Rapid Enrichment of Phosphopeptides from Tryptic Digests of Proteins Using Iron Oxide Nanocomposites of Magnetic Particles Coated with Zirconia as the Concentrating Probes

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Abstract: Iron oxide nanocomposites of magnetic particles coated with zirconia were used as affinity probes to selectively concentrate phosphopeptides from tryptic digests of α- and β-caseins, milk, and egg white to exemplify the enrichment of phosphopeptides from complex samples. Phosphopeptides, in quantities sufficient for characterization by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS), were enriched by the affinity probes within only 30 s. The affinity probe–target species conjugates were separated from the sample solution simply by applying an external magnetic field. The detection limit for tryptic digest of β-casein using this approach is ~45 fmol. Furthermore, we combined this enrichment method with a rapid enzymatic digestion method, that is, microwave-assisted enzymatic digestion using magnetic particles as the microwave absorbers, to speed up the tryptic digest reactions. Thus, we alternatively enriched phosphoproteins on the zirconia-coated particles followed by mixing with trypsin and heated the mixture in a microwave oven for 1 min. The particles remaining in the mixture were used as affinity probes to selectively enrich phosphopeptides from the tryptic digestion product by pipeting, followed by characterization using MALDI MS. Using the bifunctional zirconia-coated magnetic particles as both the affinity probes and the microwave absorbers could greatly reduce the time for the purification and characterization of phosphopeptides from complex samples.

Keywords: Zirconia · iron oxide · magnetic particles · phospho-peptides · MALDI MS · concentrating probes

Introduction

Phosphorylation plays a vital role in regulating biological functions and characterization of phosphoproteins from complex samples necessary for clarifying regulatory mechanisms in a biological system. Biological mass spectrometric methods such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and electrospray ionization mass spectrometry (ESI MS) have been used for the analysis of tryptic digests of phosphoproteins. However, the presence of non-phosphorylated peptides in the digestion product generally suppresses the ion signal of phosphorylated peptides in the mass spectra. Therefore, enriching phosphopeptides from complex samples becomes a required step prior to MS analysis. Several affinity based methods such as immobilized metal ion affinity chromatography (IMAC)[1–13] and metal oxide affinity chromatography (MOAC) have been developed for selective enrichment of phosphopeptides.[14–19] IMAC beads immobilized with Fe(III) or Ga(III) are commonly used for the purification of phosphopeptides.[1–13] Metal oxides such as titania-coated adsorbents[14–20] have been demonstrated to be effective materials for the enrichment of phosphopeptides because they seem to have higher selectivity for phosphopeptides because of their reduced nonspecific binding compared to IMAC and the results are more reproducible. We previously have reported the use of magnetic particles coated with titania[14] as the concentrating probes to selectively enrich phosphopeptides from tryptic digests of proteins. The magnetic affinity probes–target species conjugates can be readily isolated from the sample solution by employing an external magnetic field.

Kweon and Hakansson[17] recently demonstrated that zirconium dioxide microtips can effectively enrich phosphorylated peptides from complex samples. They stated that zirconia microtips have a similar performance to titania microtips but have superior selectivity for singly phosphorylated peptides. To improve the trapping and reduce the time required for enrichment, we prepared nanocomposites iron oxide magnetic particles coated with zirconia as the affinity probes for the enrichment of phosphopeptides from tryptic digests of proteins. Furthermore, we employed pipeting to vigorously mix small volumes of samples containing the affinity particles and the tryptic digests of proteins. This approach has recently been demonstrated to be effective for rapid enrichment of phosphopeptides by alumina-coated magnetic particles with high-trapping efficiency in our group.[21] Phosphopeptides in quantities sufficient for MALDI MS analysis could be enriched by the affinity probes by pipeting the sample for only 30 s. These affinity probes have a high surface-to-volume ratio, giving them a high-trapping capacity for their target species, and their magnetic property enables easy isolation by positioning an external magnetic field. In this study, we explored the use of

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composite iron oxide particles coated with zirconia as affinity probes to selectively concentrate phosphopeptides from tryptic digests of proteins.

To reduce the time required for the entire analysis, we employed microwave-assisted enzymatic digestion to reduce the digestion time. Several research groups[22−29] have endeavored to speed up enzymatic digestion using microwave heating, and the time required was reduced from several hours to 10 min. We recently showed that using magnetic particles in microwave-assisted enzymatic digestion could further reduce the time to 30 s∼1 min.[30] This improvement mainly results from two features of these particles. They are good absorbers for microwave radiation.[31−33] Therefore, their presence in the digestion solution can accelerate the heating rate. Second, proteins attached to the surfaces of magnetic particles denature to give unfolded proteins which are more susceptible to enzymatic digestion. We herein demonstrate that zirconia-coated magnetic particles also function as microwave absorbers in addition to their use as affinity probes for phosphorylated species. After phosphoproteins were trapped by the zirconia-coated magnetic particles in a sample solution, the magnetic affinity probe−phosphoprotein conjugates with added trypsin were heated in a microwave oven to carry out a fast enzymatic digestion. After digestion, the particles remaining in the solution were used as the affinity probes for the enrichment of phosphopeptides, followed by MADLI MS analysis. The feasibility of using zirconia-coated magnetic particles as affinity probes and microwave absorbers for rapid purification and characterization of phosphopeptides was demonstrated.

Experimental Section

Reagents. Iron(III) chloride hexahydrate, sodium sulfite, ammonium bicarbonate, trifluoroacetic acid (TFA), and phosphoric acid (85%) were purchased from Riedel-de Haën (Seelze, Germany), while hydrochloric acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Ammonium hydroxide solution (25%), nitric acid, and tetraethoxysilane (TEOS) were obtained from Fluka (Seelze, Germany). α-Casein (from bovine milk), β-casein (from bovine milk), dithiothreitol (DTT), iodoacetic acid (IAA), and trypsin (from bovine pancreas, TPCK treated) were purchased from Sigma (St. Louis, MO). 2,5-Dihydroxybenzoic acid (DHB) and zirconium butoxide (80%) were purchased from Aldrich (Steinheim, Germany), while ethanol was obtained from Showa (Tokyo, Japan). Nonfat milk and chicken eggs were purchased from a local grocery store.

Preparation of Fe$_3$O$_4$ Magnetic Nanoparticles. Iron(III) chloride (FeCl$_3$·· 6H$_2$O, 6.5 g) was dissolved in hydrochloric acid (2 M, 12 mL) and was diluted with water to 100 mL. The mixture was degassed using a pump. Sodium sulfite (50 mL, 0.08 M) solution was slowly added to the solution under nitrogen with stirring at room temperature. Ammonia (5%, 50 mL) was added slowly with vigorous stirring at room temperature under nitrogen. The mixture was left to react for 30 min in a water bath at 70 °C. After cooling to below 45 °C, the iron oxide magnetic nanoparticles were rinsed with deionized water twice and ethanol three times and were resuspended in ethanol (80 mL) to give a suspension of ∼22 mg/mL.

Preparation of Nanocomposites of Magnetic Particles. The iron oxide particles (22 mg/mL, 10 mL) obtained above were aggregated on the edge of a sample vial by applying an external magnetic field and the solution was removed by pipet. The particles remaining in the vial were resuspended in ethanol (40 mL). Ammonia (25%, 1.5 mL), water (6 mL), and TEOS (1.5

mL) were added sequentially to the suspension. The mixture was vigorously mixed under sonication for 20 min prior to continuously stirring for 12 h in a water bath at 40 °C. The iron oxide particles were then rinsed with ethanol five times and were resuspended in ethanol (40 mL).

Preparation of Zirconia-Coated Iron Oxide Magnetic Particles. Zirconium butoxide (0.73 mL) was added to a vial containing nitric acid solution (nitric acid (0.2 mL)/water (10 mL)). A white precipitate appeared immediately. The vial containing the mixture was wrapped with aluminum foil and was stirred for 24 h at room temperature until the solution became clear. A solution containing silica-coated iron oxide particles (5 mL) was added to 5 mL of zirconia sol obtained above and was mixed under sonication for 1 h before being continuously stirred for 24 h in a water bath at 30 °C. The particles were rinsed with ethanol and water and were redispersed in water (10 mL). The suspension was stirred for 6 h in a water bath at 60 °C to strengthen the cross-linking of zirconia with silica. The iron oxide particles coated with zirconia were then rinsed with water six times followed by resuspension in water (5 mL).

Procedures for Enrichment of Phosphopeptides. Tryptic digestion products either derived from nonfat milk or α- or β-casein were used as the samples. The proteins in the nonfat milk were denatured prior to tryptic digestion by mixing the milk (0.25 mL) with aqueous ammonium bicarbonate solution (0.25 mL, 50 mM) containing urea (8 M), followed by incubation at 38 °C for 30 min. DTT solution (50 μL, 90 mM) in aqueous ammonium bicarbonate (50 mM) was added and the mixture was maintained at 55 °C for 1 h. After cooling to room temperature, the sample solution was mixed with IAA solution (50 μL, 200 mM) prepared in aqueous ammonium bicarbonate (50 mM) in an ice bath. The sample vial was wrapped with aluminum foil and was mixed by vortex for 2.5 h at room temperature, when it was ready for tryptic digestion. The milk solution (0.6 mL) was incubated with trypsin (1 mg/mL, 10 μL) in aqueous ammonium bicarbonate (50 mM) at 38 °C for 16 h. α- (or β-) Casein and trypsin were prepared in aqueous ammonium bicarbonate (50 mM). α- (or β-) Casein (2 mg/mL, 500 μL) and trypsin (2 mg/mL, 10 μL), at a weight ratio of 50:1, were incubated at 37 °C for 18 h. The digestion product (5 μL) was acidified by diluting in TFA solution (0.15%, 44 μL).

The zirconia-coated magnetic particles from 2.5-mL suspension were isolated and resuspended in TFA solution (0.15%, 1.5 mL). To perform an enrichment experiment, the acidified digestion solution (49 μL) was mixed with the suspension (25 mg/mL, 1 μL) of magnetic particles coated with zirconia in 0.15% TFA solution and was vigorously mixed by pipeting in and out of a sample vial for 30 s. The particles conjugated with their target species were then aggregated on the vial wall by positioning a magnet to outside of the sample vial so that the solution could be easily removed by pipet. The isolated particles were rinsed with 0.15% TFA solution (60 μL × 2) in acetonitrile/deionized water (1/1, v/v), mixing vigorously by pipeting in and out of the mixture in the vial, to wash out any unbound impurities.[21] The rinsed particles were mixed with 2,5-DHB solution (30 mg/mL, 0.7 μL), acetonitrile/deionized water (2/1, v/v) containing 0.5% phosphoric acid. The mixture was deposited on a MALDI sample plate. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

Procedures for Enrichment of Phosphoproteins from Egg White Followed by Direct On-Particle Tryptic Digestion under Microwave Heating. Egg white (10-fold diluted) was denatured
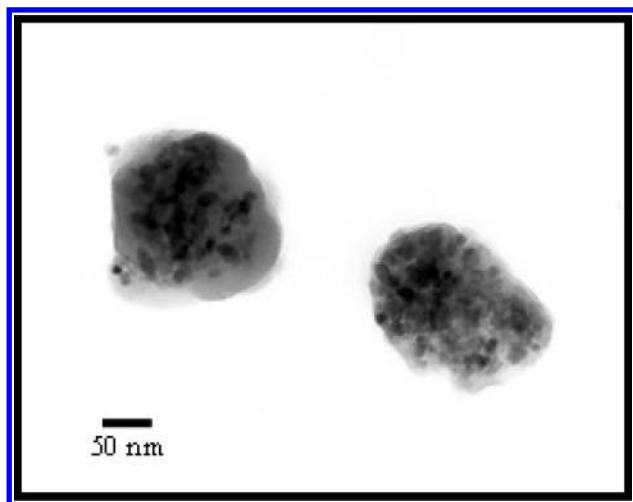
Figure 1. TEM image of iron oxide nanocomposites of magnetic particles coated with zirconia.

using the procedure for denaturing milk samples described above. The denatured sample (5 μL) acidified by 0.15% TFA solution (44.5 μL) was mixed with the suspension containing the magnetic particles coated with zirconia (25 mg/mL, 0.5 μL) by pipeting in and out of the mixture for 1 min. The particle–target species conjugates were rinsed twice with 0.15% TFA (60 μL) prepared in acetonitrile/deionized water (1/1, v/v). The rinsed particles were mixed with trypsin (0.3 mg/mL, 3 μL) in aqueous ammonium bicarbonate (50 mM) under sonication continued for 15 s. The mixture was heated in a domestic microwave oven (power: 900 W) for 1 min to carry out on-particle tryptic digestion.[30] After digestion, the mixture was acidified by 0.15% TFA (47 μL) and was mixed vigorously by pipeting in and out of the vial for 30 s. The particles were aggregated on the vial wall by an external magnetic field while the solution was removed by pipet. The particles were rinsed twice with 60 μL of acetonitrile/deionized water (1/1 v/v) containing 0.15% TFA and were mixed with 2,5-DHB (30 mg/mL, 0.7 μL) containing 0.5% phosphoric acid. The suspension was deposited on a MALDI sample plate. After evaporation of the solvent, the sample was ready for MALDI MS analysis.

Instrumentation. All mass spectra were obtained using a Biflex III (Bruker Daltonics, Germany) time-of-flight mass spectrometer equipped with a 337-nm nitrogen laser, a 1.25-m flight tube, and a sample target having the capacity to load 384 samples simultaneously. Reflectron mode, using 16.0- (IS1) and 19.0-kV (IS2) accelerating voltage, was operated, while the laser power was carefully adjusted during analysis to maintain optimized mass resolution. The TEM image was obtained using a JEOL 2000FX (Japan) transmission electron microscope (TEM).

Results and Discussion

The TEM image of the iron oxide nanocomposites of magnetic particles coated with zirconia, seen in Figure 1, show that the diameter of the particles is ~170 nm. Several iron oxide nanoparticles (shown as dark spots) were incorporated in the particles after coating with zirconia. The particles must be suspended well during preparation to prevent them from aggregation, which would give a reduced surface/volume ratio and might result in undesirable trapping capacity for their target species.

Figure 2a presents the direct MALDI mass spectrum of the tryptic digest of β-casein (9×10^{-7} M) prior to enrichment. The low-intensity peaks, numbers 1 and 2, were phosphorylated peptides derived from β-casein. Table 1 lists the corresponding sequences of these ions. After enrichment using the magnetic particles coated with zirconia as affinity probes for 30 s, three peaks with higher intensity, marked 1, 2, and 3, appeared, which are phosphopeptides derived from β-casein appearing in the MALDI mass spectrum (see Figure 2b and Table 1). We investigated whether the results were improved by extending the enrichment time to 1 h. Figure 2c presents the MALDI mass spectrum obtained after incubating the sample with the affinity probes under vortex-mixing for 1 h. A similar result to that displayed in Figure 2b was obtained. The same peaks, numbers 1, 2, and 3, appeared in the mass spectrum but with a signal abundance of these ions ~4 times higher than that in Figure 2b. Nevertheless, the results indicated that phosphopeptides sufficient for MALDI MS analysis could be enriched within only 30 s. As the concentration of the sample is lowered to 9×10^{-9} M, there are still two phosphorylated peptides (nos. 1 and 2) appearing in the mass spectrum (Figure 2d) after enrichment. When the concentration is lowered to 9×10^{-10} M, phospho-peptide marked with number 1 at m/z 2062.12 still appears in the mass spectrum (Figure 2e). These results indicate that the selectivity and affinity of the affinity probes for phosphopep-tides are quite good. Furthermore, within only 30 s, phospho-peptides in quantities sufficient for MALDI MS analysis are enriched.

Figure 3a presents the direct MALDI mass spectrum of the tryptic digest of α-casein (7×10^{-6} M) prior to enrichment. Among the peaks, there are only three phosphorylated peptide ions revealed, marked with asterisks at m/z 1660.39 (S1/#106–119), 1927.29 (S1/#43–58), and 1951.59(S1/#104–119), derived from α-casein. After enrichment by our affinity particles, several phosphopeptide ions appeared in the MALDI mass spectrum (Figure 3b and Table 2). The peaks marked with numbers 3, 4, 6, 7, and 8 are derived from α-S1-casein, while the peaks marked numbers 5, 9, and 10 are derived from α-S2-casein. The corresponding peptide sequences of these ions are listed in Table 2. All the ions revealed in the mass spectrum are phosphopeptides derived from α-casein. As the concentration of the tryptic digest of α-casein is lowered to 7×10^{-9} M, there are still several phosphopeptide ions appearing in the mass spectrum (see Figure 3c and Table 2), numbers 1, 2, and 4 at m/z 1237.37, 1660.84, and 1952.09, respectively. At such a low concentration, all these phosphopeptides are singly phospho-rylated peptides. Furthermore, peaks numbers 1 and 2 in Figure 3c do not appear in Figure 3b. Two factors, that is, ionization efficiency and binding affinity, may be involved in affecting the mass spectral pattern. At low concentrations of samples, we generally observe that the peaks derived from singly phosphorylated peptides dominate the mass spectra[14,21] be-cause they are easily ionized. Below certain concentrations, the ions of multiply phosphorylated peptides are easily suppressed by the presence of singly phosphorylated peptides owing to their poorer ionization efficiency. On the other hand, multiply phosphorylated peptides are supposed to have higher binding affinities with the affinity probes than singly phosphorylated peptides. Thus, at high sample concentrations, the amount of enriched multiply phosphorylated peptides is sufficient to compensate their poorer ionization efficiency. Therefore, the peaks derived from multiply phosphorylated peptides dominate the mass spectra after enrichment by the affinity probes. Thus,
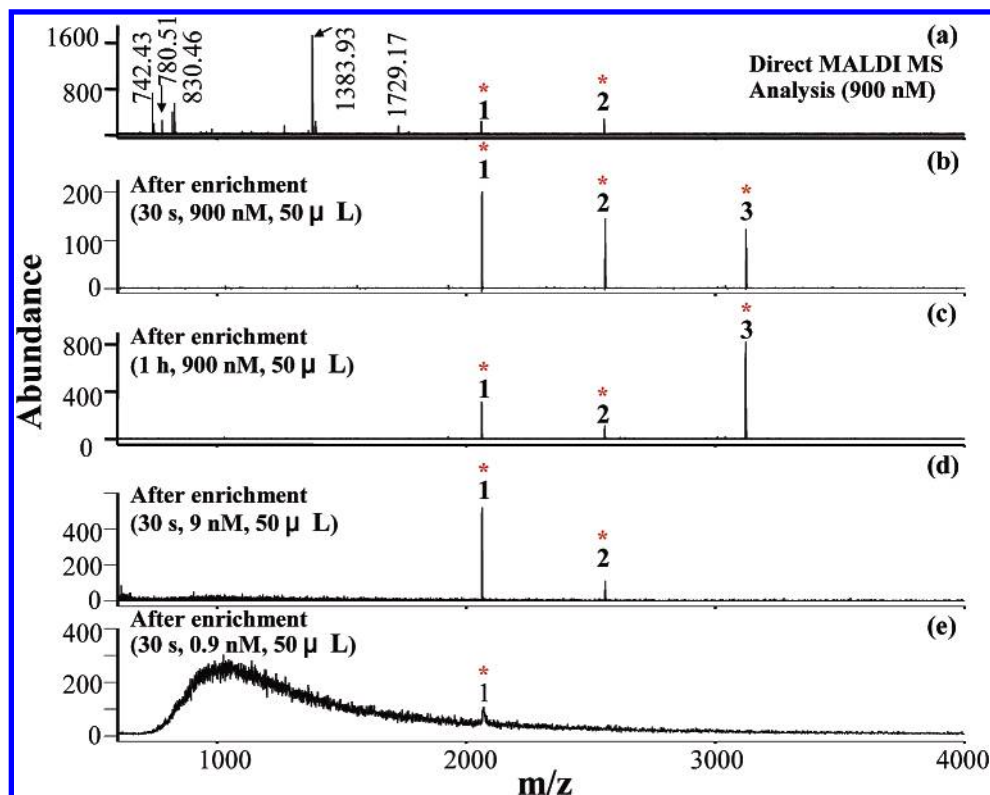
Figure 2. (a) Direct MALDI mass spectrum of the tryptic digest product of β-casein (9×10^{-7} M) prior to enrichment. MALDI mass spectra of the samples obtained using the magnetic particles coated with zirconia to selectively enrich target species from the tryptic digestion products of β-casein at the concentrations of (b) 9×10^{-7} M (50 μL), (c) 9×10^{-7} M (50 μL), (d) 9×10^{-9} M (50 μL), and (e) 9×10^{-10} M (50 μL). Phosphopeptide ions are marked with asterisks. The enrichment time was 30 s for obtaining b, d, and e, while it was 1 h for obtaining c.

Table 1. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of the Tryptic Digest of β-Casein after Enrichment by Iron Oxide Nanocomposites of Magnetic Particles Coated with Zirconia[a]

No.	Theoretic m/z	Observed m/z	Sequences
1	2061.83	2062.12	FQsEEQQQTEDELQDK (33-48)
2	2556.09	2556.14	FQsEEQQQTEDELQDKIHPF (33-52)
3	3122.27	3122.23	RELEELNVPGEIVEsLsssEESITR (1-25)

[a] Red "s" stands for phosphorylated serine.

Table 2. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of the Tryptic Digest of α-Casein after Enrichment by Iron Oxide Nanocomposites of Magnetic Particles Coated with Zirconia[a]

No.	Theoretic m/z	Observed m/z	Sequences
1	1237.47	1237.37	TVDMEsTEVF (α -S2/ 138-147)
2	1660.79	1660.84	VPQLEIVPNsAEER (α -S1/ 106-119)
3	1927.69	1927.77	DIGsEsTEDQAMEDIK (α -S1/ 43-58)
4	1951.95	1952.09	YKVPQLEIVPNsAEER (α -S1/ 104-119)
5	2618.91	2619.04	NTMEHVsssEESIIsQETYK (α -S2/ 2-21)
6	2703.50	2704.04	LRLKKYKVPQLEIVPNsAEERL (α -S1/ 99-120)
7	2720.91	2721.04	QMEAEsIsssEEIVPNsVEQK (α -S1/ 59-79)
8	2935.16	2935.29	EKVNELsKDIGsEsTEDQAMEDIK (α -S1/ 35-58)
9	3008.03	3008.21	NANEEEYSIGsssEEsAEVATEEVK (α -S2/ 46-70)
10	3087.33	3088.17	sTsEENSKKTVDMsTEVFTKKTL (α -S2 /129-153)

[a] Red "s" stands for phosphorylated serine.

we generally observe multiply phosphorylated peptides in the MALDI mass spectra when high concentrations of phosphopeptide are enriched by affinity probes (see Figure 3b and Table 2). The peaks in Figure 3b marked numbers 3, 5, 7, 8, 9, and

Table 3. Peptide Ion Peaks Observed in the MALDI Mass Spectrum of the Tryptic Digest of Egg White after Enrichment by Iron Oxide Nanocomposites of Magnetic Particles Coated with Zirconia[a]

Theoretic m/z	Observed m/z	Sequences
2088.91	2089.23	EVVGsAEAGVDAASVSEEFR (340-359)
2902.31	2902.74	FDKLPGFGDsIEAQCGTSVNVHSSLR (59-84)
3843.79	3844.19	ISQAVHAAHAEINEAGREVVGsAEAGVDAASVSEEFR (323-359)

[a] Red "s" stands for phosphorylated serine.

10 are multiply phosphorylated peptides. However, as the concentration of the phosphopeptide sample is decreased, singly phosphorylated peptides at a lower mass region dominate the MALDI mass spectra after enrichment. On the other hand, multiply phosphorylated peptides have higher detection limits in MALDI MS analysis than singly phosphorylated peptides owing to their poorer ionization efficiency. For this reason, when the concentrations of multiply phosphorylated peptides are lowered below $\leqslant \sim 10^{-9}$ M, the peaks derived from multiply phosphorylated peptides do not appear in the mass spectra. Thus, we generally observed multiply phosphorylated peptides appearing in the mass spectra obtained from high concentrations of sample ($\geqslant \sim 10^{-6}$ M), while singly phosphorylated peptides usually dominate the mass spectra at low concentrations. We have observed similar results when using titania- and alumina-coated magnetic particles as affinity probes for phosphopeptides in our previous studies.[14,21]

To demonstrate the feasibility of this approach for real samples, we employed zirconia-coated magnetic particles to trap phosphopeptides from the tryptic digest of a nonfat milk
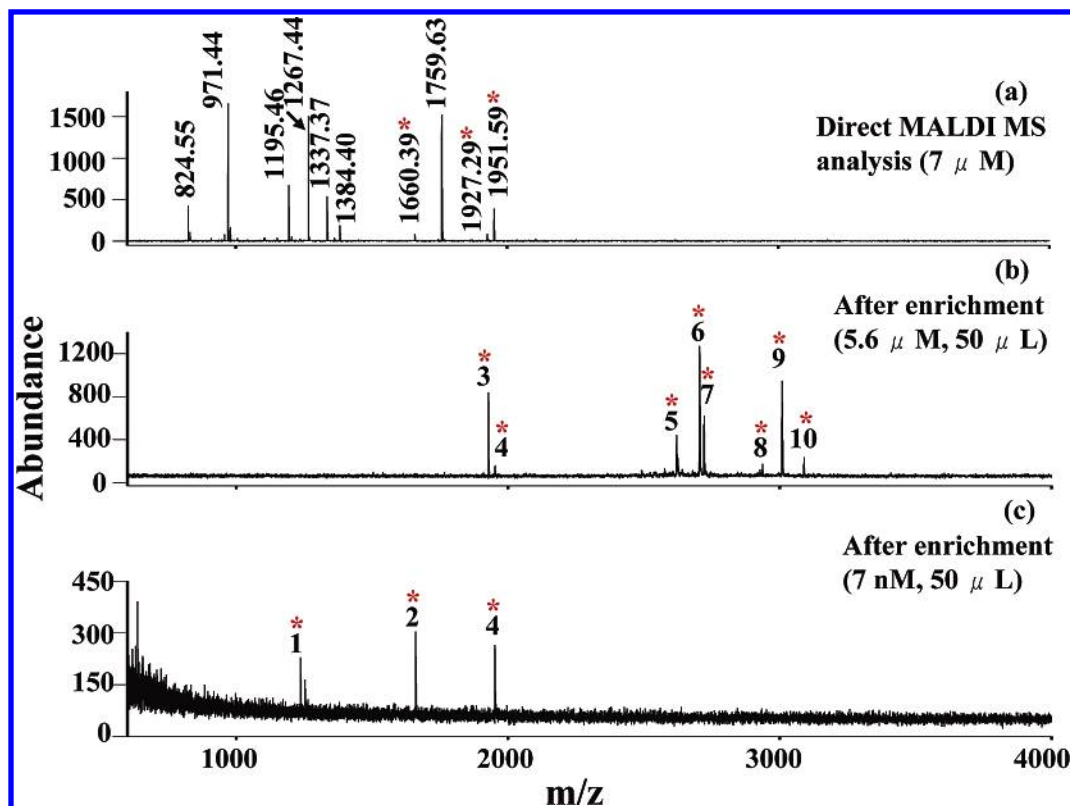
Figure 3. (a) Direct MALDI mass spectrum of the tryptic digest product of α-casein (7 × 10⁻⁶ M) prior to enrichment. MALDI mass spectra of the samples obtained using the magnetic particles coated with zirconia to selectively enrich target species from the tryptic digestion products of α-casein at the concentrations of (b) 5.6 × 10⁻⁶ M (50 μL) and (c) 7 × 10⁻⁹ M (50 μL). Phosphopeptide ions are marked with asterisks.

sample, which commonly contains abundant proteins including phosphoproteins, that is, α- and β-caseins. Figure 4a presents the direct MALDI mass spectrum of the tryptic digest of the milk sample (100-fold diluted). Among the peaks, only two, with low intensities, are generated from α-casein (S1/#104−119) and β-casein (#1−25), marked with asterisks at m/z 1951.99 and 3122.41, respectively. Without enrichment, only a few phosphopeptide ions are revealed in the MALDI mass spectra. Figure 4b displays the MALDI mass spectrum of the sample enriched from the tryptic digest of the milk sample (100-fold diluted, 50 μL). The peaks at m/z 1539.49 (α-S2/#126−137), 1927.81 (α-S1/#43−58), 2704.19 (α-S1/#99−120), and 3008.42 (α-S2/#46−70) marked with asterisks are derived from α-casein, while the peak at m/z 3122.72 (#1−25) is derived from β-casein. The peak at m/z 3040.78 is the fragmentation of the peak at m/z 3122.72 by loss of a phosphate group. In addition to the peak at m/z 2704.19, the other peaks appearing in the mass spectra are multiply phosphorylated peptides (see Table 2). The singly phosphorylated peptide ion at m/z 1951.99 does not appear in Figure 4b. Presumably, the reasons are similar to those discussed above, that multiply phosphorylated peptides enriched from high concentrations of samples generally dominate the mass spectra, while singly phosphorylated peptide ions appear in the mass spectra as the concentration decreases. On the basis of the value provided by the milk company, 100 mL of milk contains 3 g of proteins. Furthermore, the percentage of α- and β-caseins in the milk protein is about ~66%. Thus, we estimated that the concentration of caseins used for obtaining Figure 4 is ~8 × 10⁻⁶ M, which is denoted as high concentrations as indicated earlier. Thus, the signal of

the singly phosphorylated peptide at m/z 1951.99 was suppressed in the mass spectrum after enrichment. Nevertheless, the results indicate that our particles can be used to selectively enrich phosphopeptides from a very complex sample.

The results demonstrated that a contact time of 30 s was enough to enrich sufficient phosphopeptides from a tryptic digest of proteins for MALDI MS analysis. However, the tryptic digestion took 18 h. Therefore, the time required for the entire analysis was quite long although we have greatly reduced the time for enrichment. If the digestion time can be further reduced, this rapid enrichment approach will be practical. We recently developed microwave-assisted tryptic digestion using magnetic particles as the microwave absorbers and affinity probes and demonstrated that the time required for enzymatic digestion could be reduced to 1 min.[30] Egg white contains abundant phosphoproteins, for example, ovalbumin. We initially used our affinity probes to selectively enrich phosphoproteins directly from a denatured egg white sample. After enrichment, the particle−target species conjugates were mixed with trypsin and were heated in a microwave oven for 1 min to achieve tryptic digestion. After digestion, the particles remaining in the sample were used again to enrich the target phosphopeptides from the digest product by pipeting the sample in and out of the vial for 30 s. Figure 5 displays the MALDI mass spectrum obtained using our affinity probes to selectively enrich phosphopeptides from the tryptic digest of a diluted egg white sample, which was obtained from the on-particle tryptic digestion under microwave heating for 1 min. The mass spectrum is surprisingly simple. Only three major peaks marked with asterisks at m/z 2089.23, 2902.74, and
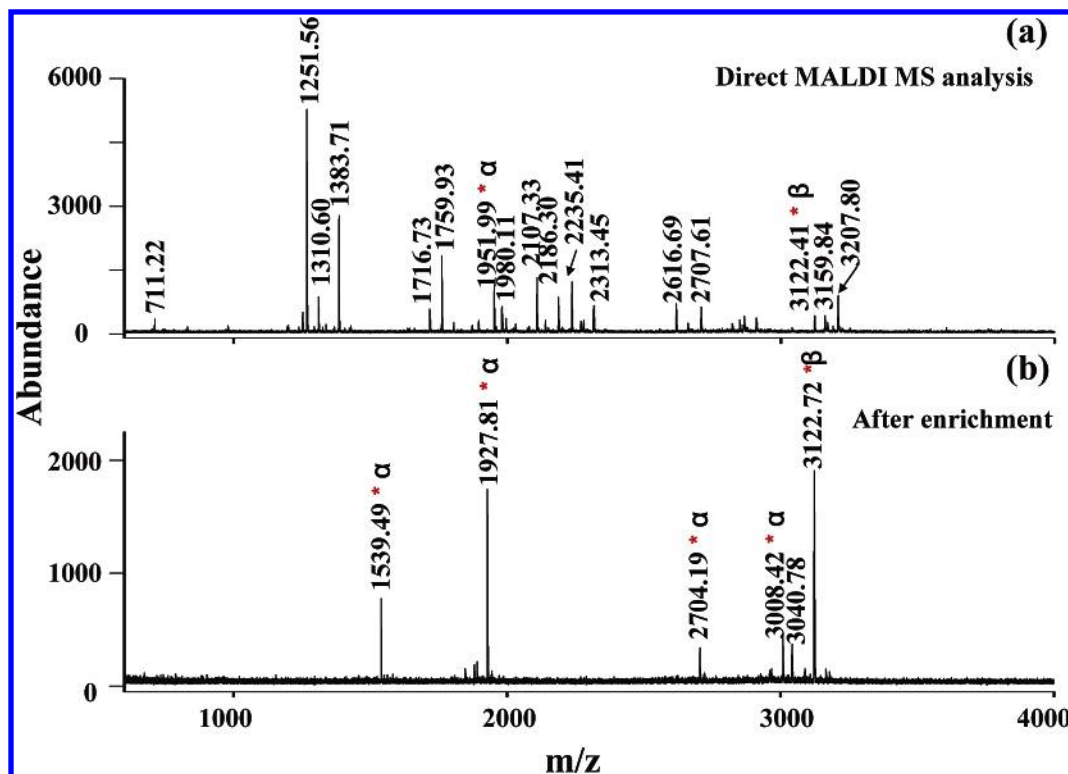
Figure 4. (a) Direct MALDI mass spectrum of a nonfat milk sample (100-fold diluted). (b) MALDI mass spectrum of the sample obtained using the magnetic particles coated with zirconia to selectively enrich target species from the tryptic digestion product of the nonfat milk sample (100-fold diluted). Phosphopeptide ions are marked with asterisks.
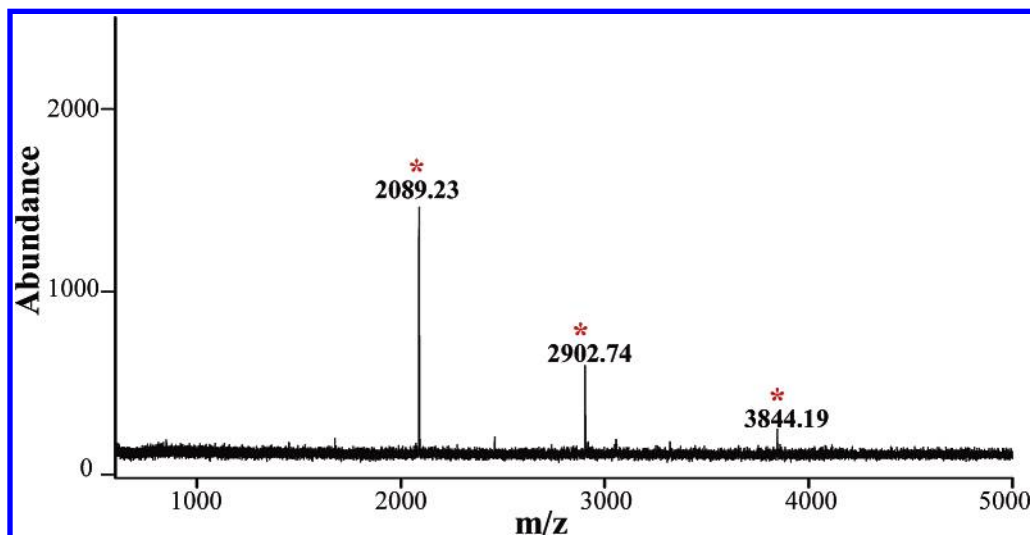

Figure 5. MALDI mass spectrum of the sample obtained using the magnetic particles coated with zirconia to selectively enrich target species from the tryptic digest of the egg white sample (10-fold diluted). The tryptic digest was obtained from on-particle protein tryptic digestion under microwave heating for 1 min. The proteins trapped on the particles were initially enriched from a diluted egg white sample by the magnetic affinity probes. Phosphopeptide ions are marked with asterisks.

3844.19, corresponding to the phosphorylated peptides derived from ovalbumin, appear in the mass spectrum. The corresponding peptide sequences of these ions are listed in Table 3. There are no non-phosphopeptide ions appearing in the same mass spectrum. The results demonstrated that our affinity probes have a quite good selectivity for phosphopeptides. Furthermore, the combination of on-particle tryptic digestion under microwave heating with the rapid enrichment method can dramatically reduce the time required for analysis.

Conclusions

We demonstrated that the iron oxide nanocomposites of magnetic particles coated with zirconia are effective affinity probes for phosphopeptides. Without requiring any desalting steps, this approach can be directly used to selectively concentrate phosphopeptides from tryptic digests of proteins. In only 30 s, phosphopeptides sufficient for characterization by MALDI-MS can be enriched from very low concentrations (~0.9 nM) and low volumes (50 μL) of sample solutions. Furthermore,

the combination of this rapid enrichment method with on-particle tryptic digestion under microwave heating has greatly reduced the time required for entire analysis. The advantages include short analysis time, ease of use, high specificity, and high sensitivity for the enrichment of phosphorylated peptides from complex sample solutions. The results obtained from this approach are quite promising. It should be useful for characterization of phosphopeptides from other complex samples.

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