

Identification of the modified amino acid residue in the modified heme protein using LC/MS/MS

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Introduction

An amino acid in a heme protein can be modified by a reaction with hydrogen peroxide, and the protein may lose its physiological activity. Moreover, it has been reported that the tryptophan which was inserted near the heme in myoglobin can be oxidized by an organic acid peroxide. In this study, we found that a methionine residue is modified in a mutant of another heme protein. However, among

the peptide fragments obtained by the peptidase digestion of the modified protein, the two peptides which contain methionine was constructed with the same set of amino acids and exhibited the same molecular weight. We identified the modified amino acid in this mutant protein by using LC/MS/MS.

Materials and Methods

The mutant of the heme protein used in this study was a mixture of non-modified and modified proteins, where about 50% of the native protein was oxidized. After digesting the mutant of the heme protein with trypsin, the obtained peptide fragments were analysed on the mass spectrometer. Preliminary analysis was first by ESI-TOF mass spectrometer. Secondary analysis was then performed by LC/MS/MS using the following analytical conditions; Nexera

UHPLC system was connected to a LCMS-8030 triple quadrupole mass spectrometer (Fig. 1). Chromatographic separations were carried out using an ODS column, Shim-pack XR-ODS II (150 mm x 2.0 mm, 2.2 μ m). The sample was eluted at 0.2 mL/min with a binary gradient system and applied to MS/MS with an ESI source, and then analyzed with positive polarity.

Analytical Conditions

HPLC: Nexera UHPLC system (Shimadzu Corporation, Kyoto, Japan)

Column: Shim-pack XR-ODS II 150 mm x 2.0 mm, 2.2 μ m

Mobile phase A: 0.1% Formic Acid B: Acetonitrile

Flow rate: 0.2 mL/min

Time program: B conc.2%(0 min)-50%(40.0 min)-90%
(41.0-45.0 min) -2%(45.01-55 min)

Injection volume: 10 μ L

Column temperature: 40°C

Mass spectrometer: LCMS-8030 (Shimadzu Corporation, Kyoto, Japan)

Ionization: ESI, Positive

DL temperature: 250°C

Nebulizer gas: 2.0 L/min

temperature: 400°C

Drying gas: 15 L/min



High Speed Mass Spectrometer

Polarity Switching Scanning Speed
• 15 msec • Max. 15000 u/sec

Fig. 1 LCMS-8030 triple quadrupole mass spectrometer

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Results

ESI-MS analysis

The tryptic digested sample of the heme protein was analyzed by ESI-TOF MS, JMS-T100LC AccuTOF (JEOL Ltd., Tokyo, Japan). Many peaks were detected in the solution of the peptide fragments obtained by the tryptic digestion (Fig. 2). Peptide F2 (sequence: IFIMK) and peptide F8 (sequence: MIFIK) exhibit a same molecular weight at m/z 651 (M+H), but differ in sequence. Another peak was detected at m/z 667, which was 16u-larger than the original peak. The peptide equivalent to m/z 667 (M+H) was not obtained from the solution of the peptide fragments obtained by tryptic digestion of the original heme protein, suggesting that this additional peak was due to the oxidized species of peptide F2 or F8. However, since MS/MS was not available in the ESI-TOF MS analysis, it was not able to identify the modified peptide.

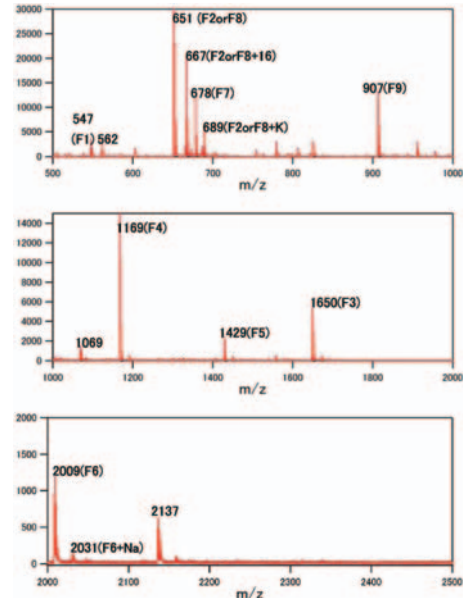


Fig. 2 ESI-MS spectra of the tryptic digested sample

LC/MS analysis

We analyzed the sample was by LC/MS/MS. Full scan MS measurement was performed, and all the peptide fragments obtained by tryptic digestion and longer than five amino acids were detected (Fig. 3).

Two peaks at m/z 326 (M+2H) correspond to the mass of the peptides F2 or F8, and the peak at m/z 334 (M+2H) to the modified peptide.

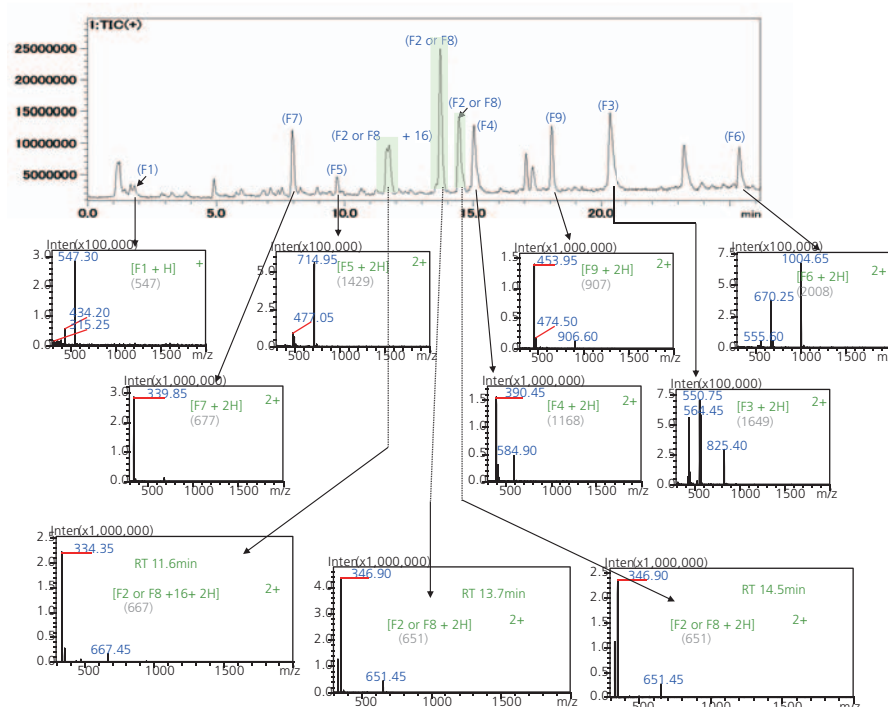


Fig. 3 LC/MS results of the peptide sample

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LC/MS/MS analysis

In order to analyze the sequence of the peptide exhibiting these peaks, product ion scan (MS/MS) measurements were performed. For acquisition of optimal MS/MS data by one injection, the product ion scan was set to have multiple scans with three collision energies (CE=15, 30, 45 v).

With the ultra high-speed scanning capacity (up to 15000u/sec), the LCMS-8030 mass spectrometer was able to acquire sufficient data points from each peak, even when acquiring data in full scanning mode.

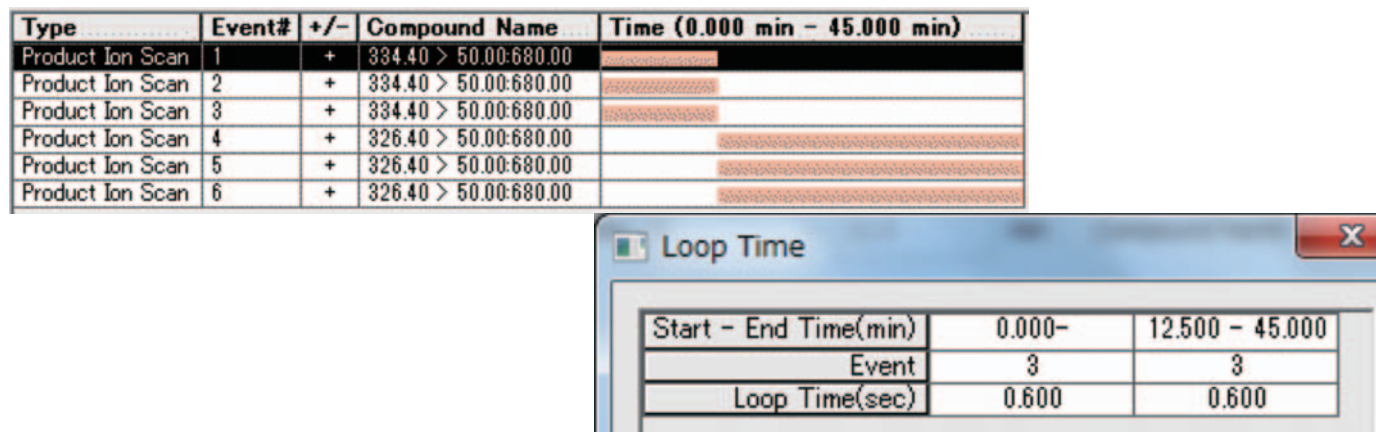


Fig. 4 Method settings for multiple product ion scan

Two of the three unknown peaks were identified as 8-ethylhydroxyetizolam and alpha-hydroxyetizolam as they are known metabolites. However the third unknown peak, which was detected the same MRM transition as that of metabolites of these two compounds, was not identified. Next, sample (B) was re-acquired with MRM triggered

automatic MS/MS and product ion scans. These product ion scan spectra were searched against a hypnotics MS/MS library and the six previously identified peaks were assigned a high hit score. In the same manner as described here, this method is highly applicable to the screening of drugs of abuse in biological samples.

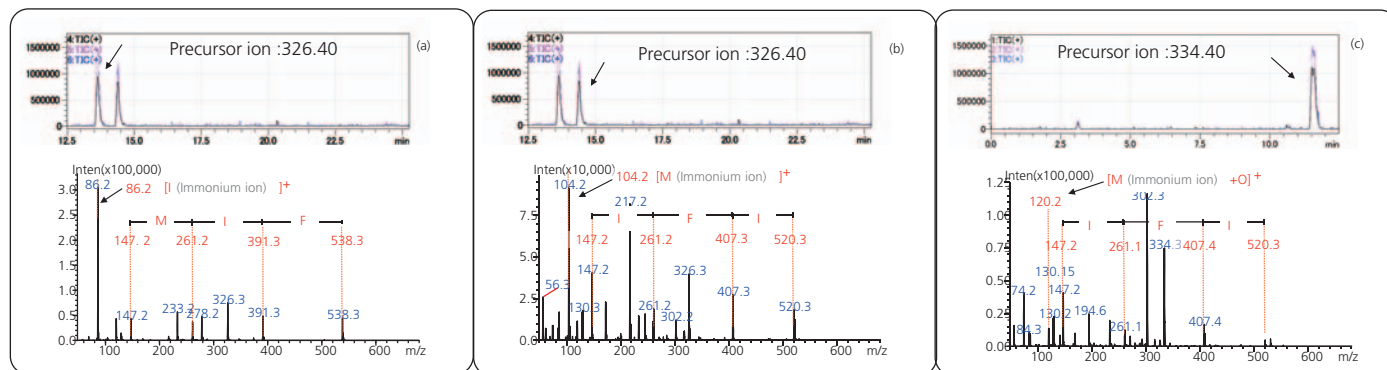


Fig. 5 MS/MS analysis: (a) MS/MS of m/z 326.4, peak 13.7 min (b) MS/MS of m/z 326.4, peak 14.5 min (c) MS/MS of m/z 334.4, peak 11.6 min
Data indicates the peptide sequences were: (a) was IFIMK (F2), (b) was MIFIK (F8), (c) was MIF(O)IK (modified F8).

The results of product ion scan data showed that MS/MS spectra @ collision energy 15 V had good information for peptide sequencing and that the two peaks of m/z 326

were F2 and F8, respectively, and that the peak of m/z 334 was oxidized F8 (MIFIK).

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Conclusions

- Peptide mapping of the heme protein was carried out by LC/MS/MS
- The modified amino acid in this mutant protein was identified by product ion scan spectrum using LC/MS/MS.
- Ultra high-speed scanning capacity of LCMS-8030 mass spectrometer was very useful for multiple product ion scan.