Exopeptidase Degradation for the Analysis of Phosphorylation Site in a Mono-phosphorylated Peptide with Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

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The utility of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) coupled with a peptide ladder sequencing method employing exopeptidase degradation for the analysis of phosphorylation site in a mono-phosphorylated peptide is investigated. MALDI-TOFMS analysis of time-dependent exopeptidase digestion using carboxypeptidase W and aminopeptidase M of the mono-phosphorylated 33-48 fragment isolated from a β -casein tryptic digestion mixture allowed for the sequencing analysis from both the C-terminus and N-terminus. Negative ion detection MALDI-TOFMS made it possible to clearly measure the peptide ladder of mono-phosphorylated peptide by the strong negative charge localized at the phosphoric acid group. Since exopeptidase activity was suppressed by the existence of a phosphorylated amino acid residue, the termination exopeptidase degradation therefore suggested the existence of a phosphorylated amino acid residue at that site. This peptide ladder sequencing method using exopeptidases was effective for the identification of the site of a phosphorylated amino acid residue by a simple MALDI-TOFMS analysis in the negative ion detection mode.

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Introduction

Phosphorylation of serine, threonine and/or tyrosine residues is one of the most common and important post-translational modifications of proteins for regulation of many cellular processes such as signal transduction, gene expression, cell cycle and apoptosis. The analysis of phosphorylation sites is therefore very important in understanding the role of the modifications in intracellular signaling. Mass spectrometry is a powerful tool for identifying phosphorylation sites with high sensitivity and throughput.¹⁻⁵ Tandem mass spectrometry is the most useful for identifying a phosphorylation site in the phosphopeptide, and is applied for phosphorylated peptides generally separated from other peptides by HPLC or more selective enrichment procedure such as an immobilized metal affinity chromatography⁶⁻¹⁰ and an immunoprecipitation using anti-phosphorylated amino acid residue antibodies.^{11,12} The fragmentation of HPO₃ or H₃PO₄ from a phosphorylated peptide is more frequent than backbone cleavages, and fragment ions containing phosphates often provide a pair of peaks with a mass shift of 80 Da or 98 Da as y-type or b-type ions. However, a phosphopeptides with strong negative charges are often difficult to detect directly with the positive ion detection mode under general conditions. Janek *et al.* have compared the detection sensitivity of positive and negative ions of synthesized phosphopeptides, and they found that phosphopeptides, which

have a low ionization efficiency in the positive ion mode, gave relatively sensitive anions in comparison with other peptides in the negative ion mode.¹³ Furthermore, phosphopeptides produced by the enzymatic digestion of model proteins can be detected easily in peptide mass fingerprints obtained in the negative ion mode.

Several methods for peptide sequencing determination have been developed except for using tandem mass spectrometry. Protein ladder sequencing reported by Chait *et al.* is an approach to N-terminal protein sequencing combined with a multiple steps of Edman degradation chemistry using a MALDI-TOFMS determination.¹⁴ Although this method can identify N-terminal sequence with only one mass mapping by MALDI-TOFMS, it is limited to the N-terminus of peptides. Sequence information from the C-terminus of peptide has been obtained by chemical degradation methods,^{15,16} but those methods have been difficult on the operation. A different approach to chemical sequencing uses enzymatic reactions. Several carboxypeptidases have been demonstrated to be attractive to analyze the C-terminal sequencing by concentration-dependent digestion¹⁷ and time-dependent digestion.^{18,19}

In the present study, the peptide ladder sequencing strategy employing exopeptidase digestion coupled with MALDI-TOFMS has been applied to the analysis of phosphorylation site in a mono-phosphorylated peptide. The authors used the 33-48 fragment (FQpSEEQQQTEDELQDK) isolated from β -casein tryptic digest as a model mono-phosphorylated peptide.

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Experimental

Materials

Alkaline phosphatase, α -cyano-4-hydroxycinnamic acid (α -CHCA) and β -casein were purchased from SIGMA Chemical Co., (St. Louis, MO, USA). Sequence-grade modified trypsin was obtained from Promega (Madison, WI, USA). Carboxypeptidase W was obtained from Seikagaku Co. (Tokyo, Japan) and aminopeptidase M was purchased from Roche Diagnostics Co. (Tokyo). Distilled and ion-exchanged water was prepared by CPW-100 ULTRA PURE WATER SYSTEM (ADVANTEC TOYO Co., Tokyo), and other reagents and solvents were of HPLC grade.

Apparatus

MALDI-TOFMS analysis was performed using Voyager RP (Applied Biosystems, Framingham, MA, USA) and AXIMA-CFR (Shimadzu, Kyoto, Japan) equipped with an N₂ laser (337 nm) under the linear mode. The accelerating and grid voltages were 25 kV and 18 kV, respectively. The matrix solution used in both positive and negative ion modes was a fresh solution of α -CHCA in acetonitrile containing 0.1% TFA at a concentration of 10 mg/mL. Peptide samples in a water/acetonitrile mixture (3:7, v/v) containing 0.1% TFA were mixed with the matrix solution at a ratio of 1:1, and a 1- μ L aliquot of the mixture was applied to the sample plate. Spectra were calibrated internally or externally using average [M+H]⁺ or [M-H]⁻ ions of two peptide standards, angiotensin I and the ACTH 7-38 fragment (American Peptide Company, Inc., Sunnyvale, CA, USA), which were included in the matrix solution.

A Nanospace SI-1 solvent delivery system (Shiseido, Tokyo) equipped with a UV detector set at 215 nm was used to the purification of the phosphorylated 33-48 fragment of bovine β -casein and time course of the enzymatic degradation using exopeptidases. Liquid chromatographic separation was performed on a Jupiter 5u C18 300 Å (5 μ m, 2.0 mm i.d. $\times$ 150 mm, Phenomenex, Torrance, CA, USA) at a flow rate of 200 μ L/min with a 10 to 60% linear gradient of mobile phase B over 30 min. Mobile phase A was water/acetonitrile (9:1) containing 0.1% TFA and mobile phase B was water/acetonitrile (1:9) containing 0.1% TFA.

Preparation of mono-phosphorylated peptide fragment

β -Casein (4 nmol) was dissolved in 100 μ L of 50 mM ammonium bicarbonate, and 1.6 μ g of sequence-grade modified trypsin in 8 μ L of 50 mM ammonium bicarbonate. After incubation overnight at 37°C, tryptic peptide fragments were separated by HPLC, and the mono-phosphorylated peptide fragment (33 - 48) was isolated, whose molecular weight was measured by both the positive and negative MALDI-TOFMS. Dephosphorylated 33-48 fragment was prepared as follows. β -Casein (4 nmol) was dissolved in 100 μ L of 50 mM ammonium bicarbonate, and 10 μ L of alkaline phosphatase solution (1 U/ μ L). After incubation overnight at 30°C, tryptic digestion was carried out by the same manner as described above. And the fraction was measured by the positive MALDI-TOFMS.

Ladder sequencing procedure using exopeptidase

The phosphopeptide or dephosphorylated peptide purified by HPLC was cleaved enzymatically at every amino acid residue according to the following procedure. To the target peptide (10 pmol) was dissolved in 15 μ L of 50 mM ammonium acetate buffer (pH 4.0), 1.2 μ g of carboxypeptidase W (substrate/enzyme ratio: ca. 20/1) was added. To prepare the C-

terminal peptide ladder, 3- μ L aliquots of the reaction mixture were withdrawn and each mixed with 3 μ L of 0.2% TFA to terminate digestion after incubation at 37°C for various amounts of time. Each sample, differing in their incubation times, was analyzed by MALDI-TOFMS. The N-terminal peptide ladder was prepared as follows. The peptide (10 pmol) was dissolved in 12 μ L of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM MgCl₂, to which was added 64 μ L of aminopeptidase M solution (20 unit/1 mL). After incubation at 37°C for various amounts of times, 11- μ L aliquots were mixed with 8 μ L of 0.2% TFA and purified using ZipTipC18 before analysis by MALDI-TOFMS.

Result and Discussion

Isolation of phosphopeptides produced by tryptic digestion

The detection sensitivity of phosphopeptides is different from that of other peptides when MALDI mass spectrometry in positive and negative ion modes were used,¹³ because every peptide fragment produced by enzymatic cleavage has a different charge and set of physicochemical properties. Peptide mapping by HPLC monitored at 210 - 220 nm based on the UV absorbance of the peptide backbone can detect phosphopeptides with similar sensitivity as it can other peptide fragments. Comparison of HPLC peptide maps obtained by enzymatic digestion with and without alkaline phosphatase treatment can identify phosphopeptides in a complex peptide mixture. Figure 1 shows peptide maps obtained by tryptic digestion of β -casein with (upper chromatogram) or without (lower chromatogram) alkaline phosphatase cleavage. In the dephosphorylated peptide map, one peak at a retention time of 10 min disappeared from the HPLC chromatogram when compared with the native peptide map, converging to the next peak at a retention time of 10.4 min. In the positive ion MALDI mass measurement, the 10-min peak gave a protonated peptide molecule at m/z 2063 corresponding to the mono-phosphorylated 33-48 fragment, and the 10.4-min peak gave a mass peak at m/z 1983 corresponding to the dephosphorylated peptide (data not shown). And the 10-min peak gave also a deprotonated peptide molecule at m/z 2061 with highly sensitivity in the negative ion detection mode. In addition, to confirm the relationship between those two peaks, the peak corresponding to the protonated molecule at m/z 2063 was treated by alkaline phosphatase, giving a new peptide ion at m/z 1983, which is consistent with the dephosphorylated 33-48 peptide (data not shown). These results suggest that the 10- and 10.4-min peaks are the mono-phosphorylated 33-48 fragment and its dephosphorylated counterpart, respectively. Finally, comparison of the peak intensities on the HPLC chromatogram reveals that the 33-48 fragment of β -casein is partially mono-phosphorylated at a ratio of about 1:1.

Peptide ladder sequencing using exopeptidases

The isolated mono-phosphorylated 33-48 fragment was time-dependently digested to form C- and N-terminal peptide ladders using carboxypeptidase W and aminopeptidase M, respectively. The phosphorylated group in this peptide was sufficiently stable during digestion by aminopeptidase M and carboxypeptidase W. The C-terminal ladder was obtained time-dependently using incubation times from 0 to 300 min as shown in Fig. 2(A). The peptide including a lysine residue, gave an intense peak, while the peak intensities of other peptides were lower because of the strong negative charge of the phosphate group. When the same sample is measured in the negative ion mode, the peak intensity of each of the ladder peptides was higher, simplifying the

Fig. 1 HPLC peptide-mapping of β -casein tryptic digest. Lower chromatogram, β -casein tryptic digest; upper chromatogram, β -casein tryptic digest after dephosphorylation using alkaline phosphatase.

peptide ladder sequencing as shown in Fig. 2(B). Although the lack of peaks corresponding to FQpSEEQQTEDE and FQpSEEQQTEDELQ was observed in the positive ion mode, both peaks were barely detected in the negative ion mode. The mono-phosphorylated 33-48 fragment used for this analysis included two possible phosphorylated amino acid residues, Ser 35 and Thr 41. The results of Figs. 2(A) and (B) indicate that there may be no phosphate group at the latter location. The use of aminopeptidase M as an exopeptidase produces an N-terminal peptide ladder.¹⁹ However, the enzymatic reaction was blocked N-terminal to the serine residue, which may be phosphorylated on its side-chain hydroxyl group (Fig. 2(C)). The same result was obtained for the N-terminal peptide ladder in negative ion mode, although the detection sensitivity of the phosphorylated peptide ladder was relatively higher than in the positive ion mode, as shown in Fig. 2(D). This phosphorylated amino acid residue is known to resist cleavage by aminopeptidase M, carboxypeptidase W and other exopeptidases.²⁰ These results suggest that the phosphorylated amino acid residue is Ser 35, agreeing with previous reports on the database.

To investigate the resistance of phosphorylated amino acid residues to exopeptidase treatments, the dephosphorylated 33-48 fragment was subjected to peptide ladder sequencing using both carboxypeptidase W and aminopeptidase M. Figure 3(A) illustrates the C-terminal peptide ladder sequencing of the dephosphorylated peptide with an m/z 1982. Although low intense peaks exist within the ladder, the peaks corresponding to the dephosphorylated peptide are detected in the range from Thr 41 to Lys 48. On the other hand, as shown in Fig. 3(B), the N-terminal peptide ladder clarified the site of the phosphorylated amino acid residue, because the dephosphorylated peptide can produce the N-terminal peptide ladder in the range from Phe 33 to Glu 37 in a shorter reaction time than in Figs. 2(C) and (D). The results suggest that the phosphorylated amino acid residue resists exopeptidase treatment in the production of the peptide ladder and that Ser 35 is partially phosphorylated in the native β -casein molecule.

Fig. 2 Enzymatic peptide ladder sequencing of the mono-phosphorylated 33-48 fragment obtained from β -casein tryptic digest. (A) C-terminal peptide ladder sequencing in the positive ion mode; (B) C-terminal peptide ladder sequencing in the negative ion mode; (C) N-terminal peptide ladder sequencing in the positive ion mode; (D) N-terminal peptide ladder sequencing in the negative ion mode.

In conclusion, peptide ladder sequencing using aminopeptidase M and carboxypeptidase W is a very simple method, requiring only MALDI-TOFMS coupled with an enzymatic reaction and not any special instruments such as MS/MS. Negative ion detection is also useful for phosphopeptide analysis, and helps to simplify amino acid sequence analysis. This enzymatic peptide ladder sequencing method described in this study will be useful for characterization of other post-translational modifications such as a glycosylation.

Fig. 3 Enzymatic peptide ladder sequencing of the dephosphorylated 33-48 fragment by the positive MALDI-TOFMS. (A) C-terminal peptide ladder sequencing; (B) N-terminal peptide ladder sequencing.

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