Characterization of Phosphopeptide and Triphenylphosphonium Derivative by Tandem Mass Spectrometry

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Phosphorylation is one of the most common process of post-translational modification of various proteins and peptides, and plays important roles in the regulation of these biomolecules *in vivo*. Many signal transduction pathways include cascades of phosphorylation events.[1] However, recognizing the presence and location of the phosphate group in peptides is often difficult by traditional methods.[2-5] The phosphoester bonds of serine, threonine, and tyrosine are not stable in chemical processes of Edman degradation; the phosphate groups of these residues are easy to be hydrolyzed. Although it is possible to convert phosphoserine specifically to S-ethylcystein that can be detected in the Edman process, phosphothreonine and phosphotyrosine are not amenable to this approach.[6-7]

Fast atom bombardment (FAB) and FAB collisionally-induced dissociation tandem mass spectrometry (FAB-CID-MS/MS) have been used to determine the molecular weight of peptides and proteins from biological matrices and to elucidate the amino acid sequence with great success.[8-9] In FAB-MS, a mixture of peptides is ionized in the ion source by bombardment with energetic xenon atoms to produce protonated molecular ions $(M+H)^+$ exclusively. In FAB-CID-MS/MS, any one of the $(M+H)^+$ ions can be selected by MS-I to pass into a collision cell where collision with a helium atom causes fragmentation of the $(M+H)^+$ ion. The resulting sequence-characteristic fragment ions are mass analysed by scanning MS-II.

Detection and sequence determination of phosphopeptides also have been accomplished by FAB and FAB-CID-MS/MS, but with varying degrees of success.[10] The limited success for the tandem mass spectrometry of phosphopeptides is mainly due to limited amount of available sample material. When phosphopeptides are purified from any of biological matrices, the recovered sample materials are usually in the range of low picomoles. Also *in vitro* synthesis and purification of those phosphopeptides are not as easy as that of unmodified peptides. Therefore, tandem mass spectral information is not much available and only a few examples have been reported for MS/MS of phosphopeptides.

We have synthesized a series of biologically active phosphopeptides and their behavior in tandem mass spectrometry are under investigation. Here, we report the first example of the sequence determination of phosphopeptide by tandem mass spectrometry and the mass spectral fragmentation of its chemically derivatized peptide is also discussed.

Experimental

Phosphorylated and non-phosphorylated peptides were synthesized in the Protein and Carbohydrate Facility, University of Michigan (Ann Arbor, MI, USA). The peptides were purified by HPLC to >98% purity, thoroughly characterized by amino acid analysis and automated Edman degradation.

The FAB-MS of peptides was carried out in MS-I of a JEOL HX110A/HX110A (JEOL Ltd., Akishima, Japan) high resolution tandem mass spectrometer ($E_1B_1E_2B_2$ configuration), installed at the Korea Basic Science Center (Taejon, Korea). The accelerating voltage was 10 kV with a mass resolution of 1 : 1000 (10% valley). The JEOL Cs^+ ion gun was operated at 25 kV. Five scans were acquired and averaged between m/z 0-1000 in 10 sec. The instrument was calibrated by using $(CsI)_nCs^+$ cluster ions. Mass spectra were acquired with a JEOL DA7000 data system running on a HP 9000 computer.

Tandem mass spectrometry (FAB-CID-MS/MS) was carried out by using MS-I and MS-II of the JEOL HX110A/HX 110A. CID of protonated peptide molecules, selected by MS-I, took place in the field-free region between B_1 and E_2, thus operating both MS-I and MS-II as double focusing instruments. The collision cell potential was held at 3.0 kV and the ion collision energy was 7.0 kV. Helium collision gas was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 50%. The MS-I was operated at a resolution adjusted so that only the C_{12}-species of the protonated peptide molecule to be analyzed was transmitted. The MS-II was operated at a resolution of 1 : 1000 and was calibrated with a mixture of CsI, NaI, KI, RbI, and LiCl.

To prepare the samples for FAB-MS analysis, each peptides was dissolved in water/acetonitrile (1 : 1) solution and the concentration was adjusted to 0.1 nmol/μl. A volume of 1 μl of this solution was mixed on the probe tip with 1 μl of matrix, glycerol or dithiothreitol (DTT) : dithioerythritol (DTE), 5 : 1.

Results and Discussion

Peptides containing phosphorylated Ser and Tyr yield strong molecular ions in both positive and negative ion modes of FAB-MS. It has been reported that phosphotyrosine-containing peptides show somewhat more intense fragments due to the loss of HPO_3 (80 Da) and HPO_4 (96 Da) from the $(M+H)^+$ or $(M-H)^-$ than phosphoserine-containing peptides in their conventional FAB mass spectra.[11-12] The target phosphopeptide ($LKRAY_{(P)}LG$-amide, MW 899), where the tyrosine (Y) is phosphorylated, however, shows a protonated molecular ion exclusively in the positive ion FAB mass spectrum (Figure 1) without any loss of HPO_3 and HPO_4. The FAB mass spectrum is well matched to that of nonphosphopeptide (LKRAYLG-amide, MW 819); the mass shift of 80 Da corresponds to the phosphate group (Figure 2).

In FAB-CID-MS/MS, peptides containing phosphotyrosine lose HPO_4 from $(M+H)^+$, but the daughter fragments retain the phosphate simplifying the location of phosphorylated residue; the loss of H_3PO_4 from the $(M+H)^+$ is not strong

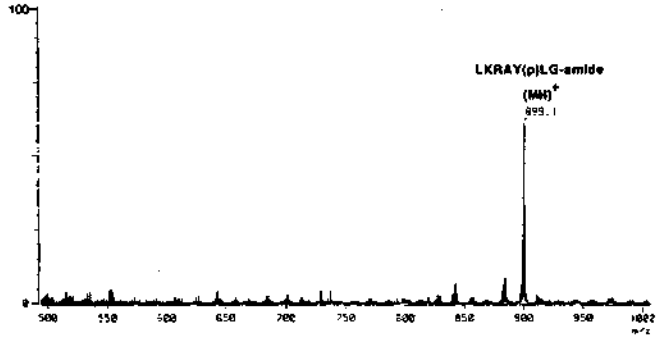

Figure 1. The positive ion FAB mass spectrum of the phosphopeptide, LKRAY$_{(p)}$LG-amide, MH^+ 899.

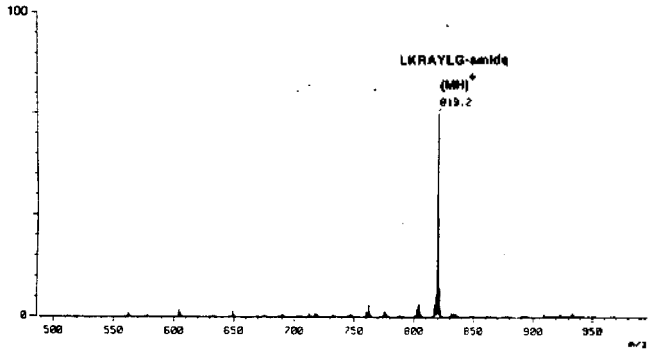

Figure 2. The positive ion FAB mass spectrum of the nonphosphopeptide, LKRAYLG-amide, MH^+ 819.

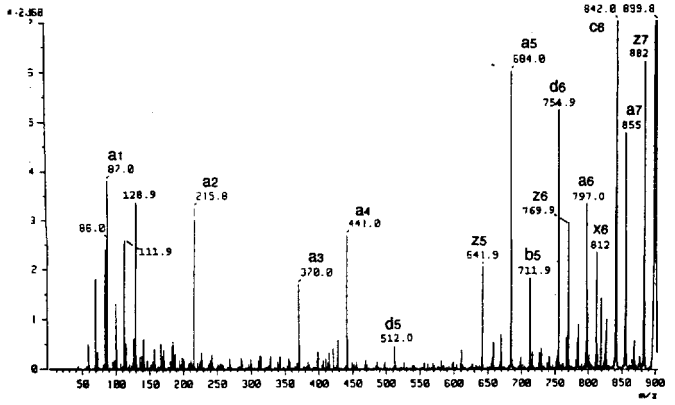

Figure 3. The FAB-CID-MS/MS spectrum of the $(M+H)^+$ ion of LKRAY$_{(p)}$LG-amide, m/z 899.

and the sequence ions observed in the MS/MS spectra arise predominantly from the intact molecular ion. The peptide sequence information then can be deduced by interpretation of the fragmentation pattern, and it is important to differentiate between the N-terminal fragments (a, b, c) and the C-terminal fragments (x, y, z).[13]

Because the basic residues (L, K, and R) at the N-terminal provide the most likely sites for protonation, this peptide is expected to give predominantly N-terminal-containing fragment ions (a, b, or c ions). The MS/MS spectrum (Figure 3) shows a complete series of a_n fragment ions.[14] In addition, the presence of peaks for d_5 and d_6 allows the assignment

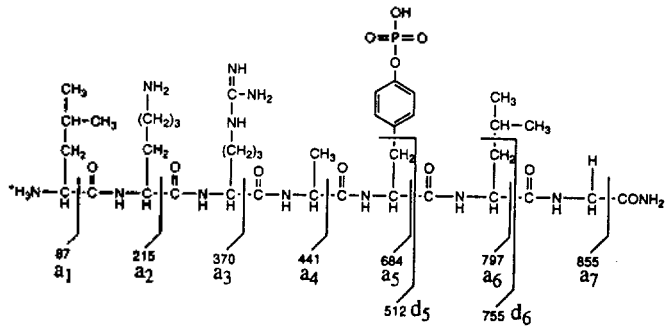

Figure 4. The structure of LKRAY$_{(p)}$LG-amide, indicating fragmentation exhibited in the spectrum shown in Figure 3.

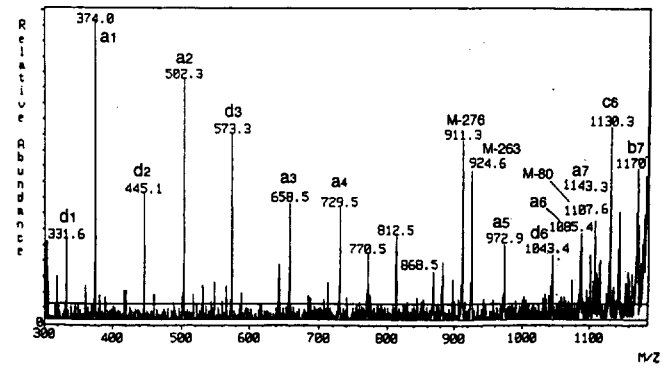

Figure 5. The FAB-CID-MS/MS spectrum of the M^+ ion of ethyl-TPP derivative, $^+PPh_3CH_2CH_2$-LKRAY$_{(p)}$LG-amide, m/z 1187.

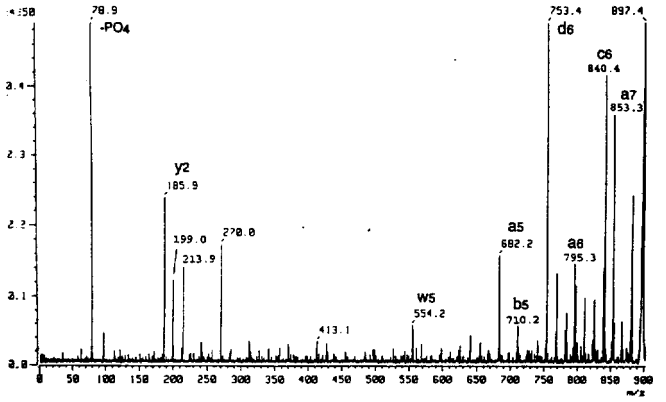

Figure 6. The FAB-CID-MS/MS spectrum of the $(M-H)^-$ ion of LKRAY$_{(p)}$LG-amide, m/z 897.

of tyrosine and leusine residues, respectively. Thus, in this case, the native phosphopeptide produces a MS/MS spectrum that can be interpreted to give the complete amino acid sequence (Figure 4).

The N-terminally ethyl-triphenylphosphonium (ethyl-TPP) derivative ($^+PPh_3CH_2CH_2$-peptide)[15] yields more complete series of fragment ions in the MS/MS spectrum (Figure 5); as expected, the spectrum contains all a_n ions and most d_n ions, the latter prove the structure of R-groups of amino acid. Therefore, the d_n ions provide confirmation of the a_n

peak assignments. In this case, the mass spectra of both the free and the derivatized peptides provide sufficient information to determine the amino acid sequence of phosphopeptides. Nevertheless, prelocation of positive charge at the N-terminal by ethyl-TPP to produce exclusively N-terminal ions is very helpful for complete structure determination.

The negative ion MS/MS spectrum (Figure 6) looks less informative than that of the positive ion, yielding a mixture of few N- and C-terminal fragment ions. However, the strong peak at m/z 80 in the negative ion spectrum proves the presence of phosphotyrosine and helps to distinguish phospho- from sulfopeptides.

In conclusion, based on the results of tandem mass analysis for the synthesized phosphopeptide, $LKRAY_{(p)}LG$-amide, characterization of any biologically active phosphopeptides using FAB and FAB-CID-MS/MS is feasible and promising. During the analysis, the phosphate group does not interfere with the formation of molecular ions and the sequence related fragment ions during FAB and FAB-CID-MS/MS. In addition, the use of ethyl-TPP derivative to prelocate a positive charge at the N-terminal of peptide appears to simplify the MS/MS spectrum and provides complete series of N-terminal fragment ions from which one can deduce the sequence.

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