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# New findings for in-gel digestion accelerated by high-intensity focused ultrasound for protein identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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## Abstract

New findings in sample treatment based on high-intensity focused ultrasound (HIFU) for protein digestion after polyacrylamide gel electrophoresis separation are presented. The following variables were studied: (i) sample volume; (ii) sonotrode diameter; (iii) previous protein denaturation; (iv) cooling; (v) enzyme concentration; and (vi) protein concentration. Results showed that positive protein identification could be done after protein separation by gel electrophoresis through peptide mass fingerprint (PMF) in a volume as low as 25 μL. The time needed was less than 2 min and no cooling was necessary. The importance of the sonotrode diameter was negligible. On the other hand, protein denaturation before sonication was a trade-off for the success of procedure here described. The protein coverage was raised from 5 to 30%, and the number of peptides matching the proteins was also increased in a percentage ranging 10–100% when the classical overnight treatment is compared with the proposed HIFU procedure. The minimum amount of protein that can be identified using the HIFU sample treatment by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was 0.06 μg. The lower concentration of trypsin successfully used to obtain an adequate protein digestion was 3.6 μg/mL.

*Keywords:* Protein digestion; High-intensity focused ultrasound; MALDI-TOF-MS

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## 1. Introduction

Proteins used as biomarkers are changing (i) disease screening/treatment and (ii) strategies for developing new drugs [1]. In addition, emerging applications of protein biomarkers such as bacterial or virus identification [2–4] can help significantly to reduce morbidity and mortality across the globe. Furthermore, protein biomarkers discovery can help governments to beat bioterrorism since bacteria are the common weapons used by bioterrorist. Protein biomarkers identification can be actually done by three different strategies: (i) proteins are separated and isolated by gel electrophoresis and then the proteins are subjected to enzymatic digestion *in situ* in the gel, to form a pool of peptides that are used later to identify the protein [5]; (ii) mixtures of proteins are digested in solution using enzymes and later the peptides produced are separated by liquid chromatography and are used to identify the proteins [6] and (iii) the solution containing the protein mixture is passed through a separation chromatographic column and then isolated proteins are passed through a column filled with immobilized enzyme, peptides are then formed and used for the identification of the proteins [7]. Identification of protein biomarkers can be done through the peptides obtained with one of the three approaches described above by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [8]. High analysis cost is, however, the *Achilles Heel* of LC-MS/MS. An alternative to LC-MS/MS is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), which can be routinely used in conjunction with the first aforementioned sample treatment for protein identification, this is, the *in-gel* enzymatic protein digestion. In addition, the second and third sample treatments also described above can be used with MALDI-TOF-MS in conjunction with preparative chromatography. In MALDI-TOF-MS, the samples are sublimated and ionized from a crystalline

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matrix [3,9] and then accelerated by electric potentials into a mass analyser. The protein identification is done by direct comparison between the experimental masses from the peptides produced by the protein digestion and those produced by the *in silico*, theoretical, digestion of all the proteins, which are included in a particular database. In most cases the set of masses of the peptides formed as a result of the sample treatment is enough for the unambiguous identification of the protein. This methodology is known as peptide mass fingerprinting (PMF) [10]. The PMF of any protein is identified using special search programs, known as search engines, such as MASCOT [http://www.matrixscience.com/search_form_select.htmL], or PROTEIN PROSPECTOR [http://prospector.ucsf.edu/].

Modern protocols for *in-gel* protein digestion have been drastically changed after the introduction of the enhancement of the enzymatic activity with the use of high-intensity focused ultrasound (HIFU). This is a recent developed technology to date in phase of internationalization [8,11]. The HIFU methodology has allowed enzymatic digestion of proteins in seconds whilst former approaches needed from 4 to 12 h to complete the enzymatic process. Although the HIFU methodology has proven to be efficient in many ways, is still a novel procedure that deserves to be further investigated.

As can be seen in Fig. 1, the classic sample treatment for protein identification is tedious and time-consuming. The first approach for the HIFU application to protein identification procedures based on PMF was focused on the reduction of the enzymatic digestion time from overnight to seconds [8]. Now, to simplify the sample handling, the research on HIFU application is focused on the elimination of the reduction and alkylation steps, which usually takes 1 h time and are used to facilitate protein digestion.

In the present work, different variables such as: (i) sonication amplitude; (ii) sonication power; and (iii) total sample volume [12–14] are investigated, but this time omitting the alkylation and reduction steps in the treatment for protein identification by MALDI-TOF-MS. Furthermore, recent achievements suggest that the shape of the sample container can even affect effectiveness of ultra-sonication [15]. We demonstrate that is necessary a minimum volume for a correct performance of the sample treatment and that alkylation and reduction steps cannot be suppressed in the sample treatment even if HIFU is used. Furthermore, the importances of variables such as probe diameter are highlighted.

Finally, in order to test the parameters studied on a biological sample, the adenylyl-sulphate reductase alfa-subunit from *Desulfovibrio desulfuricans* ATCC 27774 was identified from a complex protein mixture.

## 2. Experimental

### 2.1. Apparatus

Gel electrophoresis was performed with a Bio-Rad (Hercules, CA, USA) 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 (i) sample drying and (ii) sample pre-concentration. Biogen Cientifica (Madrid, Spain) centrifuges and vortex models Sky Line and Spectrafuge Mini were used throughout the sample treatment, when necessary. An ultrasonic cell disruptor from Dr. Hielscher (Teltow, Germany) model UP 50H 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. An incubator from P-Selecta (Barcelona, Spain) was used to perform classical protein enzymatic digestion. A Simplicity 185 from Millipore (Milan, Italy) was used to obtain Milli-Q water throughout all of the experiments.

### 2.2. Standards and reagents

A protein mixture containing glycogen phosphorylase b (97 kDa), bovine serum albumin (BSA; 66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), was purchased from Amersham Biosciences (Buckinghamshire, UK, part number 17-0446-01). BSA (>97%) was purchased from Sigma (Steinheim, Germany). All material was used without further purification. Trypsin enzyme, sequencing grade was purchased from Sigma. α-Cyano-4-hydroxycinnamic acid (α-CHCA) *puriss* for MALDI–MS from Fluka (Buchs, Switzerland) was used as MALDI matrix and Sequazyme Peptide Mass Standards Kit (part number P2-3143-00) from PE Biosystems (Foster City, USA) 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%), 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%), sodium dodecyl sulphate (SDS) from Merck (Darmstadt, Germany); and α,α,α-tris-(hydroxymethyl)methylamine + tris(hydroxymethyl)aminomethane, ultrapure grade from Aldrich (Steinheim, Germany).

### 2.3. Sample treatment

Protein amounts comprising between 3.7 and 0.06 μg were dissolved in sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) [16]. Since protocols for protein identification require many different steps, the classic sample treatment used throughout this work is schematically shown in Fig. 1 along with the intended effects for each individual step. The classic digestion sample treatment differs from the HIFU protocol in the incubation time necessary for the enzymatic digestion process: overnight versus 2 min. HIFU was performed with the 0.5 mm or 1 mm sonotrode diameter at amplitude of 70%.