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Sonoreactor-Based Technology for Fast High-Throughput Proteolytic Digestion of Proteins

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Abstract: Fast (120 s) and high-throughput (more than six samples at once) in-gel trypsin digestion of proteins using sonoreactor technology has been achieved. Successful protein identification was done by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, MALDI-TOF-MS. Specific identification of the adenylyl-sulphate reductase alfa subunit from a complex protein mixture from *Desulfovibrio desulfuricans* ATCC 27774 was done as a proof of the methodology. The new sample treatment is of easy implementation, saves time and money, and can be adapted to online procedures and robotic platforms.

Keywords: Protein digestion · sonoreactor · MALDI-TOF-MS

1. Introduction

Rapid protein identification is nowadays an issue of primary importance for the medical, biochemical, and analytical communities. For instance, protein biomarker discovery for medical diagnostic or for pharmacological purposes is becoming one of the hottest subjects among many researchers.[1]

The sample handling for protein identification through peptide formation is a complex and time-consuming procedure with many different steps, which must be done carefully to achieve reliable results. The whole procedure can take as long as 4−12 h, since the protein enzymatic digestion is typically done overnight. Therefore, new analytical methodologies have recently emerged with the aim to make protein digestion as fast and confident as possible.[2] The current procedures used to speed up enzymatic kinetics for protein digestion are mainly based on (1) microwave energy, named microwave-assisted protein enzymatic digestion, MAPED,[3,4] or on (2) ultrasonic energy, called high-intensity focused ultrasound, HIFU.[5,6] There are remarkable differences between both approaches,[2] the HIFU being the fastest: 30 s versus 20 min for an in-solution or an in-gel protein digestion using the enzyme trypsin.[2]

Technological improvements have been done in sonochemistry engines in recent years, and a new device is now available for researchers, the sonoreactor[7] (disclaimer: specific company, product, and equipment names are given to provide useful information; their mention does not imply recommendation or endorsement by the authors). This instrument offers some advantages over the ultrasonic probe and the ultrasonic bath since it combines their benefits but not their drawbacks. A comparison of the sonic energy provided by common probes, baths, and the sonoreactor is shown in Figure 1, where it can be seen that the sonic energy generated by the sonoreactor is lower than the one given by an ultrasonic probe but higher than the energy produced by a common ultrasonic bath. This fact is critical, because it allows in-gel protein digestion without the gel degradation that occurs when an ultrasonic probe is used. Gel degradation may limit the applicability of the in-gel HIFU methodology for protein identification by electrospray ionization tandem mass spectrometry, ESI-MS/MS, because the solutions obtained can be too viscous to be used with ESI. Furthermore, common ultrasonic probes permit handling samples only one at a time, whereas sonoreactor technology offers high sample throughput, since many samples can be treated at once.

In the present work, we report on the applicability of sonoreactor technology for the fast high-throughput in-gel enzymatic digestion of proteins and on their identification by peptide mass fingerprint, PMF, using MALDI-TOF-MS. In addition, the new sample treatment was successfully applied to the identification of the adenylylsulphate reductase alfa-subunit from a complex protein mixture from *Desulfovibrio desulfuricans* ATCC 27774. Adaptation of sonoreactor technology to online procedures and robotic platforms could also have promising applications in the proteomics field.

2. Experimental Section

2.1. Apparatus. Gel electrophoresis was performed with a Biorad (Hercules, CA) model powerpac basic following the manufacturer instructions. Protein digestion was realized in safe-lock tubes of 0.5 mL from Eppendorf (Hamburg, Germany). A vacuum concentrator centrifuge from UniEquip (Martinsried, Germany) model UNIVAPO 100H with a refrigerated aspirator vacuum pump model Unijet II was used for (1) sample drying and (2) sample preconcentration. Biogen Cientifica (Madrid, Spain) centrifuges and vortex models Sky Line and Spectrafuge Mini were used throughout the sample treatment, when necessary. A sonoreactor model UTR200 from Dr. Hielscher (Teltow, Germany) was used to accelerate enzymatic protein digestions. An ultrasonic bath from Elma (Singen, Germany), model Transsonic TI−H-5, was used to facilitate peptide and protein solubilization. A Simplicity 185 from

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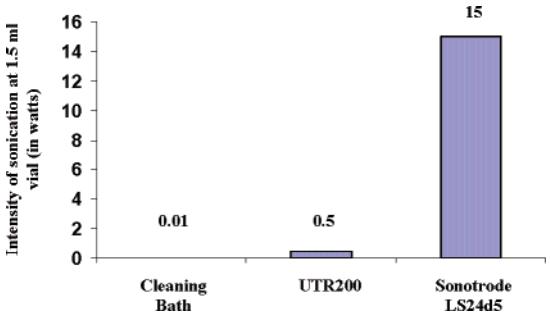

Figure 1. Comparison of the ultrasonic energy provided by common probes, baths, and the sonoreactor. Reproduced with permission from Dr. Hielscher AG (http://www.hielscher.com.)

Millipore (Milan, Italy) was used to obtain Milli-Q water throughout all of the experiments.

2.2. Standards and Reagents. Standard protein mixture of phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin, (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were purchased from Amersham Biosciences (Buckinghamshire, United Kingdom, part number 17-0446-01). α-Lactalbumin from bovine milk (≥85%) and trypsin, sequencing grade, were purchased from Sigma (Steinheim, Germany). All materials were used without further purification. α-Cyano-4-hydroxycinnamic acid (α-CHCA) puriss for MALDI-MS from Fluka (Buchs, Switzerland) was used as MALDI matrix. ProteoMass Peptide MALDI-MS Calibration Kit (MSCAL2) from Sigma was used as mass calibration standard for MALDI-TOF-MS.

The following reagents were used for gel preparation and protein digestion: methanol, acetonitrile, iodoacetamide (IAA), and DL-dithiothreitol (DTT) (99%) were purchased from Sigma; formic acid for mass spectrometry, acetic acid (> 99.5%), and ammonium bicarbonate (>99.5%) were from Fluka; bromophenol blue, glycine, glycerol, and trifluoroacetic acid (TFA, 99%) were from Riedel-de-Haën (Seelze, Germany); coomassie blue R-250, β-mercaptoethanol (>99%), and sodium dodecyl sulfate (SDS) were from Merck (Darmstadt, Germany); α,α,α-tris-(hydroxymethyl)methylamine and tris(hydroxymethyl)aminomethane, ultrapure grade, were from Aldrich (Steinheim, Germany); and ammonium persulphate (PSA) and *N*,*N*,*N*′,*N*′-tetramethylethylenodiamine (TEMED) were from Sigma (Steinheim, Germany).

2.3. Sample Treatment. Amounts of protein ranging from 0.06 to 3.7 μg were dissolved in sample buffer for sodium dodecyl sulfate−polyacrilamide gel electrophoresis, SDS-PAGE. The protein spots in the SDS-PAGE were cut and treated according to the protocol schematized in Figure 2. After washing, reduction, alkylation, and drying steps, the gel slices were incubated with trypsin (0.37 μg) in an ice bath for 45 min to rehydrate it and to allow trypsin penetration. In-gel protein digestion was then performed with the sonoreactor operating at 50% amplitude for 60 s. Next, trypsin protein digestion was stopped by the addition of 20 μL of formic acid 5%. Finally, the samples were evaporated to dryness in a vacuum concentrator centrifuge and then were resuspended with 10 μL of formic acid 0.3%.

2.4. MALDI-TOF-MS Analysis. A MALDI-TOF-MS model voyager DE-PRO biospectrometry workstation equipped with a nitrogen laser radiating at 337 nm from Applied Biosystems (Foster City, United States) was used to obtain the PMF. MALDI mass spectra were acquired as recommended by the manufacturer. Prior to MALDI-TOF-MS analysis, the sample was mixed with the matrix solution. α-CHCA matrix was used throughout this work and was prepared as follows: 10 mg of α-CHCA was dissolved in 1 mL of Milli-Q water/acetonitrile/TFA (49.95:49.95:0.1). Then, 10 μL of the aforementioned matrix solution was mixed with 10 μL of sample and the mixture was shaken in a vortex for 30 s. One microliter of each sample was spotted on two different wells on the 100-well MALDI-TOF-MS sample plate and was allowed to dry for 5 min.

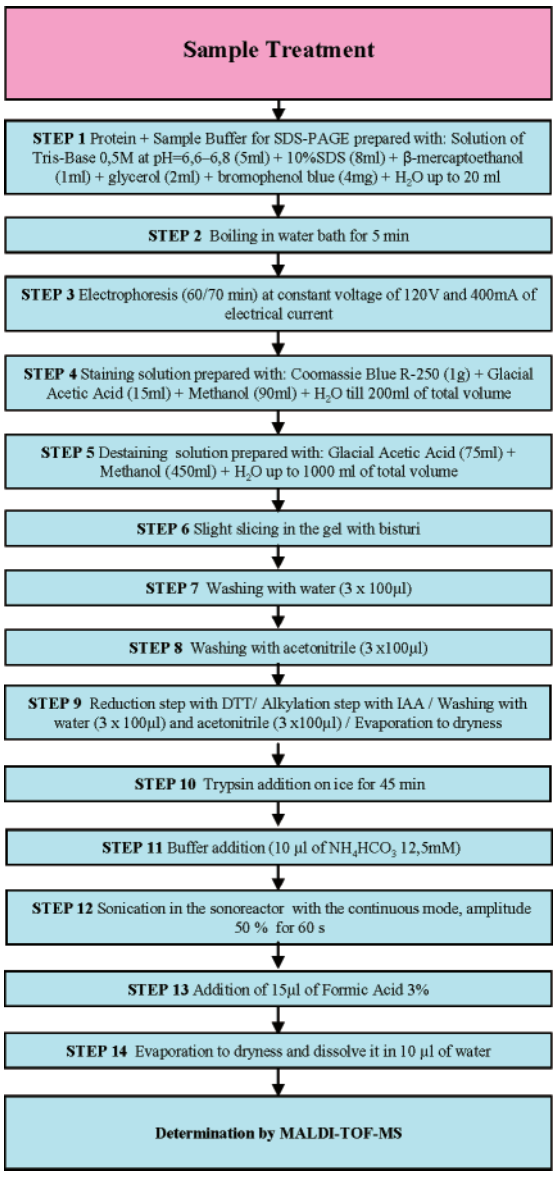

Figure 2. Comprehensive diagram of the sample treatment protocol.

Measurements were done using the reflector positive ion mode, with a 20 kV accelerating voltage, 75.1% grid voltage, 0.002% guide wire, and a delay time of 140 ns. Two close external calibrations were performed with the monoisotopic peaks of the Bradykinin, Angiotensin II, $P_{14}R$, and ACTH peptide fragments (*m*/*z*: 757.3997, 1046.5423, 1533.8582, and 2465.1989, respectively). Mass spectral analysis for each sample was based on the average of 500 laser shots. Monoisotopic peaks were manually selected from each of the spectra obtained. Peptide mass fingerprints were searched with the MASCOT and PROTEIN PROSPECTOR search engines with the following param-

eters: (1) SwissProt database; (2) molecular weight (MW) of protein: all; (3) one missed cleavage; (4) fixed modifications: carbamidomethylation (C); (5) variable modifications: oxidation (M); (6) peptide tolerance up to 100 ppm after close-external calibration. A match was considered successful when the protein identification score is located out of the random region and when it appears in the first position.

2.5. Protein Samples from Complex Mixtures. *Desulfovibrio desulfuricans* ATCC 27774 was cultured in lactate−nitrate medium.[8] To separate cells from the medium, the culture was centrifuged 30 min at 3000*g*. The pellet was then resuspended in 10 mM Tris-HCl pH 7.6 (1 mL buffer/1 mg cells) and was ruptured in a French press at 9000 psi. The bacterial extract was centrifuged at 19 000*g* for 30 min and was ultracentrifuged at 180 000*g* for 60 min. The soluble extract obtained was subjected to an anioionic exchange column (DEAE 52, Whatman). The fraction eluted between 100 and 250 mM Tris-HCl was analyzed. Four aliquots containing 0.9 μg of total protein were run in a 12.5% SDS-PAGE gel, and then, in-gel digestion of the protein observed at ca. 75 KDa was done, by duplicate, with the sonoreactor.

3. Results and Discussion

3.1. Optimization of Sonoreactor Performance. To test the applicability of sonoreactor for in-gel protein digestion, the following parameters were optimized: (1) trypsin/protein ratio; (2) digestion time; (3) sonoreactor amplitude, and (4) protein concentration.

(1) Trypsin/Protein Ratio. Slices of SDS-PAGE gel with 0.5 μg of α-lact protein were digested with decreasing concentrations of trypsin enzyme ranging from 0.6 μM to 0.008 μM. In-gel digestion (25 μL) was carried out with the sonoreactor operating in continuous mode at 70% amplitude for 60 s. Protein identification was done when 0.6 μM (375 ng in 25 μL) of trypsin was used. Lower amounts of trypsin were insufficient to perform the digestion, since no protein identification was obtained (see Figure I of Supporting Information). The optimum trypsin concentration is consistent with the one obtained for the HIFU procedure[5,6] and suggests that the cavitation phenomena[9] produced by the sonic power assists and enhances the enzymatic digestion of proteins rather than increases the trypsin efficiency in terms of cleavage capability.

(2) Digestion Time. To optimize the digestion time, different slices of SDS-PAGE gel with 0.5 μg of α-lactalbumin and BSA were digested in the sonoreactor, by duplicate, with 0.6 μM of trypsin at 70% amplitude for times comprised between 30 and 180 s. As it can be seen in Figure II (Supporting Information), correct identification of BSA was possible for all the times studied and no differences were observed in terms of the number of peptides matched or the sequence coverage. In the case of α-lactalbumin, a digestion time of 60 s was required for its positive identification out of the random region and no differences were observed for sonication times of 60, 120, and 180 s. These data suggest that times needed to perform the trypsin cleavage of proteins can be slightly high for the sonoreactor when comparing them with the HIFU procedure.

(3) Sonoreactor Amplitude. To optimize sonoreactor amplitude, slices of SDS-PAGE gel with 0.5 μg of α-lactalbumin and BSA were digested in the sonoreactor, by duplicate, with 0.6 μM of trypsin for 60 s in the continuous mode with amplitudes ranging between 25% and 90%. Interestingly, the results obtained for both proteins were different. On the one hand, the number of peptides matched and the coverage were virtually the same for BSA in all the amplitudes tested, as shown in Figure III (Supporting Information). On the other hand, for α-lactalbumin the number of peptides matched and the coverage decreased as the amplitude increased. For this reason, 50% was chosen as the optimum amplitude.

(4) Protein Concentration. To test the minimum protein concentration that was needed to do the identification correctly, amounts of protein ranging from 0.01 to 5 μg were dissolved in sample buffer for SDS-PAGE electrophoresis and then bands of proteins were submitted to the ultrafast in-gel protein digestion with the sonoreactor protocol. Protein identification was only possible for amounts of protein equal or higher than 0.1 μg for a trypsin concentration of 0.6 μM (Figure IV of Supporting Information). Higher amounts of trypsin were not tried.

Finally, when the sonoreactor procedure was applied with the optimized conditions, as described above, to gels containing BSA or α-lactalbumin and in the absence of trypsin, no evidence of protein degradation products was observed, thus indicating that the treatment did not alter protein integrity (data not shown).

3.2. Proof of the Procedure. Proof of the procedure for specific protein identification using (1) protein separation from a complex mixture, (2) sonoreactor proteolytic digestion, and (3) protein identification by MALDI-TOF-MS was done from the sulfate reducing bacteria *Desulfovibrio desulfuricans* ATCC 27774 as follows: after anion exchange column, overexpression of a protein was observed by SDS-PAGE at approximately 75 kDa. The in-gel protein digestion with the sonoreactor protocol (continuous mode, 50% amplitude and 2 min of sonication time) followed by MALDI-TOF-MS determination was carried out on the gel spots containing the overexpressed protein. Reduction and alkylation steps were included in the sample treatment. The protein was correctly identified as adenylylsulphate reductase alfa-subunit in the UniProt2006 database using the Protein Prospector search engine: 15 peptides matched and 21% of coverage.

4. Conclusions

The developed method based on sonoreactor technology for ultrafast high-throughput in-gel protein digestion represents an important advance in the proteomic area. This method is faster than the classical in-gel protein digestion protocol and also than the in-gel protein digestion with ultrasonic cell disruptors, since it allows simultaneous digestion of six samples in 1 or 2 min. This fact can be related with a better homogenization in the operation conditions during the sonication step and also with lower gel degradation. Therefore, the new methodology represents a good alternative to the classic and HIFU protocols.

The optimization demonstrates that the best sonoreactor conditions for protein digestion are 50% amplitude and 1 min of sonication time. This time, when necessary, can be higher. The minimum amount of protein that can be digested with this protocol giving enough peptides for a good identification by PMF in a MALDI-TOF-MS is 0.1 μg.

Finally, the identification of the adenylylsulphate reductase alfa-subunit from a complex mixture obtained from the sulfate-reducing bacteria *Desulfovibrio desulfuricans* ATCC 27774 demonstrates that (a) parameters tested on standard samples can be also applied to complex biologic samples and (b) the method provides important advances in fast protein recognition.

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Supporting Information Available: Figure 1, effect of the amount of trypsin used for in-gel protein digestion with sonoreactor; Figure 2, effect of the digestion time on the sequence coverage (%) obtained for α-lactalbumin and BSA (0.5 μg), after protein digestion using the sonoreactor at 70% amplitude and posterior analysis by MALDI-TOF-MS; Figure 3, effect of the sonoreactor amplitude on the sequence coverage (%) and the number of peptides matched for α-lactalbumin and BSA (0.5 μg), after protein digestion using a sonoreactor in the continuous mode for 60-s sonication time; Figure 4, MALDI-TOF-MS spectra of α-lactalbumin after in-gel protein digestion with sonoreactor (continuous mode at 50% amplitude for 60 s); a movie of the sonoreactor. This material is available free of charge via the Internet at http://pubs.acs.org.

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