Immobilized Zirconium Ion Affinity Chromatography for Specific Enrichment of Phosphopeptides in Phosphoproteome Analysis*†‡§

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Large scale characterization of phosphoproteins requires highly specific methods for purification of phosphopeptides because of the low abundance of phosphoproteins and substoichiometry of phosphorylation. Enrichment of phosphopeptides from complex peptide mixtures by IMAC is a popular way to perform phosphoproteome analysis. However, conventional IMAC adsorbents with iminodiacetic acid as the chelating group to immobilize $\text{Fe}^{3+}$ lack enough specificity for efficient phosphoproteome analysis. Here we report a novel IMAC adsorbent through $\text{Zr}^{4+}$ chelation to the phosphonate-modified poly(glycidyl methacrylate-co-ethylene dimethacrylate) polymer beads. The high specificity of $\text{Zr}^{4+}$-IMAC adsorbent was demonstrated by effectively enriching phosphopeptides from the digest mixture of phosphoprotein ($\alpha$- or $\beta$-casein) and bovine serum albumin with molar ratio at 1:100. $\text{Zr}^{4+}$-IMAC adsorbent was also successfully applied for the analysis of mouse liver phosphoproteome, resulting in the identification of 153 phosphopeptides (163 phosphorylation sites) from 133 proteins in mouse liver lysate. Significantly more phosphopeptides were identified than by the conventional $\text{Fe}^{3+}$-IMAC approach, indicating the excellent performance of the $\text{Zr}^{4+}$-IMAC approach. The high specificity of $\text{Zr}^{4+}$-IMAC adsorbent was found to mainly result from the strong interaction between chelating $\text{Zr}^{4+}$ and phosphate group on phosphopeptides. Enrichment of phosphopeptides by $\text{Zr}^{4+}$-IMAC provides a powerful approach for large scale phosphoproteome analysis.


Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, cell cycle, cytoskeletal regulation, and apoptosis. Although phosphorylation is observed on a variety of amino acid residues, by far the most common and important sites of phosphorylation in eukaryotes occur on serine, threonine, and tyrosine residues (1, 2). Because of the importance of protein phosphorylation in cellular signaling, various methods for protein phosphorylation site mapping have been developed through the years. However, this task remains a technical challenge, and there is an intense interest in development of technologies and methods for studying phosphorylation events.

MS has been widely applied as a powerful tool to characterize protein modifications including phosphorylation due to its high sensitivity and capability of rapid sequencing by tandem mass spectrometric (MS)$^2$ technique (3–6). For the phosphoproteome analysis, satisfying results often cannot be obtained by direct mass spectrometric analysis of a protein digest. This is because phosphopeptides are present at low abundance in the digest, and the mass spectrometric response of a phosphopeptide is seriously suppressed by unphosphorylated peptides. To reduce the suppression, it is crucial to purify the phosphorylated peptides from complex peptide mixtures. A number of techniques have been developed to enrich phosphorylated peptides from peptide mixtures. These techniques are immunoprecipitation (7, 8), which is less reliable and more costly; chemical reaction of the phosphate group with an enrichable tag (9, 10) or covalently linking the phosphopeptide to beads and releasing (11, 12), a labor-intensive technique whose performance is compromised by side reactions and incomplete reactions; strong cation exchange chromatography (13, 14), which often results in large scale phosphoprotein identifications but lacks enough specificities; and IMAC (15–19), which is the most frequently used enrichment technique. Recently metal oxide particles, such as titanium dioxide ($\text{TiO}_2$) and $\text{ZrO}_2$, were also reported to have high specificity for phosphopeptides (20–22).

In IMAC, metal ions such as $\text{Fe}^{3+}$ and $\text{Ga}^{3+}$ are bound to beads typically using the immobilized iminodiacetic acid as chelating typically using the immobilized iminodiacetic acid as chelating group (16, 17); phosphopeptides are selectively retained because of the affinity of the metal ions for the phosphate moiety. However the specificity of those IMAC adsor-
bents is still not high enough. Some unphosphorylated peptides (typically acidic peptides) are also strongly bound to the adsorbents, resulting in serious interference for the analysis of target phosphopeptides. The poor specificity for phosphopeptides by IMAC may be partially overcome by esterification of the acidic side chains of glutamate and aspartate residues prior to IMAC purification (23); however, this may also increase sample complexity and interfere with subsequent mass spectrometry analysis because of incomplete reactions. A better and simpler solution for this problem is to develop a new type of IMAC with higher specificity for phosphopeptides. It was reported that self-assembling monolayer treated, BSA, 2,4,6-collidine, zirconyl chloride, and POCl3 were from Sigma. Adult female C57 mice were purchased from Dalian Medical Analysis of mouse liver lysate, and then the total volume was adjusted to 1 ml by adding 10% HAc solution. With the exception that the volume was 1 ml, the washing and elution steps were the same as for standard protein digests. After lyophilization, 5 μl of 0.1% formic acid was added to redissolve the captured phosphopeptides.

Mass Spectrometric Analysis—MALDI-TOF MS experiments were performed on a Bruker Autoflex time-of-flight mass spectrometer (Bruker) in the positive ion linear mode, and each mass spectrum was typically summed with 30 laser shots. An LTQ linear ion trap mass spectrometer (Thermo Electron) with a nanospray source was used with a Finnigan surveyor MS pump (Thermo Electron). The pump flow rate was split by a cross to achieve a flow rate of 200 nl/min. The columns were in-house packed with C18 AQ beads (5 μm, 120 Å) from Michrom BioResources (Auburn, CA) using a pneumatic pump. The separation of phosphopeptides enriched from the tryptic digest of mouse liver lysate was performed using a 75-min linear gradient elution. The mobile phase consisted of mobile phase A, 0.1% formic acid in H2O, and mobile phase B, 0.1% formic acid in acetonitrile. The LTQ instrument was operated in positive ion mode. A voltage of 18 kV was applied to the cross. About 1 μl (20 μg) of redissolved peptides was loaded onto the C18 capillary column using a 75-μm-inner diameter × 220-mm-long capillary column as sample loop. For the detection of phosphopeptides the mass spectrometer was set to perform a full scan MS followed by three data-dependant MS/MS (MS2). Subsequently an MS/MS/MS (MS3) spectrum was automatically triggered when the most three intense peaks from the MS/MS spectrum corresponded to a neutral loss event of 98, 49, and 33 ± 1 Da for the precursor ion with 1+, 2+, and 3+ charge states, respectively.

Database Searching and Data Analysis—The peak lists for MS2 and MS3 spectra were generated from the raw data by Bioworks version 3.2 (Thermo Electron) with the following parameters: mass range was 600–3500, intensity threshold was 1000, and minimum ion count was 10. The generated peak lists were searched by the Sequest program included in Bioworks against the non-redundant mouse protein database (NCBI) (1, pml.MOUSE.3.21.fasta), which included 51,446 entries. The MS/MS spectra were searched with a precursor ion mass tolerance of 2 Da and fragment ion mass tolerance of 1 Da, full tryptic specificity

EXPERIMENT PROCEDURES

Reagents and Materials—α-Casein, β-casein, trypsin (from bovine pancreas, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated), BSA, 2,4,6-collidine, zirconyl chloride, and POCl3 were from Sigma. Adult female C57 mice were purchased from Dalian Medical University (Dalian, China).

Preparation of Protein Samples—For standard proteins, α-casein, β-casein, and BSA were digested in 100 mM NH4HCO3 (pH 8.1) buffer with trypsin at a protein/enzyme ratio of 50:1 by weight and incubated with trypsin at 37 °C for 16 h. Before the digestion, BSA was reduced with DTT and carboxamidomethylated with iodoacetamide. Other standard proteins were digested directly. The protein extract from mice liver was prepared according to a procedure described in detail in our previous reports (3, 26). The Bradford protein assay was used to quantify the concentration of the extracted proteins. The trypsin digestion of the protein extract was the same as that of BSA.

Preparation of Zr4+-IMAC beads—GMA-EDMA polymer beads were synthesized according to a previous report (27). The GMA-EDMA polymer beads were aminated by reaction with 29% ammonium hydroxide solution for 3 h at 40 °C. Then the resulting beads were incubated in a solution of 40 mM POCl3 and 40 mM 2,4,6-collidine in anhydrous acetonitrile for 12 h at ambient temperature to prepare phosphonate-modified beads. After rinsing with CH3CN and water, the beads were incubated in 50 mM ZrOCl2 solution to charge GMA-EDMA polymer beads with Zr4+ ion overnight under gentle stirring. Finally, the Zr4+-IMAC beads were washed with 200 mM NaCl in 10% HAc and deionized water to remove nonspecifically adsorbed Zr4+ cation and dried in vacuum at 60 °C.

Phosphoproteome Analysis—For enrichment of phosphopeptides from standard protein digest, 1 μl (2 pmol) of sample solution was mixed with an aliquot of 10 μl of Zr4+-IMAC bead suspension (10 mg/ml in 100% ACN solution), and then 10% HAc was added to reach a volume of 100 μl. After incubation for 30 min with vibration, beads were centrifuged at 35,000 × g for 10 min and washed with 100 μl of a solution of 10% HAc containing 200 mM NaCl and 100 μl of a solution of 10% HAc, respectively. Finally 100 μl of NH3·H2O (12.5%) was added to elute the captured phosphopeptides. The supernatant containing phosphopeptides was collected and lyophilized to dryness. For all samples for direct analysis with MALDI-TOF MS, 5 μl of dihydroxybenzoic acid solution (25 mg/ml) containing 1% H3PO4 was mixed with lyophilized phosphopeptides, and 0.5 μl of the resulting mixture was deposited on the MALDI target for MALDI MS analysis. For phosphoproteome analysis of mouse liver lysate, 20 mg of Zr4+-IMAC beads were mixed with a digest of 100 μg of mouse liver lysate, and then the total volume was adjusted to 1 ml by adding 10% HAc solution. With the exception that the volume was 1 ml, the washing and elution steps were the same as for standard protein digests. After lyophilization, 5 μl of 0.1% formic acid was added to redissolve the captured phosphopeptides.

Database Searching and Data Analysis—The peak lists for MS2 and MS3 spectra were generated from the raw data by Bioworks version 3.2 (Thermo Electron) with the following parameters: mass range was 600–3500, intensity threshold was 1000, and minimum ion count was 10. The generated peak lists were searched by the Sequest program included in Bioworks against the non-redundant mouse protein database (NCBI) (1, pml.MOUSE.3.21.fasta), which included 51,446 entries. The MS/MS spectra were searched with a precursor ion mass tolerance of 2 Da and fragment ion mass tolerance of 1 Da, full tryptic specificity.

The abbreviations used are: GMA-EDMA, poly(glycidyl methacrylate-co-ethylene dimethacrylate) (GMA-EDMA)1 beads with POCl3 followed with immobilization of Zr4+ using ZrOCl2 solution. The obtained new IMAC beads were first evaluated by using tryptic digests of α- and β-casein as samples, and then they were applied for phosphoproteome analysis of mouse liver.

1 The abbreviations used are: GMA-EDMA, poly(glycidyl methacrylate-co-ethylene dimethacrylate); Xcorr, cross-correlation value; ΔCn, delta Cn value.
GMA-EDMA Phosphonate Zirconium for Phosphoproteome Analysis

was applied, two missed cleavages were allowed, and the static modification was same alkylation of Cys with iodoacetamide (+57).

Fig. 1. Scheme for the preparation of Zr$^{4+}$-IMAC beads.

For the searching with MS/MS data, dynamic modifications were set for oxidized Met (+16) and phosphorylated Ser, Thr, and Tyr (+80). For the searching with MS/MS/MS data, besides the above settings, dynamic modifications were also set for water loss on Ser and Thr (+18). For the identification of phosphoproteptides based only on MS/MS or MS/MS/MS spectra, the following criteria were used for filtering the database searching results: cross-correlation value (Xcorr) >1.9, 2.2, and 3.75 for singly, doubly, and triply charged ions, respectively; delta Cn value ($\Delta$Cn) >0.1. For the phosphopeptide identifications derived from MS/MS or MS/MS/MS spectra, the following criteria were used: Xcorr > 1.5, 2.0, and 2.5; $\Delta$Cn > 0.1. Manual validation was further carried out for peptides passing the above criteria. Criteria used for manual validation included the following. (a) The phosphoric acid neutral loss peak to phosphoserine and phosphothreonine must be the dominant peak. (b) The spectrum must be of good quality with fragment ion clearly above the base-line noise. (c) Sequential members of the b- or y-ion series were observable in the mass spectra. (d) For multiply phosphorylated peptides, the peptides derived from MS$^2$ must be confirmed by MS$^3$ spectra in the same MS cycle. The phosphoproteins identified by the same phosphopeptide(s) were grouped; if the group contained more than one phosphoprotein, then only one was kept according to the method described by He et al. (28) as all proteins in each group are highly homologous, generally belonging to the same superfamily, or just different alternative splicing isoforms.

RESULTS

Preparation and Evaluation of Zr$^{4+}$-IMAC Beads—GMA-EDMA polymer beads have a neutral hydrophilic surface, and so the nonspecific adsorption of biomolecules is very weak. The surface of GMA-EDMA beads also possesses chemically active sites, *i.e.* epoxide groups, for chemical modification that make derivatization with other functional groups easy. In our previous studies, GMA-EDMA monolithic polymers prepared in an HPLC column or capillary column were used as a support for affinity chromatography (29, 30) and enzyme reactor (31). Here GMA-EDMA beads were used in the preparation of Zr$^{4+}$-IMAC adsorbents for phosphopeptide enrichment. As shown in Fig. 1, epoxide groups on the polymer surface were first transferred to amino groups by incubation in ammonium hydroxide solution, then phosphonate groups were introduced by reaction of amino groups with POCl$_3$, and finally Zr$^{4+}$ was immobilized by incubation of the modified beads in ZrOCl$_2$ solution. Compared with inorganic supports such as silica beads, GMA-EDMA polymer beads have the advantage of good chemical stability in a wider pH range. They are inert at extreme pH values. This is important for phosphopeptide enrichment because samples are typically loaded at low pH, and the captured phosphopeptides are eluted at high pH.

Because of its well-characterized five phosphorylation sites at serine residues, bovine $\beta$-casein was used to evaluate the performance of prepared Zr$^{4+}$-IMAC beads for enrichment of phosphopeptides. A trypsin digest from 2 pmol of $\beta$-casein was pretreated by the Zr$^{4+}$-IMAC beads. After thoroughly washing, the captured phosphopeptides were eluted for MALDI-TOF MS analysis. For comparison, direct analysis of a $\beta$-casein digest was also performed by MALDI-TOF MS. The obtained spectra are presented in Fig. 2. The direct analysis resulted in detection of many intense peaks of non-phosphopeptides besides two phosphopeptide peaks with weak intensity (Fig. 2A), whereas the analysis of the peptides eluted from Zr$^{4+}$-IMAC beads yielded only five dominant phosphopeptide peaks (Fig. 2B), $\beta1$ (m/z 2061) and $\beta2$ (m/z 2556) are singly phosphorylated peptides, and $\beta3$ (m/z 3122) is a quadruply phosphorylated peptide. The other three peaks represent $\beta3$ losing one H$_3$PO$_4$, losing two H$_3$PO$_4$, and its doubly charged ion, respectively. The sequences of phosphopeptides are given in Supplemental Table 1. The non-phosphopeptides were not observed in Fig. 2B, indicating the high specificity of Zr$^{4+}$-IMAC beads for phosphopeptides.

The same amount of sample was also pretreated by GMA-EDMA beads and phosphonate-modified GMA-EDMA beads, respectively, using the same procedures as those used for Zr$^{4+}$-IMAC beads, and the eluted samples were also analyzed by MALDI-TOF MS to investigate the nonspecific adsorption of peptides on the raw and intermediate beads. No peaks were observed for the eluted fractions in both cases (Fig. 2, C and D), also indicating that there was not obvious nonspecific adsorption of peptides on either GMA-EDMA beads or phosphonate-modified GMA-EDMA beads. Obviously neither GMA-EDMA nor phosphonate-modified GMA-EDMA beads have affinity for phosphopeptides; the capture of phosphopeptides by Zr$^{4+}$-IMAC beads mainly results from the strong interaction between chelating Zr$^{4+}$ and phosphate groups on the phosphopeptides.
Tryptic digests of $\alpha$-casein were also pretreated by Zr$^{4+}$-IMAC beads using the procedures described above, and the obtained MALDI mass spectra before and after Zr$^{4+}$-IMAC enrichment are shown in Fig. 3, A and B. In total 12 phosphopeptides from the tryptic digest of $\alpha$-casein were observed after Zr$^{4+}$-IMAC enrichment (Fig. 3B), whereas only nine phosphorylated peptides with weak signals were observed by direct analysis (Fig. 3A). This further demonstrated that the enrichment of phosphopeptides by Zr$^{4+}$-IMAC is very effective. For the comparison, enrichment of phosphopeptides from a tryptic digest of $\beta$-casein was also performed by using the commercial IMAC resin POROS 20 MC chelated with Fe$^{3+}$. The enrichment of phosphopeptides by Fe$^{3+}$-IMAC was performed according to the procedures by Ficarro et al. (23) and improved protocol by Kokubu et al. (19) except that NH$_3$H$_2$O (12.5%) was used as elution buffer instead of 50 mM Na$_2$HPO$_4$ (pH 9.0) and 0.1% phosphoric acid. Only six phosphopeptides were detected by MALDI in the former case (data not shown). In the latter case with the improved protocol, the performance of Fe$^{3+}$-IMAC was improved dramatically; 10 phosphopeptides were observed as shown in Fig. 3C. But compared with the mass spectra of phosphopeptides using Zr$^{4+}$-IMAC beads, fewer phosphopeptide peaks were detected, and the selectivity of Fe$^{3+}$-IMAC for phosphopeptides is much lower than that of Zr$^{4+}$-IMAC.

**Purification of Phosphopeptides from Peptide Mixture Using Zr$^{4+}$-IMAC Beads**—The previous enrichment of phosphopeptides by using Zr$^{4+}$-IMAC beads was performed with peptides derived from a single protein, but phosphoproteins are often low abundance components in real biological protein samples. To further investigate the capability of the Zr$^{4+}$-
IMAC beads to enrich low abundance phosphopeptides, an α-casein or β-casein trypic digest was mixed with a BSA trypic digest in different molar ratios of 1:0, 1:1, and 1:100 (α-or β-casein versus BSA) as semicomplex samples. The phosphopeptides were enriched by the beads and then analyzed by MALDI-TOF MS. Peaks of 14 phosphopeptides, varying from singly to multiply phosphorylated peptides, were found when the molar ratio of α-casein and BSA was at 1:1 (Fig. 4B); the results are similar to the results obtained from using only α-casein as sample (Fig. 4A). When the molar ratio of α-casein and BSA further decreased to 1:100, the data obtained (Fig. 4C) do not differ drastically from those obtained at 1:0 and 1:1. In total 13 peaks were observed; only one phosphopeptide (YLGEYLVpNSAEER where pS is phosphoserine), which is a weak peak in the mass spectra shown in Fig. 4, A and B, disappeared. Considering that the concentration of α-casein digest is 100 times lower than that of BSA, the specificity of Zr4+-IMAC beads for isolation of phosphopeptides is very high.

As phosphorylation sites in α-casein and β-casein are all at serine residues, the previous results demonstrated that Zr4+-IMAC beads have high specificity to phosphoserine peptides. To evaluate whether the Zr4+-IMAC beads can selectively enrich other type of phosphopeptides, a singly tyrosine phosphorylated peptide, RRLIEDApYAARG (where pY is phosphotyrosine; molecular weight, 1599; Upstate), was added to a trypic digest of β-casein in a 1:1 molar ratio. The phosphopeptides were enriched by the Zr4+-IMAC beads and then analyzed by MALDI-TOF MS. When only mixture of β-casein digest and the phosphotyrosine peptide was pretreated, five dominant peaks, representing four phosphoserine peptides from β-casein (β1, β3, β4, and β5) and one phosphotyrosine peptide (pY), can be observed (Fig. 5A). For the complex sample with the addition of BSA digest in a 1:100 molar ratio, the five peaks including the phosphotyrosine peptide (pY) also can be easily observed (Fig. 5B). The results further demonstrate that Zr4+-IMAC beads do have high specificity for enrichment of different types of phosphopeptides without bias.

The ability to specifically enrich phosphopeptides in the presence of a huge amount of unphosphorylated peptides is a key issue for phosphoproteome analysis. However, Fe3+-IMAC lacks enough specificity to selectively capture phosphopeptides from a complex peptide mixture. Larsen et al. (20) investigated the performance of Fe3+-IMAC to enrich phosphopeptides from the digest mixture of phosphoproteins (α-casein, β-casein, and ovalbumin) with non-phosphoproteins (serum albumin, β-lactoglobulin, and carbonic anhydrase) at different ratios. It was found that phosphopeptides could be selectively captured in the digest mixture of phosphoproteins to non-phosphoproteins at a molar ratio of 1:1. However, when the ratio decreased to 1:10, a significant number of non-phosphorylated peptides were observed, and the number of detected phosphopeptides decreased quickly. When the ratio decreased to 1:50, the peaks of phosphopeptides could hardly be observed. Similar results were also
Selective enrichment of phosphopeptides from the mixture of the tryptic digest of β-casein and a standard phosphotyrosine peptide (pY) using Zr$^{4+}$-IMAC beads. A, β-casein digest with phosphotyrosine peptide (2 pmol, respectively); B, β-casein digest and phosphotyrosine peptide (2 pmol, respectively) were mixed with 200 pmol of BSA tryptic digest. The sequence of the phosphotyrosine peptide was RRLIEDAEpYAARG.

Application of Zr$^{4+}$-IMAC Beads for Phosphoproteome Analysis of Mouse Liver—To further evaluate their performance for the capture of phosphopeptides, Zr$^{4+}$-IMAC beads were applied to analyze the phosphoproteome of mouse liver. Phosphopeptides from 100 μg of mouse liver lysate digest were enriched, and one-fifth was loaded onto a capillary C$_{18}$ column and analyzed by an LTQ mass spectrometer. Three replicate LC-MS runs were conducted for each sample. The acquired MS/MS spectra were searched by the Sequest program, and the search results were filtered with Xcorr (>1.9, 2.2, and 3.75 for 1+, 2+, and 3+ charged peptides) and ΔCn (>0.1) criteria. For the three replicate analyses, in total 1681 unique peptides were identified with an average of 109 non-phosphopeptides and 539 phosphopeptides including 137 singly, 174 doubly, and 227 triply phosphorylated phosphopeptides in each run. Among 1681 unique peptides, 87.2% were phosphopeptides, and only 12.8% were non-phosphopeptides. The very low percentage of identified non-phosphopeptides indicated that the nonspecific adsorption of non-phosphopeptides on Zr$^{4+}$-IMAC beads is weak.

The criteria used to filter MS$^3$ database search results in this study were originally used for processing unmodified peptide data (28, 32), and they were also used recently for processing phosphorylated peptide data (33). To the best of our knowledge, the criteria are the highest for processing phosphorylated peptide data searched by the Sequest program. However, because of the poor quality of the spectra for phosphopeptides, the scores of a spectrum passing the criteria do not necessarily mean true identification. Other confirmation is required to increase the confidence of the identification. The fragment ion generated by phosphate loss in the MS/MS stage can be further fragmented to generate an MS/MS/MS (MS$^3$) spectrum. It was observed previously that MS$^3$ spectra are useful for the validation of phosphopeptides identified from MS$^2$ spectra (3, 33, 34). In this study, the acquired MS$^3$ spectra were also searched by the Sequest program. Because the peptide identifications derived from MS$^3$ were used to confirm the identifications derived from MS$^2$, relatively poor spectra should be allowed. Therefore, relatively low criteria with Xcorr (>1.5, 2.0, and 2.5 for 1+, 2+, and 3+ charged peptides) and ΔCn (>0.1) were used to filter the MS$^3$ database search results, resulting in the identification of 244 unique phosphopeptides and three non-phosphopeptides. Comparing the phosphopeptides identified from MS$^2$ and MS$^3$ spectra, it was found that the sequences of 50 unique phosphopeptides were the same in both cases (34 singly phosphorylated and 16 multiply phosphorylated). The sequences of these peptides and their Xcorr scores are listed in Supplemental Table 2. The majority of these peptides have very high Xcorr scores. After manually checking, it was found that their spectra were of high quality, and so their identifications were considered as positive. These results indicated that confirmation of phosphopeptide identifications by MS$^3$ data is very effective. Fig. 6 is an example for identification of triply charged phosphopeptides from Septin-2, IYHLPDAGPSDEDEDKFEQTR, in which Ser$^{218}$ is phosphorylated. From the spectra it can be seen that b- and y-ion series are consistent with the theoretically predicted peaks in both MS$^2$ and MS$^3$ spectra, and in the MS$^3$ spectrum the peak at m/z 807.13 represents the triply charged form of the selected precursor ion at m/z 839.86 by losing an H$_3$PO$_4$ group. This phosphorylated site has not been previously reported in the literature. The high quality of MS/MS and MS/MS/MS spectra showed the high performance of Zr$^{4+}$-IMAC beads for the capture of phosphopeptides.
In addition to the 50 phosphopeptides, there should be more phosphopeptides identified only from MS2. Without the confirmation with MS3 data, these identifications should be carefully manually validated. Many multiply phosphorylated peptide identifications were also observed after filtering with Xcorr and \( \Delta \text{Cn} \) criteria. However, until now strictly universal validation criteria have not been established and defined. There are too many subjective factors for interpretation of the spectra of multiply phosphorylated peptides; therefore only singly phosphorylated peptide identifications were validated manually in this study. After manual validation, an additional 92 singly phosphorylated peptides were finally identified from MS2 spectra. Some MS3 spectra may yield significantly more structural information than the corresponding MS2 spectra, which are usually dominated by phosphate loss. It was reported that phosphopeptides can also be confidently identified by MS3 spectra (3, 33, 34). The MS3 database search results were also filtered with the strict criteria, i.e. Xcorr (>1.9, 2.2, and 3.75 for 1+, 2+, and 3+ charged peptides) and \( \Delta \text{Cn} \) (>0.1). The singly phosphorylated peptides were further manually validated, resulting in the identification of 11 phosphopeptides. The sequences and Xcorr scores for the phosphopeptides finally identified from MS2 and MS3 are given in Supplemental Tables 3 and 4, respectively. Combined with the phosphopeptides identified by MS2 and confirmed by MS3 (Supplemental Table 2), in total 153 phosphopeptides (163 phosphorylated sites) corresponding to 133 phosphorylated proteins from mouse liver were identified.
Among them, 137 peptides were singly phosphorylated, 13 were doubly phosphorylated, and three were triply phosphorylated. (The MS² and MS³ spectra with labeled fragment peaks for each peptide are listed in Supplemental Material 2.)

To characterize the phosphorylation sites identified in our study, we used Scansite (35) to define possible phosphorylation motifs in our data set. By searching the data with a high stringency cutoff filter, 38 of the confidently localized sites could be assigned with kinase motifs. Most of them are from the basophilic serine/threonine kinase group (Supplemental Table 5). To further investigate the reliability of the results, PhosphoSite (Cell Signaling Technology) was used to distinguish known phosphorylation sites. Among the 163 phosphorylated sites identified, 73.0% (119 sites) have not been reported previously, and 27.0% (44 sites) were already reported in the literature. Many new phosphorylation sites identified by MS²/MS³ spectra are known phosphorylation sites, including 33 sites reported in 50 phosphopeptides, 137 peptides were singly phosphorylated, 13 were doubly phosphorylated, and three were triply phosphorylated. (The MS² and MS³ spectra with labeled fragment peaks for each peptide are listed in Supplemental Material 2.)

The specificity of phosphopeptide enrichment is crucial for successful phosphoproteome analysis: the fewer non-phosphopeptides present, the higher the sensitivity for the detection of phosphopeptides. Phosphopeptide enrichment by Zr⁴⁺-IMAC beads followed with LC-MS/MS (MS²/MS³/MS⁴) analysis allowed the identification of 153 phosphopeptides from 133 phosphoproteins from 100 μg of lysate of mouse liver tissue by three separate runs. Among the 153 identified phosphopeptides, 137 peptides were singly phosphorylated peptides. Besides using Zr⁴⁺-IMAC, different types of Fe³⁺-IMAC adsorbents were also applied for phosphoproteome analysis of mouse liver in our laboratory, including a capillary column packed with commercial IMAC beads (POROS 20 MC beads) (3), a self-prepared Fe³⁺-IMAC silica monolithic capillary column (33), and a self-prepared Fe³⁺-IMAC mesoporous molecular sieve MCM-41 (36). Correspondingly a total of 26, 29, and 33 singly phosphorylated peptides were identified with a single LC-MS run after manual validation, respectively. The number of identified singly phosphorylated phosphopeptides by the Zr⁴⁺-IMAC approach (an average of 61 singly phosphorylated peptides for each run) was significantly higher than that by any of the above mentioned Fe³⁺-IMAC approaches. The high performance is largely because Zr⁴⁺-IMAC beads can, with high specificity, enrich phosphopeptides, effectively avoiding the suppression of non-phosphopeptides during MS detection. As many as 50 unique phosphopeptides were identified from both MS/MS and MS/MS/MS spectra with the Zr⁴⁺-IMAC approach. This also indicated that phosphopeptides could be efficiently enriched by Zr⁴⁺-IMAC beads and therefore high quality MS/MS/MS spectra were acquired.

The specificity of Fe³⁺-IMAC could be improved by esterification of acidic groups on peptide molecules prior to IMAC purification (23, 37). Moser and White (18) have applied this approach to phosphoproteome analysis of the rat liver phosphoproteome. It was found that only about 30% of identified peptides were phosphorylated if no esterification was performed, and the percentage increased to 85% when esterification was conducted. In our case by using Zr⁴⁺-IMAC beads, 87.2% of the peptides identified from MS² spectra were phosphorylated peptides. The percentage of identified phosphopeptides by using Zr⁴⁺-IMAC beads was much higher than the equivalent method using Fe³⁺-IMAC beads and was similar to the method using a combination of peptide esterification with Fe³⁺-IMAC beads. This means that the specificity of Zr⁴⁺-IMAC beads was much higher than that of Fe³⁺-IMAC and was similar to that of Fe³⁺-IMAC beads combined with peptide esterification. The disadvantage of peptide esterification prior to IMAC purification is that the products resulting from side reactions and incomplete reactions may increase sample complexity and interfere with subsequent mass spectrometry analysis. The use of Zr⁴⁺-IMAC beads is much simpler because no additional sample pretreatment is required.

Metal oxides such as TiO₂ and ZrO₂ beads were introduced as affinity materials to selectively enrich phosphopeptides (20, 21). The microcolumns or microtips packed with these metal oxides beads have been proven to have higher selectivity for trapping of phosphopeptides than those packed with conventional Fe³⁺-IMAC beads. The mechanisms of ZrO₂ and Zr⁴⁺-IMAC beads for selective capture of phosphopeptides are similar. Both of them are based on the strong interaction between the zirconium atom and phosphate groups on phosphopeptides. We also demonstrated that ZrO₂ nanobeads have high specificity for phosphopeptides (38). ZrO₂ nanobeads were also used to enrich phosphopeptides from the digest of mouse liver lysate in our laboratory. After LC-MS (MS² and MS³) analysis of the enriched phosphopeptides and manual validation of database search results, in total 141
phosphorylated peptides including 48 singly phosphorylated phosphopeptides were identified. The number of validated singly phosphorylated peptides was much lower than that in this study using Zr⁴⁺-IMAC. To further compare the specificities of ZrO₂ and Zr²⁺-IMAC, the percentage of phosphopeptides in the MS/MS database search results (filtered with Xcorr and ΔCn criteria but without manual validation) obtained using ZrO₂ nanobeads was calculated to be 72%, which was lower than that of Zr⁴⁺-IMAC (87%). The above results demonstrated that Zr⁴⁺-IMAC has higher selectivity for phosphopeptides than do ZrO₂ nanobeads. The different performance between ZrO₂ beads and GMA-EDMA phosphate Zr⁴⁺ beads (Zr⁴⁺-IMAC beads) may be due to two reasons. First, GMA-EDMA beads do not have strong Lewis acid sites on the surface as do ZrO₂ beads; therefore the surface of GMA-EDMA beads is more biocompatible, and there is less nonspecific adsorption. Second, there is a spacer arm between the polymer support and Zr⁴⁺ that allows the phosphate groups on phosphopeptides to have a better chance to access Zr⁴⁺ on GMA-EDMA beads.

Recently TiO₂ has been used as an alternative to IMAC for the selective enrichment of phosphopeptides both from simple protein samples to complex biosamples (21, 39–42). Larsen et al. (20) probed the performance of a TiO₂ microcolumn to enrich phosphopeptides from the mixture of phosphoproteins (α-casein, β-casein, and ovalbumin) with non-phosphoproteins (serum albumin, β-lactoglobulin, and carbonic anhydrase) at different molar ratios. It was found that when the molar ratio of phosphopeptide to non-phosphopeptide decreased to 1:10, the number of non-phosphorylated peptides could be well detected; when it decreased to 1:50, a number of non-phosphorylated peptides were also detected. However, when the ratio decreased to 1:100 in our case, peaks of non-phosphorylated peptides were hardly observed (as shown in Figs. 4 and 5), indicating a much more selective binding of the phosphorylated peptides on the Zr⁴⁺-IMAC beads than on the TiO₂ beads.

Kweon and Hakansson (21) investigated the performance of ZrO₂ and TiO₂ microtits for trypsin and Glu-C proteolytic digests of α-casein and β-casein and compared binding specificity and recovery of phosphopeptide by ZrO₂ with those by TiO₂. It was demonstrated that ZrO₂ microtits display overall performance similar to TiO₂ microtits. However, more selective isolation of singly phosphorylated peptides was observed with ZrO₂ compared with TiO₂, whereas TiO₂ preferentially enriched multiply phosphorylated peptides. Because Zr⁴⁺-IMAC has higher selectivity than ZrO₂ in our case, Zr⁴⁺-IMAC may also have higher selectivity than TiO₂. However, further experiments are needed to prove this allegation. Because there is no spacer arm on metal oxide beads, one disadvantage of using metal oxide beads for phosphopeptide enrichment is the presence of steric hindrance.

In conclusion, a new generation of IMAC absorbent for high specific enrichment of phosphopeptides for phosphoproteome analysis has been presented. The IMAC beads were prepared by immobilization of Zr⁴⁺ on the phosphate-modified GMA-EDMA polymer beads. Zr⁴⁺-IMAC beads were demonstrated to have high specificity to phosphopeptides by using standard phosphoproteins as well as a real biological sample. Esterification prior to IMAC purification is not necessary for Zr⁴⁺-IMAC because of its excellent specificity. Zr⁴⁺-IMAC beads prepared in this study have both the high selectivity of the Zr⁴⁺ ion and the high biocompatibility and chemical stability of the GMA-EDMA polymer and are ideal IMAC adsorbents for purification of phosphopeptides in phosphoproteome analysis.

* This work was supported by National Natural Sciences Foundation of China Grant 20675081, China State Key Basic Research Program Grant 2005CB522701, China High Technology Research Program Grant 2006AA02A309, the Knowledge Innovation program of the Chinese Academy of Sciences (Grant KJCX2.YW.H09) and the Knowledge Innovation program of the Dalian Institute of Chemical Physics (to H. Z.), and National Natural Sciences Foundation of China Grant 20605022 (to M. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES