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# Direct MALDI-MS/MS of Phosphopeptides Affinity-Bound to Immobilized Metal Ion Affinity Chromatography Beads

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**Immobilized metal ion affinity chromatography (IMAC) is a useful method to selectively isolate and enrich phosphopeptides from a peptide mixture. Mass spectrometry is a very suitable method for exact molecular weight determination of IMAC-isolated phosphopeptides, due to its inherent high sensitivity. Even exact molecular weight determination, however, is not sufficient for identification of the phosphorylation site if more than one potential phosphorylation site is present on a peptide. The previous method of choice for sequencing the affinity-bound peptides was electrospray tandem mass spectrometry (ESI-MS/MS). This method required elution and salt removal prior to MS analysis of the peptides, which can lead to sample loss. Using a matrix-assisted laser desorption/ionization (MALDI) source coupled to an orthogonal injection quadrupole time-of-flight (QqTOF) mass spectrometer with true MS/MS capabilities, direct sequencing of IMAC-enriched peptides has been performed on IMAC beads applied directly to the MALDI target. The utility of this new method has been demonstrated on a protein with unknown phosphorylation sites, where direct MALDI-MS/MS of the tryptic peptides bound to the IMAC beads resulted in the identification of two novel phosphopeptides. Using this technique, the phosphorylation site determination is unambiguous, even with a peptide containing four potentially phosphorylated residues. Direct analysis of phosphorylated peptides on IMAC beads does not adversely affect the high-mass accuracy of an orthogonal injection QqTOF mass spectrometer, making it a suitable technique for phosphoproteomics.**

Reversible phosphorylation/dephosphorylation of proteins is one of the most common posttranslational modifications and plays a key role in many signal transduction, regulatory, and metabolic pathways.[1] Phosphorylation also has other functions, including storage of phosphate in proteins and binding of metal ions, and may also serve to maintain the structure of an enzyme without regulating its activity.[2] To understand more fully the molecular basis of these processes, it is necessary to identify the specific sites of phosphorylation.

Recently, several mass spectrometric techniques capable of providing sequence information have emerged that offer advantages over earlier protocols (radiolabeling proteins with $^{32}P$ or $^{33}P$, followed by separation of the radiolabeled peptide by high-performance liquid chromatography) due to their inherent sensitivity and speed compared to Edman sequencing. Moreover, protein radiolabeling is not required. Both matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization mass spectrometry (ESI-MS) have been successfully used for mass spectrometric identification of the exact site of phosphorylation in peptides.[3] The mass spectrometric analysis of phosphopeptides, however, is hampered by the electronegativity of the phosphoryl group, resulting in low ionization efficiency, which makes the analysis of multiphosphorylated peptides especially challenging. Another difficulty is the analysis of phosphopeptides in the presence of a large number of nonphosphorylated peptides, which often suppress ionization of phosphopeptides.[4,5] This suppression effect is particularly serious if the stoichiometry of phosphorylation is low. Methods for specific detection of phosphorylation and methods reducing suppression effects are, therefore, very valuable.

An attractive method is the removal of interfering components by selective enrichment of phosphopeptides using affinity techniques such as immobilized metal ion affinity chromatography (IMAC)[6,7] followed by sample elution. NanoESI-MS/MS or LC−MS/MS can then be utilized to determine the exact location of the phosphoryl group. High concentrations of phosphate salts, however, are typically necessary for elution of the phosphopeptides from the IMAC media. Removal of phosphate salts requires an additional purification step prior to ESI-MS and further increases the possibility of sample loss. Direct enzymatic sequencing of

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phosphopeptides bound to IMAC media has been reported using MALDI,[8] but some enzymes did not work well in the presence of IMAC beads.[8,9]

Recently, MALDI coupled to orthogonal quadrupole-TOF instruments has become available, combining the speed and robustness of MALDI with the sequencing capability of MS/MS. In this paper, we report the combination of IMAC sample enrichment with direct MALDI-MS/MS by analyzing IMAC beads placed directly on the MALDI target. This allows sequencing of the enriched phosphopeptides, without the necessity for prior elution of the peptides from the beads and the concomitant risk of sample loss.

## EXPERIMENTAL SECTION

**Materials.** The phosphorylated form of the kinase domain of insulin receptor 4 peptide (KDIR4) (Thr-Arg-Asp-Ile-pTyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys) was purchased from Anaspec (San Jose, CA) and required no further purification. A stock solution was prepared at a concentration of 1 $\mu$g/$\mu$L with HPLC grade water as the solvent.

The His-tagged histone mRNA stem loop binding protein from *Drosophila melanogaster* (dSLBP)[10] was expressed in a baculovirus expression system, purified over a Ni−nitrilotriacetic acid (NTA) resin column (Qiagen, Chatsworth, CA), and eluted in 20 mM Tris-HCl, pH 8.5, 100 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol. The final concentration was 1 $\mu$g/$\mu$L. A 20-$\mu$L aliquot of the protein was digested with porcine trypsin (Promega Corp., Madison, WI) at an enzyme-to-substrate ratio of 1:50 for 4 h at 37 °C. A 10-$\mu$L aliquot of the digest was purified over a Poros R2 resin (Applied Biosystems), microtip[11] and eluted with 5 $\mu$L of 20% acetonitrile/0.2% formic acid. Compact reaction columns (CRCs) and 35-$\mu$m frits were purchased from USB Corp. (Cleveland, OH).

**Immobilized Metal Ion Affinity Chromatography.** The NTA-agarose (Sigma Chemical Co., St. Louis, MO) was used as the resin for all IMAC experiments. Affinity binding was carried out either in CRCs or, when sample was limited, in microtips made from Eppendorf Geloader tips.[11] A typical column volume for a standard was 10 $\mu$L, while biological samples had a column volume of 5 $\mu$L. The IMAC method used was based on that of Posewitz and Tempst.[12] Briefly, each column was loaded with 6× column volumes of 50 mM $Fe(NO_3)_3$ (Sigma Chemical Co.) in 12 mM HCl and then washed with 3× column volumes of $H_2O$ and 3× column volumes of 0.1% acetic acid. Peptides were diluted 1:1 v/v with 100 mM acetic acid prior to incubation.[8] A 10-$\mu$L aliquot of standard peptide was loaded on the IMAC CRC; a 2.5-$\mu$L aliquot of dSLBP (~80 pmol) was passed through the IMAC minicolumn. The peptides were then incubated with the beads for 30 min to 2 h, in an Eppendorf Thermomixer at 400 rpm for CRCs or simply passed through the minicolumn five times, for biological samples. After incubation, the beads were washed with 3× column volumes of 0.1% acetic acid, 3× column volumes of 0.1% acetic acid/30% acetonitrile, and then 3× column volumes of 0.1% acetic acid. The beads were never allowed to dry out.

**Mass Spectrometry.** MALDI/TOF-MS was performed on a Bruker Instruments Co. (Billerica, MA) Reflex III, with pulsed ion extraction. MALDI-MS/MS was performed on an Applied Biosystems Division, Perkin-Elmer Corp. (Foster City, CA) API QSTAR-Pulsar (QSTAR), with argon used as the collision gas.

For experiments on the Reflex III, recrystallized α-cyano-4-hydroxycinnamic acid (HCCA; Aldrich, Milwaukee, WI) was used as the matrix. The solvent for HCCA was 45:45:10 ethanol/water/formic acid and was used as a saturated solution. For QSTAR experiments, premixed DHB solution (Agilent Technologies Inc., Palo Alto, CA) was used without further dilution. A 0.5-$\mu$L aliquot of the settled beads was spotted on the target, followed by 0.5 $\mu$L of matrix, and the solution was allowed to dry at room temperature.

The experimental scheme is presented in Figure 1.

**Database Searching.** For searching and interpreting MALDI-MS/MS spectra, Mascot software[13] was used. Searches considered only the *D. melanogaster* genome, and a mass accuracy of 50 ppm in the parent ion mass and 0.1 Da in the product ions masses was entered. The nomenclature of Roepstorff and Fohlman, later modified by Biemann, was used to identify all product ions.[14,15]

## RESULTS AND DISCUSSION

**Direct MALDI-MS/MS of the KDIR4 Peptide Bound to IMAC Beads.** The KDIR4 phosphorylated peptide was used to evaluate the potential of performing MALDI-MS/MS directly on peptides affinity-bound to IMAC beads. Typically, beads settled in the center of the target spot, while matrix crystallized in a circular ring on the circumference of the spot, as shown in Figure 1B. The best signal often was obtained by irradiating the DHB crystals, rather than irradiating the IMAC beads themselves, suggesting that the matrix solution releases the peptides from the beads during the spotting process.

The most abundant peak in the MALDI-MS spectrum, at $m/z$ 1702.901, is the protonated molecule ($[M + H]^+$) of the standard peptide (Figure 2A). Loss of 98 Da from the $[M + H]^+$ ion gives an $[M + H - 98]^+$ peak of 20% relative abundance. Loss of 80 Da from the $[M + H]^+$ ion gives a peak of less than 1% relative abundance. Neutral loss of 98 Da, with less abundant loss of 80 Da, is thought to be characteristic of serine/threonine phosphorylation, not tyrosine phosphorylation.[16] Based on the MALDI-MS spectrum, the location of the phosphoryl group would most likely be assigned to one of the two threonine residues.

The KDIR4 peptide was subjected to collision-induced dissociation (CID), to determine whether sequence information could be obtained and to see whether the exact location of the phosphorylation site could be determined. As shown in Figure 2B, loss of 98 Da from the parent ion is a prominent dissociation pathway for phosphorylated peptides in low-energy CID MALDI-


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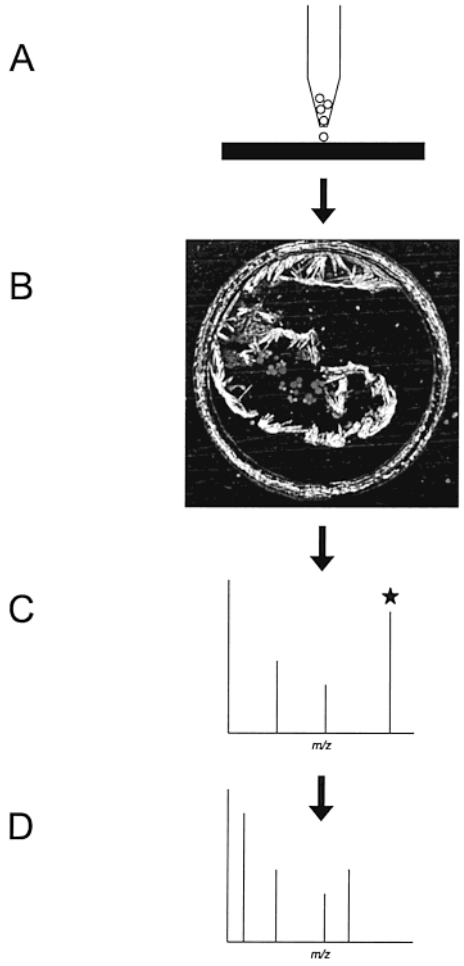


Figure 1. Experimental scheme of direct MALDI-MS/MS of peptides affinity-bound to IMAC beads. (A) An aliquot of IMAC beads is placed directly on the MALDI target, followed by an aliquot of matrix solution. (B) Image of IMAC beads spotted on MALDI target with DHB. The beads can clearly be observed in the center of the target spot, while the matrix crystals are in a ring surrounding the IMAC beads. (C) Simulated MALDI-MS spectrum of phosphorylated peptides affinity-bound to IMAC beads, with the ion selected for MS/MS indicated with ★. (D) Simulated MALDI-MS/MS spectrum of the peptide indicated in (C).

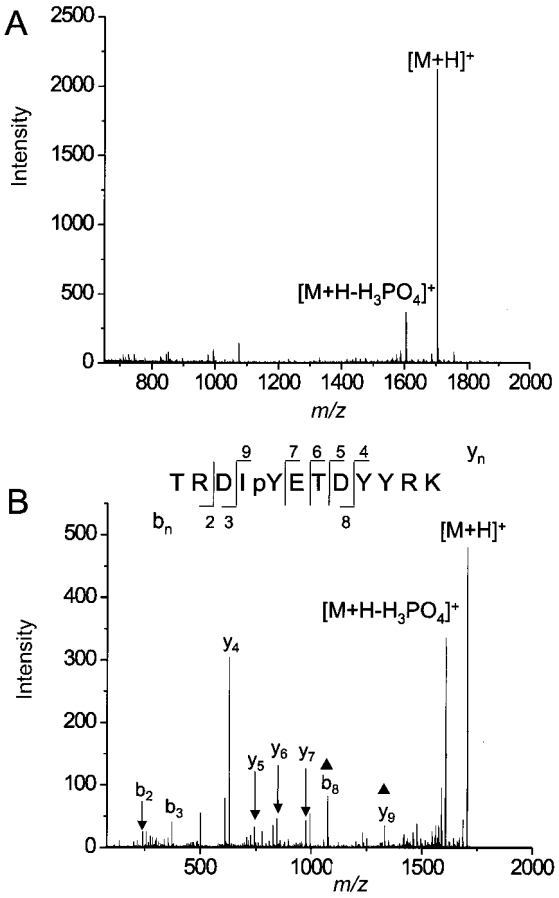


Figure 2. (A) MALDI-MS spectrum obtained from the KDIR4 peptide bound to IMAC beads applied directly to the target. (B) MALDI-MS/MS spectrum of the $[M + H]^+$ ion from Figure 1A, with sequence-specific (y and b) ions labeled. Product ions designated as ▲ contain the phosphorylated residue.

MS/MS, and this is the most abundant product ion in the spectrum. Additionally, most of the other peaks with a signal-to-noise ratio (S/N) greater than 10 can be assigned to sequence-specific b or y ions.

To locate the phosphorylated amino acid, the b and y ion series are examined. With MALDI, phosphorylated tyrosine residues usually remain intact during MS/MS,[16,17] and an increase in mass of 80 Da from the masses of the unphosphorylated b and y series ions should be observed for all product ions containing the phosphorylated tyrosine. The mass of the $y_6$ ion ($m/z$ 845.486) thus indicates that Thr[7], Tyr[9], and Tyr[10] are not phosphorylated. The $b_2$ ion, at $m/z$ 373.216, shows that Thr[1] does not contain the phosphoryl group. The $y_9$ ion, however, at $m/z$ 1330.651, is 80 Da higher than expected for the unphosphorylated peptide, confirming that the phosphoryl group is located on Tyr[5].

If only MALDI-MS information were available, the predominant $[M + H - 98]^+$ ion might have led to the incorrect assumption that one of the two threonine residues was phosphorylated. The KDIR4 peptide contains three acidic residues and two unmodified threonine residues, both of which have been reported to increase the abundance of the $[M + H - 98]^+$ ion in peptides containing a phosphotyrosine residue.[17,18] It should be noted that this is due to loss of $HPO_3$ from the tyrosine and $H_2O$ from a different residue.[19] This KDIR4 peptide study clearly illustrates the need for sequence information of IMAC-bound phosphorylated peptides to determine phosphorylation sites.

**Direct MALDI-MS/MS of dSLBP Peptides Bound to IMAC Beads.** The histone mRNA stem loop binding protein from *Drosophila* (dSLBP) was known to be phosphorylated from a shift in mobility of the protein in SDS−PAGE after alkaline phosphatase treatment (data not shown), but the sites of the phosphorylation were not known. The protein was digested with trypsin, and the digest was analyzed by MALDI-TOF/MS, as shown in Figure 3A. Many unphosphorylated tryptic peptides from dSLBP were observed; however, no phosphorylated peptides (i.e., peptides with $[M + H]^+$ ions 80 Da higher in mass than the calculated tryptic fragments derived from the protein sequence) were detected in the MALDI mass spectrum, probably due to suppression effects.

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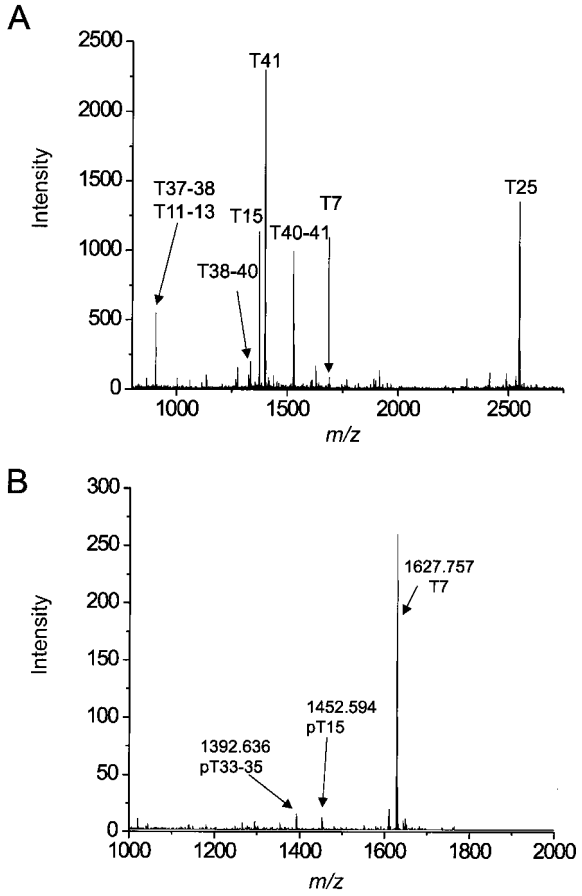


Figure 3. (A) MALDI/TOF-MS spectrum obtained from the tryptic digest of dSLBP protein. Tryptic peptides are labeled as T*n*, where *n* indicates the tryptic cleavage site counting from the N-terminus. (B) MALDI-MS spectrum obtained from the tryptic digest of dSLBP protein after isolating the potentially phosphorylated peptides on IMAC beads and placing an aliquot of the beads directly on the target. Based on the molecular weight, potentially phosphorylated tryptic peptides are indicated as pT*n*.

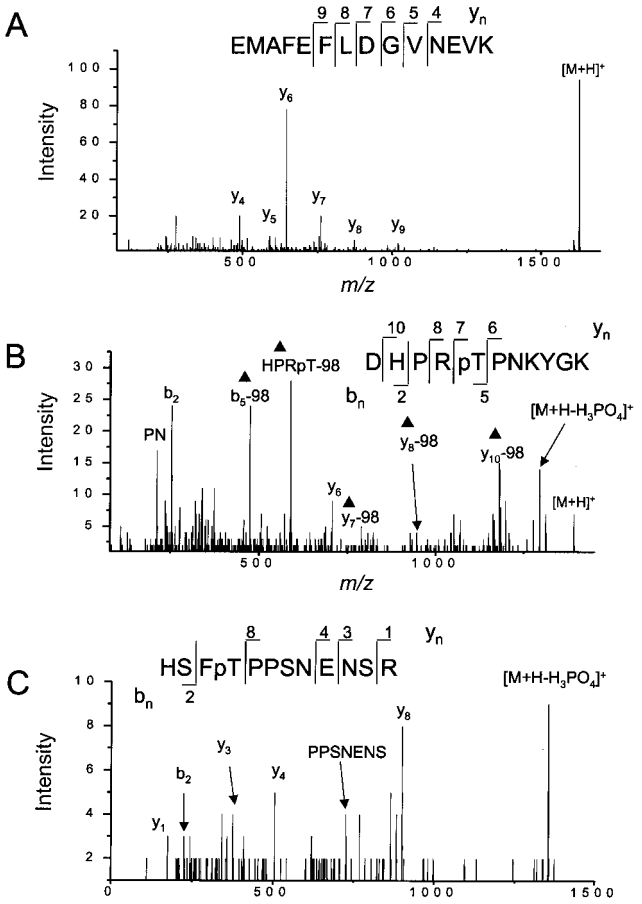


Figure 4. MALDI-MS/MS spectra of the three most abundant peptides affinity-bound to IMAC beads from the direct MALDI-MS analysis of a dSLBP digest from Figure 3B. MALDI-MS/MS spectrum of *m*/*z* (A) 1627.757 (B) 1392.636, and (C) 1452.594. Product ions designated as ▲ contain the phosphorylated residue. Sequences matched to each spectrum are shown; peptide bond cleavages corresponding to the sequence ions observed are indicated.

For phosphopeptide enrichment, the tryptic digest was passed through an IMAC column. Figure 3B shows the spectrum obtained by performing direct MALDI-MS on the peptides affinity-bound to IMAC beads. The most abundant peak in the spectrum, at *m*/*z* 1627.757, had previously been observed in the MALDI-TOF/MS spectrum (Figure 3A) and was assigned as the tryptic peptide T7 ($Glu^{86}$-$Lys^{99}$). No potentially phosphorylated peptide from dSLBP could be assigned to *m*/*z* 1627.757. Several less abundant ions (*m*/*z* 1392.636 and 1452.594) were also detected which were not observed in the MALDI/TOF-MS spectrum of the tryptic digest.

The ion signal at *m*/*z* 1452.594 might be from phosphorylated T15 ($His^{117}$-$Arg^{128}$), and the ion signal at *m*/*z* 1392.636 might correspond to phosphorylated T33−35 ($Asp^{226}$-$Lys^{236}$). The assignment of the ion at *m*/*z* 1392.636 as T33−35 is tentative, because it would require that two tryptic cleavage sites were missed. The tryptic peptide T33−35 only has one possible residue that could be phosphorylated, $Thr^{230}$. The tryptic peptide T15, however, has four potential phosphorylated residues: $Ser^{118}$, $Thr^{120}$, $Ser^{123}$, and $Ser^{127}$. In contrast to the model phosphorylated peptide, KDIR4, loss of 98 Da was not observed from either potentially phosphorylated peptide in the mass spectrum. Without the sequence information from MS/MS experiments, the identity of the peptides and the locations of the phosphorylation sites cannot be unambiguously determined.

Figure 4A−C shows the MALDI-MS/MS spectra for the three parent ions of *m*/*z* 1627.757, 1392.636, and 1452.594, respectively. No loss of either 98 or 80 Da was observed from the ion of *m*/*z* 1627.757, indicating that the peptide was probably not phosphorylated. The ion of *m*/z 1392.636 showed losses of both 80 and 98 Da, confirming that it was phosphorylated. The MALDI-MS/MS spectrum of the *m*/*z* 1452.594 ion showed a prominent loss of 98 Da, confirming that this peptide was also phosphorylated.

The ion at *m*/*z* 1627.757 was unambiguously assigned as unphosphorylated T7, based on a y ion series (from $y_4$ to $y_9$) (Figure 4A). Although this peptide is not phosphorylated, previous work has shown that peptides with a high content of acidic residues can nonspecifically bind Fe ions[12] and be retained on IMAC media. The tryptic peptide T7 contains three glutamic acid residues, and one aspartic acid residue, which probably interacted with the iron on the IMAC column.

The MALDI-MS/MS spectrum of *m*/*z* 1392.636 confirms the assignment of the peptide to phosphorylated tryptic peptide T33−35. Evidence for the location of the phosphoryl group on $Thr^{230}$

is found in the y ion series (Figure 4B). The $y_6$ ion did not contain this phosphothreonine residue and was observed at the expected $m/z$ calculated from the unphosphorylated peptide. The $y_7$, $y_8$, and $y_{10}$ ions appear 18 Da lower than the masses calculated from the sequence of the unphosphorylated peptide, corresponding to loss of 98 Da from the phosphorylated peptide. The mass difference between $y_6$ and $y_7$ is 83 Da, which corresponds to dehydroaminobutyric acid (the residue remaining after $\beta$-elimination of a phosphate group from phosphothreonine),[20] thus confirming that loss of phosphoric acid occurred from $Thr^{230}$.

Even with a MALDI-MS/MS spectrum with very low S/N, the ion of $m/z$ 1452.594 was identified as phosphorylated T15, based on sequence-specific ions (Figure 4C). The MALDI-MS/MS data allowed identification of the exact phosphorylation site, even though this peptide contains four potentially phosphorylated residues. The $y_8$ ion, which contained unmodified $Ser^{123}$ and $Ser^{127}$, showed that neither of these residues was phosphorylated. Additionally, a $b_2$ ion, containing unmodified $Ser^{118}$, was present in the MALDI-MS/MS spectrum. This showed unambiguously that $Thr^{120}$ contained the phosphoryl group, even though no product ions containing dehydroaminobutyric acid were observed.

Analyzing peptides from IMAC beads placed directly on the MALDI plate does not compromise either the enrichment or sensitivity of the IMAC method. The unphosphorylated form of tryptic peptide T15 was observed in the original MALDI-TOF/MS spectrum (Figure 3A) but was not detected in its phosphorylated form. It was only after the digest had been passed through the IMAC column (Figure 3B) that phosphorylated pT15 was observed. If the protein were 100% pure, were 100% phosphorylated at all sites, and no losses occurred during digestion and the IMAC procedure, then the determination of both sites of phosphorylation was performed on a *maximum* of 2 pmol of protein applied to the target. The results of the enrichment experiments, however, demonstrate that the protein was not completely phosphorylated and that multiple isoforms of dSLBP were present in this *Drosophila* extract. These results thus demonstrate that the unambiguous identification of phosphorylation sites can be accomplished on subpicomole amounts of protein.

For identifying IMAC-isolated peptides from unknown phosphoproteins on a proteomic level, automated interpretation of MALDI-MS/MS spectra would be desirable. It is critical to have high mass accuracy for database searching of product ion spectra.[21] One of the advantages of combining a MALDI source with a quadrupole time-of-flight instrument is the inherent high mass accuracy. All parent ions had a mass accuracy of ±14 ppm, while product ions had a mass accuracy of ±45 ppm. This mass accuracy was obtained with external calibration and is comparable to that achieved without applying beads directly to the MALDI target. Analyzing phosphorylated peptides directly from IMAC beads, therefore, does not compromise mass accuracy in both the MALDI-MS and MALDI-MS/MS modes, and baseline resolution between isotope peaks was always obtained. Mascot software was able to correctly interpret the MALDI-MS/MS spectra and identify the protein in all cases. In addition, the Mascot software was able to unambiguously identify the phosphorylation site in both phosphorylated dSLBP peptides.

## CONCLUSIONS

In this study, we report a powerful technique for isolation, identification, and sequencing of phosphorylated peptides. This technique combines direct analysis of phosphorylated peptides bound to IMAC beads with sequencing by MALDI-MS/MS using an orthogonal injection QqTOF. Analysis of beads placed directly on the MALDI target eliminates possible sample loss associated with eluting peptides from the beads and removing salts in the elution buffer. In contrast to enzymatic sequencing of phosphorylated peptides affinity-bound to IMAC beads,[8] direct MALDI-MS/MS sequencing of phosphorylated peptides affinity-bound to IMAC beads is rapid, sensitive, and does not show any amino acid residue dependency

This new method, which provides enrichment with minimal sample handling, has been applied to a protein with unknown phosphorylation sites. Direct MALDI-MS/MS of peptides from a tryptic digest of this protein, bound to IMAC beads without any additional purification or desalting steps, resulted in the identification of two novel phosphopeptides. Even with four potential phosphorylation sites on one of these peptides, location of the phosphoryl group is unambiguous.

When direct MALDI-MS and MALDI-MS/MS analysis of phosphorylated peptides bound to IMAC beads is performed, the high mass accuracy of the QqTOF mass spectrometer is maintained. With this high mass accuracy, currently available proteomics software is able to automatically interpret the MALDI-MS/MS spectra and unambiguously identify both the phosphoprotein and the phosphorylation site. The combination of direct analysis of affinity-bound phosphorylated peptides on IMAC beads and MALDI-MS/MS on a QqTOF mass spectrometer is ideally suited for phosphoproteomics.

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