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Optimization and application of microwave-assisted acid hydrolysis for rapid quantification of protein oxidation markers using LC–MS

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ABSTRACT

Simple and efficient microwave-assisted acid hydrolysis (MAAH) of proteins was used for rapid quantification of α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) as major protein oxidation markers. The precursor amino acid residues corresponding to AAS and GGS in oxidized proteins were derivatized by reductive amination with sodium cyanoborohydride ($NaCNBH_3$) and *p*-aminobenzoic acid (ABA) followed by MAAH to generate the marker derivatives AAS–ABA and GGS–ABA. The quantification was performed using electrospray ionization liquid chromatography–mass spectrometry (ESI LC–MS). The important parameters for hydrolysis were optimized, which include the temperature, the reaction time, the acid concentration and volume as well as the microwave power. Compared to the conventional acid hydrolysis of 18–24 h using 6–12 M HCl at 110 °C applied commonly in the literature and also in this work, MAAH of proteins can be completed as fast as in only 2–10 min and, additionally, with a 3–5 times higher yield of the final derivatization products. Furthermore, a better agreement between the ratio of the detected derivatization products and the theoretical yields from the studied protein has also been achieved, which indicates that MAAH may serve as a more reliable method of acid hydrolysis for this purpose than that with conventional thermal heating. The MAAH method is demonstrated to be a time-saving, reproducible and efficient technique for studying AAS and GGS as protein oxidation markers using LC–MS.

1. Introduction

Either as functional biomolecules in biological environment or as nutrients during storage and processing of foods, proteins are vulnerable to oxidation by reactive oxygen and oxidative species. Protein oxidation can affect greatly its functionalities because exact conformation and pattern of folding are closely associated with its activity and function in a biological system [1] and food quality [2,3]. Of special interest is that recent studies have indicated that protein oxidation plays a major role in a number of human diseases and aging [1,4]. Oxidative damage to proteins by reactive oxygen species can result in mainly introducing carbonyl groups into amino acid residues followed by cleavage of the polypeptide backbone, cross-linking and other modification of the side chains of amino acids [5]. Reactive oxygen and nitrogen species are formed during normal metabolism and in higher fluxes under pathological conditions. Cells can detoxify some of the reactive species, e.g. by reducing protein hydroperoxides to un-reactive hydroxides [6]. Oxidized proteins are often functionally inactive and their unfolding is associated with enhanced susceptibility to proteinases. Thus cells can generally remove oxidized proteins by proteolysis. However, certain oxidized proteins are poorly handled by cells, and together with possible alterations in the rate of production of oxidized proteins, this may contribute to the observed accumulation and damaging actions of oxidized proteins during aging [1,4,7] and other neurodegenerative diseases such as Parkinson's disease [8], Alzheimer's disease [8,9], rheumatoid arthritis [10], amyotrophic lateral sclerosis [11] and diabetes mellitus [12]. Currently, protein oxidation is increasingly gaining more attention of medical, biological and food scientists.

Previously, the quantification of the total protein carbonyls through the dinitrophenylhydrazine (DNPH) method [13] with photometric detection has been the most common technique for assessing protein oxidation. However, the associated problem is that this approach does not provide any specific information about the chemical structures and formation mechanisms, which are essential evidence to understand the oxidation pathway in vivo. Two carbonyls, α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), have been recently highlighted as biomarkers of oxidative damage to proteins [14]. AAS and GGS are oxidative deamination products of lysine and arginine/proline residues respectively (Fig. 1). For instance, both compounds have been

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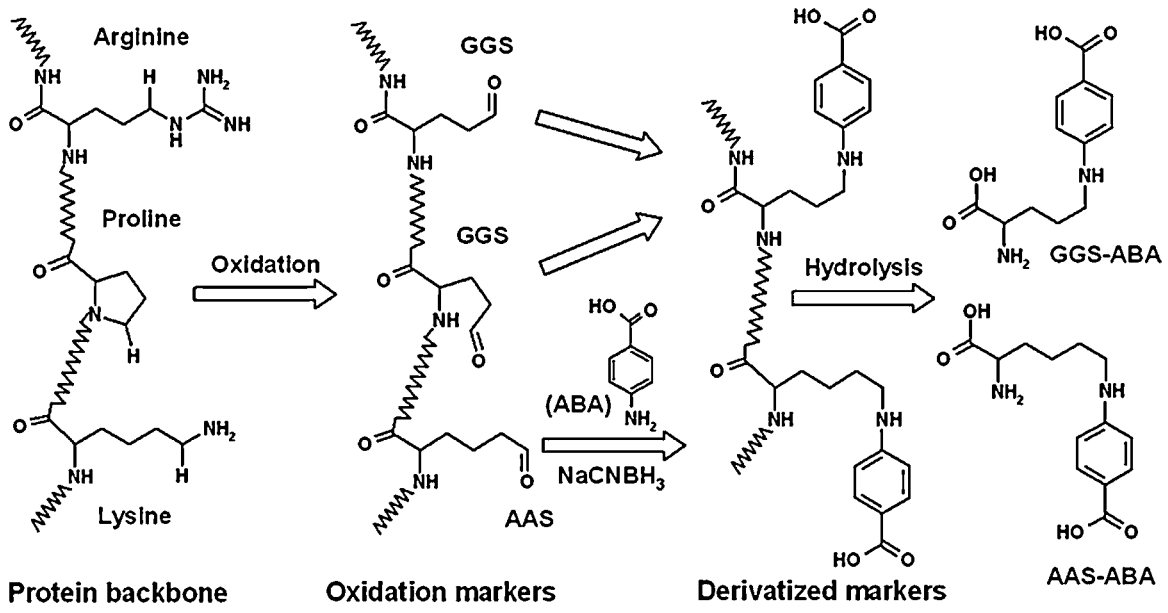

Fig. 1. Formation of the marker molecules GGS and AAS during protein oxidation and their derivatization followed by acid hydrolysis to obtain the derivatized oxidation markers GGS–ABA and AAS–ABA. Please note that, after oxidation, the proline residue produces the same aldehyde GGS as that of an arginine residue, which consequently results in a same product after derivatization with ABA. To simply the drawing, only the common product is illustrated.

reported as major carbonyl products of metal-catalyzed oxidation of plasma and liver proteins [14,15]. In medical research, these semialdehydes have been used as biomarkers of oxidative stress and indicators of serious age-related disorders. It has been reported that the quantification of these reactive semialdehydes has been proven to be a difficult task in the literature [15,16] because AAS and GGS in proteins need to be protected through chemical derivatization. Otherwise, the aldehyde groups would be destroyed during the subsequent acid hydrolysis to obtain the small molecule markers. Several derivatization methods have been described for the analysis of AAS and GGS in biological samples [14,15,17,18] using GC–MS, HPLC or LC–MS. In most cases, the sample preparation takes generally about two days. This is mainly due to the slow acid hydrolysis of 18–24 h using 6–12 M HCl at 110 °C under the conventional thermal heating condition [17,26], which has certainly limited its applications. Besides this, other two fast acid hydrolysis methods should also be mentioned if only the hydrolysis of un-derivatized proteins or peptides into free amino acids is concerned. It was realized at further elevated temperatures of 158 °C in 23–45 min with 7 M HCl and 10% trifluoroacetic acid [19] or 176 °C in 13 min with 2.5 M mercaptoethanesulfonic acid vapor [20] respectively. Although the stability of most amino acids under the same condition was satisfactory [20], there has been no reports whether these two methods are suitable for studying the protein oxidation marker semialdehydes AAS and GGS (concerning the aldehyde groups) and their derivatives AAS–ABA and GGS–ABA (formed with *p*-aminobenzoic acid, ABA) mentioned above, mainly due to the unavailability of the standards of the relevant compounds (markers and their derivatives). The semialdehydes (without derivatization) and their derivatives may not survive these hydrolysis conditions at these high temperatures. In addition, the high-temperature method also requires the reaction vials to be evacuated (high vacuum) and sealed by pulling glass tubes with such as an oxygen flame [20]. This makes it inconvenient and especially for high throughput applications.

On the other hand, as an alternative, the utilization of microwave heating to accelerate chemical synthesis reactions has been investigated in the past two decades [21]. A microwave-assisted method is particularly promising to implement the hydrolysis of organic compounds in both acid and alkaline media. In addition, the use of microwave irradiation to accelerate enzymatic reactions has also been extensively explored. For example, microwave-assisted acid hydrolysis (MAAH) has been recently applied to degrade proteins rapidly into amino acids [22] or peptides [23], followed by mass spectrometric analysis for protein sequencing and identification in proteomics studies. For complete degradation of proteins into amino acids, besides the comparable amino acid recoveries obtained using both the microwave-assisted method and the traditional direct oven heating, microwave-assisted acid hydrolysis has demonstrated a promising speed of more than two orders of magnitude faster than the conventional approach [22]. The microwaves are high frequency electromagnetic energies, which are strongly absorbed by polar molecules. The absorption results in rapid and intensive dielectric heating. The reactions under microwave conditions using closed vessels can be performed at elevated temperatures and pressures. For instance, the temperature of the solvent submitted to microwave irradiation can be raised above its boiling point [24]. For a complex macromolecule containing polar functional groups, a strong localized heating can be expected to occur at these polar targets under microwave irradiation. This could result in hydrolysis, degradation, extraction, and/or release of some of the constituents of the matrix, which opens a new analytical possibility for obtaining information on the chemical composition of such macromolecules. The dramatically accelerated reaction/interaction can be solely due to increased temperatures and a faster rate of achieving the desired local temperature, or it is associated with augmented agitation resulting from the dipolar rotation of molecules, which leads to significantly enhanced reaction efficiencies [25]. It should be noted that, as for many other experimental parameters, an optimization of temperature, duration and microwave power is essential to obtain the right condition for a microwave-assisted reaction. Overheating with microwave will generally result in serious side reactions to interfere the analysis. In this study, we will introduce and optimize microwave-assisted acid hydrolysis to hydrolyze the oxidized proteins, through which the derivatized semialdehydes AAS–ABA and GGS–ABA (Fig. 1) will be freed from the protein backbone without being destroyed. Several important parameters such as temperature, irradiation time, acid concentration and microwave power were optimized and their effects will be discussed. The result will

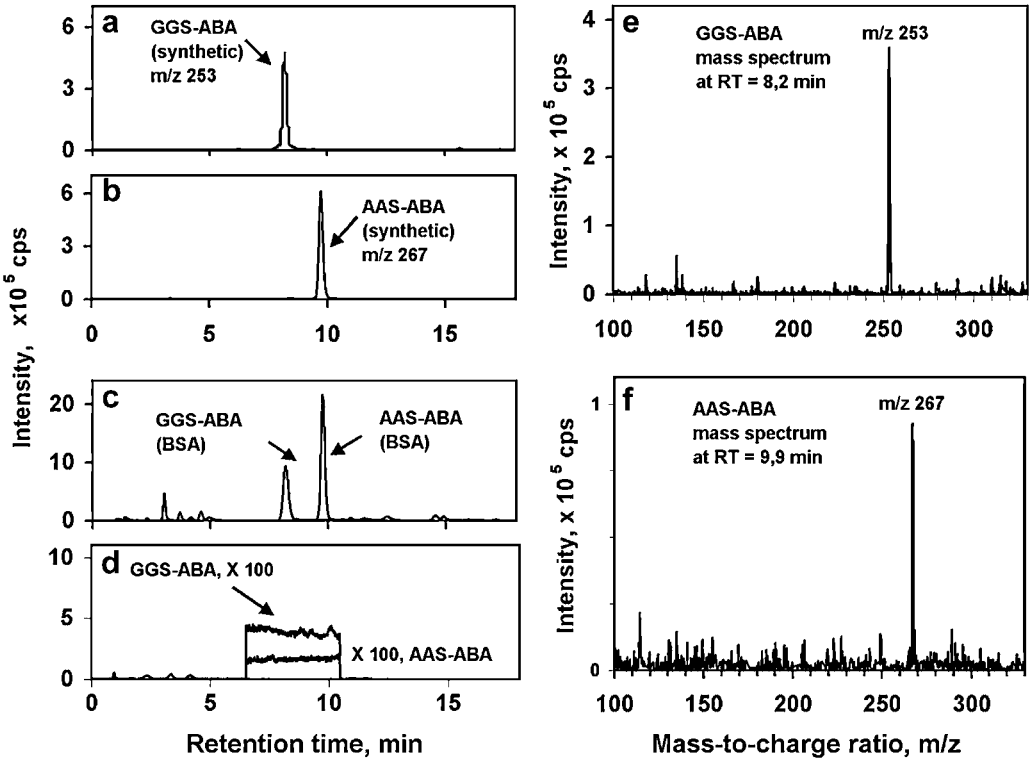

Fig. 2. ESI LC–MS selected ion monitoring (SIM) chromatograms of GGS–ABA (*m*/*z* 253) and AAS–ABA (*m*/*z* 267) obtained from (a) synthetic GGS–ABA, (b) synthetic AAS–ABA, (c) oxidized protein BSA and (d) non-oxidized BSA (blank). The corresponding full-scan mass spectra from the oxidized BSA are given in (e) for GGS–ABA (RT = 8,2 min) and (f) for AAS–ABA (RT = 9,9 min) respectively.

also been compared with that from the most commonly applied acid hydrolysis method using 6–12 M HCl at 110 °C (18–24 h) under the conventional thermal heating condition, which is also the only available report about obtaining AAS–ABA and GGS–ABA from oxidized and derivatized proteins [17,26].

2. Experimental

2.1. Materials and reagents

All chemicals were of analytical grade, except methanol of HPLC grade. N-α-acetyl-L-lysine, N-α-acetyl-L-ornithine, 2-(N-morpholino) ethanesulfonic acid (MES), sodium dodecyl sulfate (SDS), diethylenetriaminepentaacetic acid (DTPA), trichloroacetic acid (TCA) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich. α-Aminobenzoic acid (ABA), sodium cyanoborohydride ($NaCNBH_3$) and H_2O_2 (30% aqueous solution) were from Fluka. De-ionized water was purified with a Milli-Q system (Millipore Co.). Egg shell membrane isolated from the fresh hen eggs was washed thoroughly with de-ionized water, chopped into small pieces, and dried with filter paper before use.

2.2. Synthesis of AAS–ABA and GGS–ABA

The synthesis was carried out according to the literature [17,26] with some modifications. As the reaction intermediate semialdehydes, N-acetyl-L-AAS and N-acetyl-L-GGS were first synthesized from N-α-acetyl-L-lysine and N-α-acetyl-L-ornithine using lysyl oxidase activity from egg shell membrane [27]. Briefly, 10 mM N-α-acetyl-L-lysine or N-α-acetyl-L-ornithine were incubated with 5 g of egg shell membrane in 50 mL of 20 mM sodium phosphate buffer at pH 9.0 and 37 °C for 24 h with constant stirring. The egg shell membrane was then removed after centrifugation, and the pH of the solution was adjusted to 6.0 using 1 M HCl. The resulting substances were reductively aminated with 3 mmol of ABA in the presence of 4.5 mmol of $NaCNBH_3$ at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed with 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C in a rotary evaporator to dryness. The resulting AAS–ABA and GGS–ABA were purified using thin layer chromatography using ethyl acetate/acetic acid/water (20:2:1 v/v) as the elution solvent.

2.3. Oxidation of protein and its derivatization

The oxidation and subsequent derivatization of proteins were carried out according to the procedure described in the literature [17,26]. To obtain the oxidized protein as a model analyte for this study, BSA (20.0 mg/mL) was incubated with 1.0 mM H_2O_2 and 100 μM $FeCl_2$ in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for two weeks with shaking in the dark.

This was followed by derivatization to protect the semialdehydes from destroying during acid hydrolysis. Briefly, an aliquot (200 μL) of protein suspension was dispensed in Eppendorf tubes. The protein was precipitated with 2 mL of cold 10% TCA and subsequent centrifugation at 4500 g for 30 min. The resulting pellets were treated again with 2 mL of cold 5% TCA, and the protein was again precipitated after centrifugation at 4500 g for 5 min. Pellets were then treated with 0.5 mL of 250 mM 2-(N-morpholino) ethanesulfonic acid buffer at pH 6.0 containing 1% sodium dodecyl sulfate and 1 mM diethylenetriaminepentaacetic acid, 0.5 mL of 50 mM ABA in 250 mM MES buffer at pH 6.0, and 0.25 mL of 100 mM $NaCNBH_3$ in 250 mM MES buffer at pH 6.0. The mixture reacted for 90 min while the Eppendorf tubes were immersed in a water bath at 37 °C and stirred regularly. All solutions employed for the derivatization procedure were freshly made on sampling days. The derivatization reaction was stopped by adding 0.5 mL of cold 50% TCA, followed by a centrifugation at 9000 g and 4 °C for 10 min. The pellets were then washed twice with 1 mL of 10% TCA and 1 mL of ethanol/diethyl

ether (1:1 v/v) respectively. Centrifugations at 9000 g and 4 °C for 15 min were performed after each washing step.

2.4. Microwave-assisted acid hydrolysis

Each protein pellet was transferred to 0.5 mL 6 M HCl in 4 mL glass screw-vials with Teflon sealing (Anton Paar, Graz, Austria). In order to prevent further oxidation by free oxygen, the vials were flushed with argon before closure. MAAH was performed using a laboratory microwave system Multiwave 3000 (Anton Paar, Graz, Austria) with the Rotor 16MG5, which is suitable for $16 \times 4 = 64$ samples in one run. The samples were always placed at the same positions of the rotor because a slight difference might be observed at another position. In each hydrolysis cycle, eight vials were placed simultaneously for the samples and eight others only containing 6 M HCl were used as the dummy load in order to balance the heat adsorption. The best control of the reaction temperature was achieved when a loading pattern of 16×1 was used, as every vessel would be monitored by the infrared (IR) temperature sensor. The MAAH operation parameters including power (300–800 W), IR-measured microwave temperature (80–120 °C) and irradiation time (2–50 min) were optimized after a series of designed experiments. For the MAAH experiments, the maximum output power of the microwave oven was set up first. Each experiment started automatically with a fixed ramp time (typically 5 min) of the temperature by raising the microwave output power to reach the desired IR-measured temperature. Then, for a given microwave irradiation time, the actual power was adjusted automatically by a microcomputer in order to maintain this constant temperature, which was measured every 2 s by an IR-measured temperature sensor during the experiments. When the MAAH process was completed and the vials were cooled down to room temperature. The hydrolysates were evaporated to dryness under nitrogen gas stream followed by reconstitution in 200 μL de-ionized water and were analyzed by LC–MS/MS system within 24 h.

For comparison, the conventional acid hydrolysis was performed in a closed glass vial at 110 °C for 24 h according to the literature [17,26]. The follow-up sample preparation is the same as for the MAAH experiments.

For all investigations of protein oxidation, derivatization and hydrolysis, two parallel experiments were always performed and each sample was measured twice. The average area of these four determinations was taken for quantitative comparison during the optimization.

2.5. LC–MS and HPLC analysis

10 μL samples were injected into an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a reversed-phase (RP) C_8 column (Alltech Grom-Sil 120 Octyl-5 Cp with guard column, 5 μm, Ø 2.0 mm × 150 mm) eluted with a 300 μL/min binary gradient containing the mobile phases (A) methanol/0.1% AcOH and (B) water/0.1% AcOH respectively. The gradient started with 2% A for 2 min, then within 11 min to reach 25% A. After holding for 5 min, the mobile phase A was changed back to 2%, and the column was conditioned for 8 min before next injection. Mass spectrometric analysis was carried out on a triple quadrupole API 2000 mass spectrometer (Applied Biosystems/Sciex, Concord, Canada) equipped with a positive electrospray ionization (ESI) interface under either full-scan (50–700 amu) mode for identification or selected ion monitoring mode (m/z 253 for GGS–ABA and 267 for AAS–ABA) for quantification. Each sample was measured twice and the average peak area was taken for the quantitative comparison. Tandem MS was also used to confirm the identity of the two target analytes when compared with that of the synthetic standards. The fluorescence detection was carried out with the excitation and emission wavelengths set at 283 and 350 nm, respectively.

3. Results and discussion

3.1. Detection of AAS–ABA and GGS–ABA by LC–MS/MS

For LC–MS method developments and verification of the analytes, two reference standards of GGS–ABA and AAS–ABA were synthesized and purified according to the literature [17,26]. After TLC separation of the reaction mixture, the spot was collected and characterized using LC–MS/MS and HPLC with fluorescence detection (excitation and emission wavelength at 283 and 350 nm) [17,26]. For instance, in the product ion mass spectrum of the protonated GGS–ABA ($[M+H]^+$, m/z 253), the observation of the product ions at m/z 116 due to loss of a neutral molecule of the derivatization reagent *p*-aminobenzoic acid ABA (molar mass 137), at m/z 120 as a typical fragment $[NH_2–C_6H_4–C{\equiv}O]^+$ derived from ABA), at m/z 207 due to loss of formic acid HCOOH and at m/z 235 loss of H_2O is a further indication that the desired derivative GGS–ABA was formed. Similar confirmation was also obtained for that of AAS–ABA and the corresponding derivatization products of these marker molecules from the oxidized protein BSA. The typical selected ion monitoring (SIM) LC–MS chromatograms of GGS–ABA (RT = 8.2 min, m/z 253) and AAS–ABA (RT = 9.9 min, m/z 267) from the synthetic standards and the oxidized protein BSA are given in Fig. 2. Further studies by varying the HPLC separation conditions have indicated that there were no co-eluting interferences to these two compounds. Therefore, the LC–MS quantification of the two products were performed under the SIM mode with m/z 253 and 267 respectively in the follow-up optimization experiments. The resulted peak areas were normalized for quantitative comparison. The acid-hydrolyzed protein BSA without derivatization was also injected into LC–MS as a blank control, which showed no interfering peaks at m/z 253 and 267.

3.2. Optimization of microwave-assisted acid hydrolysis

The parameters such as the microwave power, the ramp time, the HCl acid volume and its concentration were optimized firstly after a series of brief studies and maintained constant at 500 W, 5 min, 0.5 mL and 6 M, respectively, throughout this study. The investigation focused mainly on the 2 most influential parameters temperature and time. The effect of the microwave irradiation times at 2, 5, 10, 20, 30, 40 and 50 min and the temperatures at 80, 90, 100, 110 and 120 °C on the acid hydrolysis process was investigated. The effect of the microwave irradiation duration on the formation of GGS–ABA and AAS–ABA at different temperatures is given in Fig. 3. For both analytes at each temperature, a maximum yield of formation is observed. However, in all cases, the yield would start to decrease with prolonged irradiation after the maximum, which may be due to further degradation or oxidation of GGS–ABA and AAS–ABA. By increasing the temperature from 80 to 110 °C, the optimized reaction time decreases from 30 min to 10 min for GGS–ABA and to only 2 min for AAS–ABA respectively (Fig. 3). A similar effect of the IR-measured microwave temperature on the acid hydrolysis is given in Fig. 4 as demonstrated for 5, 10 and 30 min of the fixed irradiation durations, respectively. It is noticeable that the required temperature to reach the maximum yield increases when the fixed microwave irradiation time decreases from Fig. 4c to a. The trend in Fig. 4c indicates that a maximum may also be reached at the temperature lower than 80 °C if the irradiation time is pre-fixed to 30 min. Furthermore, both the prolonged reaction time and the increase temperature will lead to unwanted reactions, resulting in dropping of product yields. As

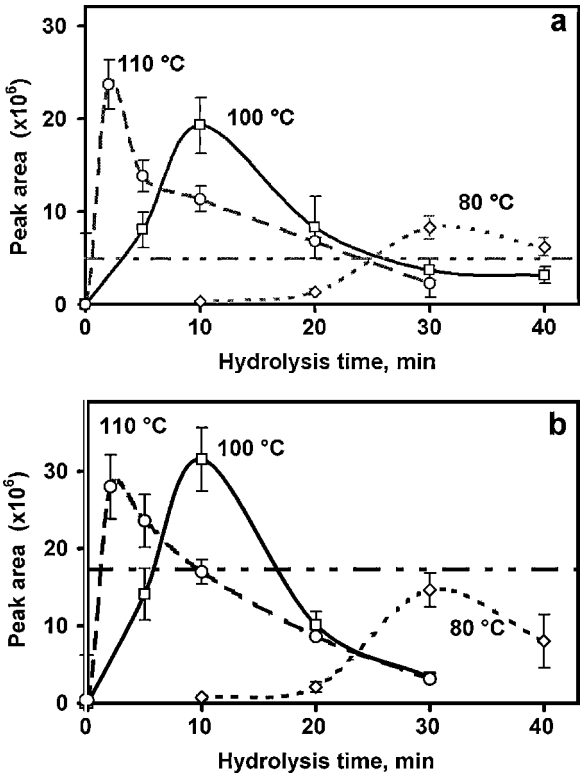

Fig. 3. Effects of the microwave irradiation time on the formation of (a) GGS–ABA and (b) AAS–ABA at the temperatures of 80, 100 and 110 °C respectively. The error bars indicate the average of four determinations (two independent experiments, each measured twice). For comparison, the horizontal dash lines in both figures indicate the amounts of products formed during the acid hydrolysis using conventional thermal heating at 110 °C for 24 h in this study.

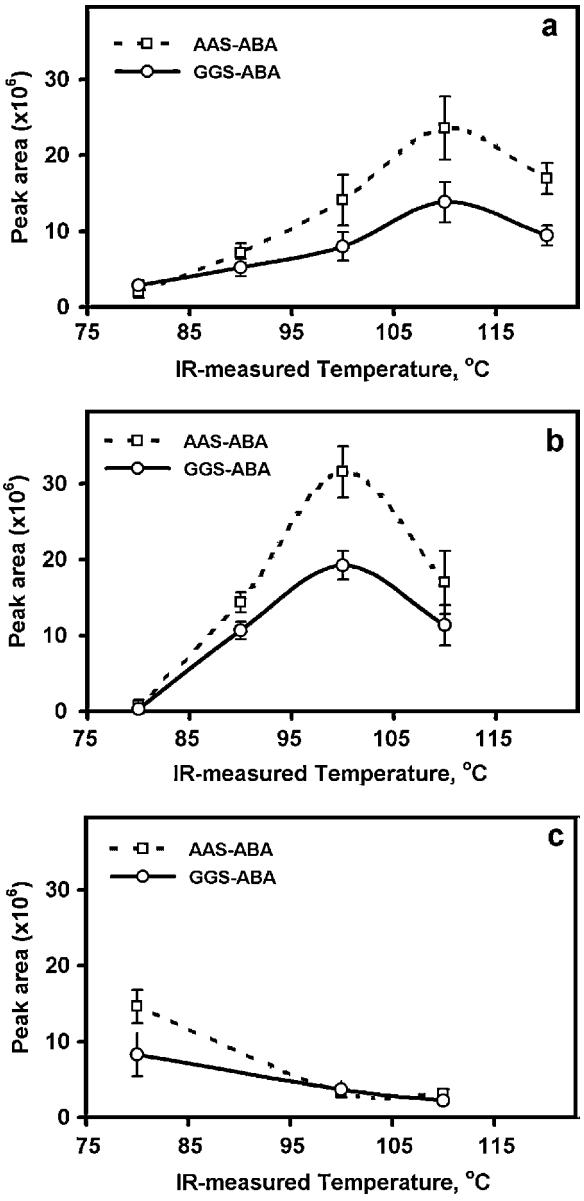

Fig. 4. Effects of the IR-measured microwave temperatures on the acid hydrolysis at the irradiation time of (a) 5, (b) 10 and (c) 30 min, respectively. The error bars indicate the average of four determinations (two independent experiments, each measured twice).

a summary, the optimized conditions to obtain GGS–ABA are at 110 °C/2 min and AAS–ABA at 100 °C/10 min respectively using a 500 W microwave.

It should be noted that the significant advantages with microwave are the dramatically shortened reaction time and also, in the meantime, the improved yield of formation of the analytes. The reaction time at 110 °C changes from 24 h with a conventional thermal heating (according to this work and also in the literature [17,26]) down to 2 min with MAAH. This is at least 500–700 times faster. As a result, the whole sample preparation time for studying protein oxidation markers can be reduced from 2 days to about 2–3 h. In addition, using a proper commercial microwave device, it is also possible to prepare dozens of samples in one run. For instance, the Multiwave 3000 microwave system from Anton Paar (Graz) used in this study with the rotor 16MG5, it supports a batch experiments of $16 \times 4 = 64$ 4-mL vials. This will not be possible using a method with the conventional thermal heating. The throughput may be further enhanced by implementing the MAAH step online for automatic sample preparation followed by LC–MS analysis if it is combined with a dedicated liquid handling and microwave device. Moreover, the significant improvement of the product formation is also achieved. For comparison, the results of 6 M HCl acid hydrolysis under the conventional heating condition (110 °C for 24 h) are also given in Fig. 3 as indicated by the horizontal dash lines. Obviously, the improvement of the yield at the optimization is about 5 times for GGS–ABA at 110 °C (2 min) and 2 times for AAS–ABA at 100 °C (10 min) respectively. In other words, microwave-assisted acid hydrolysis can result in much efficient sample preparation in a very broad parameter range, if only to maintain the efficiency of a conventional heating method.

It should be mentioned that the concentration ratio of the two marker molecules AAS–ABA and GGS–ABA is also an important parameter to judge the two hydrolysis techniques. A method resulting in a ratio close to the truth will be more reliable than others. Using both microwave and conventional heating, the yield of AAS–ABA is always slightly higher than that of GGS–ABA, which is also observed in the literature [12]. This might relate to the actual molar ratio of the two amino acid residues from BSA. The protein consists of 583 amino acid residues and, if it is completely hydrolyzed, the molar percentages of lysine (to produce AAS) and arginine/proline (to produce GGS) are about 10.1 and $3.9 + 4.8 = 8.7\%$, respectively. This was estimated by assuming that the involved amino acid residues are similarly oxidized and reasonably derivatized with ABA because they are close structural analogues only differed by a CH_2 group in the alkyl side-chains. A summary of the concentration ratios between AAS–ABA and GGS–ABA under the conventional thermal heating, the optimized

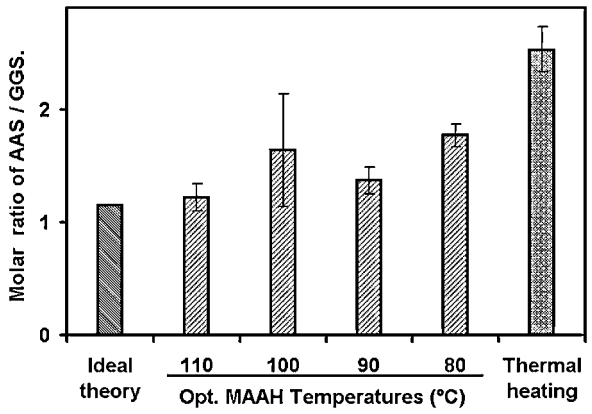

Fig. 5. Comparison of the molar ratios of the amounts of AAS–ABA and GGS–ABA obtained under the optimized microwave-assisted acid hydrolysis conditions at different temperatures and the conventional thermal heating as well as the ideal theoretical estimation of the protein BSA. The error bars indicate the average of four determinations (two independent experiments, each measured twice).

MAAH, and the ideal theoretical condition is given in Fig. 5. Noticeably compared to the theoretical ratio of 1.2, the experiments using thermal heating result in a significant high value of 2.5. This may indicate either overestimated AAS–ABA or underestimated GGS–ABA, which is either due to the incomplete hydrolysis or subsequent reactions (such oxidation or hydrolysis of the derivatization products). Interestingly, the ratios of the AAS–ABA and GGS–ABA result from the MAAH studies are more in agreement with the theoretical estimation, especially the microwave-assisted hydrolysis at slightly higher temperatures and shorter reactions times such as 110 °C and 2 min. This might indicate that MAAH produces more reliable product ratios and recoveries of the corresponding marker molecules than the thermal heating for this purpose. It should also be noted that it has been previously suggested that direct thermal heating of a protein solution in acid without microwave irradiation may initially result in protein aggregation, which probably interferes the hydrolysis [23b and c]. It is believed that a noticeable effect might result from the precipitation/adsorption of proteins on the glass wall. Heating by microwave is a highly efficient process and saves considerable energy and time primarily because the fundamental mechanism of microwave involved an agitation of dipolar molecules or ions that could oscillate under the influence of an oscillating electric or magnetic field [28]. Microwave, unlike conventional thermal heating, provides uniform heating to drive a reaction throughout a reaction mixture. Besides improving reaction efficiency, microwave heating in reactions can also enhance the selectivity and lead to less side-products. In contrast, with conventional heating and especially high temperature acid hydrolysis, the exposure and adsorption of protein samples on the inner wall of the vessel (thus, not wetted with the acid solution) could significantly affect the hydrolysis. Furthermore, with microwave-assisted hydrolysis, the sample throughput can be substantially increased using a rotor with multiple vials such as that used in this work if desirable. The successful implementation of microwave-assisted acid hydrolysis in this study will considerably help to make the proposed method to investigate the protein oxidation markers after derivatization more acceptable by more scientists because of the dramatically shortened sample preparation and improve sensitivity as discussed above.

4. Conclusion

Our results have shown that microwave-assisted acid hydrolysis of protein can be applied successfully to study the protein oxidation markers α-aminoadipic semialdehyde and γ-glutamic semialdehyde after derivatization of reductive amination, which is to prevent the degradation of the aldehyde groups of the marker molecules during acid hydrolysis. For the same acid hydrolysis, the MAAH method requires only 2–10 min at 100–110 °C, which is a few hundreds times faster than the conventional method with thermal heating. This dramatically reduces the whole sample preparation time from at least 2 days to a few hours, which is now more acceptable for scientists in medical diagnosis, molecular biology or food quality control to explore further. Using MAAH, high percent yields (2–5 times more) of the marker molecules with a ratio close to the theoretical expectation are obtained when the markers are freed from the oxidized protein through acid hydrolysis, which results in significant better recovery and more reliable estimate of protein oxidation. Furthermore, by applying a proper microwave system with multiplex sample vials, up to 60 samples can be prepared parallelly in one batch run, which further improves the analyzing throughput. This study has demonstrated that MAAH is a time-saving, efficient and reproducible method in hydrolysis of oxidized proteins containing derivatized aldehyde groups.

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