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# Weak Acid Hydrolysis of Proteins

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Weak acid hydrolysis of proteins (WAHP) is a process where a weak acid solution is used at a high temperature to hydrolyze proteins. While high-temperature solutions of strong acids result in the complete hydrolysis of proteins into individual amino acids,[1] WAHP predominantly cleaves aspartyl residues at the C-terminus and occasionally aspartic acid residues at the N-terminus[2,3] but is ineffective at room temperature. Optimal cleavage is observed after 2 h at pH 2.0 and 108 °C.[3] Recent investigations have shown that WAHP can be expedited by exposing the digestion mixture to microwave radiation.[4,5] A previous article described microwave-assisted WAHP (MAWAHP) and confirmed the identities and relative abundances of hydrolyzed peptides using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in positive ion reflectron mode.[6]

The present paper describes the use of a positive ion linear mode MALDI-MS in analyses of WAH hydrolysates of myoglobin. The experimental procedure was the same as that published previously[6] except that the mass spectra were obtained in linear mode, which is more sensitive than reflectron mode. Figure 1 shows MALDI mass spectra of myoglobin after hydrolysis in 2% formic acid. Microwave-assisted digestion (Figure 1(a)) was performed for 1 h at three different temperatures (37 °C, 50 °C, and 100 °C). Digestion was also performed in boiling water without microwave irradiation (Figure 1(b)) at incubation times of 20, 40, 60, and 120 min. Protein peaks were observed in the mass spectra of myoglobin that had been hydrolyzed by MAWAH at 37 °C and 50 °C for 1 h and in the mass spectrum of myoglobin that had been hydrolyzed in boiling water for 20 min.

Supplementary Figures S1 and S2 show enlarged mass spectra from *m/z* 1000 to *m/z* 8000 with complete assignments of the MAWAH hydrosylates of myoglobin and the

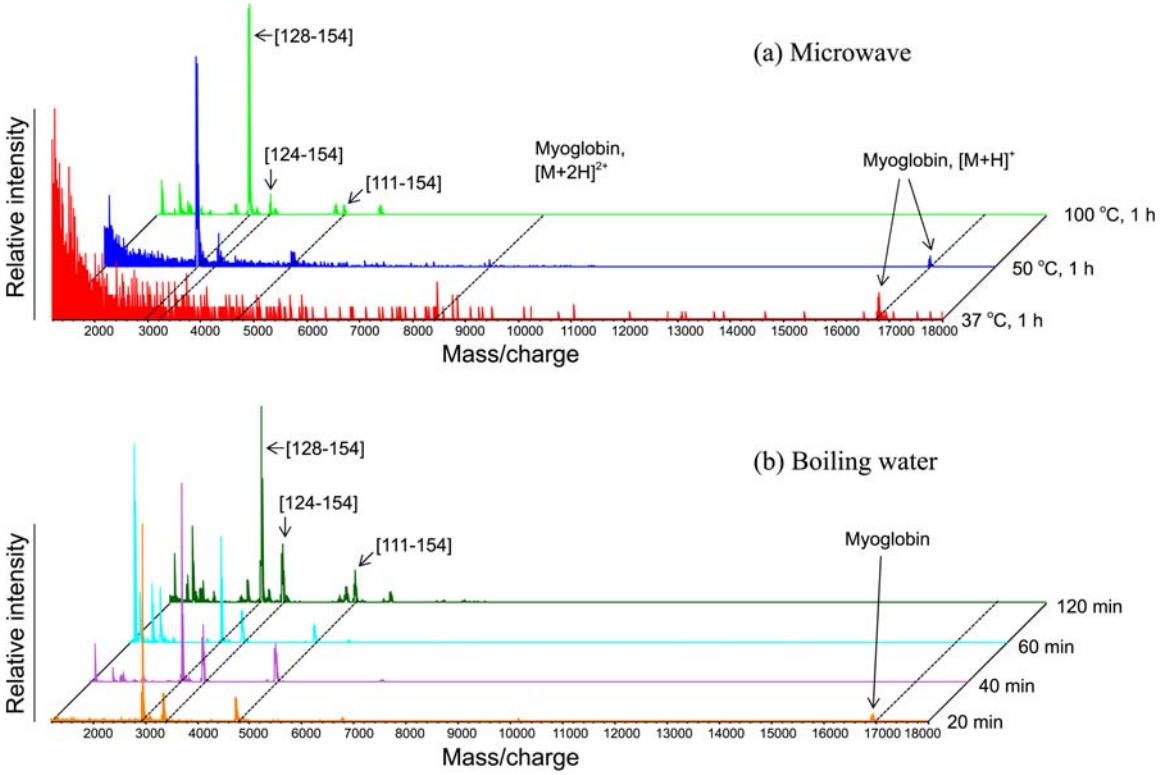


**Figure 1.** MALDI mass spectra obtained from 29 pmol of horse heart myoglobin hydrolyzed in a 2% formic acid (a) with microwave irradiation for 1 h at 37 °C, 50 °C, and 100 °C, and (b) in boiling water without microwave irradiation for 20, 40, 60, and 120 min.

**Table 1.** Summary of weak acid hydrolyzed myoglobin using microwave and boiling water under various conditions[a]

| Conditions of weak acidic cleavage | Microwave, 1 h | | | Boiling water | | | |
|---|---|---|---|---|---|---|---|
| | 37 °C | 50 °C | 100 °C | 20 min | 40 min | 60 min | 120 min |
| Total number of identified peptides | 0 | 4 | 22 | 5 | 10 | 20 | 23 |
| Number of identified peptides with missed cleavages | 0 | 3 | 12 | 5 | 7 | 12 | 14 |
| Ratio of missed cleavages | 0 | 75.0% | 54.5% | 100% | 70.0% | 60.0% | 60.9% |
| Number of identified peptides with cleavages at the N-terminal aspartic acid | 0 | 0 | 7 | 0 | 0 | 2 | 7 |
| Ratio of cleavage at N-terminus of aspartic acid to cleavage at C-terminus of aspartic acid | 0 | 0 | 31.8% | 0 | 0 | 10% | 30.4% |
| Sequence coverage | 0% | 28.8% | 100% | 89.6% | 100% | 100% | 100% |

[a]Complete list of the identified peptides can be found in Supplementary Table S1.

WAH hydrosylates of myoglobin in boiling water, respectively. Table 1 and Supplementary Table S1 show a summary of the WAH results and a complete list of the identified peptides from the two different hydrolysis conditions, respectively. No peptide peaks, except the protein peak, were observed in the mass spectrum of myoglobin hydrolyzed in a microwave oven for 1 h at 37 °C. In contrast, 4 and 22 hydrolyzed peptides were identified after 1 h of microwave irradiation at 50 °C and 100 °C, respectively. This suggests that maintaining 100 °C is critical for WAHP digestion, even with microwave assistance. The four peptide peaks detected in the 1 h hydrosylates of MAWAH originated from the C-terminal side of myoglobin. The N-terminal of each peptide was generated from a cleavage at the C-terminus of aspartic acid. Thus, the initial cleavages resulting from MAWAH of myoglobin were at the C-terminus of aspartic acid. As hydrolysis progressed, additional peptides, generated from cleavages at the N-termini of aspartic acid residues, were observed. A similar trend was observed in hydrosylates obtained from acid hydrolysis in boiling water. As shown in Figures S1(C) and S2(C-D), all of the peptides generated from cleavages at the N-terminus of aspartic acid appeared with corresponding peptides generated from cleavages at the C-terminus of aspartic acid, the latter of which were more abundant. This strongly suggests that cleavage at the N-terminus of aspartic acid occurs only if the aspartic acid has already been cleaved at its C-terminus.

As shown in Table 1, 7 of 22 peptides were generated from cleavages at the N-terminus of aspartic acid (~31.8%) after 1 h of MAWAH. A similar cleavage ratio (~30.4%) was observed after 120 min of WAH in boiling water. Therefore, the hydrolysis efficiency of MAWAH after 1 h at 100 °C matched that of WAH in boiling water after 2 h. The numbers of identified peptides, 22 and 23, were also similar in these two conditions, respectively.

The ratios of missed cleavages in all of the hydrolyzed myoglobin samples were higher than 50%. The cleavage ratio at the N-terminus of aspartic acid increased with MAWAH temperature and WAH duration in boiling water. This indicates that complete cleavage at the C-terminus of aspartic acid would never be achieved without partial cleavage at the N-terminus.

In conclusion, the use of a positive ion linear mode MALDI-MS, compared to reflectron mode, provided in-depth information in the analyses of WAH hydrolysates of myoglobin, mainly due to inherent better sensitivity of linear mode than reflectron mode. MALDI-MS analyses in positive ion linear mode demonstrated that cleavages at the N-terminus of aspartic acid always occur after cleavage of the C-terminus, and the hydrolysis efficiency of MAWAH after 1 h at 100 °C is similar to that of WAH after 2 h in boiling water.

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**Supporting Information.** Additional supporting information may be found in the online version of this article.

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