Efficient enrichment and identification of phosphopeptides by cerium oxide using on-plate matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis

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An efficient and simple method for enrichment and identification of phosphopeptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using cerium oxide is presented. After pretreatment of tryptic digests of phosphoproteins with CeO2, nonphosphopeptides are discarded and phosphopeptides are enriched. By applying the separated CeO2 on a target plate and analysis using MALDI-TOF MS, peaks of phosphopeptides and their correspondingly series of dephosphorylated peptides are observed in the mass spectra. Thus, the phosphopeptides are very easy to identify with the mass difference, which are all 80 Da between adjacent peaks in the same series, and clear background in the spectra owing to elimination of signal suppression from large amounts of nonphosphopeptides. Furthermore, the phosphopeptides can be dephosphorylated completely after a further NH4OH elution. Tryptic digest products from several standard proteins are pretreated using CeO2 to demonstrate the efficiency of this method. Phosphopeptides from a very small quantity of human serum are enriched and analyzed, and proteins also identified by searching against a database using Mascot on MALDI-TOF/TOF fragments, which indicates that this method may be employed in complex samples for further application. Copyright © 2011 John Wiley & Sons, Ltd.

Reversible protein phosphorylation is one of the most important posttranslational protein modifications, which affects an estimated one-third of all proteins and plays a critical role in the regulation of many cellular processes including signal transduction, cell proliferation, differentiation and transcription. Various technologies for protein phosphorylation site mapping have been developed in recent years. Nevertheless, this task is still a challenge because of the substoichiometric concentration of phosphorylation, as well as there being no single method sufficient for comprehensive phosphoproteome analysis so far.

Nowadays, mass spectrometry, particularly tandem mass spectrometry (MS/MS), has become a powerful tool to characterize protein phosphorylation due to its rapid sequencing capability. Significant progress has also been made in the area of protein phosphorylation identification by mass spectrometry, but satisfactory results can rarely be obtained by direct mass spectrometric analysis of a protein digest. The reason is that phosphopeptides are present at low abundance in the digest, so the mass signals of phosphopeptides are seriously suppressed by abundant nonphosphorylated peptides. As a result, numerous methods have been developed for phosphopeptide enrichment before analysis by mass spectrometry, including immunoprecipitation, chemical tagging after a β-elimination reaction, immobilized metal affinity chromatography (IMAC), metal oxides of titanium (TiO2) and zirconium (ZrO2), etc. Phosphate groups on amino acids are unstable to collision-induced dissociation (CID). The loss of 98 Da or 80 Da provides the phosphopeptide signature, by which protein phosphorylation identification is performed in the mass spectral analysis. For example, a neutral loss scan with MS/MS is used to identify precursor ions that display this signature loss. However, this approach has a disadvantage that it is time-consuming and reduced in sensitivity. Several dephosphorylation approaches have been discovered and used to analyze phosphoproteins, which are based on a characteristic mass shift owing to loss of phosphate. Phosphatase is commonly used and recently hydrofluoric acid, cerium oxide have been developed for dephosphorylation. However, it is difficult to determine which signals correspond to phosphorylated peptides because of the weak signals of phosphopeptides compared with strong signals of abundant nonphosphopeptides, and parallel experiments before and after dephosphorylation are necessary.

Here we present an efficient and simple method to identify phosphorylation by cerium oxide using on-plate matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) analysis. This approach can enrich the phosphopeptides efficiently to eliminate mass signal suppression from nonphosphopeptides. Meanwhile, signals of

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phosphopeptides can be easily distinguished using the mass difference between phosphorylated and dephosphorylated peaks directly by on-plate MALDI-TOF MS analysis and the proteins from which the phosphopeptides originate can be identified by searching against a database using Mascot on MALDI-TOF/TOF fragments.

EXPERIMENTAL

Materials and methods

The proteins α-casein, β-casein (from bovine milk), bovine serum albumin (BSA), ovalbumin, trypsin (TPCK-treated, from bovine pancreas) and the chemicals cerium oxide, glutamic acid, 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Urea, ammonium bicarbonate, dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from BioRad (Hercules, CA, USA). All the chemicals listed above were of analytical grade. HPLC grade trifluoroacetic acid (TFA) and acetonitrile were obtained from Sigma (St. Louis, MO, USA). Protein digests were diluted with loading buffer containing 2% TFA, 65% ACN (v/v) solution saturated with glutamic acid (pH 1.5). Glutamic acid in sample loading buffer is demonstrated to be able to reduce nonspecific binding of nonphosphorylated peptides.[20] The sample solution (10−4 mol/L, 2 μL) was mixed with 10 μL of a suspension of CeO2 nanoparticles (10 mg/mL in loading buffer). Then 30 μL loading buffer was added to the mixture and the resulting solution was incubated with vibration for 15 min at room temperature. This was subsequently centrifuged at 20 000 g for 2 min. The CeO2 nanoparticles with trapped phosphopeptides were deposited at the bottom of the tube, and the supernatant was discarded using an Eppendorf pipette. Subsequently, the isolated nanoparticles were rinsed with 100 μL loading buffer with vortex for 10 min to remove the nonspecifically adsorbed peptides. After isolation by centrifugation at 20 000 g for 2 min, the supernatant was removed again. Then, the isolated nanoparticles were rinsed once more with 100 μL solution of 0.1% TFA and 30% ACN under vortex for another 10 min. After isolation by centrifugation and discarding of the supernatant, the isolated nanoparticles were mixed with 2 μL DHB matrix solution, and 0.5 μL of the mixture was deposited directly on the target and dried to form a layer of mixture; then 0.5 μL DHB matrix was deposited again on the layer for MALDI-TOF MS analysis. For comparison, the trapped phosphopeptides on CeO2 nanoparticles in another group with the same treatment after the second rinse and isolation were eluted using 50 μL NH4OH (pH 11.5) under ultrasonication for 30 min. After centrifugation, the supernatant was collected and lyophilized to dryness. Likewise, 2 μL DHB matrix solution was introduced into the tube and 0.5 μL of the mixture was deposited on the MALDI target for MALDI-TOF MS analysis.

Sample preparation

α-Casein and β-casein (1 mg) were dissolved in 1 mL ammonium bicarbonate (50 mM, pH 8.2) and digested for 18 h at 37°C with trypsin at an enzyme-to-protein ratio of 1:40 (w/w), respectively. BSA and ovalbumin (4 mg) were dissolved in 1 mL of denaturing buffer containing 8 M urea, 50 mM ammonium bicarbonate and 5 mM DTT, respectively. The disulfide bonds of protein were reduced by incubation for 2 h at 37°C. Then 10 mM IAA was added and incubated for an additional 45 min at room temperature in the dark. After that, the mixture was diluted 10-fold with 50 mM ammonium bicarbonate and incubated for 18 h at 37°C with trypsin at an enzyme-to-protein ratio of 1:40 (w/w). All of the proteolytic digests were then stored in a refrigerator at −30°C for further use.

Human serum samples from the Second Affiliated Hospital of Dalian Medical University were collected in PET vacuum blood collection tubes and centrifuged for 5 min at 2000 g. Serum from the upper phase was aliquoted and stored at −80°C for further use.

Instrumentation

An Autoflex time-of-flight mass spectrometer (Bruker, Bremen, Germany) was used to obtain all MALDI-TOF mass spectra. This instrument is 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 signals with good resolution and signal-to-noise (S/N) ratio. All mass spectra in this work were acquired by searching against the SwissProt database using Mascot (Matrix Sciences, London, UK). Transmission electron microscopy (TEM) was performed using a FEI Tecnai G2 Spirit at an acceleration voltage of 120 kV, and X-ray diffraction (XRD) patterns were recorded on a Rigaku RINT D/Max-2500 powder diffraction system using Cu Ka radiation.
RESULTS AND DISCUSSION

CeO₂ material was purchased from Sigma-Aldrich and used directly without further treatments. The structure of the CeO₂ sample was characterized by TEM and XRD (data shown in Fig. 1 and Fig. S1 (see Supporting Information)). As seen from TEM and calculated from the XRD pattern, the CeO₂ nanoparticles are estimated to have a relatively uniform diameter of about 26 nm.

Different experimental conditions were investigated and optimized for enrichment and identification for phosphopeptides. The proportions between protein digests and CeO₂ nanoparticles were varied to obtain the best enrichment effect. It is found that as for the protein digest sample (10⁻⁶ mol/L, 2 μL), the CeO₂ solution at 10 mg/mL with 10 μL is proper to obtain strong signals of phosphopeptides and low nonspecific binding of nonphosphopeptides. Effects of incubation time for reaction between protein digests and CeO₂ nanoparticles were also investigated. After incubation for 15 min, the best signal intensities were achieved, which indicated that the interaction between CeO₂ and the phosphoryl groups in phosphopeptides arrived at equilibrium.

The tryptic digest of β-casein was selected to evaluate the performance of CeO₂. Figure 2(a) shows the MALDI mass spectrum obtained by the direct analysis of the tryptic digest of β-casein. The intensities of the three phosphopeptides are weak compared with nonphosphorylated peptides. After enrichment by CeO₂ nanoparticles, as shown in Fig. 2(b), nonphosphopeptide signals that appear in Fig. 2(a) have disappeared, which means that these peptides are removed after the pretreatment, whereas the signals of phosphopeptides are significantly improved after the phosphopeptides have been enriched by CeO₂ nanoparticles. Furthermore, the mass peaks of dephosphorylation are also present in the spectrum. As for singly phosphorylated peptides, the mass loss between peaks of phosphopeptide and dephosphorylation is 80 Da. As for multiply phosphorylated phosphopeptides, a series of dephosphorylated peaks appear in the spectrum. The peaks at m/z 2061.83 and 2556.09 in the mass spectra are phosphopeptides containing one phosphoryl group, and their correspondingly dephosphorylated peaks at m/z 1981.86 and 2476.13 are also present in the spectrum. The peak at m/z 3122.27 is a multiply phosphorylated peptide which has four phosphorylation sites. In Fig. 2(b), four peaks appear in front of the peak of m/z 3122.27, and the mass differences between two adjacent peaks are all 80 Da. This means that there are completely dephosphorylated peaks and all partly dephosphorylated peaks present in the enriched sample. Therefore, just simply from Fig. 2(b), we can easily detect the phosphopeptides in β-casein and the number of phosphorylation sites. The phosphopeptides are labeled and their sequences are listed in Table S1 (see Supporting Information). Figure 2(c) shows the mass spectrum of elution from the trapped phosphopeptides by NH₄OH solution. All the phosphopeptides are dephosphorylated completely. These results demonstrate that using CeO₂ nanoparticles and NH₄OH elution is an efficient method to enrich and dephosphorylate phosphopeptides, which avoids suppression of nonphosphopeptides.

The phosphopeptides of β-casein possess phosphorylation sites all at serine residues. To evaluate the enrichment performance of CeO₂ on other phosphorylated peptides at different amino acid residues, a tyrosine phosphorylated peptide (RRLIEDAEpYAARG; pY represents phosphotyrosine) was added to the tryptic digest of β-casein in the same molar ratio and then the enrichment process was performed and analyzed by MALDI-TOF MS. As shown in Fig. 2(d), apart from the same peaks of phosphopeptides and dephosphorylated peaks of β-casein as in Fig. 2(b), one phosphotyrosine peptide (pY) and its corresponding dephosphorylated peak can be easily observed. This indicates that CeO₂ can trap and dephosphorylate other types of phosphopeptides as well. Figure 2(e) shows the result after elution from the trapped phosphopeptides by NH₄OH solution, from which we can see only the completely dephosphorylated peptides present in the spectrum.

The tryptic digest mixtures of β-casein (10⁻⁶ mol/L) and BSA at different molar ratios were further applied to evaluate the ability of CeO₂ to isolate phosphopeptides in the presence of large amounts of nonphosphopeptides. Figure 2(f) shows the MALDI mass spectrum for the direct analysis of a digest mixture at the molar ratio of 1:200 in which the molar amount of BSA is 200 times that of β-casein. The spectrum is dominated by signals of nonphosphopeptides and no signals from phosphopeptides of β-casein are detected. As a comparison, Fig. 2(g) shows the MALDI mass spectrum of the products isolated from the digest mixture with the molar ratio of 1:200 after pretreatment with CeO₂ nanoparticles and detected on the MALDI plate directly without elution. Phosphopeptides and their correspondingly dephosphorylated peaks are displayed in the spectrum with a clear background, which indicates the high specificity of CeO₂ to phosphopeptides. Figure 2(h) shows the MALDI-TOF MS result of the mixture after NH₄OH elution of the CeO₂ nanoparticles. The sensitivity of this enrichment method was also determined. Phosphopeptides from 5 fmol of a tryptic digest of β-casein were pretreated using CeO₂ and 1.25 fmol deposited on the MALDI plate can be detected in the mass spectra (Fig. S2, see Supporting Information). The approach was also employed for the analysis of a tryptic
The tryptic digest of ovalbumin was further used here to test if the presence of urea, DTT, and IAA in the sample interferes with the trapping of the phosphopeptides by CeO₂ nanoparticles. Since ovalbumin is a diphosphorylated protein containing a disulfide bond, a denaturant like urea was used to denature ovalbumin. DTT was added to open the disulfide bond and IAA was added to carboxyamidomethylate the resulting cysteine residues. Figure 3(a) shows the MALDI-TOF MS result for a tryptic digest of ovalbumin directly without CeO₂ pretreatment, from which we can see a strong background of nonphosphopeptides and two small phosphopeptide signals.

As a comparison, the phosphopeptides and their dephosphorylated peaks are dominant in the spectrum after pretreatment by CeO₂, as shown in Fig. 3(b). Figure 3(c) is the result after NH₄OH elution of the CeO₂ nanoparticles, and the completely dephosphorylated peptides appear in the spectrum. These results demonstrate that the presence of urea, DTT and IAA in the sample does not compromise the performance of CeO₂ to capture phosphopeptides.

Human serum is the most commonly used sample for clinical diagnosis and nowadays usually used in studies on biomarker discovery. Thousands of peptides are degraded from larger proteins by endogenously proteolytic enzymes or secreted from cells and tissues. Phosphorylated peptides are

Figure 2. MALDI mass spectra of β-casein (10⁻⁶ mol/L, 2 μL): (a) direct analysis; (b) analysis of the phosphopeptides on CeO₂ nanoparticles directly after pretreatment; (c) analysis of the elution of CeO₂ nanoparticles by NH₄OH; (d) analysis of CeO₂ nanoparticles directly after pretreatment of mixture of tryptic digest of β-casein (10⁻⁶ mol/L, 1 μL) and standard phosphotyrosine peptide (pY) with molar ratio at 1:1; (e) analysis of the mixture elution of CeO₂ nanoparticles by NH₄OH; (f) direct analysis of tryptic digest mixtures of β-casein (10⁻⁶ mol/L, 1 μL) and BSA (2.5 × 10⁻⁵ mol/L, 8 μL) with molar ratio at 1:20; (g) the trapped phosphopeptides using CeO₂ nanoparticles from tryptic digest mixtures of β-casein and BSA with molar ratio at 1:20; and (h) analysis of the elution of CeO₂ nanoparticles by NH₄OH.
present in serum with very low abundance, so it is a challenge to enrich and identify them directly from the complex serum samples. We employed CeO₂ nanoparticles to enrich and identify phosphopeptides directly from human serum; 0.5 μL serum sample was used and the MALDI-TOF MS results are displayed in Fig. 4. Figure 4(a) shows the result after the pretreatment of phosphopeptide enrichment by CeO₂ and detected directly on the MALDI target plate. The insert shows the enlarged signals, from which the peaks can be distinguished clearly. Eight peaks are present in Fig. 4(a) at m/z values of 1309.55, 1380.59, 1389.51, 1460.56, 1465.66, 1536.69, 1545.62 and 1616.66 Da. The eight peaks can be grouped into four pairs. The difference between the two peaks in each pair is about 80 Da, as listed in Table S1 (see Supporting Information), which indicates that there are four phosphopeptides and their corresponding dephosphorylated peaks in the products. Therefore, using the difference of 80 Da, the signals of phosphopeptides can be definitely identified and abundant nonphosphopeptides are removed after phosphopeptide enrichment by CeO₂. So it is an efficient and simple way to enrich and identify phosphopeptides by CeO₂ pretreatment. Figure 4(b) shows the result after elution of CeO₂ nanoparticles by NH₄OH, and all the four phosphopeptides are dephosphorylated completely.

MALDI-TOF/TOF mass spectra for the four phosphopeptides and their corresponding dephosphorylated peptides were also recorded to obtain their sequence of amino acid residues. The b- and y-ion series are displayed in the MALDI-TOF/TOF mass spectra. The spectra of the precursor ion with m/z at 1545.62 Da and its dephosphorylated peptide with m/z at 1465.66 Da are shown in Fig. 5. In Fig. 5(a), the dominant peak with m/z at 1447.74 Da is the neutral loss peak with the precursor ion losing a H₃PO₄ group of 98 Da by CID, which illustrates that the peptide was indeed phosphorylated. The series of y-ion fragments are present almost completely in the spectrum. The fragment ions in Fig. 5(b) are in the same sequence as those in Fig. 5(a) but with no phosphorylation, which indicates that this precursor ion is the dephosphorylated peptide of the phosphorylated peak with m/z at 1545.62 Da. Data from these TOF/TOF spectra were searched against the SwissProt database using the Mascot search engine. These peptides are identified from a protein of fibrinogen, which is a phosphoprotein with a composition of α, β, γ chains and only the α chain is phosphorylated. Therefore, by using enrichment with cerium oxide and mass spectrometric analysis, the identification of phosphopeptides can be performed. Furthermore, by searching against a database using Mascot on MALDI-TOF/TOF mass spectra, exact proteins can be identified from the sample.

CONCLUSIONS

An efficient method for the enrichment and identification of phosphopeptides by CeO₂ nanoparticles was presented in this study. CeO₂ is demonstrated to be efficient to capture phosphopeptides by analysis of tryptic digests of several model phosphoproteins. Meanwhile, dephosphorylated signals also appear after pretreatment with CeO₂ nanoparticles. Therefore, in the MALDI mass spectrum, only phosphopeptides and their corresponding dephosphorylated peptides are present with a clear background without nonphosphopeptides. With further NH₄OH elution of the CeO₂ nanoparticles, complete

Figure 3. MALDI mass spectra of tryptic digest of ovalbumin (10⁻⁶ mol/L, 2μL): (a) direct analysis; (b) analysis of the phosphopeptides on CeO₂ nanoparticles directly after pretreatment; and (c) analysis of the elution of CeO₂ nanoparticles by NH₄OH.

Figure 4. MALDI mass spectra of human serum (0.5 μL): (a) analysis of the phosphopeptides on CeO₂ nanoparticles directly after pretreatment and (b) analysis of the elution of CeO₂ nanoparticles by NH₄OH.
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dephosphorylation can be achieved. Therefore, it is a very simple and efficient method to identify phosphopeptides in the sample and even figure out the number of phosphorylated sites in the phosphopeptide, and exact proteins can be identified by searching against the database on fragments of the peptides. Efficient enrichment and identification of phosphopeptides directly from human serum sample imply the potential application of this method.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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REFERENCES


