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Analysis of peptide mixtures through convenient isotopic labeling and electrospray ionization-mass spectrometry

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Abstract

We have developed a labeling method for sequencing of mixed peptides through modification of peptides and multistage electrospray ionization-mass spectrometry (ESI-MS). Mixed peptides were selectively phosphonylated on amino group through reaction of 2-bromoethyl phenylphosphinate (BEPP-H) or methyl/methyl-d_3 (1:1) phenylphosphinate (MPP-X) with peptides in the presence of tetrachloromethane and triethylamine under mild conditions to provide the labeled phosphonamidate peptides, and their primary ESI mass spectra showed doublet and triplet peaks. The triplet and doublet peaks were corresponding to the protonated molecules of the modified peptides having and having no one lysine residue, respectively, and the singlet peaks were from the non-peptides. Further, tandem mass spectra (MS/MS) of the readily identified protonated molecules unambiguously gave sequence of each member in mixed peptides.

Keywords: Peptide sequencing; Electrospray ionization-mass spectrometry; Phosphonamidate peptide; Isotopic labeling; 2-Bromoethyl phenylphosphinate; Methyl/methyl-d_3 (1:1) phenylphosphinate

1. 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]. Since the amount of peptides present from nature products and synthesized compounds for biological and medicinal screening is rather small, the structure elucidation should require only the minimum amount, mass spectrometry is a choice for this purpose. Most work on peptide sequence determination is performed with relatively soft ionization techniques such as fast atom bombardment (FAB) [2], matrix assisted laser desorption/ionization (MALDI) [3] and electrospray ionization (ESI) [4]. Many methods, such as Edman method [5], peptide ladder sequencing [6], C-terminal chemical [7,8] and enzymatic [9,10] sequencing, have been developed for sequence analysis of peptides, however, they require pure samples and chemical or enzymatic degradation prior to mass spectrometry, making them inconvenient for small amounts of peptides, especially peptide mixtures. Multistage mass spectrometry provides opportunities for analysis of peptide mixtures. When doing multistage MS, the mixtures of peptides can be separated after ionization by the first stage of mass spectrometry. In the next stages, the selected peptide ion collides with the collision gas (collision induced dissociation, CID) or a metal surface (surface induced dissociation, SID) [11], and the resulting MS/MS spectrum can be used to deduce the amino acid sequence of the peptides. Unfortunately, the complexity of the fragments makes the interpretation of peptide sequences difficult. Glish and co-workers [12] proposed that alkali-cationized peptides could be cleaved sequentially from their C-termini in ion-trapping instruments using low collision energies. However, some drawbacks make this method unpractical such as the lower sensitivity of the cationized peptides and the complex dissociation pathways when the residues are less than three in mass spectrometry. Some isotopic labeling methods have been developed and applied to quantification of proteomics [13]. In our previous research, the phosphonylated peptides simplified multistage mass spectra of the protonated and sodiated molecules relative to the unmodified peptides that allow ready sequencing of the original peptides [14,15]. As the continuum of this pro-

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gram, we would like to report a labeling method for analysis of peptide mixtures through N-phosphonylation of peptides at their N-termini using bromine (Br) or 50% deuterium (D)-labeling phosphonylating reagents and multistage ESI-MS.

2. Experimental

2.1. Materials

The peptides were commercially available from Shanghai Glsynthesis Company (∼70% pure), and they were used to phosphonylate and then analyze by ESI-MS/MS without further purification. Dichloro(phenyl)phosphine (DCPP), deuterated methanol (CD_3OD) and 2-bromoethanol were purchased from Aldrich. Methanol and ethanol were rendered anhydrous by refluxing with Mg.

2.2. Chemistry

^{31}P NMR chemical shifts were reported in ppm downfield (+) or upfield (−) from external 85% H_3PO_4 as reference. 2-Bromoethyl phenylphosphinate (BEPP-H) and methyl/methyl-d_3 phenylphosphinate (1:1) (MPP-X) were prepared as previously described [14,15].

2.2.1. Synthesis of 2-bromoethyl phenylphosphinate and methyl/methyl-d_3 (1:1) phenylphosphinate

Three millimoles of 2-bromoethanol or a mixture of 1.5 mmol of methanol and 1.5 mmol of deuterated methanol (CD_3OD) was added dropwise to 1 mmol of dichloro(phenyl)phosphine (DCPP) in 4 mL of dichloromethane at room temperature under nitrogen atmosphere, the reaction completed within 30 min, and ^{31}P NMR showed that DCPP almost quantitatively transferred into BEPP-H. The crude product BEPP-H was obtained after removal of gaseous HCl, dichloromethane and an excess of alcohol(s), and it can be used without further purification (^1H NMR showed that the purity was more than 95%). ^{31}P NMR of BEPP-H: 28.33 ppm. ^{31}P NMR of MPP-X: 30.99 ppm.

2.2.2. General procedure for synthesis of the labeled phosphonamidate peptides [14,15]

Several peptides (∼1 μg for each member) were mixed and dissolved in mixed solution, 80 μL of ethanol, 10 μL of triethylamine and 10 μL of tetrachloromethane, and then 1.5–2 equiv. of BEPP-H or MPP-X (∼1 μL) was added to the solution at 0 °C, the solution was stirred for 45 min, the corresponding phosphonamidate peptides were obtained, and the resulting solution was directly analyzed by ESI-MS/MS.

2.3. Mass spectrometry

The mass spectra were obtained using a Bruker ESQUIRE∼LC ion-trap spectrometry equipped with a gas nebulizer probe. Nitrogen was used as drying gas 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. The scan range was from m/z 103 to 1100 using positive ion mode. The selected ions $[M+H]^+$ were analyzed by multistage tandem ESI mass spectrometry through collision with helium. The amount and injection rate of the sample were ∼ng and 0.4 mL/h, respectively. The used time was within 6 min during analysis.

Table 1
Sequence of the original peptides and mass numbers for the protonated molecules of the Br-labeling peptides

Peptide no.	Sequence	$[M+H]^+$
1	GG	379, 381
2	AA	407, 409
3	AAA	478, 480
4	VFA	582, 584
5	VEQH	758, 760

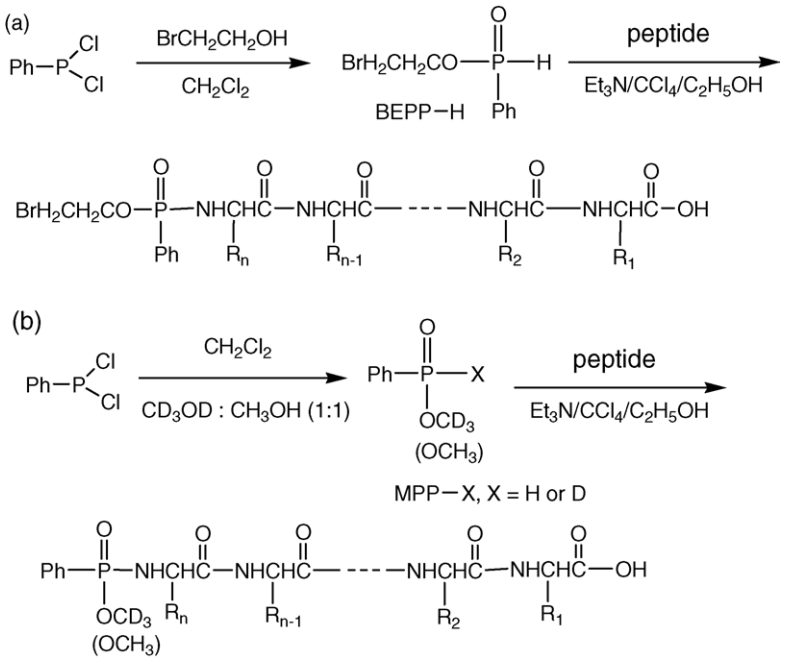
3. Results and discussion

3.1. Our strategy for analysis of peptide mixtures

Some problems are usually met for analysis of peptides as follows: (1) the samples are impure from natural products even if they are isolated by HPLC; (2) the peptide samples are contaminated; (3) peptide library contains much non-peptide impurity. Mass spectra of all these samples show many peaks, it is very hard and troublesome if every peak is analyzed by tandem mass spectrometry. Since the primary structures of proteins are usually identified through the sequential procedure in which a protein is cleaved into small peptides followed sequencing for every member of them. Here, we chose impure peptide mixtures as mode samples to develop a new and general method for analysis of peptide mixtures, which could be helpful for determination of protein primary structures. We think that the peptides are labeled by isotopic reagents such as bromo, H/D (molar rate 1:1) mixed agents before the samples are analyzed and their mass spectra can show multiple peaks for each member of peptides. Herein, some mixed peptides were phosphonylated by BEPP-H or MPP-X (Scheme 1). We believe that the following results may be observed (Fig. 1): (1) ESI-MS spectra of the phosphonylated peptides at N-termini should show multiple peaks because of isotopic labeling, and the modified peptides without Lys residue show doublet peaks, while the compounds containing one Lys residue exhibit triplet peaks because of diphosphonylation at two amino groups (imino group for proline) from N-termini of a peptide and side chain of a lysine residue; (2) the non-peptide samples cannot be phosphonylated because of the absence of amino or imino group, so their mass spectra show singlet peaks.

3.2. Analysis of Br-labeling peptide mixtures modified by BEPP-H

Mixed peptides, Gly–Gly, Ala–Ala, Ala–Ala–Ala, Val–Phe–Ala and Val–Glu–Gln–His, were effectively phosphonylated by BEPP-H to provide sample 1 (Table 1) according to the route as shown in Scheme 1a, and the sample was determined by positive ion ESI-MS. Some singlet and doublet peaks were observed in Fig. 2, the singlet peaks were put away



Scheme 1. Synthetic route of the labeled phosphonamidate peptides: (a) the preparation of the BEPP-peptides; (b) the preparation of the MPP-peptides.

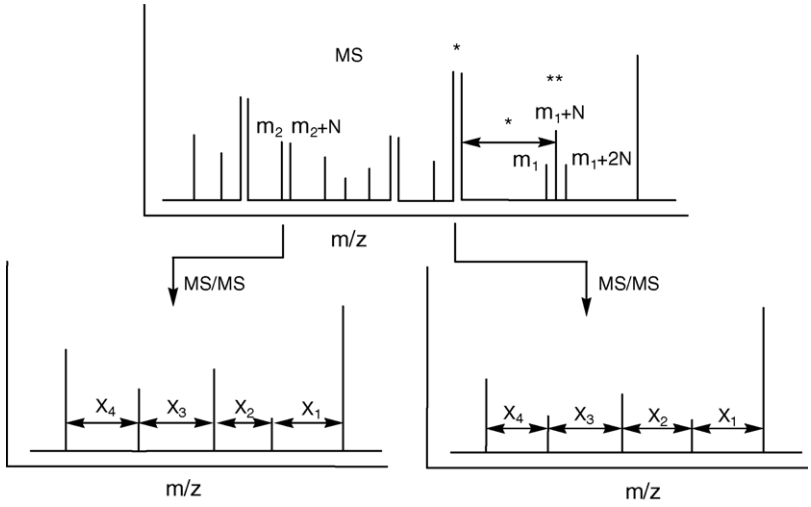


Fig. 1. Sequencing strategy of mixed peptides. The singlet, doublet and triplet peaks from non-peptides, monophosphonylated and diphosphonylated peptides, respectively (the symbols (* and **) represent mono- and diphosphonylated products of peptides containing one lysine residue, respectively).

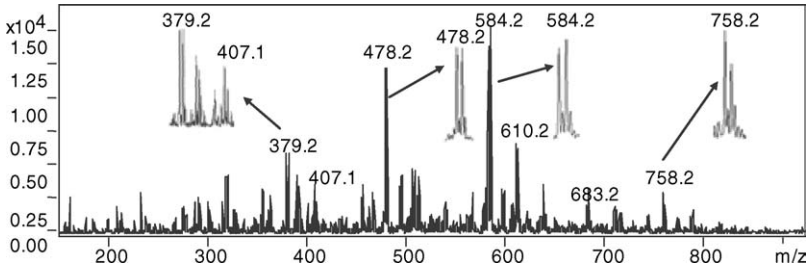


Fig. 2. The full mass spectrum of Br-labeling peptide mixtures (BEPP–Gly–Gly, –Ala–Ala, –Ala–Ala–Ala, –Val–Phe–Ala, –Val–Glu–Gln–His).

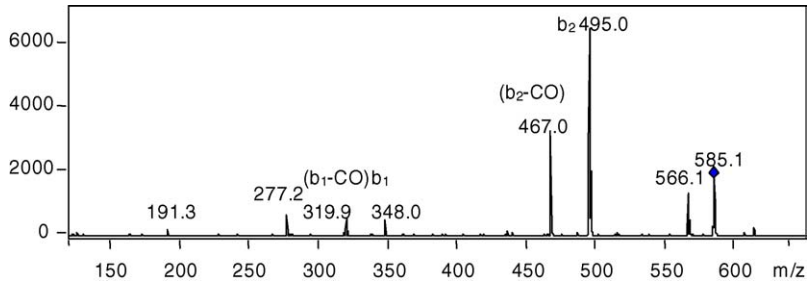


Fig. 3. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 584 (BEPP–Val–Phe–Ala).

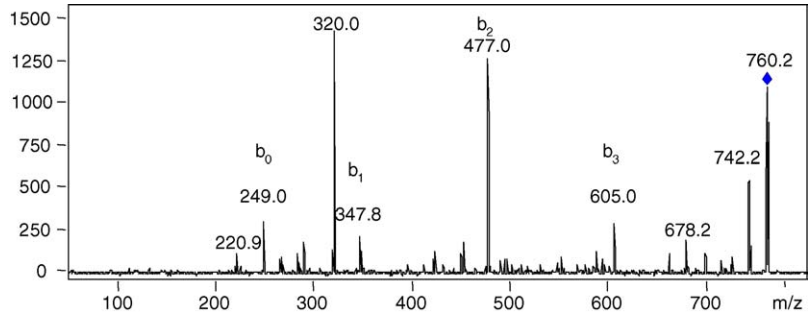


Fig. 4. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 760 (BEPP–Val–Glu–Gln–His).

because they were from the non-peptides, and the ions corresponding to the doublet peaks were chosen as the precursors and determined by MS/MS. For example, the doublet peaks at m/z 582 and 584, one (m/z 584) of the both was selected to obtain the ESI-MS/MS spectrum (Fig. 3), in which b_2 (m/z 495), b_1 (m/z 348) and $[BEPP + Val - CO]^+$ (m/z 320) ions were observed (Fig. 3), so the precursor ion $[M+H]^+$ was assigned to BEPP–Val–Phe–Ala. Similarly, MS/MS of the protonated molecule $[M+H]^+$ at m/z 760 produced the b_n fragment ions at m/z 605 (b_3), 477 (b_2), 347 (b_1) and 249 (b_0) (Fig. 4), the results clearly showed that the modified peptide was BEPP–Val–Glu–Gln–His. Other peptide derivatives could be confirmed using similar procedure (Table 1), so mixed peptides were easily identified.

The free peptides containing one lysine residue can be diphosphonylated by BEPP-H for the presence of amino group on the side chain of the lysine residue, so peptide mixtures, Gly–Gly, Ala–Ala, Val–Phe–Ala, Ala–Lys–Val and Val–Gly–Lys–His, were treated to give sample 2 (Table 2) according to the procedure in Scheme 1a, and the primary ESI-MS spectrum of the sample is shown in Fig. 5. We observed that the doublet and triplet peaks were corresponding to the mono- and diphosphonylated products, and the doublet peaks could be analyzed using similar procedure above. One of the triplet peaks was chosen for further fragmentation, unfortunately, the secondary ESI mass spectrometry exhibited complex mass peaks, which was difficult to identify sequence of the precursor ion. On the left of the triplet peaks, we found the corresponding doublet peaks whose mass number was 246 less than the triplet peaks, so this means that the doublet peaks were from the corresponding monophosphonylated peptide. Next, we determined the doublet peaks, and their secondary mass spectra gave useful sequence information of peptides. For example, the doublet peaks (m/z 565, 567, intensity ratio 1:1) on the left of the triplet peaks (m/z 809, 811, 813, intensity ratio 1:2:1) in Fig. 5 were observed, MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 565 gave the b_n ions (m/z 447 (b_2), 318 (b_1) and 249 (b_0)) (Fig. 6). The result showed the existence of the tripeptide Ala–Lys–Val derivative. Similarly, BEPP–Val–Gly–Lys–His was identified (Fig. 7).

Table 2
Sequence of the original peptides and mass numbers for the protonated molecules of the Br-labeling peptides

Peptide no.	Sequence	$[M+H]^+$ (with one Br)	$[M+H]^+$ (with two Br)
1	GG	379, 381 (doublet)	
2	AA	407, 409 (doublet)	
3	VFA	582, 584 (doublet)	
4	AKV	565, 567 (doublet)	809, 811, 813 (triplet)
5	VGKH	758, 760 (doublet)	1004, 1006, 1008 (triplet)

3.3. Analysis of 50% D-labeling peptide mixtures modified by MPP-X

We also labeled mixed peptides using methyl/methyl-d_3 (1:1) phenylphosphinate as the phosphonylating agent according to the procedure in Scheme 1b. Peptide mixtures, Gly–Gly, Gly–Phe, Ala–Ala–Ala and Glu–Cys–Gly, were labeled to give sample 3 (Table 3) that was determined by positive ion ESI-MS, some doublet peaks were observed (Fig. 8), and the corresponding phosphonamidate peptides were identified by ESI-MS/MS. For example, the ESI-MS/MS spectrum of the ion at m/z 462 gave b_n fragment ions at m/z 387 (b_2) and 284 (b_1) (Fig. 9), so the precursor ion could be assigned to MPP–Glu–Cys–Gly.

We also labeled peptide mixtures, Gly–Gly, Ala–Ala, Val–Phe–Ala, Ala–Lys–Val and Val–Gly–Lys–His, using

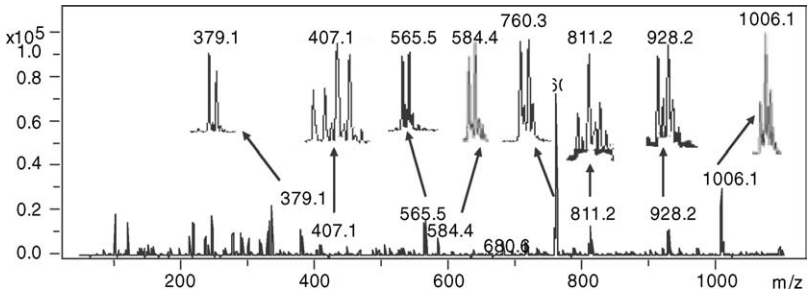


Fig. 5. The full mass spectrum of Br-labeling peptide mixtures (BEPP–Gly–Gly, –Ala–Ala, –Val–Phe–Ala, –Ala–Lys–Val, –Val–Gly–Lys–His).

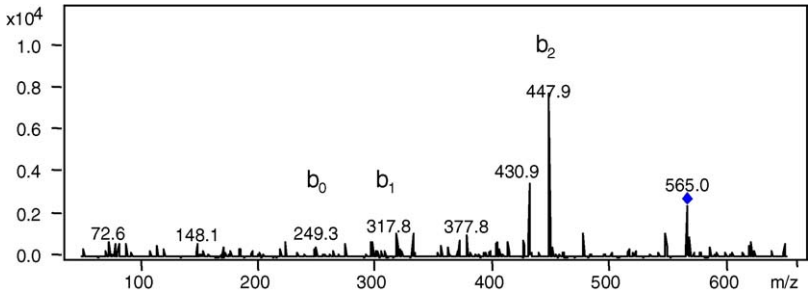


Fig. 6. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 565 (mono-BEPP–Ala–Lys–Val).

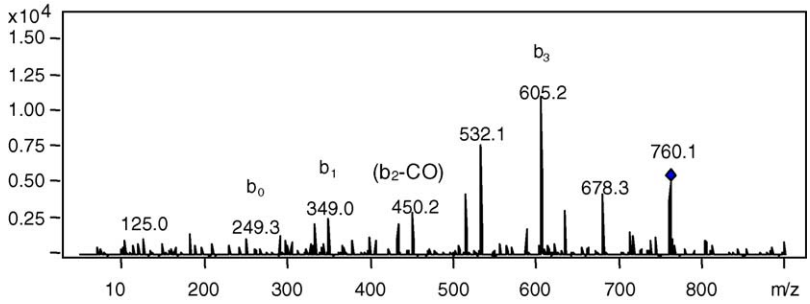


Fig. 7. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 760 (mono-BEPP–Val–Glu–Lys–His).

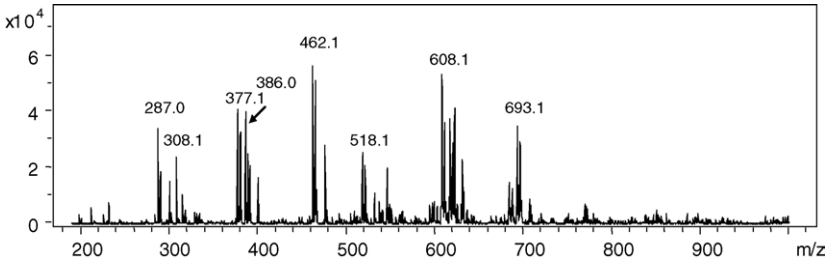


Fig. 8. The full mass spectrum of 50% D-labeling peptide mixtures (MPP–Gly–Gly, –Gly–Phe, –Ala–Ala–Ala, –Glu–Cys–Gly).

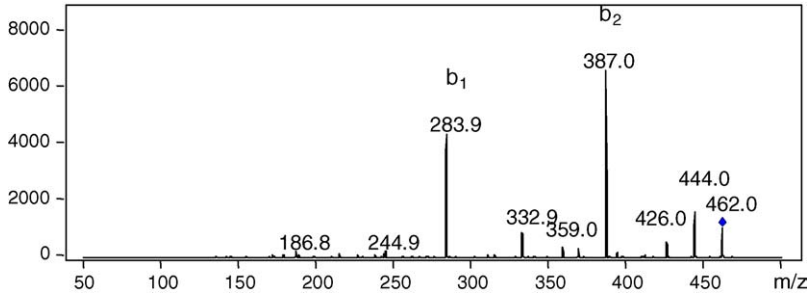


Fig. 9. MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 462 (MPP–Glu–Cys–Gly).

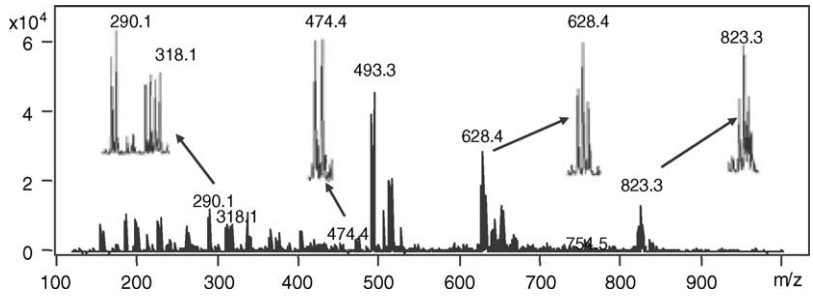


Fig. 10. The full mass spectrum $[M+H]^+$ of D-labeling peptide mixtures (MPP–Gly–Gly, –Ala–Ala, –Val–Phe–Ala, –Ala–Lys–Val, –Val–Gly–Lys–His).

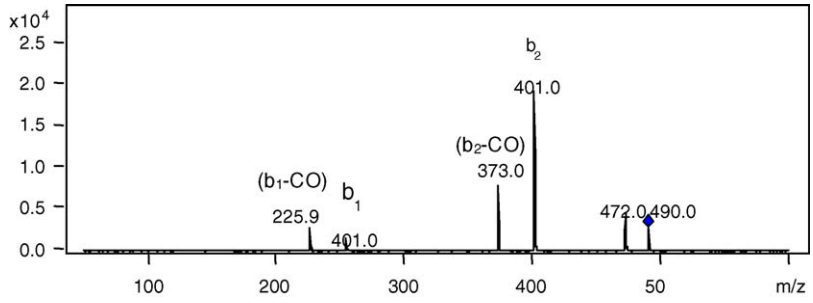


Fig. 11. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 490 (MPP–Val–Phe–Ala).

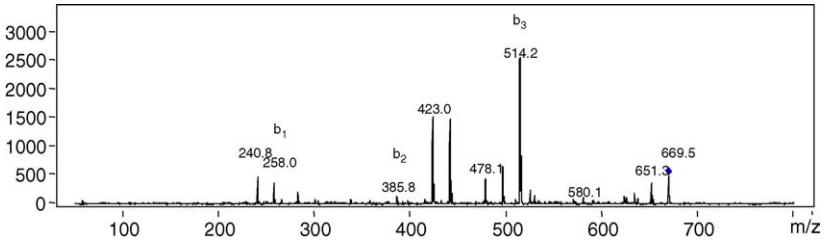


Fig. 12. ESI-MS/MS spectrum of the precursor ion $[M+H]^+$ at m/z 669 (MPP–Val–Gly–Lys–His).

Table 3
Sequence of the original peptides and mass numbers for the protonated molecules of the 50% D-labeling peptides

Peptide no.	Sequence	$[M+H]^+$
1	GG	287, 300
2	GF	377, 380
3	AAA	386, 389
4	ECG	462, 465

similar procedure above to provide the corresponding phosphonamidate peptides (sample 4) which could be readily analyzed by ESI-MS/MS (Table 4 and Fig. 10). For example, Fig. 11 shows ESI-MS/MS spectrum of the ion at m/z 490, MPP–Ala–Lys–Val was assigned according to the fragment ions b_2 (m/z 401) and b_1 (m/z 254). The triplet peaks at m/z 820, 823, 826 in Fig. 10 are corresponding to the diphosphonylated peptide containing one lysine residue, we could sequence the corresponding monophosphonylation peptide at m/z 669 by ESI-MS/MS (Fig. 12), which was assigned to the protonated MPP–Val–Gly–Lys–His. Modified peptides by MPP-X could give much simpler spectra than those by BEPP-H (compare Fig. 12 with Figs. 6 and 7), so the method using MPP-X as phosphonylating agent might be a better choice for modification of peptide mixtures.

Table 4
Sequence of the original peptides and mass numbers for the protonated molecules of the 50% D-labeling peptides

Peptide no.	Sequence	$[M+H]^+$ (with 1D)	$[M+H]^+$(with 2D)
1	GG	287, 290 (doublet)	
2	AA	315, 318 (doublet)	
3	VFA	490, 493 (doublet)	
4	AKV	471, 474 (doublet)	625, 628, 631 (triplet)
5	VGKH	666, 669 (doublet)	820, 823, 826 (triplet)

4. Conclusions

Mixed peptides were labeled via N-terminal phosphonylation by 2-bromoethyl phenylphosphinate or methyl/methyl-d_3 (1:1) phenylphosphinate to provide the corresponding phosphonamidate peptides under mild conditions, and mass spectra of the derivatives showed some doublet and triplet peaks, while non-peptides or contaminations in samples displayed the singlet peaks. Next, MS/MS spectra of the doublet peaks produced predominantly b_n-type ions, so various peptides including lysine-containing peptides were unambiguously identified. The rapid,

convenient and selective N-terminal labeling method provided can generally be used for analysis of peptide mixtures and further extension to proteins.

Acknowledgments

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