

Studying the effect of microwave heating on the digestion process and identification of proteins

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Abbreviations: α -CHCA α -cyano-4-hydroxycinnamic acid; FA formic acid; IAA

iodoacetamide; MIAA methyl iodoacetamide; IDP incomplete digestion percentage; NMCP numbers of missed cleavages; MCS missed-cleavage site.

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ABSTRACT

The impact of microwave irradiation on the **in-solution digestion** processes and the detection limit of proteins are systematically studied. Kinetic processes of many peptides produced through the trypsin digestion of various proteins under microwave heating at 50 °C were investigated with MALDI-MS. This study also examines the detection limits and digestion completeness of individual proteins under microwave heating at 50 °C and at different time intervals (1, 5 and 30 min) using LC-MS. We conclude that, if the peptides without missed cleavage dictate the detection limit, conventional digestion will lead to a better detection limit. The detection limit may not differ between microwave and conventional heating if the peptides with missed cleavage sites and strong intensity are formed at the very early stage (i.e., less than 1 min) and are not further digested throughout the entire digestion process. The digestion of *E. coli* lysate was compared under conventional and short time (microwave) conditions. The number of proteins identified under conventional heating exceeded that obtained from microwave heating over heating periods less than 5 min. The overall results show that the microwave-assisted digestion is not complete. Although the sequence coverage might be better, the detection limit might be worse than that under conventional heating.

1. INTRODUCTION

Proteins are involved in vital biological processes including immunity, enzymatic catalysis, ion transport, and signal transduction [1]. Further, proteins have been used as biomarkers for bacterial or virus identification, disease screening and medical diagnosis [2-5]. Therefore, effective and accurate protein identification is a crucial challenge in the ever-growing field of proteomic research. The process of protein digestion prior to MS analysis

has a large influence on the accuracy of protein identification. Peptide analysis offers advantages over protein analysis due to their ease of separation, fewer charge states, lower molecular mass and higher measurement accuracy. Reducing the enzymatic kinetics of protein digestion from hours (e.g., 16 h at 37 °C) to minutes is an issue of interest. Over the years, several novel techniques have been proposed for fast protein digestion including the use of infrared energy [6], alternating electric fields [7], thermal and chemical denaturation [8, 9], micro-spin columns, high pressure [10] and on-target sample treatment [11]. Other approaches have sought to identify large numbers of proteins from complex samples using immobilized-trypsin on micro reactors, trypsin-coated nanofibers or other various solid supports such as polymer particles [12], beads [13], membranes [14], magnetic microspheres [15, 16], porous silicon matrixes [17, 18] and monolithic materials [19, 20].

Modern approaches such as microwave- and ultrasound-assisted protein enzymatic digestion have been extensively explored to increase throughput and reproducibility [21]. Russell et al. reported accelerated protein digestion in organic solvents [22]. Pramanik et al. used microwave irradiation to reduce protein digestion time to 10 min [23]. Many subsequent studies have used this fast and convenient approach to identify and quantify several known proteins and also to analyze clinical samples. Lill et al. reviewed the heating principle of microwave-assisted proteomics [24], and Sun et al. demonstrated the microwave-assisted enzymatic digestion of complex protein mixtures [25], Capelo et al. reported acceleration of enzymatic protein digestion using ultrasonic energy [26]. The same group later reported the use of sonoreactor-based technology to speed up the kinetics of enzymatic digestion of proteins [27]. Different ultrasonic devices have been used to enhance performance of single proteins by accelerating the reduction, alkylation and digestion steps [27-29]. Li et al. reported the in-solution proteolysis of standard proteins using an ultrasonic water-bath [30]. Elevated temperature and pressure might accelerate digestion under microwave- and

ultrasound-assisted protocols, however the exact mechanism is still unknown. An efficient proteolytic digestion protocol in targeted proteomics will minimize the amount of time needed to detect proteins, with low detection limits and optimum digestion reproducibility. The ideal denaturing and digestion protocol would be one where complete digestion of the protein occurs quickly, resulting in maximum peptide signals, followed by a “steady-state” condition where no change in peptide concentrations is observed. Such a protocol would be robust in that small changes in digestion time would not be an important factor, resulting in optimum digestion reproducibility. Incomplete digestion may also compromise quantitative proteomics [31].

Previous studies evaluated the digestion efficiency and completeness of various protocols by determining the sequence coverage and/or by comparing the number of identified peptides [32, 33]. However, these parameters might be misleading as they may also arise due to incomplete digestion. Hence incomplete digestion percentage (IDP) and numbers of missed cleavages (NMCP) should also be considered in evaluating the digestion efficiency. Kim et al. studied the effects of temperature on ultrasonic bath-assisted tryptic digestion using MALDI-TOF-MS and reported that high sequence coverage and the identification of additional peptides resulted from the elevated number of miss-cleaved peptides [34]. Hustoft et al. evaluated the efficiency of protein identification of various accelerated methods with conventional digestion by comparing the sequence coverage and the peak area of intact proteins. They suggested using overnight digestion to produce peptides from enzyme-resistant proteins [35]. These studies did not investigate the digestion processes of individual peptides and the effect of digestion completeness on the limit of detection.

Although mis-cleaved products can sometimes enhance the quality of an identification workflow, there are circumstances in which they can compromise quantitative proteomics. We have previously studied the effect of microwave heating on the digestion process under

various solvents, reaction times, enzyme to protein molar ratios, and microwave temperatures [36]. MS-based kinetic analysis of tryptic digestion of proteins by microwave and conventional heating at similar temperatures showed no nonthermal effect in microwave-assisted digestion. We also explained how conventional heating at high temperatures (50 °C) could accelerate digestion, similar to microwave irradiation [37, 38]. In this study we evaluate the completeness of digestion under various conditions (ultrasonic water bath, microwave and water bath heating at 50 °C). The impact of digestion completeness on detection limit was further studied for the microwave heating method.

2. MATERIALS AND METHODS

2.1. Standards and Reagents

All reagents were of analytical grade and used as received. Water was purified using an ion-exchange Millipore system (Milli-Q, Bedford, MA, USA). LC-MS grade formic acid (FA), trifluoroacetic acid (TFA) and 4-Nitrophenyl-iodoacetate were acquired from Fluka (Buchs, SG, Switzerland). HPLC grade acetonitrile (ACN), dichloromethane and methanol were obtained from J. T. Baker (Phillipsburg, NJ, USA). Urea was purchased from Wako (Osaka, Japan). Angiotensin III (RVYIHPF Mr = 930.51) was obtained from Bachem (Bubendorf, Switzerland). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Methylamine (40% m/m aqueous solution) was ordered from Alfa Aesar (Ward Hill, MA, USA). Ammonium bicarbonate (NH₄HCO₃), α-cyano-4-hydroxycinnamic acid (α-CHCA), dithiothreitol (DTT), iodoacetamide (IAA), tris(hydroxymethyl)-aminomethanhydrochloride (Tris-HCl), and standard proteins such as lysozyme (chicken egg white), transferrin (human), β-lactoglobulin (bovine milk), BSA (albumin from bovine serum), cytochrome c (bovine heart), myoglobin (horse heart) and

Luria-Bertani (LB) medium were obtained from Sigma (St. Louis, MO, USA). Methyl-iodoacetamide (MIAA) was synthesized according to the reported procedure [39].

2.2. Apparatus used for accelerated protein digestion

Microwave irradiation

The microwave-assisted protein enzymatic digestions were performed in a CEM Discover microwave system (Matthews, NC, USA), equipped with a noncontact infrared sensor which monitors the temperature of the sample vial surface. This system has a single-mode cavity design, with temperature feedback control. The maximum output power is 300 W and the operating frequency is 2.5 GHz. The reaction temperature was set at 50 °C using a sensor controlled magnetron and the output power was maintained at 100 W. The digestion was performed at 50 °C for different time points (1, 5, 10, 20, 30, 45 and 60 min). The actual internal temperature of the reaction solutions was measured directly using a fiber-optic sensor probe (OPsens-PicoM system, Quebec, Canada).

Ultrasonic bath

Ultrasound-assisted enzymatic reactions were performed in an ultrasonic water bath, model LEO-803 (Leo, Taipei, Taiwan), with maximum output power of 80 W at 46 KHz. The temperature of the reaction was maintained at 50 °C and the digestion was done at different (1, 5, 10, 20, 30, 45 and 60 min) time intervals.

Water bath heating device

Enzymatic protein digestions were also done using a water bath model Brason 5210-DTH. The digestion was performed at 50 °C for 1, 5, 10, 20, 30, 45 and 60 min.

2.3. Protein digestion

Conventional/accelerated protein digestion

Protein standards (2 µg) were dissolved in 25 mM ammonium bicarbonate, denatured with 3 µL of 6 M urea solution and reduced by 5 mM DTT at 37 °C for 1 h. Cysteine residues

were alkylated by adding 25 mM IAA and the reaction was performed in the dark at room temperature for 1 h. DTT solution was added to consume any unreacted IAA reagent. All digestion solvents were adjusted to pH 8.5. For conventional digestion, proteins in 25 mM NH_4HCO_3 were digested by trypsin at a 1:25 trypsin to protein molar ratio (E/S) and angiotensin III was spiked into the solution prior to incubation at 37 °C for 16 h in a water bath. To accelerate protein digestion, protein solutions were digested with trypsin at a protease to protein molar ratio of 1:25 (mole/mole) at 50 °C using (i) microwave (ii) ultrasonication and (iii) water bath. Accelerated protein digestion was monitored at different time intervals (1, 5, 10, 20, 30, 45 and 60 min) by MALDI-MS.

Accelerated protein digestion using MIAA-labeled peptides as internal standards

In this method, proteins obtained after reduction and IAA or MIAA alkylation were diluted with 25 mM ammonium bicarbonate. The MIAA alkylated protein samples were digested with trypsin for 16 h. The IAA alkylated protein samples mixed with the MIAA-labeled digestion solution were digested using three different accelerated protein digestion methods including (i) microwave (ii) ultrasonication and (iii) water bath at 50 °C. In-solution digestion was performed by adding trypsin into the mixtures at a protease to protein molar ratio of 1:25 (mole/mole). The digestion was quenched by the addition of 0.1% TFA. Protein digestion was monitored at different time intervals (1, 5, 10, 20, 30, 45, and 60 min) using MALDI-MS.

2.4. Bacterial sample preparation

Bacteria were cultured in LB broth media at 37 °C for 24 h. The cells were harvested, washed three times with water, boiled in water for 10 min, lyophilized to dryness, and then stored at -20 °C. The lyophilized bacteria were suspended in ammonium bicarbonate (25 mM pH 8.5) at a concentration of 0.5 mg/mL. Proteins were extracted from bacterial cells using a MICROSON S-3000 sonicator (Misonix, Farmingdale, NY, USA) for 5 min (20-s sonication

with a 10-s pulse duration). The resulting suspension was centrifuged at 10000g and the supernatant was collected and then dried using a SpeedVac vacuum concentrator. Protein content was estimated by Bradford's method [40] using bovine serum albumin (BSA) as a standard. Various concentrations (1, 0.5, 0.05, 0.01 and 0.001 $\mu\text{g}/\mu\text{L}$) were used in mass spectrometric analysis to identify *E. coli* proteins.

2.5 Mass spectrometry and database search

MALDI-TOFMS

Positive ion MALDI-TOF mass spectra were acquired using an Autoflex time-of-flight mass spectrometer (Bruker Daltonic, Bremen, Germany) equipped with a 337-nm nitrogen laser (6.7 Hz, 3-ns pulse width). Spectral data were obtained in the reflectron mode with an acceleration voltage of 20 kV. A MALDI matrix was prepared by dissolving CHCA in a solvent mixture of 50% v/v ACN and 0.1% v/v TFA. The MS analysis of each protein digest was conducted immediately after the enzymatic reaction. An aliquot of the sample solution (0.5 μL) was mixed with an equal volume of the matrix solution and then applied on the target plate and dried before MALDI-MS analysis. Each mass spectrum was derived from 100 summed scans. Flex Control (Ver.3.0) and Flex Analysis (Ver.2.4) software were used for data acquisition. Each MALDI-TOF MS analysis was performed in triplicate and error bars denote standard deviations.

Liquid chromatography coupled with nano electrospray-ionization/mass spectrometry

Protein digests were analyzed using a nano LC/ESI-MS ion trap mass spectrometer (HCT ultra ETD II Bruker Daltonics, Bremen, Germany) equipped with a nano liquid chromatography (Agilent1100 series, Agilent Technologies, Germany). A home-made reverse phase C_{18} chromatography column (10 cm $\times$ 75 μm , 5 μm) was used. Peptides were eluted at a flow rate of 200 nL/min with an injection volume of 1 μL . The gradient using mobile phase A (0.1% v/v FA in 99.9% v/v H_2O) and mobile phase B (0.1% v/v FA in 99.9%

v/v ACN) over 60 min was as follows: The initial elution condition was 5% B for 5 min and ramped to 80% solvent B for 50 min and held for 5 min. Analyses were performed in the positive ion mode. A full MS scan was acquired from m/z 350 to m/z 1800, with the intensity threshold set to 2×10^5 and the collision energy of 1.3 V was set to MS/MS for the top three precursor ions.

Data from LC/MS/MS experiments were processed using the MASCOT search engine with the following parameters: (i) Swiss-prot database; (ii) taxonomy: mammalia for standard proteins and Eubacteria for *E. coli* samples; (iii) two missed-cleavage sites; (iv) peptide charge +1, +2, +3; (v) the mass tolerances of the precursor and fragment ions were set to 1.2 Da and 0.8 Da respectively; for bacteria mass, the tolerance was set at 200 ppm and the MS/MS was set to 0.5 Da (vi) carbamidomethylation of cysteine as a fixed modification; (vii) oxidation of methionine as a variable modification; (viii) expectation cutoff value was set at 0.05 and the false discovery rate was less than 6 %.

3. RESULTS AND DISCUSSION

The digestion completeness of various proteins under microwave heating at 50 °C was compared with conventional overnight heating at 37 °C. We calculated the percentages of digested peptides relative to the internal standards (MIAA-labeled peptides and angiotensin III) at different time intervals for 1 h. The results were analyzed by MALDI-MS to determine the amount of target peptides produced at each time interval. LC-MS was used to investigate the differences in the detection limit for various digestion conditions.

3.1. Comparison of accelerated/conventional protein digestions using MIAA-labeled peptides as internal standards

Previous work has presented a quantitative analysis of cysteine-containing peptides using the IAA and MIAA labeling approach [41]. MIAA-labeled peptides in tryptic digests

were used directly as internal standards for the quantitative determination of the digests. The peak ratios of the labeled peptides were determined using MALDI-MS in triplicate. The internal standard (MIAA-labeled peptides) was not affected by the present method. Although the digestion kinetics of a protein has been studied based on a single peptide, some peptides might be produced faster than the others. Therefore, in this study, we have selected various IAA and MIAA-labeled peptide fragments for quantitative analysis and to compare the digestion completeness between microwave and conventional conditions. We also compare two other accelerated methods: water and ultrasonic bath heating at 50 °C. The digestion efficiencies of four standard proteins (lysozyme, BSA, β -lactoglobulin and transferrin) under accelerated digestion (IAA-labeled) methods (50 °C for 1 h) and conventional digestion (MIAA-labeled) at 37 °C for 16 h were monitored using the IAA/MIAA labeling approach. The temperature of 50 °C was used because it has been proven to facilitate fast protein digestion.[37] If we assume that the conventional digestion is complete, the digestion completeness of peptides obtained under accelerated conditions can be summarized as follows (data not shown): (i) The three heating methods at 50 °C reveal similar digestion efficiency. Our intention is less to compare these methods than to understand digestion completeness. (ii) The intensity ratios between the IAA/MIAA-labeled peptide pairs of lysozyme at m/z 993/1007 and m/z 1325/1339 were found to be 0.4-0.5 at 1 min. These signals show an increase in intensity ratio (0.9) at 30 min and finally increase to 1 (100% completeness) at 1 h. (iii) The intensity ratio of BSA peptide pair (IAA/MIAA) at m/z 1419/1433 was 1 at 10 min, implying complete digestion, whereas other peptide pairs at m/z 1880/1894 and m/z 1724/1738 showed only 70% digestion at 1 h. (iv) The intensity ratio between the peptide pair at m/z 1715/1729 for β -lactoglobulin was 1 at 1 min, indicating fast and complete digestion. (v) The intensity ratios of transferrin peptide pairs (IAA/MIAA) at m/z 997/1011 and m/z 2171/2185 were close to 1 at 1 h, whereas other peptide pairs at m/z

887/901 and m/z 1586/1600 revealed intensity ratios of 0.6-0.8. These peptides investigated contain no missed cleavage sites. The results indicate that few peptides achieve complete digestion in one hour. Furthermore, only the β -lactoglobulin peptide showed complete digestion over a very short time period (1 min), but with no peptides of lysozyme, BSA or transferrin indicating complete digestion over such a short period.

3.2. Comparison of accelerated/conventional protein digestions using Angiotensin III as internal standard

By using MIAA-labeled peptides as internal standards, the method can monitor only thiol-group-containing peptides. To monitor the digestion process of various peptides with or without missed cleavages, we have adopted a semi-quantitative approach using angiotensin III as an internal standard.

To illustrate the digestion completeness of the four standard proteins, the abundances of digested peptides relative to the internal standard (angiotensin III) were measured at different time points up to 1 h. Figure 1 shows the peak ratios of peptides vs angiotensin III obtained from the lysozyme digestion under microwave, ultrasonic and water bath heating at 50 °C and water bath heating at 37 °C (i.e., the conventional approach). The relative intensity of the peptide at m/z 2181 with residues 15-33 (R15-33) and one missed-cleavage site (MCS) gradually decreased (at 10 min) by producing two product peptides at m/z 874 (R15-21) and 1325 (R22-33) without missed cleavages. The relative intensities for the two peptides without missed cleavages leveled off after 30 min. The time plots present similar trends for the three digestion products obtained from the accelerated digestion methods (microwave, ultrasonication and water bath at 50 °C for 1 h) (Fig. 1A-C). However, the product produced from digestion under conventional heating at 37 °C (Fig. 1D) was much less than those shown in Figs. 1A-C. This difference is clearly attributable to the temperature effect as higher temperatures should accelerate the digestion rate. The time plots demonstrate that quickly

formed peptides are not the final product and will be further digested into shorter peptides. This phenomenon was also observed for many peptides of other proteins including BSA, β -lactoglobulin and transferrin.

The relative intensities of protein digests were further compared between the microwave-assisted (50 °C, 1 hr) and conventional digestion (37 °C, 16 hr). To simplify the comparison, a peptide whose peak intensity relative to angiotensin III remained stable was chosen as a reference peak. For instance, the relative intensities of the lysozyme peptide at m/z 1428 were 96% at 50 °C for 1 h and 102% at 37 °C for 16 hr. Therefore, this peptide was selected as the reference peak and the intensities of other digested peptides were normalized against this peak. Table 1 summarizes the relative intensities of lysozyme peptides obtained from accelerated and conventional digestions. Related peptides are grouped together. Several peptides which were detected at 1hr under the fast digestion condition did not appear following overnight digestion. The intensities of the related peptides without MCS increased for overnight digestion at 37 °C. The results show that the peptides with MCSs were digested into those without MCS when the digestion was carried out under the conventional conditions (e.g., the peptide pairs: R15-33/R15-21, R62-73/R62-68, and R115-125/R117-125). Some peptides with MCSs (R1-14, R22-45, R34-61 etc.) were only detected during the short digestion time (less than 10 min.). The intensities of the peptides derived from these fast disappearing peptides remain stable under accelerated and conventional conditions (R22-33, R34-45). Sequential digestion for a peptide with 3 MCSs were also observed. The peptide R115-129 decreased in intensity by producing peptide R115-125 with 1 MCS. The peptide R115-125 was further digested into the peptide R117-125 without missed cleavage sites. The results show that fast digestion tended to yield peptides with missed cleavage sites. Many of these peptides were not observed in conventional digestion. Instead, smaller peptides derived from the further digestion of these peptides were detected. Tables S1-S3 list the relative

intensities of peptides obtained from the digestion of BSA, transferrin and β -lactoglobulin under microwave-assisted/conventional conditions. Similar conclusions to that deduced from lysozyme could be also drawn from the results.

Table 2 shows more digestion information of lysozyme obtained from conventional heating (16 hr) and early-stage (5 min) accelerated digestion. Sequence coverage, number of peptides identified, number of miss-cleaved peptides and incomplete digestion percentage are compared. The incomplete digestion percentage is defined as the percentage of summed intensities of peptides with MCS relative to the total intensities of all peptides detected. The 5 min digestion time is within the time scale of most published accelerated digestion experiments. Lysozyme produced fewer peptides (12) under conventional heating than those (17) obtained from the three accelerated digestion methods. However, the number of miss-cleaved peptides and the incomplete digestion percentage of lysozyme from accelerated digestion (~10 and ~50%) are greater than those from conventional digestion (~3 and ~5%). From these results we can conclude that the fast accelerated digestions are indeed incomplete due to the production of more peptides with MCS.

The peptides with MCS do not necessarily decrease with digestion time. It is well known that certain amino acid sequence patterns may cause missed cleavages in the tryptic digestion of proteins. Three types of digestion processes were observed for the peptides with MCSs. The summary is based on the results shown in Table 1 and Supporting Tables S1-S3, and the kinetic plots for the digestion during the first hour. (1) The abundances of peptides do not change throughout the first hour of accelerated digestion or following overnight conventional digestion. (2) The peptide signals reach maximum level at very short time (1 min) and decrease with digestion time. (3) The abundances of peptides increase with time but decrease or become zero under conventional conditions. Figure 2 illustrates examples of the 3 processes observed for the BSA digestion products. The peptide at m/z 1639 (R437-451,

KVPQVSTPTLVEVSR) has one MCS and its relative intensity remains steady during digestion from 1 min to 1 hr (Fig. 2A). This peptide was not further digested after overnight reaction at 37°C. The amino acid preceding this sequence is arginine. It is well known that missed cleavages occur when the two basic residues (arginine and lysine) are adjacent to each other [42]. The relative intensities of the peptide (R233-241) at m/z 1001 (Fig. 2B) with one missed cleavage was maximized at 1 min and then decreased over time probably by producing product peptides with no missed cleavages. Figure 2C shows that the intensity of the peptide (R168-183) at m/z 2045 gradually increased over 1 hr. The intensity became zero under the conventional condition (Table S1).

3.3. Detection limit and digestion completeness of proteins at different concentrations and reaction times

This study also examined the detection limits and digestion completeness of individual proteins (BSA, cytochrome c, and myoglobin) at 50 °C and at different time intervals (1, 5 and 30 min) using LC-ESI-MS. We compare the results between the microwave and conventional overnight digestion. The digestion completeness of a protein was monitored based on the relative intensities of various peptides containing different numbers of missed cleavages. Table 3 lists the abundances of some related peptides obtained from the digestion of cytochrome c under microwave heating for various times and under conventional heating for 16 hr. A similar phenomenon was observed for the ESI experiments when compared with the MALDI experiments. Many peptides with missed cleavages that were observed under microwave heating were not detected under the conventional conditions. The intensity of peptides without missed cleavages was found to increase when the microwave heating time was increased from 1 min. to 30 min. and further increased or remained stable after 16 hr of conventional heating.

Table 4 presents the detection limit and digestion information (i.e., number of missed cleavages and incomplete digestion percentages) for cytochrome c at different time intervals and different concentrations at 50 °C. It is clear that the number of identified miss-cleaved peptides and the incomplete digestion percentages were relatively high under microwave digestion as compared to overnight heating. The incomplete digestion also affects the detection limit. For this protein, 2 peptides R41-54 and R93-100 without missed cleavages were identified by conventional heating through the analysis of a sample corresponding to 10 fmole /1μL of cytochrome c (1 μL injected). No signal was detected for the digestion at this concentration under microwave heating at 50 °C. The two peptides observed under conventional heating had strong MS signals and were not completely generated under microwave heating (Table 3). Therefore, they could not be detected at low concentrations. The detection limit (10 fmole/μL) under conventional heating was better than that (100 fmole/μL) under microwave heating.

Supporting Table S4 shows the detection limit and digestion information for myoglobin at different time intervals and different concentrations at 50 °C. Similar conclusions were drawn from the results of myoglobin and cytochrome c. Many peptides with missed cleavages that were observed under microwave heating were not detected under the conventional conditions. Peptides R17-31, R32-42, and R134-139 without missed cleavages were found to have the strongest intensities under the conventional conditions. The detection limit under microwave heating at 1 min was 100 fmole/μL, worse than that (10 fmole/μL) under the conventional conditions. When the microwave-assisted digestion was carried out for 30 min, the detection limit could be improved to 10 fmole/μL and the detected peptides were R32-42 and R134-139.

Table 5 lists the number of peptides and miss-cleaved peptides obtained from the digestion of BSA. Microwave-assisted digestion for 1 min seems to provide sufficient

performance even when 1 fmole of sample was analyzed. One peptide at m/z 1639 (R437-451) with one MSC was detected. This peptide, along with a peptide at m/z 1479 (R421-433) without MSC, was detected when the digestion was carried out for 30 min. Both of these two peptides were also detected with conventional digestion. The peptide at m/z 1639 (R437-451) with one MSC was also observed in MALDI experiments and had similar abundances under microwave and conventional heating (Fig. 2A). As mentioned earlier, this peptide should be generated quickly and could not be further digested. Therefore, two heating modes gave similar limits of detection.

A protein mixture (BSA, cytochrome c and myoglobin) was also digested under microwave heating at 50 °C for 5 min and under conventional heating. The digestion completeness of these methods was evaluated. Proteins were mixed in equal concentrations (1:1:1 molar ratio) and the relative percentages of various peptides were monitored using nano-LC/ESI-MS. Supporting [Table S5](#) shows the number of peptides identified, number of miss-cleaved peptides and incomplete digestion percentages of the protein mixture under conventional and microwave heating. For microwave-assisted digestion, 5 min was used as the basis for comparison because it is considered a fast reaction and has better digestion efficiency than 1 min digestion. Incomplete digestion percentages of all proteins under classical overnight heating were lower than those under microwave-assisted digestion. The numbers of peptides identified from cytochrome c and myoglobin under conventional digestion were similar slightly less than those obtained from individual protein digestion. However, much fewer peptides were observed when the proteins were digested under microwave heating than those observed when the proteins were digested separately. Previous analysis of the digestion of protein mixtures [35] reached similar conclusions. The identification of fewer peptides might be due to the competition for the trypsin cleavage sites, when the mixture of proteins was digested together. This phenomenon is more severe when

the digestion takes place under microwave heating. Therefore, a longer digestion time (as in conventional experiments) should give a more complete reaction.

The sequence coverage (data not shown) for the digestion of individual or mixed proteins under various conditions depends on the proteins investigated. Some have higher coverage under the conventional condition and some have higher coverage under microwave irradiation. As expected, in all cases the sequence coverage decreases with protein concentrations. The detection limit for protein digestion was obviously affected by the degree of digestion completeness. If the peptides without missed cleavage dictate the limit of detection, conventional digestion will certainly lead to a better detection limit. The detection limit may not differ between microwave and conventional heating if the peptides with missed cleavages and strong signals are produced at the very early stage (less than 1 min) and are not further digested throughout the entire digestion process.

3.4. Digestion of *Escherichia coli* proteins

Further experiments with *E. coli* lysate investigated the completeness of the overnight and short time (microwave) digestion. Enzymatic cleavage of *E. coli* proteins was investigated under conventional heating at 37 °C for 16 h and microwave heating at 50 °C for different time intervals (1 and 5 min) by LC/ESI-MS. The peptides identified using a simplified analysis protocol are shown in Fig. 3. From the various concentrations of protein extract (ca. 1 to 0.001 µg/µL based on Bradford assay) analyzed, we chose results from the lowest concentration (0.001 µg/µL) to compare the digestion completeness of these methods. The comparison at low concentrations should more easily reveal the difference (if any) among the methods. Table 6 compares the total number of *E. coli* proteins identified from the triplicate experiments under conventional heating and CEM microwave for 5 min and 1 min. The number of proteins identified under conventional heating (24) was larger than those obtained from CEM microwave heating for 5 min (15) or 1 min (4). Among the peptides

identified under conventional digestion, **seven** peptides contain MCSs, while **nine** peptides contain MCSs for microwave digestion for 5 min. Only **four** peptides were identified under 1 min microwave heating, indicating very incomplete and inefficient digestion. Overall, the results are consistent with the digestion of individual/mixed standard proteins.

4. CONCLUDING REMARKS

The detection limit and completeness of trypsin-catalyzed protein digestion under microwave heating was evaluated and results were compared against those obtained using conventional heating at 37 °C for 16 h. The model proteins, protein mixtures and *E. coli* protein extracts were digested under microwave and conventional heating. The conventional digestion method was observed to result in more complete digestion than the accelerated method did. Other accelerated methods, including ultrasonic water bath and normal water bath heating, also lead to incomplete digestion. Because the peptides produced from the fast microwave-assisted and conventional overnight digestion differ, the detection limit of the corresponding proteins was affected by the digestion process. If the peptides without missed cleavage dictate the limit of detection, conventional digestion will certainly lead to a better detection limit. The detection limit may not differ between microwave and conventional heating if the peptides with missed cleavage and strong signals are formed at the very early stage (less than 1 min) and are not further digested throughout the entire digestion process. When the digestion of the *E. coli* protein extract was analyzed near the detection limit, conventional digestion also identified more peptides and proteins than microwave-assisted digestion. Many research papers have proposed microwave-assisted digestion methods to replace the conventional method. Microwave-assisted digestion may be used for protein analysis if the sample amount is sufficient and fast analysis is a major concern. However, conventional

digestion may be more favorable when complete digestion or better detection limit is called for. **Future work will include the improvement in detection limit for fast digestion.**

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Table 1. Relative intensities of various peptides of lysozyme under microwave-assisted digestion at 50 °C for 1 h and conventional digestion at 37 °C for 16 h.

^a Sequence of peptides	Molecular weight (m/z)	Number of missed cleavages	Relative intensity (microwave)	Relative intensity (conventional)
(1-14)	1636.8*	3	0%	0%
(14-21)	1030.5	1	7%	9%
(15-33)	2181.0	1	5%	0%
(15-21)	874.4	0	55%	86%
(22-33)	1325.6	0	24%	25%
(22-45)	2735.2*	1	0%	0%
(34-61)	3163.4*	1	0%	0%
(34-45)	1428.6	0	100%	100%
(46-61)	1753.8	0	65%	66%
(62-73)	1491.6	1	22%	0%
(62-68)	993.3	0	99%	134%
(74-97)	2636.2	1	3%	0%
(74-96)	2508.1	0	4%	7%

(97-112)	1804.8	1	48%	7%
(98-112)	1676.7	0	43%	71%
(115-129)	1819.9	3	3%	0%
(115-125)	1333.6	1	84%	22%
(117-125)	1045.5	0	22%	111%

^aPeptides observed throughout the first hour of digestion. * Marked peptides indicate that the peptides disappear within 10 min of digestion. The relative intensities of all peptides were calculated using a peptide at m/z 1428 as the reference peak.

Table 2. Comparison of lysozyme digestion under conventional heating at 37 °C for 16 h and accelerated digestion (water bath, microwave and ultrasonic water bath) at 50 °C for 5 min.

	Conventional digestion at 37 °C for 16 h	Water bath at 50 °C for 5 min	Microwave at 50 °C for 5 min	Ultrasonic water bath at 50 °C for 5 min
Sequence coverage	81±0%	85±0%	86±0%	83±1%
Number of identified peptides	12±1	17±1	17±2	16±1
Number of miscleaved peptides	3±0	10±1	10±2	9±1
Incomplete digestion percentage (IDP)	5±1%	42±1%	49±1%	55±4%

Table 3. Comparison of various peptide fragments of cytochrome c under microwave and conventional heating.

Cytochrome C			5 pmol/μL (Abundance × 10 ⁵)			
			CEM microwave 50 °C			Conventional 37 °C
Sequence of peptides	Molecular weight (m/z)	Number of missed cleavages	1 min	5 min	30 min	16 h
R27-40	1561.03	2	1.7	n.d.	n.d.	n.d.
R27-39	1434.04	1	n.d.	n.d.	21.0	n.d.
R29-40	1295.85	1	28.0	11	12.6	n.d.
R29-39	1167.72	0	24.0	35.0	50.0	80.0
R40-56	1826.05	2	0.7	15.0	20.0	n.d.
R40-54	1583.89	1	9.3	27.0	8.0	20.0
R41-56	1697.91	1	27.8	23.0	16.5	n.d.
R41-54	1455.73	0	9.6	30.0	40.0	70.0
R55-74	2380.11	2	n.d.	0.7	n.d.	n.d.
R57-74	2137.22	1	1.2	4.0	30.0	0.4
R57-73	2009.25	0	1.1	2.8	4.5	65.0

R81-89	1034.78	2	0.8	n.d.	n.d.	n.d.
R81-88	906.52	1	38.0	35.0	35.0	n.d.
R81-87	778.51	0	n.d.	33.0	35.6	45.0
R89-100	1433.98	2	14.0	6.8	50.0	10.0
R90-100	1305.91	1	5.2	6.5	8.5	9.0
R90-101	1433.96	2	9.7	6.8	35.0	n.d.
R93-105	1507.04	2	1.5	n.d.	2.0	n.d.
R93-101	1090.78	1	10.2	26.5	22.5	n.d.
R93-100	963.58	0	5.2	35.0	45.0	105.0

n.d.: not detected

Table 4. Comparison of cytochrome c digestion at different concentrations under conventional heating at 37 °C for 16 h and microwave heating at 50 °C for various times.

Cytochrome c		5 pmole/ μ L	1 pmole/ μ L	100 fmole/ μ L	10 fmole/ μ L
Conventional digestion at 37 °C (16 h)	No. of peptides identified	12	9	2	2
	No. of miss-cleaved peptides	6	4	0	0
	Incomplete digestion (%)	14.1	31.2	0	0
Microwave heating at 50 °C (1 min)	No. of peptides identified	21	12	2	-
	No. of miss-cleaved peptides	17	8	1	-
	Incomplete digestion (%)	76.4	50.1	66.2	-
Microwave heating at 50 °C (30 min)	No. of peptides identified	22	12	3	-
	No. of miss-cleaved peptides	16	7	0	-
	Incomplete digestion (%)	58.0	48.8	0	-

Table 5. Comparison of BSA digestion under conventional and microwave heating conditions.

BSA		100 fmole/ μ L	10 fmole/ μ L	1 fmole/ μ L
Conventional digestion at 37 °C (16 h)	No. of peptides identified	28	13	2
	No. of miss-cleaved peptides	10	5	1
	Incomplete digestion (%)	24.51	35.22	21.11
Microwave at 50 °C (1 min)	No. of peptides identified	26	5	1
	No. of miss-cleaved peptides	14	3	1
	Incomplete digestion (%)	43.48	53.98	100
Microwave at 50 °C (30 min)	No. of peptides identified	27	13	2
	No. of miss-cleaved peptides	13	5	1
	Incomplete digestion (%)	41.27	23.97	33.3

Table 6. Comparison of *E. coli* proteins identified under conventional heating at 37 °C for 16 h, and microwave heating at 50 °C for 5 min and 1 min.

<i>Escherichia coli</i>				Conventional at 37 °C for 16 h		Microwave at 50 °C for 5 min		Microwave at 50 °C for 1 min	
1	Protein name	Protein Description	Mr	SQ%	x	SQ%	x	SQ%	x
2	OMPC	Outer membrane protein C	41200	15	4(1)	16	4(1)	3	1(0)
3	NMPC	Putative outer membrane porin protein NmpC	40277	14	5(1)	20	5(1)	3	1(0)
4	FTNA	Bacterial non-heme ferritin	19468	7	1(0)	7	1(0)	7	1(0)
5	LPP	Major outer membrane lipoprotein Lpp	8375	33	2(0)	33	2(0)		
6	RL7	50S ribosomal protein L7/L12	12288	19	2(0)	9	1(0)		
7	OMPA	Outer membrane protein A	37292	10	3(1)	2	1(1)		
8	TNAA	Tryptophanase	53139	3	1(0)	16	4(1)		
9	G3P1	Glyceraldehyde-3-phosphatedehydrogenaseA	35681	21	5(1)	20	3(2)		
10	ASPA	Aspartate ammonia-lyase	52950	4	2(0)	2	1(0)		
11	CH10	10 kDa chaperonin	10381	28	2(0)	28	2(0)		
12	OMPX	Outer membrane protein X	18648	5	1(0)	12	1(1)		
13	RS10	30S ribosomal protein S10	11728	24	2(1)	14	1(1)		
14	DLDH	Dihydrolipoyl dehydrogenase	50942	3	1(0)	3	1(0)		
15	DBHA	DNA-binding protein HU-alpha	9529	14	1(0)			14	1(0)

16	GRCA	Autonomous glycyl radical cofactor	14332	29	3(1)				
17	GRPE	Protein GrpE	21798	7	1(0)				
18	CH60	60 kDa chaperonin	57464	1	1(0)				
19	CYDA	Cytochrome bd-I ubiquinol oxidase subunit 1	58338	1	1(0)				
20	PFLB	Formate acetyltransferase 1	85588	1	1(1)				
21	TIG	Trigger factor	48149	6	2(0)				
22	RL9	50S ribosomal protein L9	15759	35	4(0)				
23	RS11	30S ribosomal protein S11	13950	12	1(0)				
24	EFTS	Elongation factor Ts	30518	9	2(0)				
25	DNAK	Chaperone protein DnaK	69146			5	2(1)		

SQ%: Sequence coverage; x: Number of identified peptides and the number of peptides with missed cleavage sites shown in parenthesis.

Figure Captions:

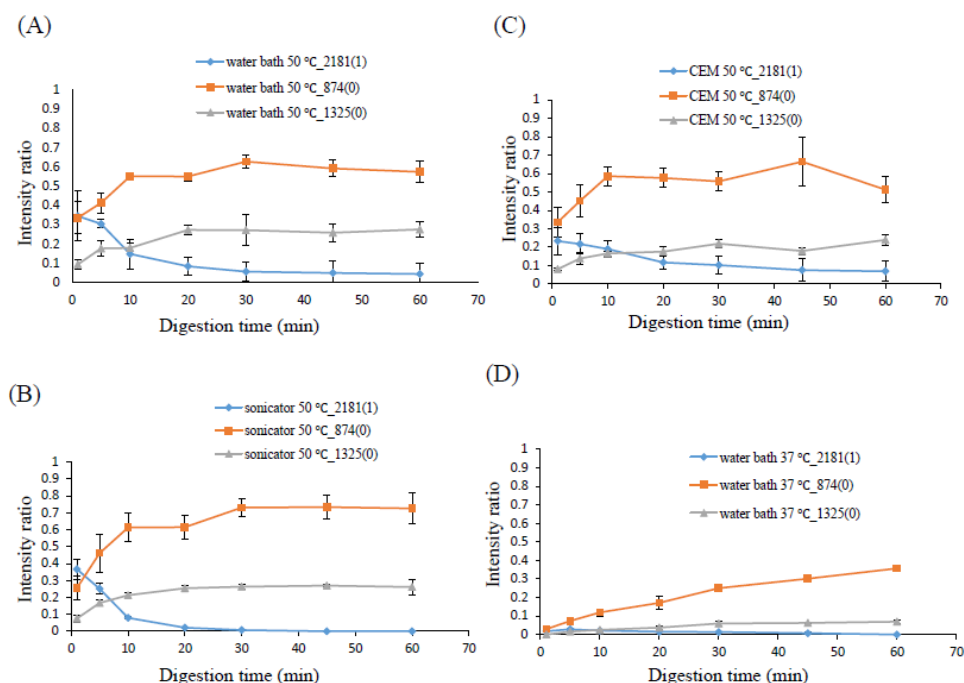


Figure 1. Comparison of relative intensities of lysozyme peptides at m/z 2181 (R15-33), m/z 874 (R15-21) and m/z 1325 (R22-33) with one, zero and zero missed-cleavage site, respectively, using angiotensin III as the internal standard, under four conditions: water bath heating (50 °C) (A), ultrasonic water-bath (50 °C) (B), CEM microwave (50 °C) (C), and water-bath heating (at 37 °C) (D) at various time intervals for 1 h.

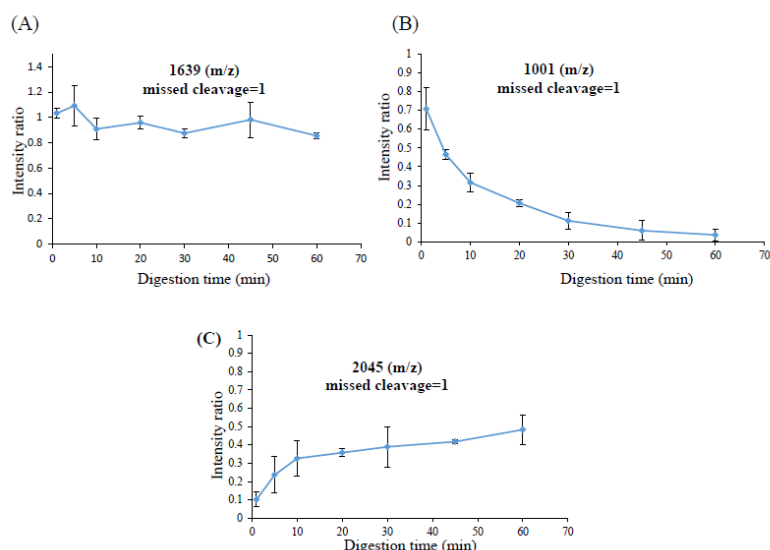


Figure 2. Comparison of relative intensities of BSA peptides at (A) m/z 1639 (R437-451 with one MCS), (B) m/z 1001 (R233-241 with one MCS), and (C) m/z 2045 (R168-183, with one MCS) vs the internal standard (angiotensin III at m/z 931), under microwave heating at 50 °C for various reaction times.

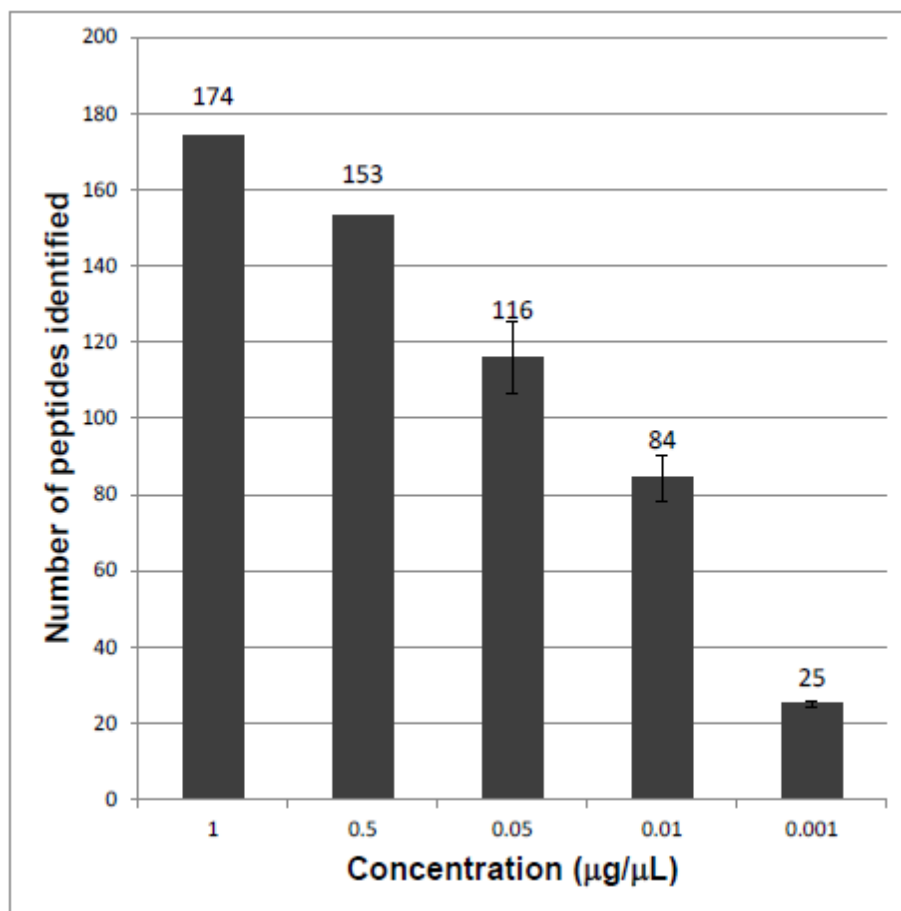


Figure 3. Number of peptides identified from various concentrations (1 to 0.001 µg/µL) of bacterial cells using LC-MS. Error bars when indicated represent standard deviation of three replicates.