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1	Article Title	**Conductive carbon tape as a sample platform for microwave-based MALDI MS detection of proteins and phosphoproteins**	
2	Article Sub- Title		
3	Article Copyright - Year	**Springer-Verlag 2011 (This will be the copyright line in the final PDF)**	
4	Journal Name	Analytical and Bioanalytical Chemistry	
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35		Received	26 March 2011
36	Schedule	Revised	18 June 2011
37		Accepted	20 June 2011

38	Abstract	In this study, we developed a novel microwave-assisted protein preparation and digestion method for matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry analysis and identification of proteins that involves using conductive carbon tape as a sample platform for sample preparation (reduction and alkylation) and digestion under microwave heating and as a plate for MALDI analysis. This method allows for the enzymatic digestion products of proteins to be directly analyzed by MALDI mass spectrometry and results in a marked reduction in sample loss. Our protocol requires only a small volume (1 μL) of reaction solvent, which increases the frequency of enzyme-to-protein contact, thereby resulting in more efficient digestion of sample than conventional in-solution digestion methods. To test this protocol, we used magnetic iron (II, III) oxide nanoparticles as concentrating probes to enrich phosphopeptides from a mixture of peptides in enzymatically digested protein samples. We found that the one-pot on-tape-based protein preparation and digestion under microwave heating combined with the on-tape-based enrichment method not only dramatically reduced the time required for phosphopeptides analysis but also allowed for the simultaneous identification of phosphoproteins. The advantages of our protocol include ease of use, high digestion efficiency, high specificity, and rapid (15 min) identification of proteins and enrichment of phosphopeptides in a mixture of enzymatically digested protein samples.

39	Keywords separated by ' - '	Carbon tape - Microwave-assisted protein preparation and digestion - Protein identification - Phosphopeptide - MALDI - Enrichment
40	Foot note information	The online version of this article (doi:10.1007/s00216-011-5198-6) contains supplementary material, which is available to authorized users.

Electronic supplementary material

ESM 1
(PDF 776 kb)

Anal Bioanal Chem
DOI 10.1007/s00216-011-5198-6

ORIGINAL PAPER

Conductive carbon tape as a sample platform for microwave-based MALDI MS detection of proteins and phosphoproteins

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Received: 26 March 2011 / Revised: 18 June 2011 / Accepted: 20 June 2011

Abstract In this study, we developed a novel microwave-assisted protein preparation and digestion method for matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry analysis and identification of proteins that involves using conductive carbon tape as a sample platform for sample preparation (reduction and alkylation) and digestion under microwave heating and as a plate for MALDI analysis. This method allows for the enzymatic digestion products of proteins to be directly analyzed by MALDI mass spectrometry and results in a marked reduction in sample loss. Our protocol requires only a small volume (1 μL) of reaction solvent, which increases the frequency of enzyme-to-protein contact, thereby resulting in more efficient digestion of sample than conventional in-solution digestion methods. To test this protocol, we used magnetic iron (II, III) oxide nanoparticles as concentrating probes to enrich phosphopeptides from a mixture of peptides in enzymatically digested protein samples. We found that the one-pot on-tape-based protein preparation and digestion under microwave heating combined with the on-tape-based enrichment method not only dramatically reduced the time required for phosphopeptides analysis but also allowed for the simultaneous identification of phosphoproteins. The advantages of our protocol include ease of use, high digestion efficiency, high specificity, and rapid (15 min) identification of proteins and enrichment of phosphopeptides in a mixture of enzymatically digested protein samples.

Keywords Carbon tape · Microwave-assisted protein preparation and digestion · Protein identification · Phosphopeptide · MALDI · Enrichment

Electronic supplementary material The online version of this article (doi:10.1007/s00216-011-5198-6) contains supplementary material, which is available to authorized users.

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Introduction

Reversible phosphorylation is one of the most common post-translational modifications. It is a ubiquitous mechanism for the regulation of many important biological processes in eukaryote cells, including signal transduction, cell proliferation, differentiation, metabolism, and communication [1–3]. Although matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) can be used to characterize the proteolytic products of phosphoproteins, the presence of nonphosphopeptides often suppresses the signal intensity generated by phosphopeptides. Thus, isolation and enrichment of phosphorylated peptides from proteolytic peptide mixtures is the first step in phosphoproteomic analysis. Several affinity methods are widely used to enrich phosphorylated peptides from a peptide complex, including immobilized metal ion affinity chroma-

tography [4, 5], metal oxide affinity chromatography [6], and metal oxide-coated magnetic nanoparticles (Fe_3O_4/TiO_2 core/shell, Fe_3O_4/ZrO_2 core/shell, Fe_3O_4/Al_2O_3 core/shell, and Fe_3O_4/Ga_2O_3 core/shell) [7–11]. Notably, magnetic iron (II, III) oxide (Fe_3O_4) nanoparticles not only permit an easy and speedy enrichment process but can also be used to enrich phosphorylated peptides [12].

Mass spectrometry-based bottom-up approaches have been widely used to characterize proteins on a proteomic scale [13]. Efficient protein degradation or digestion is the key for the success of that approach for protein identification. Conventional chemical hydrolysis or enzymatic digestion for bottom-up experiments takes several hours to perform, thereby limiting the speed of large-scale protein identification. Therefore, several protocols have been developed to speed up the protein digestion process. One such protocol is on-particle enzymatic digestion which can be performed in less than 30 min [9, 10, 14]. Another approach involving the use of enzyme-immobilized magnetic nanospheres for on-plate digestion can reduce the digestion time to 5 min and facilitate the process of protein digestion [15, 16]. Other promising approaches include microwave-assisted protein enzymatic digestion and acid hydrolysis [9, 10, 15, 17–19]. Sun and coworkers recently employed microwave-assisted protein preparation and enzymatic digestion and found that the time required for protein in-solution processes was only 6 min and that for in-gel processes was only 25 min [19]. However, the tradtional reduction and alkylation steps used in these studies were somewhat time-consuming.

In general, a matrix-assisted laser desorption/ionization (MALDI) sample plate cannot be placed in a microwave oven because the electric current induced by microwave radiation on the surface of the plate will readily produce sparks. Nevertheless, we hypothesized that the processes for microwave-assisted protein preparation and digestion might be made more efficient if an appropriate substance capable of electric conductivity and withstanding microwave irradiation could be found. Carbon tape is widely used as electrode material [20–23]. Furthermore, carbon tape can be used directly in a microwave oven. We, therefore, investigated the feasibility of using carbon tape as a one-pot digestion method. To test this protocol, we used magnetic iron (II, III) oxide nanoparticles as concentrating probes to enrich phosphopeptides from a mixture of peptides in enzymatically digested protein samples.

Experimental

Chemicals and reagents

Magnetic iron (II, III) oxide (Fe_3O_4) nanoparticles were obtained from Alfa Aesar (Ward Hill, MA). Conductive double-sided carbon adhesive tape (8 mm wide×20 m) was purchased from SPI Supplies (West Chester, PA, USA). Ammonium bicarbonate was purchased from J. T. Baker (Phillipsburg, NJ, USA). α- and β-caseins (from bovine milk), 2,5-dihydroxybenzoic acid (DHB), L(+)-lactic acid (98%), and MiniTip™ C_{18} were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Thermo Fisher Scientific (Rockford, IL, USA). Cytochrome *c* was obtained from Protea Biosciences (Morgantown, WV, USA). Modified trypsin was purchased from Promega (Madison, WI). Dithiothreitol (DTT) was purchased from Amresco (Solon, OH, USA), and iodoacetamide (IAA) was purchased from Amersham Pharmacia (Piscataway, NJ). Acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), α-cayno-4-hydroxycinnamic acid (CHCA), and phosphoric acid (99.9%) were obtained from Fluka (Steinheim, Germany). Nonfat milk was purchased from a local supermarket.

Procedures for conventional/microwave-assisted protein preparation and digestion

A 20-μL aliquot of BSA (2 mg/mL) was evaporated using a speed vacuum concentrator and then dissolved in 20 μL of ammonium bicarbonate buffer (50 mM, pH 8.0). For conventional digestion, BSA was reduced with 10 mM DTT at 57 °C for 1 h, alkylated with 50 mM IAA at room temperature in the dark for 0.5 h, and then digested with trypsin (50:1, *w/w*) at 37 °C for 16 h [19]. For microwave-assisted digestion, BSA was reduced with 10 mM DTT at 100 °C for 5 min but was not alkylated [19]. BSA was then digested with trypsin (50:1, *w/w*) in the presence of microwave radiation using a domestic 900-Watt microwave oven at different heating times (15, 30, 60, 90, and 120 s). Following that, different protein-to-trypsin ratios (20:1, 50:1, and 100:1, *w/w*) with various digestion solvents (50 mM of ammonium bicarbonate, pH 8.0, containing ACN (10%, 20%, and 30%)) needed for microwave-assisted digestion were compared. The incubation time was 60 s, and the microwave power was 900 W for those experiments. After optimization microwave-assisted enzymatic digestion, different sample preparation (reduced with 10 mM DTT at 100 °C for 5 min and not alkylated, or reduced with 10 mM DTT at 900 W heating for 5 min and then with or without alkylated by 50 mM IAA at 900 W heating for 5 min) needed for microwave-assisted protein preparation were compared. For each measurement, the micro-centrifuge tube was placed in a plastic rack, taking care to keep the cap open during heating. As described previously [24, 25], a container with 1,000 mL of water was placed beside the sample vials to absorb the extra microwave energy. After enzymatic digestion, the tryptic peptide solvent was evaporated using a speed vacuum concentrator and then dissolved in 20 μL of 0.1% TFA. The peptide solution (1 μL) was directly mixed

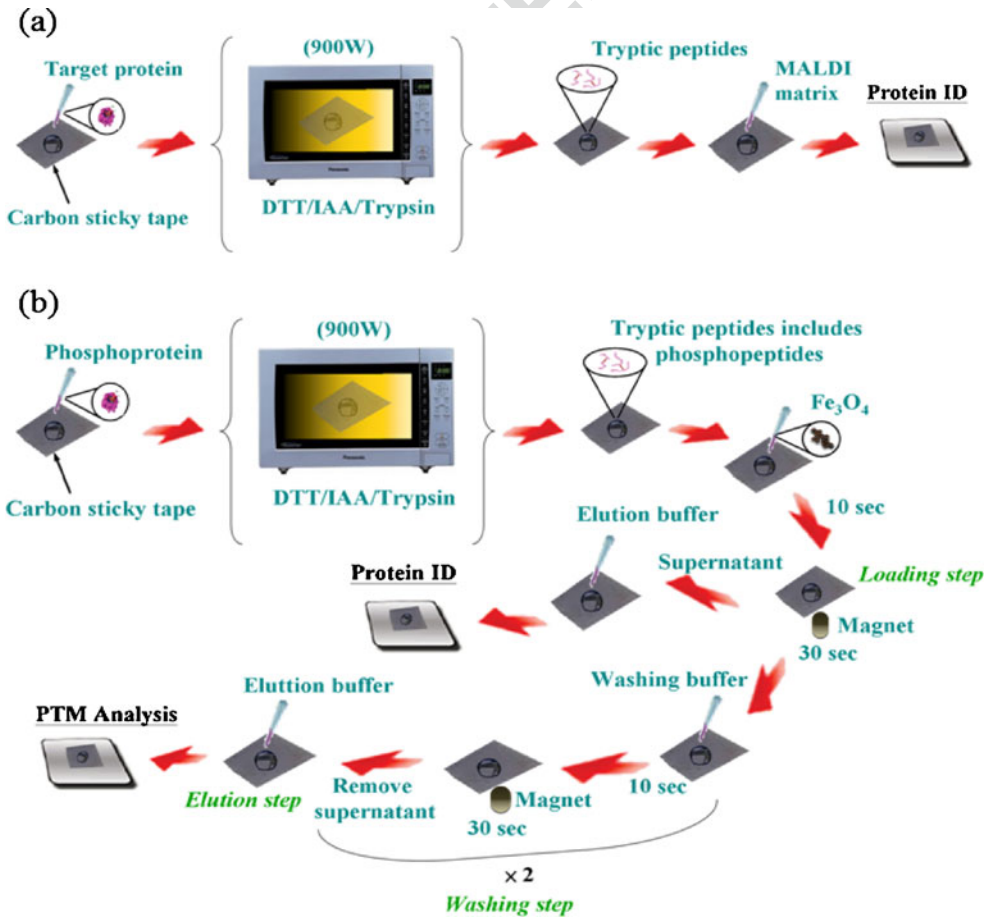
with 1 μL of 10 mg/mL CHCA solution in 50% ACN/water with 0.1% TFA in a 96×2-well Teflon MALDI plate and dried at room temperature. The mixture was then analyzed by mass spectrometry.

Procedures for direct on-tape protein preparation and digestion under microwave heating

BSA or cytochrome *c* (2 mg/mL, 20 μL each) were evaporated using a speed vacuum concentrator and then dissolved in 20 μL of ammonium bicarbonate buffer (50 mM, pH 8.0). Low-volume (1 μL) protein solutions were deposited on the conductive double-sided carbon adhesive tape (4×4 mm), reduced by 1 μL of 10 mM DTT under microwave heating (900 W) for 3 min, alkylated by 1 μL of 50 mM IAA under microwave heating (900 W) for 3 min, and digested by 1 μL of trypsin (50:1, *w/w*) under microwave heating (900 W) for 60 s (Scheme 1a). For each procedure, the carbon tape was placed on a plastic rack, and a container with 1,000 mL of water was placed beside the carbon tape to absorb the extra microwave energy [24, 25]. After enzymatic digestion, the tryptic peptides were directly mixed with 1 μL of 10 mg/mL CHCA solution in 50% ACN with 0.1% TFA on the carbon tape. Then, the carbon tape was directly transferred onto a MALDI sample plate and dried at room temperature. The mixture was then analyzed by mass spectrometry. Besides, the BSA (2 mg/mL, 1 μL) was used for the sample desalting test. After microwave-based on-tape protein preparation and digestion, the tryptic peptides were dried at room temperature. Ten microliters of 0.1% TFA was deposited onto the carbon tape to dissolve the tryptic peptides followed by desalting with MiniTip™ C_{18}.

Desalting of BSA digests with MiniTip™ C_{18}

The 10 μL MiniTip™ pipette tips contained a C18 spherical silica (50–60 μm, 200 Å pore size) sorbent bed bonded at the working end of the tip and were used for the desalting of the peptides. Referring to the instructions provided by the manufacturer, the tips were first wetted with 10 μL of 0.1% TFA in 70% ACN, and then were equilibrated with 10 μL of 0.1% TFA. The peptides dissolved in 10 μL of 0.1% TFA and then were bound to MiniTip™ by fully depressing the pipette plunger to a dead



Scheme 1 **a** Schematic diagram of on-tape-based protein preparation and digestion under microwave heating. **b** Schematic diagram of on-tape-based phosphopeptide enrichment

stop. Samples were aspirated and dispensed for ten cycles. Then, the tips were washed with 10 μL of 0.1% TFA twice. The peptides on the tips were eluted out using 1 μL of 0.1% TFA in 70% ACN onto another new carbon tape (4×4 mm). After desalting, the peptides were directly mixed with 1 μL of 10 mg/mL CHCA solution in 50% ACN with 0.1% TFA on the carbon tape. Then, the carbon tape was directly transferred onto a MALDI sample plate and dried at room temperature. The mixture was then analyzed by mass spectrometry.

Procedures for enrichment of phosphopeptides from proteolytic peptide mixture by direct on-tape preparation and digestion under microwave heating

The modified phosphopeptide enrichment method for MALDI MS was performed as described previously [12]. Briefly, 1 μL of α-casein (10 pmol) or protein mixture containing α- and β-casein, and BSA (10 pmol each) underwent microwave-assisted on-tape-based protein preparation and digestion followed by drying at room temperature. Then, 1 μL of 0.1% TFA was deposited onto the carbon tape to dissolve the tryptic peptides and then mixed with 1 or 2 μL (for protein mixture) solution of magnetic iron oxide nanoparticles (1.6 mg/mL in 0.1% TFA). The mixture was mixed by gently pipetting the solution up and down for 10 s. The magnetic iron oxide nanoparticles were then isolated by positioning a magnet under the carbon tape. The supernatant was then transferred to another carbon tape using a pipette (loading step) and then dried at room temperature. Elution buffer (1 μL) was then subsequently deposited on the carbon tape for MALDI analysis of the supernatant. The isolated particles were washed twice with 1.5 μL of 100 mg/mL lactic acid solution in 50% ACN with 0.1% TFA and were isolated again using a magnet. Elution buffer (1 μL) was added to the retained particles to release the phosphopeptides from the magnetic iron oxide nanoparticles (elution step). All carbon tapes were then transferred to the MALDI sample plate and dried at room temperature. The mixture was then analyzed by mass spectrometry (Scheme 1b). For the analysis of non-phosphorylated or phosphorylated peptides in the loading and elution steps, 30 mg/mL DHB solution in 50% ACN with 1% phosphoric acid was used as a matrix (elution buffer).

Procedures for enrichment of phosphopeptides from tryptic products of nonfat milk by direct on-tape preparation and digestion under microwave heating

Prior to analyze the phosphopeptides from nonfat milk, the sample was diluted 200-fold, 500-fold, and 1,000-fold with ammonium bicarbonate buffer (50 mM, pH 8.0) to make a final volume of 100 μL. Then, 100 μL of ammonium bicarbonate buffer (50 mM, pH 8.0) was added to μL of sample solution followed by direct on-tape preparation and digestion under microwave heating. After enzymatic digestion, the peptide mixture underwent phosphopeptides enrichment with 3.2 μg of magnetic iron oxide nanoparticles (1.6 mg/mL in 0.1% TFA) as described in the section preceding. The eluted peptides were analyzed by mass spectrometry (Scheme 1b).

Mass spectrometry and data analysis

All MALDI time-of-flight mass spectrometry (TOF MS) spectra were obtained using a Voyager DE^{pro} mass spectrometer (Applied Biosystems, Framingham, MA, USA) equipped with a nitrogen laser operated at 337 nm. Spectral data were acquired in the reflector mode with an accelerating voltage of 20 kV. A total of 5,000 laser shots for each mass spectrum were accumulated in positive-ion mode. Laser power was set to optimize the signal-to-noise (S/N) ratio and the resolution of mass peaks of the analyte. Mass calibration was performed using a mixture of peptides from Applied Biosystems, including angiotensin I, ACTH (1–17), and ACTH (7–38) as external standards. All TOF MS spectra were analyzed using Data Explorer software (version 4.0, Applied Biosystems) and reported as monoisotopic masses. A Bruker Daltonics Ultraflex III (Germany) TOF/TOF MS equipped with a Smartbean laser was employed for obtaining tandem mass spectrometry (MS/MS) spectra. The following voltage parameters were employed: ion source 1, 8.00 kV; ion source 2, 7.10 kV; lens, 3.71 kV; reflector 1, 29.50 kV; reflector 2, 13.70 kV; left 1, 19.00 kV; left 2, 2.75 kV. BSA and cytochrome *c* were identified by the MALDI-TOF MS spectra by searching against the NCBI database for exact matches using Mascot v2.2 (Matrix Sciences, London, UK). Fragment peaks (S/N >3) resulting from precursor ions (S/N>3) of phosphorylated peptide candidates were submitted via Biotools (v. 3.2) to Mascot v2.2 (Matrix Sciences, London, UK). An in-house database containing BSA (GI:1351907), cytochrome *c* (GI:119388048), α-S1-casein (GI:115646), α-S2-casein (GI:115654), and β-casein (GI:115660) was used, and the MS and MS/MS tolerances were set to±0.5 and 0.8 Da, respectively. Carbamidomethyl cysteine was set as a fixed modification, while oxidation (HW) and phosphorylation (ST) modifications were set as variable modifications. The other parameters for searching were enzymes of trypsin; two missed cleavages.

Results and discussion

Optimization of microwave-assisted protein preparation and digestion

Microwave irradiation is known to accelerate the chemical hydrolysis or enzymatic digestion of proteins [9, 10, 15,

17–19]. Although the method reduces the protein digestion time, the protein preparation using conventional methods takes at least 1 h. Previously, Sun and coworkers demonstrated that protein can be reduced in the presence of DTT at 100 °C for 5 min [19]. That observation suggests that the protein preparation time can be accelerated if protein is exposed to a high-energy source prior to enzymatic digestion, such as microwave radiation. In this study, we compared how different protein/enzyme ratios (*w/w*), various digestion solvents, and different protein preparations affect protein digestion under microwave irradiation. We then developed a platform for rapid protein identification and phosphopeptide enrichment using one-pot on-tape-based MALDI MS analysis.

We initially compared the efficiency of a conventional protocol with that of a microwave-assisted protocol at different heating times (15, 30, 60, 90, and 120 s; Fig. 1a). The efficiency of protein digestion using a conventional protocol was equivalent or lower to that of microwave-assisted digestion when the microwave radiation power was set at 900 W and incubation time was set at 30 or 60 s, respectively. Our findings were consistent with those reported by Sun et al. [19]. We then evaluated different protein-to-enzyme ratios (*w/w*) 20:1, 50:1, and 100:1 to assess the ratio associated with the most efficient microwave-assisted digestion (Fig. 1b). The result revealed that the different protein to enzyme rations (*w/w*) did not affect the efficiency of protein digestion. Then, keeping the protein/trypsin ratio (*w/w*) at approximately 50:1, the microwave power at 900 W, and incubation time at 60 s, we compared the protein digestion efficiency of various digestion solvents (Fig. 1c). The sequence coverage decreased significantly when the percentage of ACN was increased from 10% to 30%. This observation suggests that a higher percentage of ACN not only enhances the degradation of protein samples but also affects the enzyme activity. Using 50 mM ammonium bicarbonate (pH 8.0) as digestion solvent, a protein/enzyme ratio (*w/w*) of 50:1 and microwave heating for 60 s (900 W), we attempted to further improve the digestion by testing different preparation procedures (Fig. 1d). Without alkylation, the results show that the sequence coverage had no significant difference when proteins prior to enzymatic digestion were reduced by DTT at 100 °C for 5 min compared with that reduced by DTT at 900 W heating for 5 min. The sequence coverage of proteins that had been reduced by DTT and then alkylated by IAA in the presence of microwave radiation for 5 min was significantly greater than that of non-alkylated samples described above. This observation suggests that the disulfide bond rearrangement promoted by strong acids (0.1% TFA) [26, 27] or under the conditions of enzymatic cleavage in the presence of a slightly alkaline solution (50 mM ammonium bicarbonate, pH 8.0) [27] could be blocked using IAA for protein alkylation prior to enzymatic digestion, thereby increasing the efficiency of protein digestion. The protein preparation time was only 10 min and the digestion efficiency using microwave radiation was better than that of conventional in-solution digestion (Table 1).

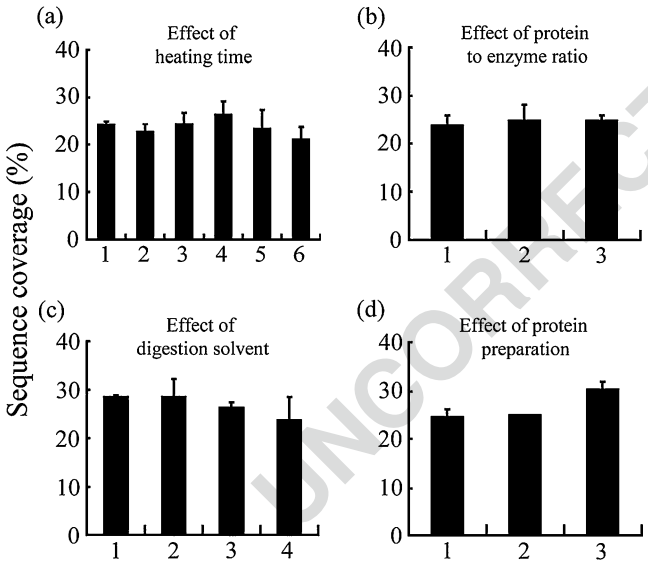


Fig. 1 Comparison of sequence coverage of identified peptide ion peaks of BSA by MALDI-TOF MS analysis: **a** samples were prepared and digested using conventional protocol (sample 1) or microwave-assisted protein preparation and digestion protocol under protein/enzyme 50:1 (*w/w*) at different microwave heating (900 W) times (15, 30, 60, 90, and 120 s) (samples 2–6); **b** samples were prepared using microwave-assisted protein preparation and digestion protocol under microwave heating (900 W) for 60 s using different protein to enzyme ratios of 20, 50, 100 (*w/w*; samples 1–3); **c** samples were dissolved in ammonium bicarbonate (50 mM, pH 8.0) without (sample 1) or with ACN (10%, 20%, and 30%; samples 2–4) and then prepared using microwave-assisted protein preparation and digestion protocol under protein/enzyme 50:1 (*w/w*) and microwave heating (900 W) for 60 s; **d** samples were prepared using microwave-assisted protein preparation and digestion protocol under protein/enzyme 50:1 (*w/w*) and microwave heating (900 W) for 60 s followed by reduction by DTT at 100 °C (5 min) (sample 1) or 900 W (5 min) without or with alkylation by IAA at 900 W (5 min) (samples 2–3)

Application of microwave-assisted protein preparation and digestion on conductive carbon tape

Recently, Li and coworkers employed trypsin-immobilized magnetic nanospheres to develop a rapid and effective on-plate digestion method for analysis and identification of proteins [16]. The proteins could be efficiently digested within 5 min under 50 °C incubation. In the current study, we hypothesized that protein preparation and digestion could be further enhanced using an on-plate procedure under microwave irradiation. In order to approach this new idea, an appropriate substance capable of microwave irradiation and electric conductivity was needed as a sample platform. Carbon tape is widely used as an electrode

JnlID 216_ArtID 5198_Proof# 1 - 28/06/2011

t1.1 **Table 1** Conventional and microwave-assisted protein preparation and digestion protocols

Protocol	Solution	Conventional min	Microwave-assisted protein preparation and digestion min
Reduce	10 mM DTT	60	5
Alkylate	50 mM iodoacetamide	30	5
Digest	50/l (*w*/*w*) of sample to trypsin, 50 mM ammonium bicarbonate (pH 8.0)	960	1
Total time		1150	11

material [20–23] and can also be subjected to microwave irradiation. Considering those two unique features, we assessed the efficiency of carbon tape as a platform for microwave-assisted protein preparation and digestion prior to analysis and identification of proteins (Scheme 1a).

The reaction volume for the on-tape procedure was only 1–2 μL. The reaction time for reduction and alkylation using our protocol was only 3 min compared with 5 min for the micro-centrifuge tube procedure described above. Figure 2 compares the sequence coverage of BSA and cytochrome *c* after conventional digestion with that after on-tape microwave-assisted protein preparation and digestion protocols. Microwave-assisted protein preparation and digestion significantly increased the digestion efficiency. We attribute the efficiency of the one-pot digestion method to the volume of reaction solvent, which increased the frequency of enzyme-to-protein contact, and to the fact that the sample did not have to be transferred, thereby minimizing the amount of loss of proteolytic peptides. Both of the microwave irradiation-based and high temperature-based processes sped up digestion efficiency [16]. However, microwave irradiation differs from high temperature on the mechanism. The microwave reactions increased polar molecular movement, and hence enhanced interactions between reactive entities more than high temperature reactions [28]. Compared with the high temperature-based enzyme digestion, the microwave irradiation-based enzyme digestion may have resulted in higher frequency of enzyme-to-protein contact. Furthermore, we found that sequence coverage of BSA was similar for the sample with or without desalting in our study (Fig. 3). The results indicate that the on-tape microwave-assisted protein preparation and digestion protocol is rapid and efficient for protein identification.

Application of on-tape-based microwave-assisted protein preparation and digestion for phosphoprotein analysis and phosphopeptide enrichment

Figure S1 (Electronic Supplementary Material) displays the procedures for on-tape-based preparation and digestion under microwave heating, the enrichment of phosphopeptides using magnetic iron (II, III) oxide nanoparticles as concentrating probes, and the co-crystallization procedure with elution buffer for subsequent MALDI MS analysis. Figure 4a displays the direct MALDI mass spectrum of α-casein (10 pmol, 1 μL) after on-tape-based preparation and digestion under microwave heating (Scheme 1a) prior to enrichment. Only one phosphopeptide peak (marked with asterisk) at *m/z* 1951.9 appeared in this mass spectrum; the remaining peaks were nonphosphopeptides (Table 3). Figure 4c displays the MALDI mass spectrum obtained using 1.6 μg of magnetic iron (II, III) oxide nanoparticles as concentrating probes to selectively trap phosphopeptides from α-casein (10 pmol, 1 μL) after the elution step followed by on-tape-based preparation and digestion under microwave heating (Scheme 1b). Several phosphopeptide peaks (marked with asterisks) appeared in the mass spectrum from the elution

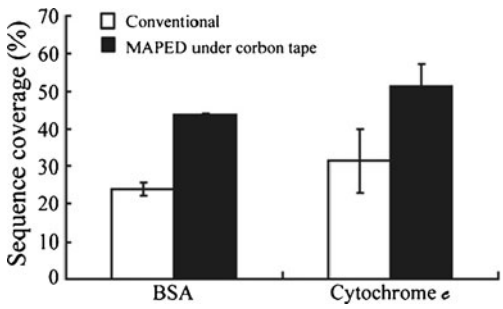


Fig. 2 Comparison of the sequence coverage of identified peptide ion peaks for BSA and cytochrome *c* by MALDI-TOF MS analysis, respectively. *Open bars* were prepared and digested using conventional protocol and *solid bars* were prepared using on-tape-based protein preparation and digestion under microwave heating protocol

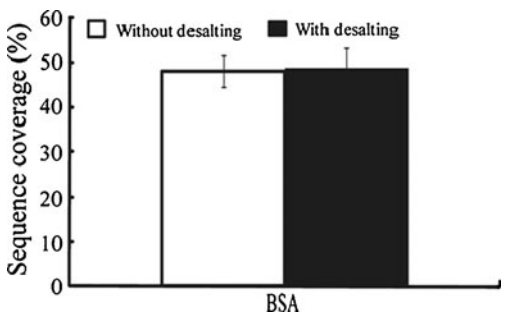


Fig. 3 Comparison of the sequence coverage for BSA in MALDI-TOF MS analysis without (*open bar*) or with (*solid bar*) MiniTip™ C_{18} desalting

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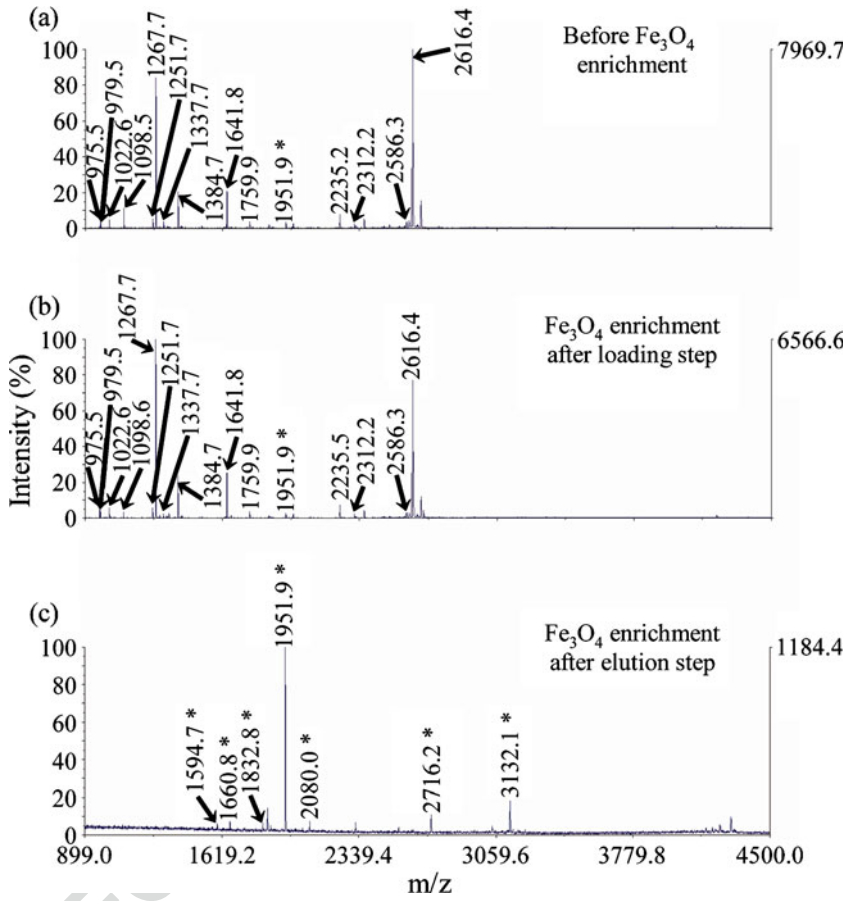

Fig. 4 MALDI mass spectra of tryptic digest products of α-casein (10 pmol) **a** before enrichment and application of 1.6 μg of magnetic iron (II, III) oxide nanoparticles, **b** after the loading step, and **c** after the elution step. The matrix solution contained 30 mg/ml DHB in 50% ACN/water with 1% phosphoric acid. Phosphopeptide ions are marked with an *asterisk*

step. Moreover, the S/N ratio of the observed phosphopeptide ions increased significantly after magnetic iron (II, III) oxide nanoparticle enrichment (Table 2). In addition, we compared the peptide ion peaks observed in MALDI mass spectrums of tryptic digest of α-casein before magnetic iron (II, III) oxide nanoparticles enrichment (Fig. 4a) with those observed after the loading step (Fig. 4b). The number of peptide ion peaks did not differ significantly. These phosphorylated peptide candidates were further confirmed by MALDI-TOF/TOF MS analysis (Electronic Supplementary Material Figs. S2–S8). The results suggest that the combination of one-pot on-tape-based protein preparation and digestion under microwave heating combined with the enrichment method not only dramatically reduces the time required for phosphopeptide analysis but can also be used for protein identification simultaneously (Table 3).

t2.1 **Table 2** Phosphopeptides observed in the MALDI-TOF mass spectra of the tryptic digest of α-casein S1 (α-S1) and α-casein S2 (α-S2)

t2.2	Observed *m/z*	Theoretic *m/z*	Peptide sequences[a]	S/N ratio		t2.3
				Before Fe_3O_4 enrichment	After Fe_3O_4 enrichment	
t2.4	1594.7	1594.7	TVDMEpSTEVFTKK (α-S2-(153–165))	–	10.7	
t2.5	1660.8	1660.7	VPQLEIVPNpSAEER (α-S1-(121–134))	–	13.3	
t2.6	1832.8	1832.8	YLGEYLIVPNpSAEER (α-S1-(104–119))[b]	–	14.3	
t2.7	1951.9	1951.9	YKVPQLEIVPNpSAEER (α-S1-(119–134))	74.3	247.3	
t2.8	2080.0	2080.0	KYKVPQLEIVPNpSAEER (α-S1-(118–134))	–	18.0	
t2.9	2716.2	2716.2	NAVPITPTLNREQLpSTpSEENSKK (α-S2-(130–152))	–	23.3	
t2.10	3132.1	3132.1	KNTMEHVpSpSpSEESIIpSQETYKQEK (α-S2-(16–39))	–	34.8	

pS refers to a phosphorylated serine unit, *en dash* no peptide was identified

[a] Searched from http://tw.expasy.org

[b] Sequences are cited from ref. [6]

t3.1 **Table 3** Overview of observed peptide ion peaks in the MALDI-TOF mass spectra of the tryptic digest of α-casein S1 (α -S1) and α -casein S2 (α-S2)

t3.2	Observed *m/z*	Theoretic *m/z*	Peptide sequences[a]	MALDI MS using Fe_3O_4 after the loading step	MALDI MS using Fe_3O_4 after the elution step
t3.3	975.5	975.5	TKVIPYVR (α-S2-(213–220))	▲	
t3.4	979.5	979.5	FALPQYLK (α-S2-(189–196))	▲	
t3.5	1022.6	1022.6	VIPYVRYL (α-S2-(215–222))	▲	
t3.6	1098.6	1098.6	AMKPWIQPK (α-S2-(204–212))	▲	
t3.7	1251.7	1251.7	TKVIPYVRYL (α-S2-(213–222))	▲	
t3.8	1267.7	1267.7	YLGYLEQLLR (α-S1-(106–115))	▲	
t3.9	1337.7	1337.7	HIQKEDVPSER (α-S1-(95–105))	▲	
t3.10	1384.7	1384.7	FFVAPFPEVFGK (α-S1-(38–49))	▲	
t3.11	1594.7	1594.7	TVDMEpSTEVFTKK (α-S2-(153–165))		▲
t3.12	1637.0	1637.9	YLGYLEQLLRLKK (α-S1-(106–118))	▲	
t3.13	1641.8	1641.7	FFVAPFPEVFGKEK (α-S1-(38–51))	▲	
t3.14	1660.8	1660.7	VPQLEIVPNpSAEER (α-S1-(121–134))		▲
t3.15	1759.9	1759.9	HQGLPQEVLNENLLR (α-S1-(23–37))	▲	
t3.16	1832.8	1832.8	YLGEYLIVPNpSAEER (α-S1-(104–119))[b]		▲
t3.17	1951.9	1951.9	YKVPQLEIVPNpSAEER (α-S1-(119–134))	▲	▲
t3.18	2080.0	2080.0	KYKVPQLEIVPNpSAEER (α-S1-(118–134))		▲
t3.19	2235.5	2235.2	HPIKHQGLPQEVLNENLLR (α-S1-(19–37))	▲	
t3.20	2312.2	2312.2	FFVAPFPEVFGKEKVNELSKK (α-S1-(38–57))	▲	
t3.21	2586.3	2586.3	HIQKEDVPSERYLGYLEQLLR (α-S1-(95–115))	▲	
t3.22	2616.4	2616.4	RPKHPIKHQGLPQEVLNENLLR (α-S1-(16–37))	▲	
t3.23	2716.2	2716.2	NAVPITPTLNREQLpSTpSEENSKK (α-S2-(130–152))		▲
t3.24	3132.1	3132.1	KNTMEHVpSpSpSEESIIpSQETYKQEK (α-S2-(16–39))		▲

pS refers to a phosphorylated serine unit

[a] Searched from http://tw.expasy.org

[b] Sequences are cited from ref. [6]

Application of on-tape-based microwave-assisted protein preparation and digestion for phosphopeptide enrichment from protein mixture

Protein mixture (1 μL) containing α- and β-casein, and BSA (10 pmol each) was subjected to on-tape-based preparation and digestion under microwave heating. Phosphopeptides were then enriched using 3.2 μg of magnetic iron (II, III) oxide nanoparticles as concentrating probes (Scheme 1b). We also used 1.6 μg of magnetic iron (II, III) oxide nanoparticles as concentrating probes for phosphopeptide enrichment, but found that 1.6 μg was not enough to trap the phosphopeptides (data not shown). On the basis of that result, we estimated that more than 1.6 μg of magnetic iron (II, III) oxide nanoparticles would be required for effective enrichment of 10 pmol of phosphoprotein using the on-tape-based enrichment procedure. Figure 5a displays the direct MALDI mass spectra of tryptic peptides in the protein mixture (containing α- and β-casein, and BSA 10 pmol each). Only two phosphopeptide peaks (marked with asterisks) at *m/z* 1951.8 and 3122.2 appear in this mass spectrum; the remaining peaks are nonphosphopeptides. The peak at *m/z* 1951.8 represents phosphopeptide derived from α-casein, and the peak at *m/z* 3,122.2 represents phosphopeptide derived from β-casein (Table 4). Figure 5b displays the MALDI mass spectrum obtained using the 3.2 μg of magnetic iron (II, III) oxide nanoparticles as concentrating probes to selectively trap target species from the protein mixture (containing α- and β-casein, and BSA 10 pmol each) that had been subjected to on-tape-based preparation and digestion under microwave heating after the elution step (Scheme 1b). Several phosphopeptide peaks (marked with asterisks) appeared in the mass spectrum from the elution step. The peaks at *m/z* 1594.7, 1660.8, 1832.8, 1951.9, 2080.0, 2716.2, and 3132.1 represent phosphopeptides derived from α-casein, while the peaks at *m/z* 2061.8, 3122.2, and 3477.4 represent phosphopeptides derived from β-casein (Table 4). Furthermore, the S/N ratio of observed phosphopeptides increased significantly after magnetic iron (II, III) oxide nanoparticle enrichment (Table 4). These phosphorylated peptide candidates were further confirmed by MALDI-TOF/TOF MS analysis (Electronic Supplementary Material Figs. S2–S11). The

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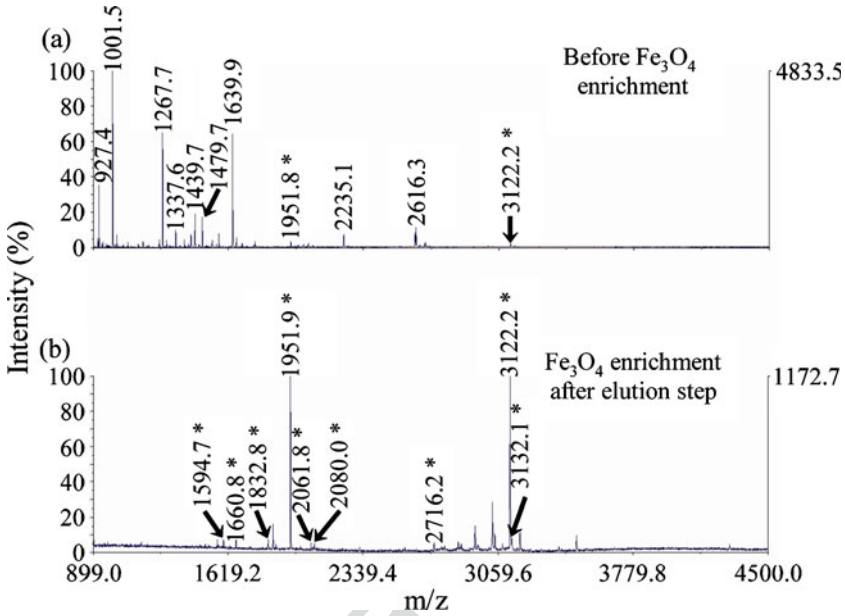

Fig. 5 MALDI mass spectra of a tryptic digest mixture containing α- and β-casein, and BSA (10 pmol each) without (**a**) or with (**b**) enrichment step using magnetic iron (II, III) oxide nanoparticles. The matrix solution contained 30 mg/ml DHB in 50% ACN/water with 1% phosphoric acid. Phosphopeptide ions are marked with an *asterisk*

results demonstrated that the use of magnetic iron (II, III) oxide nanoparticles as concentrating probes when applied directly to the carbon tape is a highly selective procedure for enrichment of phosphopeptides.

Application of on-tape preparation and digestion under microwave heating for phosphopeptide analysis in nonfat milk

In order to further demonstrate the feasibility of this approach, the nonfat milk was used as the real sample. The abundant phosphoproteins including α- and β-caseins in the nonfat milk were analyzed with the on-tap MALDI MS. MS spectra in Fig. 6a–c display the results of direct MALDI MS analysis of tryptic peptides from the nonfat milk after 200-fold, 500-fold, and 1,000-fold dilution, respectively. Only two phosphopeptide peaks (marked with asterisks) at *m/z* 1951.9 and 3122.2 were detected. The phosphopeptides at *m/z* 1951.9 and 3122.2 were generated from α-casein and β-casein, respectively (Table 4). Figure 6d–f displays the results of MALDI-MS analysis of enriched phosphopeptides from the nonfat milk using magnetic iron (II, III) oxide nanoparticles (3.2 μg) combined with the on-tap MALDI-MS analysis. For the 200-

t4.1 **Table 4** Phosphopeptides observed in the MALDI-TOF mass spectra of the tryptic digest mixture containing α-casein S1 (α-S1), α-casein S2 (α-S2), β-casein (β-C), and BSA

t4.2	Observed *m/z*	Theoretic *m/z*	Peptide sequences[a]	S/N ratio		t4.3
				Before Fe_3O_4 enrichment	After Fe_3O_4 enrichment	
t4.4	1594.7	1594.7	TVDMEpSTEVFTKK (α-S2-(153–165))	–	17.5	
t4.5	1660.8	1660.8	VPQLEIVPNpSAEER (α-S1-(121–134))	–	18.1	
t4.6	1832.8	1832.8	YLGEYLIVPNpSAEER (α-S1-(104–119))[b]	–	17.5	
t4.7	1951.9	1951.9	YKVPQLEIVPNpSAEER (α-S1-(119–134))	67.5	248.7	
t4.8	2061.8	2061.8	FQpSEEQQQTEDELQDK (β-C-(33–48))[b]	–	12.9	
t4.9	2080.0	2080.0	KYKVPQLEIVPNpSAEER (α-S1-(118–134))	–	10.3	
t4.10	2716.2	2716.2	NAVPITPTLNREQLpSTpSEENSKK (α-S2-(130–152))	–	12.1	
t4.11	3122.2	3122.2	RELEELNVPGEIVEpSLpSpSpSEESITR (β-C-(16–40))	41.8	167.4	
t4.12	3132.1	3132.1	KNTMEHVpSpSpSEESIIpSQETYKQEK (α-S2-(16–39))	–	13.2	
t4.13	3477.4	3477.4	RELEELNVPGEIVEpSLpSpSpSEESITRINK (β-C-(16–43))	–	17.6	

pS refers to a phosphorylated serine unit, *en dash* no peptide was identified

[a] Searched from http://tw.expasy.org

[b] Sequences are cited from [6]

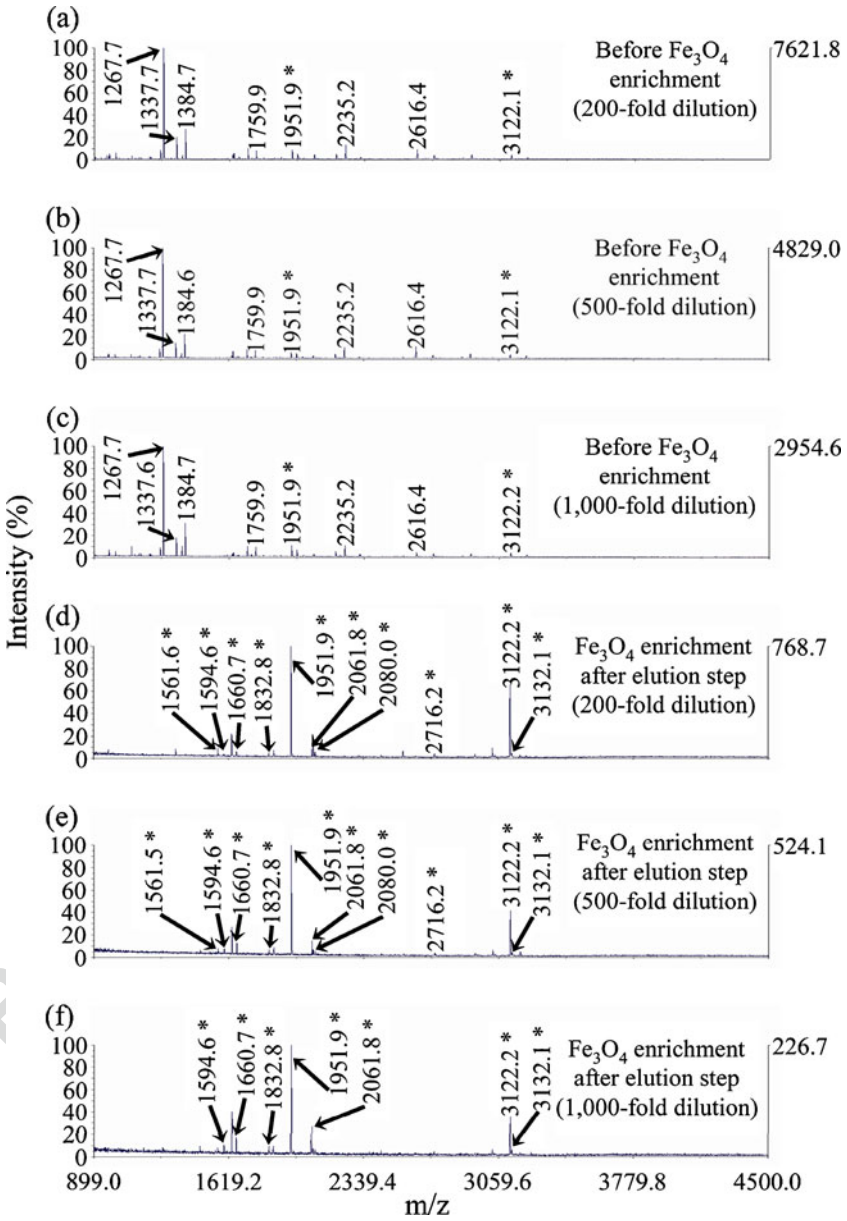

Fig. 6 MALDI mass spectra of tryptic digest of nonfat milk without (**a**, **b**, **c**) or with (**d**, **e**, **f**) enrichment step using magnetic iron (II, III) oxide nanoparticles. **a**, **d** 200-fold dilution, **b**, **e** 500-fold dilution, **c**, **f** 1,000-fold dilution. The matrix solution contained 30 mg/ml DHB in 50% ACN/water with 1% phosphoric acid. Phosphopeptide ions are marked with an *asterisk*

fold and 500-fold dilutions, ten phosphopeptide peaks (marked with asterisks) were detected (Fig. 6d, e). The phosphopeptides peaks at *m/z* 1594.6, 1660.7, 1832.8, 1951.9, 2080.0, 2716.2, and 3132.1 were produced from α-casein, while the peaks at *m/z* 2061.8, 3122.2, and 1561.5 (doubly charged ion) were generated from β-casein (Table 4). Even for the 1,000-fold dilution sample, seven phosphopeptide peaks (marked with asterisks) were observed in the MS spectrum (Fig. 6f). These phosphorylated peptide candidates were further confirmed by MALDI-TOF/TOF MS analysis (Electronic Supplementary Material Figs. S2–S11). From these results, the magnetic iron (II, III) oxide nanoparticles combined with the carbon tape for MALDI-MS analysis is a highly selective and rapid technique for phosphopeptides in complex samples.

Conclusions

In the current study, we demonstrated that the time for protein preparation and digestion could be reduced to less than 10 min and that use of carbon tape as a sample platform results in better digestion efficiency than conventional digestion protocol. The higher digestion efficiency of on-tape-based protein preparation and digestion procedures under microwave heating is attributable to several features of the reaction condition. Our protocol requires only a small volume (1 μL) of reaction solvent, which increases the frequency of enzyme-to-protein contact, thereby resulting in more efficient digestion of sample than conventional in-solution digestion methods. Furthermore, this approach can be directly used to selectively concentrate phosphopeptides

from an enzymatic peptide mixture of proteins by using magnetic iron (II, III) oxide nanoparticles as concentrating probes. In only 15 min, the combination of one-pot on-tape-based protein preparation and digestion under microwave heating with the on-tape-based enrichment method not only dramatically reduced the time required for phosphopeptide analysis but also allowed for the simultaneous identification of phosphoproteins. The advantages include ease of use, high digestion efficiency, high specificity, and short analysis time for the protein identification and enrichment of phosphopeptides from a protein mixture. The results suggest that this approach is useful for protein identification and phosphopeptide characterization.

Acknowledgment The study was funded by a grant from the National Research Council of the Republic of China.

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