaluated using a model peptide I (AAGLAIASAKDD), which contains a Lys residue in the C-terminal region. The peptide was mixed with Gmb in the presence of DMT-MM in the buffer at pH 7.0, but none of modified peptides was observed after a reaction for 30 min (Entry 1 in Table 1, Fig. 2a). After incubation for another 20 h, formation of the modified peptide was observed although its conversion rate was relatively low. Based on the molecular mass measured by LC/MS, this peptide was postulated to be a peptide containing a single Gmb moiety (Entry 2 in Table 1, Fig. 2b). MS/MS analysis of the modified peptide (Fig. 3) indicated the existence of b_1–b_9 ions in the product ion spectrum, which confirmed the Gmb modification at the N-terminal amino group. Then, to improve the conversion rate, we optimized the reaction conditions. Mechanistically, it is known that DMT-MM forms active esters with carboxylic acids, which subsequently react with the amino group(s) of the peptide to be modified. In this regard, the procedure employed in Entries 1 and 2 was considered to be unfavorable, in which formation of the active ester could not proceed efficiently in the presence of the peptide or/and the buffer salts, to lower the overall reaction yield. Therefore, we divided the procedure into two steps; firstly, Gmb was reacted with DMT-MM to obtain the active ester (Gmb-DMT) in H_2O without the peptide, and the reaction mixture was lyophilized to remove H_2O. Secondly, to this concentrated Gmb-DMT was added the peptide dissolved in the buffer suitable for selective amide bond formation at the N-terminus to complete the reaction. This procedure dramatically improved the conversion rate from 9 to 70%, without affecting the reaction selectivity (Entry 3 in Table 1, Fig. 2c). The formation of the di-modified peptide, in which both the N-terminus and the Lys side chain were modified, was also observed, but its proportion was relatively low compared to the N-terminally modified peptide.

Next, the effect of the pH condition on the N-terminal-specific modification was examined. When the reaction was performed at pH 8.0 instead of pH 7.0 using the preformed

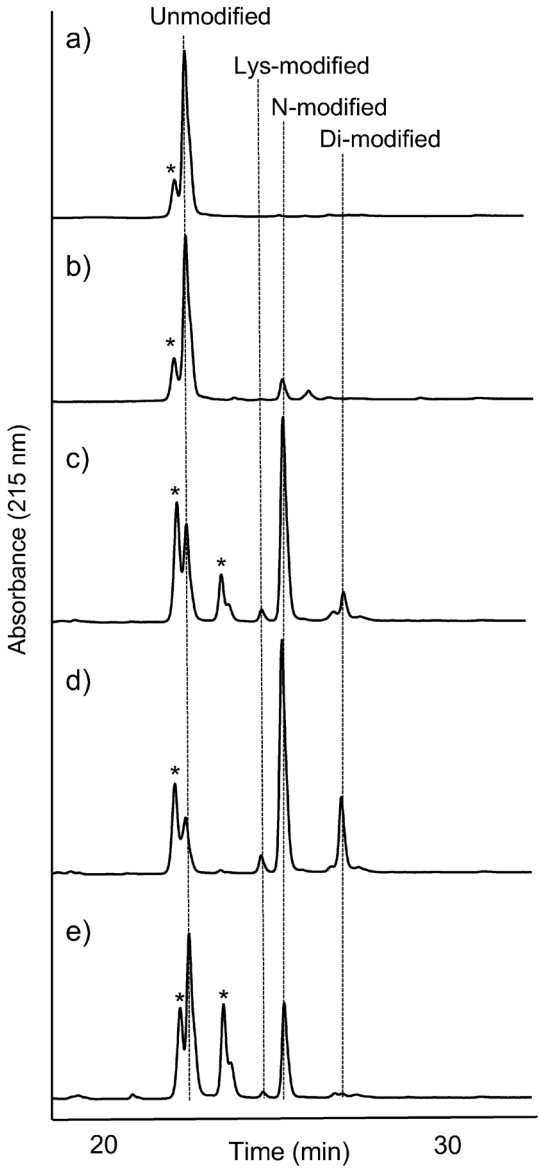

Fig. 2. HPLC profile of the modification reaction. Results of the reactions without preformation of the active ester at pH 7.0 for 30 min (a) and for 20 h (b), and those with the preformed active ester at pH 7.0 (c), pH 8.0 (d), and pH 6.0 (e) for 30 min. Asterisks indicate compounds derived from DMT-MM.

Table 1. Modification of the model peptides.

Entry	Tag[a]	Peptide[b]	Condition[c]	Preformation of the active ester	Conversion (%)[d]	N-Terminus : Lys : Both
1	Gmb	I	pH 7, 30 min	–	0	—
2	Gmb	I	pH 7, 20 h	–	9	100 : 4 : 0
3	Gmb	I	pH 7, 30 min	+	70	100 : 4 : 13
4	Gmb	I	pH 8, 30 min	+	83	100 : 6 : 30
5	Gmb	I	pH 6, 30 min	+	35	100 : 5 : 4
6	Gmb	II	pH 7, 30 min	+	49	100 : 0 : 7
7	Nic	I	pH 7, 30 min	+	11	100 : 5 : 0[e]
8	Nic	I	NHS, pH 6.0, 30 min	+	2.4	100 : 30 : 0

[a]Gmb, 4-(guanidinomethyl)benzoic acid; Nic, nicotinic acid.
[b]Peptide I, AAGLAIASAKDD; peptide II, ATQQTAAYKTLVS.
[c]Conditions: Entries 1 and 2; 70 μM peptide I, 700 μM Gmb, 700 μM DMT-MM. Entries 3–6; 70 μM peptide I or II, 700 μM Gmb-DMT. Entry 7; 70 μM peptide I, 3.5 mM Nic-DMT. Entry 8; 70 μM peptide I, 700 μM Nic-NHS at pH 6.0 for 30 min.
[d]Determined based on the UV peak areas in HPLC analysis.
[e]Determined based on the peak areas in the extracted ion (m/z 604.4 corresponding to $[M+2H]^{2+}$ for mono-modified peptides) chromatogram of LC/MS analysis.

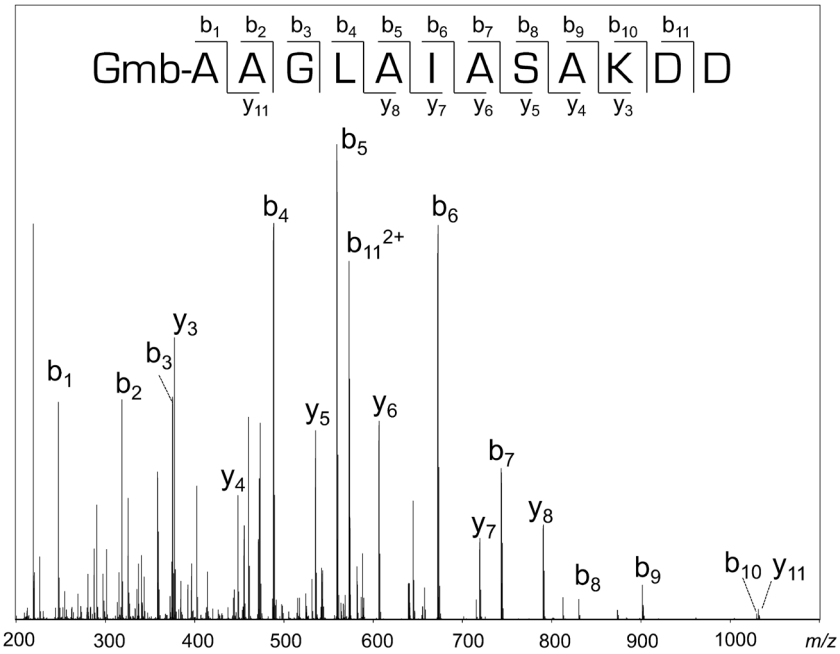

Fig. 3. Product ion spectrum of the Gmb-modified peptide I (precursor ion: $[M+2H]^{2+}$=639.3).

Table 2. Modification of the peptides obtained by enzymatic digestion of LaIT1.

No.	Peptide sequence[a]	Conversion (%)[b]	N-Terminus : Lys : Both[b]
1	DFPLSK	88	100 : 13 : 35
2	EYETCVRPRK	84	100 : 14 : 57
3	CQPPLK	93	100 : 0 : 20
4	AQICVDPK	78	100 : 0 : 10

[a]The side chain of Cys residues is carboxymethylated.
[b]Determined based on the peak areas in the TIC chromatograms of LC/MS analysis.

Gmb-DMT, the conversion rate was improved from 70 to 83% (Entry 4 in Table 1, Fig. 2d). However, the ratio of the di-modified peptide to the N-terminally modified peptide increased more than twice as much as that observed at pH 7.0. On the other hand, the reaction at pH 6.0 showed the lower conversion rate (35%) than that observed at pH 7.0, although formation of the di-modified peptide was significantly suppressed (Entry 5 in Table 1, Fig. 2e). Interestingly, the pH values of the reaction media did not affect the selectivity between the N-terminus and the Lys side chains (N-terminus : Lys=100 : 4–6). This tendency was also observed for the ketene-mediated reactions,[5] in which formation of the di-modified peptides increases with increasing conversion rates, but without affecting the selectivity. This implies that, under the conditions that lead to high conversion rates, the reaction occurs first at the N-terminal amino group of most of the peptides, followed by the modification at the Lys side chains to generate the di-modified peptides. Considering the balance between the conversion rates and the ratio of the di-modified peptide formation, the reaction at pH 7.0 was judged to be the most practical for the N-terminal selective modification using DMT-MM under current conditions. To examine the general applicability of this method, modification was performed using a peptide with different sequence (peptide II, Entry 6 in Table 1, Fig. S1) or a different tag (Nic, Entry 7 in Table 1, Fig. S1). Even using the peptide II, its N-terminal amino group was selectively modified. Likewise, high N-terminal selectivity was observed using Nic as a tag, suggesting that the method shown in this study is applicable to other carboxyl group-containing compounds. In the case of Nic, the conversion rate was lower (11%) than that using Gmb, but it would be improved by optimizing the reaction conditions, such as pH values. When this result was compared with that obtained by the NHS ester method (Entry 8 in Table 1), the latter showed a much lower conversion rate and lower selectivity. This clearly indicates that the method developed in this study is superior to the NHS ester method.

To further evaluate the effectiveness of this method, the modification was carried out for the peptides obtained by enzymatic digestion. The scorpion toxin LaIT1 was used as a model peptides, which contains several Lys residues.[12] After reduction and alkylation of cysteine residues, LaIT1 were digested with endoproteinase Lys-C, and resultant peptides were subjected to the modification. As shown in Table 2, conversion rates were satisfactory for all peptides (78–93%). Preferential introduction of Gmb into the N-terminal amino group was also achieved for all peptides (N-terminus : Lys=100 : 0–14), which were confirmed based on the observation of a series of *b*-ions in product ion spectra (Fig. S2). Relatively high ratios of di-modified peptide formation were observed for peptides 1 and 2, compared to peptides 3 and 4. This situation in peptides 1 and 2 seems similar to that observed for the modification of peptide I at pH 8.0. It is possible that the reactions of peptides 1 and 2 proceed faster than those of peptides 3 and 4 even at pH 7.0. The presence of acidic and/or aromatic residues in the N-terminal region in peptides 1 and 2 may affect the microenvironment of the terminal amino group to increase the reactivity. The use of lower pH values or shorter reaction times could reduce the di-modified peptide formation in peptides 1 and 2, although the conversion rates of peptides 3 and 4 would decrease. Furthermore, the di-modified peptide

formation does not practically interfere with the following interpretation of the product ion spectra, because they are easily distinguishable from the singly modified peptides based on the difference in the molecular mass. This is totally not the case when the peptides singly modified at the N-terminus and at the Lys side chain are mixed to give the ions having the same molecular mass with different structures. In consequence, this method is practically useful for obtaining N-terminally modified peptides without special preparation of reagents, which will facilitate the *de novo* sequencing analysis of bioactive peptides.

CONCLUSION

In this study, we demonstrated that a commercially available reagent DMT-MM can be used for N-terminal specific introduction of a charged tag in a facile manner. Unlike other methods reported to date, the reaction procedure used in this study is relatively simple and requires no special preparation of reagents. This modification method is applicable to introduction of various carboxyl group-containing compounds to the N-terminus of peptides and proteins, which will be useful not only for improvement of peptide fragmentation but also for various biochemical studies of peptides and proteins.

Abbreviations

CID, collision-induced dissociation; NHS, *N*-hydroxysuccinimide; DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; Gmb, 4-(guanidinomethyl)benzoic acid; Nic, nicotinic acid.

Acknowledgement

This study was partly supported by MEXT KAKENHI (Grant no. 25513001), Japan.

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