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Description	

Protein Carbonylation Detected with Light and Heavy Isotope-Labeled 2,4-Dinitrophenylhydrazine by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Oxidation of proteins leads to carbonylation—the formation of aldehydes or ketones—at the amino acid side chain and/or the terminal amino groups. Carbonylated proteins have been conventionally detected by UV absorption spectrometry of the stable adduct with 2,4-dinitrophenylhydrazine (DNPH). However, this routine method is limited to detection of the total carbonyl content and does not provide structural information. We developed an isotope-dilution method for the specific detection of carbonylated proteins using ¹²C₆-DNPH and ¹³C₆-DNPH. This method has the following steps: the oxidized protein or peptide is divided into two parts, and these parts are independently labeled with ¹²C₆-DNPH and ¹³C₆-DNPH; the mixtures of these two labeled solutions are subsequently measured with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The carbonylated peptide was found by searching for a doublet peak having a mass difference of 6 Da. We examined oxidized angiotensin I and oxidized lysozyme prepared by treatment with NaOCl. The oxidized angiotensin I showed four pairs of doublet peaks in the MALDI-TOF mass spectrum. The structure was determined by tandem mass spectrometry. In the case of tryptic digest of the oxidized lysozyme, two carbonylated products could be easily identified even in a complex mixture. The use of ¹³C₆-DNPH provides rapid and accurate detection of carbonylated peptides even in complex mixtures.

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1. Introduction

Protein oxidation is known to occur in a variety of diseases, and is thought to contribute to their pathology. Amino acid residues and the terminal groups in protein are susceptible to oxidation by reactive oxygen species (ROS), including superoxide anion (O₂^{•-}), peroxy radical (RO₂[•]), alkoxy radical (RO[•]), hydroxyl radical (HO[•]), hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), and singlet oxygen (¹O₂).¹⁾ For example, the oxidized proteins cause an etiology or progression of a number of human diseases, *e.g.* Alzheimer disease, Parkinson disease, arteriosclerosis.^{2)–4)} In a general mechanism, the oxidation reactions of proteins with ROS are classified into several reactions including

direct oxidation, secondary reaction *via* lipid peroxidation product, and that *via* glycation end product, which result in oxidation of amino acid side chains, terminal groups and/or cleavage of peptide chains.^{5),6)} These reactions generate carbonyl groups with aldehydes or ketones at the amino acid residues and finally induce a deterioration of protein.^{7)–9)} Detection and quantification of protein carbonylation have been studied extensively as a marker for oxidative stress.¹⁰⁾

The carbonyl group reacts readily with 2,4-dinitrophenylhydrazine (DNPH) to provide a stable 2,4-dinitrophenyl (DNP) hydrazone product. Quantification of the carbonylated protein has been usually performed by a spectrophotometric assay that measures the UV absorbance of DNP-derivatized protein.^{11),12)} This is the most widely utilized method of detecting protein carbonylation. An anti-DNP antibody is a highly sensitive tool for detection of a DNP-derivatized protein.¹³⁾ These methods are powerful techniques for quantifying the total carbonyl content of a protein or mixture of carbonylated proteins. Detection of specifically carbonylated proteins in a protein mixture was

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performed by immunoblot analysis of the proteins with two-dimensional gel electrophoresis using an anti-DNP antibody. However, this method depends on the conditions of electrophoresis-based separation, and the results are complicated by DNP-derivatized compounds without structural information. Mirzaei and Regnier reported that heavy and light isotope labeled Girard's P reagent (1-(2-hydrazino-2-oxoethyl)pyridinium chloride), a selective modifier for the carbonyl group, was used with mass spectrometry to identify carbonylated proteins in complex mixtures.¹⁴⁾ A mass spectrometry-based method has been developed to clarify the detailed structure of the carbonylated amino acid residues in the oxidized protein.

In this study, we developed an application to detect and characterize the structure of carbonylated peptides or proteins using unlabeled (¹²C₆-DNP) and ¹³C₆-labeled DNP by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. This method was applied to oxidized angiotensin I and oxidized lysozyme after reaction with NaOCl, which is known to be generated by phagocyte enzyme myeloperoxidase under physiological conditions.¹⁵⁾ Here we show that the carbonylated peptide can be easily found by searching for a mass difference of 6 Da and the ratio of peak height in the mass spectrum, even in a complex peptide mixture.

2. Experimental

2.1 Materials

Lysozyme (hen-egg) and ¹³C₆-benzene were purchased from Sigma-Aldrich Corp. (MO, USA). Angiotensin I (Human) was purchased from Peptide Institute Inc. (Osaka, Japan). Deionized water was obtained using a MilliQ system (Millipore Co., MA, USA). ZipTip C18 disposable desalting tips (ZipTip) were purchased from Millipore Co. (MA, USA). Trypsin (mass spectrometry grade) and other chemicals, including ¹²C₆-DNP, were purchased from Wako Pure Chemical (Osaka, Japan).

2.2 Synthesis of ¹³C₆-DNP

The labeled compound, ¹³C₆-DNP, was synthesized according to the routine procedure. First, ¹³C₆-benzene (0.441 g, 5.65 mmol) was brominated by refluxing at 55°C for 15 min with 0.137 mL (5.29 mmol) of Br₂ and 1 mg of Fe, and subsequently purified by distillation. The prepared bromobenzene was refluxed with 7.5 mL of H₂SO₄ and 5.0 mL of HNO₃ at 85°C. After purification by silica gel column chromatography, 316.3 mg (1.25 mmol) of 1-bromo-2,4-dinitrobenzene was obtained. A 4.0 mL solution of the obtained 1-bromo-2,4-dinitrobenzene in ethanol was added to 0.40 mL (14.4 mmol) of hydrazine hydrate in ethanol (1.5 mL) at 65°C. After cooling to room temperature, the precipitate was washed with cold ethanol and dried *in vacuo*. Finally, 201.0 mg (0.99 mol) of ¹³C₆-DNP (red crystal) was obtained.

2.3 Oxidation and modification reaction of angiotensin I, and measurement of the MALDI-TOF mass spectra

Angiotensin I aqueous solution (100 μL, 100 μM) was oxidized by adding 100 μL of 50 μM NaOCl phosphate buffer solution (pH 7.4) for 30 min on ice. Two 70 μL

aliquots of the oxidized angiotensin I solution were mixed separately with 70 μL of each 12.5 mM ¹²C₆-DNP and ¹³C₆-DNP in 2 M HCl, and incubated at room temperature for 30 min. The resulting solutions were mixed in the ratio 1 : 1 (v/v). This mixture was purified by a ZipTip according to the manufacturer's instructions. Briefly, the sample was absorbed to the ZipTip after regeneration with a 0.1% trifluoroacetic acid (TFA)/acetonitrile and 0.1% TFA aqueous solution. After washing with a 0.1% TFA aqueous solution, the peptide was eluted directly to a MALDI sample plate by 0.1% TFA and 50% acetonitrile aqueous solution. A matrix solution was prepared as 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in a 0.1% TFA and 50% acetonitrile aqueous solution, and mixed with the sample solution on the MALDI sample plate. MALDI-TOF mass spectra were obtained by an Axima CFR plus MALDI-TOF mass spectrometer (Shimadzu Co., Ltd., Kyoto, Japan) with a reflectron under positive ion mode.

Electrospray ionization (ESI)-tandem mass spectra were acquired by a LCQ Deca XP (Thermo Fisher Scientific, CA, USA) iontrap mass spectrometer with a nanospray interface under positive ion mode. The ZipTip-purified sample (20 μL) was diluted in 100 μL of 50% methanol/water, and introduced into the ion source at a flow rate of 1 μL/min with a syringe pump.

2.4 Oxidation and modification reaction of lysozyme

Lysozyme aqueous solution (200 μL, 50 μM) was oxidized by adding 200 μL of 50 μM NaOCl phosphate buffer solution (pH 7.4) for 30 min on ice. This mixture was filtered to remove NaOCl with an ultrafiltration device (Vivaspin 500, molecular weight cut off: 10 kDa, Sartorius Stedim Biotech, Goettingen, Germany) by centrifuging at 15,000 rpm and 4°C for 20 min. The filtrate was washed with a phosphate buffer and this filtration step was repeated three times. After filtering it for the third time, a phosphate buffer was added to the washed protein in the filtering device to bring the level to 200 μL. This solution was divided to two aliquots; one was mixed with 100 μL of 12.5 mM ¹²C₆-DNP in 2 M HCl, and the other with ¹³C₆-DNP in 2 M HCl. Both were incubated at room temperature for 30 min. Both solutions were precipitated with 50% ethanol/ethyl acetate at -20°C overnight. After centrifugation at 15,000 rpm for 20 min, these precipitates were suspended in 200 μL of 8 M urea. The ¹²C₆-DNP-modified and ¹³C₆-DNP-modified oxidized lysozyme samples were mixed at 1 : 3, 1 : 1, 3 : 1 (v/v) for a final volume of 80 μL.

2.5 Trypsin digestion and measurement of MALDI-TOF mass spectra

The mixtures of ¹²C₆-DNP-modified and ¹³C₆-DNP-modified oxidized lysozyme samples were diluted to 400 μL with water, and added to 8 μL of 1 M NH₄HCO₃ (pH 8.6) and 2 μL of 1 mg/mL trypsin. Trypsin digestion was performed by incubation at 37°C overnight. These digests were purified by ZipTip and measured by MALDI-TOF mass spectra as described above.

3. Results and Discussion

3.1 Carbonylated angiotensin I modified by $^{12}\text{C}_6$ -DNP and $^{13}\text{C}_6$ -DNP

Oxidized angiotensin I with NaOCl was divided into

two aliquots, one of which was reacted with $^{12}\text{C}_6$ -DNP and the other with $^{13}\text{C}_6$ -DNP. A 1 : 1 molar mixture of the $^{12}\text{C}_6$ -DNPH-modified and $^{13}\text{C}_6$ -DNPH-modified oxidized angiotensin I was prepared, and the mass spectra of the mixture were measured with the MALDI-TOF

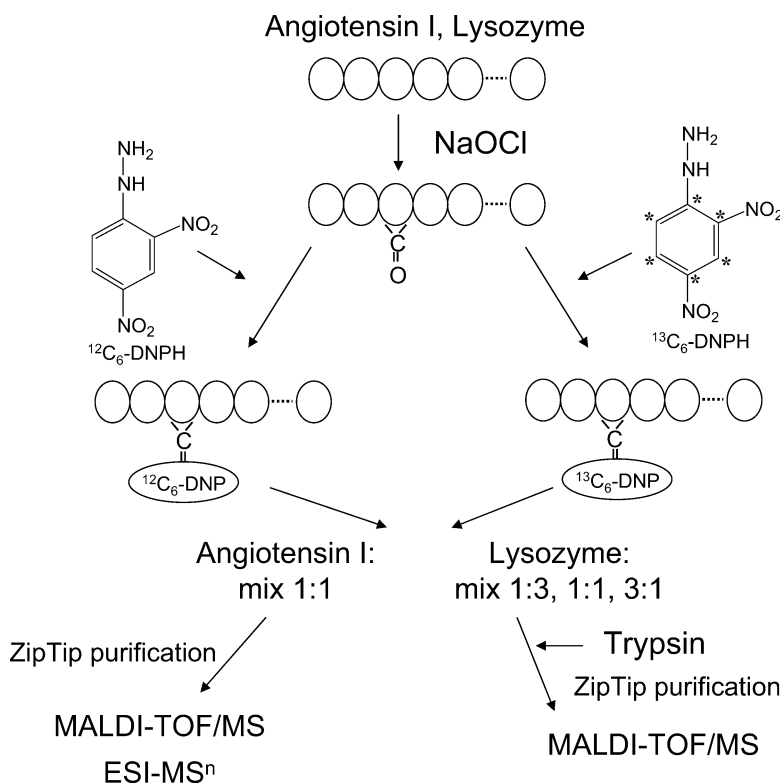


Fig. 1. Overview of the analysis for the carbonylated angiotensin I and lysozyme described in this study. 1) Angiotensin I is oxidized by NaOCl. 1 : 1 mixture of $^{12}\text{C}_6$ -DNP and $^{13}\text{C}_6$ -DNP-modified oxidized angiotensin I is measured with a MALDI-TOF mass spectrometer after ZipTip purification. The detailed structure was analyzed by an ESI mass spectrometer. 2) For lysozyme, after oxidation by NaOCl, 1 : 3, 1 : 1, and 3 : 1 mixtures of $^{12}\text{C}_6$ -DNP- and $^{13}\text{C}_6$ -DNP-modified oxidized lysozyme were digested with trypsin. The resulted peptide mixtures were measured with a MALDI-TOF mass spectrometer after ZipTip purification.

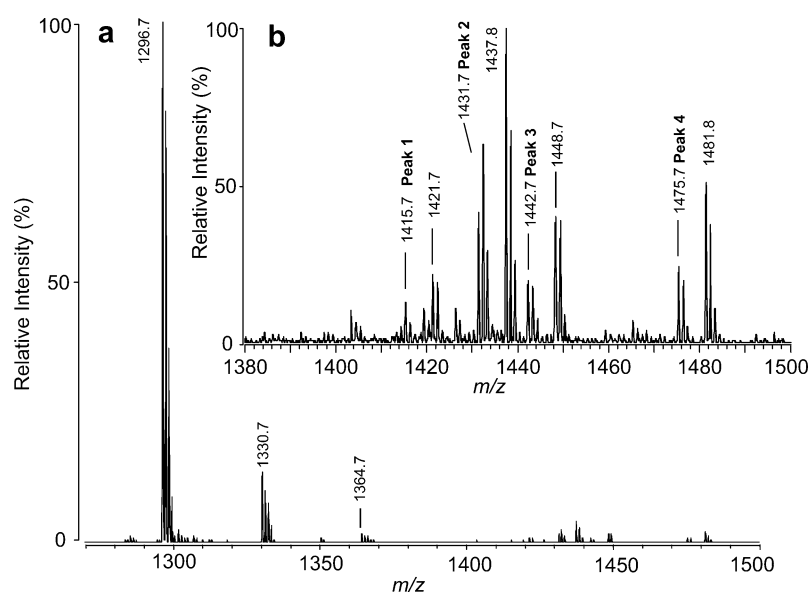


Fig. 2. a) MALDI-TOF mass spectrum of the 1 : 1 mixture of $^{12}\text{C}_6$ -DNP- and $^{13}\text{C}_6$ -DNP-modified oxidized angiotensin I. b) The enlarged spectrum showing carbonylated products. Peaks at m/z 1415.7, 1431.7, 1442.7, and 1475.7 are marked peaks 1–4.

mass spectrometer after ZipTip purification (Fig. 1). The MALDI-TOF mass spectrum shown in Fig. 2a indicates that a major product peak was observed at m/z 1330.7 besides that of a protonated ion of angiotensin I (the amino acid sequence: DRVYLHPFHL) at m/z 1296.7. This mass difference of 34 Da corresponds to chlorination of the tyrosine residue. In addition to the monochlorination of tyrosine (m/z 1330.7), the ion corresponding to dichlorination of the tyrosine residue in angiotensin I was also observed at m/z 1364.7. These observations indicate that the mono- and dichlorination on the tyrosine residue occur preferentially with NaOCl.¹⁶ Along with these products, the MALDI-TOF mass spectrum also shows four sets of doublet peaks having a mass difference of 6 Da (Fig. 2b). This result indicates that four kinds of carbonylated products of angiotensin I were generated by treatment with NaOCl.

3.2 Structure of $^{12}\text{C}_6$ -DNPH-modified oxidized angiotensin I

In general, the mass increase in the carbonylation of an amino acid and its subsequent DNPH-modification is estimated to be 194 Da (14 Da due to carbonylation and 180 Da due to DNPH-modification) on the basis of the molecular mass of the unmodified peptide. Since the molecular mass of angiotensin I is 1295.7, according to this calculation, the mass of the protonated ion of the DNPH-modified oxidized angiotensin I is 1490.7. However, this mass was not identical to any observed mass at m/z 1415.7 (peak 1), 1431.7 (peak 2), 1442.7 (peak 3), or 1475.7 (peak 4) in Fig. 2b. To elucidate these structures and the reaction mechanisms, tandem mass spectrometry is used. We first tried post-source decay analysis, but this did not work because of the extremely low quality of data.

Using an ESI-iontrap mass spectrometer, we successfully observed ESI-tandem spectra of doubly charged

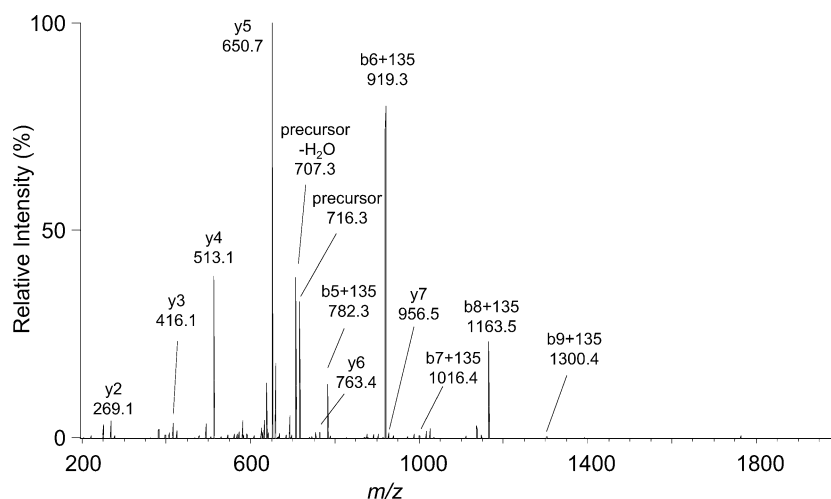


Fig. 3. ESI first generation product ion mass spectrum of $^{12}\text{C}_6$ -DNPH-modified angiotensin I. The ion at m/z 716.3 was selected as the precursor corresponding to peak 2 (Fig. 2).

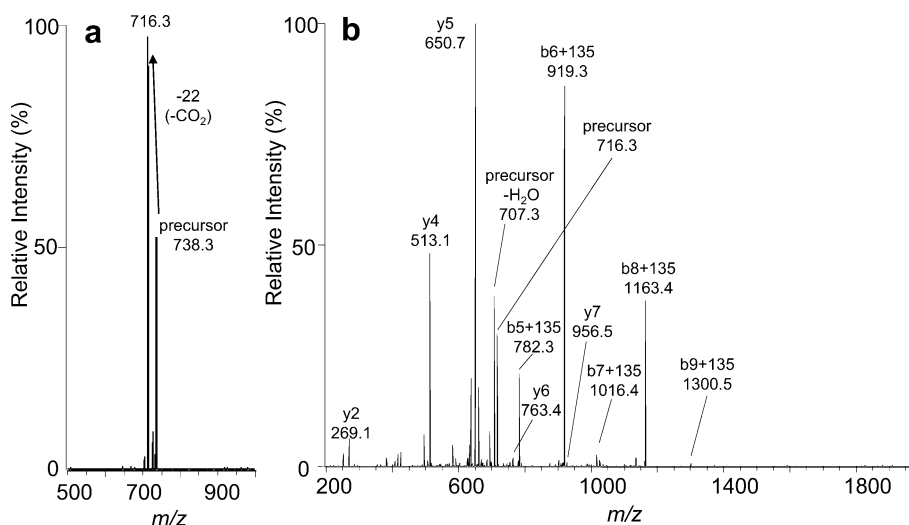


Fig. 4. a) ESI first generation product ion mass spectrum of $^{12}\text{C}_6$ -DNPH-modified angiotensin I. The ion at m/z 738.3 was selected as the precursor corresponding to peak 4 (Fig. 2). b) ESI second generation product ion mass spectrum (738.3 \rightarrow 716.3) of $^{12}\text{C}_6$ -DNPH-modified angiotensin I. The precursor ion was selected for m/z 716.3 appeared in Fig. 4a.

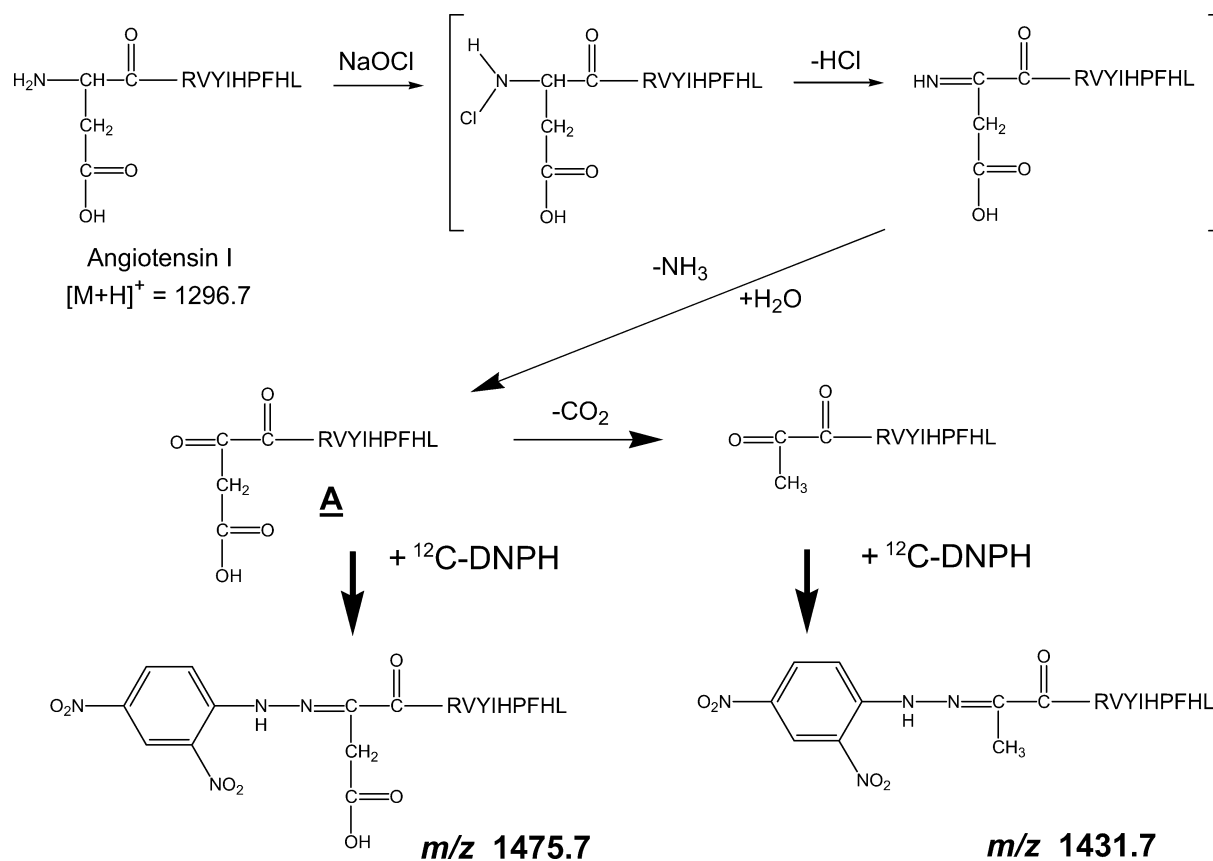


Fig. 5. Reaction scheme for generating ions at m/z 1431.7 (peak 2 in Fig. 2) and m/z 1475.7 (peak 4 in Fig. 2).

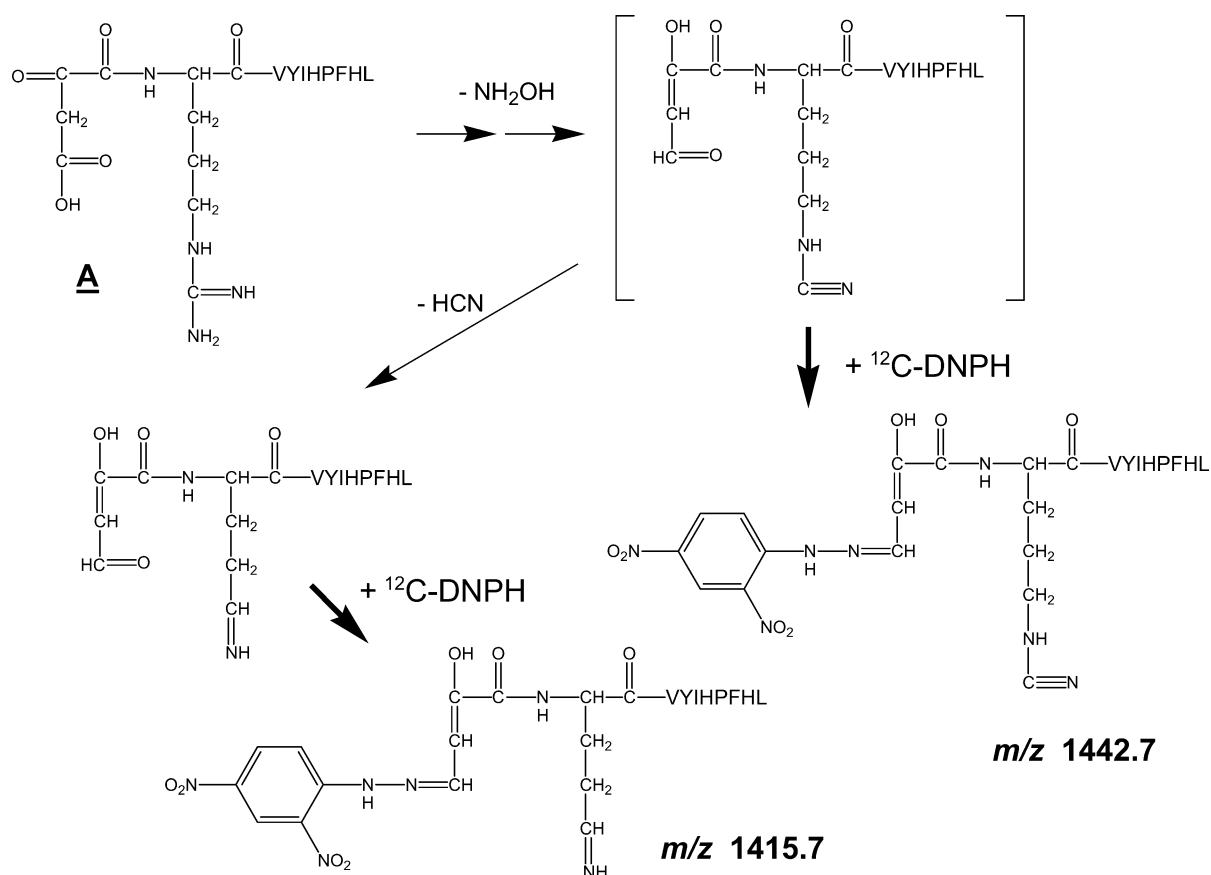


Fig. 6. Reaction scheme for generating ions at m/z 1415.7 (peak 1 in Fig. 2) and m/z 1442.7 (peak 3 in Fig. 2).

ions at m/z 716.3 and 738.3 (data not shown), which correspond to peaks 2 and 4 in Fig. 2b, respectively. The ESI first generation product ion mass spectrum of the doubly charged ion, m/z 716.3 (corresponding to peak 2), is shown in Fig. 3. The fragment ions at m/z 269.1, 416.1, 513.1, 650.7, 763.4, and 956.5 are identical to the calculated masses of y_2 to y_7 of angiotensin I, respectively. The fragment ions at m/z 782.3, 919.3, 1016.4, 1163.5, and 1300.4 correspond to b_5+135 to b_9+135 of the b-series ions at angiotensin I, respectively, indicating that the modification occurs near the N-terminal amino acids-aspartic acid, arginine, and valine. The ESI first generation product ion mass spectrum of the doubly charged ion at m/z 738.3 (peak 4) showed the ion at m/z 716.3 as the product ion corresponding to the decarboxylation from the precursor (Fig. 4a). Figure 4b shows the second generation product ion mass spectrum from the precursor ions at m/z 738.3 and 716.3, which is identical to the fragmentation profile of the first generation product ion mass spectrum of m/z 716.3 (Fig. 3). These fragmentations indicate that the structure giving peak 2 is identical to the decarboxylated form of the structure giving peak 4. Putting these results together, the reaction scheme to generate the products corresponding to peaks 2 and 4 can be summarized as shown in Fig. 5. The reaction of angiotensin I with NaOCl leads to the formation of chloramine^{6, 16, 17} at the N-terminal, followed by hydrolysis to the carbonyl group.¹⁸ The carbonyl group is generated as 1,2-diketone (compound A, in Fig. 5); thereafter, the ketonization at the N-terminal side induces a reaction with DNPH (corresponding to m/z 1475.7, peak 4), and a partial decarboxylation, yielding the product giving peak 2 (corresponding to m/z 1431.7) in Fig. 2b.

The alternative mechanism for peaks 1 and 3 in Fig. 2b can be described as follows: the mass difference

between the calculated DNP-modified angiotensin I ($1296.7+180=1476.7$ Da) and peak 3 (1442.7 Da) was rationalized by the loss of two nitrogen-containing molecules. Therefore, 1,2-diketone, the compound A shown in Fig. 6, was susceptible to further loss of an amine group. The structure of peak 3 (1442.7 Da) is possibly accounted for by the elimination of H_2NOH (33 Da) from compound A *via* the intermediate structure.¹⁹ Subsequently, the elimination of HCN (27 Da) from the cyanamide structure was expected to give peak 1 (1415.7 Da).²⁰ Figure 6 summarizes the possible reaction mechanism for generating peaks 1 and 3.

By reacting with NaOCl, angiotensin I underwent chloramination at the N-terminal, which resulted in forming the carbonyl group after the loss of HCl and hydrolysis. The reaction of $^{12}C_6$ -DNPH and $^{13}C_6$ -DNPH with the carbonyl group lead to MALDI-TOF mass spectra showing four sets of doublet peaks consisting of a 6 Da difference. By using angiotensin I as the model peptide, we could develop the feature of $^{13}C_6$ -DNPH for the selective detection of the carbonylated product in a mixture of oxidized peptide products.

3.3 Carbonylated peptide from oxidized lysozyme

We applied this method to other proteins to identify the carbonylated peptide from the digested protein. As shown in Fig. 1, oxidized lysozyme with NaOCl was divided into two aliquots, which were independently modified with $^{12}C_6$ -DNPH and $^{13}C_6$ -DNPH, and mixed at the ratios of 1 : 3, 1 : 1, and 3 : 1, respectively. These mixtures were digested with trypsin and then measured with the MALDI-TOF mass spectrometer. Since a peptide mixture generated from a protein digest shows a complex profile in the mass spectrum, we searched the carbonylated peptide by the following two steps: using the superimposed spectra of the 1 : 3, 1 : 1, and 3 : 1 mixtures, first selecting doublet peak(s) having a mass difference of 6 Da, then, retrieving the pair of $^{12}C_6$ -

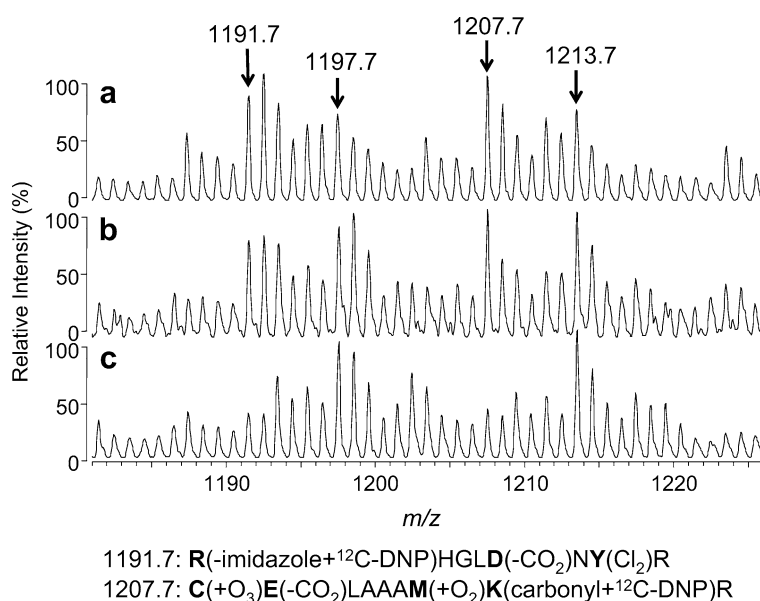


Fig. 7. MALDI-TOF mass spectra of tryptic peptide mixtures of $^{12}C_6$ -DNPH- and $^{13}C_6$ -DNPH-modified oxidized lysozyme. Portions of the mass spectra representing both the mass difference of 6 Da and peak height ratios of 3 : 1, 1 : 1, and 1 : 3 are shown as a, b, and c. The signals at m/z 1191.7 and 1207.7 were assigned as $^{12}C_6$ -DNP and oxidatively modified RHGLDNYR and CELAAAMKR, respectively. See the text for details.

DNPH- and $^{13}\text{C}_6$ -DNPH-modified peptides by checking the peak intensities indicating ratios of 1 : 3, 1 : 1, and 3 : 1.

We observed 9 peptides in the mass spectra among the 26 peptides theoretically predicted for trypsin-digestion of lysozyme. In addition to the observed 9 peptides, we found 2 carbonylated peptides that met the above criteria (Fig. 7). We could infer that cysteine and methionine residues were oxidized to cysteine sulfonic acid and methionine sulfone after reacting with NaOCl .^{21), 22)} Glutamic acid and aspartic acid were decarboxylated as in the case of angiotensin I. By considering the above reactions, the ions at m/z 1191.7 and 1207.7 were assigned as follows: the ion at m/z 1191.7 was assigned as tryptic peptide RHGLDNYR resulting from decarboxylated aspartic acid and dichlorinated tyrosine. $^{12}\text{C}_6$ -DNPH reacted with the deguaninated arginine at the ketonic carbonyl group.²³⁾ The ion at m/z 1207.7 was assigned as tryptic peptide CELAAAMKR derived from cysteine sulfonic acid, methionine sulfone, and decarboxylated glutamic acid. $^{12}\text{C}_6$ -DNPH reacted with the carbonyl group of the carbonylated lysine.²⁴⁾ The intensity ratios observed for the two peptides did not exactly match the theoretical ratios (3 : 1, 1 : 1, and 1 : 3). The exact match could be prevented by overlapping with other oxidation or digestion products and errors caused during sample preparation.

The results indicate that the oxidation of peptide simultaneously induces several independent oxidation reactions. It was difficult to identify carbonylated peptide in the digested protein because of the isolation of complex products by oxidation reaction and the complex spectra profile. Using the mass difference and the ratio of the intensities due to $^{12}\text{C}_6$ -DNPH- and $^{13}\text{C}_6$ -DNPH-modification obviates, to some extent, the difficulty of determining the carbonylated peptides in a complex mixture.

4. Conclusion

This study illustrates a mass spectrometric method for determining the carbonylated peptide and the site of carbonylation using $^{12}\text{C}_6$ -DNPH and $^{13}\text{C}_6$ -DNPH. The peptides simultaneously generate several kinds of oxidation products by oxidation reaction. This complication makes it difficult to assign modified structure in the complex mixture from the mass spectrum. The method presented here can distinguish carbonylated peptide from the purified peptide as well as a peptide mixture. This approach could be applied to the quantification of a specific carbonylated peptide in a complex mixture.

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References

- 1) B. S. Berlett and E. R. Stadtman, *J. Biol. Chem.*, **272**, 20313 (1997).
- 2) R. Stocker and J. F. Keaney, Jr., *J. Thromb. Haemost.*, **3**, 1825 (2005).
- 3) R. Sultana, D. Boyd-Kimball, H. F. Poon, J. Cai, W. M. Pierce, J. B. Klein, M. Merchant, W. R. Markesbery, and D. A. Butterfield, *Neurobiol. Aging*, **27**, 1564 (2006).
- 4) S. R. Danielson and J. K. Andersen, *Free Radical Biol. Med.*, **44**, 1787 (2008).
- 5) J. Q. Guan and M. R. Chance, *Trends Biochem. Sci.*, **30**, 583 (2005).
- 6) E. R. Stadtman and R. L. Levine, *Amino Acids*, **25**, 207 (2003).
- 7) J. Choi, A. I. Levey, S. T. Weintraub, H. D. Rees, M. Gearling, L. S. Chin, and L. Li, *J. Biol. Chem.*, **279**, 13256 (2004).
- 8) A. T. Nguyen and R. P. Donaldson, *Arch. Biochem. Biophys.*, **439**, 25 (2005).
- 9) T. A. Doser, S. Turdi, D. P. Thomas, P. N. Epstein, S. Y. Li, and J. Ren, *Circulation*, **119**, 1941 (2009).
- 10) H. A. Headlam and M. J. Davies, *Free Radical Biol. Med.*, **36**, 1175 (2004).
- 11) R. L. Levine, D. Garland, C. N. Oliver, A. Amici, I. Clement, A. G. Lenz, B. W. Ahn, S. Shaltiel, and E. R. Stadtman, *Methods Enzymol.*, **186**, 464 (1990).
- 12) E. R. Stadtman and R. L. Levine, *Ann. N. Y. Acad. Sci.*, **899**, 191 (2000).
- 13) M. Y. Aksenov, M. V. Aksenova, D. A. Butterfield, J. W. Geddes, and W. R. Markesbery, *Neuroscience*, **103**, 373 (2001).
- 14) H. Mirzaei and F. J. Regnier, *J. Chromatogr. A*, **1134**, 122 (2006).
- 15) E. L. Thomas, *Infect. Immun.*, **25**, 110 (1979).
- 16) N. M. Domigan, T. S. Charlton, M. W. Duncan, C. C. Winterbourn, and A. J. Kettle, *J. Biol. Chem.*, **270**, 16542 (1995).
- 17) C. L. Hawkins, D. I. Pattison, and M. J. Davies, *Amino Acids*, **25**, 259 (2003).
- 18) M. N. Kumara, N. S. Linge Gowda, K. Mantelingu, and K. K. S. Rangappa, *J. Mol. Catal. A: Chem.*, **309**, 172 (2009).
- 19) G. C. Wallace and J. M. Fukuto, *J. Med. Chem.*, **34**, 1746 (1991).
- 20) A. I. Lesnikovich, O. A. Ivashkevich, G. V. Printsev, P. N. Gaponik, and S. V. Levchik, *Thermochim. Acta*, **171**, 207 (1990).
- 21) L. Moulds, E. Silajdzic, N. Haroune, C. M. Spickett, and A. R. Pitt, *Proteomics*, **9**, 1617 (2009).
- 22) C. Y. Yang, Z. W. Gu, M. Yang, S. N. Lin, A. J. Garcia-Prats, L. K. Rogers, S. E. Welty, and C. V. Smith, *J. Lipid Res.*, **40**, 686 (1999).
- 23) G. Xu, K. Takamoto, and M. R. Chance, *Anal. Chem.*, **75**, 6995 (2003).
- 24) A. Temple, T. Y. Yen, and S. Gronert, *J. Am. Soc. Mass Spectrom.*, **17**, 1172 (2006).

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