

Peptide C-terminal Sequence Analysis by MALDI-TOF MS Utilizing EDC Coupling with Br Signature

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The unique Br signature was utilized for C-terminal amino acid sequencing of model peptides. C-terminal carboxyl group was selectively derivatized in peptides, containing side chain carboxyl group, using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and Br was introduced using 4-bromophenylhydrazine hydrochloride (BPH) in a one pot reaction. Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) tandem mass spectra were obtained carrying the Br signature in the y-series ions. The Br signature facilitated C-terminal sequencing and discrimination of C-terminal carboxyl groups in the free acid and amide forms.

Key Words : C-terminal sequencing, MALDI -TOF MS, Peptide, Br signature

Introduction

Mass spectrometry (MS) has been used for both N-terminal and C-terminal sequencing of peptides. Edman degradation is a well known reaction used for N-terminal sequence analysis, which can be confirmed by MS.¹ Recently, Yamaguchi *et al.* determined N-terminal sequence of proteins utilizing sulfo-NHS-SS-biotin.²

Methods for C-terminal analysis are rather limited. One approach is a sequential degradation of peptides using carboxypeptidase followed by MS analysis of the resulting peptide ladder.³ Selective C-terminal modification of peptides and proteins in the presence of side chain carboxyl groups from Asp and Glu is a challenge. In 2006, Nakazawa *et al.* developed a tagging method using imidazoline hydrazine to form oxazolone ring at the C-terminus of peptides and proteins while protecting side chain carboxyl groups.⁴ In 2009, An attempted to introduce the Br isotopic signature (⁷⁹Br 50.7%; ⁸¹Br 49.3%) to the C-terminal carboxyl group using bromophenylhydrazine to react with an active ester formed between oxazolone ring and hydroxybenzotriazole coupling reagent.⁵ The overall data analysis was somewhat complicated due to side reactions and some loss of the Br signature from the y-ions probably during the MS/MS process.

In this study, we attempted to specifically derivatize the C-terminal carboxyl group and introduce the Br signature at the same time, so that the Br signature could be used for identification of the y-series ions from the MS/MS spectra in the presence of b-ions without the Br signature. We describe a simple and straightforward method for introducing the Br signature by coupling the C-terminal carboxyl group with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimidehydrochloride followed by reaction with a nucleophile carrying a Br atom.

Experimental Section

Materials. EDC hydrochloride, BPH hydrochloride, 4-

bromobenzoic hydrazide, 4-bromophenethylamine, acetonitrile (ACN), trifluoroacetic acid, α -cyano-4-hydroxycinnamic acid (CHCA), and model peptides (vasoactive intestinal peptide fragment 1-12, HSDAVFTDNYTR, DSDPR, ASHLGLAR, WAGGDASGE, and DRVYIHPFHL) were purchased from Sigma Aldrich (St Louis, MO, USA). 2-Mercaptoethanol was from Bio-Rad (Hercules, CA, USA). Ziptip was from Millipore (Watford, UK). RKDVY, RKEVY-NH₂ and AYDFEPYR were synthesized by Merrifield solid phase method.

EDC-BPH Coupling Reaction. 10 μ L of 30 mM BPH in ACN was added to 20 μ L of 25 μ M peptide solution. 10 μ L of 30 mM EDC in ACN was then added to the mixture. The mixture was vigorously mixed by pipeting and incubated for 20 min at 40 °C. 1 μ L of 100 mM 2-mercaptoethanol was added to the mixture to quench the EDC. Then, the modified peptide was purified with Ziptip for MS analysis.

MALDI-TOF Mass Spectrometry. 10 mg of CHCA matrix was dissolved in 1 mL of 50% ACN/0.1% trifluoroacetic acid/water solution. The matrix solution was saturated with 7 mM nitrilotriacetic acid to suppress matrix cluster peaks.⁶ Then 1 μ L of the matrix solution was mixed with 1 μ L sample solution. 1 μ L of the mixture was loaded onto the MALDI plate and dried in vacuum. Mass spectra were obtained using Autoflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). A typical mass spectrum was obtained by averaging 100 laser shots. MALDI TOF/TOF spectra were obtained in the laser-induced dissociation mode by averaging 1000 shots.

Results and Discussion

Incorporating the Br Signature. It has been known that EDC reacts with carboxyl groups forming an amine-reactive *O*-acylisourea intermediate.⁷ In general, EDC reacts with a carboxyl group at pH 4.7-6.0 and the EDC coupling reaction is performed at room temperature for several hours. Our

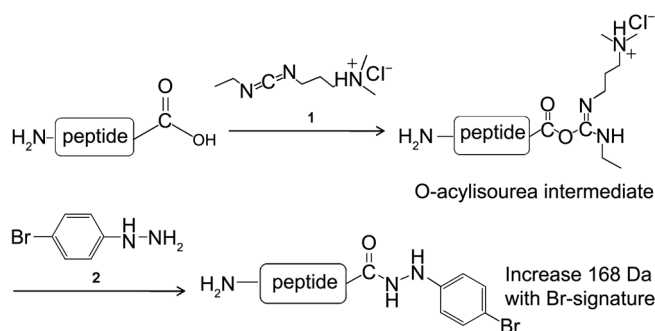


Figure 1. Reaction scheme for EDC-BPH coupling to the C-terminal carboxyl group.

approach was to introduce the Br moiety to the intermediate using a nucleophile added to the reaction mixture in advance (Fig. 1).

Selective C-terminal tagging with a moiety carrying the Br signature (51% ^{79}Br , 49% ^{81}Br) would be useful for C-terminal analysis. However, EDC coupling could take place with side chain carboxyl groups as well. EDC coupling shows a pH dependence.⁸ It was hoped that the reaction carried out at pH 3 could discern the C-terminal carboxyl group (pK_a 2) from Asp and Glu side chain carboxyl groups (pK_a 3.6-4). However, the reaction did not take place at pH 3, because it is outside the EDC activation range (pH 4.6-7.0).⁹ Thus, we hoped to selectively derivatize the C-terminal carboxyl group at higher pH by time and temperature control.

A Br-containing nucleophile was selected before time or temperature optimization. Several candidate nucleophiles with a Br atom bonded to an aromatic ring were tested. After the nucleophilic substitution, the aromatic ring should prevent, by resonance stabilization, dissociation of the Br atom. 4-Bromobenzoic hydrazide, 4-bromophenethylamine, and BPH were used as nucleophile in the coupling reaction of the VIP fragment 1-12 with EDC. 4-Bromobenzoic hydrazide did not produce any MALDI signal carrying the Br signature. When bromophenethylamine and BPH were used, the peptide peak was shifted by 181 Da and 168 Da, respectively, and showed the Br signature. However, 4-bromophenethylamine was difficult to remove by Ziptip and caused peak suppression in the MS analysis. Overall, the results were most satisfactory with BPH. Therefore, we selected BPH as nucleophile and used it in optimizing the reaction time and temperature.

Selective Tagging of the C-terminus. It would be desirable to find reaction time and temperature for tagging the C-terminal carboxyl group only. Thus we needed to investigate which carboxyl group was modified under certain reaction conditions.

Figure 2 shows the result of EDC-BPH coupling with VIP fragment 1-12 (MW: 1424.6) after 30 min at 25 °C. The control peptide peak at m/z 1425.58 (Fig. 2(a)) disappeared and a peak carrying a unique mono-Br signature was observed at m/z 1593.82 (Fig. 2(b)). The mass shift of 168.24 was consistent with 167.97 expected from the EDC-BPH tag. There are three carboxyl groups in the peptide (one C-

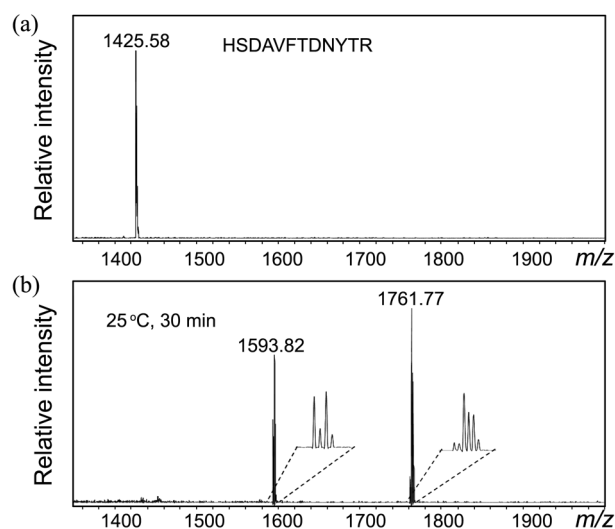


Figure 2. MALDI mass spectra showing the control peptide (a) and the peptide with one tag (1593.82) and two tags (1761.77) (b).

terminal group and two side chain groups) and we could not tell from the mass shift whether any one of them was specifically tagged or the peak represented a mixture of three peptide species carrying the tag at different sites. Another peak carrying a di-Br signature was observed at 1761.77. Anyhow, it was clear that the EDC-BPH tagging readily

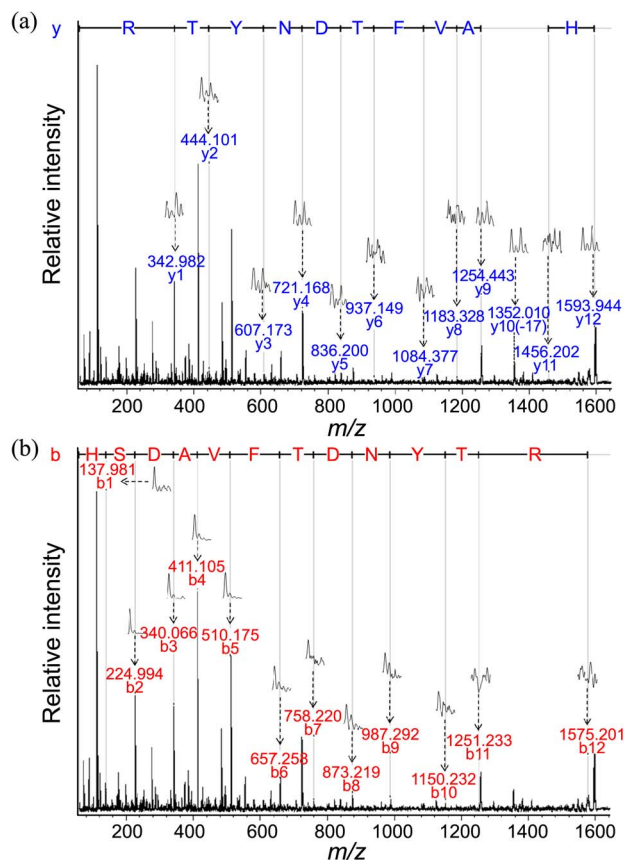


Figure 3. MALDI TOF-TOF spectrum from the VIP fragment peptide with a single tag (1593.82 peak in Fig. 2(b)) showing y-ions (a) and b-ions (b).

takes place under the mild reaction conditions.

MALDI TOF-TOF spectrum was obtained from the 1593.82 peak in Figure 2(b) to investigate where the tag was attached. Figure 3(a) shows a series of peaks carrying the mono-Br signature. The peaks with the Br-tag could be interpreted as y1, y2, y3, y4, y5, y6, y7, y8, y9, y11, and y12 ions. The y10 ion carrying the Br-tag peak appeared at m/z 1352.01, 17 Da less than expected. This introduced some ambiguity in the N-terminal side. However, nine residues from the C-terminus could be identified from the y-ions. The Br-tag at the y-1 ion clearly shows that the C-terminal amino acid is modified by the EDC-BPH coupling reaction. All the peaks with the Br-tag were consistent with the tag being at the C-terminus and not at the side chain. This was a rather encouraging result showing preferred tagging of the C-terminus over the side chain.

The b-series ions should not carry the Br-tag if the 1593.82 peak in Figure 2(b) represents a single species with the Br-tag at the C-terminus. Figure 3(b) shows a series of b-ions without the Br signature, which led to a sequence consistent with the sequence determined from the y-ions. Thus, the Br-tag is useful for distinguishing between y-ions and b-ions, and the peptide sequence determined from the y-ions can be confirmed by the sequence determined from the complementary b-series information.

The MS/MS analysis of the second peak in Figure 2(b) at 1761.77 with the di-Br signature showed that the second tag was attached to both side chain carboxyl groups with about the same frequency (result not shown). The y1, y2, y3, and y4 ions showed the mono-Br signature indicating that the Br tag was on the C-terminus. y5 and subsequent ions showed the di-Br signature, which was consistent with the 5th residue from the C-terminus being Asp. The result shows that the Br-tag can be used to determine the position of Asp and Glu residues as well as the C-terminal amino acid sequence.

Time and Temperature Control. Once it was demonstrated that the Br-tag is first added to the C-terminus, we searched for a condition for maximal tagging on the C-terminus with minimal tagging on the side chain. Considering that the EDC coupling reaction is exothermic, we increased the temperature to reduce formation of the doubly tagged peptide. Figure 4(a) shows the MALDI-MS spectrum from tagged VIP peptide after 30 min reaction at 40 °C. Only one peak was observed at 1593.89 with the mono-Br signature. After 30 min at 50 °C, the control peptide was observed at 1425.56 as well as the singly tagged peptide. A 1407.54 peak was also observed resulting from dehydration of the control peptide. Thus 40 °C seemed optimal for tagging the C-terminus without tagging side chain carboxyl groups. The same result was obtained when the reaction time was reduced to 20 min. A total of 10 peptides were derivatized for 20 min at 40 °C. In some cases, the reaction was incomplete, and in other cases the reaction proceeded to double tagging. But in most cases, the singly tagged peptide showed the strongest peak as summarized in Table 1. So, we concluded that 20 min at 40 °C is optimal for selectively tagging the C-terminus.

Table 1. Preponderance of peptides with one tag after 20 min at 40 °C

Model peptide	Molecular weight	Control	1 tag	2 tags
DSDPR	588.57	+	++	-
RKDVY	679.36	+	++	-
ASHLGLAR	823.94	-	++	-
WAGGDASGE	848.81	-	++	+
AVTYLQR	849.47	-	++	-
AYDFEPYR	1059.46	-	++	-
DRVYIHPFHL	1296.48	+	++	-
HSDAVFTDNYTR	1425.46	-	++	-

+: observed, -: not observed, ++: major peak

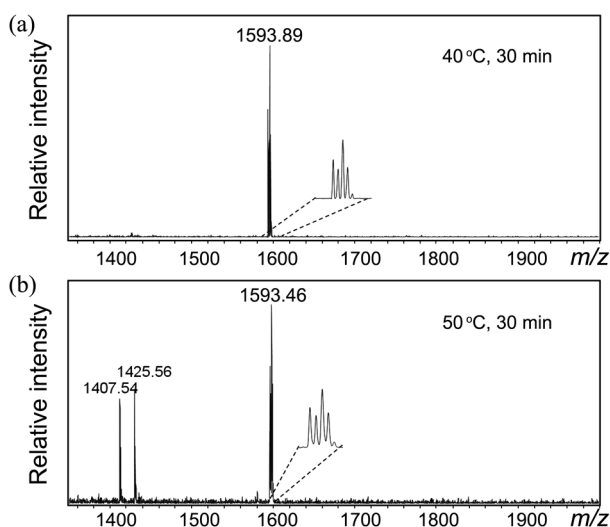


Figure 4. MALDI mass spectra from VIP fragment peptide showing only singly tagged peptide after 30 min at 40 °C (a) and the control peptide as well as the singly tagged peptide after 30 min at 50 °C (b).

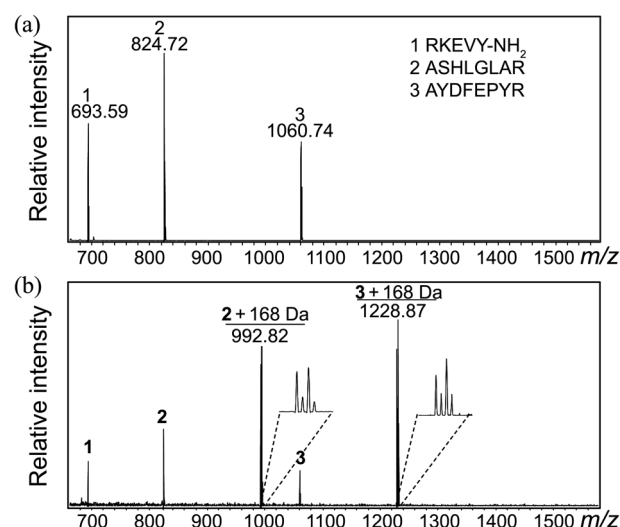


Figure 5. MALDI TOF mass spectra from a mixture of one peptide with the C-terminus in amide form (1) and two peptides in free acid form (2, 3) before (a) and (b) tagging reaction. Only peptides in free acid form are tagged.

Discrimination between Free Acid and Amide Forms. The C-terminal carboxyl group is often modified as an amide in proteins. The Br tagging would be a straightforward way of determining whether the C-terminal carboxyl group is in free acid or amide form. Figure 5 shows the MALDI mass spectrum from a mixture of three peptides, RKEVY-NH₂, ASHLGLAR, and AYDFEPYR at 20 μM concentration each. Under the reaction conditions leading to modification of the C-terminus only, RKEVY-NH₂ showed unmodified peptide at *m/z* 693.59, but not the modified peptide, indicating that the side chain carboxyl group in Glu is not tagged. The C-terminus was not modified because it was in the amide form. However, ASHLGLAR and AYDFEPYR showed tagged peptide at 992.82 and 1228.87, respectively, as well as the control peptide.

Conclusions

A Br tag, 4-bromophenylhydrazine hydrochloride, can be selectively coupled, in a one pot reaction carried out at 40 °C for 20 min, to the C-terminal carboxyl group of peptides using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride as coupling reagent. The reaction is simple and C-terminal sequencing using either y- or b-series MALDI

TOF-TOF signal is straightforward. The reaction can also be used to distinguish between carboxyl groups in free acid and amide forms.

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