Fe$^{3+}$ immobilized metal affinity chromatography with silica monolithic capillary column for phosphoproteome analysis

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Immobile metal affinity chromatography (IMAC) is a commonly used technique for phosphoproteome analysis due to its high affinity for adsorption of phosphopeptides. Miniaturization of IMAC column is essential for the analysis of a small amount of sample. Nanoscale IMAC column was prepared by chemical modification of silica monolith with iminodiacetic acid (IDA) followed by the immobilization of Fe$^{3+}$ ion inside the capillary. It was demonstrated that Fe$^{3+}$-IDA silica monolithic IMAC capillary column could specifically capture the phosphopeptides from tryptic digest of $a$-casein with analysis by MALDI-TOF MS. The silica monolithic IMAC capillary column was manually coupled with nanoflow RPLC/nanospray ESI mass spectrometer ($\mu$RPLC-nanoESI MS) for phosphoproteome analysis. The system was validated by analysis of standard phosphoproteins and then it was applied to the analysis of protein phosphorylation in mouse liver lysate. Besides MS/MS spectra, MS/MS/MS spectra were also collected for neutral loss peak. After database search and manual validation with conservative criteria, 29 singly phosphorylated peptides were identified by analyzing a tryptic digest of only 12 $\mu$g mouse liver lysate. The results demonstrated that the silica monolithic IMAC capillary column coupled with $\mu$RPLC-nanoESI MS was very suitable for the phosphoproteome analysis of minute sample.

Key words:
Immobilized metal affinity chromatography / Miniaturization / Phosphoproteome analysis / Silica monolithic column

1 Introduction

PTMs of proteins are critical to the protein function and activity. It has been estimated that approximately one-third of mammalian proteins contain covalently bound phosphate groups [1]. However, even phosphorylation being acknowledged as a crucial modification involved in many cellular events, determining the sites of phosphorylation on protein is still a challenging task. Enrichment of phosphorylated proteins and peptides is generally required before analysis because of their low abundance. A number of methods have been developed to enrich phosphopeptides [2-9], among which immobilized metal affinity chromatography (IMAC) is the most widely used method.

IMAC, employing coordinated heavy metal ions like Fe$^{3+}$ or Ga$^{3+}$, has been used for a number of years to selectively enrich the phosphopeptides from complex peptide mixtures...
It was proved that IMAC is an efficient method for isolation of phosphopeptides, mainly based on different interactions of the analytes to metal cations bound to a support material by chelating ligands. The IMAC columns commonly used were packed with particles or gels such as agarose, polymer, cellulose, and silica. Monolithic columns prepared from polymer and silica offer a convective mass transfer process with low backpressure and have recently been used successfully in proteome analyses such as C18 capillary column for a highly efficient separation of the peptide mixture, and the immobilized enzyme microreactor for fast digestion of protein mixtures. Monoliths are also good support materials for IMAC. We have prepared macroporous poly(glycidyl methacrylate–co-ethylene dimethacrylate) monolith in an HPLC column, and Cu²⁺ was immobilized on the monolithic column through iminodiacetic acid (IDA) for the purification of lysozyme from egg white. Recently, IMAC capillary columns with organic monoliths were also reported for online concentration of proteins and peptides followed by CE analysis. IMAC columns commonly used were packed with particles or gels such as agarose, poly(styrene–divinylbenzene), and silica. But IMAC with monolithic materials for the isolation of phosphopeptides, mainly based on different interactions of the analytes to metal cations bound to a support material, has not been reported yet. Therefore, silica monolith should also be an excellent support material for IMAC. But IMAC with monolithic materials for the phosphopeptide enrichment and phosphoproteome analysis has not been reported yet.

2 Materials and methods

2.1 Chemicals and materials

Adult female C57 mice were purchased from Dalian Medical University (Dalian, China); fused-silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). Tetrabutylammonium (TBA) was purchased from Fluka (Buchs, Switzerland). Water was prepared using a Milli-Q system (Millipore, Milford, MA, USA). Urea, DTT, iodoacetamide (IAA), CHAPS, and ammonium bicarbonate were all purchased from BioRad (Hercules, CA, USA). 3-Glycidoxypropyltrimethoxysilane (GLYMO), Tris, guanidine hydrochloride, sodium orthovanadate (Na3VO4), TFA, dihydroxybenzoic acid (DHB), sodium fluoride (NaF), z-casein, trypsin, and BSA were obtained from Sigma (St. Louis, MO, USA). Acetic acid, ferric chloride (FeCl3), sodium chloride, FA, and ACN were obtained from Aldrich. Ethylene glycol-bis-(2-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA), EDTA, and PMSF were purchased from Amresco (Solon, Ohio, USA). POROS 20 MC packing material was purchased from PerSeptive Biosystems (Framingham, MA, USA). All the chemicals were of analytical grade, except ACN of HPLC grade.

2.2 Preparation of protein sample

z-Casein was dissolved in 0.5 mL of 50 mM Tris-HCl buffer at pH 8.1 at 10 μM concentration. Then trypsin was added at a protein/enzyme ratio of 50:1 by weight, and the solution was incubated at 37°C for 16 h. Finally, the solution was acidified with 0.5% FA for further usage.

The protein extract from mouse livers was prepared according to the procedure performed by Jin et al. [17], with minor modification. Mice were sacrificed and their livers were promptly removed and placed in ice-cold homogenization buffer consisting of 8 M urea, 4% CHAPS w/v, 65 mM DTT, 1 mM EDTA, 0.5 mM EGTA, and a mixture of protease inhibitors (1 mM PMSF) and phosphatase inhibitors (0.2 mM Na3VO4, 1 mM NaF), and 40 mM Tris-HCl at pH 7.4. After mincing with scissors and washing to remove blood, the livers were homogenized in 10 mL of homogenization buffer per 2 g of tissue. The suspension was homogenized for approximately 1 min, sonicated for 30 s at 100 W, and centrifuged at 25 000 g for 1 h. The supernatant contained the total liver proteins. Appropriate volume of supernatant was precipitated to remove insoluble substance, lyophilized to dryness, and redissolved in denaturing solution (8 M urea, 100 mM ammonium bicarbonate, at pH 8.3) with the protein concentration determined to be 0.46 mg/mL. Then, 300 μg of this protein sample (100 μL volume) was mixed with 1 μL of 1 M DTT. The mixture was incubated at 37°C for 2.5 h, and then 5 μL of 1 M IAA was added and incubated for an additional 30 min at room temperature in the dark. The protein mixtures were diluted by 10-folds with 100 mM ammonium bicarbonate buffer (pH 8.5), and incubated with trypsin (25:1 w/w) at 37°C overnight. The solution was lyophilized to dryness, and then redissolved in 0.1% acetic acid to a concentration of 0.4 mg/mL.
2.3 Preparation of IDA monolithic silica capillary column

GLYMO-IDA-silane was synthesized according to the procedures reported before [43]. In short, IDA (4.25 g) was dissolved in 50 mL of deionized water. The solution was adjusted to pH 11.0 with 10 M NaOH and placed in an ice bath at 0°C, and then 1.4 mL of GLYMO was slowly added under stirring. The resultant solution was incubated at 65°C for 6 h, subsequently placed into an ice bath for 5 min to decrease the temperature to 0°C again, and 1.6 mL of GLYMO was added and mixed again. The temperature of the solution was raised to 65°C for a further 6 h under stirring. Similarly, another 1.7 mL of GLYMO was added to the solution and incubated overnight at 65°C under stirring. Finally, the prepared GLYMO-IDA-silane solution was adjusted at pH 6 with concentrated HCl and stored in refrigerator.

The monolithic silica capillary column was prepared in house, as reported before [26, 41, 44]. IDA modified silica monolithic capillary was realized by continuously delivering GLYMO-IDA-silane solution through the silica monolithic capillary (75 μm id x 100 mm length), and the reaction was kept at 95°C for about 12 h, then washed with 100 μL of MeOH and 100 μL of H2O.

2.4 IMAC of peptide mixtures

The IDA monolithic silica capillary column (75 μm id x 100 mm length), or the self-packed POROS 20 MC column (200 μm id x 100 mm length) was rinsed with 100 μL of 0.1% acetic acid and 100 mM EDTA, and then rinsed with 200 μL of 0.1% acetic acid. Next, the column was activated with 200 μL of 100 mM FeCl3 in 0.1 M acetic acid. Finally, the column was washed with 100 μL of 0.1% acetic acid. After loaded with α-casein digest (30 μL, 1 μM), the column was washed with 40 μL of 100 mM NaCl solution with ACN/water/glacial acetic acid (25:74:1 v/v/v) to remove nonspecifically adsorbed peptides. The column was then rinsed with an additional 30 μL of 0.1% acetic acid. The tryptic phosphopeptides were eluted from the column with 20 μL of ammoniated water (pH 10.3) into a tube, lyophilized to dryness, and analyzed by MALDI-TOF MS; or after rinsed with an additional 30 μL of 0.1% acetic acid, the IMAC capillary column was directly connected to the capillary separation column, and analyzed by μRPLC-nanoESI MS. For the phosphoproteome analysis of CS7 mouse liver, 60 μL of tryptic peptide solution from 0.2 mg/mL of protein mixture was pumped into the IMAC capillary column, which was then rinsed with 40 μL of solution containing 100 mM NaCl in ACN/water/glacial acetic acid (25:74:1 v/v/v) and 30 μL of 0.1% acetic acid, respectively, then coupled to the capillary separation column for μRPLC-nanoESI MS analysis.

2.5 MALDI and μRPLC-nanoESI MS instruments

MALDI-TOF-MS was performed on the Bruker AutoflexTM (Bruker, Bremen, Germany). The instrument was equipped with a nitrogen laser (λ = 337 nm) and its available accelerating potential was in the range of +20/–20 kV. The MALDI MS used a ground-steel sample target. All mass spectra shown were obtained in the positive ion mode. The eluted phosphopeptides were redissolved in 5 μL of 0.1% TFA, and 5 μL of DHB containing 1% phosphoric acid was added, and then 1 μL of aliquot was deposited onto the target.

A Finnigan surveyor MS pump (ThermoFinnigan, San Jose, CA) was used to deliver mobile phase. The pump flow rate was split by a cross to achieve a column flow rate of about 200 nL/min. For the capillary separation column, one end of the fused-silica capillary (75 μm id x 120 mm length) was manually pulled to a fine point of ~5 μm with a flame torch. The separation column was in-house packed with C18 AQ particles (5 μm, 120 Å) from Michrom BioResources (Auburn, CA, USA) using a pneumatic pump. The μRPLC column was directly coupled to a LTQ linear IT MS from ThermoFinnigan with a nanospray source. The mobile phase consisted of (i) 0.1% FA in water, and (ii) 0.1% FA in ACN. The LTQ instrument was operated at positive ion mode. A voltage of 1.8 kV was applied to the cross.

After being loaded with trypptic digest and washed thoroughly to remove nonspecifically adsorbed peptides, the IMAC column with captured phosphopeptides was connected directly to a separation column. Between the IMAC column and splitter, an open capillary with dimensions of 200 μm id x 100 mm length, filled with about 3 μL of phosphoric buffer (pH 6.0), was connected. The phosphoric buffer inside the open capillary was the elution buffer for phosphopeptides on the IMAC column. The captured phosphopeptides were eluted from IMAC column and retained on the separation column when the mobile phase pushed the phosphoric buffer to pass through the IMAC column. After the sample was loaded onto the separation column, the IMAC column and the open capillary were removed. After equilibration with mobile phase A, the gradient elution was then started for the separation of retained phosphopeptides on the separation column.

A 50 min and a 75 min gradient elution were applied for the separation of phosphopeptides enriched with α-casein and mouse liver protein extract, respectively. For the detection, the MS was set as a full scan MS followed by three data-dependent MS/MS events. A subsequent MS3 event was triggered upon detection of neutral loss of ~98, ~49, or ~32.7 from the precursor ions (corresponding to the neutral loss of phosphoric acid from 1+, 2+, and 3+ charge states, respectively) during any of the MS/MS scans.

2.6 Database searching

To identify the phosphopeptides in the above samples, MS2 and MS3 spectra obtained for mouse liver were searched with the SEQUEST algorithm against the newly download non-
3 Results and discussion

3.1 Preparation and evaluation of silica monolithic IMAC capillary column

Zhang et al. [35] used the monolithic rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) to prepare IMAC capillary column. After the monolith was chemically modified with IDA and immobilized with Cu\textsuperscript{2+}, the column was used for on-line concentration of some specific proteins or peptides for the subsequent separation by CE. In a similar way, Vizioli et al. [36] prepared a polymeric monolithic column by \textit{in situ} polymerization of diethylene glycol dimethacrylate, and glycidyl methacrylate for on-line capturing of histidine-containing peptides for CE analysis. To the best of our knowledge, no silica monolithic IMAC capillary column coordinated with Fe\textsuperscript{3+}, for isolation of phosphopeptides was reported yet.

There are mainly two approaches to immobilize IDA onto silica matrix [23, 43, 45, 46]. In the first approach, GLYMO is first immobilized onto the silica and then IDA was immobilized by a ring opening reaction of an epoxy group on GLYMO molecule. However, the optimum pH for the immobilization of IDA onto epoxy-activated supports is at 10–12, which is detrimental to the pore structure of the silica monolithic capillary column. Here, we choose another approach to immobilize IDA onto the silica monolithic capillary column [43]. In this approach, GLYMO-IDA-silane is synthesized before, and then the GLYMO-IDA-silane was reacted onto the surface of silica monolith, therefore, avoiding extreme pH inside the monolithic column. The IDA modified silica monolith capillary was prepared in this way, and Fe\textsuperscript{3+} was then coordinated for purification of phosphopeptides.

The performance of the prepared silica monolithic IMAC capillary column was evaluated using bovine \(\alpha\)-casein as the test protein sample. Phosphopeptides in the tryptic digest of \(\alpha\)-casein were enriched by silica monolithic IMAC capillary, as described in Section 2 and analyzed by MALDI-TOF-MS, and the obtained mass spectra of \(\alpha\)-casein digested before and after enrichment by IMAC, are presented in Figs. 1A and B, respectively. It can be seen that the spectrum in Fig. 1B is much cleaner than that in Fig. 1A. This means that the majority of nonphosphorylated peptides were removed after the enrichment by the IMAC column. Nine phosphopeptides were found in Fig. 1(B) with their sequences listed in Supplementary Table 1. The detection of phosphopeptides as major peaks in Fig. 1(B) indicates the high specificity of silica monolithic IMAC capillary column for the enrichment of phosphopeptides.

In comparison, phosphopeptides in the \(\alpha\)-casein tryptic digest were also enriched by a POROS 20 MC packed column, by the same procedure as for silica monolithic IMAC column, and the obtained MALDI-TOF-MS spectrum for the analysis of the enriched peptides is shown in Fig. 1(C). Only six phosphopeptides were found, which is not as good as that of our monolithic column. Compared with the particulate packing materials, a major advantage of silica monolith is that the IMAC column can be easily prepared in any scale from nanoliter to milliliter in batch, and the phosphopeptides can be more efficiently captured with convective mass-transfer process. The repeatability of the IMAC capillary was good, and no obvious change in performance was observed after the column was used more than 20 times.

Phosphoproteins are low abundant components in complex protein samples. In order to further investigate the capability of the IMAC monolithic column to enrich low abundant phosphopeptides, BSA tryptic digest was mixed with \(\alpha\)-casein tryptic digest in different molar ratio at 1:1 and 1:100 to simulate complex samples. The phosphopeptides were enriched by the monolithic IMAC column and analyzed by MALDI-TOF MS. The obtained results are shown in Fig. 2. It can be seen in Fig. 2(A) that nine phosphopeptides were found when the molar ratio of BSA and \(\alpha\)-casein is 1:1. When the molar ratio of \(\alpha\)-casein/BSA is further decreased to 1:100, the obtained mass spectrum, as shown in Fig. 2(B), is very similar to that of 1:1 ratio. Although there are some nonphosphopeptide peaks observed in the mass range at \(m/z\) 2700 at molar ratio of 1:100, the major peaks still belong to the phosphopeptides. Considering the concentration of phosphopeptides from \(\alpha\)-casein are 100 times lower than that of nonphosphopeptides from BSA, the specificity of the monolithic IMAC column for isolation of phosphopeptides is very high. It is interesting to note that the signal intensity of singly phosphorylated peptides, P4 and P6, decreased significantly and an extra peak P3, a doubly phosphorylated peptide, was observed by comparing Fig. 2 with Fig. 1(B). This is possibly because the interaction between Fe\textsuperscript{3+} and doubly phosphorylated peptides is relatively strong, and some acidic peptides may also have relatively strong affinity for adsorption on the IMAC column, thus the high concentration of these acidic peptides from BSA will compete.
Figure 1. MALDI-TOF mass spectra of (A) α-casein digest; (B) phosphopeptides isolated from α-casein digest by silica monolithic IMAC column; and (C) phosphopeptides isolated from α-casein digest by POROS 20 MC packed column. The peaks for phosphopeptides are marked with Pn (n = 1–11), their sequences are listed in Supplementary Table 1. Matrix, DHB containing 1% phosphoric acid.

with these singly phosphorylated peptides for adsorption on the active sites, thereby resulting in the decrease of the ion signal intensity.

3.2 On-line coupling of IMAC capillary column with μRPLC for phosphoproteome analysis

The combination of IMAC and μRPLC-nanoESI MS was often adapted to study protein phosphorylation. In this approach, the IMAC columns were used to capture the phosphopeptides from tryptic digest. The retained phosphopeptides were eluted from IMAC capillary column, concentrated by SPE C18 column, and then they were loaded onto a capillary C18 column for tandem mass spectrometric analysis. Those tedious procedures for sample preparation may result in the loss of the hydrophilic phosphopeptides. It is obviously beneficial if the retained phosphopeptides could be eluted from IMAC column onto C18 separation column directly. However, the typically used IMAC column packed with particles usually have a big column volume which is not suitable to be connected directly with the separation column with only 75 μm id [15, 17, 19, 47, 48]. In our case, the prepared silica monolithic IMAC capillary column has the dimension of 75 μm × 100 mm and volume of less than 0.44 μL, which means the silica monolithic IMAC capillary column can couple readily with μRPLC-nanoESI MS. Tryptic digest of α-casein was also used to evaluate this coupling system. After loaded with tryptic digest of α-casein, the IMAC column was washed thoroughly to remove nonspecifically adsorbed peptides, and it was then manually connected to the 75 μm separation column as described in Section 2. The retained phosphopeptides on IMAC column were eluted directly onto the separation column by phosphoric buffer, and analyzed by μRPLC-nanoESI MS under gradient elution.
Figure 2. MALDI-TOF mass spectra of phosphopeptides isolated by silica monolithic IMAC column from the mixture of α-casein and BSA with molar ratio of (A) 1:1, and (B) 1:100. The phosphopeptide peaks are marked with Pn (n = 1–11), their sequences are also listed in Supplementary Table 1. Matrix, DHB containing 1% phosphoric acid.

Typically, only MS/MS spectra were used to search the database in phosphoproteome analysis. It is known that phosphoserine and phosphothreonine, but not phosphotyrosine, lose phosphoric acid readily during the collision activation dissociation process in IT mass spectrometers. Thus, a single dominant neutral loss peak with few fragment peaks will appear in the MS/MS spectrum. The lack of informative fragment peaks in MS/MS spectrum, results in the difficulty for the unambiguous identification of phosphopeptides. Recently it was reported that MS^3 spectrum could be used to further confirm the phosphorylated peptide assignment [12, 17, 42]. In this study, an MS/MS/MS spectrum was automatically collected when one of the top three intense peaks from the MS/MS spectrum corresponded to a neutral loss of 98, 49, 32.9 Da. The α-casein digest was analyzed three times by this system. The acquired MS^2 and MS^3 spectra were submitted to SEQUEST database search, respectively, which resulted in unambiguous identification of five phosphopeptides in total as shown in Supplementary Table 1. The number of singly, doubly, and triply phosphorylated peptides are 2, 1, and 2, respectively. Compared with results obtained by MALDI analysis, phosphopeptides with four and five phosphorylation sites were not found in μRPLC-nanoESI MS analysis. This may be because these phosphopeptides are so hydrophilic that they cannot be retained on the C18 separation column and therefore, fail to be detected by μRPLC-nanoESI MS. Compared with the results previously reported by Ficarro et al. [48], that only three phosphopeptides in tryptic α-casein digest were observed with the automated IMAC-nanoRPLC-MS/MS system, our system exhibited a good performance for enriching phosphopeptides. The sensitivity of the system was evaluated by loading different
amounts of α-casein trypic digest varied from 1 fmol to 1 pmol. It was found that the identification of two singly phosphorylated peptides, VPQLEIVPNpSAEER (m/z 1660.8) and YKVPQLEIVPNpSAEER (m/z 1952.0), could be achieved even with the injection of 1 fmol sample. The LOD previously reported for the phosphoproteome analysis by LC-MS/MS is all above 50 fmol [49]. The high sensitivity of our system is due to the use of nanoliter IMAC column and the on-line coupling scheme.

The manual validation is relatively simple for singly phosphorylated peptides. Figure 3 is the mass spectra of a doubly charged singly phosphorylated peptide YKVPQLEIVPNpSAEER. From the MS/MS spectrum, it can be seen that the b- and y-ion series are consistent with the theoretically predicted peaks of the identified phosphopeptide. It is noted that the highest peak at m/z 928.01 represents the doubly charged form of the selected precursor ion at m/z 964.54 that has lost a H₃PO₄ group. It can be determined that the S¹³⁰ residue is phosphorylated. From MS/MS/MS spectrum, it can also be clearly seen that the b- and y-ion series are consistent with the theoretically predicted peaks of the identified phosphopeptide, which further confirms the identification of this peptide.

The mass spectra for multiply phosphorylated peptides are more complex. Figure 4 shows the mass spectra of doubly phosphorylated peptide DIGpSEPSTEDQAMEDIK. From the MS/MS spectrum, it can be seen that the highest peak at m/z 915.48 represents the doubly charged form of the selected precursor ion at m/z 964.54 by losing one H₃PO₄ group, and the second highest peak at 866.52 corresponding to the fragment of the precursor ion lost two H₃PO₄ groups. In the MS/MS spectrum, it can also be found that the consecutive peptide fragment peaks matched the theoretically predicted one. The highest peak with neutral loss of a H₃PO₄ group in MS/MS spectrum was further fragmented for the acquisition of MS/MS/MS spectrum. Since the selected precursor ion, lost only one H₃PO₄ group at MS² stage, the loss of another H₃PO₄ group occurred in MS³ stage, thus more fragment peaks can be observed in MS³ spectrum. After database searching, both spectra resulted in the same peptide sequence, increasing the confidence of the phosphopeptide identification.

3.3 Phosphoproteome analysis of mouse liver lysate

The silica monolithic IMAC column was further applied to the analysis of phosphoproteome of mouse liver. Tryptic digest of about 12 μg of mouse liver proteins was loaded
onto the IMAC capillary column and analyzed by μRPLC-nanoESI MS, and the obtained base peak chromatogram was shown in Fig. 5. There were about 16,000 scan events obtained during 75 min of elution time. The MS/MS spectra were searched by SEQUEST program with differential modifications of 80 Da to Ser, Thr, and Tyr residues. The obtained results were filtrated by setting the lowest Xcorr as 1.9, 2.2, and 3.75 corresponding to 1+, 2+, and 3+ charge states, respectively. Totally, 91 unique phosphopeptides were obtained with the above search process. Among them, 38 are multiply phosphorylated peptides, and 53 are singly phosphorylated peptides. After manual validation, 15 peptides were further identified as singly phosphorylated peptides. Because the purpose of our study is only to demonstrate the performance of the silica monolithic IMAC column and its coupling system, and the manual validation of multiply phosphorylated peptides is very complex with no available filter criteria widely accepted, thus the manual validation of multiply phosphorylated peptides were not performed further.

The obtained MS/MS/MS spectra were also searched by SEQUEST program. Peptide hits were filtered with the same Xcorr criteria. Based on the MS3 spectra, 22 singly phosphorylated peptides were obtained. The number was even greater than that of by searching MS/MS spectra. Among the 22 phosphopeptides, 7 phosphopeptides were matched with the results derived from MS/MS spectra. Figure 6 shows one typical MS/MS and MS/MS/MS spectra of doubly charged singly phosphorylated peptide pHSLPNSLDYAQASER, in which the S331 residue of the protein gi 51 706 331 is phosphorylated. From the spectra it can be seen that the b-and y-ion series are consistent with the theoretically predicted peaks of the identified phosphopeptide in both cases, which increase the confidence of the identification of the phosphopeptide. It is noted that the peak at m/z 879.14 represents the doubly charged form of the selected precursor ion at m/z 928.15 by losing a H3PO4 group.

As shown in Figs. 3, 4, and 6, mass spectra with rich fragment peaks were obtained for both MS/MS and MS/MS/MS spectrum. The high quality of the spectra resulted in the identification of same phosphopeptide sequence for both spectra. In this case, the function of MS3 spectrum is to confirm the identification of the phosphopeptide derived from MS2 spectrum. However, in another case as shown in Fig. 7(A), there is only one dominant peak at m/z 601.98, which represents the triply charged form of the selected precursor ion at m/z 634.55 by losing a H3PO4 group. Few other fragment peaks were observed with very low ion intensities. After database searching, the obtained highest Xcorr value for this spectrum is only 2.669, which is below the filter criteria value of 3.75 for triply charged ion. Accordingly, the phosphopeptide cannot be identified by the MS/MS spectrum. However, it is lucky that MS/MS/MS spectrum with the neutral lose peak was acquired, as shown in Fig. 7(B). After searching by SEQUEST program, phosphopeptide, KVpSVEPQDSDHQDAQPR, was identified with Xcorr value of 4.16. In total, 14 more phosphopeptides were identified based on the MS/MS/MS spectra. These phosphopeptides cannot be identified from MS/MS spectra only because of the poor spectrum quality.

As listed in Supplementary Table 2, 29 singly phosphorylated peptides were identified in this study by classification into three groups: (i) phosphopeptides derived from both
However, in the latter case, the phosphopeptides were isolated from the digest of about 300 μg protein mixture by a POROS 20 MC packed IMAC column, and the database searching with less stricter criteria by setting \( X_{corr} \geq 2 \) was performed.

4 Concluding remark

Fe\(^{3+}\)-IDA-silica monolithic capillary column was synthesized as IMAC column for the enrichment of phosphopeptides. The high specificity of the prepared IMAC column for the capture of phosphopeptides was demonstrated by analyzing standard phosphorylated protein of \( \alpha\)-casein. The Fe\(^{3+}\)-IDA-silica monolithic capillary could be manually coupled with \( \mu \)RPLC-nanoESI MS system for phosphoproteome analysis. Analysis of the tryptic digest of 12μg of mouse liver proteins by this coupling system resulted in the identification of 29 singly phosphorylated peptides. This system represents a simple and convenient way to perform phosphoproteome analysis of minute amount of sample, and full automation of the system is our future research direction.

It was also demonstrated that MS/MS/MS spectrum is beneficial for the identification of phosphopeptides in phosphoproteome analysis of mouse liver. If the acquired MS/MS spectrum has enough fragment peaks, it can result in sound identification of phosphopeptides. For this case, the information obtained in MS/MS/MS spectrum is used to confirm the identification. In another case, the MS/MS spectrum has only a dominant neutral loss peak and lacks enough informative fragment peaks from peptide backbone for phosphopeptide identification. However, fragmentation of the neutral loss peak may result in a MS/MS/MS spectrum full of informative fragment peaks. Additional 14 phosphopeptides were identified based on MS/MS/MS spectra in this study.

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5 References


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