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Investigating the Efficiency of Formic Acid and Hydrochloric Acid in Weak Acid Hydrolysis for Myoglobin

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Abstract : This study compares the efficiency of weak acid hydrolysis (WAH) using formic acid (FA) and hydrochloric acid (HCl) in the analysis of myoglobin peptides. WAH using 2% and 5% formic acid resulted in the identification of 32 peptides, with varying degrees of cleavage at the C-terminus of aspartic acid residues. HCl WAH with different concentrations demonstrated an increase in the total number of identified peptides but a decrease in fully cleaved peptides as the HCl concentration increased. Notably, deamidation was observed during HCl WAH but not in FA WAH. The addition of HCl WAH after FA WAH provided a similar pattern to HCl WAH, with slightly higher levels of hydrolysis. These findings highlight distinct cleavage patterns and deamidation effects between FA and HCl in the context of WAH.

Keywords : weak acid hydrolysis, trypsin, aspartic acid, trypsin, microwave

Introduction

In order to comprehensively analyze proteins, it is common practice to cleave them prior to subsequent analysis, such as mass spectrometry.[1-3] This is because proteins themselves are often too large to be directly analyzed by mass spectrometry. The usage of trypsin as a protease to cleave proteins is currently the most common methodology due to its high specificity, activity, and cost-effectiveness.[4,5] However, alternative cleavage methods have also been explored to enhance trypsin activity, such as using Lys-C.[6,7] Additionally, different amino acid cleavages were achieved by utilizing enzymes like Glu-C.[8] It has been reported that hydrolyzing a protein with 3 M HCl can result in the formation of polypeptide ladders with varying sizes, reaching up to the molecular mass of the protein.[9] In recent years, a new alternative cleavage method called weak-acid hydrolysis (WAH) has been introduced.[10,11] WAH selectively and predominantly cleaves the C-terminus of aspartic acid residues in proteins, with minor cleavage occurring at the N-terminus of aspartic acid.[12] The application of microwave in WAH can accelerate the cleavage process.[10] Studies have reported that microwave-assisted WAH of proteins can be performed with a small amount of weak acid (e.g., 2% formic acid) in a microwave oven for an hour incubation.[12] Since WAH selectively cleaves the C-terminal of aspartic acid residues, this method offers a simpler and faster alternative to using proteases like trypsin.

In our previous study, we discovered that a temperature of 100°C yielded the most effective results compared to temperatures of 37, 50, and 100°C.[11] Furthermore, we found that the hydrolysis efficiency of microwave-assisted WAH for 1 hr at 100°C was comparable to that of traditional WAH conducted for 2 hr in boiling water.[12] Additionally, in our previous study, we observed that the introduction of a small amount of acetonitrile during microwave-assisted WAH could reduce the occurrence of sodium adduct peaks.[11] In this study, we investigate the efficiency of WAH using FA and hydrochloric acid (HCl) and compare their effects on peptide cleavage patterns and deamidation.

Experimental

All chemicals used in the study, including horse heart myoglobin (MYG), 2,5-dihydroxybenzoic acid (2,5-DHB), HCl, phosphoric acid, formic acid, and acetonitrile (ACN), were obtained from Sigma-Aldrich (St. Louis, MO, USA). To prepare the MYG stock solution, 1 mg of MYG was dissolved in 1 mL of distilled water, resulting in a concentration of 1,000 ppm.

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All microwave-assisted acid WAH experiments were conducted in the Rapid Enzyme Digestion System microwave oven from ASTA (Gyeonggi-do, South Korea), which had an output of 800 W at 60 Hz and operated on an AC power supply of 220-240 V. During the microwave-assisted WAH, the solutions were subjected to microwave-assisted hydrolysis at a temperature of 100°C for 1 h. For the microwave-assisted WAH of MYG using formic acid, two solutions were prepared. The first solution consisted of 98 μL of MYG stock solution mixed with 2 μL of formic acid (2% formic acid solution). The second solution comprised 95 μL of MYG stock solution mixed with 5 μL of formic acid (5% formic acid solution). For the microwave-assisted WAH of MYG using dilute HCl, various 100 mL MYG solutions containing 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, or 3.0% HCl were prepared and then subjected to microwave irradiation. Additionally, sequential acid hydrolysis of MYG was also performed where weak acid hydrolysis using 2% formic was first performed, followed by acid hydrolysis using dilute HCl (0.2%–3.0% HCl).

To prepare the matrix solution for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses, 10 mg of 2,5-DHB was dissolved in 1.0 mL of a solution consisting of 50% acetonitrile (ACN) and 1% phosphoric acid in water. Each MYG hydrolyzed peptide solution was then mixed with the DHB matrix solution in a 1:1 (v/v) ratio. Subsequently, 1 μL of the mixture was loaded onto the MALDI plate and allowed to dry in the air. Mass spectrometric analysis was performed using a MALDI-TOF instrument (Voyager DE-STR; Applied Biosystems, Foster City, CA, USA) located at the CNU Chemistry Core Facility in Daejeon, South Korea. The analysis involved 500 laser shots under the following conditions: a 337-nm nitrogen laser, a pulse length of 3 ns, a pulse repetition rate of 20 Hz, and the positive ion reflectron mode. Each mass spectrum was obtained in the *m/z* range of 650–6000.

Results and Discussion

Efficiency comparison of WAH using 2% formic acid and 5% formic acid

Generally, WAH is performed using diluted weak acids, such as formic acid, acetic acid or phosphoric acid. WAH predominantly cleaves the C-terminus of aspartic acid residues. In this study, we compared the effect of formic acid concentration on WAH in terms of cleavage effectiveness and specificities by varying the concentration of formic acid from 2% to 5%.

Figure 1 displays the MALDI mass spectra of myoglobin digest obtained from 2% formic acid and 5% formic acid, revealing no noticeable difference between the two spectra. Both mass spectra provided the same number of identified peptides. Table 1 summarizes the 32 identified peptides.

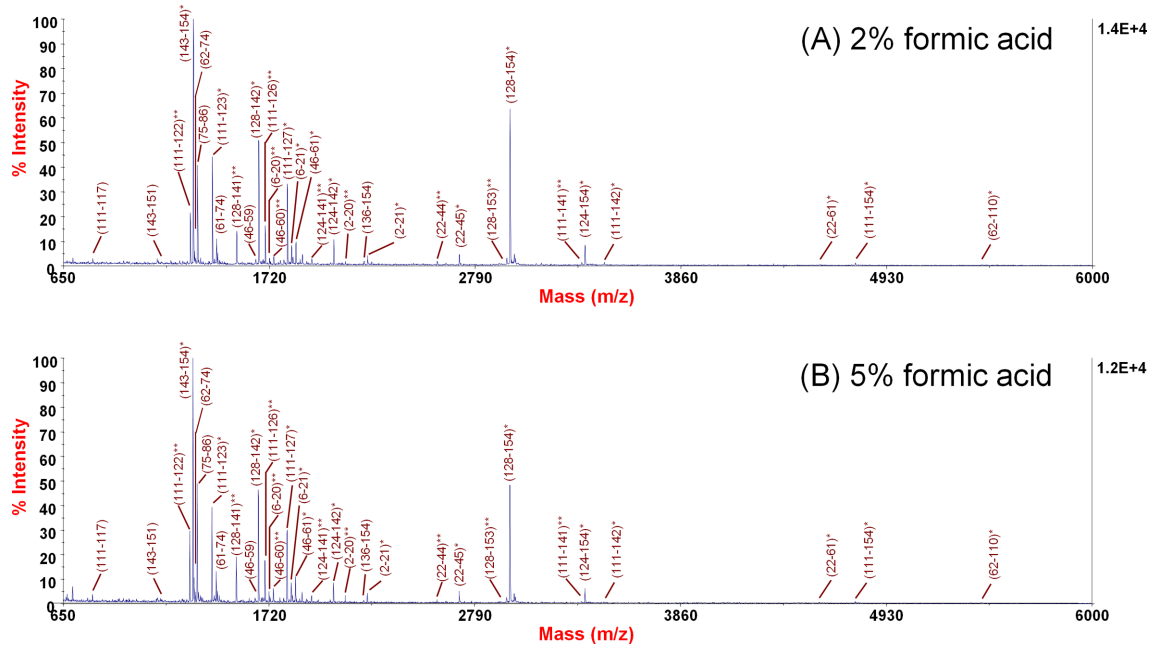

Figure 1. MALDI mass spectra of 44 pmol horse heart myoglobin hydrolyzed at 100°C by microwave irradiation for 1 h in an aqueous solution containing (A) 2% formic acid and (B) 5% formic acid. The 15 peptides from specific cleavage of microwave-assisted WAHP (e.g. C-terminal cleavage of aspartic acid residues) are marked with an asterisk, while double asterisks are marked for the 10 peptides which have the subsequent removal of an aspartic acid at the C-terminal end from the specifically cleaved peptides. The detailed peak assignments are provided in Table 1.

Table 1. Summary of the weak acid-cleaved myoglobin peptides identified from both 2% and 5% formic acid-hydrolyzed samples.

Positions		Theoretical monoisotopic *m/z* value	Experimental *m/z* value	Δmass	Peptide sequence	MC	Class[c)]
start	end						
2	20	2115.05	2115.05	0.00	(M)GLSDGEWQQVLNVWGKVEA(D)	1	UC1
2	21	2230.08	2230.05	-0.03	(M)GLSDGEWQQVLNVWGKVEAD(I)	1	C0
6	20	1742.89	1742.86	-0.03	(D)GEWQQVLNVWGKVEA(D)	0	UC1
6	21	1857.91	1857.90	-0.01	(D)GEWQQVLNVWGKVEAD(I)	0	C0
22	44	2592.39	2592.46	0.07	(D)IAGHGQEVLIRLFTGHPETLEKF(D)	0	UC1
22	45	2707.42	2707.46	0.04	(D)IAGHGQEVLIRLFTGHPETLEKFD(K)	0	C0
22	61	4582.16[a)]	4582.00	-0.16	(D)IAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEM-KASED(L)	1	C0
46	59	1647.89	1647.85	-0.04	(D)KFKHLKTEAEMKAS(E)	0	UC2
46	60	1776.93	1776.96	0.03	(D)KFKHLKTEAEMKASE(D)	0	UC1
46	61	1891.96	1891.98	0.02	(D)KFKHLKTEAEMKASED(L)	0	C0
61	74	1451.86	1451.78	-0.08	(E)DLKKHGTVVLTALG(G)	0	UN15UC36
62	74	1336.83	1336.81	-0.02	(D)LKKHGTVVLTALG(G)	0	UC36
62	110	5425.39[a)]	5425.00	-0.39	(D)LKKHGTVVLTALGGILKKKGHHEAELKPLAQSHAT-KHKIPIKYLEFISD(A)	0	C0
75	86	1346.75	1346.75	0.00	(G)GILKKKGHHEAE(L)	0	UN13UC24
111	117	802.49	802.53	0.04	(D)AIIHVLH(S)	0	UC6
111	122	1308.75	1308.77	0.02	(D)AIIHVLHSKHPG(D)	0	UC1
111	123	1423.78	1423.79	0.01	(D)AIIHVLHSKHPGD(F)	0	C0
111	126	1698.91	1698.92	0.01	(D)AIIHVLHSKHPGDFGA(D)	1	UC1
111	127	1813.93	1813.94	0.01	(D)AIIHVLHSKHPGDFGAD(A)	1	C0
111	141	3344.73	3344.65	-0.08	(D)AIIHVLHSKHPGDFGADAQGAMTKALELFRN(D)	2	UC1
111	142	3459.76	3459.72	-0.04	(D)AIIHVLHSKHPGDFGADAQGAMTKALELFRND(I)	2	C0
111	154	4767.39[a)]	4768.00	0.61	(D)AIIHVLHSKHPGDFGADAQGAMTKALELFRN-DIAAKYKELGFQG(-)	3	C0
124	141	1939.97	1939.95	-0.02	(D)FGADAQGAMTKALELFRN(D)	1	UC1
124	142	2055.00	2055.01	0.01	(D)FGADAQGAMTKALELFRND(I)	1	C0
124	154	3360.70	3360.83	0.13	(D)FGADAQGAMTKALELFRNDIAAKYKELGFQG(-)	2	C0
128	141	1549.82	1549.84	0.02	(D)AQGAMTKALELFRN(D)	0	UC1
128	142	1664.84	1664.87	0.03	(D)AQGAMTKALELFRND(I)	0	C0
128	153	2913.53	2913.53	0.00	(D)AQGAMTKALELFRNDIAAKYKELGFQ(G)	1	UC1
128	154	2970.55	2970.55	0.00	(D)AQGAMTKALELFRNDIAAKYKELGFQG(-)	1	C0
136	154	2212.18	2212.11	-0.07	(A)LELFRNDIAAKYKELGFQG(-)	1	UN8
143	151	992.58	992.60	0.02	(D)IAAKYKELG(F)	0	UC3
143	154	1324.73	1324.73	0.00	(D)IAAKYKELGFQG(-)	0	C0

[a)] Theoretical average m/z values were used.

[b)] MC = missed cleavage

[c)] 'C0' means the peptide is originated from C-terminal cleavage of aspartic acid residues. 'UC#' where # is a numerical number means the peptide has # number(s) of removed amino acid(s) at the C-terminal end of the corresponding C0 peptide. 'UN*' where * is a numerical number means the peptide has * number(s) of removed amino acid(s) at the N-terminal end of the corresponding C0 peptide. 'UN*UC#' where * and # are numerical numbers means the peptide has * number(s) of removed amino acid(s) at the N-terminal end and # number(s) of removed amino acid(s) at the C-terminal end of the corresponding C0 peptide.

Among the 32 identified peptides, 15 peptides originated from the C-terminal cleavage of aspartic acid residues (classified as C0 in Table 1), while 10 peptides resulted from the subsequent removal of C-terminal amino acids

(UC1 in Table 1). Further removal of amino acids at the C-terminal end led to the generation of 4 peptides (classified as UC# in Table 1, where # represents the number of amino acids removed at the C-terminal end). One peptide resulted from the removal of 8 amino acids at the N-terminal end from the full WAH peptide (classified as UN8 in Table 1). Two peptides (sequences of 61-74 and 75-86) were generated from unspecific cleavage, with the cleavage at the Gly74-Gly75 bond attributed to an unspecific cleavage event.

All ten UC1 peptides were observed alongside their corresponding C0 peptides. Interestingly, the intensities of UC1 peptides were greater than those of their corresponding C0 peptides in both WAH samples prepared using 2% and 5% formic acids. Furthermore, the relative intensities of the UC1 peptides to the corresponding C0 peptides were larger in the sample prepared with 5% formic acid compared to the sample prepared with 2% formic acid. This observation supports the notion that UC1 peptides are sequentially generated after their corresponding C0 peptides.

In summary, the WAH of myoglobin using formic acid (2% or 5%) resulted in the identification of 32 peptides. Among these 32 identified peptides, 15 peptides were classified as fully WAH peptides (C0 in Table 1), indicating that they were cleaved at the C-terminus of aspartic acid residues. Another 15 peptides were classified as partially WAH peptides (UC or UN in Table 1), indicating that they underwent partial cleavage either at the C-terminus or N-terminus. Additionally, two peptides were generated through unspecific cleavage. Among the 15 partially WAH peptides, 10 peptides were found to result from the removal of a single C-terminal amino acid.

WAH using HCl only

Here, a diluted HCl aqueous solution was utilized for WAH to verify the effectiveness of HCl in this process. Different concentrations of HCl ranging from 0.2% to 3.0% were employed for WAH of myoglobin. Figure 2 displays the MALDI mass spectra of the myoglobin digest obtained through WAH using 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, or 3.0% HCl, where the identified peptides are listed in supplementary Table S1.

Among the 32 identified myoglobin peptides detected from WAH using 2% FA or 5% FA, two peptides (sequences of 111-142 and 111-154) with 2 and 3 miscleavages, respectively, were not detected in any of the mass spectra using WAH with HCl. WAH with 0.2%, 0.4%, and 0.6% HCl solutions successfully identified the remaining 30 peptides. However, two peptides (sequences of 111-141 and 124-154) with two miscleavages were not detected in the HCl WAH of myoglobin using 1.0%, 2.0%, or 3.0% HCl, indicating that the highly miscleaved peptides observed in 2% FA or 5% FA WAH are cleaved differently as the HCl concentration increases. This is supported by the absence of peptides with miscleavages

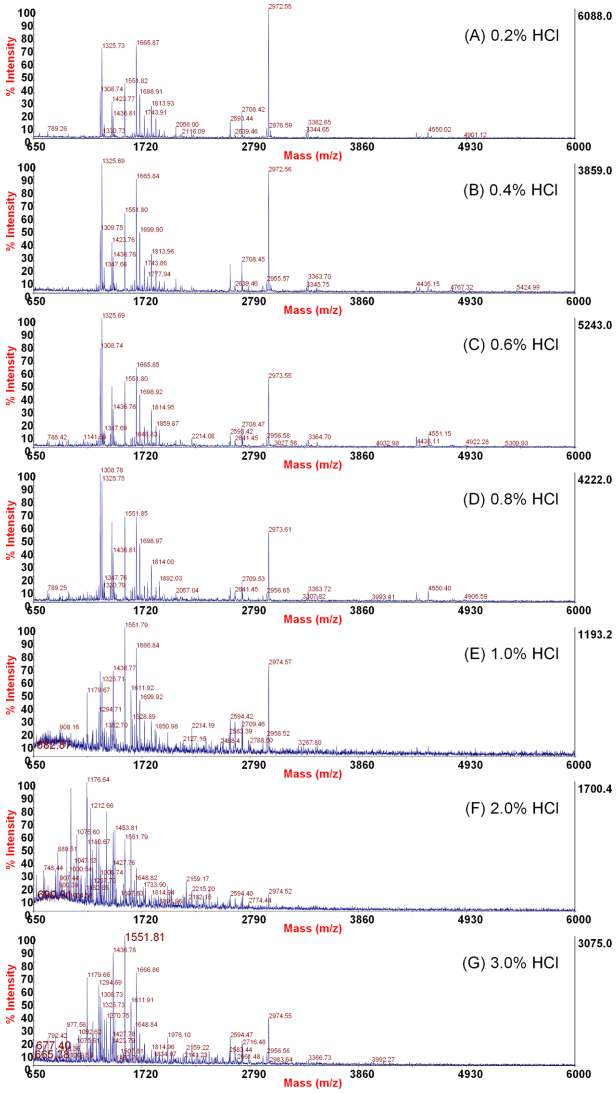

Figure 2. MALDI mass spectra of 44 pmol horse heart myoglobin from WAH using 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, or 3.0% HCl.

higher than one in the 1.0%, 2.0%, or 3.0% HCl hydrolysis. Furthermore, additional peptides were detected in the HCl WAH of myoglobin compared to FA WAH. Notably, a large peptide (sequence of 6-45 with one miscleavage from C-terminal cleavage of aspartic acid residues) at *m/z* 4550 was not detected with FA WAH, but was detected in WAH using dilute (0.2%, 0.4%, 0.6%, and 0.8%) HCl solutions. These observations suggest that FA and HCl may have different mechanisms for inducing hydrolysis.

As the percentage of HCl increased, the total number of identified peptides in the HCl WAH of myoglobin also increased, while the number of fully cleaved peptides (C0 peptides) decreased. Specifically, the total numbers of identified peptides from HCl WAH from 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, and 3.0% HCl were 45, 58, 78,

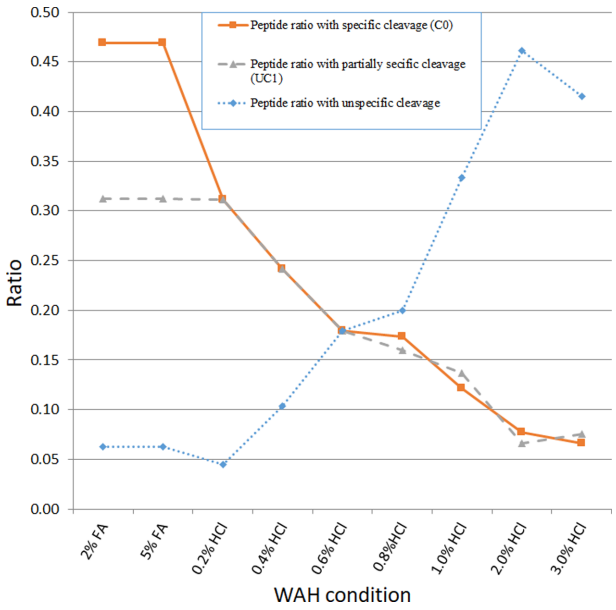

Figure 3. Ratio change of specific peptides (C0), partially specific (UC1), and unspecific peptides for both FA WAH and HCl WAH conditions.

75, 66, 91, and 106, respectively, while the numbers of C0 peptides were 14, 14, 14, 13, 8, 7, and 7. Interestingly, the number of unspecific peptides increased as the percentage of HCl increased. These findings indicate that 0.2% HCl is more effective than 5% FA in terms of hydrolyzing myoglobin.

Figure 3 illustrates the ratios of specific peptides (C0), partially specific (UC1), and unspecific peptides (UN-UC) for both FA WAH and HCl WAH conditions, showing that as the percentage of HCl increased, the ratio of specific and partially specific peptides gradually decreased while the ratio of unspecific peptides increased. In WAH of proteins using dilute HCl, the ratios of specific peptides and partially specific peptides were very similar while they were very different for 2% FA and 5% FA. The similarity of the ratios of specific peptides and partially specific peptides in WAH of proteins using dilute HCl suggests that it may be possible that the C0 and UC1 peptides were generated at the same time, while with FA C0 peptides were generated which is then followed by UC1 peptides.

Deamidation from HCl WAH

Deamidation, a spontaneous nonenzymatic reaction, leads to the conversion of asparaginyl and glutaminyl residues into aspartyl and glutamyl residues, respectively. This process can cause structural and biological alterations in peptides and protein structures.[13,14] Deamidation of asparagine is one of the most frequently occurring non-enzymatic modifications, resulting in the generation of aspartic acid or iso-aspartic acid.[15,16] Glutamine deamidation also occurs but at a slower rate compared to asparagine

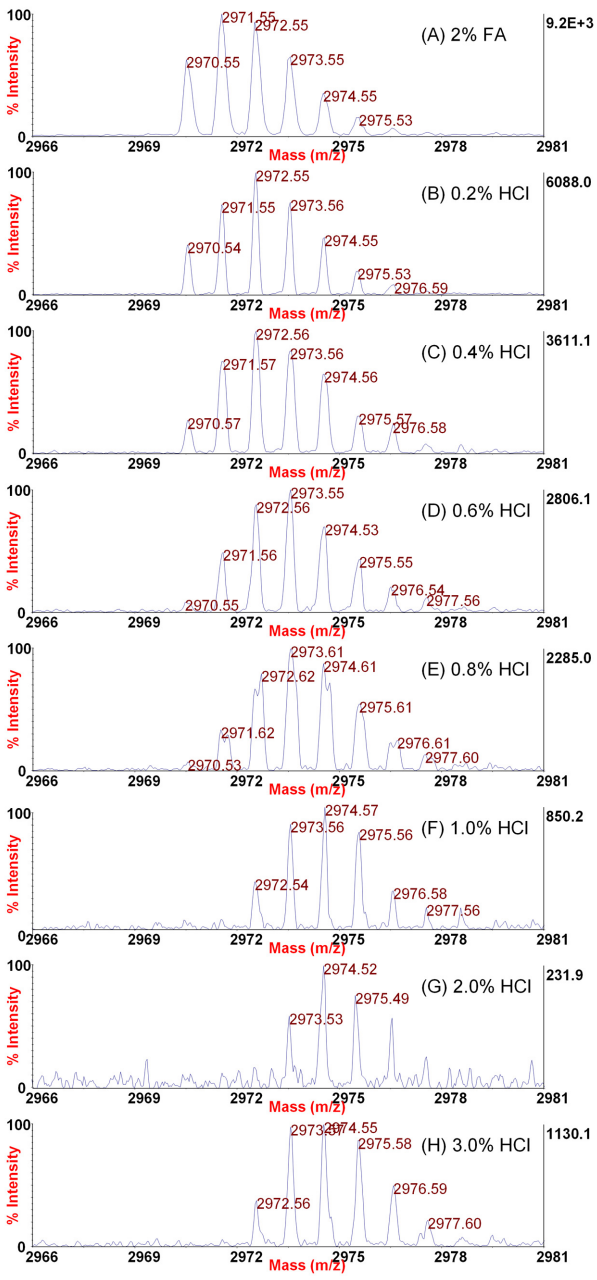

Figure 4. MALDI mass spectra of a peptide (sequence of 128-154; AQGAMTKALELFRNDIAAKYKELGFQG) obtained from HCl WAH of myoglobin using different HCl concentrations. The spectra demonstrate an increase in the deamidation degree of the peptide with higher HCl concentrations during the WAH process of myoglobin.

deamidation.[17] Deamidation commonly results in a mass increase of approximately +1 Da, corresponding to the conversion of an -NH_2 group to an -OH group. This alteration in chemical composition contributes to the observed mass change during deamidation. It has been reported that intact three-dimensional structures tend to

reduce deamidation, whereas elevated deamidation levels are observed following tryptic digestion.[18]

In this study, deamidation of both asparagine and glutamine residues was observed during HCl WAH of myoglobin while no deamidation was observed during FA WAH. Figure 4 displays the MALDI mass spectra of a specific peptide (sequence of 128-154; AQGAMTKALEL FRNDIAAKYKELGFQG, theoretical monoisotopic *m/z* value for $[M+H]^+$ of 2970.55), demonstrating the progressive increase in deamidation degree with increasing HCl concentration during WAH of myoglobin. Generally, higher levels of deamidation were observed with higher HCl concentrations, displaying a gradual increase in deamidation as the HCl concentration increases. The base peak undergoes a gradual shift, such as *m/z* 2971.55 for 2% FA, *m/z* 2972.55 for 0.2% and 0.4% HCl, *m/z* 2973.55 for 0.6% and 0.8% HCl, and *m/z* 2974.55 for 1.0%, 2.0%, and 3.0% HCl, indicating the deamidation levels of 0, 1, 2, and 3 sites, respectively. Since the peptide contains three potential deamidation sites (two Q's and one N), it is presumed that all three amino acids were deamidated in higher concentrations (1%, 2%, and 3%) of HCl.

Even at 0.2% HCl, a certain level of deamidation was observed in peptides containing asparagine or glutamine.

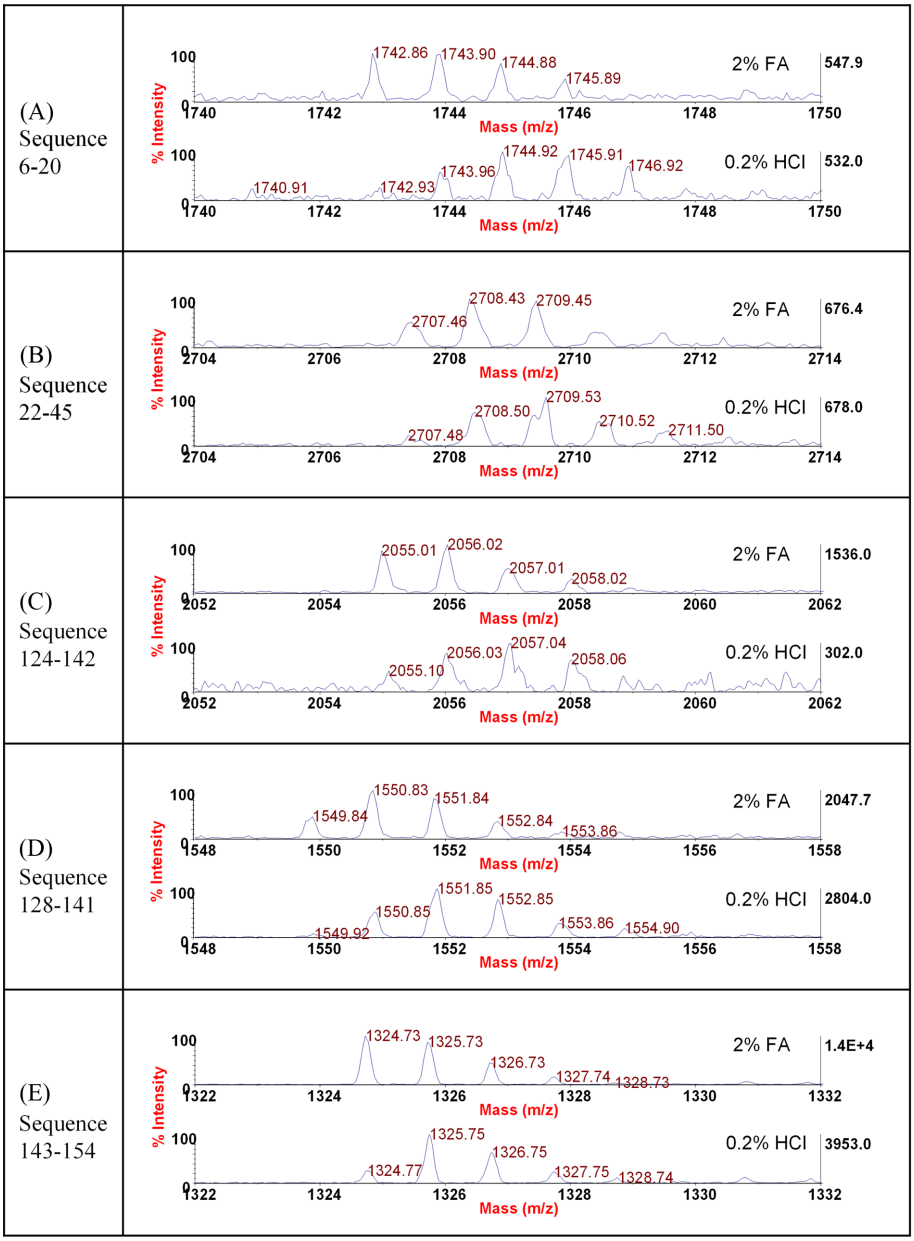

Figure 5. MALDI mass spectra of myoglobin peptides showing deamination from 0.2% HCl WAH, compared to 2% FA WAH.

The peptides that displayed deamidated forms are indicated in supplementary Table S1. Figure 5 presents representative MALDI mass spectra of peptides exhibiting deamidation from 0.2% HCl WAH in comparison to 2% FA WAH. A mass shift of one Dalton was observed due to deamidation.

WAH using FA followed by HCl

To investigate the impact of HCl on the FA WAH myoglobin peptides, HCl hydrolysis experiments were conducted using various concentrations of HCl ranging from 0.2% to 3.0% on the 2% FA WAH myoglobin peptides. Figure 6 displays the mass spectra of myoglobin digest obtained from 2% FA WAH, followed by subsequent treatment with 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, or 3.0% HCl, where the list of the identified peptides is provided in supplementary Table S2. The notation 'FA→HCl' will be used to indicate the sequence of 2% FA WAH followed by HCl treatment.

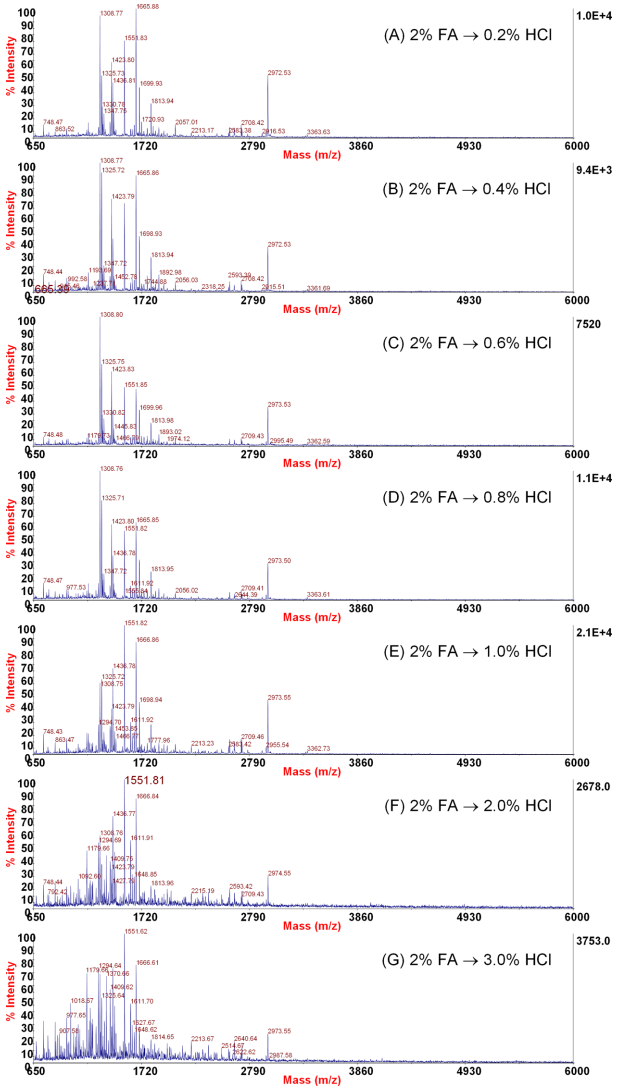

Figure 6. MALDI mass spectra of myoglobin digest from 2% FA WAH followed by 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, or 3.0% HCl.

Among the 32 identified peptides from 2% FA WAH of myoglobin, three peptides (sequences of 2-20, 2-21, and 111-142) were not detected in any of the mass spectra obtained from FA→HCl WAH. Instead, corresponding peptides resulting from further cleavage were observed. It is believed that the additional HCl hydrolysis in the FA→HCl process eliminated the missed cleavages, completing the WAH process. With increasing concentrations of HCl in FA→HCl, more peptides were detected, including those resulting from partial cleavages and unspecific cleavages. The total numbers of identified peptides in FA→HCl WAH, using 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 2.0%, and 3.0% HCl were 68, 83, 77, 90, 114, 103, and 110, respectively, while the numbers of C0 peptides were 13, 12, 11, 12, 12, 8, and 9.

Figure 7 shows the ratios of specific peptides (C0), partially specific (UC1), and unspecific peptides (UN-UC) at FA WAH and FA→HCl WAH conditions. Figure 7 exhibits similarities to Figure 3, with the main distinction being the notable decrease in the ratio observed from the 5% FA WAH to the 2% FA→0.2% HCl WAH, compared to the ratio change from the 5% FA WAH to the 0.2% HCl WAH. Additionally, there is a slight decrease in the UC1 ratio from to the C0 ratio in the 2% FA→0.2% HCl WAH. The significant ratio change is due to the additional hydrolysis using 0.2% HCl on 2% FA WAH peptides. The slight decrease of the UC1 ratio from the C0 ratio suggests that aspartic acid N-terminal cleavage of C-terminal

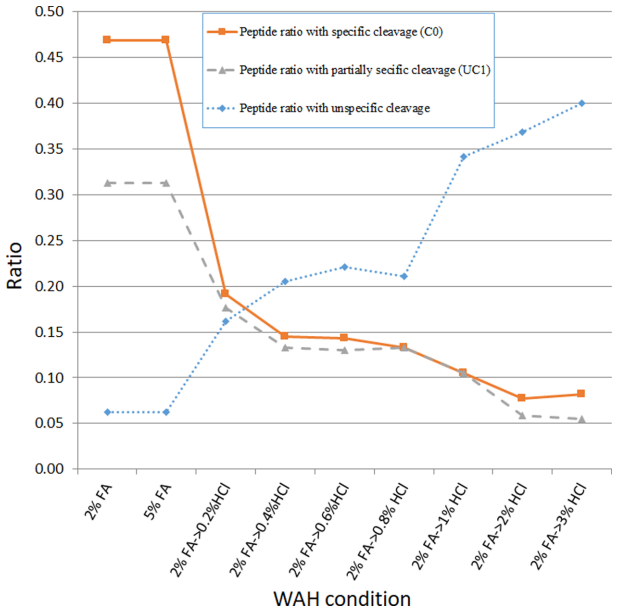

Figure 7. Ratio change of specific peptides (C0), partially specific (UC1), and unspecific peptides for both FA WAH and FA→HCl WAH conditions.

cleaved aspartic acid is further cleaved from 2% FA→ 0.2% HCl WAH, compared to 0.2% HCl WAH.

Conclusion

In this study, the efficiency of WAH using FA and HCl was compared. WAH using 2% and 5% formic acid resulted in the identification of 32 peptides, with 15 peptides fully cleaved at the C-terminus of aspartic acid residues (C0), 10 peptides partially cleaved (UC1), and 2 peptides generated through unspecific cleavage. The intensities of UC1 peptides were greater than those of C0 peptides, and this trend was more pronounced in the sample prepared with 5% formic acid. WAH using HCl with different concentrations showed an increase in the total number of identified peptides as the concentration increased, but a decrease in the number of fully cleaved peptides. Deamidation was observed during HCl WAH but not in FA WAH. The additional HCl WAH after FA WAH provided a similar pattern to HCl WAH, with slightly higher hydrolysis. Overall, FA and HCl showed different cleavage patterns and deamidation effects in WAH.

Supporting information

Supplementary information is available at https://drive.google.com/file/d/1JUFMTUdewQDSGFfZ_q1hyngK7MjC4stA/view?usp=sharing

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