Vol. 10, No. 3, 2019
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Effect of Microwave Irradiation Time on Microwave-Assisted Weak Acid Protein Hydrolysis

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Received June 17, 2019; Revised July 06, 2019; Accepted July 09, 2019
First published on the web September 30, 2019; DOI: 10.5478/MSL.2019.10.3.79

Abstract : Horse heart myoglobin (MYG) and bovine serum albumin (BSA) were hydrolyzed by microwave-assisted weak-acid hydrolysis for 10, 20, 30, 40, 50, and 60 min using 2% formic acid (FA) at 100°C. Generally, the number of identified peptides increased with increasing irradiation time, indicating that the duration of microwave irradiation is linked to the efficiency of hydrolysis. For MYG, irradiation for 60 min provided the highest number of identified peptides, the greatest sequence coverage values and the highest MASCOT score values among the investigated irradiation times. Irradiation of BSA for 50 min, however, yielded a greater number of peptides than irradiation for 60 min due to the generation of miscleaved peptides after microwave irradiation for 50 min.

Keywords : weak acid hydrolysis, MALDI-MS, microwave, myoglobin, albumin

Introduction

Proteins can be hydrolyzed into peptides by enzyme or chemical digestion, to facilitate their characterization by mass spectrometry. Microwave irradiation is used to accelerate protein hydrolysis.[1] As an example of non-enzymatic hydrolysis, weak-acid hydrolysis of protein (WAHP)[2] involves the hydrolysis of proteins in a weakly acidic solution (e.g., pH 2.0) for 2 h at 108°C.[3] Microwave irradiation is reported to expedite WAHP.[4] WAHP induces cleavage at the C-terminus, followed by the N-terminus, of aspartic acid residues.[5] We previously investigated the effects of temperature and acetonitrile (ACN) concentration on microwave-assisted WAHP, and found that a reduced temperature (e.g., 80 or 90°C) and 10% ACN could also be used for microwave-assisted WAHP.[6] In this study, the effect of microwave irradiation time on the efficiency of WAHP was investigated using horse heart myoglobin (MYG) and bovine serum albumin (BSA). The proteins were subjected to microwave-assisted weak-acid hydrolysis in 2% formic acid (FA) for 10, 20, 30, 40, 50, and 60 min at 100°C. Reducing the irradiation time would increase the throughput of WAHP processes.

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Experimental

MYG, BSA, dl-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (ABC), and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma Aldrich (St. Louis, MO, USA). FA and HCl were purchased from Samchun (Gyeonggi-do, South Korea), H_3PO_4 from Oriental Chemical Industries (Seoul, South Korea), and ACN from Duksan Pharmaceutical (Gyeonggi-do, South Korea).

Stock solutions of 10 mg/mL MYG and BSA were prepared by dissolving 10 mg of MYG and BSA in 1 mL of 50 mM ABC buffer. To prepare MYG solution, 98 μL of 10-fold-diluted MYG stock solution was mixed with 2 μL of FA. The solution was placed in a microwave oven (Rapid Enzyme Digestion System; ASTA, Gyeonggi-do, South Korea) for 10, 20, 30, 40, 50, and 60 min at a power of 100 W at 100°C.

To prepare BSA solution, 10 μL of BSA stock solution was dissolved in 90 μL of 50 mM ABC buffer and denatured by ultrasonication for 5 min, followed by sequential additions of 5 μL of 30 mg/mL DTT and 20 μL of 36 mg/mL IAA with 5 min ultrasonication after each addition. After being passed through a 30 kDa filter (Amicon Ultracentrifugation Filter 30 K; Sigma Aldrich), the BSA solution was subjected to WAH, as described above for MYG.

Matrix-associated laser desorption-ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis was performed using a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA, USA), with 300 laser shots

using a 337 nm nitrogen laser, 3 ns pulse length, 20 Hz pulse repetition rate, and positive ion reflectron mode.

The FindPept (https://web.expasy.org/findpept/) and PeptideMass (https://web.expasy.org/peptide_mass/) software packages were used to identify peptides with the enzyme option of microwave-assisted formic acid hydrolysis (C-term to D). Information on MYG and BSA can be found in UniProt (http://www.uniprot.org/) with the Swiss-Prot accession number/entry names P68082/MYG_HORSE and P02769/ ALBU_BOVIN, respectively.

Peptide mass fingerprinting analysis was performed using MASCOT public server (MatrixScience, MA, USA) at http://www.matrixscience.com/search_form_select.html. The *m/z* values of the identified monoisotopic peptide peaks for each experiment were used as data input. The search were performed against SwissProt proteome database of "Other mammalia" taxonomy, with a peptide tolerance set at 0.5 Da. The MASCOT score value was obtained from the candidate with the highest score for each analysis.

Results and discussion

WAH of MYG

MYG is present in the muscle tissue of vertebrates, and in almost all mammals. Its presence in the bloodstream is indicative of muscular damage, so MYG may have potential as a marker of heart attack in patients with chest pain. MYG consists of a single polypeptide chain of 153 amino acid residues and one heme group containing a shared iron atom.[7]

Figure 1 shows the mass spectra of MYG hydrolyzed in an aqueous solution of 2% FA at 100°C by microwave irradiation for 10, 20, 30, 40, 50, and 60 min. Table 1 shows the MYG peptides identified in Figure 1. Four peaks—AIIHVLHSKHPGDFGADAQGAMTKALELFRNDIAAK YKELGFQG (residues 111–154 with three miscleavages), AQGAMTKALELFRND (residues 128–142 with zero miscleavages), AQGAMTKALELFRNDIAAKYKELGFQG (residues 128–154 with one miscleavage), and IAAKYKELGFQG (residues 143–154 with zero miscleavages)—were found under all irradiation conditions. Here, the number of miscleavages was obtained by counting the number of aspartic acid (D) amino acids in the middle of the peptide sequence. Because the four peptides generated by WAH of MYG for 10 min were from the C-terminus, this is likely the starting point of hydrolysis of the protein. Totals of 4, 6, 7, 8, 10, and 11 peptides were identified after WAH of MYG for 10, 20, 30, 40, 50, and 60 min, respectively. Both the sequence coverage values and the MASCOT scores increased with increasing irradiation

Table 1. Peptides identified by MALDI-TOF MS in MYG hydrolyzed at 100°C by microwave irradiation in a 2% FA aqueous solution for the indicated times.

Start	End	Theoretical monoisotopic m/z value	Experimental m/z value	Number of missed cleavages	Sequences	Incubation times (min)[a]					
						10	20	30	40	50	60
2	21	2230.0774	2230.0447	1	(-)GLSDGEWQQVLNVWGKVEAD(I)						O
6	21	1857.9129	1857.8638	0	(D)GEWQQVLNVWGKVEAD(I)					O	O
6	45	4546.3153	4546.7148	1	(D)GEWQQVLNVWGKVEADIAGH-GQEVLIRLFTGHPETLEKFD(K)				O	O	O
22	45	2707.4201	2707.2305	0	(D)IAGHGQEVLIRLFTGH-PETLEKFD(K)					O	O
111	127	1813.9343	1813.8777	1	(D)AIIHVLHSKHPGDFGAD(A)		O	O	O	O	O
111	154	4765.4670	4765.30	3	(D)AIIHVLHSKHPGDFGADAQGAMT-KALELFRNDIAAKYKELGFQG(-)	O	O	O	O	O	O
124	142	2054.9963	2054.8184	1	(D)FGADAQGAMTKALELFRND(I)			O	O	O	O
124	154	3360.7044	3360.7629	2	(D)FGADAQGAMTKALELFRN-DIAAKYKELGFQG(-)		O	O	O	O	O
128	142	1664.8424	1664.8424	0	(D)AQGAMTKALELFRND(I)	O	O	O	O	O	O
128	154	2970.5505	2970.5894	1	(D)AQGAMTKALELFRN-DIAAKYKELGFQG(-)	O	O	O	O	O	O
143	154	1324.7259	1324.7992	0	(D)IAAKYKELGFQG(-)	O	O	O	O	O	O
Number of miscleaved peptides						2	4	5	6	6	7
Number of identified peptides						4	6	7	8	10	11
Sequence coverage (%)						28.8	28.8	28.8	54.9	54.9	57.5
MASCOT score						48	82	99	117	151	168

a) Detection of the corresponding peptide is indicated by 'O.'

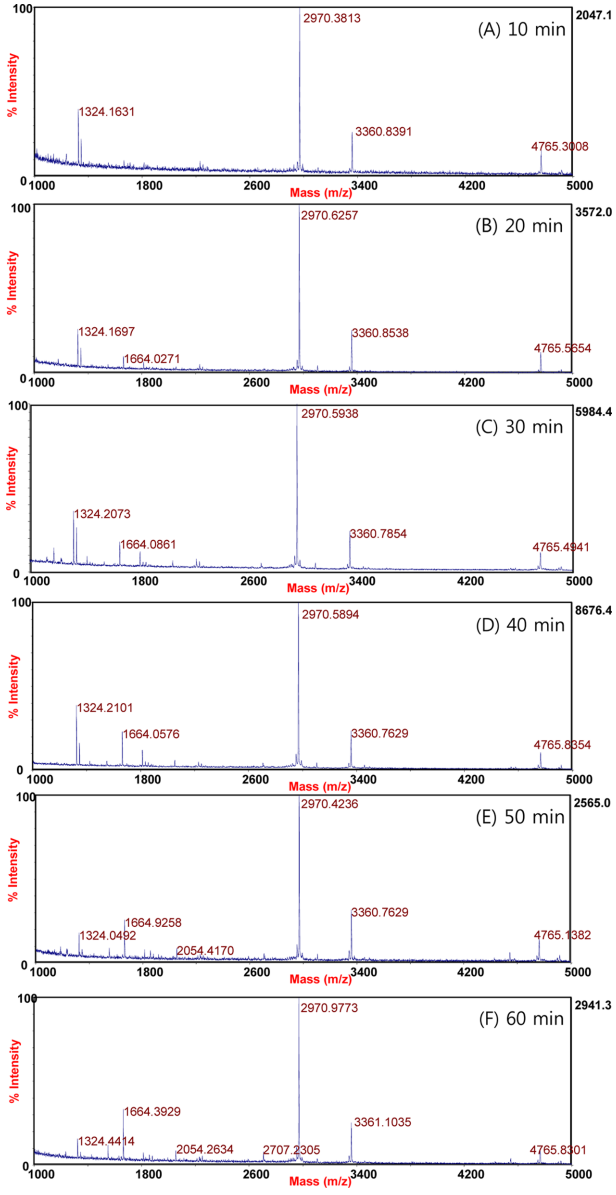

Figure 1. MALDI mass spectra of MYG hydrolyzed at 100°C by microwave irradiation in a 2% FA aqueous solution for (A) 10 min, (B) 20 min, (C) 30 min, (D) 40 min, (E) 50 min, (F) 60 min.

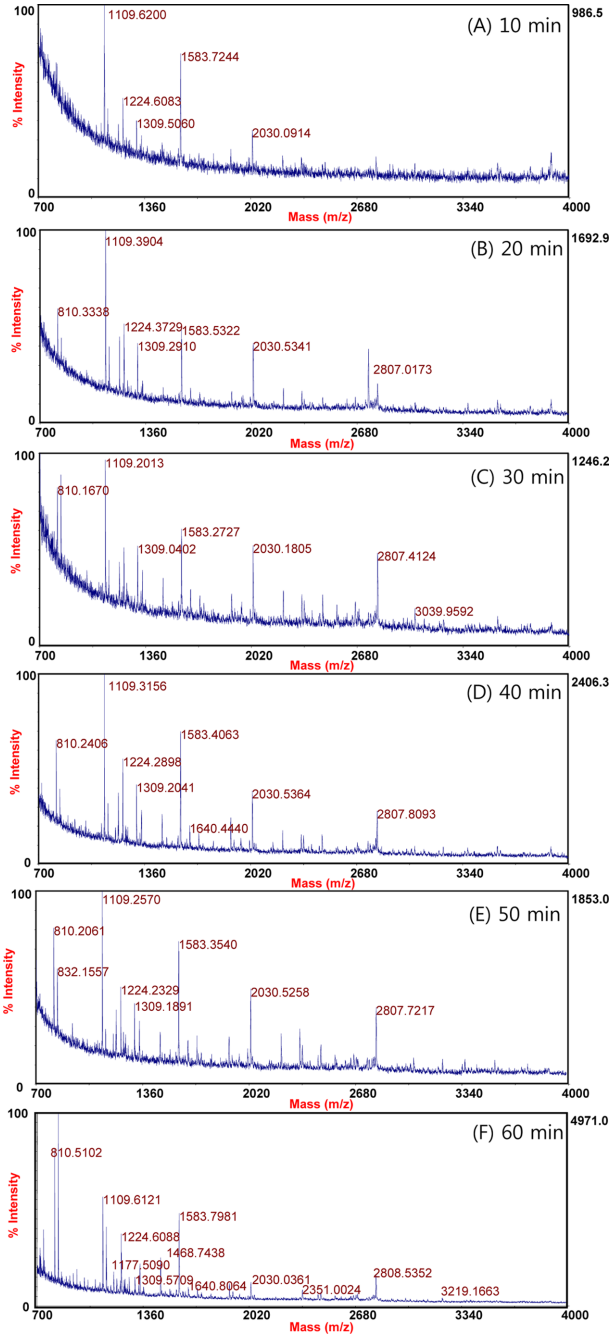

Figure 2. MALDI mass spectra of BSA hydrolyzed at 100°C by microwave irradiation in a 2% FA aqueous solution for (A) 10 min, (B) 20 min, (C) 30 min, (D) 40 min, (E) 50 min, (F) 60 min.

time. The above observations suggest that the efficiency of MYG hydrolysis increased with increasing irradiation time up to 60 min.

WAH of BSA

The BSA precursor protein comprises 607 amino acids. An N-terminal 18-residue signal peptide is removed from the precursor protein upon secretion, and so the initial protein product comprises 589 amino acid residues.[8] A further six amino acids are cleaved to yield the mature BSA protein (583 amino acids;[9] molecular weight, ~ 66,500 Da).

Figure 2 shows the mass spectra of BSA hydrolyzed in an aqueous solution of 2% FA at 100°C by microwave irradiation for 10, 20, 30, 40, 50, and 60 min. Table 1

Table 2. Peptides identified by MALDI-TOF MS in BSA hydrolyzed at 100°C by microwave irradiation in a 2% FA aqueous solution for the indicated times.

Start	End	Theoretical monoisotopic m/z value	Experimental m/z value	Number of missed cleavages	Sequences	Incubation times (min)[a]					
						10	20	30	40	50	60
25	37	1583.7924	1583.798	1	(-)DTHKSEIAHRFKD(L)	O	O	O	O	O	O
25	48	2806.463	2806.5991	1	(-)DTHKSEIAHRFKDLGEEHFK-GLVL(I)		O	O	O	O	O
26	37	1468.7655	1468.7438	0	(D)THKSEIAHRFKD(L)		O	O	O	O	O
81	96	1787.802	1787.815	0	(D)ESHAGCEKSLHTLFGD(E)				O		
97	110	1640.7948	1640.8063	0	(D)ELCKVASLRETYGD(M)		O	O	O	O	O
111	142	3888.7295	3889.3416	4	(D)MADCCEKQEPERNECFLSH-KDDSPDLPKLKPD(P)				O		
133	142	1109.6201	1109.6121	1	(D)SPDLPKLKPD(P)	O	O	O	O	O	O
136	142	810.5083	810.5102	0	(D)LPKLKPD(P)			O	O	O	O
143	148	719.3028	719.3188	0	(D)PNTLCD(E)						O
143	153	1309.5729	1309.5709	1	(D)PNTLCDEFKAD(E)	O	O	O	O	O	O
273	282	1177.5153	1177.509	2	(D)LLECADDRAD(L)					O	O
279	289	1339.631	1339.6323	2	(D)DRADLAKYICD(N)			O	O	O	O
280	289	1224.6041	1224.6086	1	(D)RADLAKYICD(N)	O	O	O	O	O	O
290	303	1697.7469	1697.7415	1	(D)NQDTISSKLKECCD(K)		O		O	O	O
304	319	1895.9895	1896.0249	0	(D)KPLLEKSHCIAEVEKD(A)	O	O	O	O	O	O
304	331	3217.5901	3217.781	1	(D)TISSKLKECCDKPLLEKSHCI-AEVEKD(F)					O	O
338	347	1254.5783	1254.5478	0	(D)VCKNYQEAKD(A)						O
389	398	1196.5041	1196.5056	0	(D)PHACYSTVFD(K)		O	O	O	O	O
389	405	2030.0164	2030.0361	1	(D)PHACYSTVFDKLKHLVD(E)	O	O	O	O	O	O
389	416	3369.64	3369.186	2	(D)PHACYSTVFDKLKHLVDE-PQNLIKQNCD(Q)					O	
580	607	3039.46	3039.7898	2	(D)KCCAADDKEACFAVEGP-KLVVSTQTALA(-)			O	O	O	
586	607	2334.2009	2334.5137	1	(D)DKEACFAVEGPKLV-VSTQTALA(-)		O	O	O	O	
587	607	2219.174	2219.7251	0	(D)KEACFAVEGPKLV-VSTQTALA(-)		O	O	O	O	
			Number of miscleaved peptides			5	8	9	11	13	10
			Number of identified peptides			6	13	15	18	19	17
			Sequence coverage (%)			13.2	23.7	22.5	31.4	29.8	24.9
			MASCOT score			63	119	143	151	178	148

a) Detection of the corresponding peptide is indicated by 'O.'

shows the BSA peptides identified in Figure 1. Six peaks—DTHKSEIAHRFKD (residues 25–37 with one miscleavage), SPDLPKLKPD (residues 133–142 with one miscleavage), PNTLCDEFKAD (residues 143-153 with one miscleavage), RADLAKYICD (residues 280–289 with one miscleavage), KPLLEKSHCIAEVEKD (residues 304–319 with zero miscleavages), and PHACYSTVFDKLKHLVD (residues 389–405 with one miscleavage)—were found under all irradiation conditions. Unlike MYG, the initially generated BSA peptides were from random positions in the primary structure. In addition, irradiation of BSA for 50 min yielded the greatest number of BSA peptides and the highest MASCOT score. Furthermore, the highest sequence coverage was observed from the irradiation for 40 min. These observations are due to the presence of several peptides with high miscleavages such as MADCCEKQEPERNECFLSHKDDSPDLPKLKPD (residues 111-142 with four miscleavages), PHACYSTVFDKL

KHLVDEPQNLIKQNCD (residues 389-405 with two miscleavages) and KCCAADDKEACFAVEGPKLVVST QTALA (residues 580-607 with two miscleavages). These miscleaved peptides present after irradiation for 40 min or 50 min had disappeared after irradiation for 60 min. Significantly reduced background noise was observed from the MALDI mass spectra of BSA irradiated for 60 min compared to the other irradiation times, implying irradiation time of 60 min is necessary to detect BSA peptides with improved signal-to-noise ratios.

Conclusion

MYG and BSA were subjected to microwave-assisted WAH for 10, 20, 30, 40, 50, and 60 min in 2% FA at 100°C. The numbers of peptides generally increased with increasing irradiation time. The highest number of peptides were identified from irradiation of MYG for 60 min; irradiation of BSA for 50 min, however, yielded a greater number of peptides than irradiation for 60 min, due to presence of several miscleaved peptides after microwave irradiation for 50 min. Conclusively, the optimum irradiation time for microwave-assisted WAH is 60 min, while a little reduced irradiation time (e.g., 40 or 50 min) could also be used as an optimum irradiation time for a certain protein such as BSA to obtain a higher sequence coverage or more peptide identification.

Acknowledgement

This work was supported by research fund of Chungnam National University.

References

1. Sun, W.; Gao, S.; Wang, L.; Chen, Y.; Wu, S.; Wang, X.; Zheng, D.; Gao, Y. *Mol. Cell. Proteom.* **2006**, 5, 769.
2. Inglis, A. S. *Methods Enzymol.* **1983**, 91, 324.
3. Li, A. *Anal. Chem.* **2001**, 73, 5395.
4. Hua, L.; Low, T. Y.; Sze, S. K. *Proteomics* **2006**, 6, 586.
5. Seo, M.; Kim, J.; Park, S.; Lee, J. H.; Kim, T.; Lee, J.; Kim, J. *Bull. Korean Chem. Soc.* **2013**, 34, 27.
6. Nam, M.; Lee, D.; Kim, Y.; Kim, J. *Mass Spectrom. Lett.* **2018**, 9, 46.
7. Ly, H. G. T.; Absillis, G.; Janssens, R.; Proost, P.; Parac-Vogt, T. N. *Angew. Chem.* **2015**, 54, 7391.
8. Shi, J.-H.; Pan, D.-Q.; Wang, X.-X.; Liu, T.-T.; Jiang, M.; Wang, Q. *J. Photochem. Photobiol.* **2016**, 162, 14.
9. Ghosh, S.; Dey, J. *J. Colloid Interface Sci.* **2015**, 458, 9.