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Zirconium Phosphonate-Modified Porous Silicon for Highly Specific Capture of Phosphopeptides and MALDI-TOF MS Analysis

Houjiang Zhou, Songyun Xu, Mingliang Ye, Shun Feng, Chensong Pan, Xiaogang Jiang, Xin Li, Guanghui Han, Yu Fu, and Hanfa Zou*

National Chromatographic R & A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

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Phosphorylation is one of the most important post-translational modifications of proteins, which modulates a wide range of biological functions and activity of proteins. The analysis of phosphopeptides is still one of the most challenging tasks in proteomics research by mass spectrometry. In this study, a novel phosphopeptide enrichment approach based on the strong interaction of zirconium phosphonate (ZrP) modified surface with phosphopeptides has been developed. ZrP modified porous silicon (ZrP-pSi) wafer was prepared to specifically capture the phosphopeptides from complex peptide mixtures, and then the captured phosphopeptides were analyzed by MALDI-TOF MS by directly placing the wafer on a MALDI target. The phosphopeptide enrichment and MALDI analysis were both performed on the ZrP-pSi wafer which significantly reduced the sample loss and simplified the analytical procedures. The prepared ZrP-pSi wafer has been successfully applied for the enrichment of phosphopeptides from the tryptic digest of standard phosphoproteins β-casein and α-casein. The excellent selectivity of this approach was demonstrated by analyzing phosphopeptides in the digest mixture of β-casein and bovine serum albumin with molar ratio of 1:100. High detection sensitivity has been achieved for the analysis of the phosphopeptides from tryptic digestion of 2 fmol β-casein on the ZrP-pSi surface.

Keywords: Zirconium phosphonate • Porous silicon • Phosphopeptide • MALDI-TOF MS

Introduction

The reversible phosphorylation of proteins catalyzed by kinases and phosphatases is a common theme in the regulation of important cellular functions such as growth, metabolism, and differentiation. Studies in protein phosphorylation may hold particular promise for dissecting signaling pathways, molecular classification of diseases, and profiling of novel kinases inhibitors.[1−3] Mass spectrometry, including electrospray ionization−mass spectrometry (ESI−MS) and matrix-assisted laser desorptipon/ionization mass spectrometry (MALDI-MS), is an important technology for the detection and characterization of protein phosphorylation and phosphopeptides.[4−8] However, the identification and characterization of phosphoprotein is still one of the most challenging tasks in contemporary proteome research because of the lower abundance of phosphoprotein, the substoichiometric nature of phosphorylation, and some technical limitations.[9] To reduce the suppressing effect of large amount of nonphosphopeptides in the digest of protein mixture containing phosphoproteins, isolation of phosphopeptides prior to mass spectrometric analysis is required. To date, several strategies have been developed to enrich the phosphopeptide prior to mass spectrometric analysis including immobilized metal (usually Fe^{3+}, Ga^{3+}) affinity chromatography (IMAC),[10−15] chemical tag after β-elimination reaction,[16−19] immunoprecipitation through phosphoprotein antibody,[20−23] particles of metal oxide such as TiO_2,[24] ZrO_2,[25] and $Al(OH)_3$,[26] and Fe_3O_4/TiO_2 core/shell nanoparticles.[27] The development of efficient methods for highly specific enrichment of phosphopeptide still is one of the most active research fields in proteome analysis.

Porous silicon (pSi) was originally used in desorption/ionization on porous silicon mass spectrometry (DIOS-MS), a novel soft ionization MS technique that does not require any matrix for the analysis of low mass compounds.[28] Various surface functionalities based on pSi have been developed to capture interest analytes for analysis by DIOS-MS.[29−33] Protein microchip array-based on macro-/nanoporous silicon combined with MALDI-TOF MS and fluorescence imaging detection has been developed by Marko-Vaga et al.[34−36]and also demonstrated as a powerful tool in high-throughput proteomics analysis. Because of its high surface area, functionalized pSi could also be used as an affinity matrix support for MALDI-TOF MS analysis where matrix was added to ionize the captured analytes. Recently, porous silicon wafer with immobilized Fe^{3+} affinity surface was prepared to analyze phosphopeptides by MALDI MS.[37] Complex peptide mixture was spotted onto the modified IMAC-pSi wafer, and nonphosphopeptides were removed from the silicon surface by thoroughly

* Corresponding author: Prof. Dr. Hanfa Zou, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China. Tel.: +86-411-84379610. Fax: +86-411-84379620. E-mail: hanfazou@dicp.ac.cn.

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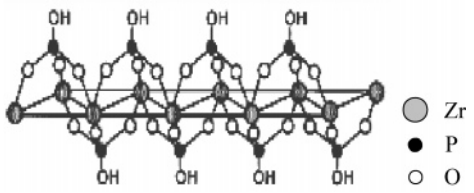

Figure 1. Layer structure of zirconium phosphonate (ZrP).

washing. After addition of matrix, the porous silicon wafer was directly placed on the MALDI target for the analysis of the captured phosphopeptides. The phosphopeptide enrichment and analysis procedures were all performed on the Fe^{3+}-terminated silicon wafer, which greatly reduced the sample loss and simplified the analysis procedure. On target purification of phosphopeptides followed by MALDI MS analysis was also reported by Dunn et al.[38] In their work, Fe^{3+}-nitrilotriacetate (NTA) was immobilized on a MALDI plate for capture of phosphopeptides. Above approaches are based on the interaction between chelated Fe^{3+} ion and phosphate group, which is well-known that some acidic peptides can be also captured. To characterize phosphoproteins more efficiently, it was necessary to develop novel affinity surface with higher selectivity for phosphopeptides.

Self-assembling monolayer and multilayer thin films of phosphate-containing organic molecules have been prepared based on the strong interaction between ZrP and phosphate group.[39−41] Also because of the strong interaction, ZrP, as the layer structure shown in Figure 1, could be used to prepare catalysis carrier,[42] biomolecule immobilization carrier,[43] and oligonucleotide microarray.[44] Here, we found that the zirconium phosphonate modified porous silicon wafer has strong affinity for phosphopeptides, and therefore, it can be used to capture phosphopeptides with high specificity. The ZrP-pSi wafer with dimension of 1 × 1 cm was prepared, and the resulted ZrP-pSi wafer was used as a MALDI sample target for on-target purification of phosphopeptides prior to MALDI analysis.

Experimental Procedures

Materials and Methods. α-Casein, β-casein, bovine serum albumin (BSA), 2,5-dihydroxybenzoic acid (2,5-DHB) and 1-1-(tosylamido)-2-phenyl-ethyl chloromethyl ketone (TPCK)-treated trypsin (E.G 2.4.21.4) were purchased from Sigma (St. Louis, MO). HPLC grade trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). $ZrOCl_2$, 2, 4, 6-collidine, and 3-aminopropyl-triethoxysilane were obtained from Sigma (St. Louis, MO). POROS 20 MC beads were purchased from PerSeptive Biosystems (Framingham, MA). Phosphorus-doped, n-type silicon single crystal (111) with low-resistivity 0.008−0.02 Ω·cm was obtained from Beijing Institute of Non-Metals (Beijing, China). All other chemicals including toluene, phosphorus oxychloride ($POCl_3$), and hydrogen fluoride (HF) were of analytical grade reagent. Acetonitrile and ethanol were of chemical reagent grade. Deionized water used for all experiments was purified with a Milli-Q water system (Millipore, Milford, MA). Matrix DHB was dissolved in acetonitrile (ACN)/water (30/70, v/v) solution containing 1% H_3PO_4 by keeping DHB at 25 mg/mL.

Fabrication of Zirconium Phosphonate-Modified Porous Silicon (ZrP-pSi) Wafer. A low-resistivity silicon wafer was cut to 1 cm × 1 cm pieces, which were then electrochemically

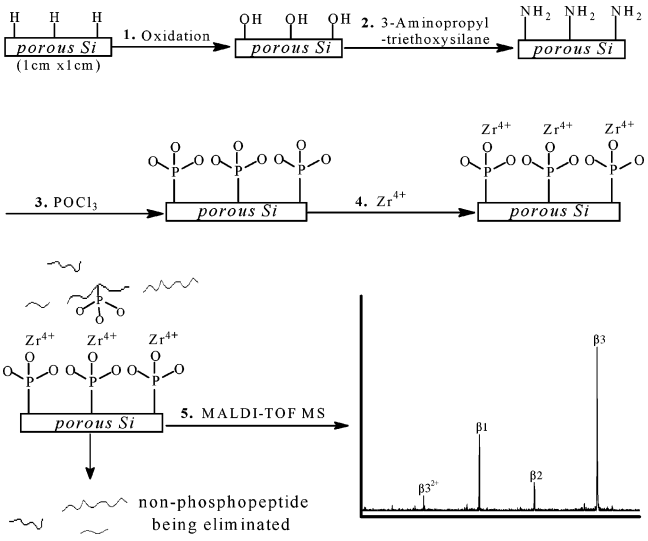

Figure 2. Scheme for preparation of ZrP-pSi to trap phosphopeptides for MALDI-TOF MS analysis.

etched in a solution of ethanol/49% HF (2:3, v/v) for 100 s in a custom-built Teflon cell, with a Pt wire as counter electrode under the illumination of a 250 W tungsten filament bulb. The current density was kept at 4 mA/cm^2. The obtained silicon wafer was further processed by rapid oxidation with 30% ozone for 1 min and then re-etched in ethanol containing 5% HF (v/v) for 60 s. The surface of the porous silicon wafer was ready for use after washing with ethanol.

The freshly etched porous silicon was incubated in 50 mL 20% HNO_3 for 2 h. After rinsing with water, porous silicon was placed in 60 mL 20% HCl at 80 °C with stirring for 6 h. It was rinsed with water until pH at 7.0. Then, pieces of the porous silicon wafer were dried in a vacuum at 110 °C overnight. The dried porous silicon was placed in 60 mL of toluene under N_2, and subsequently, 6 mL of 3-aminopropyl-triethoxysilane was added for reaction at 110 °C for 12 h under N_2. Then, the toluene was filtered, and the obtained aminopropyl-terminated porous silicon surface was washed with toluene and acetone for several times. The obtained aminopropyl-modified porous silicon surface was further transferred into phosphonate-terminated porous silicon surface with reaction in a solution of 40 mM $POCl_3$ and 40 mM 2,4,6-collidine in anhydrous acetonitrile for 12 h, and followed by rinsing with ACN and water. The resulting surface was immersed into 20 mM $ZrOCl_2$ solution to yield the ZrP-pSi wafer at room temperature overnight under gentle stirring. After that, the surface of ZrP-pSi wafer was rinsed with deionized water for several times and stored in the refrigerator at 4 °C for usage. The whole procedure for the fabrication of the ZrP-pSi wafer was shown in Figure 2. (It should be noted that HF and HNO_3 are very corrosive. Care should be taken to prevent the direct contact or inhalation of these acids; thus, all of the experiments for etching silicon were performed in the air hood under negative pressure.)

Tryptic Digestion of Protein and Peptide Mixtures. BSA (4 mg) was dissolved in 1 mL denaturing buffer solution containing 8 M urea in 50 mM ammonium bicarbonate for 3 h. The obtained protein solution was mixed with 20 μL of 50 mM DTT. The disulfide bond of protein was reduced by incubation for 2 h at 37 °C. And then 40 μL of 50 mM IAA was added, and the obtained solution was incubated for an additional 30 min at room temperature in dark. After that, the mixture was diluted

with 50 mM ammonium bicarbonate by 10-folds and incubated for 16 h at 37 °C with trypsin at an enzyme/substrate ratio of 1:40 (w/w) to produce proteolytic digest. α-Casein and β-casein (1 mg) was dissolved in 1 mL ammonium bicarbonate (50 mM, pH 8.2) and digested for 16 h at 37 °C with an enzyme-to-protein ratio of 1:40 (w/w). The digested peptide solution was lyophilized by a vacuum concentrator for usage. For the experiments to evaluate the capture specificity of phosphopeptides by ZrP-pSi, the peptide mixtures originating from tryptic digestion of β-casein and BSA at molar ratio of 1:1, 1:10, and 1:100 by keeping β-casein concentration at 1 pmol were prepared. All the peptide mixtures solution obtained were again lyophilized and redissolved in 5 μL loading solution containing 50% ACN in 0.1% TFA, 100 mM NaCl (pH 2 ∼ 3) buffer, then the 5 μL solution obtained was deposited on ZrP-pSi surface for the experiments.

The Procedures for Enrichment of Phosphopeptides by ZrP-pSi Wafer. The obtained ZrP-pSi wafer was first washed with 5 μL of 200 mM NaCl solution to remove the compounds nonspecifically adsorbed on porous silicon surface. A volume of 1 μL of tryptic digestion of phosphoprotein was diluted by 100 μL solution of 50% ACN containing 0.1% TFA and 100 mM NaCl (pH 2 ∼ 3) as loading solution, then 2 μL loading solution was directly deposited onto the ZrP-pSi wafer for incubation about 15 min. After that, the ZrP-pSi wafer was washed by 1 mL 50% ACN solution containing 100 mM NaCl and 0.1% TFA for 5 min, and followed by washing with 1 mL 50% ACN solution for 5 min, and then washed by deionzied water to remove salts and nonspecifically adsorbed compounds. Last, 2 μL of DHB solution containing 1% H_3PO_4 [45] was added onto the ZrP-pSi wafer to form the co-crystals with the trapped phosphopeptides. The ZrP-pSi wafer was directly attached to the MALDI target plate with conductive tape for the further mass spectrometric analysis. The enrichment of phosphopeptides by IMAC beads (POROS 20 MC beads) were performed according to Larsen et al.[24] with minor changes. After loading of Fe^{3+}, the IMAC beads were suspended in loading buffer of 0.1% acetic acid at concentration of 30 mg/mL. An aliquot of IMAC solution (10 μL) was mixed with 10 μL of protein digest in loading buffer, and the resulting solution was incubated for 30 min with constant rotation. After incubation, the IMAC beads were washed with aceonitrile/water/acetic acid (25:74:1, v/v/v) twice to remove nonspecifically adsorbed peptides. The bound peptides were eluted using 10 μL of NH_4OH (pH 10.5) into a tube, lyophilized to dryness. To the tube, 2 μL of DHB solution containing 1% H_3PO_4 was added, and 0.5 μL of solution was deposited on the target for MALDI-TOF MS analysis.

Instrumentation. All MALDI-TOF mass spectrometry experiments were performed on a Bruker Autoflex time-of-flight mass spectrometer (Bruker, Bremen, Germany). This instrument was equipped with a delayed ion-extraction device and a pulsed nitrogen laser operated at 337 nm. The range of laser energy was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratio (S/N). All mass spectra reported were obtained in the positive ion linear mode with delayed extraction for 90 ns and calibrated using an external calibration equation generated from the ion signal of angiotension II and insulin chain B. And each mass spectrum was typically summed with 30 laser shots.

Results and Discussions

Protein phosphorylation is very important for the regulation of the activity of proteins and attracts a lot of attentions in proteomics research field. Development of the well-defined and specific interface for the enrichment and analysis of phosphopeptides was essential to decrease sample loss, simplify analytical procedures, and to finally achieve high-throughput detection by MALDI-TOF MS. ZrP was thermally stable, chemically inert in neutral/acidic media. As the layer structure of ZrP shown in Figure 1, each zirconium coordinates to six phosphonate groups, and the phosphonate reversely binds to more than one zirconium atom. Chemistry for "self-assembled monolayer and multilayer" based on binding of phosphonate to the ZrP surface has been discussed in detail in the literature.[44] The coordination properties of free phosphate (OPO_3H_2) were very close to those of the phosphate group, and so phosphate species should also have strong interaction with ZrP surface. Therefore, we speculate that the unique and extreme strong binding of the monolayer ZrP to phosphate groups could provide a novel phosphopeptide enrichment method. On the basis of the strong multicoordination effect between ZrP and phosphate group, the ZrP-pSi wafer was expected to specifically bind with phosphate group in phosphopeptides. The unique multicoordination effect between ZrP and phosphoric species may provide higher specificity, selectivity, and sensitivity for phosphopeptides than conventional IMAC enrichment strategy.

ZrP-modified surface can be prepared by various methods such as covalent attached layer method[43] and Langmuir−Blodgett.[44] In this work, we chose the latter method for preparation of ZrP-pSi because its procedure was compatible with silicon. The whole scheme for preparation of ZrP-pSi wafer was devised as shown in Figure 2. The obtained ZrP-pSi wafer can be stored for months without an obvious loss of activity. First, we evaluated the desorption/ionization ability of ZrP-pSi wafer by depositing 0.5 μL of the tryptic digestion of β-casein and analyzing with MALDI-TOF MS. But very weak or almost no ion signal of peptide can be detected on the ZrP-pSi wafer (data not shown), so organic matrix DHB containing 1% H_3PO_4 [45] was added onto the ZrP-pSi wafer to form the co-crystals with the trapped phosphopeptides to improve the detection of phosphopeptides in all latter experiments.

To examine the effectiveness of ZrP-pSi wafer for the enrichment of phosphopeptides, we employed β-casein as the model phosphoprotein. Before deposition of sample, the obtained ZrP-pSi wafer was first washed with 5 μL of 200 mM NaCl solution to remove the chemicals nonspecifically adsorbed on the surface. Then, 2 μL of tryptic digest of β-casein (10^{-6} M in 50% ACN solution containing 0.1% TFA and 100 mM NaCl) was deposited onto the ZrP-pSi wafer. After incubation of 15 min at room temperature, the porous silicon wafer was washed with 1 mL of 50% ACN solution containing 100 mM NaCl and 0.1% TFA, and 1 mL of 50% ACN solution in sequence to remove nonphosphopeptides. The ZrP-pSi wafer was directly attached to the MALDI target plate with conductive tape for the mass spectrometric analysis, and the obtained spectrum was shown in Figure 3a. Four peaks of phosphopeptides ($\beta 3^{2+}$ at m/z 1561.14, β1 at m/z 2061.83, β2 at m/z 2556.09, and β3 at m/z 3122.27) were clearly observed. The detailed information about the detected phosphopeptides was listed in Table 1.[24,25] As a comparison, same amount of β-casein digest was deposited on the porous silicon surface and analyzed by MALDI without pretreatment procedures, and the obtained spectrum was shown Figure 3b. Both phosphopeptides (β1 m/z 2061.83,

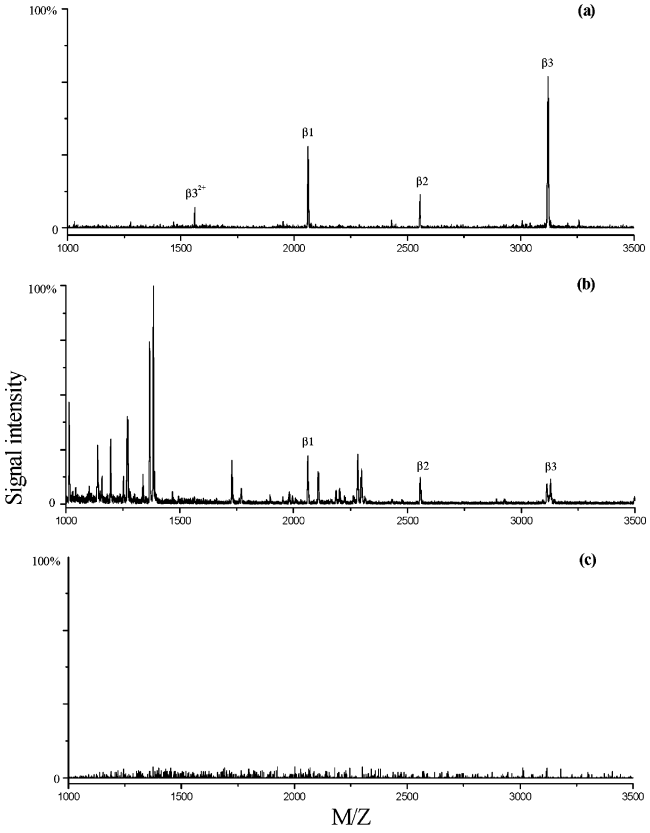

Figure 3. MS spectra of tryptic digestion of β-casein (10^{-6} M, 2μL). (a) Analysis after enrichment: sample solution was deposited on the ZrP-pSi wafer and washed with pretreatment procedures. (b) Direct analysis: sample solution was deposited on the ZrP-pSi wafer and analyzed without further pretreatment. (c) Control experiment: sample solution was deposited on the phosphonate-terminated porous silicon wafer and subjected to the same pretreatment procedures as that of (a).

Table 1. Phosphopeptides Isolated from the Tryptic Digest of β-Casein by Zirconium Phosphonate-Modified Porous Silicon Wafer

no.	$[M + H]^+$	phosphorylation site	amino acid sequence
β1	2061.83	1	FQ[pS]EEQQQTEDELQK
β2	2556.09	1	FQ[pS]EEQQQTEDELQDKIHPF
β3	3122.27	4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR

β2 m/z 2556.09, and β3 m/z 3122.27) and abundant nonphosphopeptides were observed. The presence of nonphosphopeptides resulted in the low signal-to-noise ratio of phosphopeptides. It is very obvious that phosphopeptides are specifically trapped by the ZrP-pSi wafer. To elucidate the capture mechanism, a control experiment was also conducted by depositing the same sample on the phosphate-terminated porous silicon wafer with pretreatment procedures as for Figure 3a. The obtained mass spectrum was shown in Figure 3c. No ion signal was observed, which meant no peptide was captured by phosphate-terminated porous silicon surface. Therefore, the specific capture of phosphopeptide was based on the strong interaction of phosphopeptides with ZrP.

To evaluate the ability to capture the phosphopeptides from a complex sample, the ZrP-pSi wafer was applied to trap phosphopeptides from the mixtures of tryptic digestion of β-casein and BSA with molar ratio of 1:1, 1:10, and 1:100, respectively. Figure 4a,b presented the MALDI mass spectra for the direct analysis of the tryptic digestion of β-casein and BSA at the molar ratio of 1:1 and 1:10 without pretreatment, respectively. The ion signal of phosphopeptides at β1 m/z 2061.83, β2 m/z 2556.09, and β3 m/z 3122.27 can be detected in the mass spectrum, but the ion signal was very weak in the presence of many abundant nonphosphopeptides. Figure 4d,e displayed the obtained MALDI mass spectra of the sample after using the ZrP-pSi wafer to selectively trap the phosphopeptides from the tryptic digest mixture containing β-casein and BSA with molar ratio of 1:1 and 1:10, respectively. The ion signal of phosphopeptides at β1 m/z 2061.83, β2 m/z 2556.09, and β3 m/z 3122.27 was greatly increased in comparison with those directly analyzed by MALDI-TOF MS and dominated the mass spectra. It can be seen from Figure 4c that the phosphopeptides from β-casein were difficult to be distinguished because of the presence of numerous abundant nonphosphopeptides peaks from BSA for the mixture at ratio of 1:100. However, when this sample was treated on the ZrP-pSi wafer and analyzed by MALDI, two phosphopeptides at β1 m/z 2061.83 and β3 m/z 3122.27 can still be easily detected as shown in Figure 3f. The singly phosphorylated peptide at m/z 2061.83 was very weak compared with that of multiply phosphorylated peptide at m/z 3122.27. It is obvious that the enrichment of multiply phosphorylated peptides by this approach is more efficient than that of singly phosphorylated peptides. The reason is that the interaction between the multiply phosphorylated peptide with ZrP-modified surface is stronger.

IMAC bead with Fe^{3+} is the most often used chromatographic matrix for the isolation of phosphopeptides. In comparison, IMAC beads were also applied for the enrichment of phosphopeptides from the digest mixtures of β-casein and BSA at the molar ratio of 1:1, 1:10, and 1:100, respectively. The obtained MALDI mass spectra were shown in Figure 5. As shown in Figure 5a, the three phosphopeptides could be well-detected with a clear background when the ratio of β-casein to BSA was at 1:1. However, many nonphosphopeptides appeared in the spectrum when the molar ratio increased to 1:10 in Figure 5b, and phosphopeptides were finally not detectable when the molar ratio increased to 1:100 as shown in Figure 5c. Compared with the corresponding mass spectra shown in Figure 4 where ZrP-pSi wafer was used to isolate phosphopeptides, too many nonphosphopeptide peaks were detected when IMAC beads were used especially for these with high BSA ratio. These results indicated that IMAC beads with Fe^{3+} were unable to specifically enrich phosphopeptides from a mixture containing large amount of nonphosphopeptides. Recently, porous silicon wafer with IMAC surface (IMAC-pSi) was prepared to analyze phosphopeptides by MALDI MS.[37] Decent results could be obtained when IMAC-pSi with Fe^{3+} was applied to analyze phosphopeptides in the tryptic digestion of β-casein only. However, obvious nonspecific adsorption was also observed when β-casein digest was mixed with large amount of BSA digest (data not shown). The IMAC approach is prone to lose its specificity when the concentration of nonphosphopeptides is much higher than that of phosphopeptides. The possible reason for this is that the interaction between Fe^{3+} and phosphopeptide is not specific and strong enough. Because of the multicoordination effect, the interaction between ZrP and phosphopeptide is much stronger. Therefore, the ZrP-pSi wafer was able to specifically enrich phosphopeptides even in the background of huge amount of nonphosphopeptides.

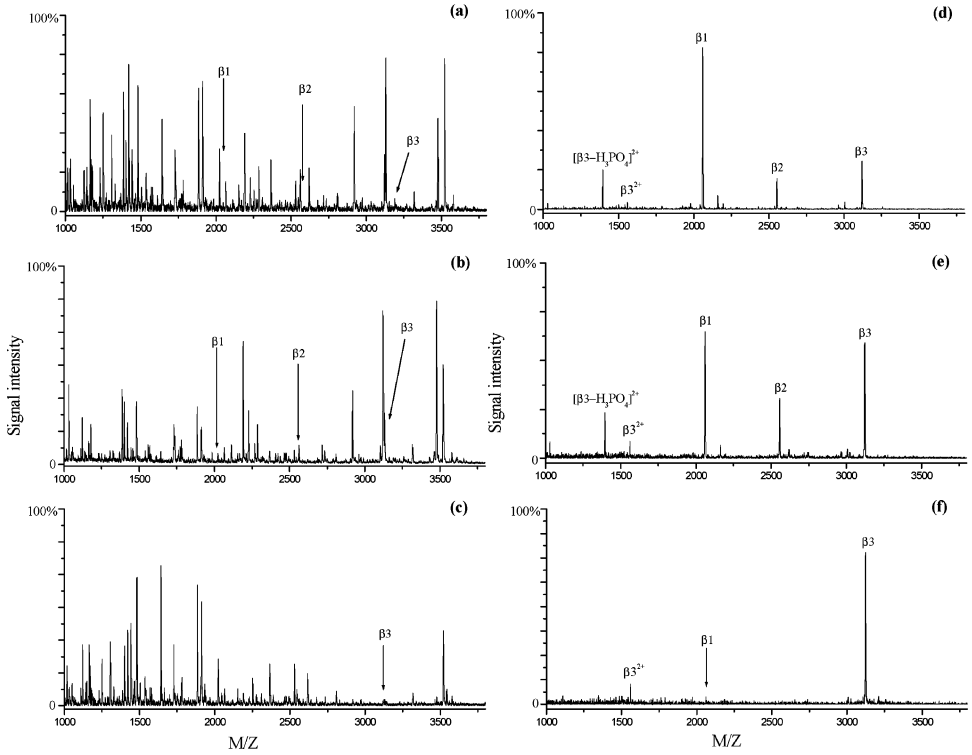

Figure 4. MALDI mass spectra for the digest mixtures of β-casein and BSA on the ZrP-pSi wafer (a−c) without and (d−f) with sample pretreatment procedures. Molar ratio of β-casein to BSA at (a and d) 1:1, (b and f) 1:10, and (c and e) 1:100.

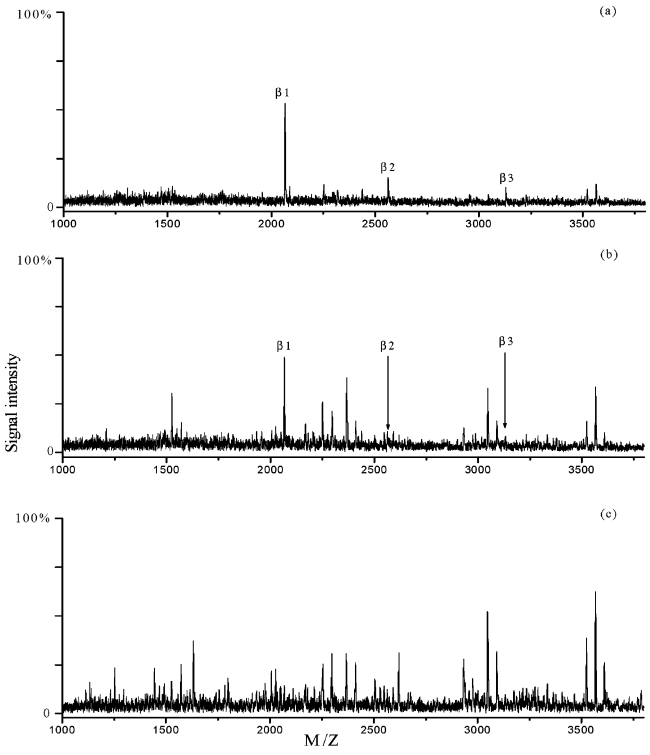

Figure 5. MALDI mass spectra for the digest mixtures of β-casein and BSA using the Fe^{3+}-IMAC beads for the selective enrichment of phosphopeptides. Molar ratio of β-casein to BSA at (a) 1:1, (b) 1:10, and (c) 1:100.

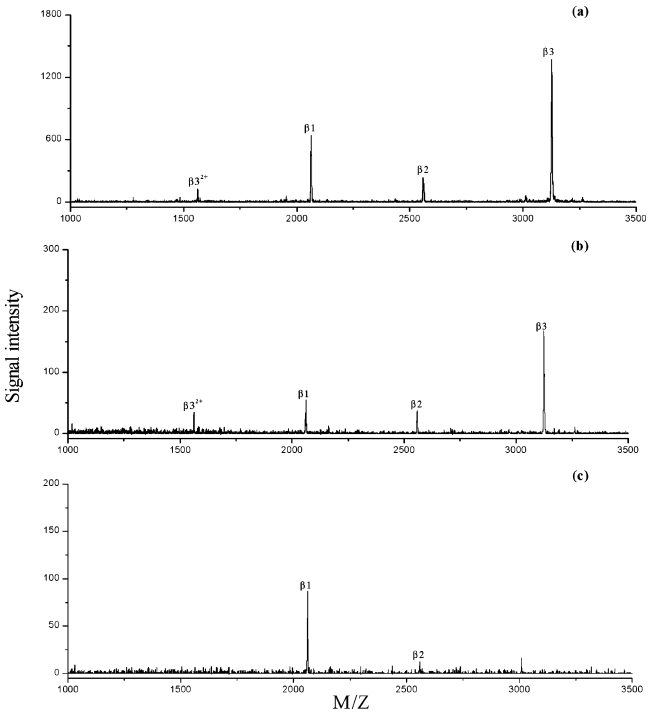

Figure 6. MALDI mass spectra for the phosphopeptides captured from tryptic digest of β-casein with amount of (a) 2 pmol, (b) 20 fmol, and (c) 2 fmol on the ZrP-pSi surface with sample pretreatment procedures, respectively.

The detection sensitivity of phosphopeptide by this approach was evaluated by deposition of different amounts of β-casein digests on the ZrP-pSi wafer followed with on-site pretreatment procedures. Panels a−c of Figure 6 show the spectra for the analysis of phosphopeptides enriched from digest of 2 pmol, 20 fmol, and 2 fmol β-casein, respectively. It can be seen that the phosphopeptides for 2 fmol β-casein can be well-detected, which indicated the high detection sensitivity of this approach. To our knowledge, the obtained detection sensitivity of phos-

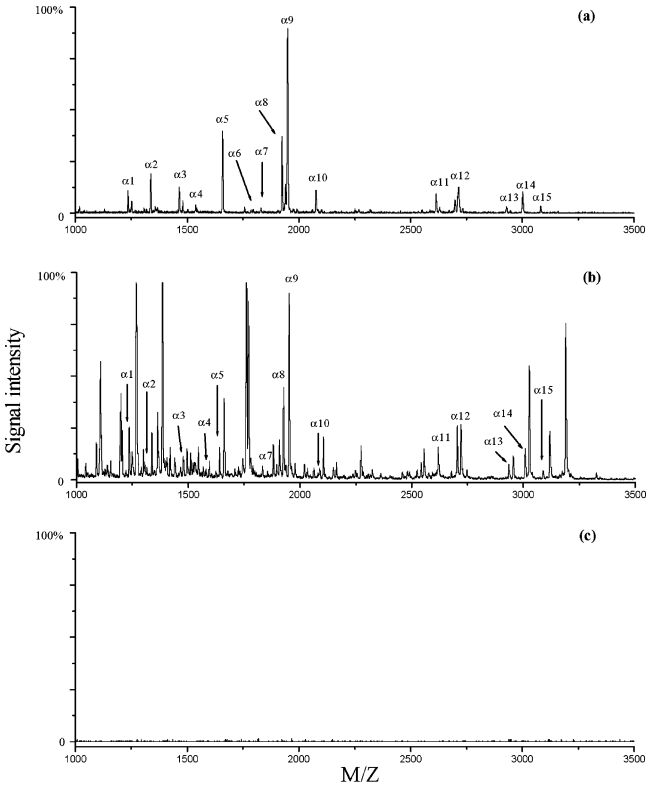

Figure 7. MS spectra of tryptic digestion of α-casein (10^{-6} M, 2 μL). (a) Analysis after enrichment: sample solution was deposited on the ZrP-pSi wafer and washed with pretreatment procedures. (b) Direct analysis: sample solution was deposited on the ZrP-pSi wafer and analyzed without further pretreatment. (c) Control experiment: sample solution was deposited on the phosphonate-terminated porous silicon wafer and subjected to the same pretreatment procedures as that of (a).

phopeptides from tryptic digest of 2 fmol β-casein might be the lowest by comparing that of other off-target enrichment method including IMAC[10,38,46,47]and Fe_3O_4/TiO_2 core/shell nanoparticles.[27] High detection sensitivity of this approach was mainly based on the fact that the phosphopeptide enrichment and MALDI-TOF MS analysis were all completed on the ZrP-pSi wafer which eliminated the sample loss that resulted from sample transferring in the conventional approach. The low detection limit may also be attributed to the strong affinity and good specificity of ZrP to capture phosphopeptides on the ZrP-pSi wafer. The reproducibility of this approach for detection of phosphopeptides on ZrP-pSi by MALDI was good. Although the absolute intensity of ion signals varied from spot to spot and position to position on one spot, the dominate ion signal of phosphopeptides could be reproducibly obtained.

The application of ZrP-pSi wafer was further employed for the analysis of tryptic digest of α-casein, which has more phosphopeptides than that β-casein does. Similarly, when α-casein digestion (10^{-6} M, 2 μL) was deposited on the ZrP-pSi wafer and pretreated with the same procedures as those for Figure 3a, the obtained mass spectrum only displayed all the 15 peaks of phosphopeptides from tryptic digest of α-casein as shown in Figure 7a. It can be seen that phosphopeptide peaks dominate the mass spectrum. Information for the phosphopeptides from α-casein, including amino acid sequence and phosphorylation sites, was shown in Table 2.[24,25] Peaks of many nonphosphopeptides from α-casein digest were detected as shown in Figure 7b with direct analysis by MALDI

Table 2. Phosphopeptides Isolated from the Tryptic Digest of α-Casein by Zirconium Phosphonate Modified Porous Silicon Wafer

no.	$[M + H]^+$	phospho-rylation site	amino acid sequence
α1	1237.50	1	TVDME[pS]TEVF
α2	1331.53	2	EQL[pS]T[pS]EENSK
α3	1466.61	1	TVDME[pS]TEVFTK
α4	1539.70	2	EQL[pS]T[pS]EENSKK
α5	1660.79	1	VPQLEIVPN[pS]AEER
α6	1832.83	1	YLGEYLIVPN[pS]AEER
α7	1847.69	1	DIG[pS]ESTEDQAMEDIK
α8	1927.69	2	DIG[pS]E[pS]TEDQAMEDIK
α9	1951.95	1	YKVPQLEIVPN[pS]AEER
α10	2079.04	1	KKYKVPQLEIVPN[pS]AEERL
α11	2619.04	4	NTMEHV[pS][pS][pS]EESII[pS]QETYK
α12	2720.91	5	QMEAE[pS]I[pS][pS][pS]EEIVPNPN[pS] VEQK
α13	2935.15	3	KEKVNEL[pS]KDIG[pS]E[pS]TEDQAMEDIKQ
α14	3008.01	4	NANEEEYSIG[pS][pS][pS]EE[pS]AEVATEEVK
α15	3087.99	5	NANEEEY[pS]IG[pS][pS][pS]EE[pS]AEVATEEVK

on the porous silicon wafer (10^{-6} M, 2 μL). And no peak appeared on the mass spectrum with control experiment as shown in Figure 7c where phosphate-terminated porous silicon wafer was used. The above-mentioned results further proved that the ZrP-pSi wafer can very efficiently capture the phosphopeptides from the digest of phosphoproteins.

Conclusions

A novel phosphopeptide enrichment approach based on the strong interaction of ZrP surface with phosphate group was presented in this study. The ZrP-pSi wafer was prepared to capture phosphopeptides, and the wafer with the captured phosphopeptides was directly placed on the MALDI target for direct analysis. High specificity of this approach was demonstrated by analysis of tryptic digest product of some model phosphoproteins. The phosphopeptide enrichment and detection steps were all accomplished on the ZrP-pSi wafer; this on-target analysis approach greatly simplifies the analytical procedures, reduces possible sample loss, and shows high sensitivity for detection of phosphopeptides. Sequence and phosphorylation sites of phosphopeptides could be determined if MALDI TOF-TOF or Q-TOF tandem mass spectrometer is used. On the basis of the unique specificity of the interaction between phosphopeptides and ZrP, a novel adsorbent with high specific affinity for phosphopeptides can be developed for large-scale phosphoproteome analysis. The ZrP-pSi wafer may be further extended to fabrication of microarray with detection by MALDI MS for high-throughput analysis of phosphopeptides and phosphoproteins.

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