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Acceleration of Microwave-Assisted Enzymatic Digestion Reactions by Magnetite Beads

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In this study, we demonstrated that microwave-assisted enzymatic digestion could be greatly accelerated by multifunctional magnetite beads. The acceleration of microwave-assisted enzymatic digestion by the presence of the magnetite beads was attributable to several features of the beads. Their capacity to absorb microwave radiation leads to rapid heating of the beads. Furthermore, their negatively charged functionalities cause adsorption of proteins with opposite charges onto their surfaces by electrostatic interactions, leading to a concentration on the surfaces of the beads of proteins present in trace amounts in the solution. The adsorbed proteins are denatured and hence rendered vulnerable to enzymatic digestion and are digested on the beads. For microwave heating, 30 s was sufficient for carrying out the tryptic digestion of cytochrome *c*, in the presence of magnetite beads, while 1 min was adequate for tryptic digestion of myoglobin. The digestion products were characterized by MALDI-MS. This rapid enzymatic digestion allowed the entire time for identification of proteins to be greatly reduced. Furthermore, specific proteins present in trace quantities were enriched from the sample on the magnetite beads and could be rapidly isolated from the sample by employing an external magnetic field. These multiple roles of magnetite beads, as the absorber for microwave irradiation, the concentrating probe, and the agent for unfolding proteins, contributed to their capability of accelerating microwave-assisted enzymatic digestion. We also demonstrated that trypsin immobilized magnetite beads were suitable for use in microwave-assisted enzymatic digestion.

Mass spectrometry has been widely used to characterize peptides generated from chemical hydrolysis or enzymatic digestion, for protein identification in proteomic analysis. Although mass spectrometry is a rapid analysis method, the enzymatic digestion takes at least several hours in conventional experimental conditions. Therefore, efforts have been made to reduce the time for enzymatic digestion to less than 1 h.[1−3] For example, on-particle enzymatic digestion can reduce the digestion time to 30 min.[1,3] Microwave irradiation gives an acceleration of enzymatic digestion of proteins[4−9] as demonstrated by Bose et al. and Pramanik et al.,[5] Chen et al.,[6] Kroll et al.,[7] Zhong et al.,[8] and Swatkoski et al.[9] In chemical hydrolysis processes, the addition of a large concentration of acid enhances denaturation, and the time required for chemical hydrolysis is generally less than that for enzymatic digestion under microwave heating. Ho and co-workers demonstrated that several organic solvents could assist the denaturation of proteins in microwave-assisted enzymatic digestion reactions.[10] Juan et al. recently employed microwave-assisted in-gel protein enzymatic digestion,[11] thereby reducing the time required from several hours to 10 min.

We supposed that the time required for microwave-assisted enzymatic digestion might be further reduced if an appropriate substance capable of absorbing microwave radiation was added to the digestion solution. Previously, magnetite beads had been reported as excellent absorbers of microwave radiation.[12−14] After examining 150 substances, Walkeiwicz et al.[12] concluded that magnetite beads were the best microwave absorbers. Kirschvink[13] concluded that the presence of a trace amount of biogenic magnetite in animals could result in damage to biological tissues under microwave irradiation. This was because the biogenic magnetite in animal tissues could absorb radiation at frequencies between 0.5 and 10.0 GHz. Holzwarth et al.[15] increased the rate of heating nonpolar solvents by microwave radiation since most nonpolar solvents do not absorb microwave radiation. These

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results imply that magnetite beads might be used as microwave absorbers to accelerate microwave-assisted enzymatic digestion. Magnetic beads have also been widely used as affinity probes[16−27] for targeting specific species because their magnetic properties facilitate isolation of the conjugate bead−target species. Proteins adsorbed on solid substrates generally denature to give unfolded proteins which are much more susceptible to proteolytic digestion.[28] Considering these two unique features, the feasibility of accelerating microwave-assisted enzymatic digestion by adding magnetite beads to the digestion solution was investigated using cytochrome *c* and myoglobin as the examples.

EXPERIMENTAL SECTION

Reagents. Trifluoroacetic acid (TFA) and hydrochloric acid were obtained from Merck (Seelze, Germany). *N*-Hydroxysuccinimide (NHS) was obtained from Fluka (Buchs, Switzerland). Dimethyl formamide (DMF) was purchased from J. T. Baker (Phillipsburg, NJ). Iron(III) chloride hexahydrate, ammonium bicarbonate, and sodium sulfite were obtained from Riedel−de Haën (Seelze, Germany), while sodium phosphate monobasic was purchased from Mallinckrodt (Paris, KY). Ammonium hydroxide and tetraethoxysilane (TEOS) were obtained from Fluka (Steinheim, Germany), and ethanol was obtained from Showa (Tokyo, Japan). Succinic anhydride, *N*-[3-]trimethoxysilyl)propyl]-ethylenediamine (80%) (EDAS), and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich (Milwaukee, WI). Cytochrome *c* (horse), myoglobin, 3-aminopropyl, triethoxysilane (APTES), *N*-(3-dimethyaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), [2-*N*-morpholinoethanesulfonic acid (MES), sodium phosphate dibasic heptahydrate (PBS), and trypsin (from bovine pancreas) were obtained from Sigma (St. Louis, MO).

Preparation of Magnetite Beads. The preparation of magnetite beads has been described elsewhere.[16] Ferric chloride (2 M) dissolved in hydrochloric acid solution (2 M, 12 mL) was diluted to 100 mL with deionized water. Freshly prepared aqueous sodium sulfite (0.08 M, 50 mL) was added slowly to the diluted ferric chloride under nitrogen. A mixture of aqueous ammonia (28%, 8 mL) and water (40 mL) was added slowly to the solution with vigorous stirring under nitrogen, and the mixture was held at 70 °C in a water bath for 15−30 min. After being cooled to below 45 °C, the magnetite nanoparticles were aggregated by placing an external magnet on the edge of the vial, and the solution was removed. The particles were rinsed several times with deionized water and water−ethanol (2:1) mixture and resuspended in ethanol (50 mL). Ethanol (7 mL), water (8 mL), TEOS (2 mL), and ammonia solution (10%, v/v) (2 mL) were added in sequence to the iron oxide particle suspension (40 mg/mL, 25 mL) with stirring at 40 °C, and stirring was continued for 12 h. The particles were rinsed with methanol several times to remove unreacted TEOS followed by resuspension in methanol (40 mL).

Preparation of Negatively Charged Magnetite Beads. APTES (98%, 8 mL) and methanol (15 mL) were added to the silanized magnetite suspension (25 mL, 40 mg/mL) obtained above, with sonication for 10 min, and the mixture was stirred for another 12 h in a water bath at 60 °C. The particles were rinsed with methanol and resuspended in 40 mL of DMF. A solution of succinic anhydride (4 g) dissolved in DMF (10 mL) was added to the suspension and stirred under nitrogen for 8 h. The particles were rinsed with DMF and water and dispersed in water to give a suspension of 30 mg/mL. The particle size of the magnetite beads with negative functionalities was ∼20 nm (see Figure 1 in Supporting Information).

Preparation of Trypsin Immobilized Magnetite Beads. The silanized magnetite beads (25 mL, 40 mg/mL) were rinsed with methanol and resuspended in methanol (40 mL). A solution of EDAS (80%, 8 mL) was added to the suspension under sonication which was continued for 10 min, and the mixture was stirred for 12 h at 60 °C. The particles were rinsed with methanol and redispersed in DMF (50 mL). Succinic anhydride (4 g) was added, and the suspension was stirred under nitrogen for 8 h. The particles were rinsed with DMF and water and dispersed in water (25 mL) to give a suspension of 30 mg/mL. The particles from the 2 mL suspension were isolated and mixed with EDC (200 mg), NHS (200 mg), and MES buffer (0.1 M, 15 mL, pH 6.8) under stirring for 1 h. After the mixture was washed with deionized water, the particles were mixed with trypsin solution (10^{-5} M, 10 mL), prepared in PBS buffer (0.1 M, pH 7.4), with stirring which was continued for 2∼3 h. The particles were rinsed with PBS buffer and resuspended in deionized water (1 mL) before use.

Microwave-Assisted Enzymatic Digestion. The mixture of proteins (10^{-5} M, 50 μL) and trypsin [0.1 mg/mL (4.29×10^{-6} M), 4 μL], a molar ratio of 29:1, was mixed with the negatively charged magnetite beads (30, 60, 150, 300, and 600 μg), previously isolated from different volumes (1, 2, 5, 10, and 20 μL) of the particle suspension (30 mg/mL), in an Eppendoff tube. The Eppendoff tube was placed on a plastic rack and heated in a domestic microwave oven for 30 s or 1 min, taking care to keep the cap open during heating. After the mixture was heated, the rack was taken out of the oven and a thermocouple probe was immediately introduced into the tube to measure the temperature. Because the solution volume in the tube was low (50 μL), the final temperature was below the boiling point of water. When a sample (50 μL) containing 600 μg of magnetite beads was microwave-heated for 1 min, the temperature was raised to ∼57 °C. However, with larger volumes (≥1 mL), the temperature increased to boiling point and care should be taken to avoid this. After microwave irradiation, the magnetite beads were isolated by applying an external magnetic field. Both the supernatant and the isolated beads were immediately analyzed by MALDI MS after microwave heating. Prior to the MALDI MS analysis, the super-

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natant (0.5 μL) was mixed with an equal volume of CHCA (25 mg/mL) prepared in acetonitrile/water containing 1% TFA (2:1, v/v), and the mixture (1 μL) was placed on a sample target. In preparing the isolated magnetite beads for analysis, to obtain the optimum crystallization of matrix with analytes, the beads from the samples containing 30, 60, 150, 300, and 600 μg of beads were mixed with 1.4, 1.4, 2, 3, and 3 μL of CHCA (25 mg/mL), respectively. The mixture (1 μL) was then placed on the sample target for MALDI MS analysis.

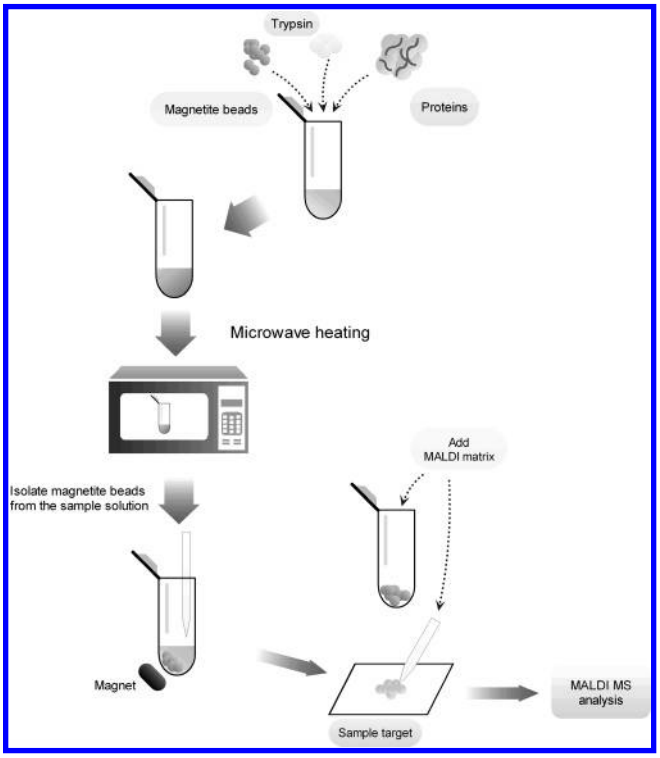
Using Magnetite Beads as Concentrating Probes Followed by Microwave-Assisted Tryptic Digestion. A nonfat milk sample (45 μL) was diluted to 0.45 mL by deionized water and filtered by a Microcon YM-10 centrifugal filter (Millipore, Billerica, MA), which could filter out molecules <10000 Da with centrifugation at 13000*g* for 30 min. The same steps were repeatedly twice to remove all the molecules <10000 Da. A milk sample spiked with cytochrome *c* was prepared by mixing the milk solution (0.5 μL) collected above, cytochrome *c* (10^{-5} M, 0.5 μL), and aqueous ammonium bicarbonate (49 μL, 25 mM, pH 8). Magnetite beads (60 μg) with negative functionalities were added to the sample solution, and the mixture was vortex-mixed for 30 min. The bead−target conjugate was isolated by magnetic separation and rinsed twice with aqueous ammonium bicarbonate. The rinsed beads were mixed with CHCA (25 mg/mL, 1.2 μL) in acetonitrile/1%TFA (2/1, v/v) for MALDI MS analysis. To another set of rinsed beads prepared in parallel was added trypsin (2 μL, 10 μg/mL). The mixture was heated in a microwave oven for 30 s, and the beads were isolated by magnetic separation. Both the supernatant and the isolated beads were prepared for immediate analysis by MALDI MS. The supernatant (0.5 μL) was mixed with an equal volume of CHCA (25 mg/mL) prepared in acetonitrile/water containing 1% TFA (2:1, v/v), and 1 μL was placed on a MALDI sample target for MALDI MS analysis. The beads were washed with CHCA solution, and a sample of the eluent (1.2 μL) was deposited on a MALDI target for MALDI MS analysis.

Instrumentation. A domestic microwave oven, with an operating frequency of 2.45 GHz and a maximum power of 900 W, was used for all the digestion reactions in this study. We did not measure the real power output. However, the temperature of the sample solutions was measured using a thermocouple immediately after the samples were removed from the oven. All the mass spectra were obtained using a Bruker Daltonics Biflex III time-of-flight mass spectrometer (Leipzig, Germany) 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 was used with 16.0 (IS1) and 19.0 kV (IS2) accelerating voltage, while the laser power was carefully adjusted during analysis to maintain optimized mass resolution. The absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia). The TEM image was obtained using a JEOL 2000FX (Japan) transmission electron microscope (TEM).

RESULTS AND DISCUSSION

Scheme 1 shows the steps for using magnetite beads to accelerate microwave-assisted enzymatic digestion. The beads were added to the digestion solution before microwave heating. Figure 1a−c displays the MALDI mass spectra of the supernatant from the microwave-assisted tryptic digest of cytochrome *c*, using

Scheme 1. Procedures for Carrying Out Magnetite Bead Accelerated Microwave-Assisted Enzymatic Digestion



different amounts of magnetite beads, heated for 30 s. With more than 150 μg of magnetic beads, no intact protein ion peaks were observed in the mass spectra, probably because all the proteins remained trapped by the beads. More beads in the digestion solution gave a more complete enzymatic digestion. The peptide residues in the mass spectrum corresponded to 83% of the sequence in cytochrome *c* (see Table 1). Figure 1d presents the MALDI mass spectrum of the supernatant obtained from the tryptic digest of cytochrome *c* with the addition of the magnetite beads (600 μg) at room temperature (~25 °C) without microwave irradiation. There are many peptide peaks at m/z > 2500 in Figure 1d, indicating incomplete digestion, while the peaks in Figure 1c show peaks at m/z < 2200. The results demonstrated that magnetite beads accelerated the enzymatic digestion. We also investigated peptide residues and proteins remaining on the surfaces of the magnetite beads. Figure 1e−g presents the MALDI mass spectra of the samples eluted from the magnetite beads, from the solutions which gave the results in Figure 1a−c. The more magnetic beads in the digestion solution, the greater is the degree of digestion of the proteins. With 600 μg of beads in solution, no intact protein molecular ions appear in the mass spectrum, indicating virtually complete digestion. The peptide residues in the mass spectrum corresponded to 86% of the sequence in cytochrome *c* (see Table 2). Figure 1h presents the MALDI mass spectrum of the sample eluted from the magnetite beads, from the solution which gave the result in Figure 1d. There is a peak marked with an asterisk appearing at m/z 5453.9 (#39−86), indicating incomplete digestion. Furthermore, several peaks with m/z > 2500 indicated that digestion was not complete. Comparison of these results with the result in Figure 1g shows

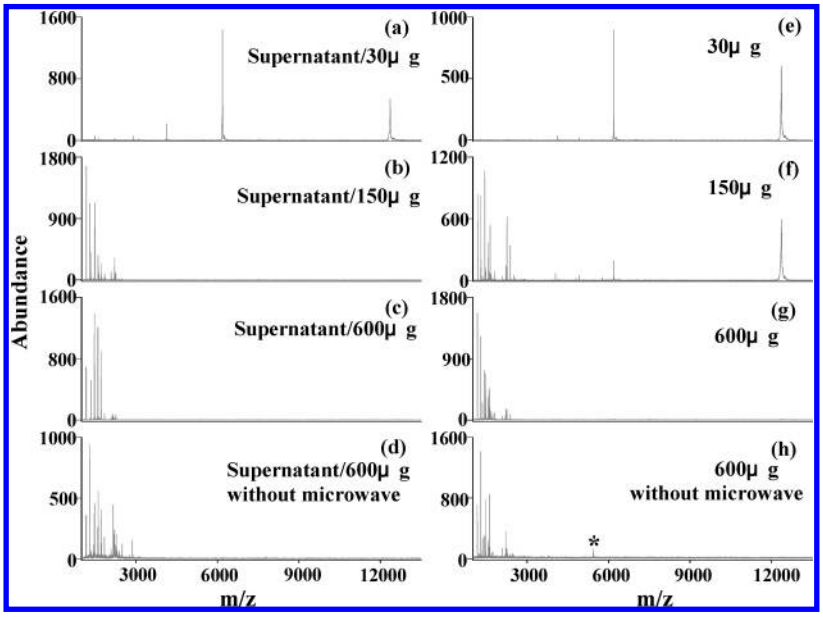


Figure 1. MALDI mass spectra of the supernatant from the tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) prepared in ammonium bicarbonate (50 mM, pH 8) with the addition of (a) 30, (b) 150, and (c) 600 μg of magnetic beads, under microwave heating for 30 s. (d) MALDI mass spectrum of the supernatant obtained from the tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) prepared in ammonium bicarbonate (50 mM, pH 8) with the addition of 600 μg of the magnetic beads, reacting at room temperature for 30 s. MALDI mass spectra of the samples eluted from the beads of (e) 30, (f) 150, and (g) 600 μg after tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) under microwave heating for 30 s. (h) MALDI mass spectrum of the sample eluted from the magnetite beads, isolated from the sample used for obtaining Figure 1d.

Table 1. Ions Observed in the Mass Spectrum Shown in Figure 1c

obsvd $[M + H]^+$	calcd $[M + H]^+$	missed cleavage	peptide sequence
1168.62	1168.62	0	TGPNLHGLFGR (28−38)
1296.35	1296.71	1	TGPNLHGLFGRK (28−39)
1350.35	1350.72	1	TEREDLIAYLK (89−99)
1470.38	1470.68	0	TGQAPGFTYTDANK (40−53)
1478.45	1478.82	2	KTEREDLIAYLK (88−99)
1478.45	1478.82	2	TEREDLIAYLKK (89−100)
1598.49	1598.78	1	KTGQAPGFTYTDANK (39−53)
1606.55	1606.91	3	KKTEREDLIAYLK (87−99)
1606.55	1606.91	3	KTEREDLIAYLKK (88−100)
1623.63	1623.79	1	EETLMEYLENPKK (61−73)
1633.45	1633.81	1	IFVQKCAQCHTVEK (9−22)
1695.23	1695.00	3	KYIPGTKMIFAGIKK (73−87)
1695.23	1695.00	3	YIPGTKMIFAGIKKK (74−88)
1712.62	1712.82	1	TGQAPGFTYTDANKNK (40−55)
1840.82	1840.91	2	KTGQAPGFTYTDANKNK (39−55)
2081.28	2081.02	1	GITWKEETLMEYLENPK (56−72)
2081.28	2081.19	4	YIPGTKMIFAGIKKKTER (74−91)
2209.72	2209.28	5	KYIPGTKMIFAGIKKKTER (73−91)

Table 2. Ions Observed in the Mass Spectrum Shown in Figure 1g

obsvd $[M + H]^+$	calcd $[M + H]^+$	missed cleavage	peptide sequence
1168.65	1168.62	0	TGPNLHGLFGR (28−38)
1296.83	1296.71	1	TGPNLHGLFGRK (28−39)
1350.66	1350.72	1	TEREDLIAYLK (89−99)
1421.80	1421.83	3	MIFAGIKKKTER (80−91)
1433.73	1433.77	1	HKTGPNLHGLFGR (26−38)
1470.68	1470.68	0	TGQAPGFTYTDANK (40−53)
1478.70	1478.82	2	KTEREDLIAYLK (88−99)
1478.70	1478.82	2	TEREDLIAYLKK (89−100)
1561.67	1561.87	2	HKTGPNLHGLFGRK (26−39)
1598.52	1598.78	1	KTGQAPGFTYTDANK (39−53)
1606.69	1606.91	3	KKTEREDLIAYLK (87−99)
1606.69	1606.91	3	KTEREDLIAYLKK (88−100)
1623.79	1623.79	1	EETLMEYLENPKK (61−73)
1633.41	1633.81	1	IFVQKCAQCHTVEK (9−22)
1675.76	1675.91	2	GGKHKTGPNLHGLFGR (23−38)
1734.82	1735.01	4	KKTEREDLIAYLKK (87−100)
1803.86	1804.00	3	GGKHKTGPNLHGLFGRK (23−39)
2080.94	2081.02	1	GITWKEETLMEYLENPK (56−72)
2080.94	2081.19	4	YIPGTKMIFAGIKKKTER (74−91)
2209.11	2209.12	2	GITWKEETLMEYLENPKK (56−73)
2209.11	2209.28	5	KYIPGTKMIFAGIKKKTER (73−91)
2367.75	2367.34	4	MIFAGIKKKTEREDLIAYLK (80−99)
2495.80	2495.44	5	MIFAGIKKKTEREDLIAYLKK (80−100)

that the addition of magnetite beads accelerate the tryptic digestion.

Figure 2 shows the increase in tryptic digestion of cytochrome *c* with increasing magnetite beads. The degree of digestion was estimated using the ratio of the sum of the intensities of the peaks at *m/z* 1168.62, 1296.83, 1433.73, 1478.45, and 1598.49 to the sum of the intensities of the peaks at *m/z* 1168.62, 1296.83, 1433.73, 1478.45, 1598.49, 4121.03, 6181.05, and 12361.09 based on the mass spectra shown in Figure 1. The peaks at *m/z* 1168.62, 1296.83, 1433.73, 1478.45, and 1598.49 are the peptide residues derived from cytochrome *c*, and they frequently appear in the mass spectra after tryptic digestion, while the peaks at *m/z* 4121.03, 6181.05, and 12361.09 correspond to triply charged, doubly charged, and singly charged cytochrome *c*, respectively. The digestion tended to completion as the amount of the magnetite beads in the digestion solution was increased to >200 μg. On the basis of this result, it was estimated that ~2.5 pmol of cytochrome *c* required 1 μg of magnetite beads for completion of the tryptic digestion under microwave heating.

The presence of organic solvents could assist protein unfolding and reduce the time required for enzymatic digestion to 10 min under microwave heating as previously reported.[10] However, enzymatic digestion was not complete with 30 s of microwave

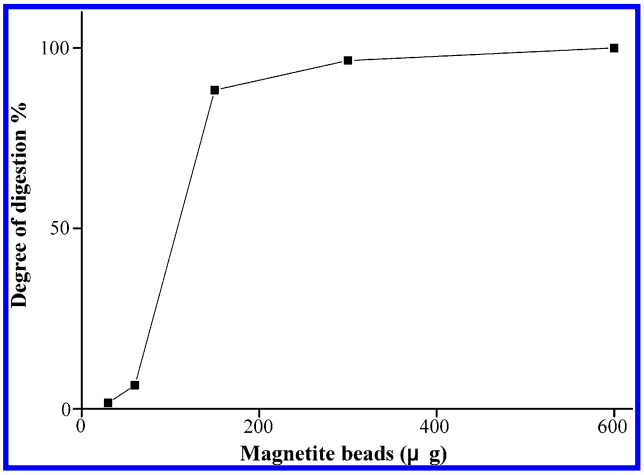


Figure 2. Degree of tryptic digestion of cytochrome *c* as a function of the amount of magnetite beads.

heating even in the presence of organic solvents (see Figure 2 in Supporting Information). The effectiveness of organic solvents combined with the use of magnetic beads was investigated. Figure 3a−c displays the MALDI mass spectra of the supernatants from the microwave-assisted tryptic digestion of cytochrome *c* in ammonium bicarbonate solution (50 mM, pH 8) containing 35% acetonitrile, in the presence of different amounts of magnetic beads heated for 30 s. With only 30 μg of beads, there are already several peptide ions revealing in the mass spectrum (Figure 3a). The digestion rate is faster than that in aqueous ammonium bicarbonate alone (Figure 1a). However, with the bead quantity increased to 600 μg, the reaction was less complete than the reactions in Figure 1b,c. There are still many peaks appearing at m/z > 3000. Figure 3d−f displays the MALDI mass spectra of the sample eluted from the magnetite beads used for obtaining Figure 3a−c, under microwave heating for 30 s. When ≥60 μg magnetite beads were added to the digestion solution, all the peptide residues eluted from the surfaces of the magnetite beads had m/z < 3000 (results not shown). Furthermore, there was no intact protein peak appearing in the mass spectra. It seems that the reaction is more complete on the surfaces of magnetite beads than in the supernatant. The extent of digestion in the solution containing organic solvent was less than that occurring on the surfaces of the beads.

Magnetite beads must have played a significant role during microwave-assisted enzymatic digestion. There may be two reasons for this. The temperature at the surface of magnetite beads would be higher than in solution since the beads are the absorbers of the radiation. This would favor protein denaturation and enzymatic digestion. Although we were unable to measure the temperature directly on the surface of the magnetite beads, we examined the temperature change of a magnetite bead suspension (600 μg in 50 μL water). During microwave heating for 3 min, the temperature of the suspension increased from room temperature (~25 °C) to 86 °C, while the temperature of water (50 μL) not containing beads increased to only 73 °C. When we used methanol instead of acetonitrile in the digestion, the results were similar to those in Figure 3 (results not shown). These results indicate that 30 s of microwave heating is sufficient for cytochrome *c* to undergo tryptic digestion in the presence of a sufficient amount of magnetite beads in aqueous ammonium bicarbonate with or without 35% acetonitrile.

It is known that proteins unfold as they adsorb to a surface of a solid substrate.[28] We have previously shown that proteins with net positive charges can be trapped on the surface of magnetite beads with opposite charged functionalities via electrostatic interactions.[16] Thus, magnetite beads play several roles during microwave-assisted enzymatic digestion: attracting proteins onto their surfaces, unfolding the proteins, and absorbing the microwave radiation. The isoelectric point (pI) of cytochrome *c* is 9.6, so cytochrome *c* has a net positive charge in the digestion solution at pH 8. Thus, during digestion, cytochrome *c* molecules readily attach to the surfaces of the magnetite beads. In order to clarify this matter, we used absorption spectroscopy to investigate the trapping capacity of the magnetite beads for cytochrome *c*. The absorption spectra of the supernatant solutions after incubation with various amounts (3, 6, and 12 mg) of magnetic beads in a sample containing cytochrome *c* (1 mL, 10^{-5} M) in aqueous ammonium bicarbonate (50 mM, pH 8) and 35% acetonitrile/ammonium bicarbonate solution (50 mM), respectively. The absorption bands are dramatically decreased after treatment (see Figure 5 in Supporting Information). With more than 6 mg beads, cytochrome *c* is totally trapped. The capacity for trapping cytochrome *c* correlates with the digestion efficiency as seen in Figures 1 and 3. When sufficient magnetite beads were employed to trap proteins onto their surfaces, the proteins trapped on the surface of the magnetite beads are readily digested. It was estimated that 1 mg of the magnetite beads could trap ~2 nmol of cytochrome *c* based on the absorption results.

Figure 4a−c displays the MALDI mass spectra of the supernatant solutions obtained from the microwave-assisted tryptic digestion solution of myoglobin prepared in aqueous ammonium bicarbonate (50 mM, pH 8) with the addition of 30, 150, and 600 μg of the magnetic beads, respectively, under microwave heating for 1 min. As the amount of the magnetite beads in the digestion solution increases, only a few ions appear in the mass spectra at m/z < 3000. The singly charged (M^+), doubly charged (M^{2+}), and triply charged ions (M^{3+}) of myoglobin ions are still observed in all the mass spectra (Figure 4a−c), indicating that the digestion reactions have not been completed. However, all the peptide residues eluted from the surface of the magnetite beads have m/z < 2200 with 600 μg magnetite beads (Figure 4f). The results demonstrate that myoglobin in the supernatant is resistant to trypsin digestion, while the protein molecules attached to the surfaces of the beads can be readily digested.

We suspect that the low degree of digestion in the supernatant is because the myoglobin is not unfolded in the aqueous solution. Organic solvents have been known to be helpful for the unfolding of proteins, so acetonitrile was added to digestion solutions to assist the unfolding of myoglobin. Figure 5a−c displays the MALDI mass spectra of the supernatant obtained from microwave-assisted tryptic digestion of myoglobin prepared in 35% acetonitrile/ammonium bicarbonate (50 mM) with the addition of 30, 150, and 600 μg of the magnetic beads, respectively, under microwave heating for 1 min. No intact protein peaks are observed in any of the mass spectra, indicating that all the proteins in the supernatant are digested to small peptide residues. This implies that myoglobin is readily denatured in the presence of organic

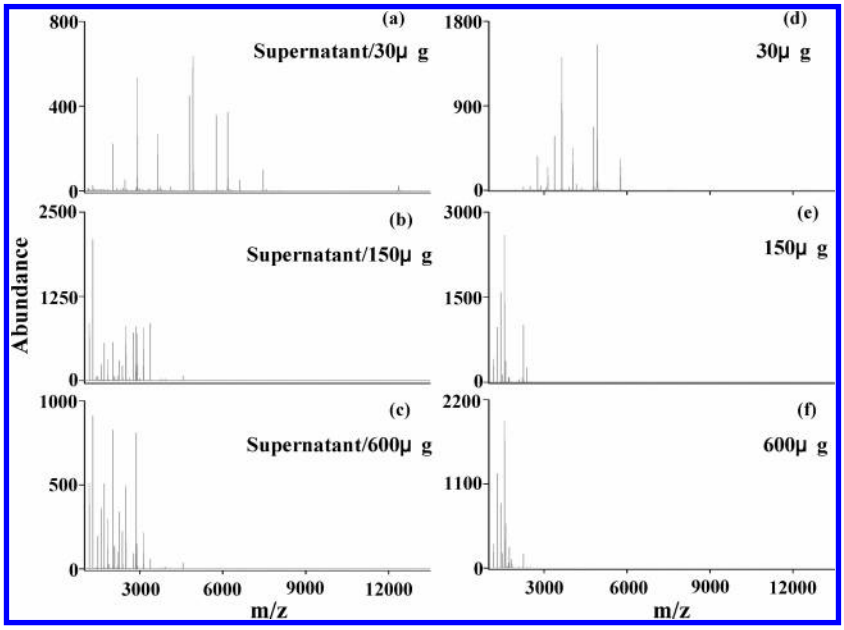


Figure 3. MALDI mass spectra of the supernatant obtained from the tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) in 35% acetonitrile/ammonium bicarbonate (50 mM) with the addition of (a) 30, (b) 150, and (c) 600 μg of the magnetic beads, under microwave heating for 30 s. MALDI mass spectra of the samples eluted from the beads of (d) 30, (e) 150, and (f) 600 μg after tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) under microwave heating for 30 s.

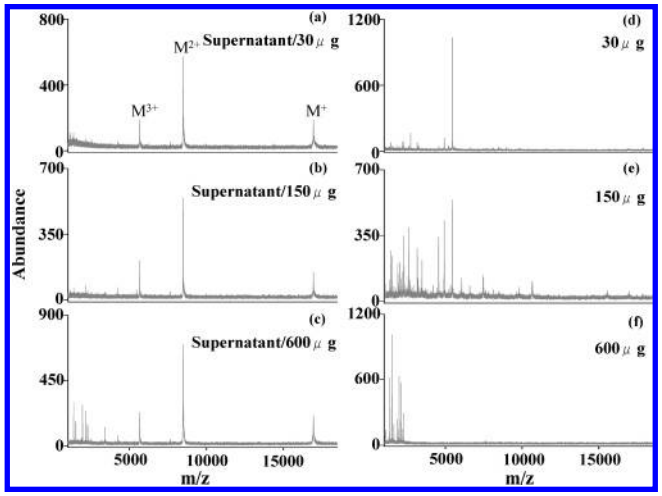


Figure 4. MALDI mass spectra of the supernatant obtained from the tryptic digest of myoglobin (10^{-5} M, 50 μL) in ammonium bicarbonate (50 mM, pH 8) with (a) 30, (b) 150, and (c) 600 μg magnetic beads, under microwave heating for 1 min. MALDI mass spectra of the samples eluted from the beads of (d) 30, (e) 150, and (f) 600 μg after tryptic digest of myoglobin (10^{-5} M, 50 μL) under microwave heating for 1 min.

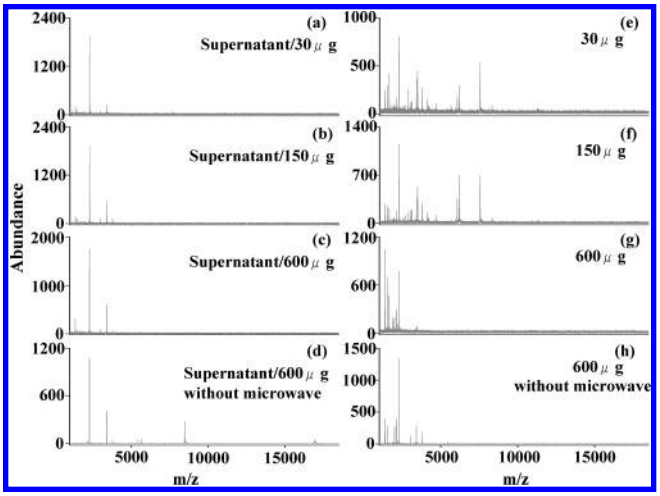


Figure 5. MALDI mass spectra of the supernatant obtained from the tryptic digest of myoglobin (10^{-5} M, 50 μL) in 35% acetonitrile/ammonium bicarbonate (50 mM) with (a) 30, (b) 150, and (c) 600 μg of the magnetic beads, under microwave heating for 1 min. (d) MALDI mass spectrum of the supernatant obtained from the tryptic digest of myoglobin (10^{-5} M, 50 μL) in 35% acetonitrile/ammonium bicarbonate (50 mM) with 600 μg magnetic beads, reacting at room temperature for 1 min. MALDI mass spectra of the samples eluted from the beads of (e) 30, (f) 150, and (g) 600 μg after tryptic digest of myoglobin (10^{-5} M, 50 μL), under microwave heating for 1 min. (h) MALDI mass spectrum of the sample eluted from the magnetite beads, isolated from the sample used for obtaining Figure 6d.

solvent and can be readily digested by trypsin. The peptide residues in the mass spectrum (Figure 5c) correspond to 54% of the sequence in myoglobin (see Table 1 in Supporting Information). Figure 5e−g presents the MALDI mass spectra of the samples eluted from the magnetite beads obtained from the samples for obtaining Figure 5a−c, respectively. The mass spectral patterns in Figure 5e−g resemble what we obtained in Figure 4d−f. That is, the addition of organic solvent does not affect the tryptic digestion on the surface of the magnetite beads. However, it helps protein unfolding in the supernatant and improves the tryptic digestion efficiency. The peptide residues in the mass spectrum (Figure 5g) correspond to 90% of the sequence in myoglobin (see Table 2 in Supporting Information). Figure 5d,h shows the results of control experiments, samples obtained from the supernatant and eluent from beads without microwave heating. Apparently, without microwave heating, there were still ions at $m/z > 5000$ appearing in the mass spectra after reaction at room temperature for 1 min. When the tryptic digestion of myoglobin was carried out in 50% methanol/ammonium bicarbonate (50 mM), the mass spectra resembled those obtained in Figure 5 (results not shown).

Absorption spectroscopy was employed to examine the trapping capacity of the magnetite beads for myoglobin in different solvent systems. The absorption spectra of the supernatant were obtained by adding various amounts (3, 6, and 12 mg) of magnetic beads to trap target species in a sample containing myoglobin (1 mL, 10^{-5} M) in aqueous ammonium bicarbonate solution (50 mM, pH 8) and 35% acetonitrile/ammonium bicarbonate solution (50 mM) (see Figure 6 in Supporting Information). It is clear that the intensity of the absorption band of myoglobin is slightly decreased after trapping by the magnetic beads. Myoglobin is not strongly adsorbed because the pI (~7.2) of myoglobin is lower than the pH of the digestion solution (pH 8.0), hence the myoglobin has a net negative charge and the bead surfaces are also negative. Thus, intact molecular ions of myoglobin still appear in the mass spectra of the supernatant (Figure 4a–c). The incomplete digestion occurring in the supernatant also implies that myoglobin is not unfolded in aqueous solution. We suspect that the presence of organic solvent in the protein solution may have unfolded the structure of myoglobin leading to increased digestion in the supernatant solution (Figure 5a–c). This can be elucidated using the absorption bands (see Figure 6 in Supporting Information). It is noticeable that the absorption bands become broad compared with those dissolved in aqueous ammonium bicarbonate. Additionally, there are very few myoglobin molecules attached to the surface of the magnetite beads by nonspecific binding. Traces of protein on the surfaces of the magnetite are readily digested by trypsin, which explains why digestion products derived from myoglobin appear in the MALDI mass spectra of the sample eluted from the beads (Figure 5e–g).

The conjugate of magnetite particles–target species can be easily isolated from the sample solution by applying an external magnetic field. This is a unique advantage of using magnetite beads as the affinity probe. As demonstrated above, enzymatic digestion can be accelerated if the proteins become attached to the surfaces of the magnetite beads resulting in their denaturation. These results imply that this approach can be a very sensitive and selective method if magnetite beads are first used to concentrate traces of proteins from the sample before carrying out the microwave-assisted enzymatic digestion. A diluted nonfat milk sample containing cytochrome *c* was used to demonstrate the selective concentrating capacity of the magnetite beads in the protein mixture. The major proteins in the milk sample were casein (~83%) and globulins (~15%), which have isoelectric points below that of the solution except for α-S2-casein (pI = 8.34). These milk proteins carry net negative charges, while cytochrome *c* (pI = 9.6) carries a net positive charge in buffer solution at pH 8. Thus, cytochrome *c* is adsorbed on the surface of the magnetite beads with negative functionalities. Figure 6a presents the MALDI mass spectrum of the diluted milk sample containing cytochrome *c*. Many protein peaks appear in the mass spectrum. Cytochrome *c* molecular ions with a weak intensity also appear in the mass spectrum. Figure 6b displays the MALDI mass spectrum of the sample obtained using magnetite beads to trap selectively their target species in the diluted milk sample containing cytochrome *c*. The peak at m/z about 12361 (M^+) appears in the mass spectrum, as well as its doubly (M^{2+}) and triply charged ions (M^{3+}). The magnetite beads–target species conjugates were mixed with trypsin to carry out enzymatic digestion under

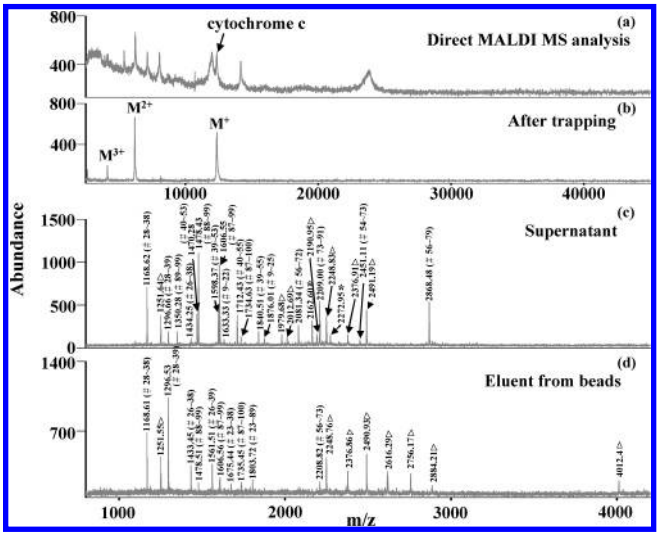


Figure 6. (a) Direct MALDI mass spectrum of a diluted nonfat milk sample containing cytochrome *c* (10^{-7} M). (b) MALDI mass spectrum of the sample obtained using the magnetite beads (60 μg) to trap target proteins from a diluted milk sample (50 μL) containing cytochrome *c* (MW = 12360, 10^{-7} M) in ammonium bicarbonate (50 μL, 25 mM). (c) MALDI mass spectra of the supernatant obtained from the tryptic digest product of magnetite bead–target proteins conjugate under microwave heating for 30 s. (d) MALDI mass spectra of the sample eluted from the beads obtained from the tryptic digest product of magnetite bead–target proteins conjugate under microwave heating for 30 s. The peaks marked with asterisks were the peptides derived from trypsin autolysis, while the peaks marked with triangular marks were presumably derived from milk proteins.

microwave heating for 30 s. Parts c and d of Figure 6 are the MALDI mass spectra of the samples obtained from the supernatant and eluent from the beads, respectively. In Figure 6c, 17 peaks were identified as peptide residues derived from cytochrome *c*, while two marked with asterisks at m/z 2162.60 and 2272.95 were derived from trypsin autolysis. No intact protein peak was observed in the mass spectra. The detailed sequences derived from cytochrome *c* were marked on each peak. The matched peptides cover 89% of the sequence of cytochrome *c*. There were six peaks marked with triangular characters from milk proteins. In Figure 6d, 10 peaks derived from cytochrome *c* appear in the mass spectrum, while 8 peaks marked with triangular characters were unidentified, presumably derived from milk proteins. The matched peptides cover 60% of the sequence of cytochrome *c*. The results indicate that the magnetite beads can be used as the concentrating probes to enrich trace proteins from a complex sample. Furthermore, the trapped proteins on the surface of the magnetite beads were readily digested under microwave heating with the assistance of the magnetite beads. A very short digestion time is sufficient to carry out the enzymatic digestion.

Alternatively, we immobilized trypsin on the surfaces of magnetite beads to simplify the digestion steps. Parts a and b of Figure 7 display the MALDI mass spectra of the supernatant obtained from the microwave-assisted tryptic digest of myoglobin prepared in 35% acetonitrile/ammonium bicarbonate aqueous solution (50 mM, pH 8) with the addition of 30 and 300 μg of the trypsin immobilized magnetic beads, respectively, under microwave heating for 1 min. The results are similar to those obtained in Figure 5. All the peaks appearing in the mass spectra are less than 4000 Da. The overall sequence coverage obtained from the

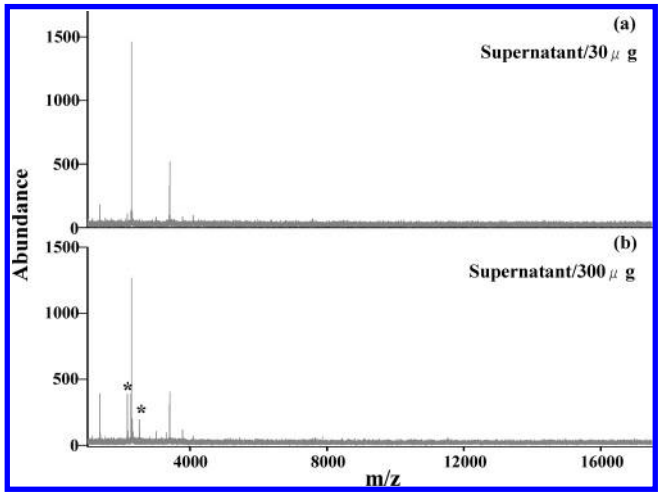


Figure 7. MALDI mass spectra of the supernatant obtained from the tryptic digest of myoglobin (10^{-5} M, 50 μL) in 35% acetonitrile/ammonium bicarbonate (50 mM) with (a) 30 and (b) 300 μg of trypsin immobilized magnetic beads, under microwave heating for 1 min. The peaks marked with asterisks were the peptides derived from trypsin autolysis.

mass spectra is 87%. When the amount of the beads was increased to 60 μg, the peak with weak intensity marked with an asterisk, generated from trypsin autolysis, starts to appear in the mass spectrum (see Figure 7 in Supporting Information). As the amount of the beads was increased to 300 μg, the peptide peaks marked with asterisks generated from trypsin autolysis became noticeable (Figure 7b). Additionally, when we used cytochrome *c* as the sample, the tryptic digestion could be carried out in aqueous ammonium carbonate (pH 8) without the addition of organic solvents (results not shown). The results were similar to those shown in Figure 1. The results indicate that microwave-assisted digestion can be carried out if trypsin molecules have been immobilized onto the surfaces of magnetite beads. Furthermore, the addition of organic solvents to the digestion solution assists protein unfolding and increases the degree of digestion.

CONCLUSIONS

We have demonstrated that magnetite beads can accelerate microwave-assisted enzymatic digestion. The beads have two major functions: (1) as the radiation absorbers for microwaves and (2) as the selective trapping probes for concentrating trace proteins. Furthermore, this approach has the advantages of sensitivity, selectivity, ease-of-use, and speed. To the best of our knowledge, this is the first report of the employment of magnetite beads for accelerating microwave-assisted enzymatic digestion. These two unique features combined with the use of MALDI MS analysis are a promising approach to protein identification. It can be further extended to affinity-based proteomic analysis since functionalized magnetite beads for targeting specific proteins have been extensively studied recently.

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SUPPORTING INFORMATION AVAILABLE

The TEM image of magnetic particles is displayed in Figure 1 of Supporting Information. MALDI mass spectra of the tryptic digest product of cytochrome *c* obtained under microwave heating for 30 s in different solvent systems, without adding magnetite beads as the irradiation absorbers, are presented in Figure 2 of Supporting Information. MALDI mass spectra of the supernatant and eluent obtained from the tryptic digest of cytochrome *c* (10^{-5} M, 50 μL) prepared in ammonium bicarbonate (50 mM, pH 8) with the addition of 600 μg of the magnetic beads, under microwave heating for different times, are also displayed in Figures 3 and 4 of Supporting Information, respectively. The corresponding sequences of the peaks appearing in Figure 5c,g are listed in Tables 1 and 2 of Supporting Information, respectively. The absorption spectra of cytochrome *c* and myoglobin trapped by different amounts of magnetite beads in different solvent systems are displayed in Figures 5 and 6 of Supporting Information, respectively. The MALDI mass spectra of the supernatant obtained from the tryptic digest of myoglobin (10^{-5} M, 50 μL) in 35% acetonitrile/ammonium bicarbonate (50 mM) with 60 μg and 150 μg of trypsin immobilized magnetic beads, under microwave heating, for 1 min are displayed in Figure 7a,b of Supporting Information, respectively. These materials are available free of charge via the Internet at http://pubs.acs.org.

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