Picolinamidination of Phosphopeptides for MALDI-TOF-TOF Mass Spectrometric Sequencing with Enhanced Sensitivity

Jong-Seo Kim,* Enshi Cui, and Hie-Joon Kim
Department of Chemistry, Seoul National University, Seoul, Korea

Two orders of magnitude matrix-assisted laser desorption/ionization (MALDI) signal enhancement of phosphopeptides has been achieved by picolinamidination of N-terminal amine group and ε-amine group of lysine residues. Due to the presence of picolinamidination tag at the N-terminal amine of peptides, MS/MS spectra with a strong b-ion series was obtained, which greatly facilitated sequencing and identification of the phosphorylation site. Phosphorylation site of a phosphopeptide could be identified from MALDI TOF/TOF spectrum obtained from a tryptic or a chymotryptic phosphopeptide, which was not even detected in the positive ion mode, without signal enhancement by picolinamidination, due to the negative charge of the phosphate group in the presence of other peptides. (J Am Soc Mass Spectrom 2009, 20, 1751–1758) © 2009 American Society for Mass Spectrometry

Identification of phosphoprotein and its phosphorylation site is an important part of proteomics research. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of phosphopeptides is a key step in investigation of a phosphoprotein [1–6]. Yet, it is limited by the low concentration of phosphoproteins in cells and the low ionization efficiency of phosphopeptides due to the negatively charged phosphate group [7, 8]. Therefore, orders of magnitude increase in the mass spectrometric signal intensity could be critical in the analysis of low abundance phosphopeptides.

Given the capability of the mass spectrometer available, two different approaches are conceivable for detection of low abundance phosphopeptides. A straightforward approach is enrichment. Immobilized metal ion affinity chromatography (IMAC) is the most widely used technique for enrichment of phosphopeptides [9–14]. Recently, enrichment methods using metal oxide instead of metal ion have been developed [15–19]. Affinity separation after binding specific functional groups via β-elimination/Michael addition has also been used [20–25].

Esterification of acidic peptides could reduce their ionization efficiency in the negative mode and thus make selective detection of phosphopeptides possible [6]. In a more direct approach, a highly basic moiety could be incorporated to the phosphopeptide by β-elimination/Michael addition to replace the negative charge of the phosphate group and to enhance the MALDI signal in the positive ion mode [26–30]. Coumarin tagging of amine could enhance the MALDI signal of phosphopeptides 30- to ~40-fold [4]. Additives in the matrix solution were also used to improve detection of phosphopeptides without enrichment and derivatization [3, 31].

A cumulative effect of peptide enrichment and signal enhancement would be desirable, since most phosphopeptides of interest exist in cells at low concentration. If the concentration of a certain phosphopeptide is 100 times lower than the detection limit of the available mass spectrometer, a 10-fold enrichment will not be sufficient. However, another 10-fold signal enhancement by chemical derivatization could bring the phosphopeptide signal above the noise level.

Recently, we showed that the MALDI signal of a peptide could be enhanced over 20-fold by picolinamidination [32]. Ethyl picolinimidate reacts with the N-terminal amino group as well as the ε-amino group of lysine residues. The picolinamidine group is strongly basic and thus significantly increases the ionization efficiency of the modified peptide. Picolinamidination would be quite useful if the added basicity could counteract the suppressed ionization efficiency of a phosphopeptide in the positive ion mode. In this paper, we show that over 100-fold enhancement of the MALDI signal from phosphopeptides and other acidic peptides could be achieved by picolinamidination. The characteristic fragmentation pattern of picolinamidinated peptides with enhanced signal intensity significantly improved tandem mass spectrometric sequencing and identification of phosphorylation site of synthetic phosphopeptides.

Address reprint requests to Dr. H.-J. Kim, Department of Chemistry, Seoul National University, San 56-1, Sillim-dong, Gwanak-gu, Seoul, 151-747, Korea. E-mail: hjkim1@snu.ac.kr

* Current address: Biological Sciences Division and Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA 99352, USA.

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Experimental

Chemicals

Picolinamide and α-cyano-4-hydroxycinnamic acid (CHCA) were obtained from Aldrich (Milwaukee WI, USA). Peptides (DRVYIHPF, ASHLGLAR, DAEFRHDSGYQVHHQK) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PyBOP, Fmoc-Glu(OtBu)-OPfp, Fmoc-Asp(OtBu)-OH, Fmoc-Ser(PO-(OBzl)OH)-OH, Fmoc-Thr(PO-(OBzl)OH)-OH, and Fmoc-Tyr(PO-(OBzl)OH)-OH used in peptide synthesis were from Novabiochem (San Diego, CA, USA). Nitrilotriacetic acid (NTA), trifluoroacetic acid (TFA), *N,N*-diisopropylethylamine, piperidine, methylene chloride, acetonitrile, triethanol amine (TEA) dimethyl sulfoxide, *N,N*-dimethylformamide, and triisopropylsilane were from Sigma-Aldrich. Triethyloxonium tetrafluoroborate was from TCI (Tokyo, Japan).

Synthesis of Ethyl Picolinimidate Tetrafluoroborate

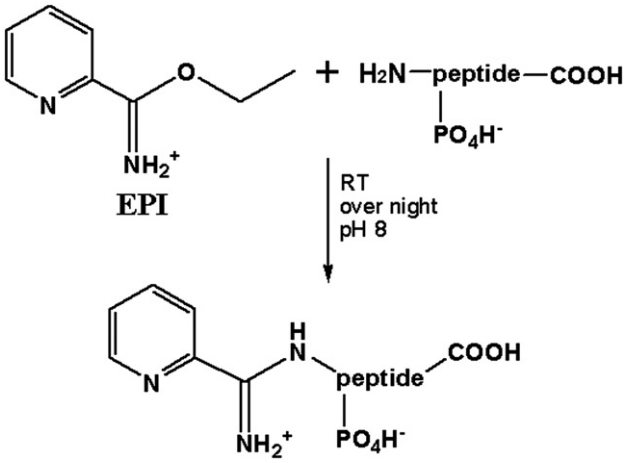
Ethyl picolinimidate (EPI) tetrafluoroborate was synthesized as reported [33]. Forty mg picolinamide (0.33 mmol) was dissolved in 670 μL of anhydrous methylene chloride and 330 μL of 1 M solution of triethyloxonium tetrafluoroborate in methylene chloride (0.33 mmol) was added. The mixture was vortex-mixed overnight at room temperature until pink color from the initial reaction completely disappeared. The precipitate was removed by centrifugation, and methylene chloride from the supernatant was removed using a vacuum centrifuge to get the desired product as white precipitate. EPI could be used for as long as 30 d if kept away from moisture, which hydrolyzes EPI.

Synthesis of Peptides

Some peptides and phosphopeptides (GD**pT**KAVEK, AEQ**pS**LKDVNK, RESG**pT**NAY, AKGQ**pY**VDY, R**pY**KVF, RYKVF, WDEGDF, AEDDVEDY, AENDVGDK) were synthesized by the solid-phase method of Merrifield. Peptides synthesized were purified by reveres-phase HPLC. After checking the molecular weight and purity by MALDI MS, the peptide was freeze-dried after evaporation of the solvent and stored in a freezer.

Picolinamidination of Peptides and MALDI Sample Preparation

A preliminary test showed that picolinamidination of phosphopeptide is slower than that of normal peptides. Therefore, the EPI concentration and reaction time were increased over the reported values [32]. Typically, 10 μL of 10 μM phosphopeptide in water was mixed with the same volume of 100 mM ethyl picolinimidate tetrafluoroborate (EPI) (23.7 mg/mL, 10,000-fold molar excess) in 500 mM TEA buffer (pH 8.0). Picolinamidination was allowed to proceed overnight at room temperature (Scheme 1) and terminated by adding 5 μL 1.0% TFA to 5 μL of the reaction mixture. To remove excess reagents for MALDI analysis, 5 μL of the final mixture was loaded on ZipTip C18 (Millipore, Bedford, MA, USA) and eluted with 5 μL matrix solution. The matrix solution was made by dissolving 10 mg CHCA in 1 mL mixture of acetonitrile and water, 1:1, containing 0.1% TFA and 7 mM NTA used to suppress matrix clusters [34]. The concentration of picolinamidinated peptide loaded on the MALDI plate was estimated to be 2.0–2.5 μM. For acidic peptides, 5000-fold excess EPI in 250 mM TEA buffer was used and picolinamidination reaction was allowed to proceed for 1–2 h at room temperature.



Scheme 1. Reaction of ethyl picolinimidate with the amine group of a phosphopeptide.

MALDI MS Analysis

One μL of the picolinamidinated peptide solution in the matrix solution was loaded on the MTP 384 polished steel plate. Mass spectra were obtained in positive ion mode using Auto Flex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm N_2 laser. Typically, signals from 100 shots were added. MALDI MS/MS data were obtained using laser-induced dissociation mode and LIFT technique. Collision-induced dissociation mode was also used for phosphopeptides.

Results and Discussion

Signal Enhancement of Acidic Peptides by Picolinamidination

Signal enhancement by picolinamidination was first tested using peptides with acidic amino acid residues, which are known to suppress signal intensity in the positive ion mode. Thus, peptides were synthesized with several aspartic acid and glutamic acid residues so that the final pI of the peptide should be less than 4. Modification by one picolinamidination increases *m/z* by 104.04.

Picolinamidination of AEDDVEDY with five acidic residues (theoretical pI 2.71) was complete in 2 h; 10:1 mixture with equal volume of the original peptide (20 μM) and the picolinamidinated peptide (2 μM) showed a small peak of the original peptide with S/N of 37 and a much stronger peak of the picolinamidinated peptide with S/N of 190 (Figure 1a). The result shows a 50-fold signal enhancement.

Signal enhancement of AENDVGDK with three acidic residues (theoretical pI 3.74) was more pronounced. The MALDI spectrum from an equimolar (2 μM) mixture of the original peptide and the picolinamidinated peptide showed the picolinamidinated peptide peak only (*m/z* 1055.5, result not shown). When 40 μM solution of the original peptide and 2 μM solution of the picolinamidinated peptide were mixed in equal volume, the picolinamidinated peptide peak was 12 times stronger than the original peptide (*m/z* 847.4 peak) indicating about 240-fold signal enhancement (Figure 1b).

The signal enhancement was confirmed at a limiting concentration of the original peptide; 2.0 μM solution of AEDDVEDY showed no peak (Figure 2a). After enrichment using ZipTip, the original peptide and its sodium adduct were observed with S/N of 5 and 18, respectively (Figure 2b). To get the mass spectrum in Figure 2b, 8 μL of 2.0 μM peptide solution was mixed with 2 uL of 250 mM TEA buffer. The mixture was loaded on ZipTip and eluted with 3 μL of the matrix solution. Thus the peaks in Figure 2b result from about 5 μM peptide in the solution loaded on the sample plate. When the peptide was treated similarly except that 40 mM EPI in 250 mM TEA buffer was used for picolinamidination, a strong peak of picolinamidinated peptide was observed at *m/z* 1059.4 with S/N of 1140 (Figure 2c). The signal enhancement was about 230-fold relative to the peptide peak in Figure 2b and 60-fold relative to the sodium adduct. Generally, over 50-fold signal enhancement was obtained from acidic peptides.

Signal Enhancement of Phosphopeptides by Picolinamidination

Picolinamidination of AEQpSLKDVNK (MW 1211.6) and GDpTKAVEK (MW 927.4) with three amino groups (N-terminal plus 2 lysines) were expected to increase *m/z* by 312.1 to 1523.7 and 1239.5, respectively. Both MALDI spectra in Figure 1c and d obtained from a mixture with equal volume of unmodified (20 μM) and picolinamidinated peptide (2 μM) at 10:1 M ratio show triply picolinamidinated peptide peaks as dominant peaks. Smaller di-picolinamidinated peptides were observed at *m/z* 1419.7 and 1135.5. Loss of the phosphate group (79.97 Da) was also observed. It might come partly from the dephosphorylated peptide impurity even though the peptide was purified by HPLC after synthesis. LC/MS analysis of the peptides before picolinamidination reaction showed 1%~2% of the dephosphorylated peptides (−79.97 Da), and this percentage did not change significantly after picolinamidination as measured by LC/MS (result not shown). The relatively high intensity of the dephosphorylated peptide peaks in Figure 1c and d might reflect the presence of dephosphorylated peptide impurity in the original sample and/or in the derivatization reaction products and laser-induced dissociation in this case even though

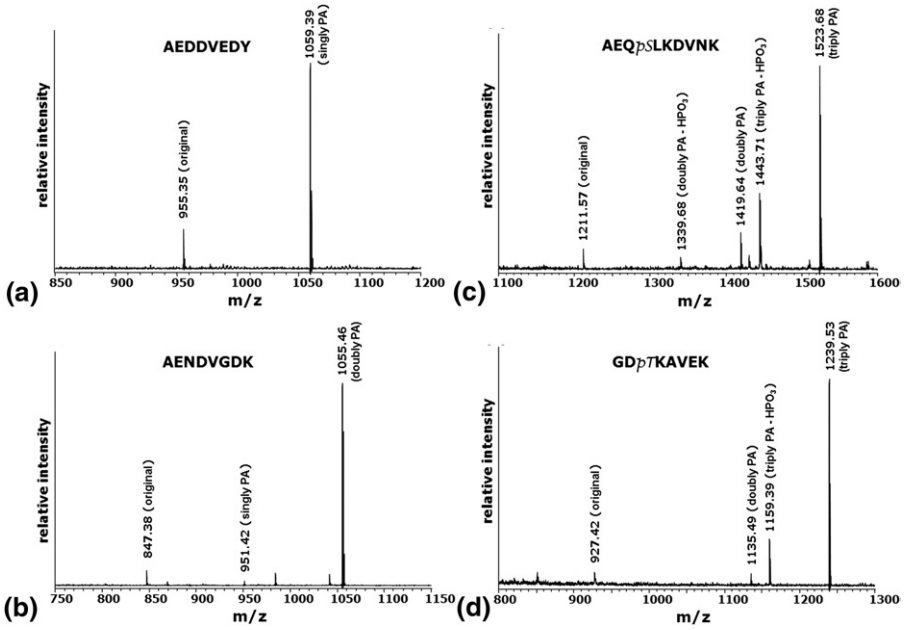


Figure 1. MALDI spectra obtained from a mixture of the original peptide and the picolinamidinated peptide in 10:1 (**a**), (**c**), (**d**), and 20:1 M ratio. (**a**) AEDDVEDY, (**b**) AENDVGDK, (**c**) AEQpSLKDVNK, (**d**) GDpTKAVEK. PA: picolinamidinated.

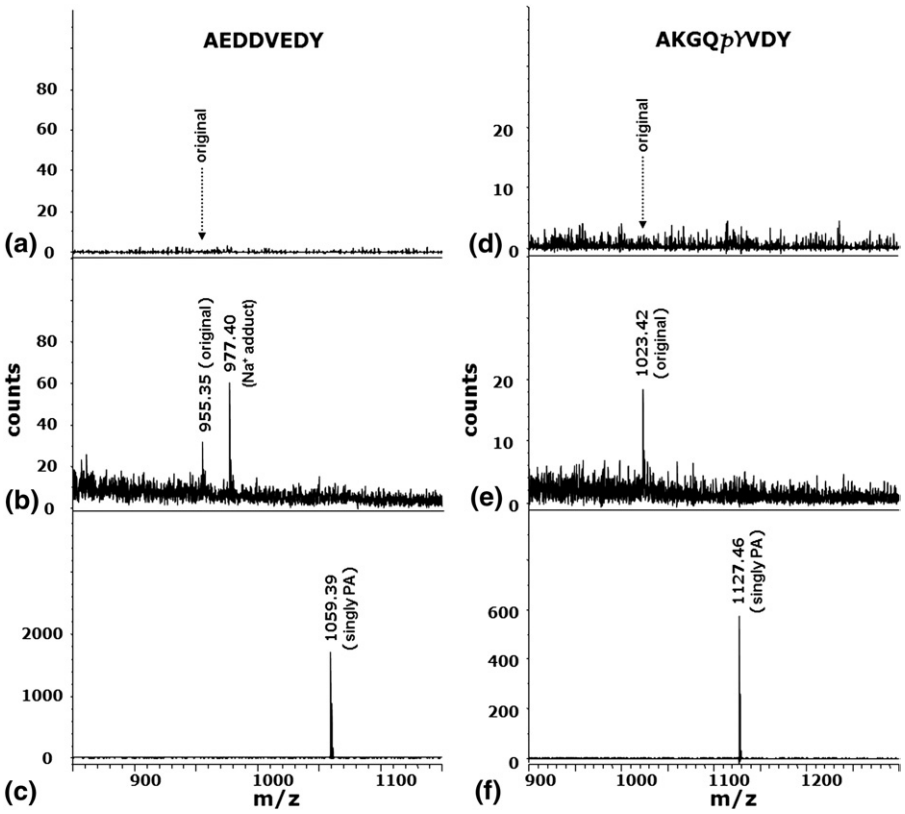


Figure 2. MALDI spectra from acidic peptide AEDDVEDY (**a**)–(**c**) and phosphopeptide AKGQpYVDY (**d**)–(**f**) at limiting concentration. (**a**) 2.0 μM, without picolinamidination, showing no signal, (**b**) enriched with ZipTip to 5.3 μM, without picolinamidination, showing peptide at *m/z* 955.35 and sodium adduct at *m/z* 977.40, (**c**) 5.3 μM, with picolinamidination, showing singly picolinamidinated peptide at *m/z* 1059.39, (**d**) 0.5 μM, without picolinamidination, showing no signal, (**e**) enriched with ZipTip to 1.3 μM, without picolinamidination, showing original peptide at *m/z* 1023.42, (**f**) 1.3 μM, with picolinamidination, showing singly picolinamidinated peptide at *m/z* 1127.46.

loss of 98 Da (H_3PO_4) is more often observed from phosphopeptides.

The original peptides present in 10-fold excess were much lower in signal intensity than the picolinamidinated peptides. The signal intensity of triply picolinamidinated AEQpSLKDVNK (*m/z* 1523.7) was 10 times higher than that of the original peptide present in tenfold excess (Figure 1c). The result shows that the MALDI ionization yield of phosphopeptide was enhanced about 100-fold upon picolinamidination. A similar or even greater signal enhancement (150-fold) was observed from GDpTKAVEK (Figure 1d).

The signal enhancement was again confirmed at a limiting concentration of a phosphopeptide AKGQpYVDY. The original phosphopeptide was not detected at 0.5 μM concentration (Figure 2d). After 3-fold concentration with ZipTip, a small peak with S/N ratio of 4 was observed at *m/z* 1023.4 (Figure 2e). After reaction, singly picolinamidinated phosphopeptide peak (*m/z* 1127.5) with S/N ratio of 410 was obtained (Figure 2f). The result again shows about 100-fold signal enhancement. GDpTKAVEK also showed over 100-fold signal enhancement (result not shown). These results show that at least 100-fold signal enhancement could be achieved by picolinamidination from a variety of phosphopeptides.

Signal Enhancement in a Peptide Mixture

To demonstrate signal enhancement of phosphopeptide in a peptide mixture, a solution containing five synthetic tryptic peptides (GDpTKAVEK, AEQpSLKDVNK, AENDVGDK, ASHLGLAR, DAEFRHDSGYQVHHQK) at 2 μM concentration each was used. The MALDI spectrum in Figure 3a from the mixture without picolinamidination showed the two arginine(R)-containing peptides (ASHLGLAR and DAEFRHDSGYQVHHQK at *m/z* 824.5, S/N 270 and 1953.9, S/N 840, respectively), but not the two phosphopeptides (GDpTKAVEK, AEQpSLKDVNK) and the acidic peptide without R (AENDVGDK). If we assume that AENDVGDK and GDpTKAVEK were just under the noise (S/N = 1), we can say that their signal is 270 times weaker than the ASHLGLAR signal.

After picolinamidination, both phosphopeptides (*m/z* 1239.9, S/N 360 and 1524.3, S/N 58) and the acidic peptide (*m/z* 1055.7, S/N 460) were observed (Figure 3b) indicating a significant enhancement of the signal intensity. The DAEFRHDSGYQVHHQK signal became much weaker after picolinamidination probably due to suppression by other enhanced peaks.

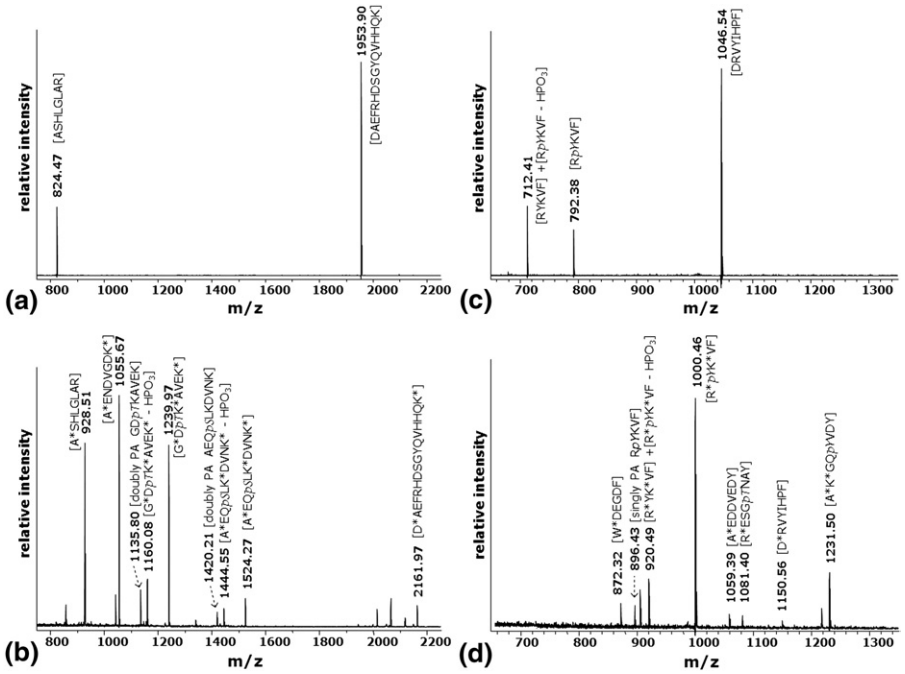


Figure 3. MALDI spectra from a mixture of five synthetic tryptic peptides (**a**), (**b**), and from seven synthetic chymotryptic peptides (**c**), (**d**), without picolinamidination (**a**), (**c**), with picolinamidination (**b**), (**d**), and a mixture of at 0.25 μM concentration each upon loading on the sample plate, (**c**) without picolinamidination, (**d**) with picolinamidination. Asterisk indicate site of picolinamidination.

Figure 3c shows MALDI spectrum from a synthetic chymotryptic peptide mixture (RESGpTNAY, AKGQpYVDY, RpYKVF, RYKVF, WDEGDF, AEDDVEDY, DRVYIHPF; 1 μM each). Of the seven peptides, only three were observed. All three had R and one was a phosphopeptide. After picolinamidination, the two phosphopeptides (RESGpTNAY and AKGQpYVDY), and the two acidic peptides (WDEGDF and AEDDVEDY), not detected before were observed (Figure 3d). The signal RpYKVF dephosphorylated seems to be added up to the signal intensity of RYKVF, before and after picolinamidination. Probably picolinamidinated RYKVF and DRVYIHPF were suppressed by picolinamidinated RpYKVF.

It appears from the results presented above that the effect of introducing positive charge by picolinamidination is most pronounced with phosphopeptides. Initially, we reported 20- to 35-fold signal enhancement using ordinary peptides [32]. Signal enhancement of phosphopeptides is an order of magnitude greater. Pashkova et al. reported that coumarin tag increases MALDI signal of ordinary peptides 2- to 10-fold, while phosphopeptide signal is increased 30- to 40-fold [4]. They attributed the signal enhancement to an increased hydrophobic interaction between coumarin and the CHCA matrix, which would be more pronounced in hydrophilic phosphopeptide. Signal enhancement by picolinamidination is about an order of magnitude greater than by coumarin tag probably due to the combined effect of the increased basicity upon picolinamidination and increased interaction with the matrix in terms of co-crystallization and energy transfer.

Sequencing Picolinamidinated Tryptic Peptide by MS/MS

Having demonstrated that acidic peptide and phosphopeptide signal could be significantly elevated so that peptides not detected at all yield a strong signal upon picolinamidination, we attempted to sequence these peptides by tandem mass spectrometry. The *m/z* 1055.7 peak from the acidic peptide, AENDVGDK, in Figure 1b was selected. Figure 4a shows TOF/TOF spectrum from the *m/z* 1055.67 peak. All of the fragment ions in the y-series (y_1–y_7) and b-series (b_1–b_7) were observed. The correct sequence was readily determined from these ions.

The y_7 peak was the strongest. Beardsley and Reilly and coworkers reported that peptide bond of the N-terminal amino acid of an amidinated peptide is preferentially dissociated in the MS/MS process, and, therefore, b_1 and y_{n-1} ions are the most abundant among fragment ions [35, 36]. In the case of a picolinamidinated tryptic peptide like AENDVGDK, where the C-terminal lysine residue is picolinamidinated, all of the y-series ions should have a picolinamidination tag at the C-terminal and thus would yield a strong signal. The b-series ions carry a picolinamidination tag at the N-terminus. Thus, both the y-series and the b-series ions are observed. This high intensity of all y-series and the b-series peaks would be very useful in tandem mass spectrometric sequencing of tryptic peptides following picolinamidination. Peptides with C-terminal arginine yield strong signal without picolinamidination. Therefore, signal enhancement of peptides with C-terminal

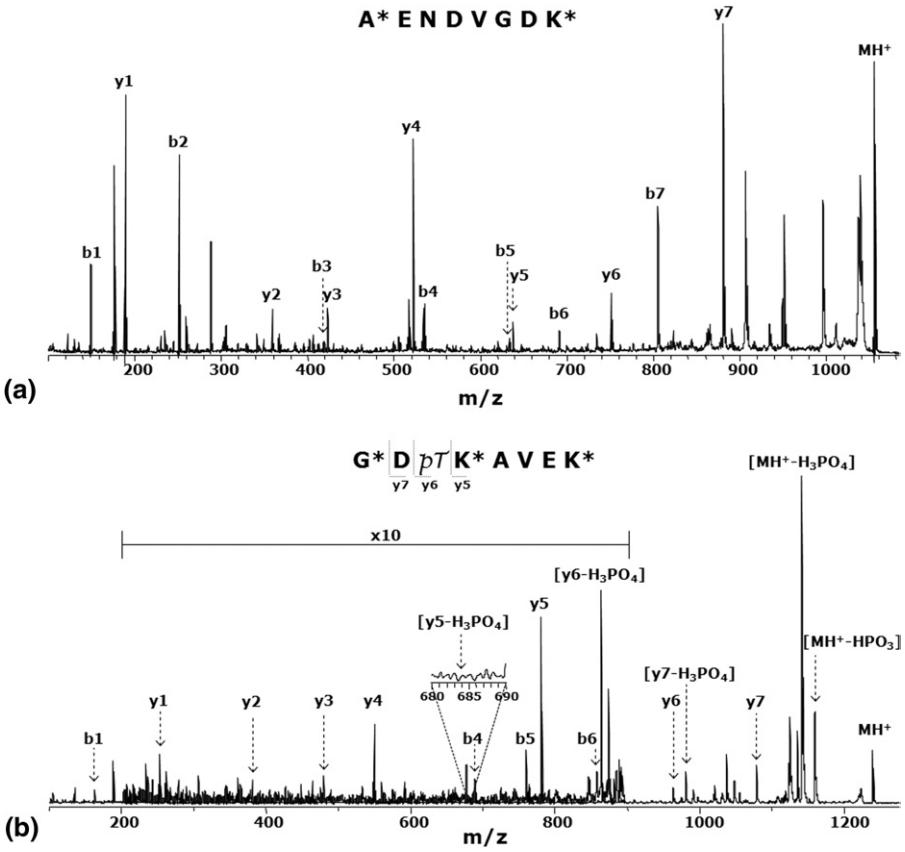


Figure 4. MALDI TOF/TOF spectrum of picolinamidinated tryptic peptide, (**a**) AENDVGDK, showing strong y-series ions, (**b**) GDpTKAVEK, showing fragments with loss of phosphoric acid from y_7 and y_6, but not from y_5 and lower y-series ions.

lysine will complement the proficiency of analyzing peptides with C-terminal arginine in peptide sequencing as well as in peptide mapping.

It would be highly desirable to be able to identify the phosphorylation site from a phosphopeptide by MS/MS experiment. TOF/TOF experiment was performed on the picolinamidinated phosphopeptide, GDpTKAVEK, in Figure 1d obtained by loading 1 pmol peptide on the plate. The MS/MS spectrum in Figure 4b shows that pairs of peaks, phosphorylated and fragments showing a loss of phosphoric acid, are obtained from the parent peptide, y_7 and y_6 ions, but not from y_5 and lower y-series ions. The result clearly indicates that the sixth residue from the C-terminus, T, is phosphorylated. Identification of the phosphorylation site by the MS/MS experiment was possible by the increased ion yield upon picolinamidination.

Sequencing Picolinamidinated Chymotryptic Peptide by MS/MS

Chymotryptic peptides do not have C-terminal arginine or lysine that could be picolinamidinated. Yet, since only the N-terminal amine group is picolinamidinated, b-series ion signal should be enhanced while y-series ion should be suppressed. So, we can easily deduce the sequence information from the MS/MS spectrum showing predominant b-series ions. Figure 5a shows MALDI TOF/TOF spectrum of picolinamidinated chymotryptic peptide with five acidic residues, AEDDVEDY; b_2 through b_7 ions have acidic residues. Yet, due to the picolinamidination tag at the N-terminus, all b-series ions were detected and the correct sequence could be deduced from these ions; y-series ions without the picolinamidination tag were less intense.

Figure 5b shows MALDI TOF/TOF spectrum of picolinamidinated phosphopeptide, RESGpTNAY; b-series ions were predominant over y-series ions, which were hardly detected. Dephosphorylated peaks were observed from b_6 and b_5, but not from b_4 and lower b-series ions. The result clearly shows that the 5th residue from the N-terminus, T, is the phosphorylation site. This data illustrate that the picolinamidination could be useful for chymotryptic peptides as well as for tryptic peptides.

Conclusions

Picolinamidination of amino groups in peptides dramatically enhances MALDI signal intensity of phosphopeptides. The signal enhancement could be utilized for sequencing and phosphorylation site analysis from

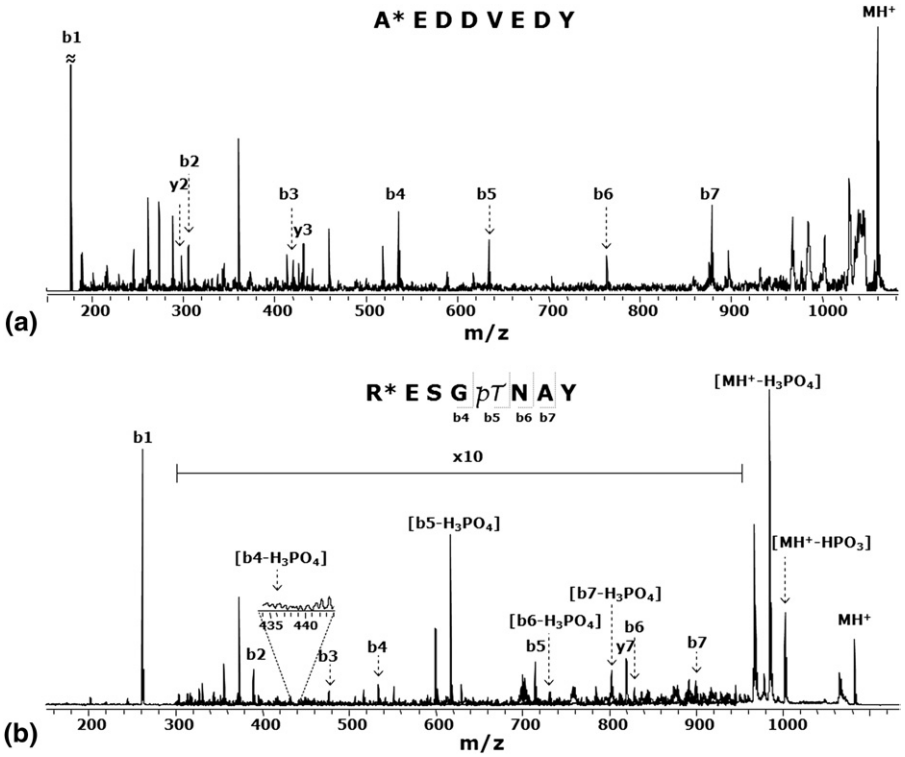


Figure 5. MALDI TOF/TOF spectrum of picolinamidinated chymotryptic peptide, (**a**) AEDDVEDY, showing predominant b-series ions over y-series ions, (**b**) RESGpTNAY, showing fragments with loss of phosphoric acid from b_6 and b_5, but not from b_4 and lower b-series ions.

either tryptic or chymotryptic phosphopeptides at limiting concentrations by MALDI TOT-TOF MS. It is hoped that picolinamidination would help analyze low concentration phosphopeptides of importance in proteomics either by itself or in combination with enrichment techniques.

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