

# Immobilized Zirconium Ion Affinity Chromatography for Specific Enrichment of Phosphopeptides in Phosphoproteome Analysis\*<sup>§</sup>

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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 stoichiometry 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. *Molecular & Cellular Proteomics* 6:1656–1665, 2007.

Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, cell cycle, cytoskeletal regulation, and apo-

ptosis. 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 ( $\text{MS}^n$ ) 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 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-

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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 and multilayer thin films of phosphate-containing organic molecules can be prepared based on the strong interaction between phosphate groups on target molecules with zirconium ( $Zr^{4+}$ ) phosphonate on the surface of a solid matrix (24–26). Taking advantage of the strong interaction, we have developed a porous silicon wafer with its surface chemically modified with zirconium phosphonate for highly specific capture of phosphopeptides followed with direct MALDI-TOF MS analysis (26). It was observed that the selectivity of the zirconium phosphonate-modified surface was higher than that of conventional  $Fe^{3+}$ -IMAC beads. Although the chip-based technique is very convenient for MALDI analysis, the amount of captured phosphopeptides is not adequate for LC-MS/MS analysis in phosphoproteomics research. Here a novel  $Zr^{4+}$ -IMAC adsorbent was prepared by modifying poly(glycidyl methacrylate-co-ethylene dimethacrylate) (GMA-EDMA)<sup>1</sup> beads with  $POCl_3$  followed with immobilization of  $Zr^{4+}$  using  $ZrOCl_2$  solution. The obtained new IMAC beads were first evaluated by using tryptic digests of  $\alpha$ - and  $\beta$ -casein as samples, and then they were applied for phosphoproteome analysis of mouse liver.

#### EXPERIMENT PROCEDURES

**Reagents and Materials**— $\alpha$ -Casein,  $\beta$ -casein, trypsin (from bovine pancreas, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated), BSA, 2,4,6-collidine, zirconyl chloride, and  $POCl_3$  were from Sigma. Adult female C57 mice were purchased from Dalian Medical University (Dalian, China).

**Preparation of Protein Samples**—For standard proteins,  $\alpha$ -casein,  $\beta$ -casein, and BSA were digested in 100 mM  $NH_4HCO_3$  (pH 8.1) buffer with trypsin at a protein/enzyme ratio of 50:1 by weight and incubated 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 livers 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  $Zr^{4+}$ -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% ammo-

nium hydroxide solution for 3 h at 40 °C. Then the resulting beads were incubated in a solution of 40 mM  $POCl_3$  and 40 mM 2,4,6-collidine in anhydrous acetonitrile for 12 h at ambient temperature to prepare phosphonate-modified beads. After rinsing with  $CH_3CN$  and water, the beads were incubated in 50 mM  $ZrOCl_2$  solution to charge GMA-EDMA polymer beads with  $Zr^{4+}$  ion overnight under gentle stirring. Finally the  $Zr^{4+}$ -IMAC beads were washed with 200 mM NaCl in 10% HAc and deionized water to remove nonspecifically adsorbed  $Zr^{4+}$  cation and dried in vacuum at 60 °C.

**Enrichment of Phosphopeptides by  $Zr^{4+}$ -IMAC Beads**—For enrichment of phosphopeptides from standard protein digest, 1  $\mu$ l (2 pmol) of sample solution was mixed with an aliquot of 10  $\mu$ l of  $Zr^{4+}$ -IMAC bead suspension (10 mg/ml in 100% ACN solution), and then 10% HAc was added to reach a volume of 100  $\mu$ l. After incubation for 30 min with vibration, beads were centrifuged at  $35,000 \times g$  for 10 min and washed with 100  $\mu$ l of a solution of 10% HAc containing 200 mM NaCl and 100  $\mu$ l of a solution of 10% HAc, respectively. Finally 100  $\mu$ l of  $NH_3 \cdot H_2O$  (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  $\mu$ l of dihydroxybenzoic acid solution (25 mg/ml) containing 1%  $H_3PO_4$  was mixed with lyophilized phosphopeptides, and 0.5  $\mu$ l of the resulting mixture was deposited on the MALDI target for MALDI MS analysis. For phosphoproteome analysis of mouse liver lysate, 20 mg of  $Zr^{4+}$ -IMAC beads were mixed with a digest of 100  $\mu$ g 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  $\mu$ l 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  $C_{18}$  AQ beads (5  $\mu$ m, 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  $H_2O$ , and mobile phase B, 0.1% formic acid in acetonitrile. The LTQ instrument was operated in positive ion mode. A voltage of 1.8 kV was applied to the cross. About 1  $\mu$ l (20  $\mu$ g) of redissolved peptides was loaded onto the  $C_{18}$  capillary column using a 75- $\mu$ m-inner diameter  $\times$  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 ( $MS^2$ ). Subsequently an MS/MS/MS ( $MS^3$ ) 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  $33 \pm 1$  Da for the precursor ion with 1+, 2+, and 3+ charge states, respectively.

**Database Searching and Data Analysis**—The peak lists for  $MS^2$  and  $MS^3$  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 of the mouse International Protein Index (IPI) (ipi.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

<sup>1</sup> The abbreviations used are: GMA-EDMA, poly(glycidyl methacrylate-co-ethylene dimethacrylate); Xcorr, cross-correlation value;  $\Delta$ Cn, delta Cn value.

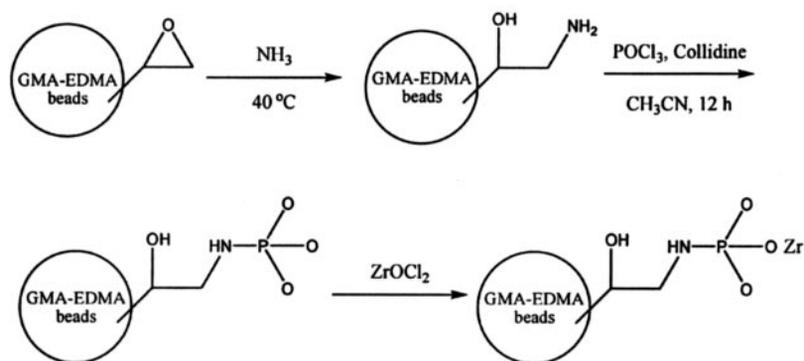


FIG. 1. Scheme for the preparation of Zr<sup>4+</sup>-IMAC beads.

was applied, two missed cleavages were allowed, and the static modification was set for alkylation of Cys with iodoacetamide (+57). 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 phosphopeptides 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/MS spectra used to validate the MS/MS identifications, 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<sup>2</sup> must be confirmed by MS<sup>3</sup> 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.

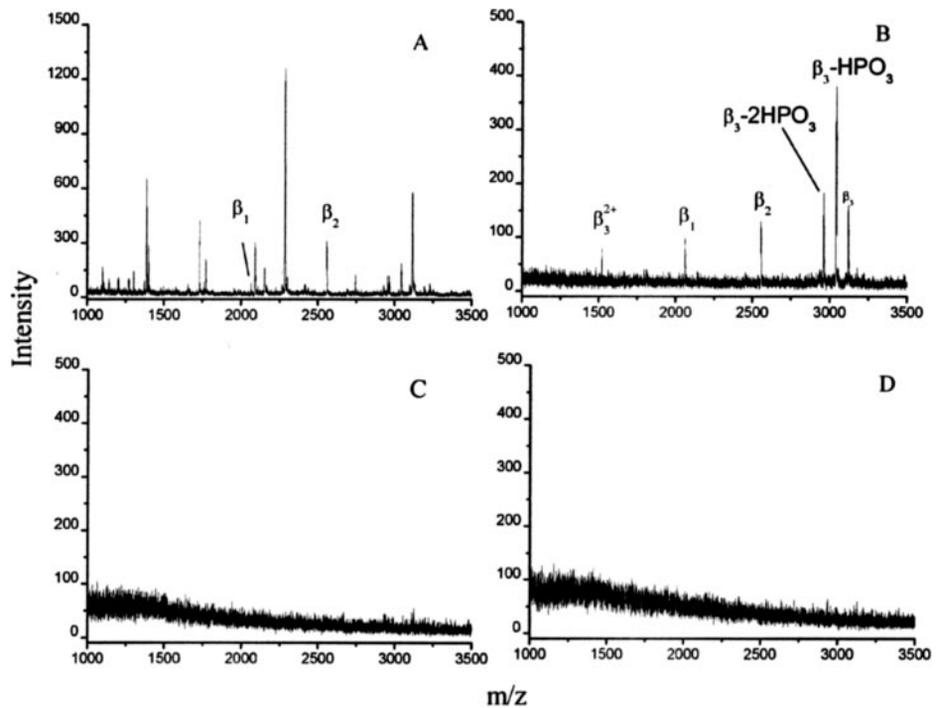
## RESULTS

**Preparation and Evaluation of Zr<sup>4+</sup>-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<sup>4+</sup>-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<sub>3</sub>, and finally Zr<sup>4+</sup> was immobilized by incubation of the modified beads in ZrOCl<sub>2</sub> 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<sup>4+</sup>-IMAC beads for enrichment of phosphopeptides. A tryptic digest from 2 pmol of  $\beta$ -casein was pretreated by the Zr<sup>4+</sup>-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<sup>4+</sup>-IMAC beads yielded only five dominant phosphopeptide peaks (Fig. 2B).  $\beta$ 1 (*m/z* 2061) and  $\beta$ 2 (*m/z* 2556) are singly phosphorylated peptides, and  $\beta$ 3 (*m/z* 3122) is a quadruply phosphorylated peptide. The other three peaks represent  $\beta$ 3 losing one H<sub>3</sub>PO<sub>4</sub>, losing two H<sub>3</sub>PO<sub>4</sub>, 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<sup>4+</sup>-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<sup>4+</sup>-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<sup>4+</sup>-IMAC beads mainly results from the strong interaction between chelating Zr<sup>4+</sup> and phosphate groups on the phosphopeptides.

FIG. 2. MALDI mass spectra of tryptic digest of  $\beta$ -casein. A, direct analysis; B, analysis after pretreatment by  $Zr^{4+}$ -IMAC beads; C, analysis after pretreatment by GMA-EDMA polymer beads; D, analysis after pretreatment by GMA-EDMA phosphonate polymer beads. The loading amount of tryptic digest of  $\beta$ -casein was 2 pmol.



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  $\alpha$ -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  $NH_3 \cdot H_2O$  (12.5%) was used as elution buffer instead of 50 mM  $Na_2HPO_4$  (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  $Zr^{4+}$ -

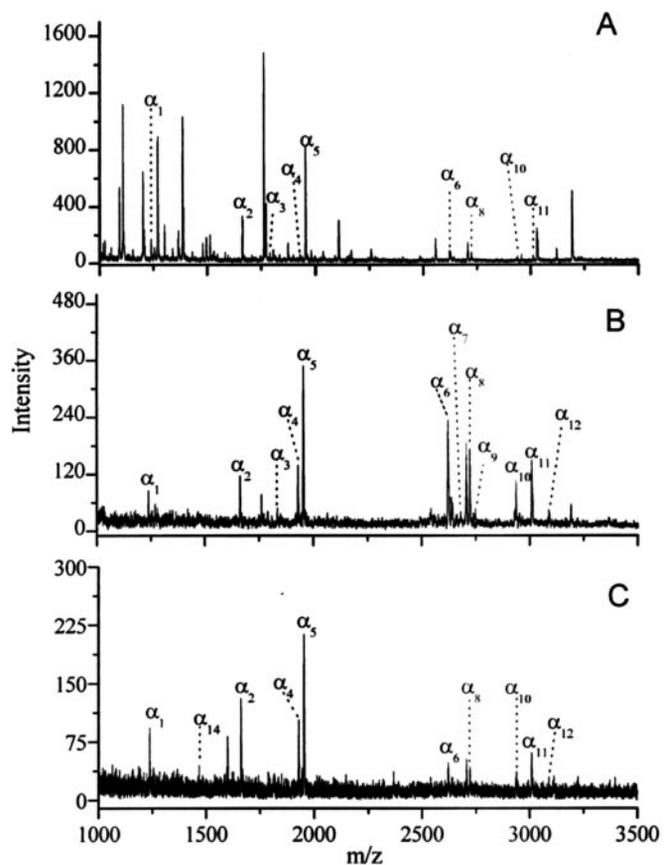
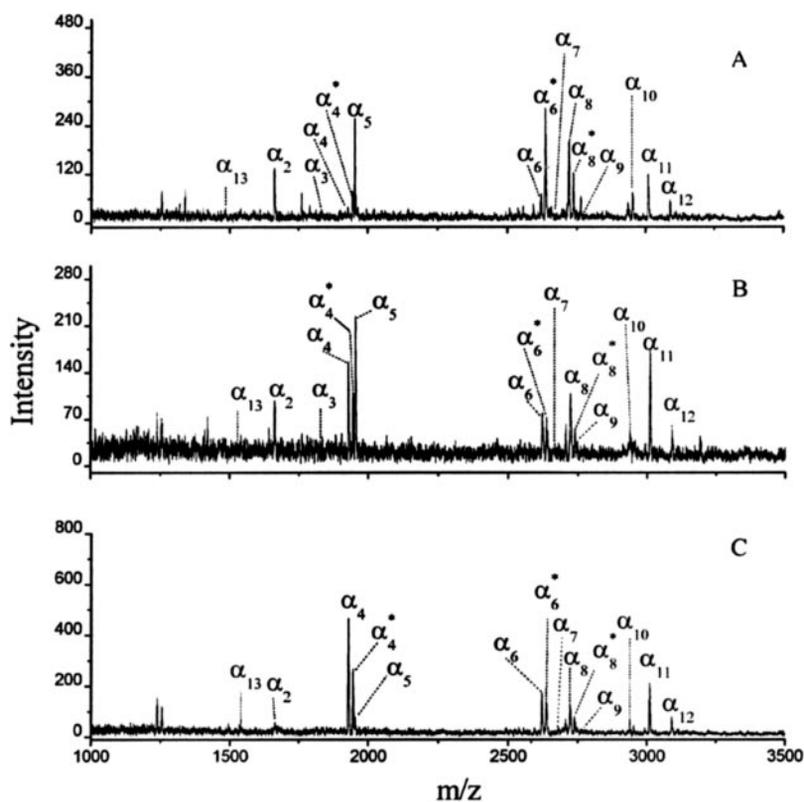


FIG. 3. MALDI mass spectra of the tryptic digest of  $\alpha$ -casein. A, direct analysis; B, analysis after enrichment by  $Zr^{4+}$ -IMAC beads; C, analysis after enrichment by  $Fe^{3+}$ -IMAC. The loading amount of tryptic digest of  $\alpha$ -casein was 2 pmol.

FIG. 4. Selective enrichment of phosphopeptides from the mixture of the tryptic digest of  $\alpha$ -casein and BSA in different molar ratios: 1:0 (A), 1:1 (B), and 1:100 (C). The loading amount of tryptic digest of  $\alpha$ -casein was 2 pmol. \*, phosphopeptides with oxidized Met residue.



IMAC beads to enrich low abundance phosphopeptides, an  $\alpha$ -casein or  $\beta$ -casein tryptic digest was mixed with a BSA tryptic digest in different molar ratios of 1:0, 1:1, and 1:100 ( $\alpha$ - or  $\beta$ -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  $\alpha$ -casein and BSA was at 1:1 (Fig. 4B); the results are similar to the results obtained from using only  $\alpha$ -casein as sample (Fig. 4A). When the molar ratio of  $\alpha$ -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 (YLGEYLIVPNpSAEER 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  $\alpha$ -casein digest is 100 times lower than that of BSA, the specificity of  $Zr^{4+}$ -IMAC beads for isolation of phosphopeptides is very high.

As phosphorylation sites in  $\alpha$ -casein and  $\beta$ -casein are all at serine residues, the previous results demonstrated that  $Zr^{4+}$ -IMAC beads have high specificity to phosphoserine peptides. To evaluate whether the  $Zr^{4+}$ -IMAC beads can selectively enrich other type of phosphopeptides, a singly tyrosine phosphorylated peptide, RRLIEDAepYAARG (where pY is phosphotyrosine; molecular weight, 1599; Upstate), was added to a tryptic digest of  $\beta$ -casein in a 1:1 molar ratio. The phosphopeptides were enriched by the  $Zr^{4+}$ -IMAC beads and then

analyzed by MALDI-TOF MS. When only mixture of  $\beta$ -casein digest and the phosphotyrosine peptide was pretreated, five dominant peaks, representing four phosphoserine peptides from  $\beta$ -casein ( $\beta 1$ ,  $\beta 3$ ,  $\beta 4$ , and  $\beta 5$ ) 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  $Zr^{4+}$ -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,  $Fe^{3+}$ -IMAC lacks enough specificity to selectively capture phosphopeptides from a complex peptide mixture. Larsen *et al.* (20) investigated the performance of  $Fe^{3+}$ -IMAC to enrich phosphopeptides from the digest mixture of phosphoproteins ( $\alpha$ -casein,  $\beta$ -casein, and ovalbumin) with non-phosphoproteins (serum albumin,  $\beta$ -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

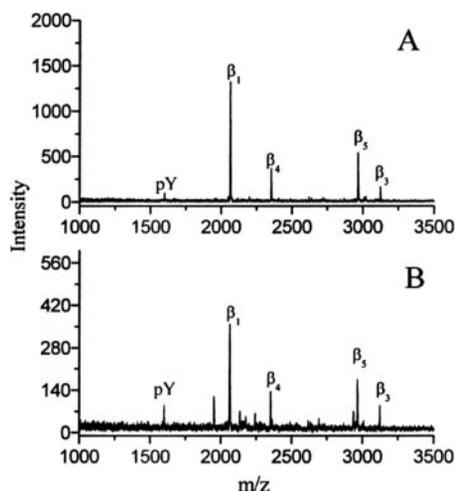


FIG. 5. Selective enrichment of phosphopeptides from the mixture of the tryptic digest of  $\beta$ -casein and a standard phosphotyrosine peptide (pY) using  $Zr^{4+}$ -IMAC beads. A,  $\beta$ -casein digest with phosphotyrosine peptide (2 pmol, respectively); B,  $\beta$ -casein digest and phosphotyrosine peptide (2 pmol, respectively) were mixed with 200 pmol of BSA tryptic digest. The sequence of the phosphotyrosine peptide was RRLIEDAEpYAARG.

observed in our previous works (26). Phosphopeptides cannot be specifically captured by  $Fe^{3+}$ -IMAC from a digest mixture of  $\beta$ -casein and BSA with a ratio of 1:10. However, as can be seen from Fig. 4, phosphopeptides in a complex peptide mixture can be specifically isolated and well detected by the  $Zr^{4+}$ -IMAC beads prepared in this study. Those results demonstrated that  $Zr^{4+}$ -IMAC has much higher selectivity for enrichment of phosphopeptides than conventional  $Fe^{3+}$ -IMAC. The ability of  $Zr^{4+}$ -IMAC to specifically enrich phosphopeptides from the peptide mixture in which the concentrations of non-phosphorylated peptides were several orders of magnitude higher than that of phosphorylated peptides is largely a result of the extremely strong interaction between chelating  $Zr^{4+}$  and phosphate groups in phosphopeptides. As was mentioned before, the interaction is so strong that stable self-assembling monolayer or multilayer thin films of phosphate-containing organic molecules could be prepared this way (24–26).

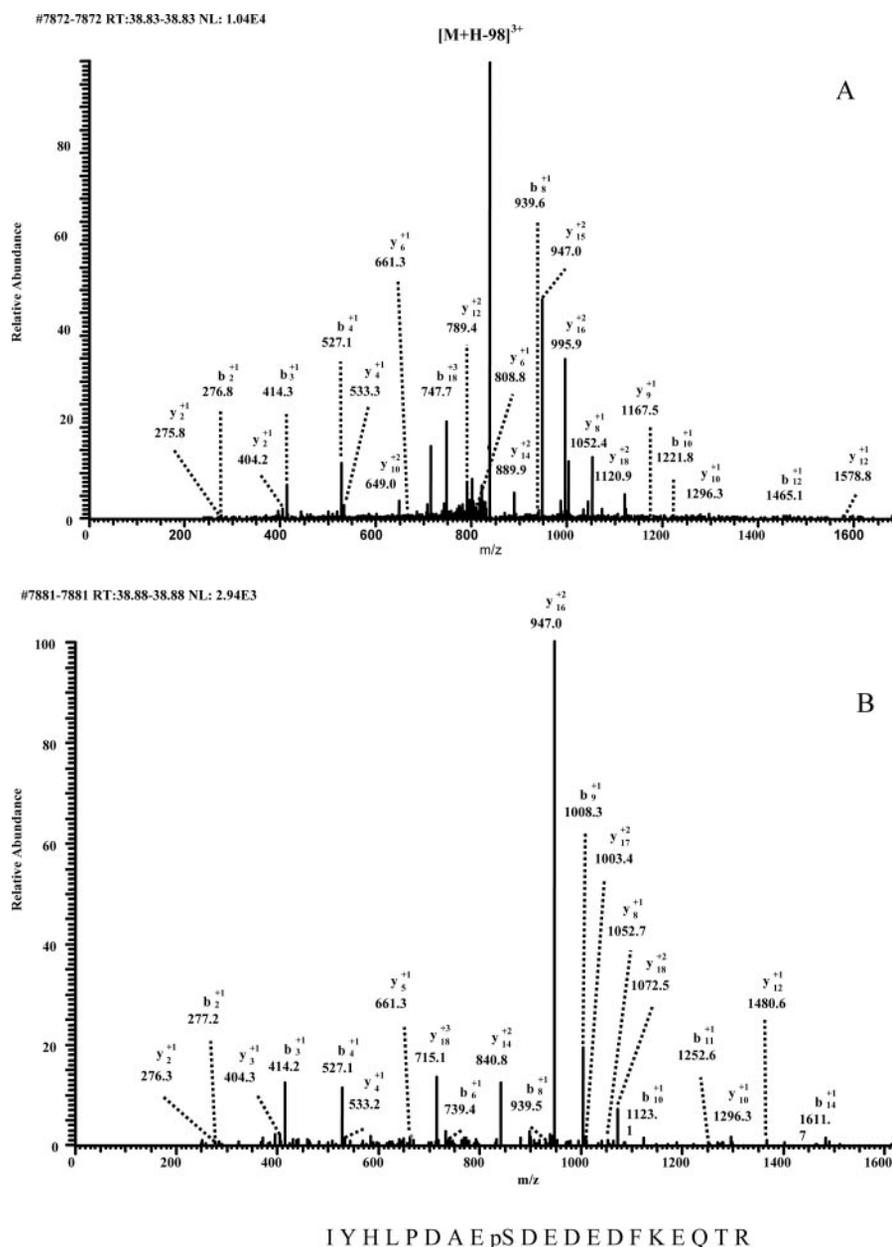
**Application of  $Zr^{4+}$ -IMAC Beads for Phosphoproteome Analysis of Mouse Liver**—To further evaluate their performance for the capture of phosphopeptides,  $Zr^{4+}$ -IMAC beads were applied to analyze the phosphoproteome of mouse liver. Phosphopeptides from 100  $\mu$ g of mouse liver lysate digest 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.<sup>2</sup> 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  $\Delta$ Cn

<sup>2</sup> The raw data for the three LC-MS/MS runs obtained by the LTQ are available upon request.

(>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<sup>2</sup> 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<sup>3</sup>) spectrum. It was observed previously that MS<sup>3</sup> spectra are useful for the validation of phosphopeptides identified from MS<sup>2</sup> spectra (3, 33, 34). In this study, the acquired MS<sup>3</sup> spectra were also searched by the Sequest program. Because the peptide identifications derived from MS<sup>3</sup> were used to confirm the identifications derived from MS<sup>2</sup>, 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  $\Delta$ Cn (>0.1) were used to filter the MS<sup>3</sup> database search results, resulting in the identification of 244 unique phosphopeptides and three non-phosphopeptides. Comparing the phosphopeptides identified from MS<sup>2</sup> and MS<sup>3</sup> 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<sup>3</sup> data is very effective. Fig. 6 is an example for identification of triply charged phosphopeptides from Septin-2, IYHLPDA-EpSDEDEDFKEQTR, in which Ser<sup>218</sup> 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<sup>2</sup> and MS<sup>3</sup> spectra, and in the MS<sup>2</sup> 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  $H_3PO_4$  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.

FIG. 6. A, MS<sup>2</sup> spectrum of the doubly charged form of a singly phosphorylated peptide identified from the tryptic digest of lysate of mouse liver. The dominant peak at *m/z* 807.13 represents the doubly charged neutral loss peak (after loss of the H<sub>3</sub>PO<sub>4</sub> group) for its selected precursor ion (*m/z* 839.86). The identified phosphopeptide was IYHLPDAEpSDEDFKEQTR. B, MS<sup>3</sup> spectrum of the doubly charged neutral loss peak at *m/z* 807.13. The b- and y-ion series shown both in A and B are indicated to verify the identification of the peptide.



In addition to the 50 phosphopeptides, there should be more phosphopeptides identified only from MS<sup>2</sup>. Without the confirmation with MS<sup>3</sup> data, these identifications should be carefully manually validated. Many multiply phosphorylated peptide identifications were also observed after filtering with Xcorr and  $\Delta Cn$  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 MS<sup>2</sup> spectra. Some MS<sup>3</sup> spectra may yield significantly more structural information than the corresponding MS<sup>2</sup> spectra,

which are usually dominated by phosphate loss. It was reported that phosphopeptides can also be confidently identified by MS<sup>3</sup> spectra (3, 33, 34). The MS<sup>3</sup> 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  $\Delta Cn$  (>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 MS<sup>2</sup> and MS<sup>3</sup> are given in Supplemental Tables 3 and 4, respectively. Combined with the phosphopeptides identified by MS<sup>2</sup> and confirmed by MS<sup>3</sup> (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 MS<sup>2</sup> and MS<sup>3</sup> 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 from novel phosphorylation sites. Among the 163 phosphorylated sites identified, 73.0% (119 sites) have not been reported previously, and 27.0% (44 sites) were known phosphorylation sites, including 33 sites reported in mouse liver and 11 phosphorylated sites reported in other mammalian such as human and rat (see Supplemental Tables 2–4). There were 64 phosphorylation sites in 50 phosphopeptides determined by both the MS<sup>2</sup> and MS<sup>3</sup> spectra. It was found that 42.2% (27 sites) of these sites are known phosphorylation sites. For the phosphorylation sites determined by only MS/MS spectra, the percentage of known phosphorylation sites was down to 16.3%. And for those determined by only MS/MS/MS spectra, the percentage was 18.2%. Obviously 42.2% is a very high percentage, indicating that the phosphopeptides identified both by MS<sup>2</sup> and MS<sup>3</sup> spectra are of high confidence. Based on the fact that high quality MS<sup>2</sup> and MS<sup>3</sup> spectra could be acquired for these phosphopeptides, their concentrations in the sample may be higher than others. In other words, their parent phosphoproteins may be relatively high abundance proteins. That probably is one of the important reasons that many of these phosphorylation sites were already reported in the literature. Many new phosphorylation sites from low abundance phosphoproteins identified by only MS<sup>2</sup> or MS<sup>3</sup> should also be very useful for related biological research in the future.

#### DISCUSSION

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 Zr<sup>4+</sup>-IMAC beads followed with LC-MS/MS (MS/MS/MS) 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 Zr<sup>4+</sup>-IMAC, different types of Fe<sup>3+</sup>-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 Fe<sup>3+</sup>-IMAC silica monolithic capillary column (33), and a self-prepared Fe<sup>3+</sup>-IMAC meso-

porous 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 Zr<sup>4+</sup>-IMAC approach (an average of 61 singly phosphorylated peptides for each run) was significantly higher than that by any of the above mentioned Fe<sup>3+</sup>-IMAC approaches. The high performance is largely because Zr<sup>4+</sup>-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 Zr<sup>4+</sup>-IMAC approach. This also indicated that phosphopeptides could be efficiently enriched by Zr<sup>4+</sup>-IMAC beads and therefore high quality MS/MS/MS spectra were acquired.

The specificity of Fe<sup>3+</sup>-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 Zr<sup>4+</sup>-IMAC beads, 87.2% of the peptides identified from MS<sup>2</sup> spectra were phosphorylated peptides. The percentage of identified phosphopeptides by using Zr<sup>4+</sup>-IMAC beads was much higher than the equivalent method using Fe<sup>3+</sup>-IMAC beads and was similar to the method using a combination of peptide esterification with Fe<sup>3+</sup>-IMAC beads. This means that the specificity of Zr<sup>4+</sup>-IMAC beads was much higher than that of Fe<sup>3+</sup>-IMAC and was similar to that of Fe<sup>3+</sup>-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 Zr<sup>4+</sup>-IMAC beads is much simpler because no additional sample pretreatment is required.

Metal oxides such as TiO<sub>2</sub> and ZrO<sub>2</sub> 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 Fe<sup>3+</sup>-IMAC beads. The mechanisms of ZrO<sub>2</sub> and Zr<sup>4+</sup>-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 ZrO<sub>2</sub> nanobeads have high specificity for phosphopeptides (38). ZrO<sub>2</sub> nanobeads were also used to enrich phosphopeptides from the digest of mouse liver lysate in our laboratory. After LC-MS (MS<sup>2</sup> and MS<sup>3</sup>) 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  $Zr^{4+}$ -IMAC. To further compare the specificities of  $ZrO_2$  and  $Zr^{4+}$ -IMAC, the percentage of phosphopeptides in the MS/MS database search results (filtered with Xcorr and  $\Delta Cn$  criteria but without manual validation) obtained by using  $ZrO_2$  nanobeads was calculated to be 72%, which was lower than that of  $Zr^{4+}$ -IMAC (87%). The above results demonstrated that  $Zr^{4+}$ -IMAC has higher selectivity for phosphopeptides than do  $ZrO_2$  nanobeads. The different performance between  $ZrO_2$  beads and GMA-EDMA phosphonate  $Zr^{4+}$  beads ( $Zr^{4+}$ -IMAC beads) may be due to two reasons. First, GMA-EDMA beads do not have strong Lewis acid sites on the surface as do  $ZrO_2$  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  $Zr^{4+}$  that allows the phosphate groups on phosphopeptides to have a better chance to access  $Zr^{4+}$  on GMA-EDMA beads.

Recently  $TiO_2$  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  $TiO_2$  microcolumn to enrich phosphopeptides from the mixture of phosphoproteins ( $\alpha$ -casein,  $\beta$ -casein, and ovalbumin) with non-phosphoproteins (serum albumin,  $\beta$ -lactoglobulin, and carbonic anhydrase) at different molar ratios. It was found that when the molar ratio of phosphoprotein to non-phosphoprotein 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  $Zr^{4+}$ -IMAC beads than on the  $TiO_2$  beads.

Kweon and Hakansson (21) investigated the performance of  $ZrO_2$  and  $TiO_2$  microtips for trypsin and Glu-C proteolytic digests of  $\alpha$ -casein and  $\beta$ -casein and compared binding specificity and recovery of phosphopeptide by  $ZrO_2$  with those by  $TiO_2$ . It was demonstrated that  $ZrO_2$  microtips display overall performance similar to  $TiO_2$  microtips. However, more selective isolation of singly phosphorylated peptides was observed with  $ZrO_2$  compared with  $TiO_2$ , whereas  $TiO_2$  preferentially enriched multiply phosphorylated peptides. Because  $Zr^{4+}$ -IMAC has higher selectivity than  $ZrO_2$  in our case,  $Zr^{4+}$ -IMAC may also have higher selectivity than  $TiO_2$ . 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 adsorbent for high specific enrichment of phosphopeptides for phosphopro-

teome analysis has been presented. The IMAC beads were prepared by immobilization of  $Zr^{4+}$  on the phosphonate-modified GMA-EDMA polymer beads.  $Zr^{4+}$ -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  $Zr^{4+}$ -IMAC because of its excellent specificity.  $Zr^{4+}$ -IMAC beads prepared in this study have both the high selectivity of the  $Zr^{4+}$  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.

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