technical notes Journal of proteome research

Specific On-Plate Enrichment of Phosphorylated Peptides for Direct MALDI-TOF MS Analysis

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Received August 13, 2007

Abstract: An on-plate specific enrichment method is presented for the direct analysis of peptides phosphorylation. An array of sintered TiO_2 nanoparticle spots was prepared on a stainless steel plate to provide porous substrate with a very large specific surface and durable functions. These spots were used to selectively capture phosphorylated peptides from peptide mixtures, and the immobilized phosphopeptides could then be analyzed directly by MALDI MS after washing away the nonphosphorylated peptides. β-Casein and protein mixtures were employed as model samples to investigate the selection efficiency. In this strategy, the steps of phosphopeptide capture, purification, and subsequent mass spectrometry analysis are all successfully accomplished on a single target plate, which greatly reduces sample loss and simplifies analytical procedures. The low detection limit, small sample size, and rapid selective entrapment show that this on-plate strategy is promising for online enrichment of phosphopeptides, which is essential for the analysis of minute amount of samples in high-throughput proteome research.

Keywords: phosphorylated peptides • enrichment • TiO_2 nanoparticles • MALDI-TOF MS

Introduction

Mass spectrometry, including matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and electrospray ionization mass spectrometry (ESI MS), has played an important role in protein profiling research.[1] MALDI is a standard ionization technique to transfer neutral solid-state samples to gas-phase ions for further analysis by mass spectrometry.[2] Several alternative substrates and matrices have been introduced over the recent years to add additional functions on MALDI target plates. Plates covered with specific solid-phases would present special affinities to target biomolecules. For example, polymer-coated target plates have been introduced to capture specific proteins from complex samples, based on ion exchange or reverse-phase mechanisms, and then the captured proteins could be further analyzed by mass spectrometry.[3–6]

Phosphorylation is one of the most important post-translational modifications of proteins, and this reversible procedure regulates a wide range of biological processes: transmitting signals, labeling proteins for degradation, and controlling cellular growth and metabolism.[7–9] To understand these biological functions at a molecular level, a characterization of phosphorylated proteins is required. Recently, MS-based techniques were proven to be efficient tools for phosphorylation sites mapping.[9–12] However, the low abundance of phosphorylated peptides and the signal suppression of fruitful nonphosphorylated peptides make the analysis still difficult. Furthermore, compared with unmodified peptides, the positive ions formation of phosphopeptides is much less efficient due to the increased acidity, which largely hampers the identification of phosphopeptides by MS.[13] Therefore, separation and enrichment of phosphorylated peptides from proteolytic digest mixtures are crucial steps. To date, several strategies have been developed to meet this requirement. One strategy based on the specific affinity between antibodies and phosphorylated amino acids has been established for enriching proteins phosphorylated on tyrosine.[14,15] However, this technique is limited by the high cost of antibodies. Another commonly used strategy is immobilized metal ion affinity chromatography (IMAC),[16–21] which remains a standard method for specifically enriching phosphorylated peptides prior to MS nowadays, where Fe^{3+} or Ga^{3+} ions are commonly used. The IMAC approach is based on ionic interactions; thus, nonspecific enrichment is unavoidable. It has been reported that peptides rich in glutamic, aspartic, cysteine, and histidine residues are often copurified.[8] Alternatively, metal oxides such as ZrO_2,[9,10] TiO_2,[7,11] and Al_2O_3[8,22] have been used to selectively concentrate phosphopeptides, where the phosphate functional groups can bind to the surface of metal oxide particles. Because of their stability over a wide pH range, acidic buffers can be employed to avoid nonspecific binding. Recently, a comprehensive study shows that this so-called metal oxide affinity chromatography technique could provide information that is complementary to IMAC.[23] On the basis of these strategies, a number of works have been reported using on-column or in-solution techniques.[7,12] However, additional procedures must be employed in these works, such as desalting usually necessary for on-

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10.1021/pr0705284 CCC: $37.00 © 2007 American Chemical Society

Published on Web 11/30/2007

column enrichment systems, centrifugation for in-solution enrichment systems, and sample manipulation from the separation to the detection devices for both systems. These procedures inevitably introduce possible sample loss and impede the integration of analytical procedures. To characterize phosphoproteins more efficiently, it is preferable to develop a simple online approach where the enrichment procedure could meet the requirement of high-throughput proteome analysis, especially when the sample available is limited.

Herein, a novel plate-based technique for fast enrichment of phosphopeptides from substoichiometric mixtures is described to address these issues. MALDI target plates are covered with an array of sintered TiO_2 nanoparticle spots. The spot is first used as a solid phase extractor to adsorb interacting molecules, and then as a stable support of the MALDI matrix for the ionization of those retained molecules, which are further analyzed by mass spectrometry. The TiO_2 spot has a very large specific to geometric area ratio, giving it a high-trapping capacity for its target species. The phosphopeptides can then be directly collected on the target plate efficiently without any other additional procedures. As a result, this in situ enrichment approach is very effective, sensitive, and especially suitable for online analysis coupled with sample separation. The tryptic digests of β-casein and drinking milk were employed as model samples to examine the feasibility of this highly specific on-plate enrichment method. The TiO_2 array-based affinity target plate provides a very efficient tool to specifically analyze phosphopeptides of a biological sample, for example, in the study of post-translational modifications.

Experimental Section

Reagents. Phosphoric acid (≥85%) and acetic acid (≥99%) were purchased from Shanghai Feida Chemical reagents Ltd. Ammonium hydroxide solution (25∼28%) was obtained from Shanghai No.4 Reagent Company, Kunshan. Titanium dioxide nanoparticles were obtained from Degussa (P25, Germany). Acetonitrile (ACN, 99.9%) and trifluoroacetic acid (TFA, 99.8%) were purchased from Merck (Darmstadt, Germany), while ammonium bicarbonate, 2,5-dihydroxybenzoic acid (DHB, 98%), β-casein (from bovine milk, 90%), bovine serum albumin (BSA, 95%), myoglobin (from horse heart, 95%), trypsin (from bovine pancreas), and PHOS-Select iron affinity gel were obtained from Sigma (St. Louis, MO). All these reagents were used as received without further purification. Drinking milk was purchased from a local grocery store. Deionized water (18.4 MΩ·cm) used for all experiments was obtained from a Milli-Q system (Millipore, Bedford, MA).

Preparation of a Stable Suspension of Commercial TiO_2 Nanoparticles. The P25 TiO_2 particles were heated at 300 °C for 2 h and then separated in a mortar for 2–3 h. During the separation procedure, 1 mL of 10% acetic acid (in water) was added for every 1 g of TiO_2 particles drop by drop to keep them wet. After being separated, the nanoparticles were suspended in an aqueous solution of ethanol (89% (v/v), TiO_2 concentration was 100 mg/mL), followed by sonication for 1 h. The resulting TiO_2 suspension could keep stable for several months. Before use, this suspension was diluted in water by 25×. Of course, one cannot assume the absence of nanoparticle aggregation in the suspension, but the present protocol provides enough dispersion to obtain a homogeneous suspension that can be easily manipulated.

Preparation of TiO_2-Modified Target Plate. The diluted suspension of TiO_2 nanoparticles was dropped as an array of spots (∼2 μL for each spot) on a stainless steel plate and dried at room atmosphere and temperature. The resulting modified target plate was subsequently heated in an oven at 400 °C for 1 h (the oven temperature was ramped from room temperature to 400 °C in 30 min) and naturally cooled-down and stored in a desiccator.

Tryptic Digestion of BSA, Myoglobin, β-Casein, and Drinking Milk. One milligram of BSA, myoglobin, or β-casein was dissolved in 1 mL of ammonium bicarbonate aqueous solution (25 mM, pH ∼8) separately, denatured at 100 °C for 5 min, and digested with trypsin for 12 h at 37 °C (enzyme-to-protein ratio of 1:30 (w/w)). Thirty microliters of drinking milk was diluted in 900 μL of NH_4HCO_3 aqueous solution at 25 mM. This solution was then centrifugated at 16 000 rpm for 15 min, and the supernatant was saved for tryptic digestion. After denaturation at 100 °C for 5 min, the supernatant was incubated for 12 h at 37 °C with 30 μg of trypsin for proteolysis. This tryptic digest product from milk was diluted by 25× in a DHB buffer (DHB at 20 mg/mL in ACN/water/TFA, 50/49.9/0.1% (v/v)) or deionized water before analysis.

On-Plate Extraction of Phosphopeptides. The digest products of BSA, β-casein, and drinking milk were diluted in given volumes of 2,5-DHB buffer (DHB at 20 mg/mL in ACN/water/TFA, 50/49.9/0.1% (v/v)) to adjust the hydrolysates to lower pH and required concentrations. Then, the digests were adsorbed on the TiO_2-modified target plate for either 30 or 5 min and washed with a solution of 2,5-DHB (DHB at 20 mg/mL in ACN/water/TFA, 50/49.9/0.1% (v/v)). Finally, 0.5 μL of 400 mM ammoniac aqueous solution was added to desorb the phosphorylated peptides bound to the TiO_2. A total of 0.3μL of TFA solution (2% in water (v/v)) was added on the sample spot and dried at room atmosphere before the deposition of 0.5μL of DHB matrix (20 mg/mL in ACN/water/H_3PO_4, 50/49/1% (v/v)) overlayer. The phosphorylated peptides captured on the TiO_2-modified target plate were analyzed by MALDI-TOF MS. The reproducibility was examined by repeating each experiment at least three times. For comparison, the digests of BSA, β-casein, and drinking milk diluted in deionized water at different concentrations were analyzed on a blank plate using a DHB matrix. A volume of 0.5μL of each sample was dropped on the plate and dried at ambient atmosphere followed by the deposition of 0.5 μL of DHB matrix.

Immobilized Metal-Affinity Chromatography (IMAC). IMAC purification of phosphorylated peptides was performed according to previous reports[7,24] with minor changes. Briefly, samples were diluted in a washing/loading buffer of 250 mM acetic acid/30% ACN, and 100 μL of each sample was added to 20 μL of IMAC beads (PHOS-Select iron affinity gel; Sigma), previously washed three times using the washing/loading buffer. After incubation at room temperature with vigorous shaking for 30 min, the supernatant was removed, and the resin was washed three times with 100 μL of washing/loading buffer to remove nonspecifically adsorbed peptides. The bound peptides were then eluted using 20 μL of 400 mM NH_4OH into a tube, and 0.5 μL of the solution was dropped on a blank target plate. After drying at ambient atmosphere, 0.3 μL of 2% TFA and 0.5 μL of DHB matrix were subsequently added before MALDI-TOF MS analysis.

MALDI-TOF MS and Data Analysis. All the MALDI-TOF mass spectrometry experiments were performed on an Applied Biosystems 4700 proteomics analyzer (Applied Biosystems) equipped with a Nd:YAG laser operating at 355 nm, a repetition rate of 200 Hz, an acceleration voltage of 20 kV, and a delayed

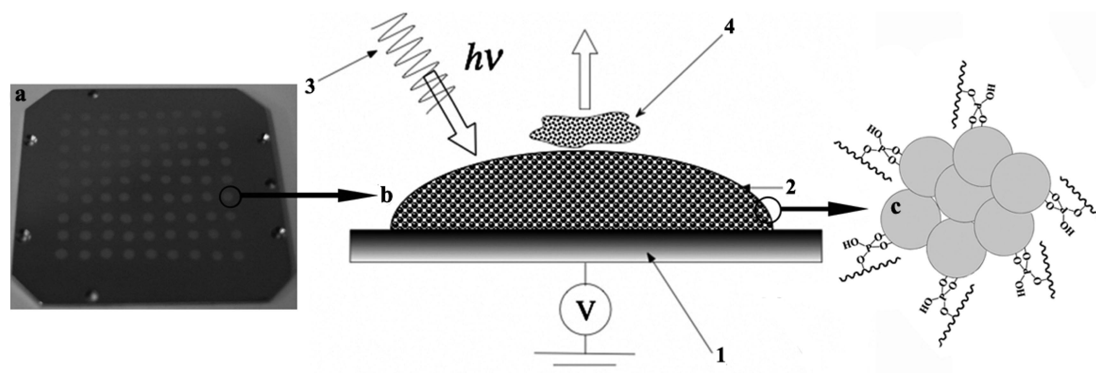

Figure 1. (a) Picture of a self-made sintered TiO_2-modified target plate with 81 spots. (b) Component drawing of the TiO_2 array-based MALDI plate. (1, steel plate; 2, sintered TiO_2 layer; 3, UV laser; 4, released matrix and sample). (c) Schematic illustration of the coordination interaction between phosphophate groups and TiO_2 nanoparticles.

ion-extraction device. All mass spectra were obtained in the positive ion reflector mode with an accumulation of 2000 laser shots and calibrated using an external calibration equation generated from the ion signal of tryptic digest of myoglobin. The laser intensity was adjusted to obtain good resolution and signal-to-noise ratio (S/N) generally from 6000 to 7000 instrument units varied with different samples. In all experiments, DHB (20 mg/mL) in 49% ACN and 1% phosphoric acid was used as the matrix to help ionization. Mass spectrometric data analysis was performed with Data Explorer (TM) Software and the FindMod tool available on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://expasy.org/). The masses and intensities of special peaks were read out using the Data Explorer (TM) Software, and then sent to the FindMod tool for database searching, where the experimentally measured peptide masses were compared with the theoretical peptides calculated from a specified Swiss-Prot/TrEMBL entry to finish the peak identification.

Results and Discussion

On-Plate Enrichement of Phosphorylated Peptides Based on a TiO_2 Nanoparticle Modified Target Plate. Drops of a suspension of nanoparticles are applied on a plain stainless steel plate to form an array of spots (Figure 1a). After solvent evaporation, the particles are heated at 400 °C for sintering, which is an important procedure to ensure the highest sorbing capacity and form a stable frit support phase for samples and matrix. When the TiO_2 frit spots are exposed to the samples, the phosphorylated peptides are selectively captured on the modified target plate. Afterward, a washing step is employed to effectively remove the nonspecifically bound peptides. A solution of 2,5-DHB (DHB at 20 mg/mL in ACN/water/TFA, 50/49.9/0.1% (v/v)) is used as elution solution, which has been proven capable of specifically displacing nonspecifically bound acidic peptides from the TiO_2.[7] This solution is also used to dilute the digest products of proteins to avoid adsorption of nonphosphorylated peptides. Following the washing step, an aqueous ammoniac solution is added to desorb the captured phosphorylated peptides from the TiO_2 substrate. After the addition of an organic acid overlayer, part of the matrix is ablated and released in gas phase with samples under the irradiation of UV laser (Figure 1b), and then the released ions are driven by electric fields to a mass spectrometer for measurements.

This on-target enrichment strategy was first studied by extracting phosphorylated peptides from the β-casein digest with varied concentrations in 30 min. β-Casein is a major protein component of milk, containing 5 different phosphorylated sites on serine, and has been widely used as a model sample in the study of phosphoproteome analysis.[7,9–12,25] With the well-studied sequence, the theoretical masses of the tryptic digest peptides derived from β-casein could be calculated, and the detected peaks on mass spectra could be identified by comparison with theoretical results. Figure 2a shows the mass spectrum of the tryptic digest of β-casein at a concentration of 8.5×10^{-7} M (90% of purity) obtained on the TiO_2-modified plate. Almost all the peaks observed are related to phosphorylated peptides. The peaks 12, 14, and 19 (m/z 2061.8, 2556.1, and 3122.3) are derived from phosphopeptides of β-casein, while the peaks marked 3, 4, 6, 7, 9, and 10 (m/z 1466.6, 1539.7, 1594.7, 1660.8, 1927.7, and 1952.0) are derived from phosphopeptides of α-casein. Here, not only all the five phosphorylated sites of β-casein are identified from three phosphopeptides, but also six phosphorylated peptides of α-casein are clearly observed due to the substoichiometric impurity present in β-casein (about 10% or less in the sample). The detailed sequence information is listed in Table 1. This result indicates that the plate-based in situ phosphopeptides enrichment method is really feasible. The peaks 1, 2, and 5 (m/z 1031.4, 1278.6, and 1561.7) could not be directly identified during the database searching. However, we observe that each isotope peak of them is separated by 0.5 m/z units, respectively (Figure 2a, inset), indicating a characteristic of doubly charged ions. By calculation, we suspect that they could be attributed to the doubly charged ions of the three phosphopeptides 12, 14, and 19. Though it is unusual to see doubly charged ions with such a small molecular weight in MALDI mass spectrum, a similar phenomenon has been reported previously.[11] We can also observe the metastable loss of H_3PO_4 from parent ions during the fly procedure in TOF-tube, which further supports the identification of phosphorylation in proteins. The peaks marked 11 and 13 (m/z 1968.3 and 2462.6) are assigned to the dephosphorylated fragments of peptide 12 and 14, respectively, and the peaks 16, 17, and 18 (m/z 2841.8, 2935.3, 3028.8) are related to the dephosphorylated fragments of peptide 19, a multiply phosphorylated peptide. Because of the reduced kinetic energy, the mass difference between the metastable peaks and their respective parent ions is ~93.5 Da instead of 98 Da as expected for in-source fragments.[26]

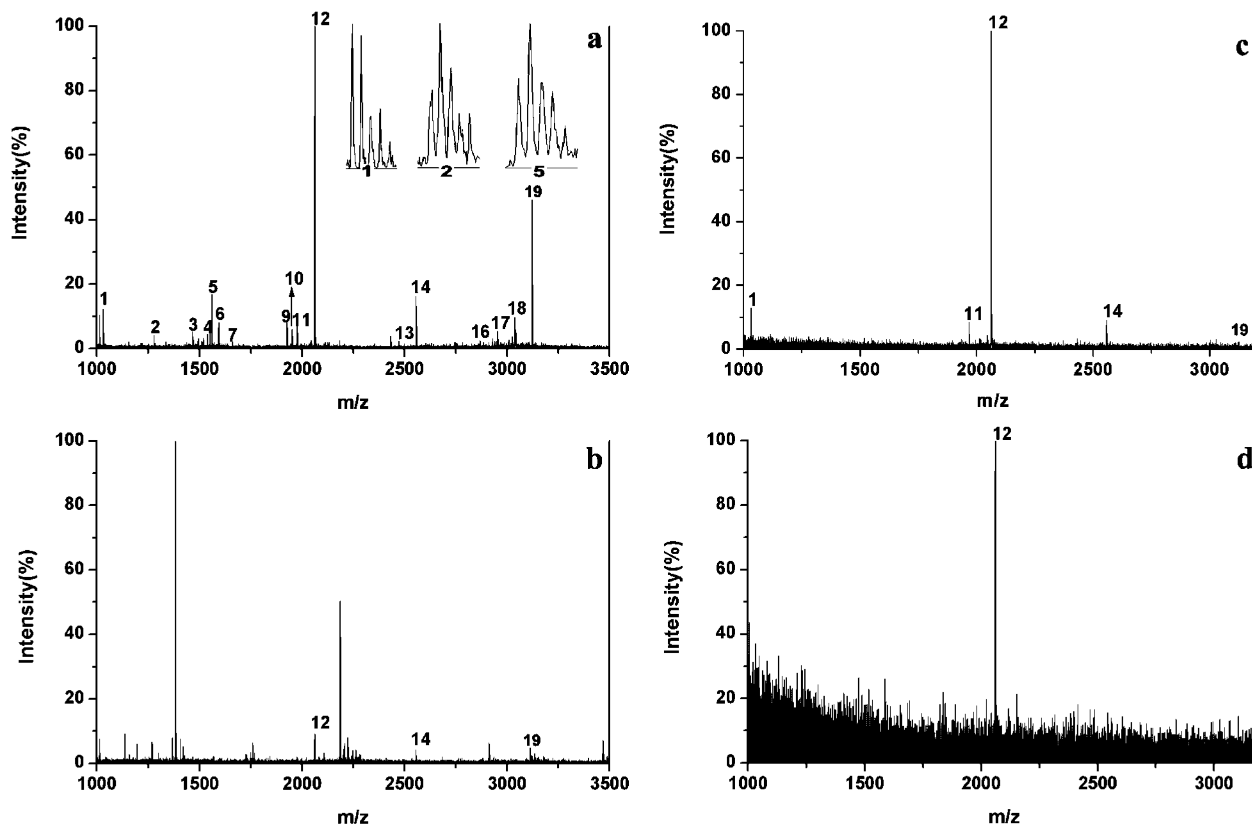

Figure 2. Mass spectra obtained using the TiO_2-modified target plate to selectively enrich phosphorylated peptides from the tryptic digests of β-casein in 30 min at concentrations of 8.5×10^{-7} M (a), 8.5×10^{-9} M (c), and 1.7×10^{-9} M (d), respectively. Mass spectrum of the tryptic digests of β-casein (8.5×10^{-7} M) obtained on a regular plate without pre-enrichment (b).

Table 1. Detailed Information of the Observed Phosphorylated Peptides Obtained from Tryptic Digestion of α-Casein S1 and S2, and β-Casein[a]

peak number	observed *m/z*	number of phosphate groups	aa	amino acid sequence
No. 3	1466.6	1	α-S2 (153–164)	TVDMESTEVFTK
No. 4	1539.7	2	α-S2 (126–137)	EQLSTSEENSKK
No. 6	1594.7	1	α-S2 (153–165)	TVDMESTEVFTKK
No. 7	1660.8	1	α-S1 (106–119)	VPQLEIVPNSAEER
No. 8	1832.8	1	(α-S1)[b]	YLGEYLIVPNSAEER
No. 9	1927.7	2	α-S1 (43–58)	DIGSESTEDQAMEDIK
No. 10	1952.0	1	α-S1 (104–119)	YKVPQLEIVPNSAEER
No. 12	2061.8	1	β (33–48)	FQSEEQQQTEDELQDK
No. 14	2556.1	1	β (33–52)	FQSEEQQQTEDELQDKIHPF
No. 19	3122.3	4	β (1–25)	RELEELNVPGEIVESLSSSEESITR

[a] The phosphorylation sites are underlined. [b] A new sequence variant of the α-S1 casein in the region 104–119.[7]

As a comparison, the same β-casein digest (8.5×10^{-7} M, 90%) analyzed by MALDI-MS without pre-enrichment is shown in Figure 2b. Only three weak peaks of phosphorylated peptide–ions are detected (marked with 12, 14, and 19), in the presence of numerous nonphosphopeptide signals. Therefore, it is clear that phosphorylated peptides could be selectively trapped by the TiO_2 phase on the modified target plate. Furthermore, when the concentration of sample is as low as 8.5×10^{-9} M, several phosphopeptide-related peaks (1, 11, 12, 14, and 19) are observed in the mass spectrum after on-plate enrichment by the sintered TiO_2 nanoparticles (Figure 2c). This result shows that the on-target enrichment technique is sensitive to phosphopeptide samples at low concentrations. When the concentration is lowered down to 1.7×10^{-9} M, the peak of phosphopeptide (12 at *m/z* 2061.8) can still be observed in the mass spectrum (Figure 2d).

Highly Specific Enrichment of Phosphorylated Peptides from Complex Peptide Mixtures. The selectivity of this on-plate enrichment method was further evaluated by capturing phosphorylated peptides from complex mixtures of tryptic digests of BSA and β-casein with ratios of 1:1, 15:1, and 100:1 (mol/mol) in 30 min. The concentration of BSA was fixed at 2.6×10^{-6} M in the mixtures. Figure 3a presents the mass spectrum of the mixed tryptic digest of BSA and β-casein at ratio of 1:1 without pre-enrichment. Only two phosphopeptide–ion signals (12 and 19) are observed in the mass spectrum at *m/z* 2061.8 and 3122.3, the other abundant ion signals pertaining to nonphosphopeptides. In contrast, after enrich-

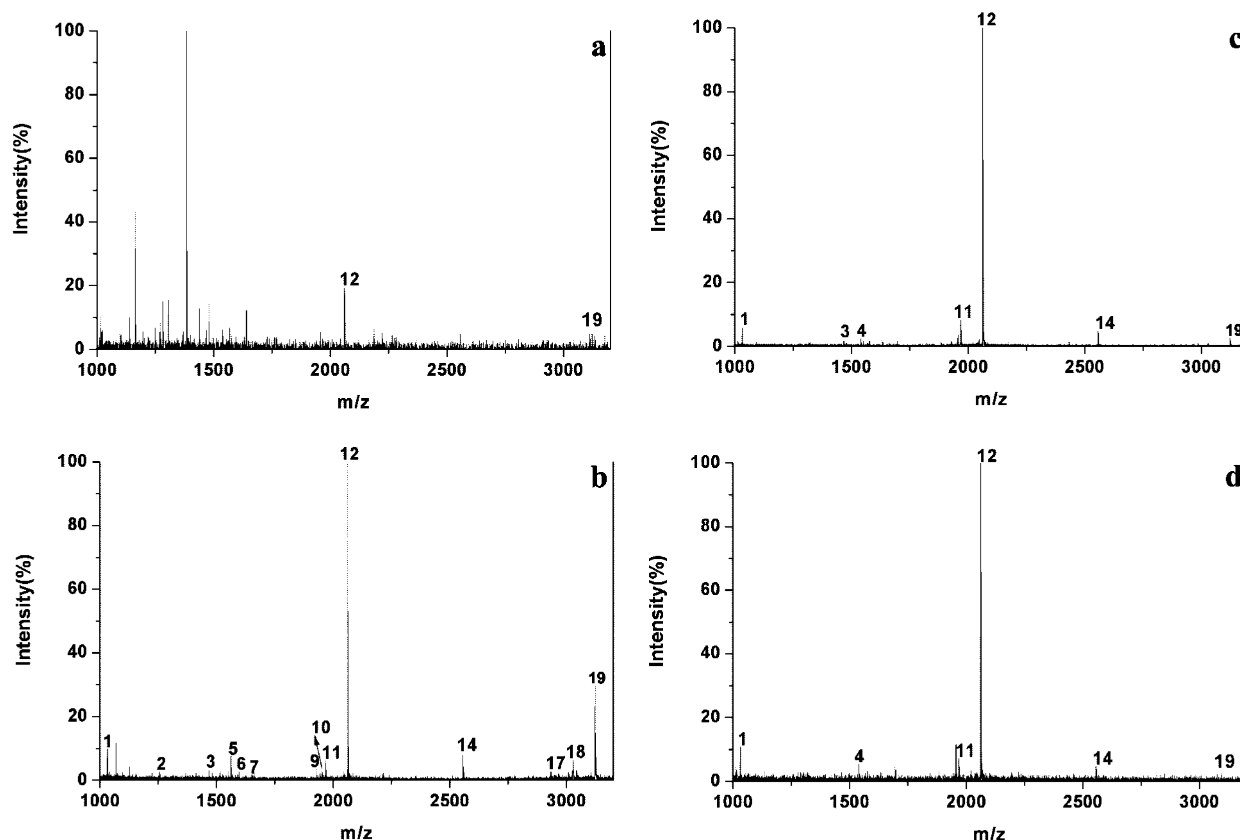

Figure 3. (a) Mass spectrum of the mixed tryptic digests of BSA and β-casein with the ratio of 1:1 obtained on a regular target plate without pre-enrichment. Mass spectra obtained using the TiO_2 nanoparticle modified plate to selectively concentrate phosphorylated peptides from the digest mixtures of BSA and β-casein with ratios (mol/mol) of 1:1 (b) 15:1 (c), and 100:1 (d), respectively.

ment by the TiO_2-modified target plate, the signals related to phosphopeptides are highly enhanced and dominate the mass spectrum as shown in Figure 3b. Three phosphorylated peptides (12, 14, and 19) of β-casein and five phosphopeptides (3, 6, 7, 9, and 10) of α-casein are clearly detected. The other peaks are assigned to doubly charged ions (1, 2, and 5) and dephosphorylated fragment ions (11, 17, and 18). Panels c and d of Figure 3 display the mass spectra obtained after the on-plate enrichment of phosphopeptides from the tryptic digest mixtures of BSA and β-casein at ratios of 15:1 and 100:1, respectively. Three peaks (12, 14, and 19) of phosphopeptides at m/z 2061.8, 2556.1, and 3122.3 are selectively detected in the presence of numerous abundant nonphosphopeptides from BSA.

For comparison, a commercial IMAC product (PHOS-Select iron affinity gel) was employed to enrich phosphopeptides from the same digest mixtures of BSA and β-casein with ratios of 1:1, 3:1, and 15:1 (mol/mol), where the concentration of BSA was fixed at 2.6×10^{-6} M. IMAC is one of the most popular techniques used for the isolation of phosphopeptides, which has been utilized either in batchwise or in column formats to develop semiautomatic or online analysis of phosphoproteome.[24,27] The resulting mass spectra are presented in Figure 4. Three phosphorylated peptides (12, 14, and 19) derived from β-casein are clearly identified from the digest mixture of BSA and β-casein at a ratio of 1:1 (Figure 4a). The peaks of phosphopeptides dominate the mass spectrum, and the phenomenon of metastable loss of H_3PO_4 is well-observed. However, the selectivity is limited when excessive nonphosphopeptides are included in the samples. Only two peaks related to phosphopeptides (12 and 19) appear in the mass spectrum with abundant nonphosphopeptide signals when the ratio of BSA to β-casein is at 3:1 (Figure 4b). As the ratio is further increased to 15:1, almost no signals from phosphopeptides could be detected (Figure 4c). Compared with the corresponding results in Figure 3, one can see that the TiO_2-modified plate shows a better selective affinity to phosphorylated peptides. Considering the less specific interaction between Fe^{3+} and phosphopeptides,[7,9] strategies have been developed as improvements over commercially available techniques recently.[28]

To further test the advantages of this on-plate enrichment strategy, a complex sample from the tryptic digest of commercial bovine milk, which contains fruitful phosphorylated proteins such as α- and β-casein, was employed. The milk producer indicates a protein concentration of at least 3%, and it has been reported that the casein content is about 66% (w/w) in milk proteins,[10] so that the concentration of casein used in this experiment is approximately 10^{-6} M. Figure 5a shows the mass spectrum of the tryptic digest of drinking milk without pre-enrichment. Only one weak ion signal of phosphopeptide at m/z 2061.8 appears together with abundant nonphosphopeptide signals. After the on-plate enrichment of 30 min by the sintered TiO_2 nanoparticles, all the dominating peaks are related to phosphorylated peptides (Figure 5b), including peptides 12, 14, and 19 from β-casein and 4, 7, 8, 9, and 10 from α-casein (Table 1).

Rapid in Situ Enrichment of Phosphorylated Peptides. To speed-up the analysis and avoid sample loss, it is important to develop an online method to concentrate phosphopeptides following the separation steps prior to MALDI MS to provide

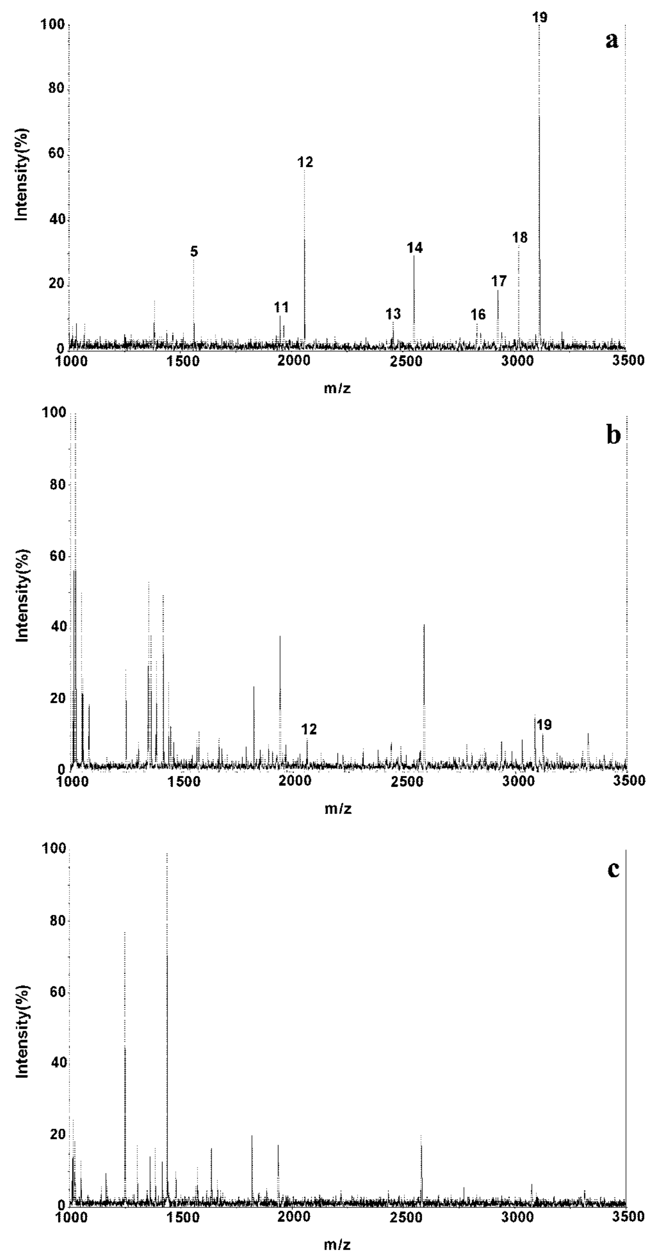

Figure 4. Mass spectra obtained using the IMAC beads to selectively enrich phosphopeptides from the tryptic digest mixtures of BSA and β-casein with ratio of (a) 1:1, (b) 3:1 and (c) 15:1 (mol/mol).

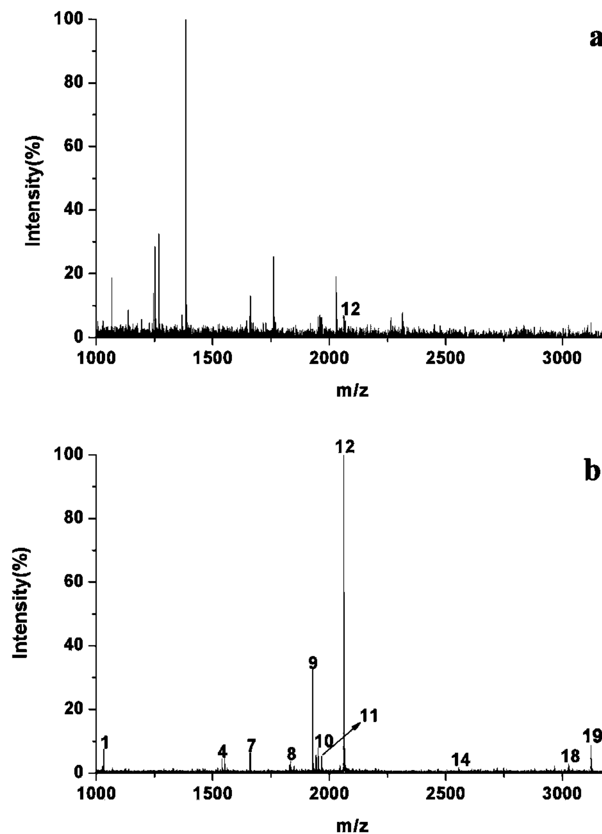

Figure 5. (a) Mass spectrum of the tryptic digests of commercial bovine milk obtained on a regular target plate without pre-enrichment. (b) Mass spectrum obtained using the TiO_2 nanoparticle modified plate to selectively concentrate phosphorylated peptides from the tryptic digest of the bovine milk in 30 min.

high-throughput protein profiling especially when only a limited amount of sample is available. Here, the TiO_2 microarray-based on-plate enrichment method is a promising strategy to achieve this goal. Accordingly, 2 μL of tryptic digest of β-casein at different concentrations was deposited onto the TiO_2 spot on the modified target plate, subsequently incubated at room temperature for only 5 min. After the washing step, the captured phosphorylated peptides were directly analyzed by MALDI-MS. The resulting mass spectra are shown in Figure 6. When the amount of tryptic digest on one TiO_2 spot is 1.5 pmol, the obtained spectrum (Figure 6a) is quite good as 11 peaks related to phosphopeptides, including 3 peaks of phosphopeptides (12, 14, 19) from β-casein, 2 peaks of phosphopeptides (3, 10) from α-casein, and all the 6 dephosphorylated peaks (11, 13, 15 (m/z 2748.3), 16, 17, and 18) of parent ions 12, 14, and 19 are clearly visible. The detection limit of this on-plate strategy is about 30 fmol (Figure 6b), which is comparable with the recently reported methods based on chromatography,[7,8,29] microchip,[30] on-plate solid extraction,[13] chemical modification,[31] or magnetic beads.[10–12,22] These strategies generally exhibit high selectivity and sensitivity at femtomole level (about 10–50 fmol)[10,11,22] with a possible combination with ESI or MALDI MS measurement,[27,29] whereas the present on-plate approach is a rapid in situ enrichment technique for direct MALDI MS analysis. By integrating the steps of phosphopeptide capture, purification, and subsequent mass spectrometry analysis all on a single plate, the analytical procedures are simplified and the possible sample loss is reduced. In this way, the separation fractions from LC could be directly deposited onto the TiO_2 spots for phosphopeptide enrichment prior to MALDI MS analysis.

The low detection limit and high selectivity of this on-plate enrichment toward phosphopeptides could be attributed to an intense coordination reaction occurring between TiO_2 nanoparticles and phosphate groups.[7] The dilution/elution buffer also plays an important role, which includes a strong acid to prevent ionic interaction, an organic solvent at high concentration to avoid hydrophobic interaction, and DHB to prevent nonphosphopeptides adsorption. It has been proven that DHB could enhance the selective enrichment of phosphopeptides on TiO_2 by competing with nonphosphorylated peptides for the active binding sites.[7] Moreover, the very large specific to geometric area ratio of the TiO_2 frit nanoparticle phase on the target plate is another important factor to enhance the phos-

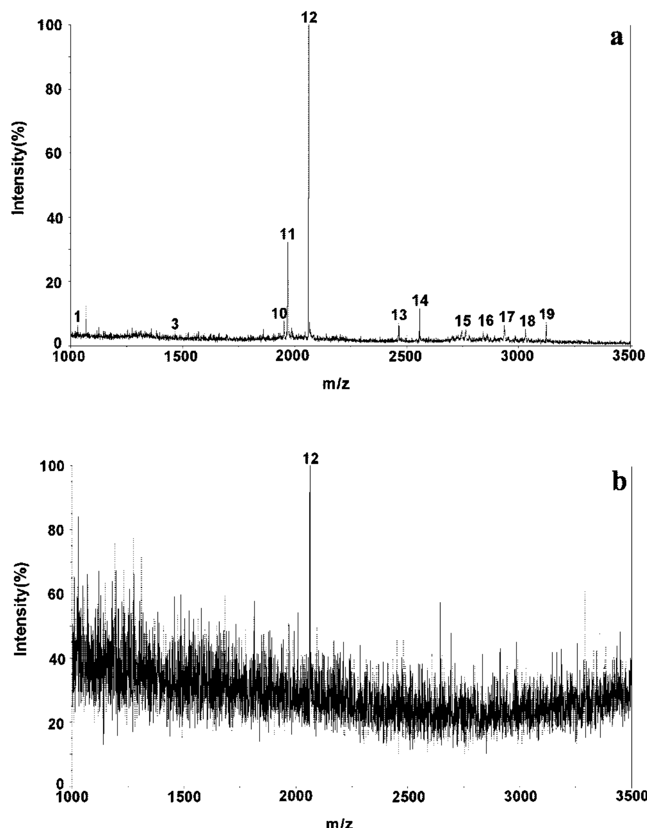

Figure 6. Mass spectra of the tryptic digest of β-casein at (a) 1.5 pmol and (b) 30 fmol after incubation on the TiO_2 nanoparticle spots for 5min at room temperature.

phopeptide concentration efficiency. Indeed, for a sintered nanoparticle layer of thickness h, the ratio of specific surface area/geometric surface area is $3h/r$ for a hard sphere model where r is the radius of the nanoparticles. For example, for a layer thickness of 1 μm and a nanoparticle radius of 10 nm, the specific surface area is 300× larger than the geometric area. Experimentally, the effective area enhancement is smaller than predicted as the relatively large peptides may not be able to penetrate all the way through the nanoscale structure of the TiO_2 film. Nevertheless, the present TiO_2-based spot provides an enhanced adsorption capacity toward the target molecules.

Conclusions

In this paper, a novel on-plate specific enrichment approach based on the interaction between phosphopeptides and TiO_2 nanoparticles is proposed. A target plate modified with the sintered TiO_2 nanoparticles array has been prepared to specifically concentrate phosphopeptides from complex peptide mixtures without any additional procedures such as on-column separation, desalting, or centrifugation. The captured phosphopeptides on the plate could be directly analyzed by MALDI MS after the addition of an organic acid overlayer. We have demonstrated that the TiO_2 array-based target plate exhibits high sensitivity and selectivity to phosphopeptides, while significantly simplifying the analytical procedures and reducing possible sample loss. With these advantages, the on-plate strategy could be very useful for high-throughput analysis of phosphopeptides and phosphoproteins coupled with LC or 2D gel separation. Full automation of the system for real-world proteome analysis is planned as the next step. It should provide a very efficient tool for the large-scale study of post-translational modifications of proteins.

Acknowledgment. This work was supported by NSFC (20775016, 20575013, 20735005), National Basic Research Program of China (2007CB714506), and Swiss-China cooperation research program.

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PR0705284