Clinical Proteomics

ISSN 1542-6416/06/02:133–144/$30.00 (Online)

Original Article

Laser-Induced Dissociation of Phosphorylated Peptides Using Matrix Assisted Laser Desorption/Ionization Tandem Time-of-Flight Mass Spectrometry

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Abstract

Reversible phosphorylation is one of the most important posttranslational modifications of cellular proteins. Mass spectrometry is a widely used technique in the characterization of phosphorylated proteins and peptides. Similar to nonmodified peptides, sequence information for phosphopeptides digested from proteins can be obtained by tandem mass analysis using either electrospray ionization or matrix assisted laser desorption/ionization (MALDI) mass spectrometry. However, the facile loss of neutral phosphoric acid (H_3PO_4) or HPO_3 from precursor ions and fragment ions hampers the precise determination of phosphorylation site, particularly if more than one potential phosphorylation site or concensus sequence is present in a given tryptic peptide. Here, we investigated the fragmentation of phosphorylated peptides under laser-induced dissociation (LID) using a MALDI-time-of-flight mass spectrometer with a curved-field reflectron. Our data demonstrated that intact fragments bearing phosphorylated residues were produced from all tested peptides that contain at least one and up to four phosphorylation sites at serine, threonine, or tyrosine residues. In addition, the LID of phosphopeptides derivatized by N-terminal sulfonation yields simplified MS/MS spectra, suggesting the combination of these two types of spectra could provide an effective approach to the characterization of proteins modified by phosphorylation.

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Key Words: Phosphorylation; MALDI; time-of-flight mass spectrometry; curved-field reflectron; N-terminal sulfonation; fragmentation.

Introduction

Reversible phosphorylation is one of the most common and important regulatory modifications of cellular proteins ***(1)***. This modification plays a crucial role in maintaining and regulating protein structure and function in a number of biological processes and signal transduction pathways. Among 6000 yeast proteins recently reported in the genomic database of budding yeast *Saccharomyces cerevisiae*, about 250 are protein kinases that are responsible for substrate phosphorylation ***(2)***. Phosphorylation usually occurs on one of three amino acid residues: serine, threonine, or tyrosine. The majority of phosphorylation occurs on serine residue (~90%), with only a small number of phosphoproteins containing phosphothreonine (~10%) and phosphotyrosine (~1%). The identification and characterization of phosphorylated proteins is a major effort in proteomics.

Mass spectrometry has become a powerful tool for protein identification, providing rapid and reliable peptide sequencing using a number of fragmentation methods. The determination of phosphorylation sites within modified proteins, however, remains a challenge. This is primarily because the most common method used to induce fragmentation in electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) mass spectrometers, low-energy collision-induced dissociation (CID), results in significant loss of neutral phosphoric acid (H_3PO_4 98 Da) or HPO_3 (80 Da) from phosphorylated amino acid residues ***(3–7)***. The facile loss of these groups could result from either gas-phase β-elimination of the phosphate ester from phosphoserine or phosphothreonine residues, or from a two-step process involving the loss of HPO_3 followed by the loss of H_2O from phosphotyrosine residues. This fragmentation pathway is generally at the expense of other pathways that would produce sequence ions; particularly those that retain the phosphate group, so that it is not possible to fix the actual position of the phosphate on the peptide sequence. In tandem instruments which utilize low energy CID, that is: quadrupole ion traps, hybrid quadrupole/time-of-flight mass spectrometers, Fourier transform mass spectrometers, and so on, ions are initially cooled through collisions with inert gas in an RF ion guide, retaining little of the internal energy derived from the ionization process as they enter the mass analyzer. Following precursor ion selection multiple low-energy collisions increase the internal energy slowly, favoring fragmentation of the weaker bonds that leads not only to the facile loss of anionic groups (such as phosphate) and other posttranslational modifications, but also to preferential cleavages at proline, aspartic acid, and glutamic acid residues ***(8,9)***.

Electron capture dissociation was introduced recently as a fragmentation method for Fourier transform mass spectrometers ***(10–12)*** and has shown considerable promise as an activation method for elucidating phosphorylation sites ***(13–15)***. For peptides (and for proteins in so-called *top down* methods ***[16]***) electron capture dissociation produces predominant *c* and $z^{\cdot}$ (or $c^{\cdot}$ and *z*) fragment ions resulting from the cleavage of the amine backbone bond of precursor ions. Fragmentation is more distributed, rather than selective for specific residues or side groups, and, therefore, an abundance of sequence-specific fragment ions are available for the determination of both sequences and the location of posttranslational modifications. Similarly, it has also been suggested that MALDI mass spectra obtained on time-of-flight mass spectrometers produce *charge remote fragmentation*, both from *post-source decay* and CID processes ***(17)***. Similar to the high-energy CID processes described previously for sector mass spectrometers ***(18)***, fragmentation is not localized at specific sites, but well-distributed and

therefore more informative of structure. The tandem time-of-flight (TOF/TOF) mass spectrometer provides a particularly effective instrumental configuration for observing *charge remote fragmentation* of MALDI ions with better mass selection than the single analyzer instruments for which post-source decay was initially developed ***(19)***. Unlike most other tandem and hybrid instruments in which precursor ions are collisionally cooled, the internal energy resulting from the initial MALDI processes can be used to drive the fragmentation. Such *laser-induced dissociation* (LID) processes can also be complemented by the inclusion of a collision chamber to produce single, high energy collisions that also lead to *charge remote fragmentation*. The motivation for this current work was the possibility that well-distributed fragmentation could also be observed for phosphorylated peptides and could provide sequence-specific fragments that were more informative for determining phosphorylation sites.

Here, we present our investigation of the fragmentation of phosphorylated peptides by LID using a MALDI-TOF/TOF mass spectrometer with a curved-field reflectron ***(20)***. Our data demonstrate that intact fragments bearing phosphorylated residues could be produced from a series of peptides with from one to four phosphorylation sites at serine, threonine, or tyrosine residues; and that these could be used effectively to map the phosphorylation sites. In addition, we utilized a method developed recently in our laboratory ***(21)*** to form N-terminal sulfonate derivatives of phosphopeptides, which led to far simpler and more interpretable mass spectra that could be used to complement the direct fragmentation sequencing.

Experimental

Materials

All chemicals used in this study were of analytical grade. 4-Sulfophenyl isothiocyanate (SPITC), sodium bicarbonate and the peptide, RELEELNVPGEIVEpSLpSpSEESITR, were purchased from Sigma (St. Louis, MO). α-Cyano-4-hydroxycinnamic acid was from Aldrich (Milwaukee, WI). The synthetic peptides, RLEpSR and RLEpTR, were prepared by the Synthesis and Sequencing Facility at the Johns Hopkins University School of Medicine (Baltimore, MD). Peptides VSSDGHEpYIYVDPMQLPY and VSSDGHEpYIpYVDPMQLPY were gifts from Dr. Akhilesh Pandey. All other peptides were synthesized with FMOC chemistry by solid phase peptide synthesis and purified by reverse-phase high-performance liquid chromatography.

Sulfonation

Phosphopeptides were derivatized with SPITC as previously described ***(21)***. In brief, the reagent solution was prepared by dissolving SPITC (10 mg/mL) in 20 m*M* $NaHCO_3$ (pH ~ 9.0). The sulfonation reaction was carried out in a 0.6-mL Eppendorf tube by mixing 9 µL of reagent solution with 1 µL of peptide solution (approx 10–100 pmol). After incubation for 30 min at 55°C the reaction was terminated by adding 1 µL of 1% trifluoracetic acid (TFA). The sample was then loaded onto a micropipet tip (C18 OMIX, Varian, Lake Forest, CA), washed with 3 × 10 µL of 0.1% TFA and followed by eluting with 10 µL of 75% acetonitrile/0.1% TFA. The solution was evaporated to dryness by SpeedVac and then resuspended with 10 µL of ddH_2O.

Mass Spectrometry

All MS and MS/MS spectra were acquired in the positive ion mode using a Kratos Analytical (Manchester, UK) AXIMA-CFR high performance mass spectrometer equipped with a pulsed extraction source, a 337-nm pulsed nitrogen laser and a curved-field reflectron. The AXIMA is in effect a tandem configuration **(Fig. 1)** that has been used both in a LID **(Fig. 1A)** and high energy CID mode with the inclusion of a collision chamber

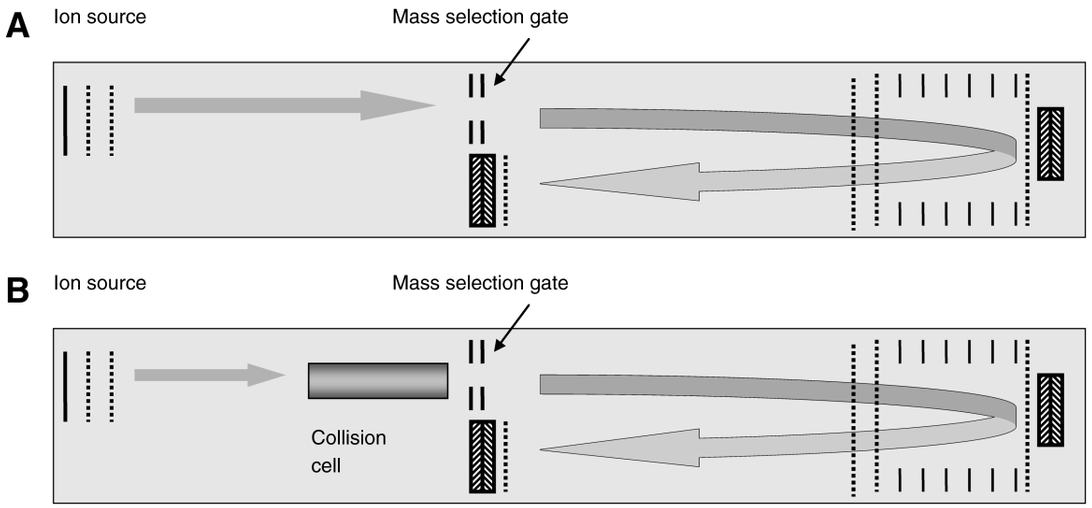

Fig. 1. Schematic of the AXIMA CFR mass spectrometer using **(A)** laser-induced dissociation or **(B)** collision-induced dissociation.

(Fig. 1B) ***(20)***. In this study, only the LID mode was used. The matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (10 μg/μL) in 50% acetonitrile containing 0.1% of TFA. A thin layer of the matrix was applied onto the sample plate first, followed by the addition of 0.5 μL of sample and 0.5 μL of matrix and was allowed to dry at room temperature.

Results and Discussion

Ten phosphopeptides were investigated and their fragmentation results are summarized in tabular form **(Table 1)**. These peptides include phosphorylation on all three target residues, serine, threonine, and tyrosine, at various positions and contain up to four phosphorylated residues within individual species. The masses of the protonated peptides range from 740.3 to 3123.6 Da. As listed in **Table 1**, all peptides tested produced nearly complete sets of *b*- and *y*-series sequence ions including these containing intact phosphorylated amino acid residues, while the loss of HPO_3 (–80 Da) and H_3PO_4 (–98 Da) from some precursors and fragments were also observed. This well-distributed fragmentation, including both sequence ions and phosphate loss, is consistent with the suggestion by Gross et al. ***(17)*** that there is considerable charge-remote fragmentation in the post-source dissociation processes that occur following laser desorption.

Figure 2 shows the fragmentation of three peptides (FQpSEEQQQTEDELQDK, RLEpTR, and VSSDGHEpYIYVDPMQLPY) containing a single phosphorylated residue. For the two peptides with phosphoserine and phosphothreonine **(Fig. 2A**,**B)** neutral loss of phosphoric acid from the molecular ion is the dominant process, whereas fragments resulting from the loss of both H_3PO_4 and HPO_3 from the precursor ion of the phosphotyrosine-containing peptide **(Fig. 2C)** were observed but not dominant. At the same time, there is extensive sequence-specific (*b*- and *y*-series) fragmentation that includes intact sequence fragments retaining the phosphate group, and sequence fragments showing the loss of 98 U (in the case of serine and threonine) or 80 mass units (in the case of tyrosine). In **Fig. 2A**, the sequence ions y_{14} and y_{15} all include the

Table 1
Product Ions in the MALDI MS/MS Spectra of Phosphorylated Peptides

Peptides	Exp. M+ H^+	Calc. M+H^+	Product ions									
			Mass	Type	Mass	Type	Mass	Type	Mass	Type	Mass	Type
SA**pS**EPSLHR	1063.5	1063.9	175.6	y_1	425.3	y_3	738.0	y_6	906.2	y_7*	983.3	MH-80
			312.2	y_2	609.1	y_5	807.9	y_7-98	965.3	MH-98		
FQ**pS**EEQQQTEDELQDK	2061.8	2062.0	390.8	y_3	977.7	y_8	1430.9	b_{11}*	1620.8	y_{13}	1916.3	y_{15}*
			503.5	y_4	1106.1	y_9	1461.6	b_{12}-98	1673.4	b_{13}*	1965.1	MH-98
			632.5	y_5	1234.3	y_{10}	1491.6	y_{12}	1689.9	y_{14}-98	1983.2	MH-80
			747.0	y_6	1315.5	b_{10}*	1560.0	b_{12}*	1787.6	y_{14}*		
			876.4	y_7	1362.3	y_{11}	1575.3	b_{13}-98	1818.5	y_{15}-98		
LPKINRSA**pS**EPSLHR	1784.9	1784.8	174.9	y_1	721.9	b_6	1063.3	y_9*	1348.5	y_{12}-98	1686.5	MH-98
			312.2	y_2	738.6	y_6	1079.0	b_{10}-98	1447.3	y_{12}*	1705.3	MH-80
			339.9	b_3	808.3	b_7	1096.5	b_{10}-80	1513.8	b_{14}-98	1767.7	b_{15}*
			425.6	y_3	880.2	b_8	1121.8	y_{10}-98	1531.5	b_{14}-80		
			452.3	b_4	906.2	y_7*	1177.0	b_{10}*	1576.0	y_{13}*		
			566.3	b_5	965.6	y_9 -98	1219.5	y_{10}*	1611.1	b_{14}*		
			609.4	y_5	976.3	y_8*	1333.5	y_{11}*	1670.1	b_{15}-98		
CLRRN**pS**DR	1099.5	1099.8	175.4	y_1	529.2	b_4	727.0	y_5*	845.8	b_7-80	1001.3	MH-98
			290.4	y_2	571.5	y_4*	729.7	b_6-80	883.7	y_6*	1082.1	b_8*
			373.3	b_3	643.4	b_5	810.6	b_6*	925.2	b_7*		
			456.7	y_3*	712.3	b_6 -98	827.7	b_7-98	984.1	b_8-98		
RLE**pS**R	740.3	740.3	157.3	b_1	269.7	b_2	468.5	b_4-98	584.5	y_4*	723.1	b_5*
			175.3	y_1	341.9	y_2 *	486.6	y_4-98	625.7	b_5-98		
			243.9	y_2-98	399.2	b_3	566.1	b_4*	642.2	MH-98		
RELEELNVPGEIVE - **pS**L**pS**p**S**p**S** EESITR	3123.6	3123.9	477.0	y_4	885.2	b_7	1479.4	b_{13}	1958.2	(b_{17}-98)*	2750.1	MH-3_ 98-80
			528.0	b_4	902.2	y_7	1609.3	b_{14}	2027.2	b_{18}-2 × 98	2830.1	MH-3 × 98
			605.8	y_5	983.4	b_8	1678.3	b_{15}-98	2044.0	y_{16}****	2847.9	MH-2 × 98-80
			657.2	b_5	1137.2	b_{10}	1790.8	b_{16}-98	2124.7	(b_{18}-98)*	2927.9	MH-2 × 98
			735.0	y_6	1266.4	b_{11}	1860.3	b_{17}-2 × 98	2140.6	y_{17}****	2945.9	MH-98-80
			771.2	b_6	1379.7	b_{12}	1888.8	b_{16}*	2732.4	MH-4 × 98	3025.8	MH-98

(Continued)

Table 1. *(Continued)*

Peptides	Exp. M+ H^+	Calc. M+H^+	Product ions									
			Mass	Type	Mass	Type	Mass	Type	Mass	Type	Mass	Type
RLE**pT**R			754.4	754.3	157.0	b_1	269.7	b_2	482.2	b_4-98	598.2	y_4*
			175.3	y_1	356.1	y_2*	500.4	y_4-98	639.1	b_5-98		
			258.3	y_2-98	399.3	b_3	580.1	b_4*	656.2	MH-98		
pYMAPYDNY	1116.4	1116.6	375.7	b_2*	462.6	b_4-80	625.7	b_5-80	741.8	y_6	1098.9	b_8*
			410.8	y_3	543	b_4*	670.8	y_5	820.9	b_6*		
			445.5	b_3*	574	y_4	705.8	b_5*	934.9	b_7*		
VSSDGHE**pY**IYVDPMQLPY-	2193.6	2194.2	583	b_6	954.7	b_8*	1231.1	b_{10}*	1542.1	b_{13}*	1916.4	b_{16}*
		712.2	b_7	987.8	b_9-80	1330.0	b_{11}*	1674.3	b_{14}*	2012.6	b_{17}*	
			747.7	y_6	1068.1	b_9*	1365.1	b_{12}-80	1802.3	b_{15}*	2095.8	MH-98
				b_8-80		b_{10}-80		b_{12}*		b_{16}-80		MH-80
			874.5		1150.9		1445.1		1835.2		2113.7	
VSSDGHE**pY**I**pY**VDPMQLPY-	2273.9	2273.7	584.0	b_6	1068.5	b_9*	1445.7	(b_{12}-80)*	1915.7	(b_{16}-80)*		
		712.1	b_7	1231.6	(b_{10}-80)*	1526.0	b_{12}**	1995.9	b_{16}**			
			747.9	y_3	1311.9	b_{10}**	1754.3	b_{14}**	2175.8	MH-98		
			955.0	b_8*	1411.0	b_{11}**	1882.1	b_{15}**	2193.7	MH-80		

* Fragments containing one phosphorylated residue.
** Fragments containing two phosphorylated residues.
**** Fragments containing four phosphorylated residues.

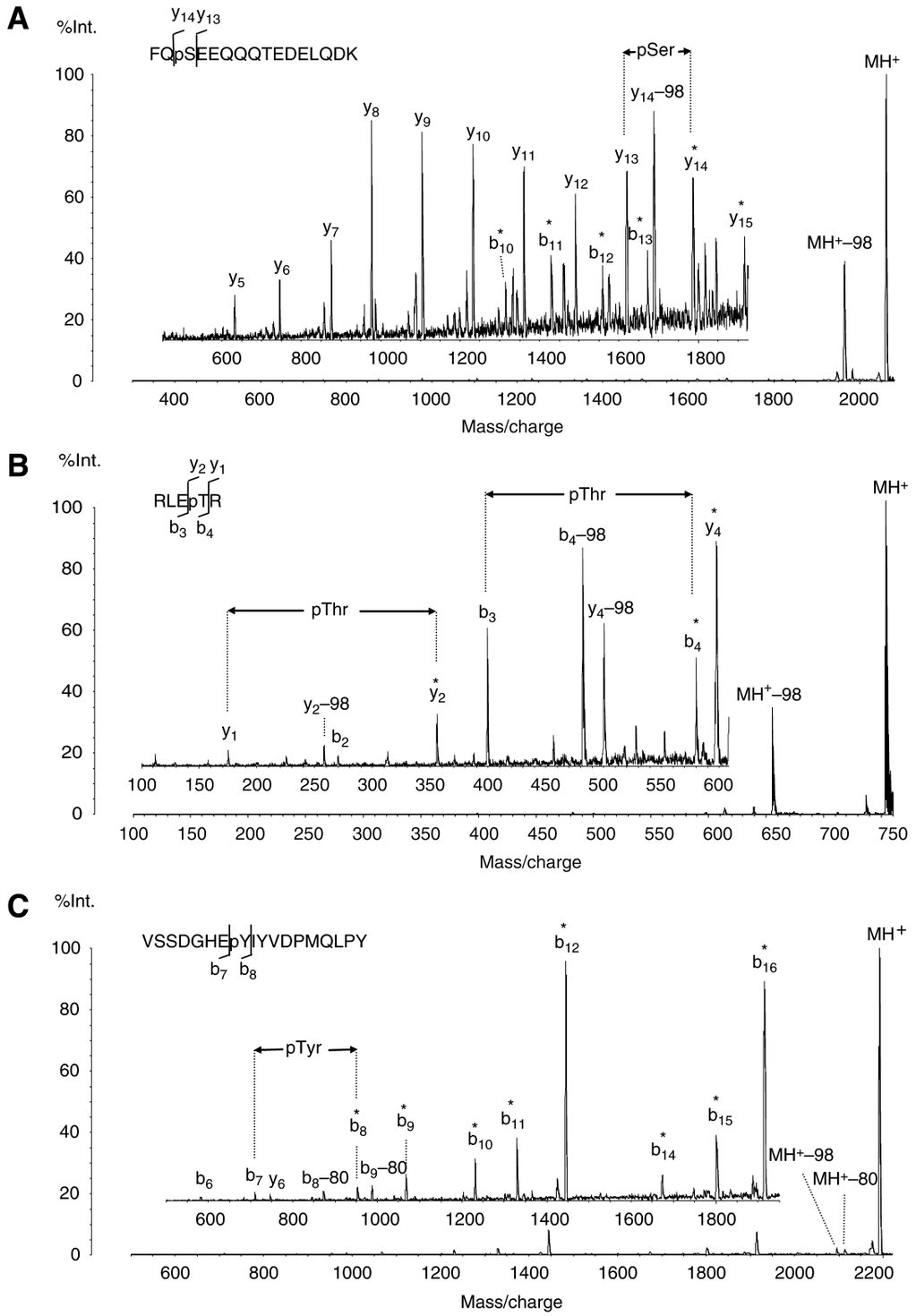

Fig. 2. MALDI MS/MS spectra of the peptides: **(A)** FQpSEEQQQTEDELQDK, **(B)** RLEpTR, and **(C)** VSSDGHEpYIYVDPMQLPY. pX represents phosphorylated serine, threonine, or tyrosine residue. Asterisks label the intact product ions containing phosphate group.

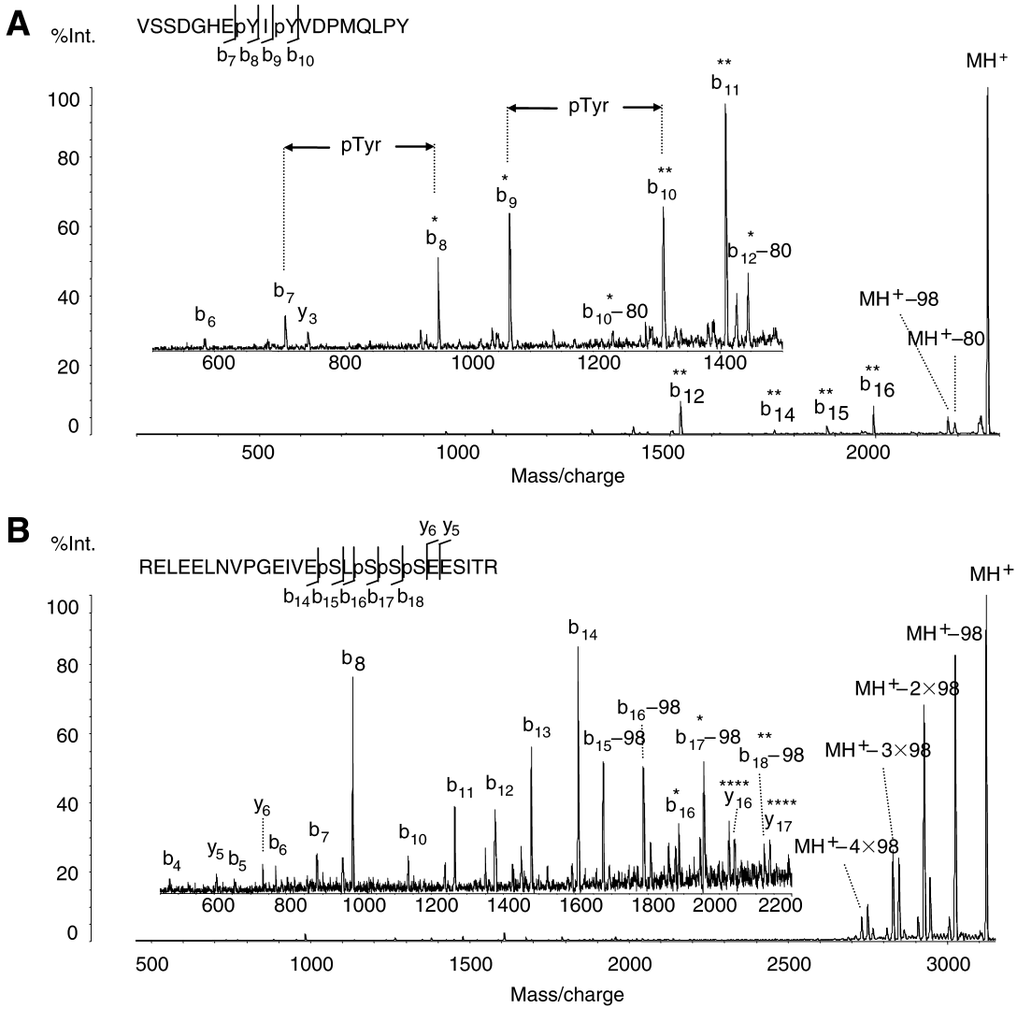

Fig. 3. MALDI MS/MS spectra of the peptides: **(A)** VSSDGHEpYIpYVDPMQLPY and **(B)** RELEELNVPGEIVEpSLpSpSpSEESITR.

phosphate group, while the ions y_5 to y_{13} do not, suggesting that the phosphate is located at the serine residue. In addition, there is also a y_{14}-98 ion; and the ions b_{10} to b_{13} include phosphate, consistent with the location of the phosphate group toward the N-terminus. In the spectrum of the peptide RLEpTR **(Fig. 2B)**, the phosphothreonine residue can be identified by comparing the masses between y_1 and y_2 ions or between b_3 and b_4 ions. The peptide VSSDGHEpYIYVDPMQLPY is relatively large but there is no difficulty in determining which one of three tyrosine residues is phosphorylated **(Fig. 2C)** by comparing the b_7 and b_8 ions, and noting that all ions from b_8 to b_{16} include phosphate.

A peptide may carry more than one phosphorylated residue **(Fig. 3)**. Where these are all phosphotyrosine residues, identification of the modification sites is less problematic because neutral losses are not dominant as we suggested in the discussion of **Fig. 2C**. Fragmentation

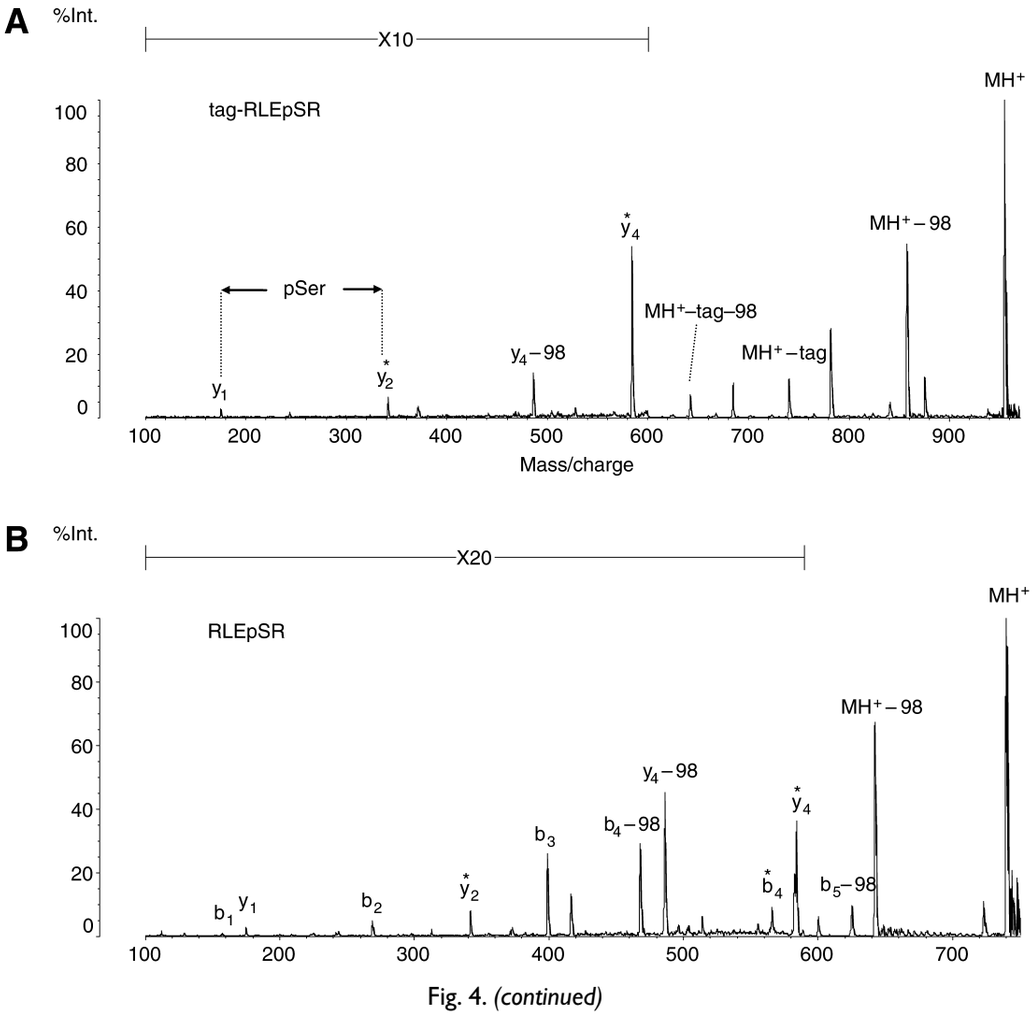

Fig. 4. *(continued)*

of the peptide VSSDGHEpYIpYVDPMQLPY **(Fig. 3A)** reveals that two internal but not the C-terminal tyrosine residues are modified by phosphorylation. This is determined by the mass differences between b_7 and b_8 and between b_9 and b_{10} ions; and the observation of several large *b* ions (b_{10} to b_{16}) containing two modified residues validates the identification. In contrast, the analysis of multiple phosphoserine-containing peptides is more challenging because the elimination of one or more phosphoryl groups from the molecular ion are dominant fragmentation channels that compete with sequence-specific fragmentation; and the irregular removal of these groups from fragments can further complicate the spectra. The peptide, RELEELNVPGEIVEpSLpSpSpSEESITR, contains five serine residues with four of them modified by phosphorylation. In addition to the parent ion, the mass spectrum **(Fig. 3B)** was dominated by several high mass fragments resulting from the consecutive release of one to four phosphoric acids from the precursor ion. At the same time, other product ions were observed in the mass range from 450 to 2200 Da with high signal/noise ratio. The detection of y_6

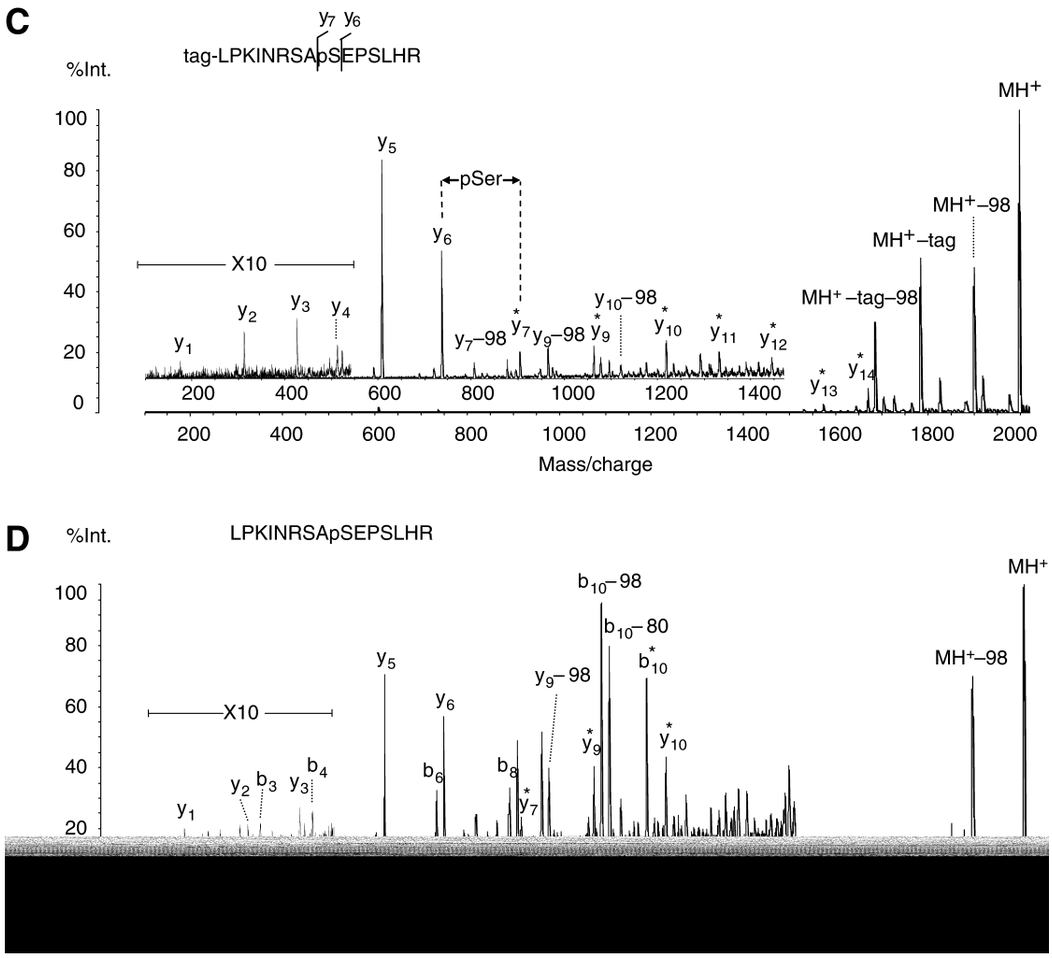

Fig. 4. MALDI MS/MS spectra of **(A)** N-terminally derivatized RLEpSR, **(B)** underivatized RLEpSR, **(C)** N-terminally derivatized LPKINRSApSEPSLHR, and **(D)** underivatized LPKINRSApSEPSLHR. Peptides were N-terminal derivatized with 4-sulfophenyl isothiocyanate as described.

and y_7 ions, containing no mass increase by phosphate group, suggests that the serine residue at the fourth C-terminal position is not modified whereas the presence of b_{16}, y_{16}, and y_{17} indicates that all other four serines were phosphorylated.

N-terminal sulfonation has been used to simplify MS/MS spectra for *de novo* peptide sequencing ***(21,22)***. Modification of the N-terminus of a peptide by a sulfonic acid group leads to the formation of *y*-series ions in the positive ion mode, as *b*-series (and other N-terminal) products are neutralized or negatively charged by the presence of the sulfonic acid group. To determine the usefulness of the sulfonation approach for phosphopeptides, we derivatized the peptide, RLEpSR, with SPITC at its N-terminus and examined the fragmentation of the peptide derivative. The MS/MS spectrum **(Fig. 4B)** of the native peptide shows that its fragmentation yielded product mixtures including *b* (b_1 to b_4) and *y* (y_1, y_2, and y_4)

ions, and some neutral loss ions (b_4-98, y_2-98, and y_4-98). In contrast, the fragmentation of the derivatized peptide **(Fig. 4A)** generated exclusively *y*-series ions in the range from 100 to 600 Da, and no N-terminal ions, indicating that N-terminal sulfonation is also capable of directing the fragmentation of phosphorylated peptides. In addition to the loss of phosphate from the molecular ion, we also observe the additional characteristic ions corresponding to MH^+-tag and MH^+-tag-98. These characteristic ions are also observed for the spectrum of the N-terminal sulfonated derivative of the peptide LPKINRSApSEPSLHR **(Fig. 4C)**. In contrast to the mass spectrum of the underivatized phosphopeptide **(Fig. 4D)**, which shows both *b*- and *y*-series ions, the simple mass spectrum of the derivatized peptide provides relatively easy location of the phosphorylation site.

Conclusions

We investigated the fragmentation behaviors of phosphorylated peptides using MALDI-TOF mass spectrometry. Our results demonstrated that LID allows the formation of intact fragments containing phosphorylated residues and provides important information for localizing phosphorylation sites. This overcomes a major problem in analyzing phosphopeptides that is the neutral loss of H_3PO_4 and HPO_3 from tandem mass spectra products. Combining with N-terminal sulfonation method, MALDI tandem TOF mass spectrometer provides a promising tool in analyzing protein and peptide phosphorylation.

Acknowledgments

The authors wish to thank Dr. Akhilesh Pandey at the Johns Hopkins University for providing two commercial peptides. This work was supported by a contract BAAHL-02-04 from the National Heart Lung and Blood Institute (Jennifer VanEyk, PI). Mass spectral analyses were carried out at the Middle Atlantic Mass Spectrometry Laboratory.

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