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Effects of Temperature and Acetonitrile on Microwave-Assisted Weak Acid Protein Hydrolysis

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Abstract : The effects of temperature and acetonitrile (ACN) concentration on microwave-assisted weak-acid hydrolysis of proteins were investigated. Myoglobin was hydrolyzed for 1 h using 2% formic acid and a microwave with different concentrations of ACN (0, 5, and 10%) at various temperatures (50, 60, 70, 80, 90, and 100°C). The numbers of peptides identified with each concentration of ACN were the same for each temperature. The greatest number of peptides (18 total) was obtained with hydrolysis at 100°C, and 6 of these were a result of additional removal of aspartic acid at the C-terminus. Hydrolysis at 80°C resulted in 13 peptides, of which only 1 was generated by the additional removal of aspartic acid, and 12 were observed with hydrolysis at 100°C. Our results demonstrate that microwave-assisted weak-acid hydrolysis of proteins can be performed successfully at 80°C, which could be beneficial for limiting side reactions and generating larger peptide sequences.

Keywords : Microwave, MALDI, Acid hydrolysis, Acetonitrile, Temperature

Introduction

Weak acid hydrolysis of proteins (WAHP) requires an elevated temperature in the presence of a weak acid.[1,2] WAHP predominantly cleaves the C-terminus of aspartic acid residues, followed by cleavage of the N-terminus of aspartic acid,[2] thereby removing aspartic acid at the C-terminal end of peptides generated by C-terminal cleavage of aspartic acid residues. Microwave radiation is often used to expedite WAHP.

WAHP is advantageous over enzyme-mediated protein digestion in terms of digestion time,[3] flexibility of the digestion conditions,[4] potential for middle-down analysis,[5] and compatibility with downstream mass spectrometric analysis.[6] While WAHP cleaves mainly cleaves the C-terminus of aspartic acid residues, trypsin cleaves exclusively at lysine or arginine residues. Since the abundance of aspartic acid in human proteome is lower than lysine or arginine,[5] WAHP of human proteome generates larger peptides than tryptic digestion.

In a previous study, we observed that maintaining a temperature of 100°C is critical for microwave-assisted WAHP digestion at 37, 50, and 100°C.[2] In this study, the effects of temperature and acetonitrile (ACN) concentration on microwave-assisted WAHP of myoglobin were investigated. Temperatures ranging from 50°C to 100°C (50, 60, 70, 80, 90, and 100°C) were investigated in 1-h microwave-assisted WAHP of myoglobin using 2% formic acid (FA) and three different concentrations of ACN (0, 5, and 10%). ACN is often added to expedite tryptic digestion.[7-9] Since myoglobin contains 8 aspartic acid residues, 19 lysine residues, and 2 arginine residues, weak-acid hydrolysis of myoglobin generates larger peptides than tryptic digestion.

Experimental

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. A stock solution of 10 mg/mL horse heart myoglobin was prepared in deionized water. To prepare the sample solutions for WAHP, 10 μL of the stock solution were mixed with 2% FA (2 μL FA in 88 μL deionized water), 2% FA/5% ACN (2 μL FA, 5 μL ACN, and 83 μL deionized water), or 2% FA/10% ACN (2 μL FA, 10 μL ACN, and 78 μL deionized water). The sample solutions were placed in a microwave oven (HST Rapid Enzyme Digestion System with 800 W

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output at 60 Hz, AC 220-240 V; Hudson Surface Technology, Fort Lee, NJ, USA) at various temperatures (50, 60, 70, 80, 90, and 100°C) for 1 h.

2,5-Dihydroxybenzoic acid (DHB) was used as a matrix-assisted laser desorption/ionization (MALDI) matrix. To prepare the DHB matrix solution, 10 mg DHB were added to 1 mL 50 % ACN and 1% phosphoric acid in water.[10] To prepare the MALDI sample spots, a 1.5-μL mixture of sample and matrix (1:1) was loaded onto the MALDI plate and dried in a vacuum chamber. Each MALDI spot contained 44 pmol hydrolyzed myoglobin peptides.

Mass spectra were obtained using the Tinkerbell RT MALDI-time-of-flight mass spectrometer (ASTA Inc., Suwon, South Korea) with a 349-nm Nd:YLF ultraviolet laser source in the positive ion linear mode. Peptide identification was performed using the FindPept (https://web.expasy.org/findpept/) and PeptideMass (https://web.expasy.org/peptide_mass/) programs with the enzyme option for microwave-assisted FA hydrolysis (C-term to D). The horse heart myoglobin information can be found using UniProt (http://www.uniprot.org) with the Swiss-Prot accession number/entry name P68082/MYG_HORSE.

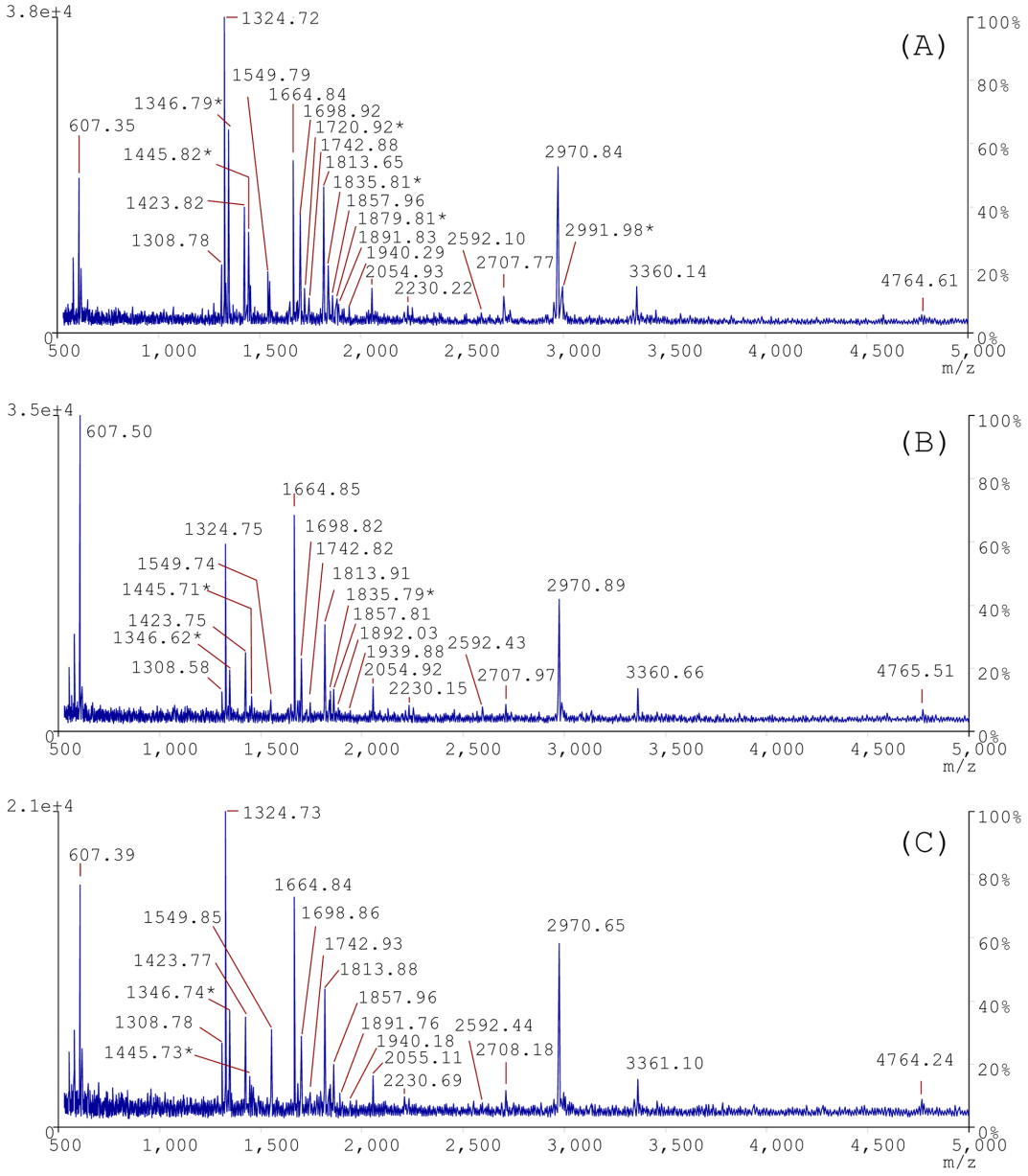

Figure 1. Matrix-assisted laser desorption/ionization mass spectra of 44 pmol horse heart myoglobin hydrolyzed at 100°C by microwave irradiation for 1 h in an aqueous solution containing 2% formic acid and (A) 0% ACN, (B) 5% ACN, and (C) 10% ACN. The sodium adduct peaks are marked with an asterisk.

Table 1. Summary of peptides identified following microwave-assisted weak acid hydrolysis of myoglobin using different concentrations of acetonitrile at different incubation temperatures.

Position		Theoretical monoisotopic *m/z* value	ACN percentage																		peptide sequence	Number of missed cleavages
			0%						5%						10%							
start	End		Temperature (°C)						Temperature (°C)						Temperature (°C)							
			50	60	70	80	90	100	50	60	70	80	90	100	50	60	70	80	90	100		
2	21	2230.08	-[a]	O[b]	O	O	O	O	-	O	O	O	O	O	-	O	O	O	O	O	-.GLSDGEWQQVLNVWGKVEAD.I	1
6	20	1742.89	-	-	-	-	O	O	-	-	-	-	O	O	-	-	-	-	O	O	D.GEWQQVLNVWGKVEA.D[c]	
6	21	1857.91	-	O	O	O	O	O	-	O	O	O	O	O	-	O	O	O	O	O	D.GEWQQVLNVWGKVEAD.I	0
22	44	2592.39	-	-	-	-	-	O	-	-	-	-	-	O	-	-	-	-	-	O	D.IAGHGQEVLIRLFTGHPETLEKF.D[c]	
22	45	2707.42	-	-	-	O	O	O	-	-	-	O	O	O	-	-	-	O	O	O	D.IAGHGQEVLIRLFTGHPETLEKFD.K	0
46	61	1891.96	-	O	O	O	O	O	-	O	O	O	O	O	-	O	O	O	O	O	D.KFKHLKTEAEMKASED.L	0
111	122	1308.75	-	-	-	-	-	O	-	-	-	-	-	O	-	-	-	-	-	O	D.AIIHVLHSKHPG.D[c]	
111	123	1423.78	-	-	O	O	O	O	-	-	O	O	O	O	-	-	O	O	O	O	D.AIIHVLHSKHPGD.F	0
111	126	1698.91	-	-	-	-	O	O	-	-	-	-	O	O	-	-	-	-	O	O	D.AIIHVLHSKHPGDFGA.D[c]	1
111	127	1813.93	-	O	O	O	O	O	-	O	O	O	O	O	-	O	O	O	O	O	D.AIIHVLHSKHPGDFGAD.A	1
111	154	4765.47	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	D.AIIHVLHSKHPGDFGADAQG AMT-KALELFRNDIAAKYKEL GFQG.-	3
124	141	1939.97	-	-	-	-	O	O	-	-	-	-	O	O	-	-	-	-	O	O	D.FGADAQGAMTKALELFRN.D[c]	
124	142	2055.00	-	-	-	O	O	O	-	-	-	O	O	O	-	-	-	O	O	O	D.FGADAQGAMTKALELFRND.I	1
124	154	3360.70	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	D.FGADAQGAMTKALELFRN-DIAAKYKELGFQG.-	2
128	141	1549.82	-	-	-	O	O	O	-	-	-	O	O	O	-	-	-	O	O	O	D.AQGAMTKALELFRN.D[c]	0
128	142	1664.84	-	-	O	O	O	O	-	-	O	O	O	O	-	-	O	O	O	O	D.AQGAMTKALELFRND.I	0
128	154	2970.55	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	D.AQGAMTKALELFRNDIAAKYKEL-GFQG.-	1
143	154	1324.73	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	D.IAAKYKELGFQG.-	0
Number of identified peptides			4	8	10	13	16	18	4	8	10	13	16	18	4	8	10	13	16	18		
Sequence coverage (%)			28.8	52.3	52.3	68.0	68.0	68.0	28.8	52.3	52.3	68.0	68.0	68.0	28.8	52.3	52.3	68.0	68.0	68.0		

a) Not detected.
b) Detection of the corresponding peptide.
c) Cleavage at the N-terminus of aspartic acid.

Results and Discussion

Microwave-assisted WAHP is commonly performed at 100°C. The current investigation was performed to determine whether the temperature can be reduced in the presence of ACN. Figure 1 shows the MALDI mass spectra of myoglobin hydrolyzed at 100°C by microwave irradiation for 1 h in an aqueous solution containing 2% FA and different concentrations of ACN (0, 5, and 10%). Table 1 shows a summary of the peptides identified by microwave-assisted weak acid hydrolysis of myoglobin using the different concentrations of ACN at hydrolysis temperatures of 50, 60, 70, 80, 90, and 100°C.

As shown in Table 1, the presence of ACN did not result in a significant change in the generation of weak-acid-hydrolyzed peptides. The numbers of identified peptides were the same among the three different concentrations of ACN at each temperature. Sodium adduct peaks differed depending on the concentration of ACN, in that the number of the sodium adduct peaks generally decreased as the concentration of ACN increased. The numbers of the identified sodium adduct peaks were 6, 3, and 2 with hydrolysis containing 0, 5, and 10% ACN, respectively at hydrolysis temperature of 100°C.

Figure 2 shows the changes in sequence coverage and number of identified peptides according to the hydrolysis temperature of microwave-assisted weak acid hydrolysis of myoglobin. As expected, higher temperatures resulted in the identification of more peptides, with the highest number of peptides observed at 100°C. In total, 13, 16, and 18 peptides were identified at 80, 90, and 100°C, respectively. Of these, 12 common peptides were a result of cleavage at the C-terminus of aspartic acid residues, while the remaining peptides were a result of the subsequent removal of aspartic acid at the C-terminal end, resulting in the same sequence coverage at 80, 90, and 100°C. The peptides exhibiting removal of aspartic acid at the C-terminal end were observed primarily at 90°C and 100°C, while at temperatures below 90°C, most of the peptides were a result of C-terminal cleavage of aspartic acid residues. This observation confirms that microwave-assisted WAHP initially cleaves the C-terminus of aspartic acid, followed by cleavage of the N-terminus of aspartic acid.[2] The peptides observed at 100°C that were not observed at 80°C were a result of N-terminal cleavage of aspartic acid residues located at the C-terminal end of peptides generated by cleavage of the C-terminus of aspartic acid residues.

The current results demonstrated that microwave-assisted WAHP can be applied at temperatures as low as 80°C. The use of a lower temperature for microwave-assisted WAHP would limit potential side reactions and allow for a middle-down proteomic analysis, in which larger peptide sequences are used for better analysis of proteins.[11] Selecting a microwave-assisted WAHP temperature of 80, 90, or 100°C could allow for controlled digestion providing a specific distribution of peptide sizes.

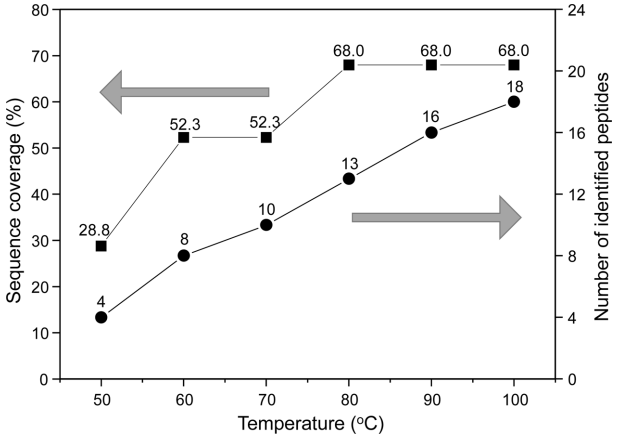

Figure 2. Dependence of sequence coverage and the number of identified peptides on hydrolysis temperature (50, 60, 70, 80, 90, and 100°C) in microwave-assisted weak acid hydrolysis of myoglobin.

Only four peptides were observed at each concentration of ACN (0, 5, and 10%) in the microwave-assisted weak acid hydrolysis of myoglobin at 50°C. Three of the four peptides (amino acids 111-154, 128-154, and 143-154) were obtained from the C-terminal end of myoglobin, and the fourth peptide had two uncleaved sites (amino acids 124-154) or a peptide from the N-terminal end of the protein (amino acids 2-21). These observations strongly suggest that weak acid hydrolysis occurred at either side of the protein sequence, similar to exoprotease activity.[12]

Conclusion

Myoglobin was hydrolyzed by microwave-assisted weak acid hydrolysis at three different concentrations of ACN (0, 5, and 10%) for 1 h using 2% FA at various temperatures (50, 60, 70, 80, 90, and 100°C). The presence of ACN did not increase the number of identified peptides, while a decreased number of sodium adduct peaks were observed with increasing amounts of ACN. The highest number of hydrolyzed peptides was observed at 100°C. However, all of the expected hydrolyzed peptides with C-terminal cleavage of aspartic acid residues were observed at 80, 90, and 100°C, suggesting that successful generation of hydrolyzed peptides can be achieved at 80°C. A reduced temperature for microwave-assisted WAHP is beneficial to generate larger peptide sequences in proteome analysis and limit side reactions, such as dephosphorylation, formylation, and water loss.[4]

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