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Microwave-assisted high-throughput acid hydrolysis in silicon carbide microtiter platforms—A rapid and low volume sample preparation technique for total amino acid analysis in proteins and peptides

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ABSTRACT

An efficient microwave-assisted high-throughput protein hydrolysis protocol was developed utilizing strongly microwave absorbing silicon carbide-based microtiter platforms. The plates are equipped with 20 bore holes having the proper dimensions for holding standard screw-capped HPLC/GC vials. Due to the possibility of heating up to four heating platforms simultaneously (80 vials), parallel microwave-assisted acid hydrolyses can be performed under carefully controlled conditions significantly reducing the overall time required for protein hydrolysis and the subsequent evaporation step required for larger volumes of acid. An extensive optimization of the hydrolysis conditions has demonstrated that 5 min irradiation at 160 °C with 6 N HCl leads to comparable results in terms of total and individual amino acid recovery as the traditional method requiring 24 h heating at 110 °C. Complete hydrolysis of several proteins and synthetic peptides was performed using 25 μg of sample material and 100 μL of 6 N HCl in a dedicated low-volume HPLC/GC vial. Since the hydrolysis and subsequent analysis can be performed from the same vial, errors caused by sample transfer can be minimized. Control experiments have demonstrated that the observed rate enhancements are the result of a purely thermal/kinetic effect as a consequence of the considerable higher reaction temperatures.

1. Introduction

Amino acid composition analysis in proteins and peptides is a classical but rather complex analytical method [1]. Prior to analysis, the proteins/peptides have to be completely hydrolyzed involving an acid-catalyzed cleavage of the peptide bonds to yield the free amino acids. The resulting hydrolyzates can then be analyzed by well-established amino acid analysis techniques employing pre- or post-column derivatization in combination with ion exchange or reversed-phase high-performance liquid chromatography to determine the amino acid composition [1]. The critical hydrolysis step is usually performed by heating the samples to be analyzed at elevated temperatures in the presence of high concentrations of acids for extended time periods [1–4]. Traditional liquid- or gas-phase hydrolysis is generally performed in sealed tubes using 6 N HCl at 110 °C for 24 h [1–4], although methanesulfonic acid (MSA) has also been employed under similar conditions in order to determine sensitive amino acids such as methionine and tryptophan [2,5].

As the acid hydrolysis step is rather time-consuming, several reports over the past 20 years have advocated the use of microwave irradiation as a tool to speed-up the hydrolysis of proteins and peptides under acidic conditions [2,6–11]. Indeed, using sealed vessel microwave irradiation in a higher temperature range hydrolysis times could often be reduced to a few minutes retaining the recovery rates attained by conventional methods [2,6–11]. Although these results are impressive, a clear scientific rationalization for the use of microwave irradiation in these chemical transformations has not been given [11]. The question has to be asked if the experienced rate enhancements are of purely thermal origin (the result of efficient dielectric heating during the irradiation processes), or if so-called nonthermal microwave effects are implicated that would involve a direct interaction of the electromagnetic field (not related to a macroscopic temperature effect) with, for example, the peptide/protein backbone or other substrates/intermediates in the reaction mixture [11,12]. Protein hydrolysis is additionally an important tool in medical and basic research for testing protein alterations and modifications on the amino acid level. The specificity, extreme sensitivity and reproducibility of the mass spectrometric assay of single amino acids after protein hydrolysis provide a powerful technique for probing chemical events that

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occur in complex biological processes. Other analytical techniques to detect or identify components of a complex protein mixture generally involve protease digestion and denaturation, followed by liquid chromatography with peptide mass fingerprinting (LC–MS) or LC–MS/MS (tandem MS). However, (i) quantification of proteomic data is still a substantial challenge, (ii) peptide masses overlap even with a high-resolution mass spectrometer and (ii) not all peptides are detected with the same intensity making it not suitable to quantitatively determine post-translational modifications of amino acids. However, due to the complex and time-consuming nature of the protein hydrolysis method, its use has so far been limited. The development of a high-throughput protocol would make this method feasible for routine testing in medical and diagnostic research applications.

In the present paper, we present a critical comparison of microwave versus conventionally heated acid hydrolysis reactions using a dedicated microwave reactor with accurate internal temperature and pressure monitoring. Based on the results of these investigations a microtiter plate platform allowing carefully controlled protein and peptide hydrolyses to be performed on a scale as low as 100 μL in standard HPLC/GC vials has been developed. The use of this platform enables the microwave-assisted hydrolysis of up to 80 proteins/peptides in parallel employing 5 min irradiation time at 160 °C. An additional advantage is that a sample transfer from the reaction vial to the analysis vial is not required.

2. Experimental

2.1. Chemicals and reagents

Bovine serum albumin (BSA, fatty acid free, Calbiochem®, Nottingham, UK) was obtained from Merck Biosciences, low-density lipoprotein (LDL) was isolated by ultracentrifugation of plasma obtained from normolipidemic blood donors with EDTA as anticoagulant. The plasma density was adjusted with KBr to 1.24 g/mL. Subsequently, a two-step density gradient was generated in centrifuge tubes (16 mm × 76 mm) by layering the density-adjusted plasma (1.24 g/mL) underneath a PBS solution (1.006 g/mL). Tubes were sealed and centrifuged at 90,000 rpm for 4 h in a 90TI fixed angle rotor (Beckman Coulter, High Wycombe, UK). After centrifugation, the clearly separated LDL-containing band was collected, desalted via a PD10 column (GE Healthcare, Chalfont St Giles, UK). The synthetic 24-mer peptide magainin-II-amide [13] and the 42-mer β-amyloid [14] were available from a previous study involving solid-phase peptide synthesis [13,14]. DL-Norleucine, phenol, and an amino acid standard were purchased from Sigma–Aldrich (Steinheim, Germany). 6 N HCl standard solution was obtained from Carl Roth GmbH (Karlsruhe, Germany).

2.2. Heating equipment and temperature monitoring

Conventional heating experiments were performed in a heating oven equipped with a sand bath (Memmert, Schwabach, Germany). Preliminary optimization of the hydrolysis reaction conditions was performed in a Monowave 300 single-mode microwave reactor (Anton Paar GmbH, Graz, Austria) equipped with a fiber optic probe for accurate internal temperature measurement [15]. For the experiments either standard 10 mL Pyrex tubes or 10 mL vessels made out of sintered silicon carbide (SiC) (Anton Paar GmbH, Graz, Austria) were utilized [16]. For the microtiter platform experiments a Synthos 3000 multimode microwave instrument was equipped with the 4 × 20 MGC rotor, capable of holding microtiter heating blocks made out of strongly microwave absorbing silicon carbide (Anton Paar GmbH, Graz, Austria) to be equipped with HPLC/GC vials [17,18]. Accurate temperature measurement inside the HPLC/GC vials during the multimode microwave experiments, was accomplished by using a multi-channel signal conditioner (TempSens signal conditioner, Opsens, Quebec, Canada), capable of using up to four OTG-F fiber optic temperature sensors (Opsens, Quebec, Canada) simultaneously [19].

2.3. Amino acid analysis

Total amino acid analysis was performed applying a Biochrom 20 amino acid analyzer (Pharmacia Biotech, Stockholm, Sweden) equipped with a LiHR column (200 mm × 4.6 mm) following standard procedures. Calibration of the instrument was performed with an external amino acid calibration standard. The single amino acids are separated applying ion exchange chromatography, mixing the eluate continuously with ninhydrine reagent at a coil temperature of 135 °C. The amino acids react with the ninhydrine (post-column derivatization) and the generated colored complexes were analyzed using a flow photometer. Primary amino acids were detected at 570 nm and secondary amino acids were analyzed at 440 nm, respectively.

2.4. LC–MS instrumentation

Mass spectrometry analysis was performed on an API 2000 (Applied Biosystems, California, US) equipped with a AAA-MS HPLC column (250 mm × 4 mm, Phenomenex, Aschaffenburg, Germany).

2.5. Procedures

2.5.1. Conventional protocol

For the conventional hydrolysis experiments a 5 mL vial was charged with 0.5 mg BSA (25 μL taken from a 20 mg/mL BSA stock solution), 100 μL (1.31 μg) of a norleucine stock solution (100 nmol/mL), and after drying 2 mL of 6 N HCl standard solution containing 0.1% (w/v) phenol were added. The vessels were flushed with nitrogen, sealed and heated in a heating oven (110 °C preset temperature of the heating oven) for 24 h. After cooling to ambient temperature the reaction mixture was transferred to analysis vials and the HCl was evaporated (Evapomix, Buchler Instruments Inc., Fort Lee, NJ, USA). The residue was dried overnight in a dessicator containing KOH to eliminate residual HCl. After drying, the remaining material was re-dissolved in 2 mL 0.2 M Li-Citrat buffer (pH 2.2, Biochrom Ltd., Cambridge, UK) and 100 μL of the obtained mixture were injected for the total amino acid analysis (see Section 2.3).

2.5.2. Single-mode microwave experiments

Reaction temperature optimization was performed in a Monowave 300 applying standard 10 mL Pyrex vessels equipped with a stir bar, 25 μL BSA stock solution (0.5 mg, 20 mg/mL), 100 μL internal standard solution (100 nmol/mL) and 2 mL HCl containing 0.1% (w/v) phenol. Fiber optic controlled runs were performed at temperatures ranging from 140 to 200 °C for 10 min hold time. The experiments at 170 °C were performed with/without flushing the reaction vessel using Ar prior to heating and an additional experiment was performed sparging the reaction mixture with Argon before heating. The same reaction mixtures as described above were used for a hydrolysis time optimization, heating the reaction mixtures at 160 °C, 170 °C and 180 °C for 1, 3, 5, 7, and 10 min, respectively. After irradiation the samples were prepared for analysis as described in Section 2.5.1 for the conventional approach. For experiments simulating conventional heating 10 mL Pyrex vials as well as 10 mL SiC vessels of identical shape were charged with the same reaction mixtures as described above and heated in the Monowave 300 instrument applying a reaction temperature of 160 °C for 1, 3 and 5 min (experiments performed in triplicates). For the downscaling experiments a 10 mL Pyrex vial was equipped

with 100 μg BSA (10 μL taken from a 10 mg/mL BSA stock solution), 20 μL (0.26 μg) of the norleucine standard solution (100 nmol/mL), and 2 mL of 6 N HCl solution containing 0.1% (w/v) phenol. The reaction mixture was heated for 5 min at 160 °C in the Monowave 300 reactor.

2.5.3. Multimode microwave experiments

Additionally, reactions were performed in standard HPLC/GC vials containing a 200 μL conical inlet, having the proper shape for being heated in the SiC microtiter platforms. The SiC platforms were placed onto the corresponding rotor (4 × 20 MGC) (Fig. S1) and were heated in a Synthos 3000 multimode microwave instrument [17,18]. In a first set of low scale experiments the conical HPLC/GC vials were equipped with 100 μg BSA (10 μL from the 10 mg/mL stock solution), 20 μL of the norleucine stock solution, 30 μL of conc. HCl (~12 M), and 40 μL of 6 N HCl containing 0.25% (w/v) phenol. The second set of the downscaling experiments was performed using 25 μg BSA (2.5 μL of the 10 mg/mL stock solution), 5 μL of the norleucine stock solution, 22.5 μL of dd H_2O, 30 μL of conc. HCl, and 40 μL of 6 N HCl containing 0.25% (w/v) phenol. Both sets of experiments were heated in the same multimode microwave experiment (Synthos 3000) heating the reaction mixtures at 160 °C for 5 min. The temperature of the microwave run was controlled by an external IR sensor integrated into the bottom of the microwave instrument, measuring the surface temperature of the SiC blocks. The actual reaction temperature was controlled by a fiber optic probe, inserted manually into a reference temperature measurement vial heated together with the other vials (Fig. S2) [19]. To reach 160 °C inside the HPLC/GC vials, filled with 100 μL of reaction mixture, the IR temperature was set to 175 °C, determined in preliminary temperature optimization experiments. After heating, the HPLC/GC vials were directly put in a dessicator for drying/removal of HCl (~3 h). Subsequently the content was re-dissolved in 120 μL Li-Citrat buffer and directly injected for analysis. As a final set of experiments several peptides were hydrolyzed, applying the optimized conditions in the Synthos 3000 (160 °C, 5 min hold time) using 25 μg of BSA, MAGAININ, and β-amyloid taken from previously prepared stock solutions. The peptides were combined with 5 μL internal standard solution and 6 N HCl containing 0.1% (w/v) phenol to obtain a final volume of 100 μL. Control experiments were performed under conventional conditions for comparative reasons using 250 μg of each protein/peptide, heating the reaction mixtures (2 mL total filling volume) at 110 °C for 24 h in a drying oven. For the analysis all samples were treated as described above (Section 2.5.1).

2.5.4. Carbamylation and hydrolysis of LDL

LDL (1 mg protein/mL) was incubated with 10 mmol/L or 50 mmol/L potassium cyanate in phosphate buffered saline (pH 7.4), containing 100 μmol/L diethylenetriaminepentaacetic acid (DTPA) for 1 h at 37 °C, to generate low- and high-carbamylated LDL. The modified LDL preparations were passed over a PD10 column to remove unreacted reagents.

After modification, internal standards (50 ng $^{13}C_6$-HCit and 1 μg $^{13}C_6$-Lysine) were added to 10 μg of carbamylated LDL. Samples were hydrolyzed using both, the conventional (110 °C, 24 h, 2 mL) as well as the microwave-assisted protocol (160 °C, 5 min, 100 μL) applying the optimized reaction conditions described in Section 2.5.3. Following hydrolysis, HCl was evaporated under a stream of nitrogen in a sample concentrator. Samples were resuspended in 100 μL Li-Citrat puffer (0.2 mol/L, pH 2.2) and derivatized with the EZ:faast Kit according to the manufactures instructions (Phenomenex, Aschaffenburg, Germany). Derivatized samples were brought to dryness under a stream of nitrogen, resuspended in 100 μL methanol, transferred into autosampler vials and used for MS-analysis.

3. Results and discussion

3.1. Optimization of microwave heating protocols

Our initial experiments focused on establishing optimum microwave conditions for the liquid-phase acid hydrolysis of bovine serum albumin (BSA) as a model protein. This large serum albumin protein (607 amino acid residues, molecular weight ~66.4 kDa) has served as a reference protein in several previous microwave-assisted protein acid hydrolysis studies [2,11], and therefore appeared very suitable for this purpose. The traditional method for the acid hydrolysis of large proteins such as BSA requires 24 h heating at 110 °C with 6 N HCl in a sealed tube in order to achieve complete hydrolysis [1–5]. In most instances, the sealed tube is evacuated in order to remove oxygen [2], although providing an inert atmosphere such as Ar or N_2 has also been shown to have the same effect [6]. In addition, a defined amount of phenol (0.1–1%) is generally added to the hydrolysis medium in order to minimize the oxidation of sensitive amino acids [2,10,11].

Initial optimization experiments were performed in a dedicated single-mode microwave reactor (Monowave 300) equipped with a fiber-optic internal temperature probe and pressure sensor to accurately monitor these important process parameters [15,16]. Hydrolysis was carried out in 10 mL sealed Pyrex reaction vials on a 2 mL scale using the magnetic stirring option in order to avoid thermal gradients not untypical in single-mode microwave reactors [20,21]. In the first set of experiments a constant reaction time of 10 min was applied, whereas the reaction temperature was increased stepwise in 10 °C increments from 140 °C to 200 °C (internal pressures from 3 to 12 bar). An evaluation of the total amount of detected of amino acids revealed that while at 140 °C and 150 °C the overall recovery of amino acids was still lower compared to the conventionally heated reference experiment (110 °C, 24 h), hydrolyses conducted at ≥160 °C provided the same total amount of amino acids as the conventionally heated run (Fig. S3 in the Supporting Information). Interestingly, a closer examination of the individual recovery rates for the amino acids contained in BSA revealed that using very high hydrolysis temperatures (190 °C, 200 °C) some of the more labile amino acids including Ser, Thr, and Cys exhibited lower recovery rates presumably due to decomposition under these comparatively harsh conditions (Fig. 1). Met and Trp could not be detected under any of the chosen conditions presumably due to oxidation/decomposition, which is a known problem for acid-based protein hydrolysis methods [1–5]. On the other hand, some amino acids such as Val, Leu, and Lys displayed somewhat higher recovery rates at these extreme temperatures compared to the reference experiment performed at 110 °C for 24 h (Fig. 1).

Based on the these results, 160–180 °C was considered as an optimum temperature range for the microwave-assisted hydrolysis of BSA, since in this temperature region the results most closely matched the data obtained using the traditional method involving conductive heating at 110 °C requiring 24 h. The microwave conditions were subsequently fine-tuned with respect to the optimum hydrolysis time. Thus, the protein and 6 N HCl were heated for 1, 3, 5, 7 and 10 min in the temperature range of 160–180 °C (Fig. S4 in the Supporting Information). These experiments demonstrated that even after only 3 min hydrolysis time at 180 °C the hydrolysis results in terms of total amino acid concentration were very close to the standard procedure at 110 °C and 24 h. For practical reasons, in particular concerning the high internal pressure of 8 bar developing at 180 °C, the ultimately chosen conditions for microwave-assisted BSA hydrolysis involved 5 min of heating to 160 °C (5 bar). It has to be mentioned that the given hydrolysis time of 5 min reflects the time period that the sample is kept at the chosen temperature of 160 °C. Including the heating and cooling

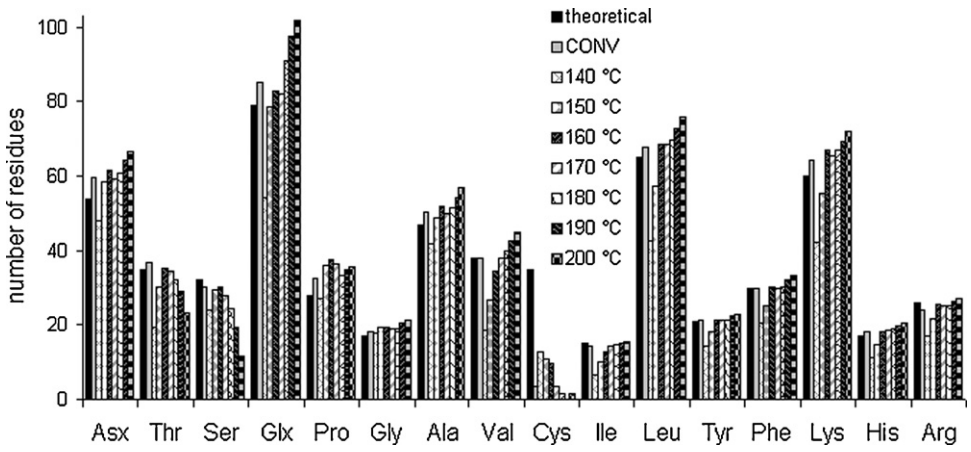

Fig. 1. Comparison of amino acid compositions of BSA following liquid-phase hydrolysis with 6 M HCl at different temperatures. The protein was subjected to conventional sealed vessel hydrolysis at 110 °C for 24 h (grey bar) and to microwave-assisted hydrolysis for 10 min at temperatures ranging from 140 °C to 200 °C. The theoretical number of residues is shown in black. The results are normalized to the Val residues (CONV). Asx shows Asn and Asp, Glx shows Gln and Glu. Met and Trp are not shown.

ramp adds ~4 min to the hydrolysis time and therefore leads to an overall processing time of ~9 min (Fig. S5 in the Supporting Information). These optimized high-temperature microwave conditions for BSA hydrolysis using 6 N HCl are similar to those previously published [2,10,11], albeit reliable temperatures measurements have not always been reported in the past.

3.2. Investigation of nonthermal microwave effects

It has been suggested that due to the very high dipole moment of an amide bond, irradiation of proteins or peptides with microwave energy may lead to a de-aggregation of the peptide backbone or to conformational changes in the protein via direct interaction of the peptide chain with the electric field [11,12]. Microwave effects of this type would not be reproducible by conventional heating at the same measured bulk reaction temperature. Before designing a miniaturized and parallel high-throughput microwave protein hydrolysis method it was therefore deemed imperative to investigate if the observed rate enhancements seen in microwave-assisted protein hydrolyses were the result of a purely thermal/kinetic effect (as a consequence of the considerable higher reaction temperatures) or involved nonthermal microwave effects not connected to a macroscopic change in temperature. For this purpose the hydrolysis of BSA with 6 N HCl was repeated in the Monowave 300 microwave reactor, but instead of a standard Pyrex reaction vial a vessel of identical geometry made from sintered silicon carbide (SiC) was employed (Fig. S6 in the Supporting Information). SiC is a strongly microwave absorbing chemically inert ceramic material that possesses high thermal conductivity and effusivity (a measure for the ability to exchange thermal energy with its surroundings) [16]. Microwave irradiation induces a flow of electrons in the semiconducting ceramic that heats the material very efficiently through resistance heating mechanisms. The use of SiC reaction vessels in combination with the Monowave 300 microwave reactor provides an almost complete shielding of the contents inside from the electromagnetic field [16]. Therefore, these experiments do not involve electromagnetic field effects on the chemistry and thus mimic a conventionally heated autoclave experiment, however retain the excellent process control features of modern microwave instruments [16].

The chosen reaction temperature for the SiC vessel experiments was 160 °C and the mixtures were heated for 1, 3, and 5 min, respectively. The temperature of the hydrolysis mixtures applying both types of vessels was controlled by a fiber-optic probe, guaranteeing identical reaction temperature profiles for both vial types (Fig. S7 in the Supporting Information). The determined amount of total amino acids as well as the recovery of individual amino acids for all experiments at 160 °C involving the same heating time was practically identical, regardless if a Pyrex vessel (microwave dielectric heating) or a SiC vessel (conductive heating) was used. Therefore, it can be safely concluded that the observed rate enhancements seen in microwave-assisted protein hydrolyses [2,6–11] are based on a purely thermal/kinetic effect as a consequence of the considerably higher reaction temperatures compared to the standard conditions. The results selecting Ser as a representative and temperature sensitive amino acid (see Fig. 1) are shown in Fig. 2 and a comparison of the total amino acid composition is given in Fig. S8 in the Supporting Information.

3.3. Miniaturization and parallelization

After having established optimum conditions for the acid-mediated rapid hydrolysis of BSA in single-mode microwave reactors our goal was to parallelize and to downscale this method. While the applied single-mode microwave technique described in Section 3.1 has provided a clear scientific rationale and optimized conditions for the use of microwave technology in acid-mediated protein hydrolysis, the single-mode method requires the use of specialized and comparatively large scale microwave process vials and does not allow an easy adaptation to a high-throughput format, since each reaction vessel needs to be irradiated separately. We have therefore translated the optimized conditions described above to a parallel method that employs a high-throughput microtiter platform made out of silicon carbide in combination with standard screw-capped HPLC/GC vials [17–19]. These recently commercialized platform allows sealed-vessel parallel microwave processing of up to ~80 reactions on a 50–2000 μL scale using multimode microwave reactors under carefully controlled reaction conditions (4 × 20 MGC rotor and Synthos 3000 reactor, Anton Paar GmbH) [17,18]. Critical for the success of these reaction platforms is the use of sintered SiC material for the manufacturing of the microtiter-based plates. Microwave irradiation leads to a rapid and homogeneous heating of the entire plate, with minimal deviations in the temperature recorded at different positions of the plate or inside the vials [19]. Because of the large heat capacity and high thermal conductivity (100 times higher than glass) of SiC, the plates are able to moderate any field inhomogeneities inside a microwave cavity and therefore avoid the problems of “hot and cold spots” frequently experienced in multimode microwave systems [19]. For the protein hydrolysis studies described herein a 5 × 4 deep-well

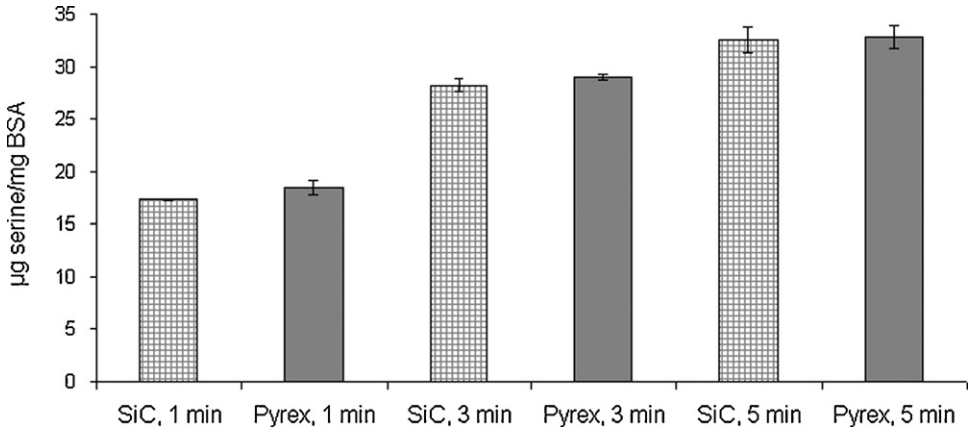

Fig. 2. Comparison of serine recoveries in the BSA liquid-phase hydrolysis with 6 M HCl at 160 °C for 1, 3, and 5 min using either Pyrex or SiC vessels. Experiments were performed in triplicates.

SiC platform that utilizes standard low volume (maximum filling volume 200 μL, conical design) screw-cap GC vials was employed (Fig. 3) [18].

In preliminary calibration experiments a fiber-optic temperature probe was inserted into one of the HPLC/GC vials in order to establish the genuine reaction temperature of the hydrolysis mixture [19]. A set surface temperature of the SiC block of 175 °C consistently resulted in an internal reaction temperature of 160 °C matching the conditions achieved in the single-mode microwave experiments (Fig. S9 in the Supporting Information). The most appropriate low-volume hydrolysis conditions using this experimental set-up involved the hydrolysis of only 25 μg of BSA with 100 μL of 6 N HCl. Compared to the standard control experiment (110 °C, 24 h) in which 0.5 mg of BSA were hydrolyzed with 2 mL of acid and 798 μg of amino acids were detected (Fig. 1), the low-volume experiment applying the high-throughput microwave microtiter plate conditions at 160 °C delivered a similar amount of 742 μg detected amino acids (Fig. S10 in the Supporting Information). The individual amino acid recoveries were very similar as well and are shown in Fig. S11 in the Supporting Information.

An additional advantage of the low-volume hydrolysis approach is that the traditional evaporation step required for larger volumes of acid can be eliminated and replaced by the use of a fast parallel sample concentration method (for example using a Speedvac or a sample concentrator) and/or by simply placing the HPLC/GC vials directly into a dessicator filled with KOH for ~3 h. Since the sample preparation step for the chromatographic amino acid determination involving the addition of 100 μL of Li-Citrat buffer (see Section 2.5.1) can be performed directly from the HPLC/GC vials any potential contamination or loss of material usually caused by transferring samples can be eliminated.

Fig. 3. Reaction platform made out of sintered silicon carbide (grey reaction block). The SiC heating platform is capable of holding up to 20 standard 200 μL screw-capped HPLC/GC vials (see insert) [17,18]. By using the corresponding rotor inside a multimode microwave reactor, four heating platforms (80 reaction vessels) can be heated simultaneously (Fig. S1 in the Supporting Information).

3.4. Hydrolysis of other proteins and synthetic peptides

In addition to BSA used as a reference protein for the hydrolysis studies described above, two small synthetic peptides recently synthesized in our laboratories (the 24-mer MAGAININ analog [13] and the 42-mer β-amyloid peptide [14]) were hydrolyzed to evaluate the optimized hydrolysis conditions. All three proteins/peptides were hydrolyzed in a single microtiter plate experiment applying 25 μg of each protein/peptide, 100 μL of 6 N HCl and 160 °C for 5 min heating conditions. For comparison purposes the three proteins/peptides were additionally hydrolyzed using the conventional heating protocol (250 μg of each protein, 110 °C, 24 h, 2 mL total filling volume). Gratifyingly, the results for both hydrolysis protocols in terms of total and individual amino acid recovery were very similar (Fig. 4a–c), confirming the general usefulness of the high-speed microwave method.

As already seen in the temperature optimization work described above (see Section 3.1 and Fig. 1), for some amino acids discrepancies between the theoretical and experimentally determined values exist that are either related to the stability of the individual amino acids or to the fact that some amino acids (or some partial sequences) are difficult to hydrolyze. For example, in the hydrolysis of the 42-mer β-amyloid peptide the recoveries for Val and Ile were somewhat lower in the microwave-assisted hydrolysis compared to the theoretical number or residues and the results obtained from conventionally heated experiments. We believe that these deviations are the result of incomplete hydrolysis of the Ile–Ile and Val–Val–Ile partial sequences in the β-amyloid peptide. This hypothesis was corroborated by time dependent hydrolysis studies performed conventionally at 110 °C for 24, 48, and 72 h (Table S1 in the Supporting Information). It is clearly seen that for Ile and Val the recoveries increase going from 24 h to 72 h indicating that there are some regions in this peptide that are difficult to hydrolyze. However, the longer hydrolysis led to a reduced recovery of the sensitive amino acid Ser. Therefore, the acid-mediated hydrolysis of proteins/peptides requires the careful consideration of both of these effects, regardless if microwave or conventional heating is used.

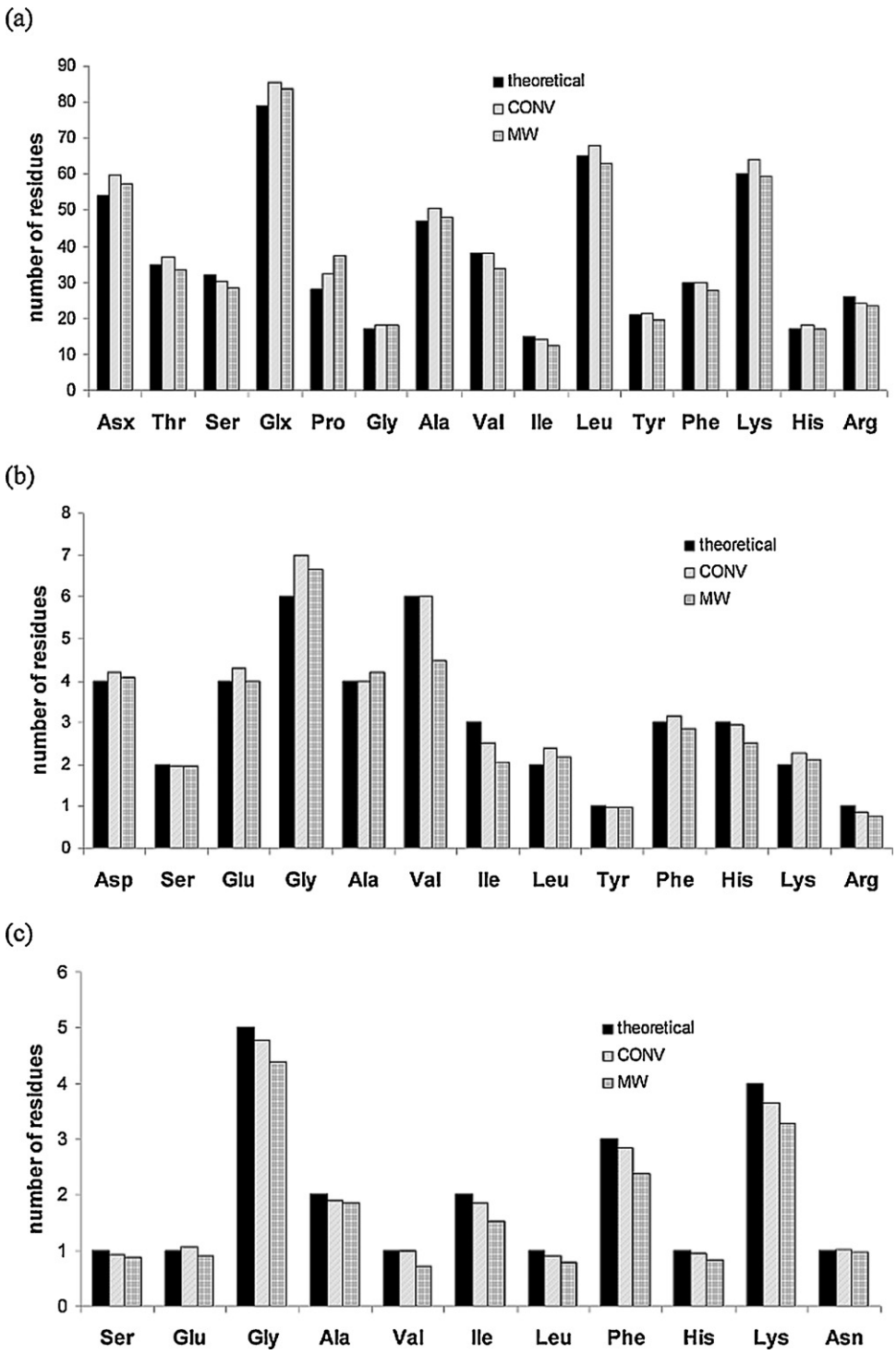

Fig. 4. Comparison of amino acid compositions of (a) BSA, (b) β-amyloid, and (c) MAGAININ peptide following liquid-phase hydrolysis with 6 M HCl at 160 °C for 5 min performed in a microtiter plate set-up (Fig. 3). The proteins/peptides were subjected to conventional sealed vessel hydrolysis at 110 °C for 24 h (CONV) and to microwave-assisted hydrolysis (MW). The theoretical number of residues is shown in black.

3.5. Quantification of post-translational protein modifications

A rapid, high-throughput quantification of post-translational protein modification would offer an application with immense potential in routine diagnostics, since chronic and inflammatory diseases are associated with increased modification of proteins at sites of pathology. For example, in renal disease, elevated levels of oxidized plasma proteins [22] and cyanate modified proteins (carbamylated proteins) [23] correlate with an increased cardiovascular risk. Of particular interest, high concentrations of carbamylated low-density-lipoproteins (cLDL) have recently been reported to accumulate in plasma of patients with chronic renal failure [23].

Herein, we compared the microwave-assisted high-throughput protein hydrolysis protocol (160 °C, 5 min) with the traditional hydrolysis protocol (110 °C, 24 h) for the quantification of LDL located carbamyllysine (homocitrulline) content of in vitro carbamylated LDL. A sample of 10 μg of carbamylated

Table 1
Comparison of conventional and microwave-assisted (microtiter plate) hydrolysis of modified LDL protein.[a]

	CONV	MW
	HCit/LDL	
Carbamyl. LDL	202.7 ± 31.1	207.4 ± 23.5

[a] HCit/LDL indicates the number of homocitrulline residues which can be found on one LDL particle. A 100% modification rate would be represented by the total conversion of all 356 lysine residues to homocitrulline. Results represent the mean of triplicate determinations ± SD.

LDL was subjected to both hydrolysis methods. The results clearly demonstrate that both, the traditional and the rapid, microwave-assisted protein hydrolysis method are equally suitable for the quantification of protein associated homocitrulline (Table 1).

4. Conclusions

In summary, we have demonstrated that acid-mediated protein hydrolyses of synthetic peptides and proteins can be carried out very efficiently under controlled single-mode microwave conditions requiring a fraction of the time compared to traditional protocols that rely on conductive heat transfer mechanisms (110 °C, 24 h). Extensive optimization studies using BSA as a reference protein have shown that complete hydrolysis can be achieved within 5 min at 160 °C using 6 N HCl as a reaction medium. While the observed rate enhancements (24 h → 5 min) are of a purely thermal/kinetic nature as a consequence of the higher reaction temperatures, the optimized conditions could be successfully translated to a miniaturized and parallel hydrolysis method using a dedicated multimode microwave reactor. For this purpose the hydrolysis was performed in a silicon carbide-based microtiter plate platform allowing carefully controlled protein and peptide hydrolyses to be performed on a scale as low as 100 μL in standard HPLC/GC vials. The use of this platform enables the microwave-assisted hydrolysis of up to 80 proteins/peptides in parallel employing 5 min irradiation time at 160 °C. The general usefulness of this protocol was demonstrated using other proteins and synthetic peptides as reference material. In addition, we have demonstrated that the microwave protein hydrolysis method is a suitable tool to rapidly determine post-translational modifications. Therefore, this procedure could be used to establish routine diagnostics for post-translational protein modifications, which have been implicated in many pathophysiologies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.062.

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