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Coupling Strong Anion-Exchange Monolithic Capillary with MALDI-TOF MS for Sensitive Detection of Phosphopeptides in Protein Digest

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Protein phosphorylation is one of the most biologically relevant and ubiquitous post-translational modifications. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for the analysis of protein phosphorylation by detection of phosphopeptides in phosphoprotein digest. Enrichment of phosphopeptides by immobilized metal ion affinity chromatography (IMAC) or metal oxide affinity chromatography (MOAC) followed with MALDI analysis is the common approach. However, the pH for loading and elution of phosphopeptides is incompatible with protein digestion as well as the preparation of the MALDI matrix solution. Therefore, some pretreatment steps, such as pH adjustment and desalting, are required, which make the approach tedious and insensitive. In this study, a strong anion-exchange (SAX) capillary monolith was prepared to enrich phosphopeptides from protein digest for MALDI-TOF MS analysis. It was found that phosphopeptides could be specifically retained on the SAX column at high pH around 8 and could be eluted by 5% formic acid. Thus, the protein digests without any pretreatment could be loaded onto the SAX column under basic pH condition; after removing nonphosphopeptides by washing, the bound phosphopeptides could be eluted directly onto a MALDI target and analyzed by MALDI-TOF MS. This approach significantly simplified the analytical procedures and reduced the sample loss. Because of the excellent MALDI MS compatible procedure and the microscale SAX column, a detection limit as low as 50 amol for the analysis of phosphopeptides from β-casein digest was achieved. To circumvent the inconvenience of the sample loading, a new simple sample introducing method based on capillary action was proposed, which further reduced the detection limit to 10 amol.

Protein phosphorylation, one of the most important post-translational modifications of proteins, plays an important role in regulating cellular processes of cell signal transduction. Thus, characterization of protein phosphorylation is of great interest for understanding regulation mechanisms. Protein phosphorylation analysis can be performed at two levels. One is the proteome level, and the other is the individual protein level. Proteome sample, typically total cell lysate, is extremely complex. Therefore, the primarily issue for phosphoproteome analysis is to reduce the sample complexity. Thus, specific enrichment of phosphopeptides from the digest of protein mixture and efficient separation of the enriched phosphopeptides prior to mass spectrometer analysis is crucial for phosphoproteome analysis.[1,2] In comparison with large-scale phosphorylation analysis at the proteome level, phosphorylation site mapping of the individual phosphoprotein is of equal importance.[3,4] The sample for the analysis of phosphorylation sites on an individual protein is not complex as only one protein is presented in the sample. In most cases of biological application, only a trace amount of protein is available for analysis. Therefore, the primary issue for phosphorylation analysis of individual proteins is to improve the detection sensitivity.

Electrospray mass spectrometry is often applied to phosphoproteome analysis because of its good compatibility with reversed-phase liquid chromatography (RPLC) for efficient separation of phosphopeptides. The online coupling of RPLC with a matrix-assisted laser desorption ionization (MALDI) mass spectrometer is difficult, and so MALDI time-of-flight mass spectrometry (MALDI-TOF MS) is typically used to analyze less complex samples. It is very suitable for the analysis of phosphorylation at individual protein level because of its high throughput and high mass accuracy. To analyze phosphorylation sites on a protein, the protein is first digested by protease (typically trypsin), and the phosphorylation sites are then determined by MS analysis of the phosphopeptides in the resulting protein digest. However, it is difficult to detect phosphopeptides with high sensitivity by MALDI-TOF MS due to the low ionization efficiency of phosphopeptides.

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To improve detection sensitivity, phosphopeptides could be chemically modified to enhance the ionization efficiency.[5,6] However, chemical derivatization is typically unspecific and introduces some chemical reagents, thus requiring further purification steps. The use of matrix additives or comatrixes to enhance phosphopeptide response in MALDI MS is a very appealing approach[7−9] as no chemical or enzymatic conversions are required. Since Kjellstrom and Jensen found the addition of 1% phosphoric acid to 2,5-dihydroxybenzoic acid (DHB) matrix could significantly improve the ionization of phosphopeptides,[8] the developed matrix system has been routinely used for MALDI MS analysis of phosphopeptides. It should be mentioned that a very important reason for the low sensitivity of MALDI MS for the detection of phosphopeptides in protein digest is the serious ion suppression by unphosphorylated peptides in the digest. Though the above two methods were proved to be effective for the improvement of ionization efficiency of phosphopeptides, they cannot effectively reduce the interference of unphosphorylated peptides. Therefore, the isolation and enrichment of phosphopeptides from protein digest prior to MALDI-TOF MS analysis is essential for protein phosphorylation analysis.

Immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) are the two most popular approaches to selectively enrich phosphopeptides from protein digest.[10] Enrichment of phosphopeptides followed with MALDI MS analysis can be performed in different ways. Phosphopeptides can be enriched by incubating IMAC or MOAC beads in protein digest followed by washing and elution. The separation of beads from solution is typically realized by centrifugation.[11,12] If magnetic beads are used, a magnetic field can be applied to isolate the beads more efficiently and conveniently.[13−15] After enrichment by beads, a small portion of phosphopeptides are then spotted onto the MALDI plate for analysis. Direct use of beads usually consumes a large amount of protein sample. To circumvent this problem, the beads can be packed into tiny column or tip for processing minute sample. For example, the commercial ZipTip$_{MC}$ tips are made by loading 0.6 μL of IMAC resin into a 10 μL pipet tip, and the enriched phosphopeptides can be directly eluted onto the MALDI plate for subsequent MS analysis. Polymer monolithic extraction tips containing trapped metal oxide nanopowders are used to enrich phosphopeptides for MALDI MS analysis.[16,17] To further simplify the analysis procedure, the enrichment of phosphopeptides can be performed on the MALDI plate.[18,19] The MALDI plate is immobilized with either IMAC materials or metal oxides for on-probe purification. In such analysis, a drop of protein digest is incubated on the MALDI plate, which is then rinsed to remove the unphosphorylated peptides prior to addition of matrix and subsequent analysis using MALDI MS. Because of the limited capacity, the on-probe purification approach is less likely to be able to detect phosphopeptides in very diluted sample. The sensitivity for the above approaches based on either IMAC or MOAC is at the low femtomole,[15−19] and the sample volume is typically varied from a few microliters to several hundred microliters. Therefore, new approaches that are able to detect phosphopeptides from protein digest at much higher detection sensitivity and require much less sample amount are in great demand.

pH is a key factor that crucially influences the detection sensitivity of the current MALDI MS approaches for the characterization of protein phosphorylation. The optimal pH for loading sample and elution of phosphopeptides in IMAC or MOAC are not compatible with that of protein digestion by trypsin and MALDI analysis. A high pH is required for protein digestion, whereas phosphopeptides can only be retained on IMAC or MOAC resin at low pH; therefore, the protein digest needs to be acidified before loading to the beads. The matrix solution for MALDI analysis is always prepared in acidic solution, whereas phosphopeptides can only be eluted from IMAC or MOAC beads by alkaline buffer. To solve this problem, the eluted phosphopeptides in alkaline buffer can be desalted by using a C18 column or using volatile alkaline buffer like ammonium hydroxide for elution of phosphopeptides followed with drying down the solution. The incompatibility of pH values makes the phosphorylation analysis by IMAC/MOAC−MALDI MS tedious and of low sensitivity. To overcome this limitation, we turn to the use of strong anion-exchange (SAX). It was reported recently that phosphopeptides could be specifically isolated from protein digest by SAX.[20,21] As compared with unphosphorylated peptides, phosphopeptides are more negatively charged and thereby are retained more strongly on SAX than unphosphorylated peptides. The loading of phosphopeptides onto SAX can be performed by using buffer with high pH at which the phosphopeptides are highly negatively charged. The captured phosphopeptides can be eluted by using buffer with low pH.[21] It is clear that the loading and elution pH values for SAX-based enrichment are perfectly compatible with both protein digestion and the MALDI matrix solution. Thus, the coupling of phosphopeptide enrichment by a capillary SAX column with MALDI MS should be an excellent platform for the analysis of protein phosphorylation in minute samples.

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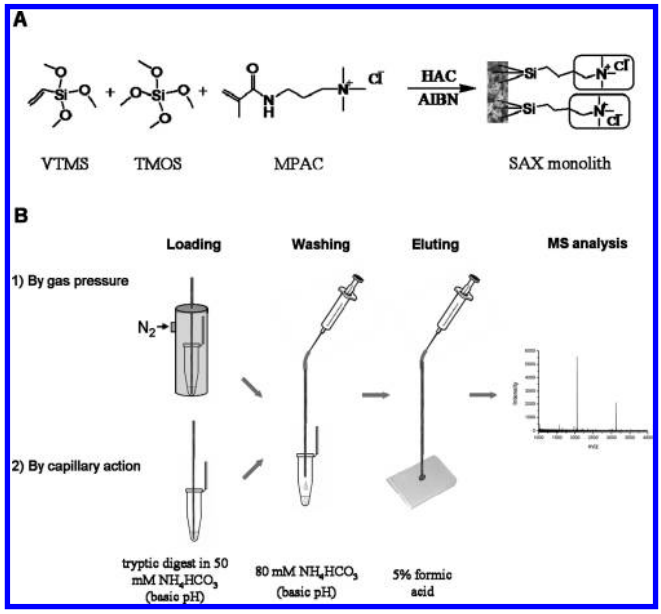

Figure 1. (A) Schematic for the incorporation of the organic monomer with alkoxysilanes to form the SAX monolith and (B) schematic of the sample purification procedures.

In this study, a new approach that couples SAX monolithic capillary-based enrichment with MALDI-TOF MS was developed for sensitive detection of phosphopeptides in protein digest. An organic−silica hybrid SAX monolithic capillary was prepared for selective enrichment of phosphopeptides. Protein digests without any pretreatment were loaded onto the SAX column at high pH, after removing nonphosphopeptides by washing, the bound phosphopeptides were eluted with 5% formic acid directly onto a MALDI target and analyzed by MALDI-TOF MS. Because of the excellent MALDI MS detection compatible procedure and the microscale SAX column, a detection limit at the low attomole level for the analysis of phosphopeptides from β-casein digest was achieved. To facilitate the sample loading, a new sample introducing method based on capillary action was proposed, which reduced the sample dilution effect and thereby further improved the detection sensitivity. To the best of our knowledge, this is the first report of the detection limit at low attomole for detection of phosphopeptides in protein digest. This system is very suitable for analysis of protein phosphorylation at the individual protein level due to its high sensitivity and simplified procedure.

EXPERIMENTAL SECTION

Materials and Chemicals. All the water used in this experiment was prepared using a Milli-Q system (Millipore, Bedford, MA). Dithiothreitol (DTT), ammonium bicarbonate, and iodoacetamide (IAA) were all purchased from Bio-Rad (Hercules, CA). α-Casein, β-casein, bovine serum albumin (BSA), trypsin (from porcine pancreas, TPCK-treated), DHB, and urea were all obtained from Sigma (St. Louis, MO). Poly(ethylene glycol) (PEG, 10 000 MW), vinyltrimethoxysilane (VTMS), [3-(methacryloylamino)propyl]trimethylammonium chloride (MPAC, 50 wt % solution in water), formic acid, and acetonitrile (ACN) were obtained from Aldrich (Milwaukee, WI). The molecular structure of the organic monomer used in this experiment is illustrated in Figure 1A. Tetramethoxysilane (TMOS) was obtained from Chemical Factory of Wuhan University (Wuhan, China). 2,2-Azobisisobutyronitrile (AIBN) was purchased from Shanghai Chemical Plant (Shanghai, China) and recrystallized in ethanol before use. Fused-silica capillaries with 150 μm i.d. were purchased from the Reafine Chromatography Ltd. (Hebei, China). All the chemicals were of analytical grade except ACN, which was of HPLC grade. Matrix DHB was dissolved in ACN/water (50/50, v/v) solution containing 1% H_3PO_4 by keeping DHB at 25 mg/mL.

Preparation of the SAX Organic−Silica Hybrid Monolithic Column. The organic−silica hybrid SAX monolithic capillary was prepared according to the "one-pot" process reported previously[22] with slight modification. The fused-silica capillary was pretreated by rinsing with 1.0 M HCl for 12 h, water for 30 min, 1.0 M NaOH for 12 h, and water for another 30 min. The capillary was then dried with nitrogen gas at room temperature for further use. To prepare the organic−silica hybrid SAX monolithic column, first a prepolymerization mixture containing acetic acid (0.01 M, 5.0 mL), PEG (540 mg), TMOS (1.8 mL), and VTMS (600 μL) was prepared and stirred at 0 °C for 1 h to form a homogeneous solution. Then MPAC (40 μL) and AIBN (2 mg) were added into 0.5 mL of the resulting precondensation mixture with 10 min of sonication. After that, the mixture was manually introduced into the pretreated capillary with a syringe, and then both ends of the capillary were sealed with rubber stoppers. The capillary was incubated at 30−45 °C for 12 h for condensation reaction and 60 °C for another 12 h for polymerization. The obtained organic−silica hybrid monolithic column was then flushed with water and methanol to remove the PEG and other residuals before use.

Tryptic Digestion of Proteins. α-Casein and β-casein (1 mg) were dissolved separately in 1 mL of ammonium bicarbonate (50 mM, pH 8.2) and digested with trypsin for 18 h at 37 °C with an enzyme-to-protein ratio of 1:25 w/w. BSA (4 mg) was dissolved in 1 mL of denaturing buffer containing 8 M urea and 50 mM ammonium bicarbonate and incubated for 3 h. To the protein solutions 20 μL of 50 mM DTT was added. The disulfide bond of the 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 the dark. After that, the mixture was diluted with 50 mM ammonium bicarbonate (pH 8.2) by 10-fold and incubated for 18 h at 37 °C with trypsin at an enzyme-to-protein ratio of 1:25 w/w to produce proteolytic digest. Then, the BSA tryptic digest was purified with a homemade C18 solid-phase cartridge and exchanged into 0.1% formic acid−water solution.

Purification of Phosphopeptides by the SAX Column. The overall procedure for purification of phosphopeptides by the SAX column included steps of sample loading, washing, and elution. Ammonium bicarbonate solution (50 mM, pH 8.2) was used as the loading buffer, while 5% formic acid solution was used as the elution buffer. For MALDI-TOF MS analysis of the eluted phosphopeptides, DHB solution (25 mg/mL in 50% ACN) containing 1% H_3PO_4 was used as the matrix solution. Two different sample loading approaches were used, as illustrated in Figure 1B. One is pressure loading by nitrogen gas. The sample, tryptic digest of phosphoprotein, was first diluted by the loading buffer to a proper concentration. Then, 20 μL of the sample was transferred to a 1.5 mL microcentrifuge tube and the tube was

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place in a high-pressure bomb connected to a nitrogen gas cylinder. A 150 μm i.d. SAX monolithic column was inserted into the bomb. The sample was introduced by applying 200 psi of nitrogen until all the sample was completely pushed into the capillary. After that, washing and eluting were carried out using a syringe. The column was washed with 80 μL of 50 mM ammonium bicarbonate solution followed by washing with 80 μL of 80 mM ammonium bicarbonate solution (pH 8.2) to remove unspecially bound peptides. The captured peptides were then eluted by 20 μL of 5% formic acid solution into a tube. An amount of 0.5 μL of this eluate was deposited onto a MALDI target plate. After it was dried, 0.5 μL of DHB solution (25 mg/mL in 50% ACN) containing 1% H_3PO_4 was added for MALDI-TOF MS analysis. When the amount of the loaded sample was less than 1 fmol, the elution procedure was different. Before elution, the washing buffer inside the column was removed by pumping gas into the column using an empty syringe. Then the bound peptides were eluted directly onto a MALDI target plate by about 2 μL of 5% formic acid solution with a syringe. The other sample loading method was by capillary action. A 5 cm $\times$ 150 μm i.d. SAX monolithic column was first dried with nitrogen gas at room temperature for 1 min, and then it was dipped into a protein digest solution with proper concentration. It can be filled with the sample solution automatically in less than 2 min due to capillary action. Except sample loading step, the other steps were the same with those above.

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 50 laser shots.

RESULTS AND DISCUSSION

Preparation and Characterization of the Sax Monolithic Capillary. The organic−silica hybrid SAX monolithic capillary column was prepared by the "one-pot" approach reported recently.[22] The characteristic of this approach is that the organic functionalities can be introduced onto the hybrid monolith by mixing the organic monomers with silane monomers concurrently followed with in situ polycondensation and polymerization. The organic monomer, MPAC, has a quaternary ammonium group, and so it provides the SAX functionality after the monolith is formed. The schematic for the incorporation of the organic monomer with alkoxysilanes for the preparation of the hybrid monolith is shown in Figure 1A. Two major reactions were involved: the polycondensation of hydrolyzed precursors of TMOS and VTMS, and the copolymerization of the precondensated siloxanes and vinyl organic monomers. The porous monolithic structure was formed during the polycondensation step. Since the formation of the through-pores is highly temperature-dependent, the polycondensation temperature was carefully optimized. The experiments indicated that a condensation temperature lower than

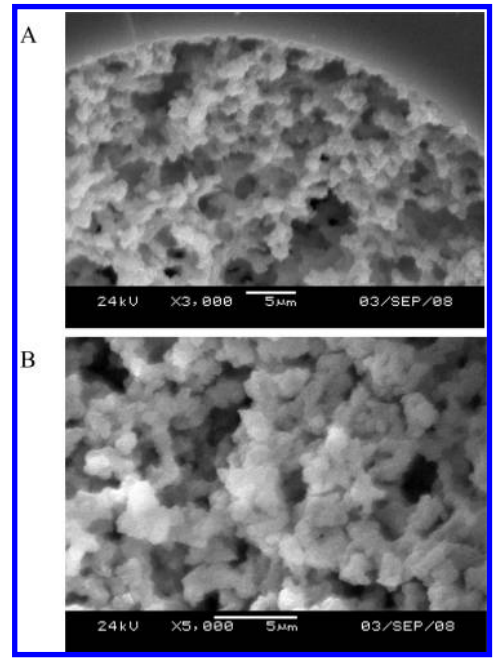

Figure 2. SEM photographs of the organic−silica hybrid SAX monolith at different magnifications: (A) 3000×; (B) 5000×.

35 °C leads to the formation of big through-pores and detachment of the hybrid monolith from the inner capillary wall, whereas a temperature higher than 42 °C resulted in poor permeability, which limited quick sample loading. It was found the column synthesized at 37 °C exhibited acceptable column permeability. The copolymerization reaction was performed at 60 °C, which is the same with most free radical polymerizations using AIBN as an initiator. The scanning electron microscopy (SEM) images of the obtained hybrid SAX column are shown in Figure 2. It can be seen that the formed organic−silica monolithic matrix was well-attached to the inner wall of the capillary and a uniform organic−silica hybrid monolithic matrix with large through-pores was obtained. The permeability of the monolithic column was examined using a μUPLC pump (Waters) with ACN and water as the mobile phase. The permeability decreased by 17% when the flushing solution changed from ACN to water, which indicates the column's swelling in aqueous buffer and shrinking in organic buffer were slight. The monolithic column also showed acceptable permeability and good mechanical stability under a pressure as high as 5700 psi at a flow rate of 40 μL/min. The SAX monolith was stable for 4 weeks of continuous use with a constant performance.

Capacity of the capillary is an important parameter. By taking advantage of capillary action, a simple method was developed to measure the total pore volume of the SAX monolithic column at a given length. An open 75 μm i.d. capillary was used as a pipet to measure the volume. First, the open capillary was calibrated. To a centrifuge tube, 1.5 μL of water was transferred by a commercial pipet. One end of the empty capillary was dipped into the water, and all of the water wicked into the capillary due to capillary action. The length of the water zone in the capillary was determined to be 34.4 cm. Thus, the open capillary pipet was calibrated to be 43.6 nL/cm, which is very close to the calculated value, 44.2 nL/cm. Before the measurement of the pore volume, the SAX monolithic capillary was dried by flowing through

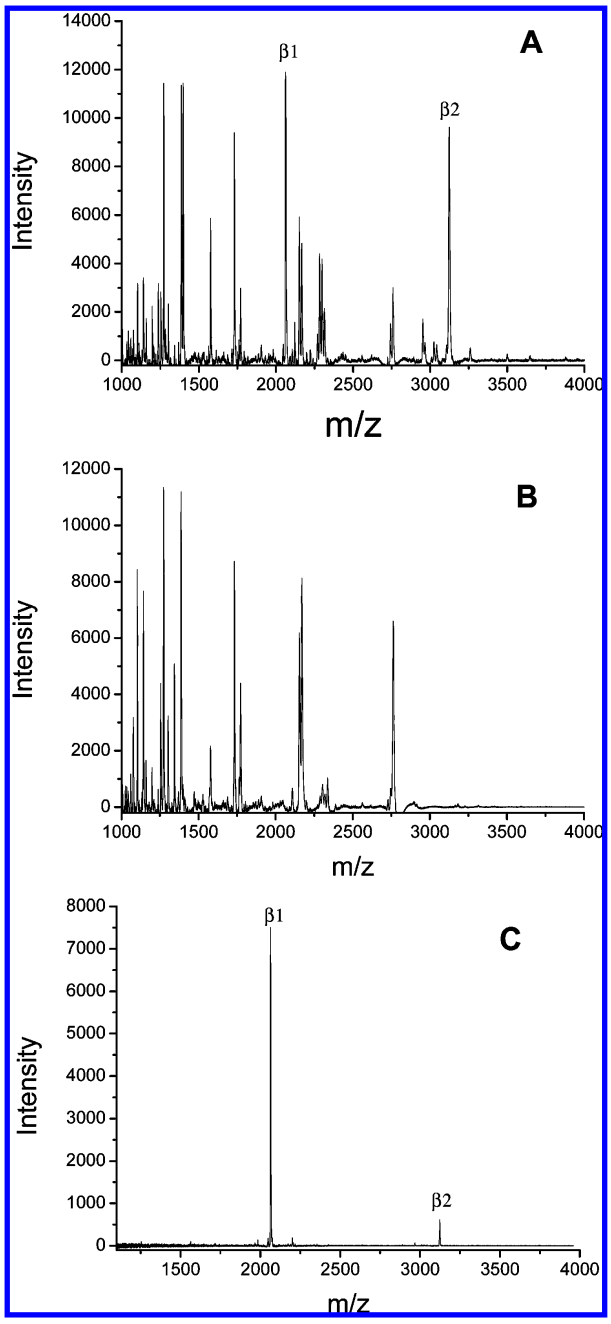

Figure 3. Mass spectra for analysis of tryptic β-casein digest: (A) direct analysis (500 fmol); analysis of (B) flow through and (C) enriched phosphopeptides from the SAX column. Tryptic β-casein digest (20 μL, 7.5 $\times$ 10^{-7} M) was loaded onto a 14 cm $\times$ 150 μm i.d. SAX monolithic column, and $^1/_{40}$ of flow through and $^1/_{50}$ of enriched phosphopeptides were taken for MALDI analysis. Refer to Table 1 for the identities of the peaks.

nitrogen gas. To a centrifuge tube, 1.5 μL of water was transferred by a commercial pipet. And then a dried SAX porous monolithic column of 14 cm $\times$ 150 μm i.d. was dipped into water, and the whole monolithic column was filled with water automatically by capillary action within several minutes. After that, the calibrated capillary was used to measure the volume of the water left in the tube. The volume of the remaining water was determined to be 475 nL. Thus, the pore volume of the 14 cm long hybrid SAX porous monolithic column was 1.025 μL. On the basis of these data, the pore volume of a SAX porous monolithic column with the same inner diameter but with different lengths could be easily determined. For example, the total pore volume for a 5 cm long monolithic column was calculated to be 366 nL.

Table 1. Overview of Observed Phosphopeptides for Tryptic Digest of α-Casein and β-Casein by MALDI MS Analysis

no.	$[M + H]^+$	number of sites	amino acid sequence[a]
		α-Casein	
α1	1237.50	1	TVDMEpSTEVF
α2	1660.79	1	VPQLEIVPNpSAEER
α3	1832.83	1	YLGEYLIVPNpSAEER
α4	1847.69	1	DIGSEpSTEDQAMEDIK
α5	1927.69	2	DIGpSEpSTEDQAMEDIK
α5#	1943.79	2	DIGpSEpSTEDQAoMEDIK
α6	1951.95	1	YKVPQLEIVPNpSAEER
α7	2619.04	4	NTMEHVpSpSpSEESIIp SQETYK
α8	2678.01	3	VNELpSKDIGpSEpSTEDQA MEDIK
α9	2703.91	5	pyroEMEAEpSIpSpSpSEEIVP NpSVEAQK[b]
α10	2720.91	5	QMEAEpSIpSpSpSEEIVPNp SVEQK
α10#	2736.91	5	QoMEAEpSIpSpSpSEEIVPNp SVEAQK
α11	2935.15	3	EKVNELpSKDIGpSEpSTEDQA MEDIK
α12	3008.01	4	NANEEEYSIGpSpSpSEEpSAE VATEEVK
α13	3087.99	5	NANEEEYpSIGpSpSpSEEpSAE VATEEVK
		β-Casein	
β1	2061.83	1	FQpSEEQQQTEDELQDK
β2	3122.27	4	RELEELNVPGEIVEpSLpSpSp SEESITR

[a] pS and oM denote phosphorylated serine and oxidized methionine, respectively. [b] The peptide signal at m/z 2703.91 represents the variant of α-S1 in the region of 74−94.

Specificity of the SAX Monolithic Capillary for Enrichment of Phosphopeptides. The tryptic digest of β-casein was first used to investigate the phosphopeptide enrichment performance of the prepared SAX monolithic column. β-Casein digest (15 pmol) diluted in 50 mM ammonia bicarbonate was loaded onto a SAX monolithic column of 14 cm length and 150 μm i.d. After washing with 80 mM ammonia bicarbonate buffer, the bound peptides were eluted by about 20 μL of 5% formic acid and 0.5 μL (1/50) of this eluate was deposited onto a target plate for MALDI analysis. Figure 3 shows the MALDI mass spectra for direct analysis of β-casein digest and analysis of the flow through and bound peptides on the SAX column. For the direct analysis, many peaks were observed in the mass spectrum, among which two peaks were identified as phosphopeptides: m/z at 2061.83 and 3122.27. The amino acid sequences for these phosphopeptides were identified to be FQpSEEQQQTEDELQDK and RELEELNVPGEIVEpSLpSpSpSEESITR, respectively (details are shown in Table 1). The two phosphopeptide peaks disappeared in the mass spectrum for the analysis of flow through, whereas only the two phosphopeptide peaks were observed in the mass spectrum for the eluted fraction from the SAX column. These results clearly indicate that the phosphopeptides were specifically captured by the SAX column. An 80 mM ammonium bicarbonate buffer was used to wash the SAX monolithic column after sample loading. All nonphosphopeptides disappeared after washing as shown in Figure 3C, which means the washing buffer could effectively remove most of nonphosphopeptides from the SAX column. With

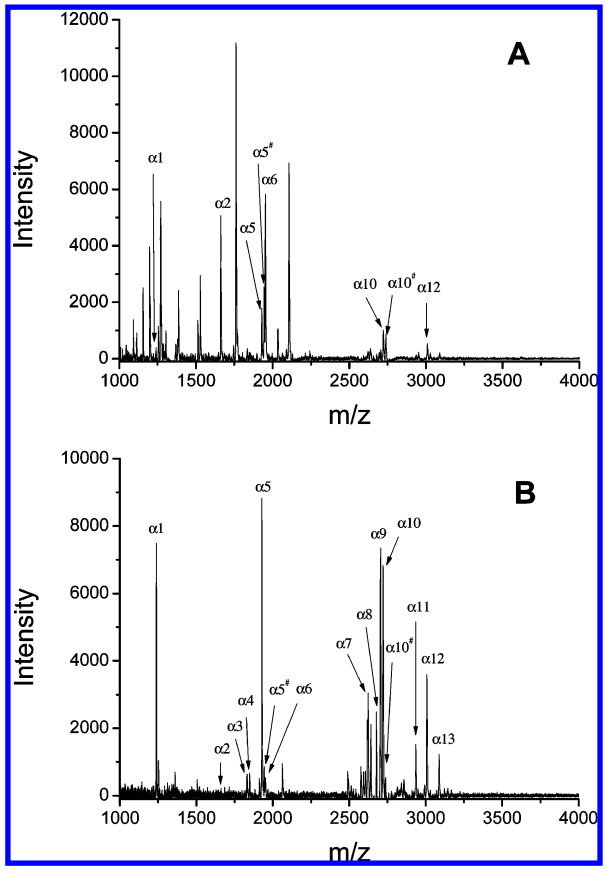

Figure 4. Mass spectra for analysis of tryptic α-casein digest: (A) direct analysis (500 fmol); (B) analysis of enriched phosphopeptides from the SAX column. Tryptic α-casein digest (20 μL, 7.5×10^{-7} M) was loaded, and $^{1}/_{50}$ of enriched phosphopeptides were taken for MALDI analysis. Other conditions were as in Figure 3. Refer to Table 1 for the identities of the peaks.

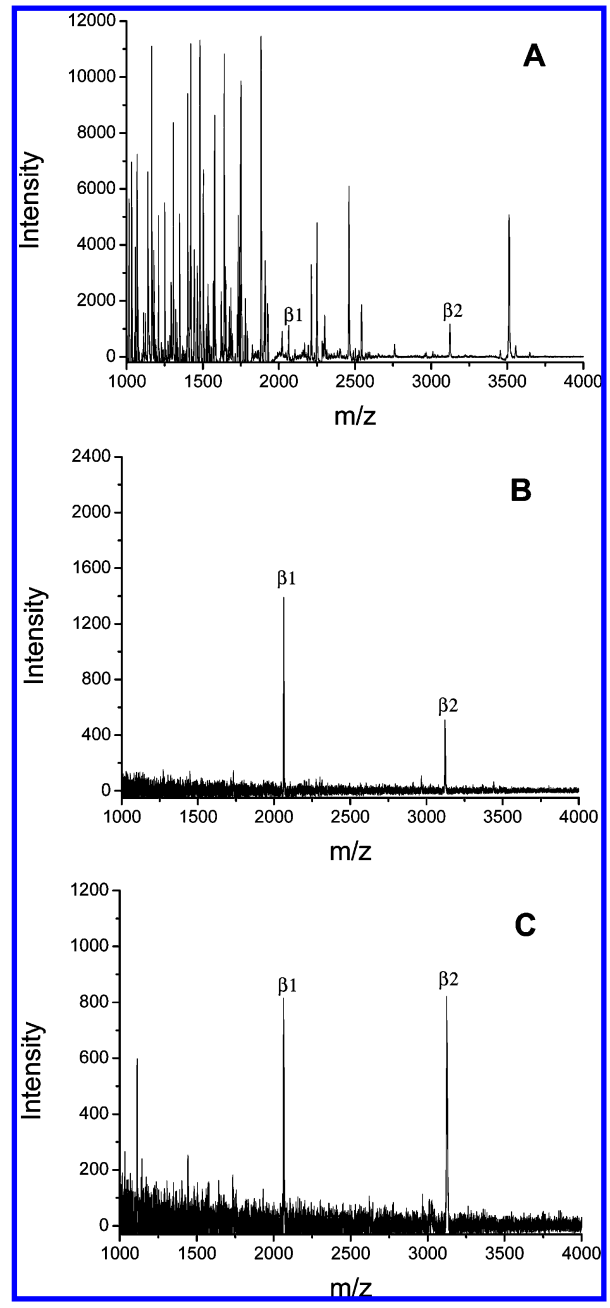

Figure 5. Specificity for enrichment of phosphopeptides by the SAX monolithic column: mass spectra for (A) direct analysis of the digest mixture of β-casein and BSA with a molar ratio of 1:10; analysis of phosphopeptides enriched by the SAX column from the digest mixture of β-casein and BSA with a molar ratio of (B) 1:10 (5 pmol of β-casein and 50 pmol of BSA were loaded onto the SAX column) and (C) 1:100 (0.5 pmol of β-casein and 50 pmol of BSA were loaded onto the SAX column).

further increased concentration of ammonia bicarbonate in the washing buffer, the loss of phosphopeptides was observed. Therefore, ammonia bicarbonate with the concentration of 80 mM was used as the washing buffer throughout of this study. The capability of this method to complex samples was demonstrated using the tryptic digest of α-casein, which has more phosphopeptides than β-casein does. A mass spectrum for direct MALDI-TOF analysis of the tryptic digest of α-casein without any pretreatment is shown in Figure 4A. Because the signals of the phosphopeptides were suppressed by the nonphosphorylated peptide ions, only eight phosphopeptides were detected. However, after extraction and enrichment of phosphopeptides by the SAX monolithic column, 15 peaks of phosphopeptides from tryptic digest of α-casein were detected and dominated the mass spectrum. Information for the phosphopeptides, including amino acid sequence and phosphorylation sites, is shown in Table 1. All the results proved that the SAX monolithic column can efficiently capture the phosphopeptides from the digest of phosphoproteins.

To further investigate the specificity of this phosphopeptide enrichment approach, more challenging samples, digest mixtures of β-casein and BSA at molar ratio of 1:10 and 1:100, were used as the test samples. Because the unphosphorylated peptides from BSA are of much higher abundance than those of phosphopeptides from β-casein, the dominant peaks in the MALDI spectrum for direct analysis of the digest mixture of β-casein and BSA at molar ratio of 1:10 were unphosphorylated peptides (Figure 5A). However, after SAX enrichment (Figure 5B), only two phosphopeptide peaks dominated in the mass spectrum, which indicates that nonphosphopeptides were effectively removed and phosphopeptides were specifically enriched. The phosphopeptides could still be specifically enriched by the SAX monolithic column when the ratio was further decreased to 1:100 (Figure 5C). The specificity of the SAX column for isolation of phosphopeptides is much better than that of the commonly used Fe^{3+}−IMAC as unphosphorylated peptides were observed in the spectrum when the ratio of phosphoprotein to unphosphorylated protein

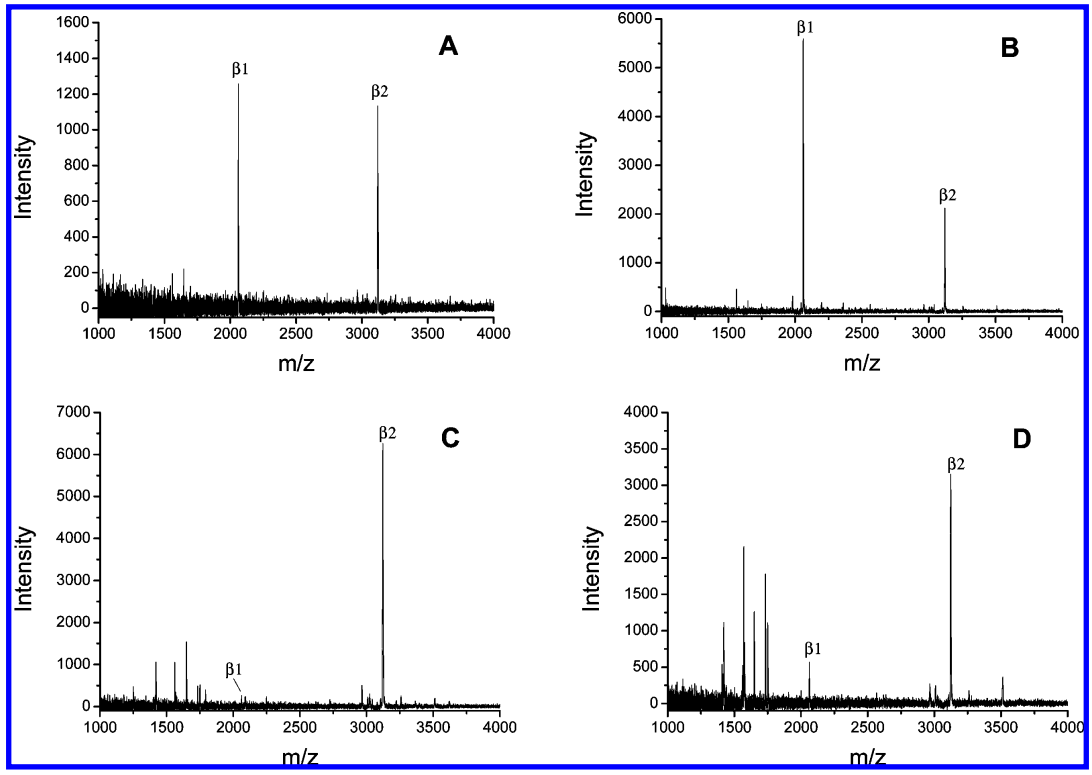

Figure 6. Mass spectra of phosphopeptides enriched by the SAX monolithic column from digest mixture of β-casein and BSA at a molar ratio of 1:10 with the presence of (A) 10 mM DTT and 20 mM IAA, (B) 1 M urea, (C) 0.5% SDS, and (D) 0.5% Triton.

was 1:1.[23] However, the specificity of the SAX column is poorer than the new Ti^{4+}−IMAC as phosphopeptides could be specifically enriched from the digest mixture of phosphoprotein and unphosphorylated protein with ratio of 1:500.[11] It should be mentioned that MALDI-TOF MS is commonly applied to analyze protein phosphorylation in less complex samples where only one or a few proteins are presented. For the analysis of such samples, the specificity of the SAX column is good enough.

Tolerance of Denaturing Agents. To improve digestion efficiency, proteins, especially those with disulfide bonds, are typically denatured prior to digestion to facilitate the access of protease to the bonds on the protein sequence. Thus, the presence of denaturants in the digest is common, and it is necessary to investigate if denaturants influence the enrichment of phosphopeptides by the SAX column. In the first step of denaturation, DTT and IAA were added to open the disulfide bonds and alkylate the sulfhydryl groups on proteins, respectively. To evaluate the performance of the phosphopeptide enrichment in the presence of DTT and IAA, the digest mixture of β-casein and BSA with ratio of 1:10 was used as the test sample. Figure 6A reveals that the spectrum was almost identical with that without addition of DTT and IAA (Figure 5B), indicating that the presence of DTT and IAA in the digest had no significant effect on the performance of the phosphopeptide enrichment. Urea, a chaotropic denaturant, is often used to denature proteins prior to digestion. It can be seen from Figure 6B that the presence of 1 M urea in the protein digest mixture had no influence on the performance of the phosphopeptide enrichment. Surfactant is another type of denaturant for protein. The effect of two surfactants, sodium dodecyl sulfate (SDS) and Triton, on the performance of phosphopeptide enrichment was investigated. It was found that the monophosphopeptide cannot bind to the SAX column with the addition of 0.5% SDS in the digestion mixture (Figure 6C). This is not strange since SDS, as an anionic surfactant, will compete with phosphopeptides for the positively charged binding sites on the SAX column and the quadruply phosphorylated peptide (β2) can prevail over SDS (monoly negatively charged) but the monophosphopeptide cannot in the competition. Triton is a nonionic surfactant. The addition of Triton in the digest mixture should not affect the retention of phosphopeptides on the SAX column. However, it was observed that the intensity of the monophosphopeptide decreased significantly (Figure 6D), which indicates that the addition of 0.5% Triton also resulted in the weak retention of phosphopeptides on the SAX column. Furthermore, the addition of Triton also deteriorated the specificity for phosphopeptide enrichment as some nonphosphopeptides appeared in the mass spectrum. The above results indicate the SAX phosphopeptide enrichment approach is not tolerant of surfactants. As the addition of urea has no obvious effect on phosphopeptide enrichment by SAX, the protein digest with urea can be submitted directly to the SAX column for phosphopeptide enrichment followed with MALDI-TOF MS detection.

Sensitivity. The major objective of this study is to develop a new sensitive platform for protein phosphorylation analysis. The sensitivity of the platform by coupling SAX enrichment with MALDI MS detection was investigated by analyzing different amounts of β-casein digest. To reduce the sample loss that resulted from the nonspecific adsorption of phosphopeptides on the column, a short column with a length of 5 cm was used. The

(23) Larsen, M. R.; Thingholm, T. E.; Jensen, O. N.; Roepstorff, P.; Jorgensen, T. J. D. *Mol. Cell. Proteomics* **2005**, *4*, 873–886.

procedures for sample loading and washing were the same as above, but the procedure for elution was modified to analyze minute samples. After washing, the SAX column was filled with 80 mM ammonium bicarbonate buffer. To alleviate the effect of salt on the sensitivity of MALDI detection, the buffer in the column was removed by using a syringe filled with air before elution. Also, instead of eluting phosphopeptides to a tube, the captured phosphopeptides were directly eluted onto a MALDI plate by 2 μL of 5% formic acid for analysis of minute samples. After drying down of the phosphopeptides in formic acid solution, 0.5 μL of matrix solution was added to the spot for MALDI analysis. As shown in Figure 7A, phosphopeptides in 1 fmol of β-casein digest could be easily detected. The amount of loaded sample was further decreased to the attomole level. It was found that there was no difficulty to find the sweet spot to give a mass spectrum with nice signal-to-noise ratio for detection of phosphopeptide when the amount of β-casein digest was as low as 50 amol (Figure 7B). To make sure the phosphopeptide signal did not come from the carryover from the last run, a blank test was performed. It can be seen from Figure 7C that no phosphopeptide peak was observed, which means the carryover was ignorable. The sensitivity of the approaches based either on IMAC or MOAC is at the low femtomole.[15−19] The higher sensitivity obtained from the SAX-based approach is mainly due to the use of an excellent MALDI MS compatible elution buffer. To elute phosphopeptides from the SAX column, 5% formic acid was used, which was totally compatible with the MALDI matrix. As a contrast, for the elution of phosphopeptides from IMAC or MOAC, an alkaline buffer must be used. If an involatile alkaline buffer is used, the eluted phosphopeptide sample needs to be desalted prior to MALDI MS analysis, which will inevitably lead to a loss of sample. If a volatile buffer like ammonia hydroxide is used, the residue buffer will form salt with the acid in the matrix which suppresses the ionization of phosphopeptides. For the on-plate enrichment approach where IMAC materials or metal oxides were immobilized on the MALDI plate, matrix was added without elution. The poor sensitivity of the on-plate approach is probably because of the plate's limited capacity due to its low surface area. The porous monolithic column has much higher surface area and so has much higher capacity and is able to enrich more phosphopeptides from diluted samples.

Sample Loading by Capillary Action. The procedure of loading sample onto the SAX monolithic column by the nitrogen gas through the high-pressure bomb is inconvenient. To overcome such a drawback, a simple sample loading method was proposed. Instead of using any outer force, capillary action of the monolithic capillary was used as the driving force to wick the sample solution into the monolithic column. A SAX monolithic column of 5 cm × 150 μm i.d. was used to test the performance of sample loading by capillarity. The SAX monolithic column was first dried by flowing through nitrogen for about 1 min. Then one end of the dried column was dipped into the sample solution. It was found the whole dried column can be filled with sample within 2 min. As the total pore volume of the SAX column was 366 nL, about 366 nL sample was introduced into the column in this way. Except the sample loading step, the other steps are the same as in the investigation of sensitivity. It was found phosphopeptides in as low as 10 amol of β-casein digest could be enriched and detected by MALDI-TOF MS (Figure 8A). The blank test (Figure 8B) confirmed that the phosphopeptide signal on the mass spectrum was not resulted from carryover. When 1 fmol of β-casein digest was directly spotted onto the MALDI plate for analysis, no peak was observed (Figure 8C). This is because the presence of 50 mM ammonium bicarbonate in the sample suppressed the ionization of peptides during MALDI MS analysis. After purification by the SAX column, the salt and nonphosphopeptides were gone; thereby the ionization efficiency of phosphopeptides was significantly improved, and so high sensitivity was achieved.

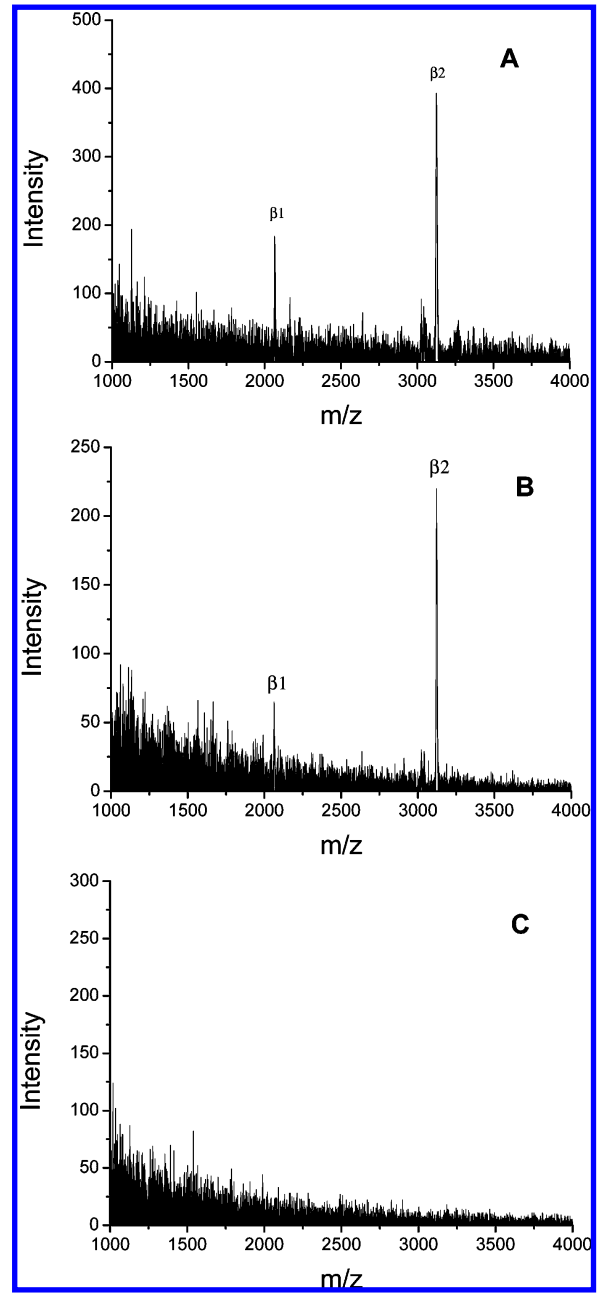

Figure 7. Sensitivity for enrichment of phosphopeptides by the SAX monolithic column followed with MALDI MS detection. Mass spectra for phosphopeptides enriched from tryptic digest of β-casein with an amount of (A) 1 fmol, (B) 50 amol, and (C) 0 mol (procedures were as A and B except no sample was loaded). Tryptic β-casein digest solutions (20 μL) with different concentrations were loaded onto a 5 cm × 150 μm i.d. SAX monolithic column, and the trapped phosphopeptides were eluted directly onto a MALDI plate.

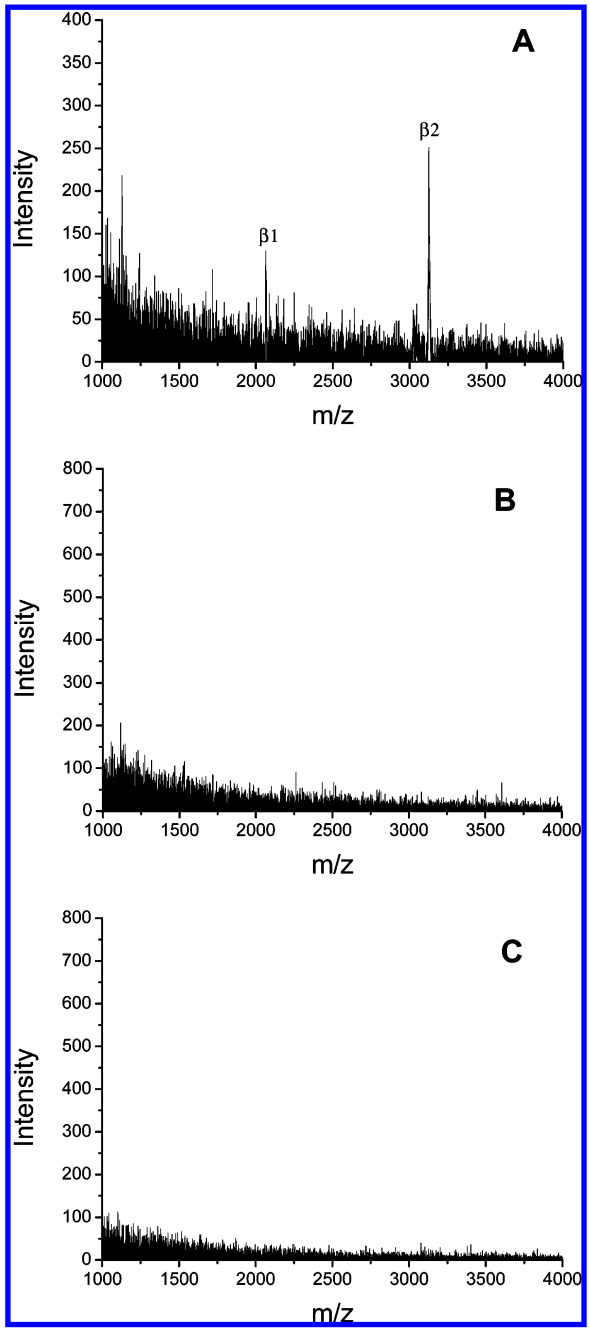

Figure 8. Sample loading by capillary action. Mass spectra of phosphopeptides enriched from β-casein digest with an amount of (A) 10 amol and (B) 0 mol (procedures were as A except no sample was loaded). (C) Mass spectra for direct analysis of 1 fmol of β-casein digest. β-Casein digest (2.8×10^{-11} M) was loaded onto a 5 cm $\times$ 150 μm i.d. SAX monolithic column by capillary action; the loaded volume was determined to be 366 nL.

By using the capillarity sample loading method, samples as minute as 0.4 μL could be easily and rapidly introduced into the SAX column. As a comparison, when pressure sample loading was used, 20 μL of protein digest was needed to be added to a tube for sample loading. This means the sample was diluted by 50-fold as compared with the direct loading by capillary action. This is the main reason that a lower detection limit was obtained for the capillarity sample loading. The volume that could be loaded by capillary action depends on the total pore volume of the monolithic column. As a porous monolithic column can be easily prepared in different sizes by using a capillary with different inner diameter and/or length, loading different volumes of sample could be easily achieved by using columns with different sizes. For example, an ultralong column could be used to load a big sample volume. In such a case, sample will be concentrated on one end of the column and the remaining large portion of the porous monolithic column will serve as a "capillary pump". To reduce the possible sample loss resulted from unspecific adsorption of the long porous matrix in the column, the captured sample should be eluted out of the column from the same end. The coupling of the porous monolithic capillary column to MALDI-TOF MS is more straightforward and more sensitive. The enriched phosphopeptides could be directly eluted onto the MALDI plate with 5% formic acid with a volume of only 2 μL. The use of a low volume of elution buffer is to make sure the eluted phosphopeptides presented in a tiny spot on the plate so that high-sensitivity detection could be achieved. By using capillary action to introduce sample, enrichment of phosphopeptides from multiple samples could be easily accomplished by using multiple SAX columns.

CONCLUSION

In this study, SAX monolithic capillary-based enrichment was coupled with MALDI-TOF MS for sensitive detection of phosphopeptides from protein digest. This platform is most fit for analyzing protein phosphorylation in less complex samples where only one or a few proteins are present. In comparison with IMAC and MOAC, the SAX-based enrichment is more MALDI MS compatible. Together with the merits of the monolithic column-based treatment including microliter treatment capability and limited sample loss, the excellent MALDI MS compatibility enabled coupling of SAX monolithic capillary-based enrichment with MALDI-TOF MS analysis, a very sensitive platform for phosphorylation analysis. For IMAC- and MOAC-based enrichment, MALDI-TOF MS analysis provides a detection limit at the low-femtomole level.[15−19] To the best of our knowledge, this paper is the first report of the detection limit at low attomole for detection of phosphopeptides in protein digest. The high sensitivity of this approach mainly attributes to the simplified analytical procedures, limited sample loss, and excellent compatibility of the elution buffer with the matrix solution as well as the microscale of the monolithic column.

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