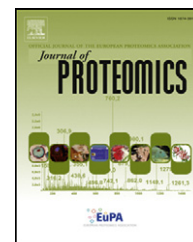


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Evaluating the potential nonthermal microwave effects of microwave-assisted proteolytic reactions

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ABSTRACT

Microwave-assisted proteolytic digestion methods have evolved into a highly effective approach and serve as an alternative to conventional overnight digestion. This approach typically exploits the unique microwave properties to facilitate the digestion of proteins into their peptides within minutes. Conventional digestion is carried out at 37 °C while microwave-assisted digestion requires much higher and sometimes inconsistent temperatures. Thus, this study aims to investigate whether the faster reaction rate is due to the microwave quantum effect or the thermal effect. Quantitative mass spectrometry was used to conduct kinetic analysis of tryptic digestion for several proteins by microwave and conventional heating. The percentages of digestion products relative to internal standards showed no significant difference between microwave and conventional heating conditions at the same digestion temperature. The optimum temperature for tryptic digestion was determined to be 50 °C. Furthermore, this study compares the digestion completeness indicators of several proteins under microwave and conventional heating. Again, the values obtained from microwave and conventional heating were similar given identical temperatures. The overall results prove that a nonthermal effect does not exist in microwave-assisted tryptic digestion. Therefore, conventional heating at high temperatures (50 °C) can be also used to accelerate digestion reactions.

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1. Introduction

Microwave heating is widely used as an efficient non-conventional energy source for causing chemical reactions [1,2]. In general, microwave reactions operate by agitating polar molecules that oscillate under the effect of the electromagnetic field. Recently, the advantages of microwave technology have also been exploited in fields including drug discovery [3], material sciences [4], proteomics [5,6], nanotechnology [7], and biochemistry [6,8,9]. Most related studies have shown that, compared to conventional heating techniques, microwave heating results in significantly reduced reaction times and enhanced product yields/purities [2]. There are two main hypotheses to explain the acceleration of chemical reactions (i.e., changes in reactivity and selectivity) under microwave irradiation. One holds that it is just a transfer of heat (i.e., a

thermal effect) while the other is based on specific radiation effect (i.e., microwave or nonthermal effect). The role of nonthermal effects in organic synthesis is highly contentious [2,8–12], with some researchers asserting that microwaves are simply a convenient means of heating [1,13], while others have produced experimental evidence of a nonthermal effect [2,14]. This problem was addressed in a recent review by Hoz et al. [2], who examined the evidence for nonthermal effects of microwaves on chemical reactions. They concluded that the microwave irradiation effect on a chemical reaction is very complex and involves thermal and nonthermal effects. Other studies have examined the nonthermal effects of microwaves on protein conformation and function [15–17].

Proteomics attempts to comprehensively elucidate biological processes by systematically analyzing the proteins expressed in a cell or tissue. The speed, accuracy, selectivity, and sensitivity

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of mass spectrometry (MS) have made it particularly useful for analyzing the tryptic digestion of proteins [18–20]. On the other hand, conventional proteolytic digestion methods are often time consuming. Several approaches were developed for rapid proteolysis through thermal denaturation [21], chemical denaturation [22], ultrasound [23], and enzymes immobilized on various supports [24,25]. In recent years, microwave-assisted proteolytic digestion has developed into a highly useful technique and an interesting alternative for conventional overnight digestion [5,6]. This technique normally exploits the unique properties of microwaves to cleave proteins into their peptides within minutes. Furthermore, microwave-assisted proteolytic digestion procedures, such as tryptic [5,26–29] and acid-mediated proteolysis [30–35], allow for the rapid preparation of samples for bottom-up analysis. Pramanik et al. [5] investigated how microwave irradiation affected protein digestion with trypsin in 10 min. That study examined proteins including cytochrome c, ubiquitin (tightly folded and resistant to denaturation), lysozyme, and myoglobin. Juan et al. [26] extended this approach to digest several known proteins in gel with trypsin under 5 min of microwave irradiation. Moreover, the protocol for microwave-assisted digestion was further optimized to analyze clinical samples [28].

Functionalized magnetic nanoparticles (NPs) were also used as efficient affinity probes and heat (microwave) absorbers for microwave-assisted proteolytic digestion methods [29,36–38]. Chen et al. developed an efficient method using magnetite beads to digest proteins in a domestic microwave device within 1 min. Wu et al. successfully applied quantum dots (QDs) as enrichment and accelerating probes for microwave enzymatic digestion of proteins [39–41]. The same group [42] proposed a washing and separation-free method via the use of TiO_2 NPs as an accelerating and affinity probe for the microwave-assisted tryptic digestion of proteins. They reported that TiO_2 NPs (which provide good heat absorption) produced higher sequence coverage (100%) than CdS QDs (95%) for cytochrome c.

Our previous study demonstrated that, under the influence of rapid microwave heating, enzymatic reactions can proceed in a solvent such as chloroform, which, under conventional digestion conditions, renders the enzyme inactive [43]. Another work investigated how the digestion process was affected by various solvents and reaction times, different enzyme to protein molar ratios, and varying microwave temperatures [44]. Microwave-assisted proteolytic digestion often yielded miss-cleaved peptides, resulting from incomplete hydrolysis reactions between enzymes and substrates. To investigate whether microwave irradiation catalyzes proteolytic digestions only by heat or by a combination of thermal and nonthermal energies, this study compares the effects of microwave irradiation and conventional heating on protein digestion given

identical reaction temperatures. Mass spectrometry is used to qualitatively and quantitatively determine the relative percentages of peptide digests vs internal standards for several proteins in various solvents, temperatures, different protein/enzyme (E/S) molar ratios and various enzymes, with and without microwave irradiation are determined. Comparisons are also made of the digestion efficiencies, number of miss-cleaved peptides (NMCP), incomplete digestion percentage (IDP), and sequence coverage of several proteins with and without microwave irradiation.

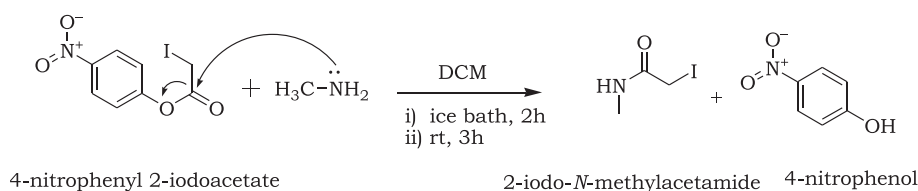
2. Experimental

2.1. Materials and chemicals

All reagents were of analytical grade and used as received. Ultrapure Milli-Q water was produced in the laboratory with a Millipore system (Bedford, MA, USA). HPLC grade acetonitrile (ACN), dichloromethane, and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Urea was from Wako (Osaka, Japan). Trifluoroacetic acid (TFA), 4-nitrophenyl iodoacetate, and glycoprotein hormone α (32–46) amide were purchased from Fluka (Buchs, SG, Switzerland). Immobilized trypsin (TPCK) was acquired from Pierce (Thermo Scientific, USA). Sequencing grade trypsin was obtained from Promega (Madison, WI, USA). α -Cyano-4-hydroxycinnamic acid (HCCA), ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), Tris buffer, lysozyme (chicken egg white), albumin (bovine serum), transferrin (human), β -lactoglobulin (bovine milk), cytochrome c (bovine heart), α -casein (bovine milk), myoglobin (horse heart) and trypsin (bovine pancreas) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylamine (40% w/w aqueous solution) was obtained from Alfa Aesar (Ward Hill, MA, USA).

2.2. Synthesis of N-methyliodoacetamide (MIAA)

MIAA was synthesized according to the reported procedure [45] with minor modifications. A volume of 43 μL aqueous methylamine (40%, 11.63 M, 2 equiv.) was added dropwise to an ice-bath cooled solution of 4-nitrophenyl iodoacetate (76.7 mg, 0.25 mmol, 1 equiv.) in anhydrous dichloromethane (5 mL) over 20 min. 4-Nitrophenol was precipitated during the reaction (Scheme 1). The reaction mixtures were stirred for an additional 2 h at 0 °C and 3 h at room temperature. The resulting crude products were filtered through a paper filter to remove 4-nitrophenol. The filtrate was dried with 1 g of anhydrous Na_2SO_4 and concentrated in a rotary evaporator at 40 °C with a water vacuum pump. Chromatography (SiO_2 ,



Scheme 1 – Synthesis of N-methyliodoacetamide.

1.5×15 cm, 3–5% EtOAc/CH₂Cl₂, gradient elution) afforded MIAA as white crystals (42 mg, 86%). The purity of the product was greater than 99% (HPLC). The product is stable at room temperature without decomposition.

2.3. Quantitative analysis

Protein samples were dissolved in 3 μ L of 6 M urea solution and 30 μ L of 10 mM DTT in ammonium bicarbonate (25 mM, 50 mM) was added into the sample solution. The pH value of all the buffers used through the entire experiments was adjusted to 8.5. The reduction was carried out at room temperature for 1 h. Cysteine residues were alkylated by adding 25 mM IAA or MIAA and the reaction was performed in the dark at room temperature for 1 h. The solution was reacted with 10 mM DTT (30 μ L) to remove an excess of the IAA or MIAA. To test the quantitative linearity, aliquots of protein samples ranging from 3 pmol to 30 fmol were derivatized with IAA and MIAA, respectively, and then mixed at molar ratios ranging from 0.1 to 10. The samples were subjected to conventional in-solution digestion prior to MALDI-MS analysis. For quantitative analysis, MIAA derivatized peptides in tryptic digests were directly used as internal standards without further purification.

2.4. Enzymatic digestion

The protein samples obtained after IAA or MIAA alkylation were diluted with 25 mM of ammonium bicarbonate. The MIAA alkylated protein samples were digested with trypsin for 18 h. Then the known amount of protein digests (containing MIAA labeled peptides) was spiked into IAA alkylated protein samples for quantitative analysis. In-solution digestion was performed by adding trypsin into the mixtures at enzyme to protein molar ratios of 1:5/1:25/1:50 and the solutions were incubated at various temperatures. For microwave-assisted digestion, sample solutions were digested with trypsin under microwave irradiation at various enzyme to protein molar ratios and temperatures. The final sample volume was 250 μ L. All the reactions were performed in a 600- μ L Eppendorf tube with stirring. The Eppendorf tube was sit in a 10-mL Pyrex tube designed for a commercial microwave system. The digestion was quenched by the addition of 0.1% TFA prior to MS analysis.

2.5. Microwave irradiation

Microwave-assisted proteolytic digestions were accomplished with a CEM Discover microwave system (Matthews, NC, USA).

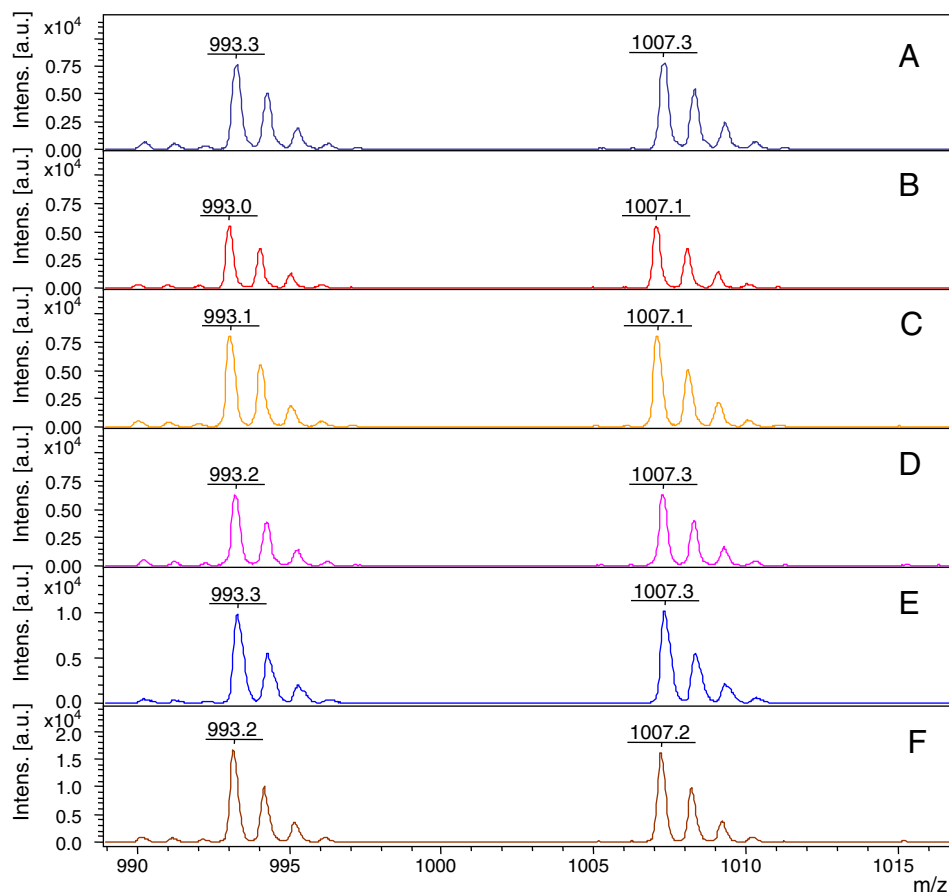


Fig. 1 – MALDI mass spectra of a mixture of the IAA- and MIAA-labeled tryptic digests of lysozyme after a second digestion at various time intervals: (A) 5 min, (B) 15 min, (C) 20 min, (D) 30 min, (E) 45 min, and (F) 60 min. The IAA-labeled tryptic digest was added into the MIAA-labeled tryptic digest after the MIAA-labeled digest was subjected to the second digestion. The original molar ratio between IAA and MIAA-labeled proteins was 1:1.

The maximum output power is 300 W and the operating frequency is 2.5 GHz. This system has a single-mode cavity design, with temperature feedback control. A noncontact infrared sensor, which monitors the temperature of the sample vial surface, is located beneath the reaction vessel. The set reaction temperature was maintained by using a sensor-controlled magnetron and by varying the microwave power in the range between 1 and 50 W. The actual internal temperatures inside the reaction solutions were measured directly by a PCM-1-100 ST fiber-optic sensor probe (Opsen, Canada).

2.6. Mass spectrometry

The MS analysis of each protein digest was conducted immediately after the enzymatic reaction. Mass spectra were obtained 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). Laser power was optimized until the best quality spectra were obtained. Spectral data were obtained in the reflectron positive mode with an acceleration voltage of 20 kV. Each mass spectrum was derived from 100 summed scans. Flex Control (Ver.2.0) and Flex Analysis (Ver.2.2) software were used for data acquisition. The MALDI matrix was prepared by saturating HCCA in 50% of ACN

solution containing 0.1% TFA. A 0.5 μ L aliquot of sample solution was mixed with an equal volume of the HCCA matrix solution, then spotted onto the target plate, and dried before MALDI-MS analysis. Each MALDI-TOF MS analysis was performed in triplicate and error bars denote standard deviations. Mass peak lists were submitted for peptide mass fingerprinting using the MASCOT search engine and searched against MSDB database (taxonomy: all entries). The searching parameters were set as follows: a mass tolerance of 200 ppm, two missed-cleavage sites, variable modifications of cysteine carbamidomethylation and methionine oxidation.

3. Results and discussion

The thiol groups of cysteine residues in proteins were alkylated using IAA and MIAA to measure protein digests quantitatively via MALDI-MS [46]. The reagents IAA and MIAA were introduced during the alkylation step of a common protein denaturation and digestion procedure [46]. Quantitative analysis using the IAA and MIAA labeling approach has been previously demonstrated. For instance, when the IAA and MIAA derivatized lysozyme peptides were mixed in various ratios ranging from 0.1 to 10, the observed relative ratios correlated well with the

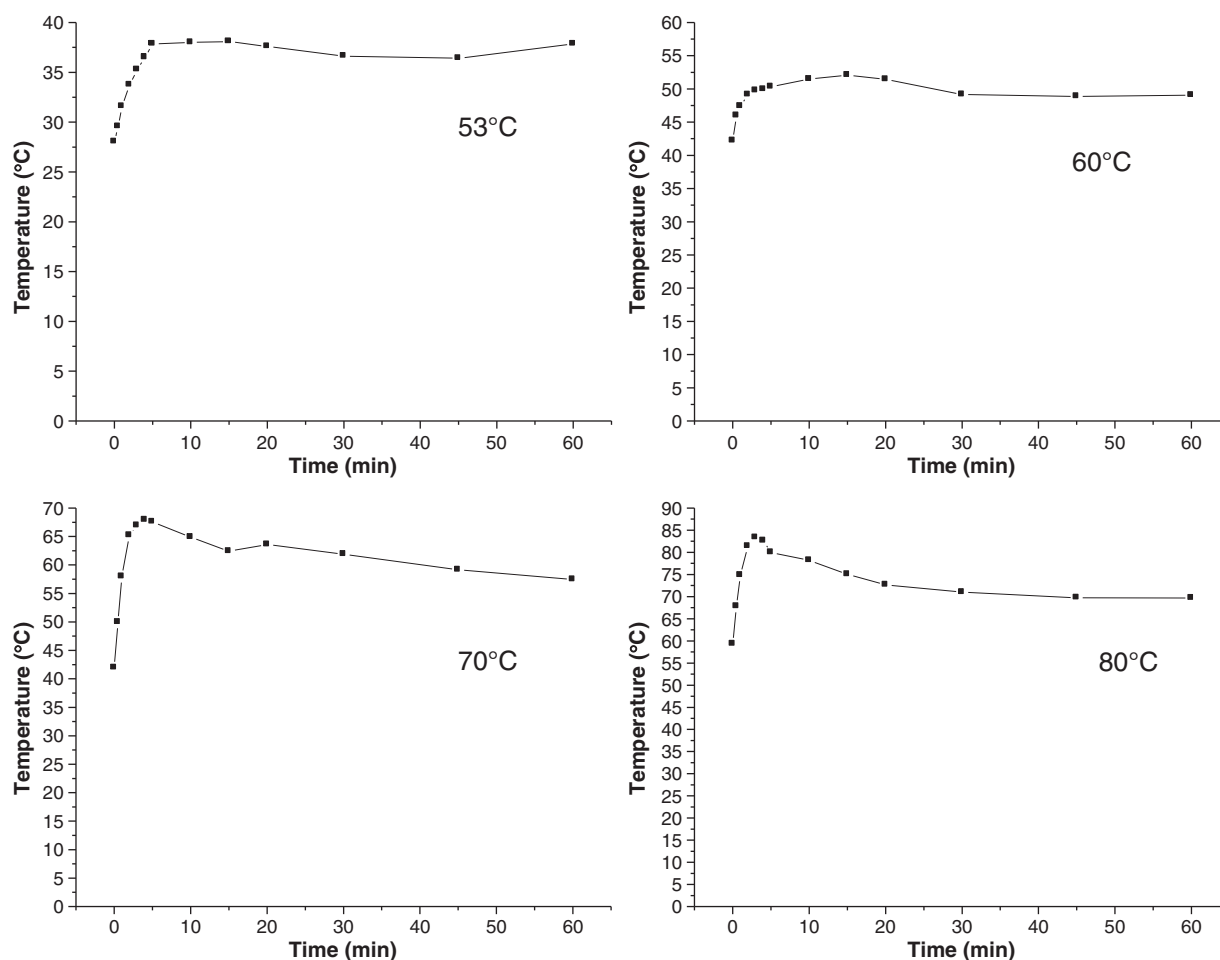


Fig. 2 – Measurement of temperature profiles using a fiber optics thermometer. Note the difference between the measured temperatures and set values (indicated in the figures) using the built-in temperature sensing devise.

theoretical ratios (see Supporting information Fig. S1). The calibration curve for peptide WWCNDGR has a good dynamic range with slope=1.16 and $R^2=0.9987$. The relative error in the ratios was less than 10%. For the quantitative analysis in this study, MIAA-derivatized peptides in tryptic digests were used directly as internal standards without further purification. The MIAA-labeled protein digest was added to an IAA-labeled protein solution during microwave-assisted or conventional digestion. To ensure that trypsin did not further digest the internal standard, two aliquots of protein solutions were labeled with MIAA and IAA, respectively and digested with trypsin at 37 °C for 18 h. The MIAA-labeled protein digest was subjected again to tryptic digestion for various time intervals. Following the second digestion, the MIAA-labeled solution was mixed with the IAA-labeled protein digest (original protein ratio 1:1). The peak ratios of the labeled peptides were determined using MALDI-TOF-MS. Panels (A)–(F) in Fig. 1 represent MALDI mass spectra of a mixture of the IAA- and MIAA-labeled tryptic digests of lysozyme after the second digestion at various time intervals. The peak ratio of IAA- and MIAA-derivatized peptides did not change, implying that the first digestion was complete and the internal standard was not digested or produced after the second tryptic digestion.

Conventional IR sensors on the outside vessel wall fail to monitor accurately reaction temperatures in microwave-assisted reactions, easily leading to a misinterpretation of results [13]. This study compared the results obtained by direct microwave heating on proteolytic digestions with the outcome of conventionally heated proteolytic digestions by monitoring the internal digestion temperature, through use of a fiber-optic thermometer. In the experiments, microwave temperature settings were 53 °C, 60 °C, 70 °C, and 80 °C, respectively. Temperatures were subsequently measured at various time intervals, i.e. 0, 0.5, 1, 2, 3, 4, 5, 10, 15, 30, 45 and 60 min. The temperature increased initially; the temperatures then stabilized less than 10 min later (Fig. 2). The temperatures were then measured in triplicate using the fiber-optic thermometer. The real average temperatures were found to be 37 °C, 49.8 °C, 61.4 °C, and 72.6 °C, respectively, i.e. lower than our microwave settings. These temperatures were then rounded off to 37 °C, 50 °C, 62 °C, and 73 °C, respectively, with these temperatures used in the direct comparison of microwave heating with conventional thermal heating.

The microwave effect was investigated by performing protein digestion (in 25 mM NH_4CO_3 buffer, at an enzyme/protein ratio of 1:25, pH 8.5) for various digestion times under

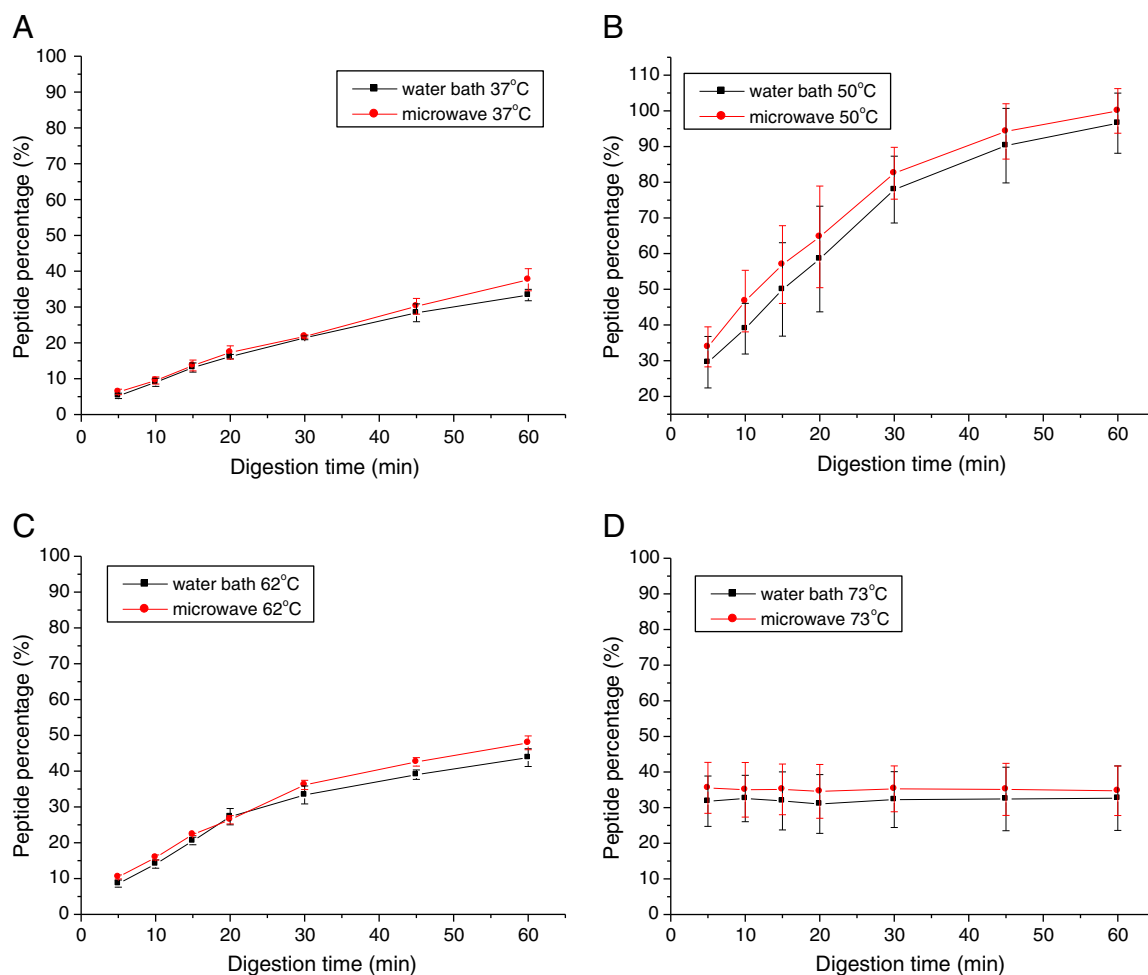


Fig. 3 – Comparison of digestion percentages of lysozyme peptide (WWCNDGR) under microwave heating and conventional heating at (A) 37 °C, (B) 50 °C, (C) 62 °C, and (D) 73 °C for different time points.

microwave heating and conventional incubation at 37 °C, 50 °C, 62 °C, and 73 °C, respectively. Four proteins (i.e. lysozyme, β -lactoglobulin, bovine serum albumin (BSA), and transferrin) were investigated. The peptides with zero missed cleavage and without interference from peptide masses near those of the target peptides were selected for quantification. The results were analyzed by MALDI-MS to determine the amount of target peptides produced for each time interval at various temperatures. The percentages of digested peptides relative to the internal standards were calculated. Fig. 3 illustrates the relative percentages of the lysozyme peptide (WWCNDGR) under microwave heating and conventional heating at various time intervals and temperatures. The peptide percentages of the microwave-assisted digestion and conventional digestion for 1 h at 37 °C, 50 °C, and 62 °C were 30–35%, 95–100%, and 40–45%, respectively. A previous study has found an optimum temperature range from 50 to 65 °C for trypsin digestion [47]. Under our conditions, the reaction at 50 °C produced an optimum digestion percentage. When the digestion temperature was 73 °C, the percentage of digestion leveled off at approximately 35% after 5 min, implying no further increase in the yield and ultimately that the trypsin activity had been destroyed. In addition to

accelerating the reaction, the high temperature decreased the enzyme activity as well. The opposite effects of rate acceleration and enzyme deactivation were also reflected in the initial peptide percentage. The 5 min percentages at 50 °C, 62 °C, and 73 °C were 30%, 10%, and 32%, respectively. The combination of two opposite trends in peptide production yielded a minimum (for instance, 10%) lying in-between 50 °C and 73 °C.

Of all the proteins examined, β -lactoglobulin was easily digested under our experimental conditions (100% digestion completeness in 5 min based on the selected peptide, see Supporting information Fig. S2). The digestion percentages of BSA at various temperatures with and without microwave irradiation are shown in Supporting information Fig. S3. The minimum caused by the two opposite trends in peptide percentages at 5 min was not obvious for BSA. It is because the digestion percentages at 62 °C and 73 °C were low and only 6% was obtained at 73 °C before the enzyme lost all its activity. Carefully comparing the conventional digestion with the microwave-assisted digestion revealed no significant difference in the peptide percentages between digestions carried out under thermal heating or microwave heating at the same temperatures. It is noted that a single peptide is not enough to

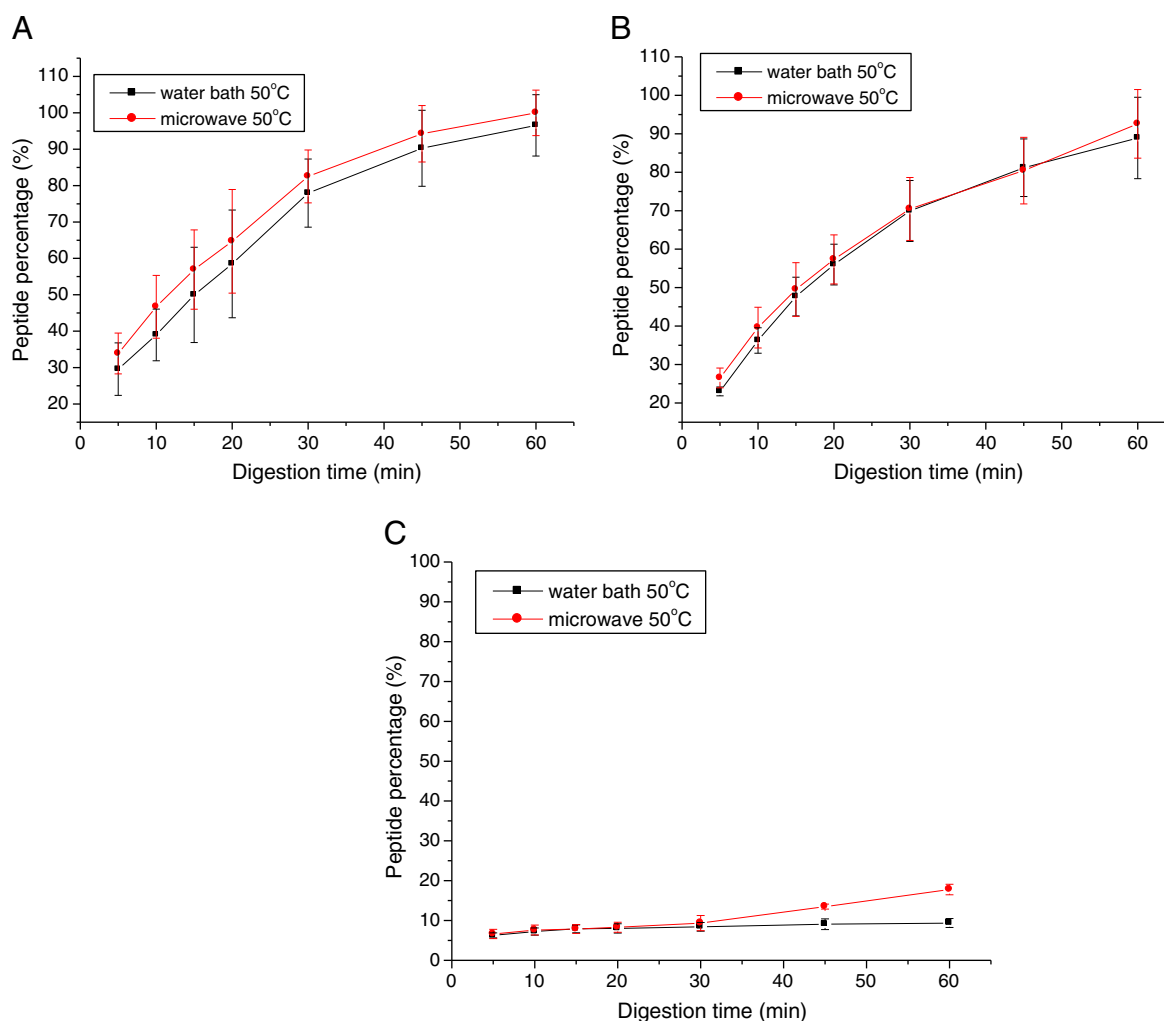


Fig. 4 – Comparison of digestion percentage of lysozyme peptide (WWCNDGR) in (A) 25 mM NH_4HCO_3 , (B) 50 mM Tris, and (C) 50 mM Tris buffer (35% ACN) solvents at 50 °C under microwave and conventional thermal heating.

describe the digestion progressing of a protein. Some peptides might be produced faster than the others. However, one peptide is enough to make comparisons between microwave irradiation and conventional heating.

This study also investigated the digestion percentage of the four proteins in various solvents under microwave/conventional heating at 50 °C for different time intervals. Fig. 4 shows the digestion percentage of lysozyme in various solvents at 50 °C under microwave and conventional heating for 0–60 min. The digestion percentage of lysozyme was 95–100% in 25 mM NH_4HCO_3 as well as 50 mM Tris buffer. Previous studies have indicated that organic solvent-containing solutions denature proteins and increase the sequence coverage of protein digests [43,44]. However, the peptide percentage of lysozyme in 50 mM Tris buffer containing 35% ACN at 50 °C under microwave and thermal heating was lower than 15% (Fig. 4C). For most studies, the digestion performance is based on qualitative analysis such as sequence coverage. Improved sequence coverage does not necessarily mean a better digestion efficiency unless quantitative analysis is carried out. Based on the results shown in Fig. 4, the digestion efficiency of lysozyme in 25 mM NH_4HCO_3 as well as 50 mM Tris buffer under microwave heating provided higher digestion percentages than organic solvent-containing

buffers do. Overall, the results under microwave heating almost resemble those obtained from conventional heating under the same conditions. Previous study in qualitative MS and SDS-PAGE analysis of the protein digestion reaches similar conclusions as those found in the current report [48]. The extensive quantitative analysis in the present study provides more accurate and convincing support for the nonexistence of microwave effect.

Fig. 5 illustrates the digestion percentages of transferrin in 25 mM NH_4HCO_3 at 50 °C under microwave and conventional heating at various E/S ratios for 0–60 min. As is expected, the digestion percentage increased significantly when the E/S ratio changed from 1:50 to 1:5. Transferrin appeared to be difficult to digest. According to the results shown in Fig. 5, conventional and microwave-assisted protein digestion somewhat differ in digestion efficiency. However, their difference is greater than those for other proteins such as lysozyme and BSA. The differences should be attributed to the experimental error.

Next, the digestion process was studied by varying the enzymes under microwave and conventional heating at different time intervals. The enzymes, including Promega trypsin, Sigma trypsin and immobilized trypsin, were applied to the digestion of proteins with and without microwave irradiation. Fig. 6 presents the digestion percentages of BSA in various

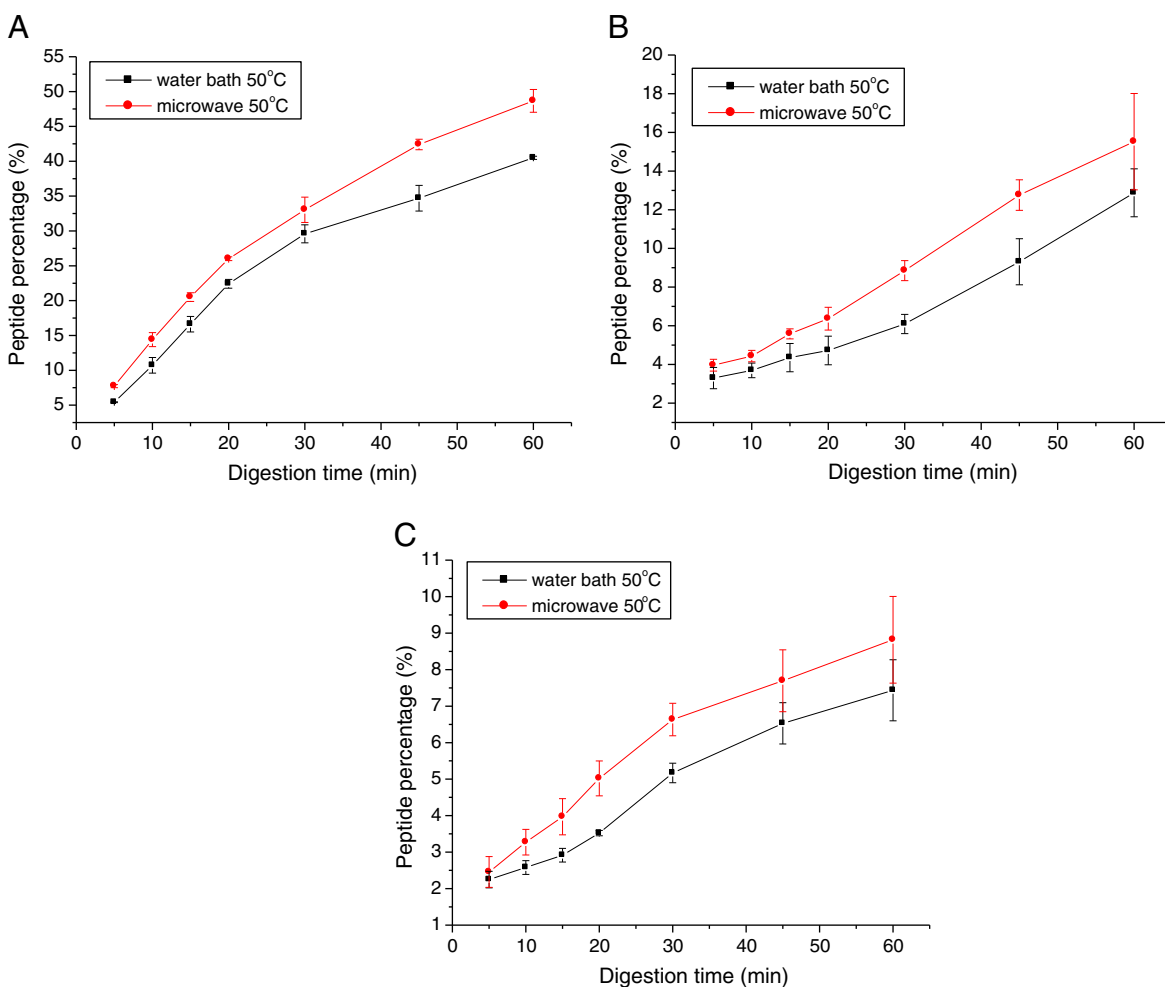


Fig. 5 – Comparison of digestion percentages of transferrin peptide (KPVEEYANCHLAR) in 25 mM NH_4HCO_3 at 50 °C under microwave and conventional heating at (A) 1:5, (B) 1:25, and (C) 1:50 E/S ratios for 0–60 min.

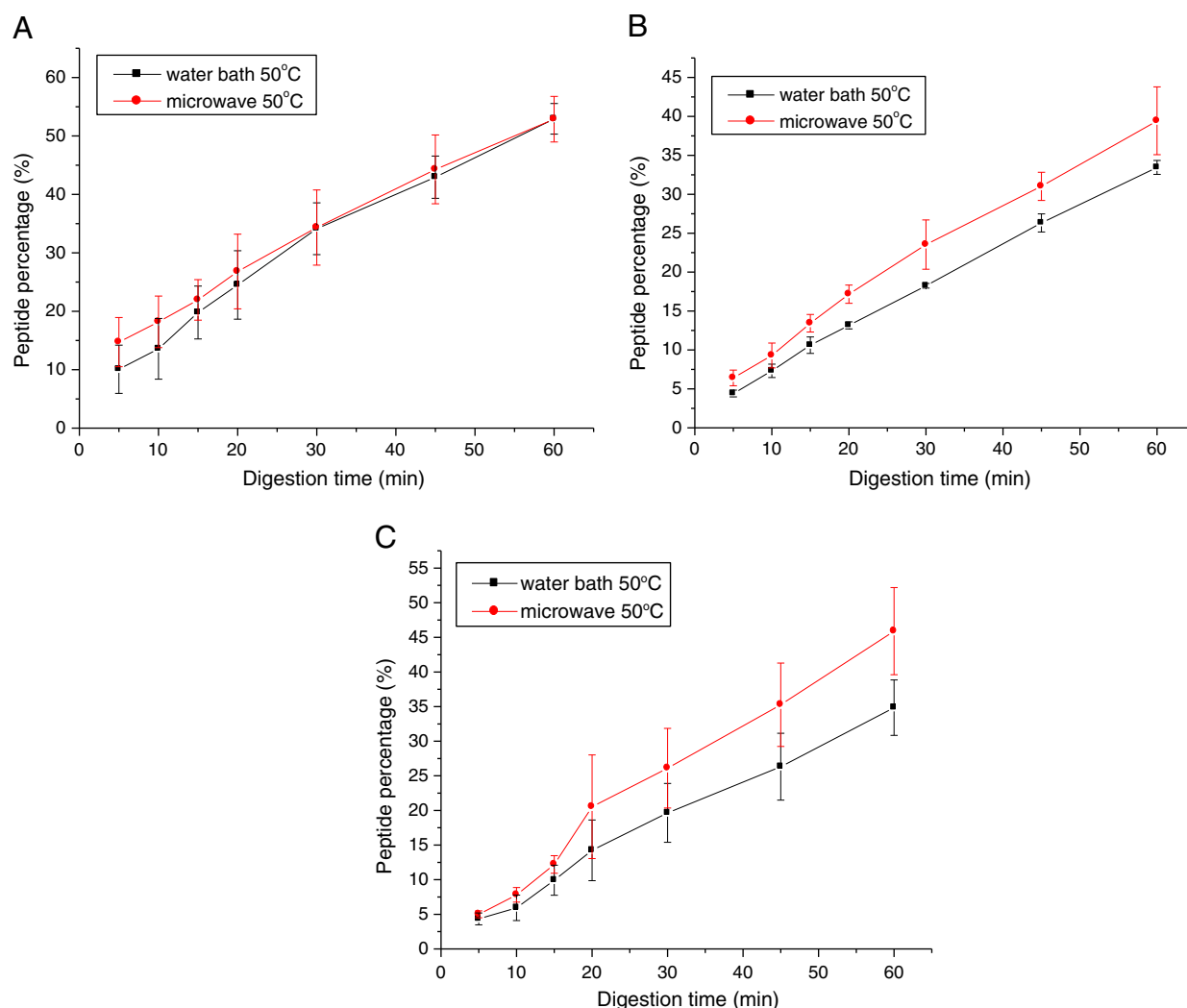


Fig. 6 – Comparison of digestion percentage of BSA peptide (RPCFSALTPDETYVPK) using (A) Promega, (B) Sigma, and (C) immobilized trypsin under microwave and thermal heating at 50 °C for 0–60 min.

enzymes under microwave and thermal heating at 50 °C for 0–60 min. According to this figure, the digestion percentage for Promega trypsin is higher than that for Sigma trypsin. Since the exact amount of immobilized trypsin is unknown, the immobilized enzyme cannot be compared with the other two enzymes. Overall results demonstrate that conventional and microwave-assisted protein digestions do not significantly differ from each other. The difference shown in Fig. 6C seems to be an experimental deviation due to the heterogeneous factor of immobilized trypsin.

This study also examined the incomplete digestion percentage (IDP), sequence coverage, number of miss-cleaved peptides (NMCP) and digestion efficiency of several proteins (cytochrome c, α -casein, BSA, myoglobin) at various temperatures with and without microwave irradiation. IDP refers to the ratio between the abundance of peptides with missed cleavage site(s) and the total abundance of peptides detected. Digestion efficiency is defined here as the ratio of the intensity of the most abundant peptide fragment ion to that of this peptide plus undigested protein. The value is only used to indicate the presence of undigested proteins. Fig. 7 illustrates MALDI-TOF mass spectra

of the tryptic fragments of cytochrome c obtained from conventional heating at 50 °C for 30 min (Fig. 7A) and microwave irradiation at 50 °C for 30 min (Fig. 7B). The spectra are similar, and the results correlate with the time-plot data. Table 1 summarizes the IDP, sequence coverage, NMCP and digestion efficiency of cytochrome c obtained from Fig. 7. The IDP and sequence coverage under microwave heating are 76 and 85%, respectively. The values under conventional heating are 73 and 88%. The numbers are comparable between conventional and microwave heating. Similarly, the number of miss-cleaved peptides is nearly the same, 14 (out of 19) vs 15 (out of 20). Tables S1–S3 in Supporting information list the values for other proteins (α -casein/BSA/myoglobin). Many studies have reported that microwave irradiation can accelerate enzymatic reactions. According to our results, the thermal effect increases the digestion rate. Moreover, although microwave-assisted reactions (at temperature greater than 37 °C) are faster than those under conventional overnight heating at 37 °C, the reactions are generally incomplete. Most related studies are based on qualitative experiments that yield good sequence coverage. In this study, combining the quantitative and qualitative results

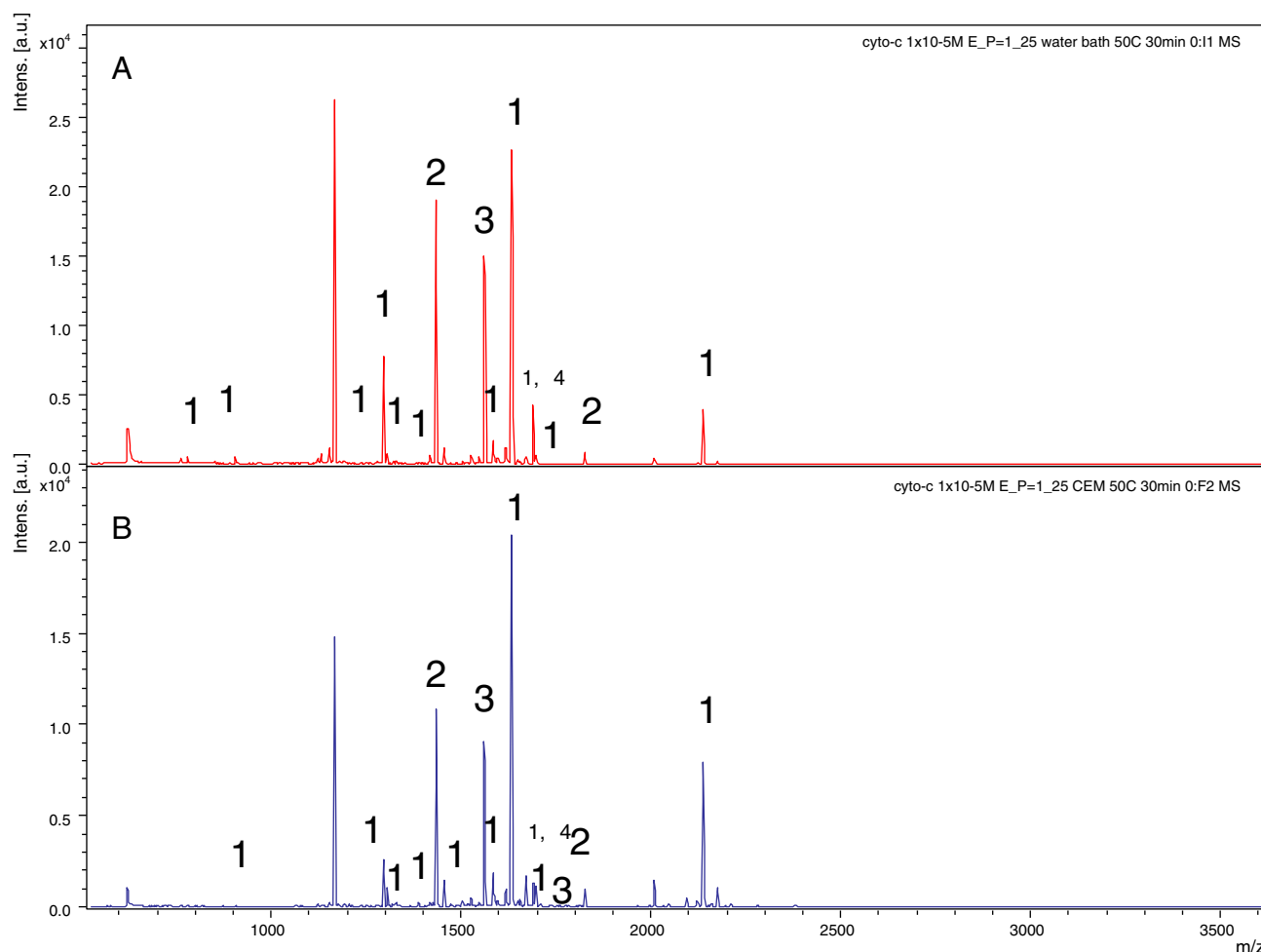


Fig. 7 – MALDI mass spectra of the tryptic digest of cytochrome c obtained from digestion in 25 mM NH_4HCO_3 with an E/S ratio of 1:25 under conventional heating at (A) 50 °C for 30 min, and microwave irradiation at (B) 50 °C for 30 min. The peaks marked 1, 2, 3 and 4 correspond to the peptides with one, two, three and four missed cleavage sites, respectively.

shows that the high sequence coverage often arises from more miss-cleaved peptides due to incomplete digestion. Under conventional heating conditions (25 mM NH_4HCO_3 , E/S of 1:25 at 37 °C for 12 h), the IDP, sequence coverage, NMCP and digestion efficiency for cytochrome c were 46%, 75%, 10 out of 17, and 100%, respectively (Table 2). The incomplete digestion percentage (46%) is significantly lower than that (76%) under microwave irradiation at 50 °C for 30 min. Moreover, the sequence coverage is lower for conventional heating than for microwave irradiation (75% vs 85%). Therefore, we conclude

that evaluation of tryptic reactions cannot be based only on sequence coverage. Incomplete digestion percentage and other quantitative analyses are required to make a reliable comparison between microwave and conventional heating. Table 2 also lists the digestion data of cytochrome c obtained from the reaction performed under conventional heating at 37 °C for 30 min. The sequence coverage was 87%, which is higher than that for 12-hour digestion. However, the reaction is certainly not complete because the digestion efficiency is 97%, suggesting the existence of undigested intact protein.

Table 1 – Comparison of cytochrome c digestion at 50 °C for 30 min under microwave and conventional heating.

	CEM (100 W)	Water bath
Incomplete digestion: (%)	76%	73%
Sequence coverage: (%)	85%	88%
Number of missed cleaved peptides (total peaks)	14 (19)	15 (20)
Digestion efficiency: (%)	100%	100%

Table 2 – Digestion of cytochrome c under conventional heating at 37 °C for 12 h and 30 min.

	12 h	30 min
Incomplete digestion: (%)	46%	79%
Sequence coverage: (%)	75%	85%
Number of missed cleaved peptides (total peaks)	10 (17)	15 (21)
Digestion efficiency: (%)	100%	97%

4. Conclusions

A critical examination of the proteins discussed herein provides no evidence of nonthermal microwave effects. The inferences of this study should be solid and convincing because the data acquired under many conditions and at many reaction time points up to 60 min were compared. Based on the results of this study, we conclude that most of the earlier observed differences between experiments performed under microwave and conventional heating conditions for proteolytic digestions are owing to the temperature effect. Moreover, the rapid enzymatic digestion is likely incomplete if the reaction is performed for a few minutes. Therefore, both microwave and heating bath can be used to accelerate tryptic digestion. Although microwave may be a convenient way of heating, temperature control is relatively difficult especially for the domestic microwave. The unstable temperature might incur higher experimental errors in quantitative analysis. Furthermore, the incomplete digestion causing less production of certain target peptides decreases sensitivity in both the qualitative and quantitative analyses.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.01.005>.

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