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Peptide sequencing through N-terminal phosphonylation and electrospray ionization mass spectrometry

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Peptides were phosphonylated at their N-termini by reacting with ethoxyphenylphosphinate in the presence of triethylamine and tetrachloromethane under mild conditions. The phosphonylated peptides were analyzed by tandem electrospray ionization mass spectrometry. N-Terminal phosphonylation selectively increased the intensities of b_n-type ions relative to other ion types. The resulting simplified mass spectra clearly show the sequential loss of amino acid residues from the C-termini of peptides, providing a convenient and rapid method for peptide sequencing. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: peptide sequencing; electrospray ionization mass spectrometry; phosphonamidate peptides; N-terminal phosphonylation; ethoxyphenylphosphinate

INTRODUCTION

Primary sequences of proteins are traditionally determined by cleaving a protein into smaller peptide fragments and sequencing the peptides using a variety of methods.[1] The most widely practiced method has been the Edman method,[2] which degrades a peptide sequentially from its N-terminus to release phenylthiohydantoin derivatives which are subsequently analyzed by HPLC. The advent of soft ionization techniques such as fast atom bombardment (FAB),[3] matrix-assisted laser desorption/ionization (MALDI)[4,5] and electrospray ionization (ESI),[6] which make it possible to generate intact macromolecular ions in the gas phase, has led to the development of numerous mass spectrometric approaches to obtain sequence and structural information of biological molecules. For example, Chait *et al*.[7] have developed a modified Edman degradation method to convert a peptide to be sequenced into a series of progressively shorter peptides, which are analyzed by MALDI-MS to obtain the sequence of the original peptide (peptide ladder sequencing). Boyd *et al*. developed a C-terminal peptide sequencing method analogous to Edman degradation by converting the C-terminal amino acid of a peptide into a thiohydantoin using a carboxyl-group activating reagent and thiocyanate anion.[8,9] C-Terminal sequencing of peptides has also been achieved through enzymatic digestion of peptides with carboxypeptidase Y followed by MALDI-MS analysis.[10,11] These methods, however, require pure peptide samples and chemical or enzymatic degradation prior to mass spectrometry, making them inconvenient for small amounts of peptides especially peptide mixtures.

Tandem MS equipped with soft ionization methods provides an increasingly popular alternative approach for peptide characterization. A peptide mixture can be separated during the first stage of mass spectrometry; during the next stages, the selected peptide ion is fragmented by either collision-induced dissociation (CID) or surface-induced dissociation (SID).[12] The resulting MS/MS spectrum can be used to deduce the amino acid sequence of the original peptide. Unfortunately, a protonated peptide is typically dissociated into a wide variety of fragment ions including the $\mathbf{a}_n$, $\mathbf{b}_n$, and $\mathbf{c}_n$ ions that correspond to the N-terminal fragments and the $\mathbf{x}_n$, $\mathbf{y}_n$, and $\mathbf{z}_n$ ions that represent the C-terminal fragments.[13,14] In general, it is difficult to predict which type of fragment ions will be formed for a given peptide, and the MS/MS spectra are often so complicated that *de novo* sequence determination is impossible. It has previously been shown that alkali-cationized peptides can be successively dissociated from their C-termini in magnetic sector mass spectrometers,[15–22] providing opportunities for peptide sequencing. The alkali-cationized peptides can also be cleaved sequentially from their C-termini to yield a series of $[\mathbf{b}_n + \mathrm{Na} + \mathrm{OH}]^+$ ions in ion-trap instruments using low collision energies.[23,24] This method is analogous to Edman

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degradation, except that the cleavage occurs from the C-terminus instead of the N-terminus, making it suitable for sequencing N-terminally blocked peptides. However, some drawbacks such as the lower sensitivity of cationized peptides and the still-very-complex dissociation pathways render this method impractical.[23–28]

Many efforts have been made to simplify the mass spectra by modifying peptides with either positively or negatively charged groups. For positive derivatization, a positively charged group such as trimethylammoniumacetyl or tris[(2,4,6-trimethoxypheny)phosphonium]acetyl is introduced to the N-terminus of a peptide.[29,30] Similarly, a negatively charged group such as the sulfonyl group and the 4-aminonaphthalenesulfonyl group has been added to the N- and C-termini of peptides, respectively.[31,32] We have previously found that the ionization efficiency of peptides can be greatly improved by introducing a neutral dialkyloxyphosphoryl group to their *N*-termini in positive-ion ESI-MS[33] and FAB-MS.[34] The dissociation efficiency also seemed to be higher; unfortunately, a variety of dissociation pathways of the phosphoryl group complicated the mass spectra and sequence interpretation. We reasoned that, since the P-C bond in phosphonamidate is relatively stable under low-collision activation conditions, an N-terminally phosphonamidylated peptide should have less fragmentation and therefore simpler MS/MS spectra. In this work, we show that peptides can be phosphonylated at their N-termini and their sequences can be unambiguously determined by ESI-MS/MS.

EXPERIMENTAL

Materials

All peptides include Ala-Gly-Phe-Leu-ValOH (MW 505), Leu-Gly-Phe-Ala-ValOH (MW 505), Gly-Leu-Val-Ala-PheOH (MW 505), Phe-Phe-Phe-Phe-PheOH (MW 753), Phe-Ala-Ser-Asp-LeuOH (MW 551), Val-Ala-Ser-Phe-LeuOH (MW 535), Val-Glu-Gln-HisOH (MW 511), Leu-Glu-His-GlnOH (MW 525) and Val-Arg-Leu-Asp-Ser-PheOH (MW 735) were commercially available from Shanghai Glsynthesis Company (~80% pure). The peptides were used in phosphonylation and ESI-MS/MS analysis without further purification. Dichloro(phenyl)phosphine (DCPP) was purchased from Beijing Chemical Factory (Beijing). Ethanol was rendered anhydrous by refluxing with Mg.

Chemistry

^{31}P NMR chemical shifts were reported in ppm downfield (+) or upfield (−) from external H_3PO_4 (85%) reference. Ethoxyphenylphosphinyl and phosphonamidyl peptides were prepared as previously described.[35]

Synthesis of ethoxyphenylphosphinate (EPP)

Ethanol (5 mmol) was added dropwise to 2 mmol of dichloro(phenyl)phosphine dissolved in 4 mL of ethyl ether at room temperature under nitrogen atmosphere. The reaction completed within 30 min, and ^{31}P NMR spectrum showed that DCPP was almost quantitatively converted intoEPP. The crude product was obtained after removal of HCl, ethyl ether, and ethanol by distillation and was used without further purification (^{1}H NMR shows that its purity is more than 95%). ^{31}P NMR of EPP: 26.99 ppm.

General procedure for synthesis of phosphonamidate peptides

Peptide (~1 mg) was dissolved in 100 μL of triethylamine, 800 μL of ethanol and 100 μL of tetrachloromethane and was treated with 1.2–1.5 equiv of EPP (~20 μL) at −5 °C. The resulting solution was stirred for 45 min and directly analyzed by ESI-MS/MS.

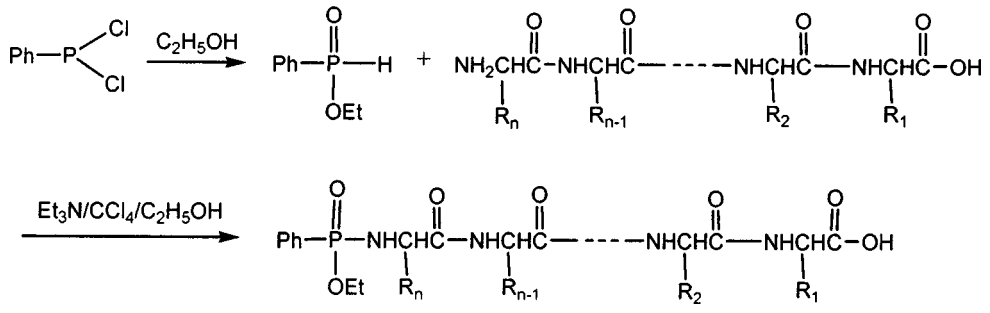
Mass spectrometric conditions

Mass spectra were recorded on a Bruker ESQUIRE ~LC ion-trap spectrometer equipped with a gas nebulizer probe. Nitrogen gas was used for drying at a flow rate of 4 L/min. The nebulizer gas fore-pressure was 7 psi. The electrospray capillary was typically held at 4 kV. Samples were dissolved in ethanol and ionized by electrospray ionization. The scan range was from m/z 100 to 1000 in positive-ion. The selected ions $[M + H]^+$ were analyzed by multistage tandem mass spectrometry through collision with helium.

RESULTS AND DISCUSSION

N-terminally ethoxyphenylphosphinylated peptides were prepared by treating the free peptides with 1.2 to 1.5 equiv of EPP in a mixed solvent containing triethylamine, tetrachloromethane and ethanol (Scheme 1).[35] The resulting solution was directly analyzed by ESI-MS/MS.

The mass spectra of the modified peptides showed intense molecular ions ($M + H^+$) usually as the base peaks. The protonated molecular ions were selected as precursor ions for CID fragmentation. ESI-MS/MS showed



Scheme 1. Synthetic route of phosphonamidate peptides.

much simpler fragmentation pattern relative to those of unmodified peptides. The spectra are usually dominated by the b_n ions, corresponding to the modified N-terminal fragments. This made sequence interpretation trivial and unambiguous in most cases. For example, Fig. 1 shows the ESI-MS^2 mass spectrum of peptide EPP-Ala-Gly-Phe-Leu-Val-OH. The spectrum consists of the $[M + H]^+$ ion at m/z 674, the $[M + H - H_2O]^+$ ion at m/z 656, b_4 ion ([M-Val-16]$^+$) at m/z 557, b_3 ion ($[M + H - LeuValOH]^+$) at m/z 444, and b_2 ion ($[M + H - PheLeuValOH]^+$) at m/z 297. The spectrum is essentially devoid of any other peaks and therefore the peptide sequence Phe-Leu-Val-OH is unambiguously assigned on the basis of the mass differences between adjacent b_n ions. Next, the b_2 ion at m/z 297 ($[M + H - PheLeuValOH]^+$) was selected for further fragmentation to obtain the ESI-MS^3 spectrum, which displayed an intense peak at m/z 240 (data not shown). This indicates the loss of a glycyl residue and the remaining fragment ion corresponds to [EPP-Ala]$^+$. Two small satellite peaks at m/z 416 and 269 were most likely due to loss of ethylene (from the phosphonyl group) or CO (from amide) from b_3 and b_2 ions, respectively (Fig. 1). Therefore, the complete amino acid sequence of the phosphonylated pentapeptide was determined as EPP-Ala-Gly-Phe-Leu-Val-OH.

We also determined the sequences of other peptides containing special amino acid residues, for example, EPP-Phe-Ala-Ser-Asp-LeuOH and EPP-Val-Glu-Gln-HisOH. Figure 2 shows the ESI-MS^2 spectrum of peptide EPP-Phe-Ala-Ser-Asp-LeuOH. A series of fragment ions resulting from sequential loss of C-terminal amino acid residues were observed at m/z 702, 589, 474, 387, and 316, corresponding to loss of LeuOH, AspLeuOH, SerAspLeuOH, and Ala-Ser-Asp-LeuOH from the original peptide, respectively. We also observed intense peaks corresponding to $[b_3 + 1]^+$ at m/z 475 and $[b_2 + 1]^+$ at m/z 388, although their mechanism of formation is currently unknown. More impressively, the ESI-MS^2 spectrum of peptide EPP-Val-Glu-Gln-HisOH produced a complete set of the b_n fragment ions at m/z 680, 525, 397, and 268 (corresponding to $[M + H]^+$, b_3, b_2, and b_1 ion, respectively), but was still relatively free of other fragment ions (Fig. 3). In addition, an intense ion $[EPP + Val - CO]^+$ at m/z 240 was observed.

Mass spectra of arginine-containing peptides often show peculiar results due to the presence of a guanidino group on its side chain.[36] In addition, some investigators reported that peptides with Asp usually displayed strong signals resulting from cleavage at the carboxy-side of Asp in mass spectrometry.[24,37] We chose to determine the sequence of peptide Val-Arg-Leu-Asp-Ser-PheOH, which contains both arginyl and aspartyl residues. The peptide was N-terminally phosphonylated and then analyzed by ESI-MS/MS. The ESI-MS^2 spectrum (Figure 4) shows $[M + H]^+$ at m/z 904, b_5 at m/z 739, b_4 at m/z 652, b_3 at m/z 537, and b_2 at m/z 424. In addition, ions corresponding to $[b_3\text{-}17]^+$ (m/z 520) and $[b_2\text{-}17]^+$ (m/z 407) were observed, which are formed due to loss of NH_3 from the arginine guanidino group. The b_4 ion (m/z 652) was the base peak, which is in agreement with the previous reports by other investigators.[24,36] The ESI-MS^3 spectrum of the b_4 ion (m/z 652) displayed the similar fragment ions to ESI-MS of the original molecule (Figure 5), which allowed the entire peptide sequence to be determined.

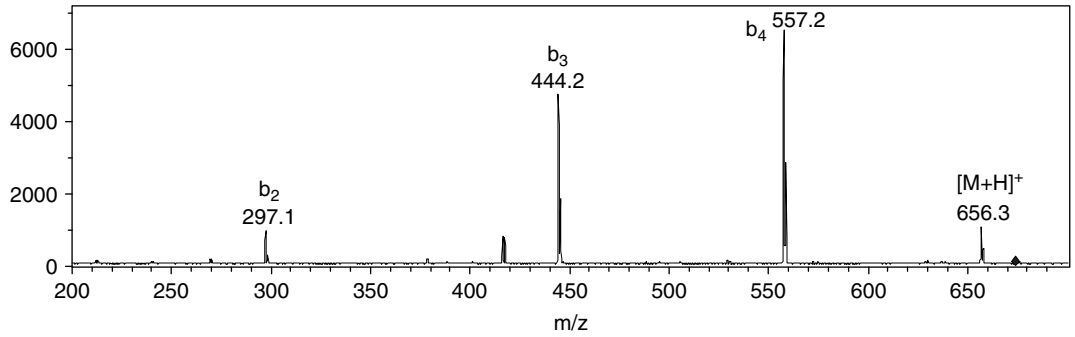


Figure 1. ESI-MS^2 spectrum of the protonated molecule $[M + H]^+$ at *m/z* 674 of EPP-Ala-Gly-Phe-Leu-ValOH.

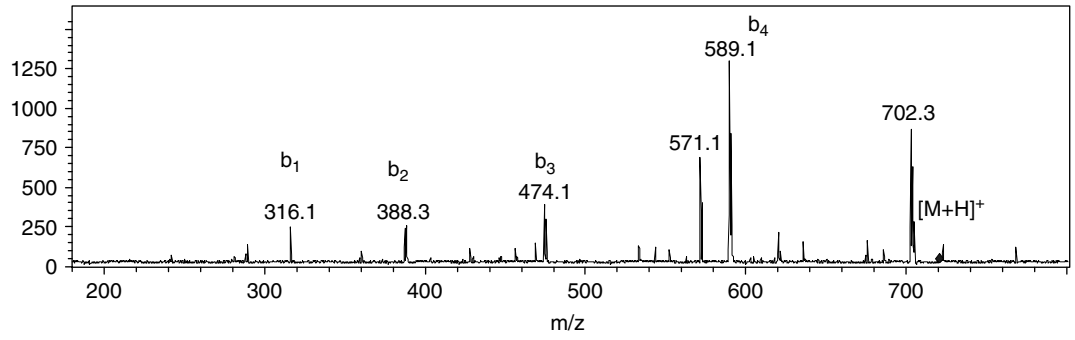


Figure 2. ESI-MS^2 spectrum of the protonated molecule $[M + H]^+$ at *m/z* 720 of EPP-Phe-Ala-Ser-Asp-LeuOH.

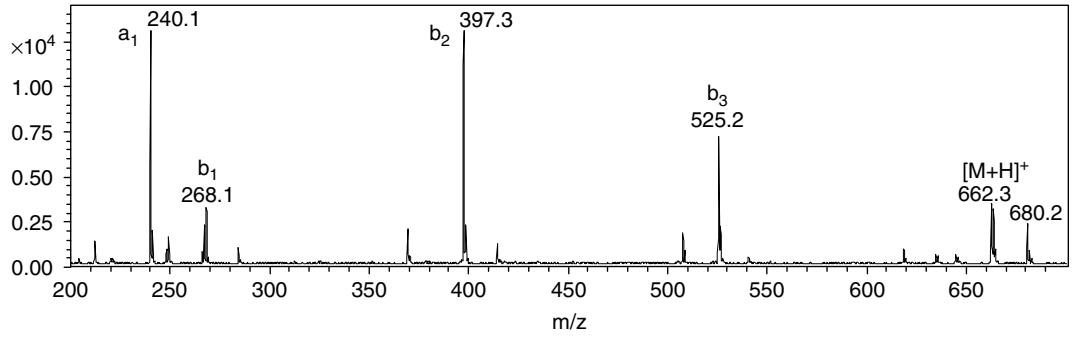


Figure 3. ESI-MS^2 of the protonated molecule $[M + H]^+$ at *m/z* 680 of EPP-Val-Glu-Gln-HisOH.

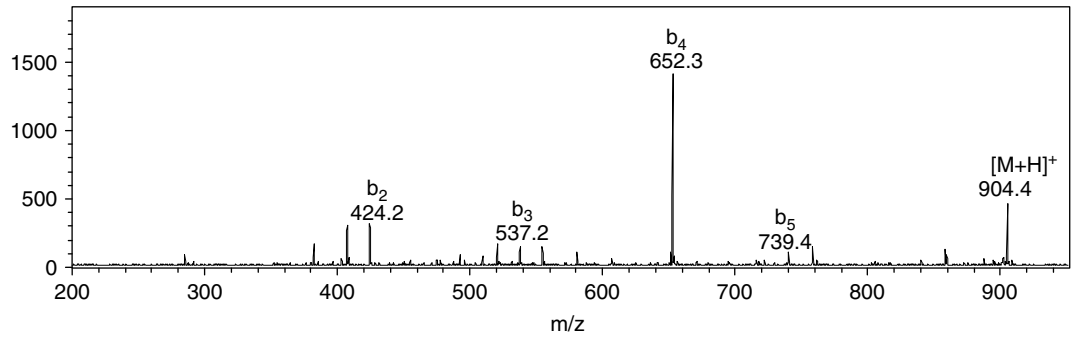


Figure 4. ESI-MS^2 of the protonated molecule at *m/z* 904 of EPP-Val-Arg-Leu-Asp-Ser-PheOH.

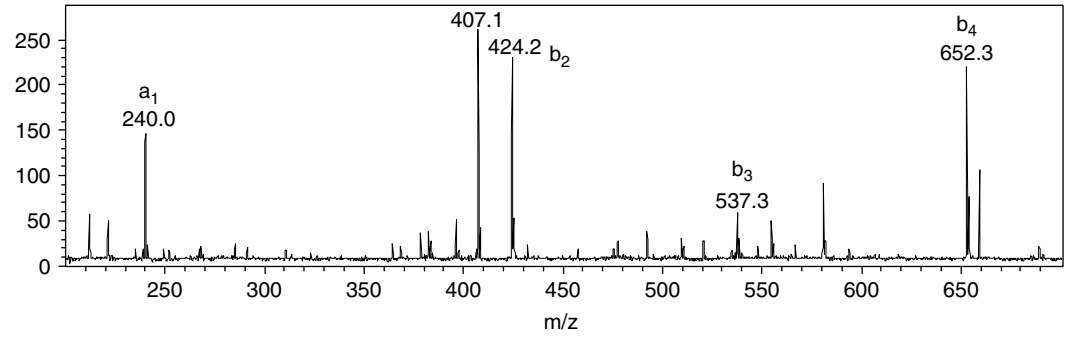


Figure 5. ESI-MS^3 spectrum of b_4 ion at *m/z* 652 in Fig. 4.

CONCLUSION

Peptides with free N-termini were readily converted into the corresponding N-terminally phosphonamidylated peptides by treatment with EPP under mild conditions. MS/MS of the resulting peptide derivatives produced predominantly the b_n-type ions and much simplified spectra that allow ready sequence determination of the original peptide. This provides a rapid, convenient, and general method for peptide sequence determination.

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