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Development of microwave-assisted acid hydrolysis of proteins using a commercial microwave reactor and its combination with LC–MS for protein full-sequence analysis

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ABSTRACT

Microwave-assisted acid hydrolysis (MAAH) can be used to degrade a protein non-specifically into many peptides with overlapping sequences which can be identified by mass spectrometry (MS) to produce a sequence map that covers the full sequence of a protein. The success of this method for protein sequence analysis depends on the proper control of the MAAH process, which is currently done using a household microwave oven. However, to meet the regulatory or good laboratory practice (GLP) requirement in a clinical or pharmaceutical laboratory, using a commercial microwave device is often required. In this paper, we report a method of performing MAAH using a CEM Discover single-mode microwave reactor. It is shown that, using an optimized protocol for MAAH, reproducible results comparable to those obtained using a household microwave oven can be generated using the commercial reactor. To illustrate the potential applications of MAAH MS for characterizing clinically relevant proteins, this method was applied, for the first time, to map the amino acid sequences of normal and sickle-cell human hemoglobin as well as bovine hemoglobin. Full sequence coverage was readily achieved from 294 and 266 unique peptides matched to the alpha and beta subunits of normal hemoglobin, respectively, 334 and 265 unique peptides matched to the alpha and beta submit units of sickle-cell hemoglobin, and 377 and 224 unique peptides matched to the alpha and beta subunits of bovine hemoglobin. This method opens the possibility for any laboratory to use a commercial laboratory equipment to perform MAAH MS for protein full-sequence analysis.

1. Introduction

Microwave-assisted acid hydrolysis (MAAH) of proteins combined with mass spectrometry (MS) has been developed as a useful tool for protein sequence analysis [1,2]. Within minutes, proteins are non-specifically digested to peptides by an acid under the microwave irradiation. The resultant peptides can be analyzed by MS. There are two methods of MAAH MS for protein sequence analysis. One method uses 6 M HCl to produce polypeptide ladders by non-specific hydrolysis of individual amide bonds [3]. By controlling the microwave irradiation time (<1 min), protein molecules produce N-terminal polypeptides and their counterparts, C-terminal polypeptides, from predominately one amide-bond breakage per molecule. Direct MS analysis of the resultant polypeptide ladders can provide the protein sequence information. For small proteins ($<20{,}000$ Da), the entire sequence can be read, and for larger proteins, terminal sequence information can be generated. Another method involves the use of 25% trifluoroacetic acid (TFA) for MAAH with a much longer microwave irradiation time (e.g., 10 min) [4]. A protein, such as bovine serum albumin (BSA), can be digested into small peptides from the hydrolysis of multiple amide bonds and thus many internal peptides are produced. By analyzing the hydrolyzed peptides using liquid chromatography tandem MS (LC–MS/MS), up to 100% protein sequence coverage can be readily achieved [5].

Until now, MAAH for protein sequence analysis has been carried out mainly with a household microwave oven [1,2]. However, for reasons such as obtaining regulatory approval and following good laboratory practice (GLP) as well as considering other issues related to reliability, oven-to-oven performance consistency and operational safety, a commercial microwave device tailored to perform MAAH is a preferred choice. Commercial microwave systems are widely used for degrading proteins into amino acids [6] and have also been reported for use in other microwave-assisted protein degradation methods such as trypsin digestion [7–9] and site-specific acid hydrolysis [10–14], where only a small number of chemical bond breakages occur which are adequate for protein identification based on MS/MS spectral match

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of one or a few peptides. In contrast, in the method of MAAH for protein full-sequence analysis, many amide bonds need to be hydrolyzed and thus more stringent experimental conditions are required in order to generate many peptides that can cover the entire protein sequence. In this paper, we report the optimized protocols for performing both HCl and TFA MAAH experiments using a commercial CEM Discover single-mode microwave reactor for protein sequence analysis. In addition, we illustrate a potential application of the TFA MAAH protocol, in combination with LC–MS/MS, for analyzing the sequences of hemoglobin variants. Determining hemoglobin variant sequences is significant in clinical applications where over 1200 variants of human hemoglobins have been discovered to date and this number keeps increasing [15].

2. Experimental

2.1. Chemicals and reagents

All chemicals and reagents, except those specifically noted, were purchased from Sigma-Aldrich Canada (Markham, ON, Canada). LC–MS-grade water and acetonitrile (ACN) were purchased from Thermo Fisher Scientific (Edmonton, AB, Canada). ACS-grade 37% HCl was from Merck (KGaA, Darmstadt, Germany).

2.2. Preparation of normal human hemoglobin

Fresh blood was collected from a healthy volunteer and stored in a tube with anticoagulant reagent (EDTA). Red blood cells were separated from plasma and other blood components by centrifuging at $1958 \times g$ for 15 min. After centrifugation, plasma and red blood cells were separated into two layers and the plasma was transferred into a 1.5-mL micro-centrifuge tube. The red blood cell solution was lysed by adding LC–MS-grade water and vortexing. Then the lysed solution was centrifuged at $20{,}800 \times g$ for 15 min. The supernatant containing hemoglobin was transferred to a 1.5-mL micro-centrifuge tube for further analysis and the cell debris was discarded.

2.3. HCl MAAH of proteins

For HCl MAAH, 40 μL of 0.25 mg mL^{-1} protein solution was mixed with 0.5 μL of 500 mM DTT and 40 μL of 6 M HCl in a 1.5 mL polypropylene vial. The household microwave oven method was adapted from that reported elsewhere with some modifications [16] (see Supplemental note N1). For the commercial microwave device method, the CEM Discover single-mode microwave reactor equipped with a fiber-optic probe (CEM Corporation, Matthews, NC) was used. HCl MAAH was carried out in the CEM device with a water bath of boiled water for a period of 30–90 s with 200 or 300 W power applied. The acid hydrolysates were cooled and dried in a SpeedVac (Thermo Savant, Milford, MA) to remove the acid. 30 μL of 250 mM NH_4HCO_3 was used to reconstitute the sample. 9 μL of 500 mM DTT was added to reduce the disulfide bonds still remaining in hydrolyzed peptides with incubation for 60 min at 37 °C. This step may be omitted for not a highly folded protein. In our previous work, we have shown that performing DTT reduction after HCl MAAH can help release more peptides that may be linked by disulfide bonds due to incomplete reduction of these bonds in a highly folded protein (e.g., prion) [16]. After reduction, the peptide solution was acidified to pH 2 with 50% TFA and diluted to 105 μL with 0.1% TFA.

2.4. TFA MAAH of proteins

For TFA MAAH, 10 μL (1 μg μL^{-1}) of the protein solution was mixed with an equal volume of 20 mM DTT in a 1.5-mL polypropylene centrifuge vial and incubated at 60 °C for 20 min. 20 μL 50% (v/v) TFA was added to the sample solution after incubation. The household microwave oven method was adapted from the literature [5] (see Supplemental note N1). For the commercial microwave device method, TFA MAAH was carried out in the CEM microwave system with a water bath of boiled water for 7.5 min or 10 min with 200 or 300 W power applied. The sample was dried in the SpeedVac. The dried hydrolysates were then reconstituted with 105 μL of 0.1% TFA.

2.5. LC-desalting or fractionation of hydrolysates

Salt removal or desalting of the hydrolysates generated by the TFA MAAH method was carried out in an Agilent 1100 HPLC system (Palo Alto, CA, USA) using a 4.6 mm i.d. × 5 cm Polaris C18 column with a particle size of 3 μm and 300 Å pore (Varian, MA, USA). After injecting 100 μL of the peptide sample, the column was flushed with 97.5% mobile phase A (0.1% TFA in water) and 2.5% mobile phase B (0.1% TFA in ACN) at a flow rate of 1 mL min^{-1} at room temperature to effectively remove the salts. The concentration of mobile phase B was then step-wise increased to 85% to ensure complete elution of the peptides from the column, followed by 15 min re-equilibration with 97.5% mobile phase A.

The fractionation of the hydrolysates generated by the HCl MAAH method was carried out on a 3 mm i.d. × 15 cm Zorbax 300 - SB C_3 column with a particle size of 3.5 μm and 300 Å pore (Agilent, Mississauga, ON, Canada) in the Agilent 1100 HPLC system. After injecting 100 μL of the peptide sample, the column was flushed with 97.5% mobile phase A (0.1% TFA in water) and 2.5% mobile phase B (0.1% TFA in ACN) at a flow rate of 0.4 mL min^{-1} for 5 min at room temperature to effectively remove the salts. The concentration of mobile phase was then step-wise increased to 30% for 9 min to elute the low molecular weight peptides from the column, followed by 15 min re-equilibration with 97.5% mobile phase A.

2.6. LC–MS/MS

The collected peptides from the desalting or fractionation step were dried in the SpeedVac to remove all the solvents and acid, reconstituted with 0.1% formic acid and then analyzed by a quadrupole time-of-flight (QTOF) Premier mass spectrometer (Waters, Manchester, U.K.) equipped with a nanoACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) [5]. 5 μL of peptide solution was injected onto a 75 μm i.d. × 150 mm Atlantis dC_{18} column with 3 μm particle size (Waters). Solvent A consisted of 0.1% formic acid in water, and Solvent B consisted of 0.1% formic acid in ACN. The peptides generated by TFA MAAH were first separated using 120 min gradients (2–6% Solvent B for 2 min, 6–25% Solvent B for 95 min, 30–50% Solvent B for 10 min, 50–90% Solvent B for 10 min, 90–5% Solvent B for 5 min at 35 °C; the column was pre-equilibrated at 2% Solvent B for 20 min) and electrosprayed into the mass spectrometer (fitted with a nanoLockSpray source) at a flow rate of 300 nL min^{-1} . Mass spectra were acquired from m/z 300–1600 for 0.8 s, followed by four data-dependent MS/MS scans from m/z 50–1900 for 0.8 s each. The peptides generated by HCl MAAH were separated using a 30 min gradient (2–7% Solvent B for 1 min, 7–25% Solvent B for 19 min, 25–50% Solvent B for 1 min, 50–90% Solvent B for 2 min, 90–90% Solvent B for 6 min, 90–95% Solvent B for 2 min; the column was pre-equilibrated at 2% Solvent B for 20 min) and electrosprayed into the mass spectrometer at a flow rate of 350 nL/min. Mass

spectra were acquired from m/z 400–1600 for 0.8 s, followed by six data-dependent MS/MS scans from m/z 50–1900 for 0.8 s each. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide. Leucine enkephalin and (Glu1)-fibrinopeptide B, a mixed mass calibrant (i.e., lock-mass), was infused at a rate of 300 nL min^{-1}, and an MS scan was acquired for 1 s every 1 min throughout the run.

2.7. Database search

Raw MS and MS/MS data were lock-mass-corrected, de-isotoped, and converted to peak list files by ProteinLynx Global Server 2.3 (Waters) [5]. Peptide sequences were identified via automated database searching of peak list files using the MASCOT search program (http://www.matrixscience.com). Database searches were restricted to the protein sequences downloaded from the SwissProt database. The following search parameters were selected for all database searching (i.e., for both HCl and TFA MAAH methods): enzyme, nonspecified; missed cleavages, 0; peptide tolerance, ±30 ppm; MS/MS tolerance, 0.2 Da; peptide charge, (1+, 2+, and 3+); variable modifications, deamidation of asparagine and glutamine. For hemoglobins, oxidation of M, Y, W and C were also selected as variable modifications in the search. We note that chlorination of amino acids has been observed when HCl MAAH was used to hydrolyze proteins into amino acids [17]. However, in our paper, using a milder condition than amino acid generation, we did not observe chlorination of peptides, which is consistent with a recent report where chlorination was not reported in HCl MAAH MS of proteins using a high resolution Orbitrap MS [18]. Thus, in our paper, we did not include chlorination modification in database search. The search results, including unique peptide sequences, ion score, MASCOT threshold score for identity, calculated molecular mass of the peptide, and the difference (error) between the experimental and calculated masses were extracted to Excel files. All the identified peptides with scores lower than the MASCOT identity threshold scores for identity were then deleted from the list. The rationale of using a threshold score to validate a peptide identification has been described previously [5].

3. Results and discussion

3.1. MAAH method development

For the household microwave oven, microwave irradiation is continuously applied to the samples at a specified power. However, in the standard mode of the CEM Discover microwave system which controls the temperature and pressure at the same time, power would be applied intermittently to maintain a pre-set temperature or pressure. The reproducibility of MAAH using the CEM system operated in this mode was found to be poor. To mimic the MAAH condition in the household microwave oven, the power-time mode of CEM was chosen in which a pre-set power is applied to the sample all the time unless the temperature or pressure exceeds the set limit. The household microwave oven has a maximum power of 1200 W, compared to 300 W in the CEM system. To compensate for the lower power of CEM, a water bath of boiled water was used for MAAH. In the setup shown in Fig. 1, 100 mL of boiled water was added to the container in CEM. The sample vials were then placed in the holder that sat in the container. Microwave irradiation was applied for a short period with a power of 200 W or 300 W. The temperature limit was set to 150 °C and the pressure limit was set to 147 psi. The fiber-optic probe was immersed in the water bath, not inside a sample vial, to monitor the temperature and pressure. In this way, the temperature and pressure could not exceed the limits and thus the microwave irradiation could be applied to the samples all the time. The actual temperature and pressure inside the sample vials were unknown; this was also true for the household microwave oven.

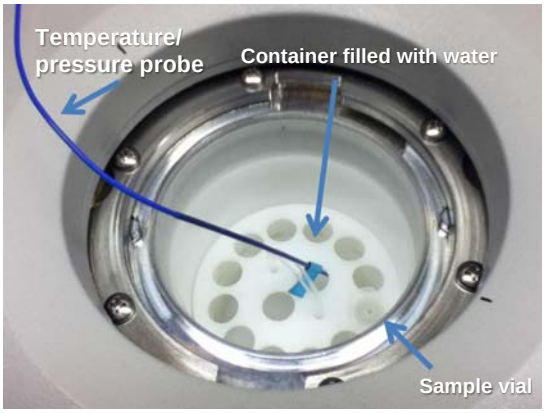

Fig. 1. Picture of the sample setup in the CEM Discover microwave system for MAAH.

3.2. TFA MAAH

Supplemental note N1 describes the performance comparison of TFA MAAH using the household microwave oven and the commercial microwave device using a standard protein, BSA. For the CEM system, comparable results to a household microwave oven were obtained using 10 min microwave irradiation at the maximum power, i.e., 300 W.

3.3. HCl MAAH

Due to low abundance of the terminal peptides compared to the internal peptides in the TFA MAAH hydrolysate, the terminal peptide sequence information may be missing, resulting in less than 100% sequence coverage [19]. Thus, if we combine the advantage of preferentially detecting terminal peptides by HCl MAAH and the advantage of detecting many internal peptides by TFA MAAH, full sequence coverage of a protein can be ensured and many sequence overlaps from the terminal and internal peptide ladders can be generated for detailed amino acid sequence analysis of a protein. However, in HCl MAAH, the microwave irradiation process needs to be carefully controlled to avoid further hydrolysis of the terminal peptides into internal peptides.

The current practice of HCl MAAH using a household microwave oven is to place a sample vial near a beaker filled with room temperature water, and the beaker sits on a rotating plate in the oven (i.e., the classic method) [2]. The water is used to absorb the major portion of the microwave radiation. However, household microwave ovens are known to have “hot” spots due to unevenly distributed microwave radiation inside the oven. It is thus necessary to find a proper location of the sample vial inside an oven and this location is always used in subsequent experiments in order to generate reproducible results. We have now developed an optimized method for HCl MAAH using a water bath similar to that used for TFA MAAH. In this water-bath method, 100 mL of boiled water is added to a plastic beaker, a floating sample rack is placed in this water bath, and one or more sample vials are then placed on the rack. The beaker is placed inside a household oven for MAAH.

We initially tested different hydrolysis times for HCl MAAH of BSA using 1200 W, and found that all the proteins were hydrolyzed to peptides (and likely amino acids) and the internal peptides were dominated even after 20 s microwave irradiation.

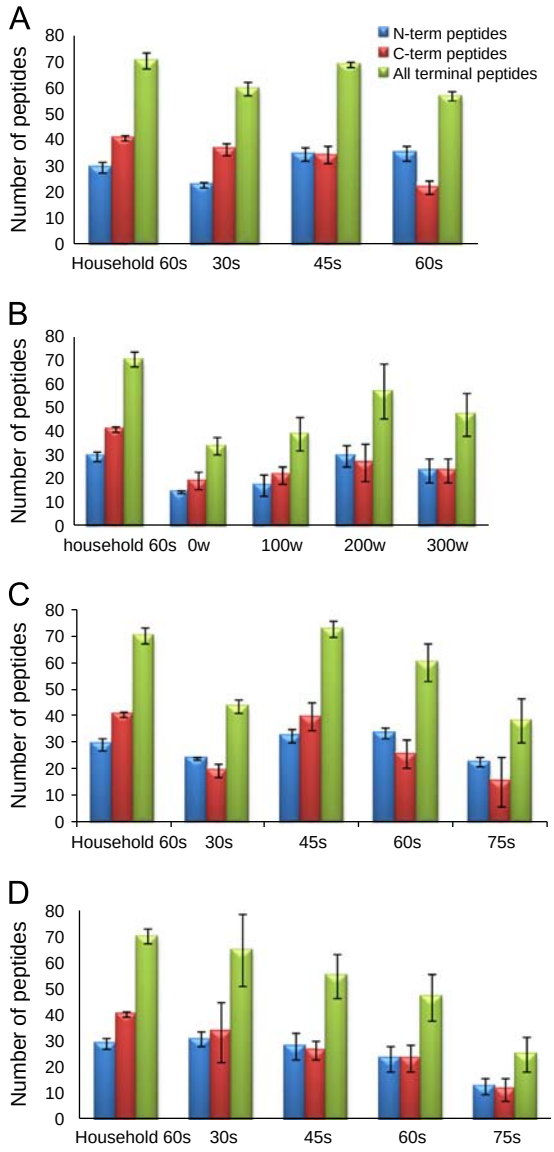

Fig. 2. Comparison of the number of terminal peptides of BSA identified by LC–ESI MS/MS in the hydrolysates generated by HCl MAAH ($n=3$) with (A) the water bath method in a household microwave oven using different microwave irradiation times, (B) the water bath method in the CEM system using different microwave power, (C) the water bath method in the CEM system using different irradiation times at 200 W, and (D) the water bath method in the CEM system using different irradiation times at 300 W. For comparison, the number of terminal peptides identified using the classic HCl MAAH method is also shown in each plot.

To slow down the hydrolysis process, a lower power (240 W) was used and different hydrolysis times (30, 45 and 60 s) were tested. Fig. 2(A) shows the numbers of terminal peptides identified. For comparison, the data obtained from the classic method (60 s irradiation at 1200 W) is also shown. From Fig. 2(A), it is clear that HCl MAAH using a water bath for 45 s at 240 W could generate comparable results with those of the classic method. We also found that the results were reproducible from the sample vials placed at different sample positions in the floating rack. In addition, the location of the beaker inside the microwave oven did not affect the results.

After we developed the water bath method for reproducible HCl MAAH using the household microwave oven, we started to transfer this method to the CEM system. In this case, a water bath of boiled water was also employed to avoid the uneven heating. First of all, we tested HCl MAAH of BSA using 60 s irradiation at 0, 100, 200, and 300 W in the CEM system and the results are shown in Fig. 2(B). Fig. 2(B) shows that the average number of identified peptides gradually increased as the power increased, reached the maximum at 200 W, and then decreased at 300 W. Next, we tested HCl MAAH of BSA at a fixed power (200 W or 300 W) using different irradiation times ranging from 30–75 s in the CEM system. The results from 200–300 W are shown in Fig. 2(C and D), respectively. These results indicate that HCl MAAH for 45 s at 200 W or 30 s at 300 W in CEM generated comparable results to those from the household microwave oven. In particular, the numbers of peptides detected and the standard derivations of the data from the 45 s at 200 W are very similar to the household microwave oven results.

The above discussion indicates that similar results could be obtained in the CEM system as those in the household microwave oven for both TFA and HCl MAAH for BSA. Previous work has shown that the same optimal conditions could be applied for MAAH of various different proteins, i.e., the MAAH conditions used are not protein-dependent [19]. To illustrate the potential utility of the MAAH MS method in a laboratory that requires the use of a commercial microwave oven such as a clinical laboratory, we applied the method to map the sequences of various hemoglobin molecules.

3.4. MS for analyzing hemoglobin variants

Currently, hemoglobin variation detection is a part of newborn screening practice in many parts of the world. This is typically done using HPLC or electrophoresis for screening a few commonly known variants such as sickle-cell hemoglobin. Once an abnormal HPLC or electrophoresis pattern is discovered, the sample is sent to a special clinical analytical laboratory equipped with various tools, including MS, to determine the hemoglobin variant. In some cases, the initial screening process may miss the detection of a hemoglobin variant (false negative) and a follow-up analysis of the patient sample is done in the special clinical analytical laboratory. Over 1200 variants have been reported [15] and new variants are still being discovered [20,21]. To apply MS to characterize a hemoglobin variant, a bottom-up approach, where an enzyme (e.g., trypsin) is used to digest the protein into several peptides, is often used to analyze the amino acid sequence of hemoglobin with 70–90% sequence coverage [22–26]. Top-down sequencing of the intact hemoglobin has also been reported for generating sequence information [27–29]. Although it provides lower sequence coverage (~50%), the top-down method can potentially provide complementary information to the bottom-up method for characterizing a specific variant [30,31]. MAAH MS is a bottom-up method. But, unlike enzyme digestion, MAAH generates many peptides with sequence overlaps that can always cover the entire protein sequence. Thus, it can potentially be a very useful method for the analysis of hemoglobin variants. We have examined the performance of the method for analyzing three different hemoglobins, i.e., bovine hemoglobin, normal and sickle-cell human hemoglobin.

Different species have different hemoglobin sequences and some interesting evolution studies have been reported recently using hemoglobin sequence information of different species [32]. We applied TFA MAAH LC–MS/MS to map the bovine hemoglobin sequence. The alpha and beta subunits of bovine hemoglobin were identified with 99–100% sequence coverage, respectively, when the MS/MS data from the hydrolysate were searched against all the bovine hemoglobin variants. Fig. 3(A and B) shows the protein sequences and sequence coverage of the two subunits. There were 801 peptides (377 unique) matched to the alpha subunit and 335 peptides (224 unique) matched to the beta subunit (see Supplemental tables T1 and T2 for the list of unique peptides

A MVLSAADKGNVKAAWGKVGGHAAEYGAEALERMFLSFPTTK
TYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHLDDLPGALSEL
SDLHAHKLRVDPVNFKLLSHSLLVTLASHLPSDFTPAVHASLDKF
LANVSTVLTSKYR

B MLTAEEKAAVTAFWGKVKVDEVGGEALGRLLVVYPWTQRFFE
SFGDLSTADAVMNNPKVKAHGKKVLDSFSNGMKHLDDLKGTF
AALSELHCDKLHVDPENFKLLGNVLVVVLARNFGKEFTPVLQA
DFQKVVAGVANALAHRYH

C MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTK
TYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNALS
ALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASL
DKFLASVSTVLTSKYR

D MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRF
FESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKG
TFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQ
AAYQKVVAGVANALAHKYH

E MVLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTK
TYFPHFDLSHGSAQVKGHGKKVADALTNAVAHVDDMPNALS
ALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASL
DKFLASVSTVLTSKYR

F MVHLTPVEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRF
FESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKG
TFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQ
AAYQKVVAGVANALAHKYH

Fig. 3. Protein sequence and sequence coverage (underlined) by the hydrolysate peptides of (A) alpha subunit of bovine hemoglobin, (B) beta subunit of bovine hemoglobin, (C) alpha subunit of normal human hemoglobin, (D) beta subunit of normal human hemoglobin, (E) alpha subunit of sickle hemoglobin, and (F) beta subunit of sickle hemoglobin. TFA MAAH was used for digesting the proteins.

identified). These peptide sequences indicate that the first methionine of the alpha subunit was removed after translation while the methionine of the beta unit was not, which is consistent with the literature [33]. Thus, the effective sequence coverage for the alpha subunit was also 100%.

For the normal human hemoglobin isolated from the red blood cells of a healthy individual, the TFA MAAH LC–MS/MS method identified 553 peptides (294 unique) matched to the alpha subunit and 420 peptides (266 unique) matched to the beta subunit (Supplemental tables T3 and T4), allowing 100% sequence coverage as shown in Fig. 3(C and D). In this case, the first methionine of both subunits was removed after translation. For the sickle-cell hemoglobin, a variant of normal hemoglobin with an amino acid substitution at residue 7 from Glu to Val in the beta subunit, the TFA MAAH method could also cover 100% sequence with many overlapped sequences from different peptides (Fig. 3(E and F)). There were 617 peptides (334 unique) matched to the alpha subunit and 407 peptides (265 unique) matched to the beta subunit (Supplemental tables T5 and T6). Fig. 4 shows the MS/MS spectra of three peptides generated by TFA MAAH from the sickle-cell hemoglobin. The sequences of these peptides cover the amino acid substitution site and thus the substitution site can be readily identified. This example illustrates the power of using multiple peptides containing the same substitution site to unambiguously identify the variant site.

To illustrate the possibility of using HCl MAAH MS to determine or confirm the terminal peptides of a hemoglobin variant, the hydrolysates of normal human hemoglobin, sickle-cell hemoglobin and bovine hemoglobin generated by the HCl MAAH method were analyzed by LC–ESI MS/MS and searched against their corresponding protein sequences. Supplemental tables T 7–12 list the unique terminal peptides identified from these hemoglobins. For bovine hemoglobin, the first methionine of the alpha subunit was removed while the beta subunit was not. For human hemoglobins,

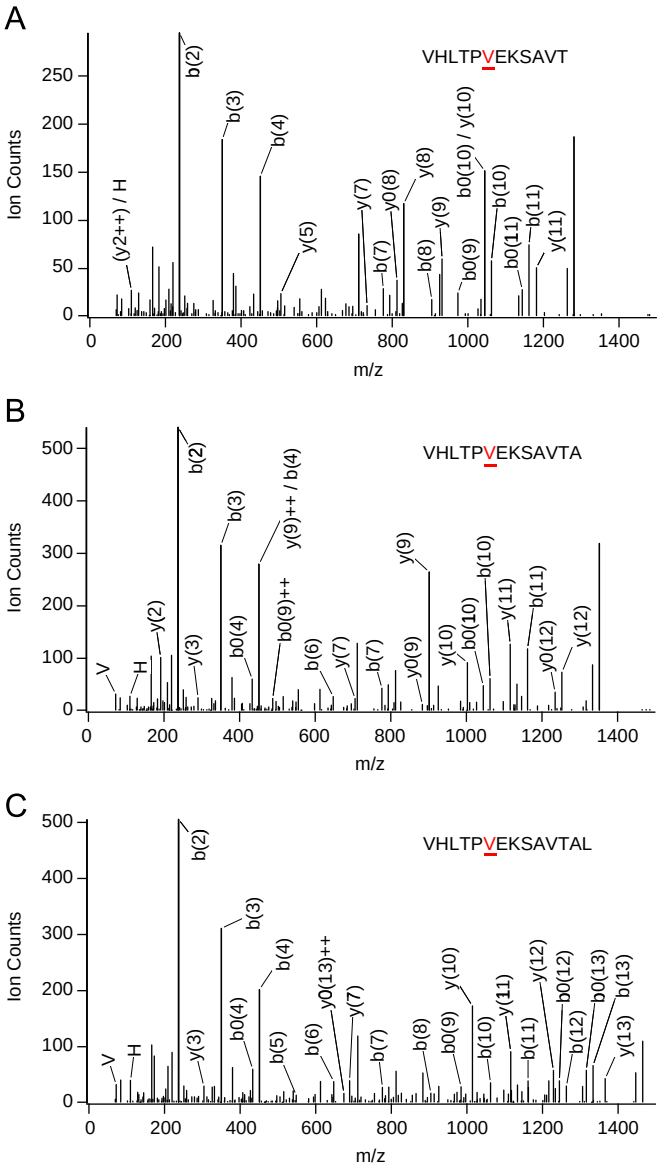

Fig. 4. MS/MS spectra (signal intensity vs. m/z) of three peptides found in the hydrolysate of sickle-cell hemoglobin generated by TFA MAAH. The peptide sequences covering the amino acid substitution site are listed.

the alpha and beta subunits had the first methionine removed. Thus the HCl MAAH data confirmed the results obtained by the TFA MAAH method.

The above results indicate that MAAH MS can be used to generate many peptides with their overlapping sequences covering the entire protein sequence of hemoglobin. The MAAH method using a commercial microwave device can be readily implemented in a clinical laboratory where identification or confirmation of hemoglobin variants is performed. It provides a complementary or alternative tool to the existing methods, such as enzyme-based bottom-up and top-down methods, for MS-based hemoglobin characterization. We note that protein identification is different from protein sequence analysis. Protein identification can be done using MAAH MS/MS for complex protein samples as we have shown elsewhere [4]. However, for protein sequence analysis, we want to detect as many peptides from a protein as possible to cover the full sequence. This method works best if the dominant protein has a purity of above 70%, as we have seen previously [5]. However, MAAH has been recently shown to be able to digest proteins separated by gel electrophoresis [34]. Since closely related

hemoglobin variants may be separated by gel electrophoresis [35], the combination of electrophoresis separation of hemoglobin variants and in-gel MAAH MS is worth investigating in the future. In addition to characterizing variants in a special clinical laboratory, the use of MAAH MS for fast screening of common hemoglobin variants will be explored. To this end, we are in the process of developing a hemoglobin sequence database and a robust search algorithm for processing MAAH MS data in a high-throughput manner.

4. Conclusions

In this paper, we report a MAAH method based on the use of a commercial microwave device that can be used in a laboratory where strict regulatory or good laboratory practice requirement is applied. We have optimized the microwave sample setup and the experimental conditions to generate reproducible results for both TFA and HCl MAAH methods. We have shown that comparable results could be obtained from MAAH MS using the CEM Discover microwave system and a household microwave oven. As an example of potential applications of the method for characterizing clinically relevant samples, we showed that MAAH MS could be used to map the protein sequence with 100% coverage for bovine hemoglobin, normal human hemoglobin, and sickle-cell hemoglobin. This method should be useful to serve as a complementary or alternative tool to the existing enzyme-based bottom-up and top-down MS methods for detailed characterization of hemoglobin variants. The application of this method for characterizing recombinant proteins, such as those used as vaccines and antibody drugs in pharmaceutical industry, will be reported in the future.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.042.

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