Skimmer CID-ion trap mass spectrometry of phosphotyrosine-containing peptides

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Abstract. Proteomic analysis is becoming a popular field in science. Analysis of protein modifications is useful in deciphering cellular functions and errors in pathways that can result in disease. There has been increased interest in the phosphotyrosine proteome. Due to the difficulty in finding the location of the tyrosine phosphorylation site in the tyrosine phosphorylated peptide or even to verify that the parent protein is a phosphotyrosyl-protein, new analytical tools are being developed. The phosphotyrosine immonium ion can be produced via skimmer CID for detection via ion trap mass spectrometry and is a useful marker for the indication of the presence of a phosphotyrosine residue. Skimmer CID analysis can also be used to differentiate phosphotyrosine-containing peptides from other phosphorylated peptides. In this study, phosphotyrosine-containing peptides were analyzed by skimmer CID in an ion trap mass spectrometer. The factors affecting the signal abundance of the phosphotyrosine immonium ion were investigated.

1. Introduction

The field of proteomics involves the study of proteins expressed by a genome at a certain time and set of conditions [1]. Studying proteins in conjunction with the genome provides vital information on how the protein is modified after it has been transcribed. In spite of this, studying proteins is more complex than studying genes. Proteins cannot be amplified by PCR for DNA amplification and protein concentrations are in flux. Proteins can exist with modifications in low stoichiometric amounts. An example of a post-translational modification that occurs on proteins is reversible phosphorylation.

Phosphorylation is an important modification involved in cellular functions including cell growth, metabolism, and transcription [2]. Phosphorylations occur primarily on serine, threonine, and tyrosine residues in eukaryotic cells. It is believed 5% of the vertebrate genome encodes for kinases and phosphatases, enzymes that aid in the addition and removal, respectively, of phosphate to these amino acid residues [3]. The most common site of phosphorylation is on serine (pS) residues (~90%) followed by threonine (pT) (~10%). Although phosphorylation occurs less frequently on tyrosine residues (pY) (~0.05%), reversible phosphorylation on tyrosine is important in signal transduction cascades. Abnormalities involving the phosphorylation of tyrosine amino acids can give rise to disease [4]. Studying the site location of tyrosine phosphorylation is beneficial to the advancement of health care.

A few different methods have been utilized in the analysis of protein phosphorylation. In one method phosphorylated proteins are metabolically labeled with [³²P] phosphate, enzymatically digested, and

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separated by HPLC. This experiment provides evidence or denial of the existence of a phosphorylated residue, but no sequence information is obtained [5,6]. Additional experiments are required to provide the primary sequence of the peptides, such as Edman degradation or mass spectrometry.

Mass spectrometric methods are desirable for characterization of phosphoryl-proteins due to high mass accuracy, sensitivity, the lack of required radioactive labeling, and the capacity to locate the phosphorylation site through tandem mass spectrometry experiments. One mass spectrometric technique used for the analysis of phosphopeptides entails detecting the neutral loss of H_3PO_4 (−98 Da) or HPO_3 (−80 Da) from serine and threonine phosphorylated peptides via MS/MS in a triple quadrupole [7,8], MALDI-time of flight (tof)-PSD [9,10], MALDI-IT [11] or quadrupole tof (q-tof) mass spectrometry [12]. Precursor scanning for specific marker ions (i.e., m/z 63 (PO_2^-) and m/z 79 (PO_3^-)) in the negative ion mode can be performed using a triple quadrupole mass spectrometer [13–15]. The drawback in this experiment is the difficulty of negative ion mode sequencing experiments. As an alternative, skimmer CID can be performed in a triple quadrupole mass spectrometer by increasing the voltages between the octopole and the skimmer lens [7,16–18] producing higher energy collisions and subsequent ion fragmentation. Detection of low mass ions via skimmer CID is useful in ion trap mass spectrometry since low mass ions are not trapped in in-trap CID experiments [19].

Precursor ion scanning for the detection of the phosphotyrosine immonium ion has been reported [20,21]. The authors concluded the quadrupole-tof (q-tof) mass spectrometer was superior to a triple quadrupole mass spectrometer due to enhanced resolution. The resolution of the q-tof mass spectrometer provides definitive assignment of m/z 216.043 as the phosphotyrosine immonium ion ($\text{Im}(\text{pY})$), whereas the triple quadrupole mass spectrometer provides only unit resolution. An additional benefit of precursor ion scanning experiments is that they are executed in the positive ion mode to allow for peptide sequencing in the same experiment without having to acidify the sample.

Precursor ion scanning for the phosphotyrosine immonium ion via q-tof-MS is an attractive method, but this method may not be available to all labs. Triple quadrupole and ion trap mass spectrometers suffer from limited resolution and therefore the inability to provide exact mass of the phosphotyrosine immonium ion. Many commercially available ion traps only trap a limited m/z range of ions when performing in-trap CID experiments. If product ions have q_z values outside the stability limit, they are ejected from the trap. This property of ion trap mass spectrometers is the reason the phosphotyrosine immonium ion may not be detected during in-trap CID experiments when the isolated peptide is greater than 760 mass units [22]. Skimmer CID can be applied to peptide ions (before in-trap CID experiments) to scan for the phosphotyrosine immonium ion. Multiple stage MS capabilities of the ion trap allows for the verification of the 216 ion by in-trap CID as the phosphotyrosine immonium ion. The skimmer CID voltage can be quickly adjusted to allow for increased ion beam transmission and detection of singly or doubly charged parent ions. This ion can then be subjected to in-trap CID to provide sequence information on the peptide. In this research, skimmer CID is used to study phosphotyrosine containing peptides with an ion trap mass spectrometer.

2. Experimental

2.1. Chemicals and compounds

Methanol (MeOH), acetonitrile (ACN), ammonium acetate (NH_4OAc), and isopropanol were purchased from Mallinckrodt Baker, Inc. (Paris, KY). Formic acid (FA) (98% min.) was obtained from EM Science (Gibbstown, NJ). Water (18 $\text{M}\Omega$) was acquired from a Nanopure Infinity Barnstead water

purifier (Dubuque, IA). Trypsin (TPCK) and TFA were obtained from Pierce (Rockford, IL). Glacial acetic acid (99.99+ %) (AcOH) was purchased from Sigma Co. (Milwaukee, WI). Custom peptides LMPYVR, LMPSVR, LMPTVR, and DENpYpYK (*gallus gallus* Syk 517–522) were synthesized by New England Peptide, Inc. (Fitchburg, MA). Peptides Ac-DpYVPML-NH₂ and Ac-IpYGEF-NH₂ were purchased from Bachem (King of Prussia, PA). Src-2 (Ac-pYpYpYIE) was purchased from AnaSpec, Inc. (San Jose, CA).

2.2. Mass spectrometry

Experiments were performed using a Bruker Esquire~LC ESI-QIT (Bruker Daltonics, Bremen, Germany). The standard Bruker ESI needle was replaced with a stainless steel (SS) tube (204 μm OD $\times$ 102 μm ID) (Small Parts, Inc Dallas, TX). The ends were bluntly cut at the Department of Physics Machine Shop (Purdue University, West Lafayette, IN). To increase the sensitivity, the stainless steel needle was tapered in an acid bath connected to a 9 V battery [23]. The tapered SS needle was glued to the ferrule inside the nebulizer needle assembly with epoxy to fix the ESI needle position. Source conditions were as follows: the capillary was set to -4600 V, the end plate voltage was set to -3100 V, the nebulizer gas (N₂) was 17 psi, and the drying gas (N₂) flow rate was 5 l/min at 325°C. For direct infusion experiments, a syringe pump (Cole-Palmer Instrument Co. 74900 Series) was coupled to the needle using PEEK tubing and a SS reducing union. The syringe pump flow rate was 60 $\mu\text{l/hr}$. Peptides were dissolved in 50 : 50 MeOH : H₂O with 0.1% FA. Peptide concentrations were: LMPYVR, LMPSVR, LMPTVR, and DENpYpYK (Syk 517–522), 10 pmol/ μl ; Ac-DpYVPML-NH₂, 11.6 pmol/ μl ; Ac-IpYGEF-NH₂ 13.3 pmol/ μl ; Src-2 (Ac-pYpYpYIG-OH) 9.7 pmol/ μl . Capillary exit offset and skimmer 1 voltages were varied to produce skimmer CID.

2.3. Nanoflow capillary LC-MS

For LC-MS experiments, an HP 1100 Binary HPLC pump (Hewlett Packard) was coupled to the ESI-QIT mass spectrometer. Mobile phases were A: 0.08% formic acid in H₂O, B: 0.068% FA in methanol. A linear gradient of 5% B–70% B was applied over 120 minutes followed by 70% B–85% B for 15 minutes. A 75 μm I.D. $\times$ 280 μm O.D. $\times$ 15 cm (length) reversed phase PepMap ¹⁸C capillary column (3 μm particles, 100 Å pores) was purchased from LC Packings, Inc. (San Francisco, CA). The column was connected to a short piece of fused silica capillary via a small Teflon tube. The fused silica connector capillary was coupled to the ESI needle through a SS reducing union (150 μm bore). Flow from the HPLC outlet was split via a stainless steel T-junction using a piece of fused silica capillary as a flow restrictor capillary to deliver a final flow rate of 250 nl/min (measured from the ESI needle). Samples were injected using a 5 μl PEEK loop from Alltech (Deerfield, IL). The data were acquired with Bruker Daltonics DataAnalysis software, version 3.0.

3. Results

The ion at m/z 216 was first observed in the analysis of a synthetic phosphopeptide with the sequence LMPYVR. This peptide fragmented under the ESI-QIT conditions set up for nanoflow capillary LC-MS experiments. Upon further investigation, it was apparent that peptide fragmentation was occurring in the region between the capillary exit and the first skimmer because the voltages were too high. This turned out to be useful because this ion (m/z 216) is not typically observed by in-trap CID of higher molecular weight peptides because it is not efficiently trapped.

Fig. 1. Direct infusion ESI limit of detection study of peptide Ac-IpYEFG-NH₂. Sample was dissolved in 50 : 50 MeOH : H₂O with 0.1% FA. The flow rate from the syringe pump was 1 μ l/min. A: The blunt tipped SS ESI needle provided a detection limit of 65 fmol/ μ l. B: The detection limit with the tapered SS ESI needle was 800 amol/ μ l.

3.1. Low level phosphopeptide analysis by direct infusion ESI-MS

Endogenous levels of phosphotyrosine-containing proteins are often much lower than phosphoserine and phosphothreonine-containing peptides. Obtaining sufficiently large concentrations of protein from biological samples can be tedious or even impractical. Because of this, sensitivity becomes a major concern. The limit of detection (LOD) for phosphotyrosine-containing peptides using a blunt ESI needle was determined to be 65 fmol/ μ l as shown in Fig. 1A. This would be too high most for biological sample analyses. A tapered ESI needle improves sensitivity for several reasons. As solvent flows from the needle it wets the entire surface of the needle, the tapered needle has a smaller surface area compared to the blunt tipped ESI needle. A tapered needle provides a more focused spray and smaller Taylor cone [24]. This results in the formation of smaller droplets and an increase in sensitivity. Using a tapered ESI needle, the LOD for the same tyrosine phosphorylated peptide was reduced to 800 amol/ μ l as shown in Fig. 1B. Lowering the detection limit of phosphotyrosine-containing peptides is necessary in proteomic analysis of biological samples.

3.2. Skimmer CID of Src-2 (Ac-pYpYpYIE) and verification of m/z 216 as the phosphotyrosine immonium ion

Figure 2A shows the skimmer CID mass spectrum for Src-2 (Ac-pYpYpYIE). The peptide was ionized by a potassium ion, a sodium ion, as well as a proton as observed by m/z 's 1070.1, 1054.2, and 1032.2, respectively. Several b and y fragment ions were observed as well. The base peak is m/z 216, which corresponds to the phosphotyrosine immonium ion. This ion verifies that the peptide contains a phosphotyrosine residue. Of course, many ions can have a mass to charge ratio of 216 mass units, so the presence of m/z 216 does not prove the existence of a phosphotyrosine residue. As a result, another experiment is required to verify the identity of m/z 216 as the phosphotyrosine immonium ion. This is accomplished by isolation and fragmentation of m/z 216 by in-trap CID.

Figure 2B shows the in-trap CID mass spectrum of m/z 216 which confirms the ion to be the phosphotyrosine immonium ion. The ions observed include the loss of a water molecule at m/z 198, the neutral loss of HPO₃ at m/z 136, and the neutral loss of phosphoric acid at m/z 118. The tropylium ion is observed at m/z 91.

3.3. Skimmer CID vs in-trap CID on the detection of the phosphotyrosine immonium ion

Several peptides containing phosphotyrosine residues were analyzed via skimmer CID and in-trap CID analysis for the detection of the phosphotyrosine immonium ion. The results are shown in Table 1.

Fig. 2. A: Skimmer CID mass spectrum from direct infusion of Ac-pYpYpYIE. B: In-trap CID mass spectrum of m/z 216, the phosphotyrosine immonium ion produced by skimmer CID.

Table 1
Detection of m/z 216 by skimmer CID and in-trap CID

Peptide	Skimmer CID	In-trap +2	In-trap +1
DENpYpYK	X	ND	ND
LMpYVK	X	ND	X
Ac-IpYGEF-NH ₂	X	ND	ND
Ac-DpYVPML-NH ₂	X	ND	ND
Ac-pYpYpYIE	X	ND	ND

Fig. 3. A: Effect of increasing skimmer CID voltage on the signal intensity of m/z 216 from the direct infusion ESI analysis of Ac-DpYML-NH₂. B: Effect of increasing concentration of peptides Ac-DpYML-NH₂ and Ac-IpYGEF-NH₂ on signal intensity of m/z 216. Samples were analyzed by LC-MS in the following concentrations each: 0.25 μg , 0.025 μg , 2.5 ng, and 0.25 ng. Samples were dissolved in water prior to injection.

All five of the phosphotyrosine-containing peptides produced a detectable phosphotyrosine immonium ion when fragmented by skimmer CID. The same five peptides (shown in Table 1) were also analyzed without skimmer CID. Singly charged peptide ions were isolated and subjected to in-trap CID. The only phosphotyrosine immonium ion observed in this experiment was for the peptide LMPYVR. When doubly charged ions were subjected to isolation and in-trap CID, no phosphotyrosine immonium ion was observed for any of the five peptides. Clearly, skimmer CID is the best fragmentation method for the detection of the phosphotyrosine immonium ion in ion trap mass spectrometry experiments.

3.4. Factors affecting signal intensity of m/z 216

Figure 3A shows the effect of varying the skimmer CID voltages for the peptide Ac-DpYVPML-NH₂. The phosphotyrosine immonium ion was observed starting when the skimmer CID voltage was set at 45 V. As can be seen, the signal intensity increased with increasing skimmer CID voltage. The maximum signal intensity of the m/z 216 phosphotyrosine immonium ion was observed at 156.6 V. The voltage for maximum signal intensity of m/z was dependent upon location of the phosphotyrosine residue within the peptide sequence. When the voltage becomes too high, the signal decreases dramatically because the ion beam is no longer focused. In addition, excess energy can cause the phosphotyrosine immonium ion to further fragment.

Figure 3B shows the signal response measured for the phosphotyrosine immonium ion with respect to increasing concentration of peptide. The peptides were injected on the reversed-phase capillary column as a mixture so that measurements for both peptides could be taken from one experiment. The peptides were resolved by LC-MS [19]. Signal intensity of m/z 216 increased for both Ac-DpYVPML-NH₂ and Ac-IpYGEF-NH₂ with increasing peptide concentration at a constant skimmer CID voltage at 95 V.

The standard phosphotyrosine-containing peptides were analyzed to determine the skimmer CID voltage required for the phosphotyrosine immonium ion to produce a relative signal abundance of at least 60%. Signal abundance of 60% was chosen because it allows the phosphotyrosine immonium ion to be clearly observed. The results of this analysis are displayed in Table 2. Peptides containing more than one phosphotyrosine residue (DENpYpYK and Ac-pYpYpYIE) produced the Im(pY) ion with lower skimmer CID voltages while the peptide with the most internal pY (LMPYVR) required the highest skimmer CID voltage to produce a relative signal abundance of the phosphotyrosine immonium ion at greater than 60%. The signal intensity of m/z 216 increased with both increasing skimmer CID voltage

Table 2
Effect of skimmer CID voltage on the intensity of m/z 216

Peptide sequence	Skimmer CID voltage (V)	Signal intensity m/z 216
LMpYVR	90.9	3.0×10^5
Ac-IpYGEF-NH ₂	73.6	5.0×10^5
Ac-DpYVPML-NH ₂	76.6	2.5×10^5
DENpYpYK	63.6	8.0×10^5
Ac-pYpYpYIE	43.6	0.5×10^5

Fig. 4. Skimmer CID mass spectrum of 100 fmol/ μ l of Ac-IpYGEF-NH₂.

as well as with increasing concentration of peptide. In addition, the location of the pY residue within the peptide sequence affected the signal intensity of m/z 216.

3.5. Limit of detection of m/z 216

The peptide with the sequence Ac-IpYGEF-NH₂, was analyzed by direct infusion ESI with a skimmer CID voltage of 73.6 V. The signal-to-noise ratio of m/z 216 observed from 100 fmol/ μ l solution of this peptide was greater than 20, as shown in Fig. 4. The LOD for m/z 216 for this peptide was 11 fmol/ μ l.

Table 3 shows the signal to noise ratio and signal intensity for the phosphotyrosine immonium ion derived from direct infusion skimmer CID analysis of the synthetic peptide LMpYVR at a skimmer CID of 90 V. The limit of detection of the phosphotyrosine immonium ion for a peptide with an internal phosphotyrosine residue was typically higher than for phosphotyrosine residues located toward the termini of the peptide. This observation was also noted by Salek et al. [25]. To achieve a signal-to-noise ratio of 20 for LMpYVR, a concentration in the pmol/ μ l range was required. This indicates higher skimmer CID

Table 3
LOD of m/z 216 from LMpYVR

Concentration	Signal intensity 216 m/z	Signal/noise
10 pmol/ μ l	5×10^5	>20
1 pmol/ μ l	8.5×10^4	>20
500 fmol/ μ l	4.6×10^4	>20
250 fmol/ μ l	2.4×10^4	4.8
125 fmol/ μ l	1.5×10^4	3
100 fmol/ μ l	ND	ND

voltages and increased concentrations may be required for certain phosphotyrosine-containing peptides to detect the Im(pY) ion at 60% abundance.

3.6. Specificity of m/z 216

Of the three main phosphorylation sites, phosphorylation on tyrosine is typically the most difficult to detect from peptide mixtures [26,27]. One way to differentiate a tyrosine phosphorylated peptide from a serine or threonine phosphorylated peptide is to perform skimmer CID. Immonium ions for phosphoserine and phosphothreonine are labile and not often observed and therefore are not good marker ions [20]. The phosphotyrosine immonium ion at m/z 216 is specific to the phosphotyrosine-containing peptide. Figure 5A shows the skimmer CID mass spectrum of a peptide with the sequence LMpYVR, protonated peptide, m/z 761. The immonium ion for phosphotyrosine is observed as the base peak. The entire y-ion series for the peptide was observed along with the b-ion series with the exception of b_1 . The expected neutral loss of HPO_3 produced an ion at m/z 681.5. Neutral ammonium ion losses were observed from both y_3 and y_4 . Figure 5B displays the skimmer CID tandem mass spectrum of a peptide with the sequence LMpSVR. The base peak in this spectrum is the protonated intact peptide, m/z 685. The ion at m/z 587.4 corresponds to the loss of phosphoric acid from the peptide. The sequential loss of leucine and methionine from the $(M+H-H_3PO_4)^+$ ion was observed at m/z 474.3 and m/z 343.3. Y_2 and y_1 ions were observed at m/z 274.2 and m/z 175.2, respectively. No ion at m/z 216 was observed because this peptide contains a serine phosphorylated residue, not a tyrosine phosphorylated residue. In Fig. 5C, the skimmer CID tandem mass spectrum for the peptide LMpTVR is shown, protonated peptide m/z 699. The base peak of this mass spectrum is m/z 601.4 which corresponds to the loss of H_3PO_4 . No y or b ions were observed for this peptide. The ion corresponding to the phosphotyrosine immonium ion at m/z 216 was also not observed because this peptide contains a threonine phosphorylated residue. As shown, the phosphotyrosine immonium ion is specific to tyrosine phosphorylated peptides.

4. Conclusion

Skimmer CID is a method capable of differentiating phosphotyrosine-containing peptides from phosphoserine, phosphothreonine, and non-phosphorylated peptides. Skimmer CID is useful in the analysis of Immobilized Metal Affinity Chromatography (IMAC) selected peptides, LC-MS of mixtures or direction infusion ESI experiments. In the analysis of phosphotyrosine-containing peptides via ion trap mass spectrometry the phosphotyrosine immonium ion is not always detected. In skimmer CID, the

Fig. 5. Skimmer CID mass spectrum of A: LMpYVR, B: LMpSVR, and C: LMpTVR. The phosphotyrosine immonium ion is observed in the skimmer CID analysis of LMpYVR.

peptides ions are fragmented before reaching the trap which allows lower mass ions to be detected, relative to in-trap CID. The presence of a phosphotyrosine residue in peptides can be confirmed by in-trap CID analysis of m/z 216. Skimmer CID of phosphoserine and phosphothreonine do not produce the phosphotyrosine immonium ion which allows the differentiation of phosphotyrosine-containing peptides from other phosphorylated peptides.

Fig. 5. (Continued.)

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