Fast and Efficient Proteolysis by Microwave-Assisted Protein Digestion Using Trypsin-Immobilized Magnetic Silica Microspheres

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Fast and Efficient Proteolysis by Microwave-Assisted Protein Digestion Using Trypsin-Immobilized Magnetic Silica Microspheres

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A fast and efficient proteolysis approach of microwave-assisted protein digestion was developed by using trypsin-immobilized magnetic silica (MS) microspheres. In the work, immobilization of the enzyme onto MS microspheres was very simple and only through a one-step reaction with 3-glycidoxypropyltrimethoxysilane (GLYMO) which provides the epoxy group as a reactive spacer. Considering that the magnetic particles are excellent microwave absorbers, we developed a novel microwave-assisted digestion method based on the easily prepared trypsin-immobilized MS microspheres. This novel digestion method combined the advantages of immobilized trypsin and the rapid-fashion of microwave-assisted digestion, which resulted in high digestion efficiency. BSA and myoglobin were used as model proteins to optimize the conditions of this method. Peptide fragments produced in 15 s could be confidently identified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Equivalent or better digestion efficiency was observed comparing to current in-solution digestion. Besides, because of the unique magnetic responsivity, the immobilized trypsin can be isolated easily with the help of an external magnet and thus used repeatedly. High activity was obtained even after seven runs of the trypsin-immobilized MS microspheres. To further verify its efficiency in proteome analysis, one reversed-phase liquid chromatography (RPLC) fraction of rat liver extract was applied. After 15 s incubation, 16 totally unique peptides corresponding to two proteins were identified. Finally, the rat liver sample was used to evaluate its worth for the application. With analysis by liquid chromatography–electrospray-tandem mass spectrometry (LC–ESI-MS/MS), comparable digestion efficiency was observed with typical in-solution digestion but the incubation time was largely shortened. This new microwave-assisted digestion method will hasten the application of the proteome technique to biomedical and clinical research.

Proteomic analysis of complex mixtures aims to obtain a global perspective of changes in protein expression in a rapid fashion. The analysis of protein mixtures is usually performed by either a bottom-up or top-down process. Top-down approaches, which isolate intact proteins from complex mixtures before identification by mass spectrometry, present great advantages over purely bottom-up approaches.[1–6] Much full-scale information of proteins, such as molecular weight, p*I*, and hydrophobicity were revealed in the isolation process. As the commercial maturity of multidimensional separation technology increased as well as advances in mass spectrometric ionization methods, the top-down approach is becoming more and more convenient in large-scale proteomic analysis.

Although protein separation and identification can be highly automated and rapid, the current conventional in-solution or in-gel protein digestion in contrast is considerably slower (from several hours to overnight) and limits large scale proteome analysis. Besides, chronic autodigestion of the enzyme and sample loss in this process also prevent further positive identification of proteins by the mass spectrum.[7] Sometimes additives such as surfactants, organic solvents, and urea are used to facilitate more complete digestion,[8,9] which will contaminate the digests and result in extra purification procedures. Thus, development of a fast and highly efficient proteolysis method which can meet these challenges is in great demand.

Microwave irradiation was recognized in the mid-1980s to be an efficient heating source for chemical reactions, where reactions that require several hours under conventional conditions can often be completed in a few minutes with very high yields and reaction selectivity.[10–12] In recent years, several groups have applied the

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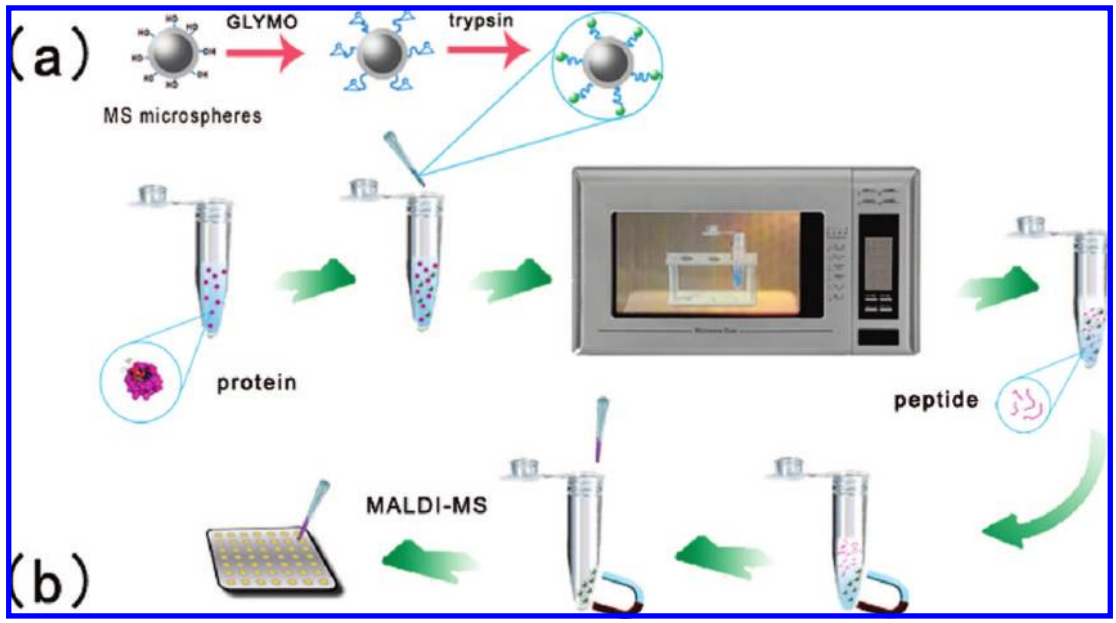

Figure 1. (a) Illustration of the synthesis of trypsin-immobilized MS microspheres. (b) Schematic diagram of microwave-assisted protein digestion by using MS microspheres.

microwave technique to protein digestion and shortened the digestion process to several minutes. Pramanik et al.[13] applied microwave technology to digest known proteins in-solution or in-gel with trypsin in 10 min, including a protein that was tightly folded and extremely resistant to denaturation (bovine ubiquitin). Juan et al.[14] also used microwave technology to digest several known proteins in-gel with trypsin in 5 min. Lin et al.[15] demonstrated that several organic solvents could assist the denaturation of proteins in the microwave-assisted digestion process. Recently, Sun et al.[16] successfully applied the microwave technology for protein mixtures by free enzyme both in-solution and in-gel. Equivalent digestion efficiency was obtained in 6 min compared with the standard overnight in-solution digestion method. There are also some reports on microwave-assisted protein acid hydrolysis.[17–19] Microwave-assisted digestion is so encouraging that continuous improvements are made. Magnetite beads have the excellent absorbers of microwave radiation. Chen et al.[20] found that microwave-assisted digestion of simple proteins such as cytochrome *c* was achieved in 30 s, in the presence of magnetite beads as the microwave absorber.

All the work mentioned above in the microwave-assisted digestion field mainly used free enzyme. Compared with free enzyme, the immobilized enzyme is more resistant to the unfolding of its native structure that may be caused by heat and pH changes.[21] Besides, reduced autolysis products allow for an effective protease concentration many times higher than in solution. A variety of methods have been reported for trypsin immobilization on various supports, such as polymer particles,[22] glass,[23] membrane,[24,25] gel beads,[26] sol−gel supports,[27,28] porous silicon matrix,[29–31] porous monolithic materials,[32–34] and magnetic materials.[35] During these developments, magnetic microspheres draw great attention due to their ease of manipulation and recovery. More particularly, they are the best microwave irradiation absorbers.[20] It is reasonably supposed that the efficiency of microwave-assisted protein digestion could be further improved if trypsin-immobilized magnetic particles were applied instead of free enzyme.

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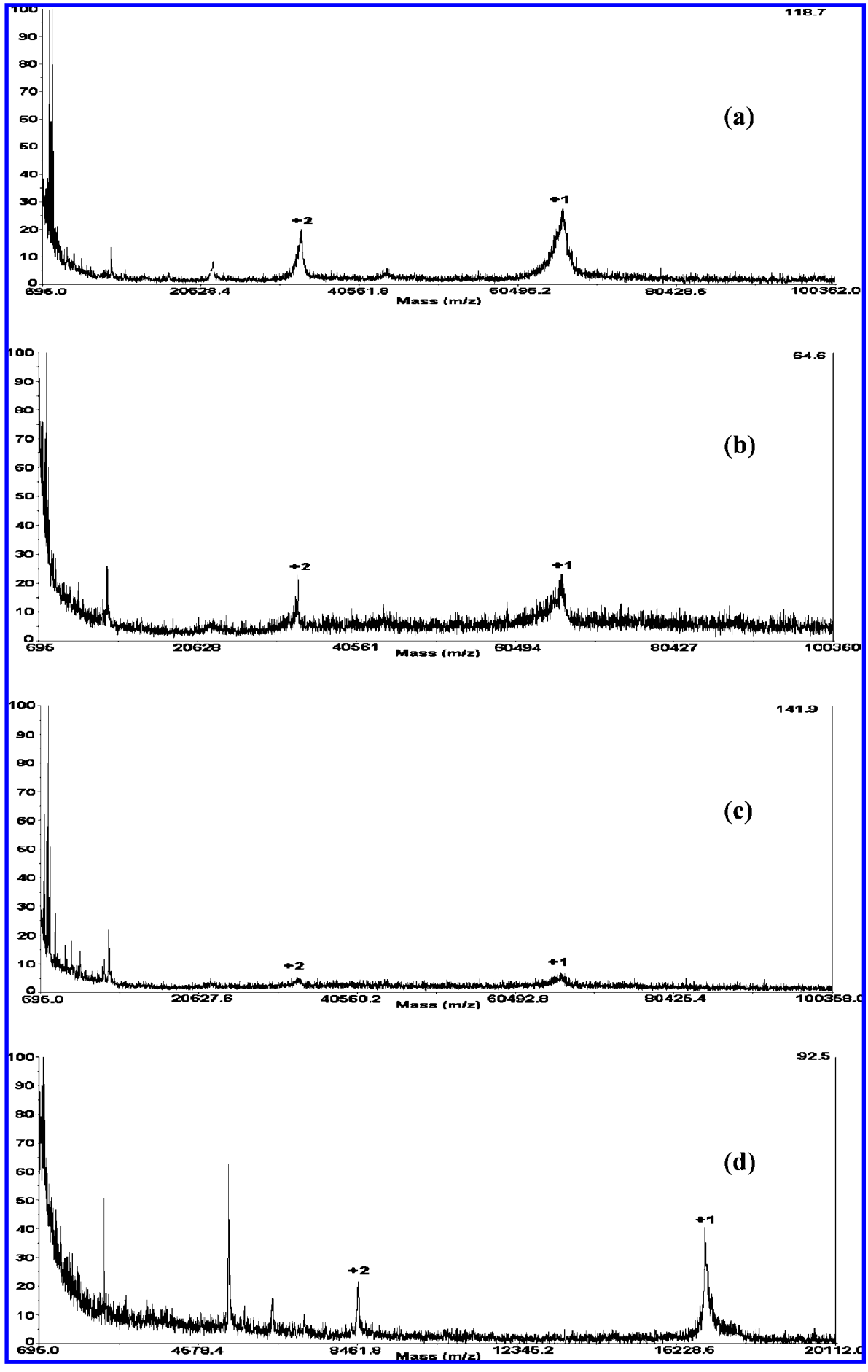
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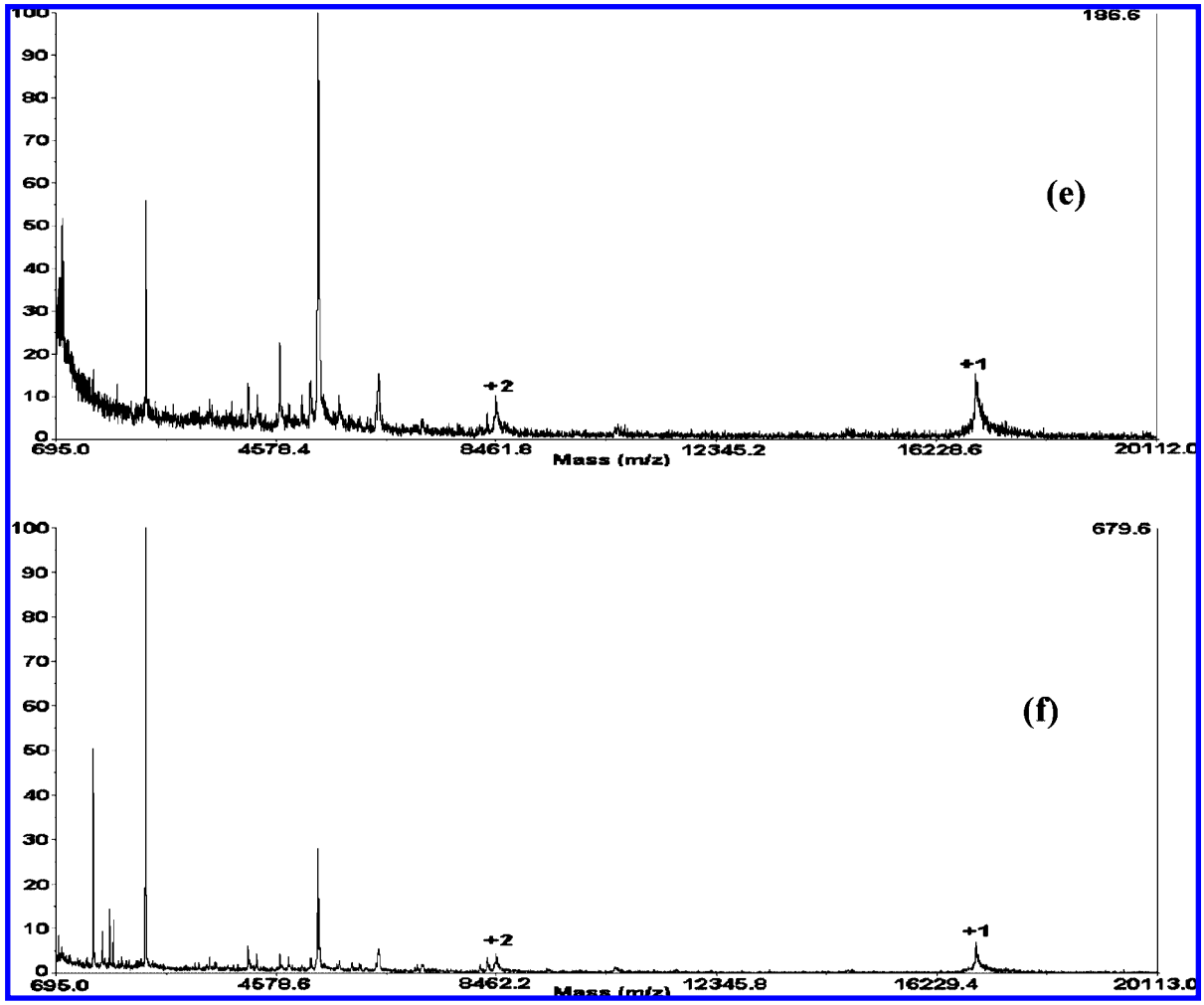

Figure 2. Influence of the amount of trypsin-immobilized MS microspheres on the microwave-assisted digestion efficiency: (a−c) digests of BSA, 0.5 μg/μL, 50 μL; (d−f) digests of myoglobin, 0.5 μg/μL, 50 μL; (a,d) 50 μg of trypsin-immobilized MS microspheres was added; (b,e) 300 μg of trypsin-immobilized MS microspheres was added; (c,f) 500 μg of trypsin-immobilized MS microspheres was added. All the incubation times were 15 s, and the power of the microwave irradiation was 700 W. The linear MALDI-TOF MS mode was applied on the ABI 4700 mass spectrometer.

In the present work, we developed a very simple and facile method for immobilization of enzyme onto MS microspheres directly. For the first time, we applied these easily prepared trypsin-immobilized MS microspheres in microwave-assisted digestion. High digestion efficiency was observed in 15 s both for standard proteins and the reversed-phase liquid chromatography (RPLC) fraction of rat liver extract. The success of the RPLC fraction of rat liver extract demonstrates that this novel digestion method speeds up the top-down proteomic technique for batch analysis of biological and clinical samples.

EXPERIMENTAL SECTION

Materials and Chemicals. 3-Glycidoxypropyltrimethoxysilane (GLYMO), TPCK-treated trypsin, BSA, and myoglobin were purchased from Sigma Chemical (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Merck (Darmstadt, Germany). Acetonitrile was HPLC grade from Fisher Scientific (Fairlawn, NJ). Water was purified using a Milli Q system (Millipore, Molsheim, France). All of other chemicals were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China).

Preparation and Characterization of GLYMO-Linked MS Microspheres. MS microspheres with core−shell structure were first synthesized as previously described.[35] In the next step, 0.05 g of MS microspheres were dispensed in toluene with 1.5 g of 3-glycidoxypropyltrimethoxysilane added. The mixture was stirred at room temperature for 1 h. Finally, the mixture was heated to 65 °C and kept at this temperature for 6 h.

Immobilization of Enzyme onto MS Microspheres. An amount of 5 mg of prepared GLYMO-linked MS microspheres were transferred to a 1.5 mL Eppendorf tube and dispersed in 500 μL of 2μg/μL TPCK-treated trypsin solution (25 mM NH_4HCO_3 as the buffer, pH ~ 8). The mixture was ultrasonicated for 1 min to form a unique suspension. Then the suspension was agitated at 37 °C for 3 h. After reaction, the supernatant solution was removed with the help of an external magnet. Retained magnetic microspheres were washed with deionized water three times and redispersed in 500 μL of 25 mM NH_4HCO_3 (pH ~ 8).

The UV absorption value of the supernatant solution was measured at $\lambda = 280$ nm and compared to the UV absorption value of the trypsin solution before immobilization to calculate the amount of trypsin immobilized on the MS microspheres.

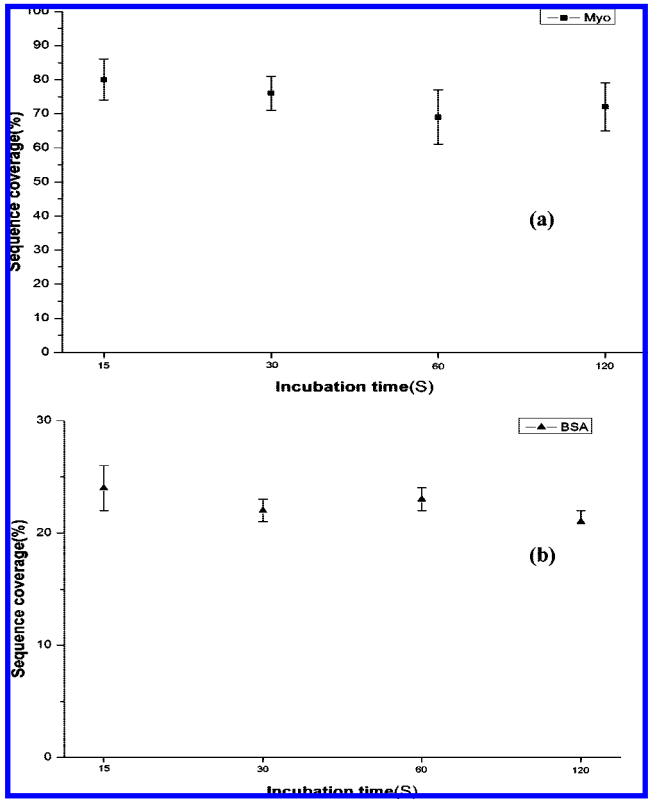

Figure 3. Sequence coverage of (a) myoglobin (0.5 μg/μL, 50 μL), (b) BSA (0.5 μg/μL, 50 μL) under different incubation time of microwave-assisted protein digestion. For each digestion, 500 μg of trypsin-immobilized MS microspheres were introduced and five replicate spots were analyzed by MALDI-TOF MS.

Microwave-Assisted Protein Digestion by Trypsin-Immobilized MS Microspheres. Proteins were dissolved in 25 mM NH_4HCO_3 (pH $\sim$ 8) to a concentration of 500 ng/μL. Low-concentration (50 ng/μL) protein solutions were prepared by dilution of the 500 ng/mL solutions with the same buffer. The suspension of trypsin-immobilized MS microspheres (10 mg/mL, 25 mM NH_4HCO_3 as buffer) was transferred to 50 μL of protein solution in a 0.6 mL Eppendorf tube. A domestic microwave oven (output power 700 W) was used to conduct the microwave-assisted protein digestion process. For each microwave operation, the Eppendorf tube was placed on a plastic rack, taking care to keep the cap open during heating. After microwave irradiation, the rack was taken out of the oven. With the use of an external magnet to retain the magnetic microspheres, the supernatant was deposited on the matrix-assisted laser desorption ionization (MALDI) plate directly. Five replicate spots were taken for every process.

Extract Protein from Rat Liver Tissue. Healthy rat liver was obtained from Liver Cancer Institute in Zhongshan Hospital, Fudan University. The liver tissue was cut into small pieces and cleaned with cold physiological saline solution (0.9% NaCl) to remove blood and some possible contaminants. Then, 0.18 g of tissue debris was rapidly mixed with lysis buffer, containing 1 mM PMSF, 0.2 mM Na_3VO_4, 1 mM NaF, complete protease inhibitor cocktail (Roche, Basel, Switzerland), and 0.1% TFA. The tissue sample was homogenized in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 15 min at 18 000*g*. The supernatant was collected. The protein concentration of the sample was 5.54 mg/mL according to the modified Bradford method described by Qu et al.[36]

RPLC Separation of Rat Liver Extract. The separation of the rat liver extract and the fraction collection were conducted on a completely automated Shimadzu LC-2010A system combined with FRC-10A fraction collector (Shimadzu Corp., Japan). Integrated LC system consisted of a quaternary low-pressure gradient pump, autosampler, online degasser, block heating-type column oven, and UV−vis detector with a D2 lamp. A Shimadzu Class-VP station was used to acquire and process data. The separation procedure was performed at 25 °C.

Table 1. Detailed Information of Peptide Fragment Identified by Microwave-Assisted Protein Digestion in 15 s[a]

myoglobin			BSA		
$[M + H]^+$ (observed)	peptide matched	MC	$[M + H]^+$ (observed)	peptide matched	MC
1606.91	K.VEADIAGHGQEVLIR.L	0	1193.68	R.DTHKSEIAHR.F	1
1271.72	R.LFTGHPETLEK.F	0	712.42	K.SEIAHR.F	0
1661.9	R.LFTGHPETLEKFDK.F	1	1249.69	R.FKDLGEEHFK.G	1
1086.61	K.HLKTEAEMK.A	1	1163.71	K.LVNELTEFAK.T	0
1102.61	K.HLKTEAEMK.A Oxidation (M)	1	927.55	K.YLYEIAR.R	0
1378.89	K.HGTVVLTALGGILK.K	0	1083.65	K.YLYEIARR.H	1
1506.99	K.HGTVVLTALGGILKK.K	1	1347.6	K.GACLLPKIETMR.E Oxidation (M)	1
1982.11	K.KGHHEAELKPLAQSHATK.H	1	906.54	K.IETMREK.V	1
1854.02	K.GHHEAELKPLAQSHATK.H	0	990.61	R.EKVLTSSAR.Q	1
735.53	K.HKIPIK.Y	1	733.47	K.VLTSSAR.Q	0
1885.1	K.YLEFISDAIIHVLHSK.H	0	1017.64	K.VLTSSARQR.L	1
1502.73	K.HPGDFGADAQGAMTK.A	0	1001.66	R.ALKAWSVAR.L	1
748.48	K.ALELFR.N	0	847.56	R.LSQKFPK.A	1
1360.81	K.ALELFRNDIAAK.Y	1	922.54	K.AEFVEVTK.L	0
941.52	K.YKELGFQG.-	1	1692.99	K.AEFVEVTKLVTDLTK.V	1
			1177.45	K.ECCDKPLLEK.S	0
			1567.82	K.DAFLGSFLYEYSR.R	0
			1439.89	R.RHPEYAVSVLLR.L	1
			1283.8	R.HPEYAVSVLLR.L	0
			1479.86	K.LGEYGFQNALIVR.Y	0
			1640.03	R.KVPQVSTPTLVEVSR.S	1
			1511.9	K.VPQVSTPTLVEVSR.S	0

[a] MC, number of missed cleavages.

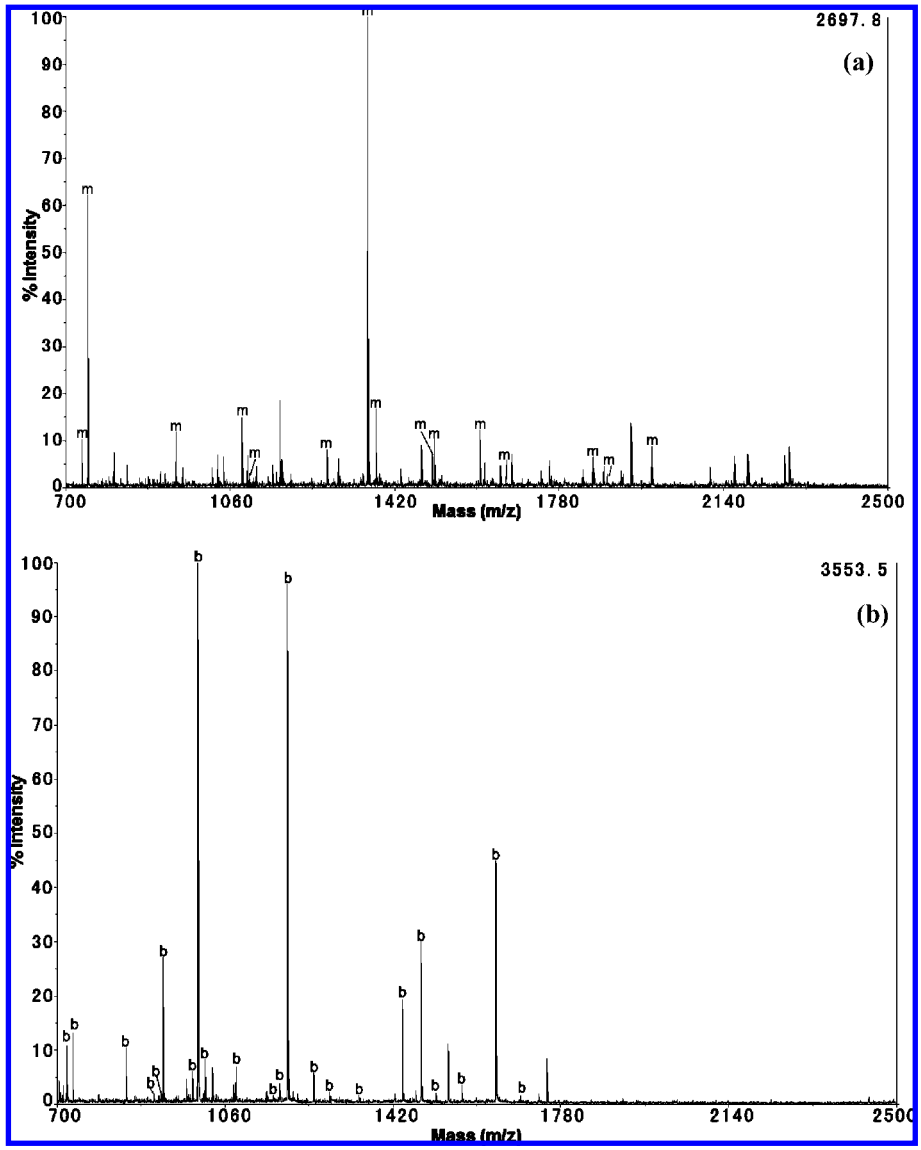

Figure 4. MALDI-TOF mass spectra obtained from (a) myoglobin (0.5 μg/μL, 50 μL), (b) BSA (0.5 μg/μL, 50 μL) digested with trypsin-immobilized MS microspheres under microwave irradiation (output power, 700 W) for 15 s. An amount of 50 μL (10 mg/mL) of suspension of MS microspheres were introduced. m, peptide from myoglobin; b, peptide from BSA. The reflector mode was used to detect low-mass peptides.

An amount of 500 μg of protein was loaded onto a reversed phase column (250 mm × 4.6 mm, 5 μm, 300 Å, C18, Hypersil, Elite HPLC, China) preceded by a 20 mm × 4.6 mm guard column. Mobile phase A consisted of 0.1% TFA in water, and mobile phase B consisted of 0.1% TFA in acetonitrile (all v/v). The flow rate was 0.7 mL/min. After a 5 min elution with 0% B, the separation was performed using gradient conditions as following: 0% B increased up to 15% B in 20 min, from 15% to 30% in 15 min, from 30% to 50% in 40 min, from 50% to 70% in 20 min, finally from 70% to 80% in 20 min, hold at 80% for 10 min and then ramped down to 0% B for equilibrium. Fifty RPLC fractions were collected every 2 min (1.4 mL) (from 5 to 15 min and from 19.5 min to 109.5 min). On column UV detection for RPLC was set at 215 nm. All the collected fractions were lyophilized and redispersed in 50 μL of 25 mM NH_4HCO_3 (pH = 8.3) for the next microwave-assisted protein digestion. The resulting peptide fragments were then sent for MALDI-time-of-flight tandem mass spectrometry (TOF MS/MS) identification.

MALDI-TOF MS Analysis and Database Searching. Sample solutions were deposited on the MALDI target using the dried droplet method. An amount of 1 μL of sample solution was spotted onto the MALDI plate and then another 0.5 μL of CHCA matrix solution (5 mg/mL, 0.1% TFA in 50% ACN/H_2O solution) was introduced. Positive ion MALDI-TOF MS spectra were acquired on a 4700 Proteomics Analyzer (Applied Biosystems). The sample was excited using an Nd:YAG laser (355 nm) operated at a repetition rate of 200 Hz and acceleration voltage of 20 kV. Before identifying the samples, the MS instrument was calibrated by an internal calibration mode with tryptic peptides of myoglobin.

All spectra were taken from signal-averaging of 800 laser shots with the laser intensity kept at a proper constant. GPS Explorer software (Applied Biosystems) with Mascot (Matrix Sciences, London, UK) as a search engine and NCBI (version of 070316)

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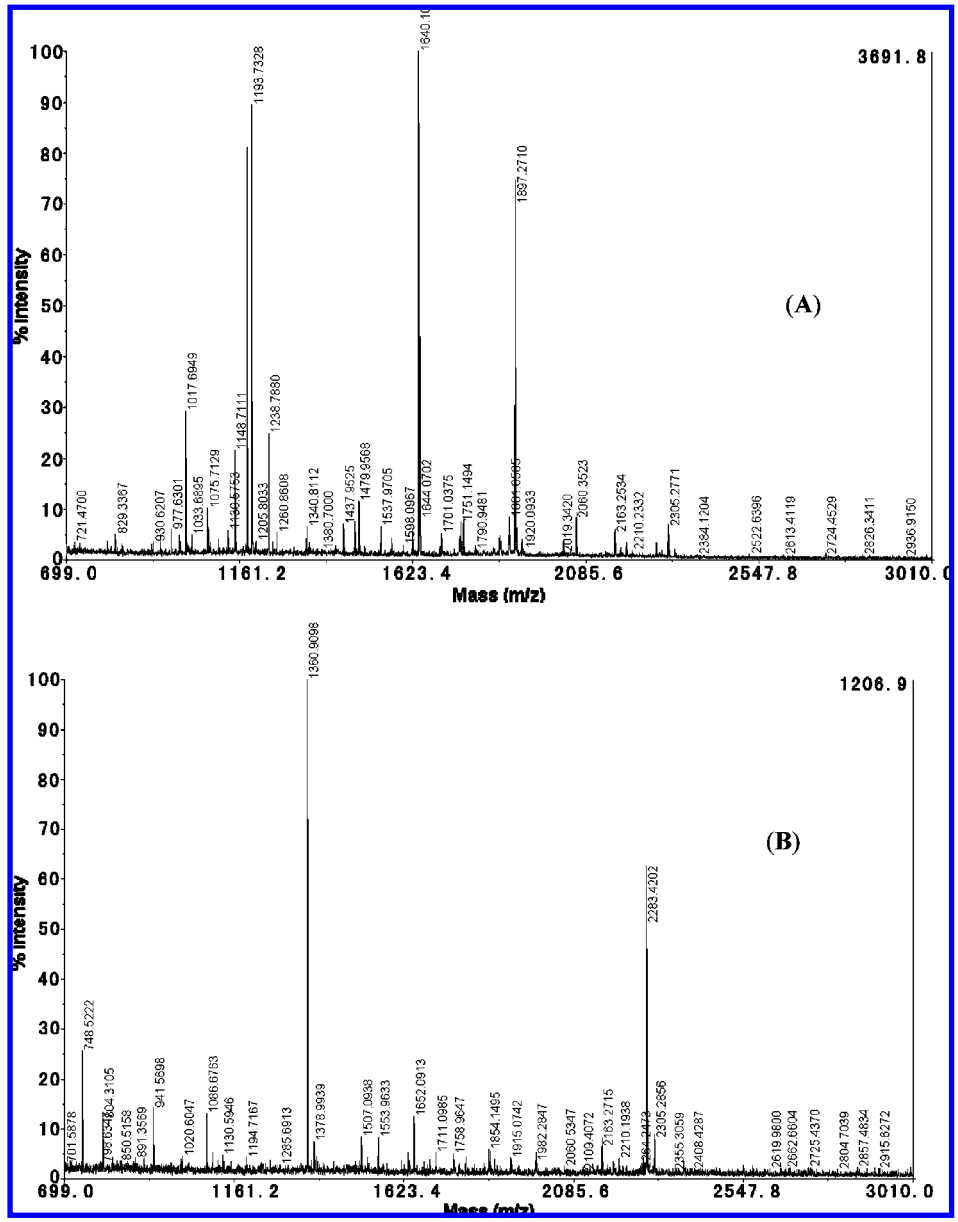

Figure 5. The MALDI mass spectra of tryptic peptides originating from 2.5 μg of proteins of (A) BSA, (B) myoglobin by trypsin-immobilized MS microspheres in microwave-assisted digestion. Conditions: 500 μg of TIMNs to 50 μL of proteins (50 ng/μL); incubation time, 15 s; incubation power, 700 W.

as a database were used to identify proteins. Standard proteins were identified with peptide fingerprint mass spectra. As for the rat liver extract, proteins were identified using the peptide fingerprint mass spectra combined with tandem mass spectra. The peptide mass tolerance was set to 100 ppm, and the tandem mass tolerance was set to 0.8 Da.

Microwave-Assisted Digestion of Real Proteome by Trypsin-Immobilized MS Microspheres. *(1) Digestion of the Rat Liver Extract.* An amount of 100 μL of the sample solution (5.54 mg/mL) was diluted to 400 μL by ammonium bicarbonate buffer (25 mM, pH $\sim$ 8). The sample was reduced by 10 mM DTT at 57 °C for 1 h and alkylated by 50 mM iodoacetamide at room temperature in the dark for 0.5 h. And then, 200 μL of the sample was digested under microwave irradiation for 15 s with 1 mg of trypsin-immobilized MS microspheres added. The other 200 μL sample was digested by trypsin (1:50) at 37 °C for 16 h.

(2) LC–ESI-MS/MS Process. The elution gradient for the RPLC column was from 5 to 100% buffer B (0.1% formic acid, 95% ACN). Eluted peptides were detected in a survey scan from 300 to 1800 amu (1 microscan) followed by eight data-dependent MS/MS scans (isolation width, 2 amu; 35% collision energy; dynamic exclusion for 1 min) in a completely automated fashion on an LTQ-Orbitrap ESI mass spectrometer. All the MS/MS database search were performed using the SEQUEST algorithm-based Bioworks 3.3 EF2 (Thermo Finnigan) from a rat database of International Protein Index (version 3.27). According to ref 46, the filtering criteria was calculated through a reverse database searching (Figure S3 in the Supporting Information) and the X_{corr} value vs charges was obtained as follows: $p < 0.01$, $> 3.56(+3)$, $> 2.52(+2)$, $> 2.0(+1)$; $\Delta Cn > 0.1$; and peptide length > 7 were also applied.

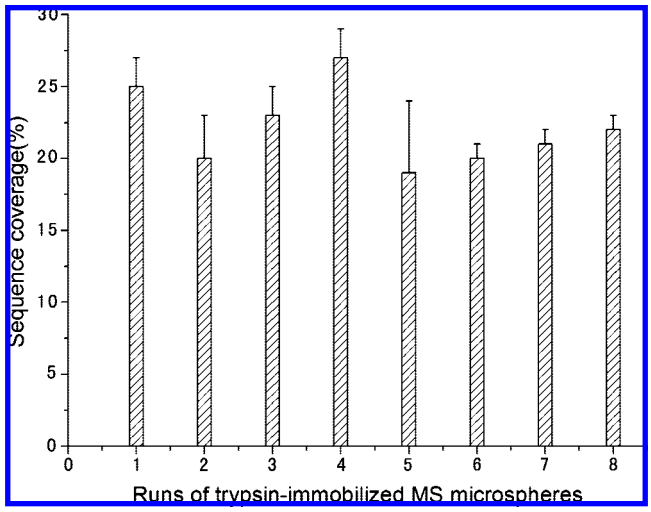

Figure 6. Reusability test of novel synthesized trypsin-immobilized MS microspheres in microwave-assisted protein digestion. With BSA as the model protein (0.5 μg/μL, 50 μL), eight consecutive runs of MS microspheres (10 μg/mL, 50 μL) were conducted. Between each operation, the MS microspheres were washed with 25 mM NH_4HCO_3 buffer solution (pH $\sim$ 8) three times.

RESULTS AND DISCUSSION

Immobilization of Enzyme onto GLYMO-Linked MS Microspheres. Magnetic polymer microspheres combine the excellent properties of polymer microspheres and unique magnetic responsivity of magnetic particles, which means that the magnetic polymer microspheres not only offer the advantage of easy handling with the help of an applied magnetic field but also permit diverse modification on their surfaces. Compared to magnetic microspheres encapsulated by organic polymers such as polystyrene, magnetic microspheres encapsulated by inorganic silica display better responsivity and more flexibility for further modification through silicon alkyl. Figure S1 displays the transmission electron microscope (TEM) images of magnetic microspheres before and after encapsulation by silicon polymer. The TEM image in Figure S1,a shows uniform and small sizes of the magnetic microspheres (about 200 nm) synthesized by the solvothermal reaction. Figure S1,b reveals that MS microspheres with well-defined core−shell structures were successfully synthesized. From the comparison of the two pictures, we can see that the thick silica shell (covering the black core in Figure S1,b, about 70 nm) was formed uniformly which provides magnetic microspheres with a silicalike surface for further modification.

GLYMO was chosen as the derivative reagent for MS microspheres. Compared to other reactive "spacer" groups such as diol, aldehyde, or tresyl groups, the epoxy group shows a better immobilization ratio and ability of the enzyme for structural recognition.[37,38] The epoxy group of GLYMO can easily attach the enzyme via functionalized groups belonging to amino acid residues. Figure 1a displays the synthesis process of GLYMO-linked MS microspheres and trypsin immobilization.

The immobilization ability of GLYMO-linked MS microspheres for trypsin immobilization was studied by measuring the UV absorption value of the supernatant trypsin after the immobilization procedure, and the amount of trypsin immobilized on the magnetic nanoparticles turned out to be about 32 μg/mg, which is higher than the commercial paramagnetic porous glass beads used in Krogh's work (18.1 μg/mg).[39]

Microwave-Assisted Protein Digestion by Trypsin-Immobilized MS Microspheres for MALDI MS Mapping. Many reactions, such as enzyme digestion, can be accelerated or made more selective when microwave, rather than heat, is applied. Figure 1b displays the flowchart of microwave-assisted protein digestion by trypsin-immobilized MS microspheres. A fixed plastic rack was placed at a set height in order to afford consistent reaction conditions. After digestion, the MS microspheres can be easily isolated from the digestion products by using a magnet. The resulting tryptic digests were then mass-analyzed by MALDI-TOF MS. To verify the efficiency of this novel microwave-assisted digestion protocol, myoglobin and BSA were chosen as model substrates for proteolysis. For

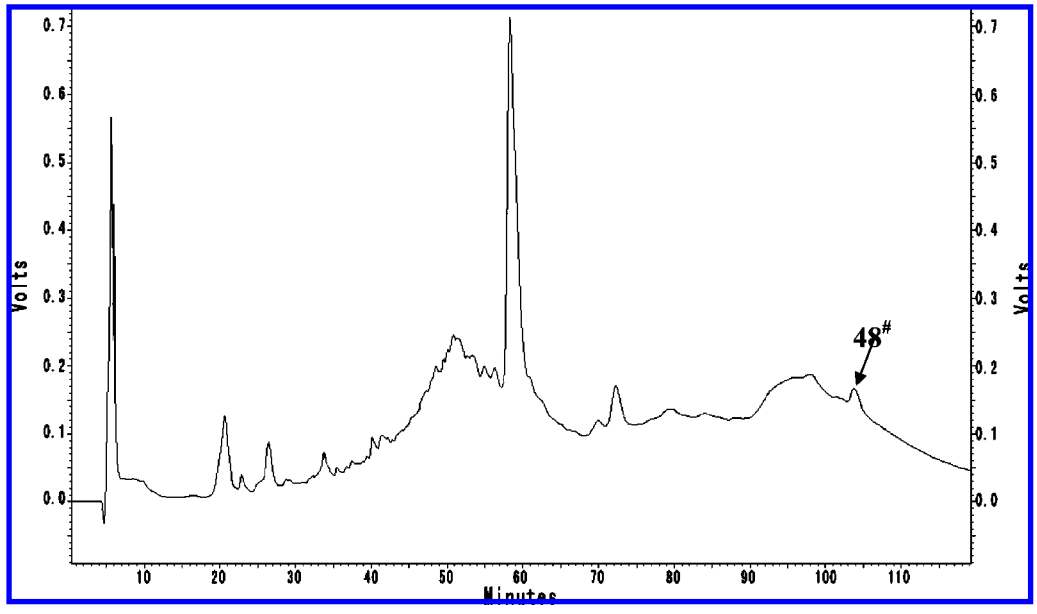

Figure 7. The RPLC chromatogram of rat liver extract. One fraction is indicated with an arrow at 103.5−105.5 min (fraction no. 48). The details of separation conditions are displayed in the Experimental Section.

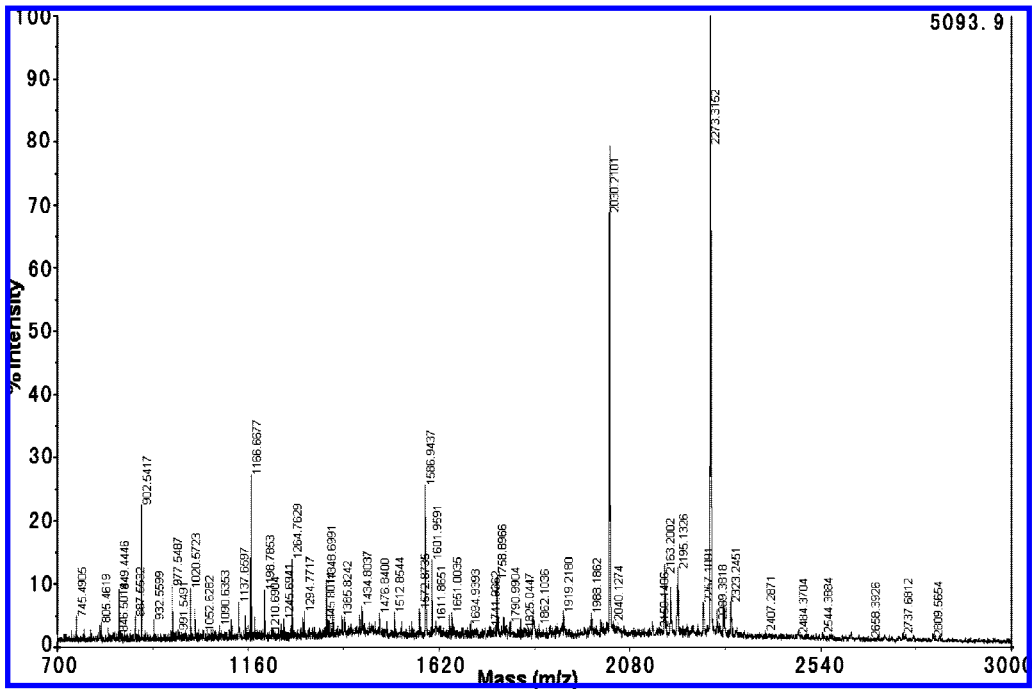

Figure 8. MALDI mass spectrum of microwave-assisted tryptic digests of RPLC fraction no. 48 of the rat liver extract in Figure 6. A ratio of 1/50 of the microwave-assisted tryptic digests were deposited on the MALDI plate using the dried-spot method. The sample was excited using an Nd:YAG laser (355 nm) operated at a repetition rate of 200 Hz and an acceleration voltage of 20 kV. The reflector mode was used to detect the low-mass peptides.

Table 2. Proteins Identified from the RPLC Fraction No. 48 Digested by Trypsin-Immobilized MS Microspheres under Microwave Irradiation for 15 s Followed by MALDI-TOF MS/MS Analysis

protein name	accession number	protein MW	protein p*I*	peptide count	protein score
ribonuclease UK114 (14.5 kDa translational inhibitor protein) (perchloric acid soluble protein)	gi 1709863	14 294.559 57	7.79	4	173
TRAF3-interacting JNK-activating modulator [*Rattus norvegicus*]	gi 62078999	59 070.359 38	8.93	12	62

comparison, the digestion of those two standard proteins was also performed by free trypsin in solution.

Generally, longer incubation time results in better digestion efficiency, but a reduction in the digestion time is an important intent in proteomic applications. Figure displays the digestion efficiency of the microwave-assisted method under different amounts of trypsin-immobilized MS microspheres. From the figure we can see that with the amount of trypsin-immobilized MS microspheres increased from 50 to 500 μg, the intact protein peaks disappeared, and more peptide peaks under or around 2000 m/z were observed. Thus, 500 μg of the trypsin-immobilized MS microspheres were added in the following experiments. Figure 3 displays the sequence coverage of BSA and myoglobin by microwave-assisted digestion under different incubation times. With trypsin-immobilized MS microspheres providing a much higher concentration of protease as well as microwave absorbance, high digestion efficiency was observed in 15 s. From 15 to 90 s, no significant increase on sequence coverage was obtained. The average sequence coverage of 80% for myoglobin and 24% for BSA was obtained, respectively. As the incubation time increased to 120 s, no significant difference was obtained. Thus, the following experiments were carried out in 15 s. Figure 4 displays the mass spectra of tryptic fragments generated from 15 s microwave-assisted digestion using trypsin-immobilized MS microspheres. The peaks marked with "m" and "a" correspond to peptides derived from myoglobin and BSA, respectively. Detailed identification results were listed in Table 1. Five duplicate experiments were carried out, resulting in a sequence coverage range of 73−86% for myoglobin and 21−26% for BSA, which indicated higher or equivalent digestion efficiency than those of current in-solution digestion procedures (Table S1).

Because of the autodigestion of free enzyme and thus the interference in MS identification, the ratio of enzyme/protein (w/w) in conventional in-solution digestion is always kept very low (1:50 to 1:30). In order to get better digestion efficiency for small quantity protein samples, a concentrating procedure is inevitable. Because the enzyme-immobilized MS microspheres can be easily removed with a magnet after digestion, it was feasible to increase the proportion of enzyme with no need to worry about the interference from trypsin autodigestion. Here we studied the digestion efficiency of 2.5 μg of protein sample with the TIMNs protocol. Figure 5 displays the MALDI mass spectra of proteins (50 ng/μL, 50 μL) digested by TIMNs with microwave irradiation in 15 s. Compared to the results of high-concentration proteins

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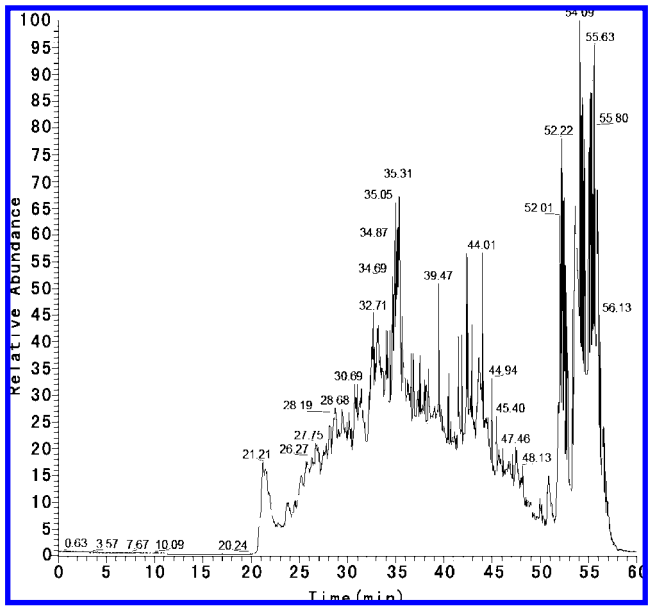

Figure 9. The total ion chromatogram for the separation of rat liver extract digests by microwave-assisted digestion using trypsin-immobilized MS microspheres. About 40 μg of protein was analyzed by RPLC−ESI-MS/MS. Digestion conditions: 100 μL of rat liver extract (5.54 μg/μL) was diluted to 400 μL by 25 mM NH_4HCO_3 buffer solution (pH ~ 8) and then digested with 700 W of microwave irradiation for 15 s with the addition of 500 μg of trypsin-immobilized MS microspheres.

(1 mg/mL, Figure 3), less peptides of BSA and myoglobin were observed but still the proteins were identified successfully. In comparison, 1 mL of the same protein samples (50 ng/μL) went through conventional in-solution digestion (12 h, trypsin/enzyme 1:30) but with no proteins identified. (Data not shown).

Because after digestion the immobilized trypsin can be isolated easily from the sample solution by an external magnet, they can be used repeatedly. Consecutive operations for BSA were conducted and the resulting products were thereafter analyzed by MALDI-TOF MS (Figure 6). Between each operation, the MS microspheres were washed with 25 mM NH_4HCO_3 buffer solution (pH 8) three times. As demonstrated in Figure 6, the immobilized enzyme still kept high activity even after seven runs of the microwave-assisted protein digestion. However, the digestion efficiency fell in the following run. That may be a result of the long time exposure of the enzyme to microwave irradiation, which might change the molecular structure of some enzymes and thus decrease their activity.

To primarily verify the digestion efficiency of this protocol for protein mixtures, two protein solutions (BSA and cytochrome *c*) were mixed in the mass ratio of 1:1. An amount of 500 μg of trypsin-immobilized MS microspheres were introduced. As indicated in Figure S2, 20 peptides totally corresponding to BSA and 9 peptides corresponding to cytochrome *c* were identified after 15 s of incubation. This indicated that this novel microwave-assisted digestion method has great potential for digest protein mixtures in a very short time.

Performance of Microwave-Assisted Protein Digestion by Trypsin-Immobilized Magnetic Microspheres in Top-Down Proteome Analysis. The top-down proteomic approach provides purified protein for subsequent identification steps either by 2D-PAGE or 2D-LC.[40] 2D-LC offers an advantage over 2D-PAGE in terms of ease of automation and protein recovery; thus, it was considered a potential way for fast and large scale characterization of proteins. However, the long digestion process seems to hamper its further development. Therefore, with our established protocol of rapid and efficient microwave-assisted protein digestion using trypsin-immobilized magnetic microspheres, the possibility of a quick top-down approach for large-scale protein analysis will be greatly enhanced. The performance of this strategy was demonstrated with analysis of RPLC fractions of the rat liver extract.

Figure 7 shows the RPLC separation process of rat liver extract protein. The RPLC fractions were collected in 2 min intervals (from 5 to 15 min and from 19.5 to 109.5 min). Each fraction was lyophilized and redissolved in 50 μL of 25 mM NH_4HCO_3 buffer followed by adding trypsin-immobilized magnetic microspheres. The digestion process was only performed for 15 s, and 1 μL of supernatant was deposited on the MALDI plate directly with the help of an external magnet. The resulting digests were analyzed by MALDI-TOF MS/MS. As a model, MALDI mass spectra of the RPLC fraction collected from 103.5 to 105.5 min (fraction no. 48, displayed with an arrow in Figure 7) was displayed in Figure 8. Numbers of peptide fragments were observed in the spectrum, which indicated that the protein mixture has been successfully digested in 15 s with the microwave-assisted digestion using the trypsin-immobilized magnetic microspheres. The searches were performed against the NCBI database (version of 070306) with the combined PMF and MS/MS method, allowing for one missed cleavage. Peptide mass spectra were collected automatically with an acquisition mass range of 700−3000 m/z. Mass tolerances of ±100 ppm for precursor ion and 0.8 Da for MS/MS were adopted. According to the Mascot search result, protein scores greater than 56 were considered significant ($p < 0.05$).Table 2 lists the proteins identified from the RPLC fraction no. 48. Proteins identified with the same peptide were grouped. After redundancy removal, 16 unique peptides corresponding to two proteins were confidently identified.

Then the real proteome, rat liver extract without any prefractionation procedure, was chosen to further evaluate the digestion efficiency of this method and compare with the solution-phase overnight protocol. The entire proteome was digested in only 15 s and went through 1D LC−ESI-MS/MS directly. Figure 9 was the total ion chromatogram of the novel microwave-assisted digestion protocol. After a database search according to the SEQUEST criteria set above (Experimental Section), 318 proteins were identified with $p < 0.01$ (Table S1). The same LC−MS/MS analysis parameters were applied for typical digests of the same rat liver extract, and 364 proteins were identified (Figure S3). These results clearly show that this novel digestion approach is comparable to conventional in-solution digestion, while the incubation time is greatly shortened to 15 s, the proteolysis efficiency has been significantly improved.

Because rat liver extract is highly complex, one dimension of LC separation was still not sufficient. If more separation techniques were combined, this fast and highly efficient digestion protocol with trypsin-immobilized magnetic microspheres will hasten the application of proteome technology for large-scale clinical analysis.

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CONCLUSIONS

In summary, a fast and efficient proteolysis approach which significantly reduced the digestion time was established by microwave-assisted protein digestion using easily prepared trypsin-immobilized MS microspheres. The method we developed for synthesis of trypsin-immobilized MS microspheres was very simple and was only a one-step reaction. Combined with microwave irradiation, the whole proteolysis can be completed in 15 s and showed higher efficiency than conventional in-solution digestion for a standard protein. The efficiency of this protocol was further demonstrated by digestion of RPLC fractions of a real proteome sample, a rat liver extract. After analysis by MALDI-TOF MS/MS, a number of peaks were observed in the mass spectrum and 16 unique peptides corresponding to two proteins were confidently identified. The real proteome sample was also used to further evaluate its worth for application. With analysis by LC−ESI MS/MS on a LTQ instrument, comparable digestion efficiency was observed with typical in-solution digestion but the incubation time was largely shortened. The methodology will be explored for the possibility of inserting rapid tryptic digestion into chromatography-based protein separation systems for online digestion and is under investigation in our laboratory.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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