Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: A new approach to protein digestion in minutes

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(RECEIVED May 3, 2002; FINAL REVISION July 19, 2002; ACCEPTED July 28, 2002)

Abstract

Accelerated proteolytic cleavage of proteins under controlled microwave irradiation has been achieved. Selective peptide fragmentation by endoproteases trypsin or lysine C led to smaller peptides that were analyzed by matrix-assisted laser desorption ionization (MALDI) or liquid chromatography-electrospray ionization (LC-ESI) techniques. The efficacy of this technique for protein mapping was demonstrated by the mass spectral analyses of the peptide fragmentation of several biologically active proteins, including cytochrome c, ubiquitin, lysozyme, myoglobin, and interferon α -2b. Most important, using this novel approach digestion of proteins occurs in minutes, in contrast to the hours required by conventional methods.

Keywords: Microwave irradiation; accelerated protein digestion; mass spectrometry; selective endoprotease reactions

Advances in recombinant DNA technology have resulted in the production of a variety of therapeutic proteins. Recent progress in genomics and proteomics research has also identified many new proteins (Hanash 2000; Pandey and Mann 2000; Washburn and Yates 2000; Washburn et al. 2001). Two ionization techniques—electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) ionization—have greatly expanded the role of mass spectrometry (MS) in molecular weight determination of intact proteins as well as their post-translationally modified variants, even with molecular masses exceeding 500 kDa (Fenn et al. 1989; Hillenkamp et al. 1991; Chait and Kent 1992; Mirza et al. 2000).

To obtain detailed structural information, proteins are selectively cleaved into smaller polypeptide fragments by

controlled chemical or enzymatic reactions. The resulting mixture is then analyzed by MALDI-MS or LC-ESI-MS (Gibson and Biemann 1984; Griffin et al. 1991; Davis and Terry 1992). This method of protein analysis is known as peptide mapping (Chowdhury et al. 1990; Yates et al. 1993; Nguyen et al. 1995). A useful source for gaining a broad understanding of the application of LC-ESI-MS to the analysis of peptides and proteins is described in the book *Applied Electrospray Mass Spectrometry* (Pramanik et al. 2002).

Each polypeptide fragment can be further studied by tandem mass spectrometry (MS/MS) for the verification of the amino acid sequence and the determination of any post-translational modification of the original protein. In addition, the peptide map data or the amino acid sequence information generated in this manner can be used in a genome or protein database search for protein identification. Thus, enzymatic cleavage for producing smaller fragments of the analyte is an important step in the characterization of the structure of proteins.

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0213702>.

In recent years microwave irradiation has been shown to accelerate organic reactions (Gedye et al. 1986; Giguere et al. 1986; Bose et al. 1990, 1997, 2002b; Erickson 1998; Lidstrom et al. 2001; Richter et al. 2001; Larhed et al. 2002). For example, reactions that require several hours under conventional conditions can be completed in a few minutes. In an effort to avoid the risk of possible explosions, Bose and coworkers (1997, 2000) under the name of microwave-induced organic reaction enhancement (MORE) chemistry have carried out reactions in an open flask using limited amounts of higher boiling solvents such as acetonitrile, *N,N*-dimethyl formamide (DMF), and chlorobenzene. Microwave irradiation has been applied by synthetic chemists to improve chemical processes or in modifying chemo-, regio-, or stereo-selectivity. A comprehensive review of this subject was published (Caddick 1995; Hong et al. 2001). Additional reports are cited in the literature illustrating the breadth of microwave chemistry, which cover such areas as heterocycles, natural products, peptides, catalytic reactions, and other medicinal compounds (Suib 1998; Erdelyi and Gogoll 2001; Kabalka et al. 2001).

In the area of peptide biochemistry, the use of microwave-assisted reactions has been very limited. Chen and coworkers (1991) used microwave irradiation to accelerate the hydrolysis of peptides and proteins with 6 M HCl in a sealed tube. In a recent report, microwave-enhanced modification of the Akabori reaction (Akabori et al. 1952) for the identification of the carboxy-terminal amino acid residue of peptides (and also the amino acid sequence of some segments of the peptide starting at the amino terminus) has been developed in our laboratory (Bose et al. 2002a). The key hydrazinolysis step of the Akabori reaction can be completed in 5 min–10 min under microwave irradiation in an open vessel, whereas the conventional technique requires heating in a sealed tube for several hours. In this report, we describe the use of microwave technology for the enzymatic digestion of proteins. Under microwave irradiation, rapid and selective enzymatic proteolysis of several proteins was achieved.

Results and Discussion

Microwave-enhanced trypsin digestion of bovine cytochrome c

Initial studies of microwave digestion of proteins were carried out on bovine cytochrome c, a globular protein relatively resistant to enzymatic cleavage under nondenaturing conditions (Mirza et al. 1993). The protein was treated with trypsin at a 1:25 protease-to-protein ratio (by weight) and the solution was subjected to microwave irradiation for various time intervals as described in Materials and Methods. The temperature of the apparatus was set at 37°C throughout the digestion. The reaction was quenched with 0.1%

trifluoroacetic acid and the products were analyzed by MALDI-MS. Dramatically, within 10 min of microwave irradiation, extensive cleavage products of the protein were generated as shown in the MALDI mass spectrum of the digested sample (Fig. 1). Most of the expected tryptic peptides were observed in the spectrum, except for the fragment corresponding to sequences 1–7. Very low abundance signals corresponding to singly and doubly charged molecular ions were observed in the spectrum (data not shown). No attempt was made to determine the amount of the undigested protein.

Figure 1B shows the coverage of cytochrome c obtained based on Figure 1A. Mass of each tryptic fragment in Figure 1A (which corresponds to a part of the cytochrome c sequence) is shown in the form of a horizontal line in Figure 1B. Tryptic fragments discussed in this paper are denoted by $T_1, T_2, T_3, \dots, T_n$. All those peaks that match the protein sequence are shown in the form of horizontal lines; they represent the extent of the coverage of the protein from the amino-terminus to the carboxyl -terminus in Figure 1B. It is important to note that a number of signals corresponding to incomplete cleavages were observed in the MALDI spectrum (Fig. 1 and Table 1). We have found previously that this type of cleavage occurs where two contiguous cleavage sites are present (K-K, R-R, K-R) or those cleavage sites are in close proximity (Pramanik et al. 1991).

A parallel experiment was conducted using a trypsin-to-cytochrome c ratio of 1:25; this time the digestion mixture was maintained at a temperature of 37°C for 6 h (classic approach) (Lee and Shively 1990). Figure 2A shows the MALDI mass spectrum of the resulting tryptic fragments and Figure 2B shows the corresponding coverage of the protein. Almost 88% of the protein coverage was achieved from this digestion; only two peptide fragments corresponding to sequences 1–8 and 23–27 were not observed (Table 2). A comparison of these two approaches of trypsin digestion of cytochrome c demonstrates that microwave irradiation for 10 min produced similar results to the classic method in 6 h of digestion.

Additional studies were conducted to compare the extent of tryptic peptides generation from cytochrome c in 12 min at 37°C under microwave irradiation versus the classic method at 37°C. Figure 3A shows the MALDI mass spectrum of the 12-min microwave-assisted trypsin digest of cytochrome c (1:25, w/w) at 37°C. Polypeptide signals exhibited in the spectrum provided 90% sequence coverage of cytochrome c. The signals correspond to singly charged (m/z 12,234) and the doubly charged (m/z 6,117) ions in spectrum (Fig. 3A) represent the presence of intact cytochrome c in the sample. Figure 3B describes the MALDI data for a 12-min trypsin digestion at 37°C without microwave for cytochrome c (1:25, w/w). This experiment shows no hydrolysis products of the protein. The observed ion peak at m/z 1,281 and the other weak lower mass ions do not

Fig. 1. (A) MALDI mass spectrum of tryptic fragments of cytochrome c after 10 min of microwave irradiation at 1:25 protease-to-protein ratio by weight and (B) tryptic fragments in A are represented in the form of horizontal lines, showing the total sequence coverage of cytochrome c.

correspond to the tryptic fragments of cytochrome c. The two abundant ions at higher masses (m/z 12,236 and 6,118) corresponded to the intact cytochrome c. Thus, microwave irradiation greatly accelerates the digestion process, providing complete mapping of the protein in minutes, whereas the classic method provides no cleavage products.

In another experiment, cytochrome c was subjected to microwave irradiation in the absence of a protease. After 20 min of microwave irradiation of the protein solution, MALDI-MS did not yield signals in the lower mass region corresponding to peptide fragments. Instead, the intact protein was detected (data not shown). This result confirms that microwave irradiation only assists and enhances the enzymatic digestion of proteins; it does not induce degradation/breakdown of the protein in the absence of a protease.

Microwave-assisted trypsin digestion of bovine ubiquitin

The study was continued with another protein, bovine ubiquitin, which is a tightly folded protein that is extremely

resistant to denaturation. The stability of the protein has been attributed to the pronounced hydrophobic core and that 90% of the residues in the polypeptide chain appear to be involved in intramolecular hydrogen bonding. In this study, microwave experiments were performed at various ratios of enzyme-to-protein concentrations. These results were compared with the data obtained from the classic methods.

Figure 4A shows the coverage of bovine ubiquitin after the protein was subjected to microwave-enhanced trypsin digestion (1:5, w/w) for 10 min at 37 °C. The protein coverage was established by the observation of the expected peptide signals in the LC-ESI mass spectrum. This result was further verified by MALDI-MS analysis. Figure 4B shows the ubiquitin coverage after 24 h of trypsin digestion by the classic methods when the sample was heated on a hot plate at 37°C. These results suggested that a similar coverage was obtained from the two different experiments (Fig. 4). Thus, microwave irradiation enhances the enzymatic digestion; within 10 min almost complete primary structural information was obtained. In addition, the microwave tryp-

Table 1. Peptides with mass values observed in MALDI mass spectrum of cytochrome *c* after 10 min of trypsin digestion with microwave irradiation

Peptide sequence	Expected mass values	Observed peptide with mass values	
T ₁ GDVEK	546.6	T ₃₋₇ = 2273	
T ₂ GK	203.2		
T ₃ K	146.2		
T ₄ IFVQK	633.8		
T ₅ CAQCHTVEK	1018.2	T ₆₋₈ = 1674.6	T ₆₋₉ = 1802.7
T ₆ GGK	260.3		
T ₇ HK	283.3	T ₈ = 1168.3,	T ₈₋₉ = 1296.3
T ₈ TGPLNLHGLFGR	1168.3		
T ₉ K	146.2	T ₉₋₁₁ = 1825.6	T ₉₋₁₃ = 3947.7
T ₁₀ TGQAPGFSYTDANK	1456.5	T ₁₀₋₁₂ = 3690.4	T ₁₀₋₁₃ = 3816.0
T ₁₁ NK	260.3	T ₁₁₋₁₅ = 3798.5	T ₁₂₋₁₄ = 2796
T ₁₂ GITWGEETLMEYLENPK	2010.2	T ₁₂ = 2010	
T ₁₃ K	146.2	T ₁₃ = 805.4	T ₉₋₁₄ = 4603.0
T ₁₄ YIPGTK	677.8	T ₁₀₋₁₄ = 4475	
T ₁₅ MIFAGIK	779.0	T ₁₅ = 788.9,	T ₁₅₋₁₆ = 906.4,
T ₁₆ K	146.2	T ₁₆₋₁₉ = 1561.2	T ₁₇₋₂₁ = 1976.8
T ₁₇ K	146.2	T ₁₇₋₂₁ = 1976.8	
T ₁₈ GER	360.4	T ₁₈₋₁₉ = 1305.3	T ₁₅₋₁₉ = 2322
T ₁₉ EDLIAYLK	964.1	T ₁₅₋₁₉ = 2322	
T ₂₀ K	146.2	T ₁₆₋₂₀ = 1689.6	T ₁₅₋₂₁ = 2865.2, T ₁₆₋₂₁ = 2104.9
T ₂₁ ATNE	433.4	T ₁₅₋₂₁ = 2865.2,	

sin digestions of ubiquitin were carried for various time intervals (10 min, 30 min, and 60 min) using lower ratios of enzyme-to-protein concentrations (1:25–1:200, w/w). The mass spectral data yielded satisfactory coverage, but increasing the reaction time (30 min and 60 min) resulted in only minor improvements. This data suggest that at longer irradiation times (>30 min) the enzyme loses its activity. However, earlier time points such as 10 min, 30 min, and 60 min at 37°C using the classic approach did not have any effect on the protein, except for a small amount of cleavage observed between amino acid residues 74 and 75.

Another protein, horse heart myoglobin (molecular mass 16,951 Da), was also used in the study. Figure 5 shows the MALDI mass spectrum of trypsin digest obtained from 10 min of microwave irradiation. The spectral data demonstrates a complete coverage of the protein. This study further establishes the efficacy of the microwave methodology.

Dithiothreitol (DTT) reduced chicken egg lysozyme (molecular mass 14,306 Da) was similarly subjected to microwave-assisted trypsin digestion (1:5, w/w). The MALDI mass spectrum (Fig. 6A) of this sample displayed the expected tryptic peptides with the exception of the fragment corresponding to sequences 74–96. When the digestion experiment was conducted at 37°C for 10 min in the absence of microwave irradiation, no tryptic fragments of lysozyme were detected in the MALDI spectrum (Figure 6B); instead, abundant ions representing the singly and doubly charged molecular ions of lysozyme were exhibited in the mass spectrum. Thus, these control experiments further confirm

the effect of microwave in accelerating the enzymatic digestion of proteins.

Application of microwave-assisted trypsin digestion of recombinant human interferon α -2b (rh-IFN α -2b) protein

The peptide mapping of rh-IFN α -2b, an antiviral/anticancer agent marketed by Schering-Plough, demonstrates the utility of microwave-assisted enzymatic digestion for a larger protein. This protein has an average molecular mass of 19,266 Da and contains four cysteines at positions 1, 29, 98, and 138; these cysteine residues are responsible for the presence of the two disulfide bonds in the protein. We have previously characterized this protein including the confirmation of cysteines 1–98 and 29–138 disulfide linkages (Pramanik et al. 1991).

The microwave-assisted trypsin digest mixture of rh-IFN α -2b using various irradiation intervals were analyzed by on-line LC-ESI-MS, using a C18 reversed phase 1-mm/15-cm column. Figure 7 represents the total ion chromatogram (TIC) after 10 min of irradiation. The individual tryptic peptides of rh-IFN α -2b are denoted by T₁, T₂, T₃,...T_n. These data agree with the cDNA-determined amino acid sequence of rh-IFN α -2b. Several peptide signals arising from incomplete cleavages were also observed in the spectrum; we have found in our earlier studies that incomplete cleavage by trypsin often occurs under normal digestion

Fig. 2. (A) MALDI mass spectrum of tryptic peptides of cytochrome c generated after 6 h of digestion (1:25, w/w) at 37°C (classic approach) and (B) tryptic fragments in A is represented in the form of horizontal lines showing the total sequence coverage of cytochrome c.

conditions. As discussed above, the rh-IFN α -2b molecule has four cysteines (at positions 1, 29, 98, and 138), which are contained in the tryptic peptides T₁, T₅, T₁₀, and T₁₇, respectively (Fig. 7). The peptide signals T₁-SS-T₁₀, T₅-SS-T₁₇, T₅-SS-T_{16,17}, and T₁-SS-T_{9,10} observed in the TIC corresponded to disulfide-linked peptides at m/z 4,616, 2,118, 2,246, and 6,049, respectively.

In a separate experiment, the reduced form of rh-IFN α -2b (obtained by treatment with DTT) was digested under microwave irradiation conditions as described above, and the sample mixture was analyzed by LC-ESI-MS. As expected, the MS data showed complete disappearance of the disulfide-containing peptide signals (m/z 4,616, 2,118, 2,246, and 6,049), whereas new intense signals due to the released peptides T₁, T₅, T₁₀, and T₁₇ appeared in the spectrum (data not shown).

Another specific enzyme, endoprotease lysine-C, has also been successfully applied with microwave irradiation to the digestion of proteins, within 10 min of microwave irradiation, >90% of the digested products of these proteins (cytochrome c, lysozyme, ubiquitin, and myoglobin) were observed in the MALDI spectra (data not shown). These re-

sults demonstrate that the application of microwave is not restricted to one specific enzyme.

Application of microwave-assisted digestion was also applied to proteins (for example, myoglobin, SA 436, YAE-1) separated on SDS-PAGE gels. The in-gel digestion of proteins with trypsin using microwave irradiation was successfully tried, and complete peptide maps of proteins were obtained (data not shown). Briefly, coomassie blue-stained protein band was excised and washed with 50% methanolic solution. The washed band was subjected to microwave irradiation for destaining in the presence of 30 μ L of 10 mM ammonium acetate buffer. Fifteen minutes of microwave irradiation with 30% power (144 W) at 37°C resulted in the complete destaining of the gel band. The colorless gel was then transferred into a new 300- μ L Eppendorf tube for trypsin digestion. The gel was cut into smaller pieces and suspended in 10 mM ammonium bicarbonate solution before it was treated with trypsin. A similar procedure as described above was followed for in-gel digestion of protein using microwave irradiation. After 15 min of digestion, the protein solution was quenched with 0.1% TFA and subjected to mass spectrometric analysis.

Table 2. Peptides with mass values observed in MALDI mass spectrum of cytochrome *c* after 6 h of trypsin digestion at 37°C (classic method)

Peptide sequence	Expected mass values	Observed peptide with mass values
T ₁ GDVEK	546.6	
T ₂ GK	203.2	
T ₃ K	146.2	
T ₄ IFVQK	633.8	T ₄₋₅ = 1633.5
T ₅ CAQCHTVEK	1018.2	
T ₆ GGK	260.3	
T ₇ HK	283.3	T ₇₋₈ = 1433.6
T ₈ TGPNLHGLFGR	1168.3	T ₈ = 1168.3, T ₈₋₉ = 1296.3
T ₉ K	146.2	T ₉₋₁₃ = 3947.7
T ₁₀ TGQAPGFSYTDANK	1456.5	T ₁₀₋₁₂ = 3690.4
T ₁₁ NK	260.3	
T ₁₂ GITWGEETLMEYLENPK	2010.2	T ₁₂ = 2010, T _{12,13} = 2010
T ₁₃ K	146.2	T ₁₃₋₁₄ = 805.4
T ₁₄ YIPGTK	677.8	T ₁₄₋₁₅ = 1438.9
T ₁₅ MIFAGIK	779.0	T ₁₅ = 778.5, T ₁₅₋₂₁ = 2866.7
T ₁₆ K	146.2	T ₁₆₋₁₈ = 616.5, T ₁₆₋₂₀ = 1781.5
T ₁₇ K	146.2	T ₁₇₋₁₉ = 1433.6, T ₁₇₋₂₀ = 1634.6
T ₁₈ GER	360.4	T ₁₈₋₁₉ = 1305.3
T ₁₉ EDLIAYLK	964.1	
T ₂₀ K	146.2	
T ₂₁ ATNE	433.4	

Kinetic studies of the trypsin digestion of IFN α -2b under microwave irradiation

A series of experiments were conducted to determine the reaction rate of the microwave-assisted trypsin digestion of the protein, IFN α -2b. Data obtained from a microwave assisted digestion experiment, in which an instrumental setting of 37°C was used (Star-6 microwave apparatus, CEM Corp.), was then compared to the results achieved by the classic method at 37°C (Fig. 8). Digestion products were sampled at intervals of 0 min, 5 min, 10 min, 20 min, and 30 min, and the resulting aliquots were then analyzed by LC-MS. At 5 min, the microwave-assisted reaction was found to be 11% complete, 17% at 10 min, 27% at 20 min, and ~32% at 30 min. Two more data points were acquired at 1 h and 2 h, respectively, indicating no further increase in the rate of the reaction. In comparison, the classic tryptic digestion method did not show reaction products until the 20-min mark, 20% at 20 min, 36% at 30 min. After 30 min the rate of digestion by the classic method continued to increase but at a slower rate, reaching 50% in 2 h and 78% in ~6 h (data not shown). In the case of the microwave-assisted reaction, the loss of enzymatic activity (at ~30 min) is possibly due to the denaturation of the protein at elevated temperature.

Microwave-assisted tryptic digestion experiments of IFN α -2b were also carried out at instrumental temperature settings of 45°C and 55°C, and aliquots were taken at 0 min, 5 min, 10 min, 20 min, and 30 min. These results were

analyzed by LC-ESI-MS to determine the amount of protein digested for each time interval. These results are plotted in Figure 8. At 5-min intervals, both digestion experiments showed ~60% of the protein digested, reaching 67%–70% digestion in 10 min–20 min. At this point, no further increase in the yield was observed, suggesting that the activity of trypsin had been destroyed. Fresh enzyme (3 μ g of trypsin) was then added to the two samples from the 45°C experiment that had been collected at 10 min and 20 min. These samples were then subjected to further microwave irradiation of 10 min–20 min resulting in 80%–90% completion of the digestion (data not shown). With elevated temperature at 45°C and 55°C, the amount of digestion produced in 10 min has been quadrupled compared to the data for the 37°C setting. The identical rate plots for 45°C and 55°C reactions indicate that a temperature of 45°C optimizes the microwave-assisted tryptic digestion of IFN α -2b. In conclusion, we have shown that microwave-assisted reactions can produce high yields in minutes, which would require hours by classic methods.

Observations on the rate of acceleration of the enzymatic digestion

To investigate the mechanism involved in the observed rate acceleration of the enzymatic cleavage of proteins under microwave irradiation, trypsin digestion of rh-IFN α -2b was

Fig. 3. MALDI mass spectra of cytochrome c after 12 min of trypsin digest (1:25, w/w) (A) with microwave irradiation and (B) without microwave irradiation.

carried out at three different microwave temperature settings, 37°C, 45°C, and 55°C (Fig. 8). Reaction samples were taken at intervals of 0 min, 5 min, 10 min, 20 min, and 30 min. The temperatures of the reaction solutions were

measured at each sampling point using a thermocouple and were found to be 49°C, 65°C, and 74°C, respectively. Each measurement was made as quickly as possible to minimize a decrease in the solution temperature. On the basis of our experiments, these temperatures were reached in ~5 min of microwave irradiation. Significantly, the temperatures of these reaction solutions were found to be much higher than their microwave settings. It is important to note that the rapid elevation of the solution temperature to >60°C greatly enhanced the digestion reaction.

In an effort to explore the feasibility that the rapid increase in temperature is responsible for the accelerated rates observed in the microwave, the above enzymatic reaction was further studied without microwave irradiation, using a heating block to maintain the desired temperature. A fresh reaction mixture was introduced into the preheated block at 60°C to simulate the rapid heating process observed in the microwave. The rates of enzymatic cleavage measured in this study fairly resembled the results obtained from the microwave experiment (Fig. 8). This suggests that the rapid increase in the reaction temperature is at least partially responsible for the large acceleration seen under microwave conditions (Lidstrom et al. 2001; Larhed et al. 2002).

Fig. 4. Total sequence coverage of ubiquitin (1:5, w/w) (A) after 10 min of microwave-assisted tryptic digestion and (B) after 24 h of trypsin digest at 37°C (classic approach).

Fig. 5. MALDI mass spectrum of the tryptic digest of myoglobin after 10 min of microwave irradiation.

Factors influencing proteolytic cleavage of proteins with enzymes

Many enzymatic reactions—including the proteolytic cleavage of proteins with trypsin and lysine c—are traditionally conducted in aqueous solution at 37°C for a few hours. The rationale appears to be (1) the slow rate of denaturation of the enzyme and the substrate protein, and (2) a reasonable rate for the recognition of ligation sites (e.g., next to an arginine or lysine component for trypsin) on the substrate protein by the enzyme resulting in selective peptide bond scission. The sheath of water molecules around proteins and intramolecular hydrogen bonding between different parts of a protein molecule play important roles in the dynamic behavior of proteins. There is growing recognition that folding and unfolding of proteins may influence the chemical interaction of proteins with ligands.

There are two schools of thought about microwave energy transfer to a reaction. One set of research workers believe that microwaves are just a convenient way of heating (Kuhnert 2002); other research workers (Mayo et al. 2002) have produced experimental data that point to a non-thermal microwave effect. It is interesting to note, however, that no microwave-assisted reaction is slower than the corresponding conductivity/convection-heated conventional reaction. Early in the development of microwave technology, it was observed that microwaves would produce notable increase in reaction rates for reactions that are slow under conventional conditions.

We have studied enzymatic cleavage of proteins using microwave energy and compared our findings with conven-

tional heating of the reaction mixture. Microwaves are non-ionizing radiation that interact in the liquid phase with ion pairs and with organic molecules that have dipole moment (such as amides, esters, and water, but not hydrocarbons such as benzene or hexane). Microwave energy is directly transferred to these species, and the energy profile is different than that involved in traditional conduction/convection heating. However, both types of enzymatic reaction could produce nearly comparable results in spite of the different energy profiles and thus allow MALDI and ESI-MS methods to be used on the protein hydrolysate produced by either method for correct protein mapping.

Our experimental observations show that ~60°C is an optimum temperature for rapid proteolysis and a reasonably low rate of denaturation. That bulk temperature for the reaction mixture can be reached by heating on a hot plate or by microwave irradiation for 1 min–2 min.

Tightly folded proteins are known to require many hours for adequate proteolysis by enzymes under conventional conditions. It is expected that these very proteins will give greatly enhanced proteolysis rates under microwave irradiation. We plan to test this possibility. Our current observations on the action of trypsin on bovine ubiquitin is in agreement with this expectation.

Conclusions

Accelerated enzymatic digestion of proteins under controlled microwave irradiation has been demonstrated. This approach accelerates the digestion process to minutes versus

Fig. 6. MALDI mass spectrum of the DTT-reduced lysozyme after 10 min of trypsin digest (1:5, w/w) (A) with microwave irradiation and (B) without microwave irradiation.

hours by traditional methods. Kinetic study experiments indicated that the microwave-assisted process is optimized at an instrumental setting of 45°C (actual bulk temperature 60°C) resulting in ~70% completion of the protein digestion in 10 min. In the case of trypsin or lysine digest, most of the expected peptide fragments were generated with >80% coverage of the protein. No nonspecific cleavage product was detected in the mixture. This mapping strategy combined with MALDI or LC-ESI-MS provides sequence and disulfide bond information, as well as the location of any modification sites with high speed and accuracy. We have successfully demonstrated the usefulness of this new method for probing primary structure of ubiquitin, cytochrome c, myoglobin, lysozyme, and a few other proteins such as IFN α -2b.

Preliminary work shows that even inexpensive domestic microwave ovens can be programmed for this novel approach to proteolytic cleavage of proteins. Further studies for a better understanding of microwave-assisted enzymatic reactions are in progress. We believe that the use of microwaves in protein identification will be an important advancement in biotechnology and proteome research.

Materials and methods

Microwave operation

Digestion of proteins was conducted in a specialized, temperature-controlled, single beam microwave applicator (Prolabo Syntheswave 402) with a maximum power of 300 W; only 10% (30 W) of the available power was used. The sample vial was placed in a three-hole Teflon insertion rack, which in turn was lowered into the irradiation chamber. The temperature of the apparatus was programmed to increase gradually from 40°C to 50°C. The actual temperature of the sample solution was measured with a Fisher thermocouple (Fisherbrand Dual-Channel Thermometer with Offsets, Fisher Scientific, Pittsburgh, PA) immediately after microwave irradiation and was found to be ~50°C.

The digestion of proteins was also conducted with a single beam, multi-inlet Star-6 Microwave Apparatus (CEM Corporation, Matthews, NC). This microwave has a maximum power output of 480 W. For our experiments, this equipment was set at 30% of the available power or 144 W. Sample vials were placed in a four-hole Teflon insert rack, which could be lowered into the glass insertion chamber. Enzyme digestion experiments were carried out at fixed instrumental temperature settings of 37°C, 45°C, and 55°C. These targeted microwave temperature settings were kept constant by the sensor-controlled on/off duty cycle of the magnetron. The infrared sensor continuously monitored the surface of the glass wall of the

Fig. 8. Plots showing the rate of trypsin digestion of IFN α -2b in the first 30 min under microwave irradiation and classic condition.

in 75 mM ammonium bicarbonate solution. A variable ratio of protease-to-protein, 1:200 to 1:5 (w/w) concentration was used. Samples were irradiated for 5 min, 10 min, 15 min, 20 min, 30 min, or 60 min with microwave at 30% (144 W) of the maximum power setting. After a preselected interval, the sample was quenched by the addition of 0.1% TFA solution and was stored in dry ice for mass spectrometric analysis.

Enzymatic digestion of rh-IFN α -2b (MW 19,269)

A 10- μ L rh-IFN α -2b protein solution (concentration of 3 μ g/ μ L) was dissolved in 90 μ L of ammonium bicarbonate buffer in an Eppendorf tube, and three sets of this solution were prepared for trypsin digestion. Trypsin was added to each of the 100- μ L solution at an enzyme-to-protein ratio of 1:50, 1:25, and 1:5 (w/w), respectively. The solution was then irradiated under microwave for 5 min and 10 min. A separate microwave experiment was conducted using the disulfide reduced form of rh-IFN α -2b where the reduction of rh-IFN α -2b was performed by adding 20-fold molar excess of DTT.

Acknowledgments

We are indebted to the National Science Foundation (CHE-9910242), Union Mutual Foundation, and the George Barasch Research Fellowship funds for partial support of this research. We thank Stevens Institute of Technology for laboratory facilities and the Schering-Plough Research Institute for use of advanced mass spectrometric instrumentation. We acknowledge Dr. John Piwinski for his support of this research project.

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