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Short communication

An assessment of the indirect high intensity ultrasonic assisted cleavage of complex proteomes with immobilized trypsin.

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ABSTRACT

The indirect high intensity ultrasonic assisted cleavage of complex proteomes using immobilized trypsin has been assessed. It has been found that the formation of aggregates between the beds supporting the immobilized trypsin is promoted. This affects the efficiency of the digestion process, which can be 100 times lower than the digestion efficiency obtained with in-solution trypsin. Through the use of isotopic peptide labelling with 18-O, it has been quantified that the digestion efficiency over serum samples can be 1.2–100 times higher for the 70% of the peptides when indirect ultrasonication is replaced by direct ultrasonication. Therefore, direct high intensity ultrasonic assisted cleavage of proteins is proposed as an alternative to be combined with immobilized trypsin.

1. Introduction

Quantification of proteins through the labelling of peptides with ^{18}O can be done following two different approaches [1–3]. In the first approach, the sample is digested in ^{18}O water. Therefore, the digestion of the protein and the labelling of the peptides take place at the same time. It has been demonstrated, however, that if the digestion of proteins is done at the same time that the peptide labelling, there is a lack in the double ^{18}O incorporation if this approach is used [4–7]. To solve this problem, some algorithms have been developed to correct this inefficient double labelling [8,9]. However, the simplest way to overcome this drawback is by doing the protein digestion and the protein labelling in two separated and consecutive steps. Thus, in the second approach, the sample is first digested in normal water, then dried and finally recomposed in ^{18}O water. This allows for a reproducible and efficient double incorporation of ^{18}O, leading to the accurate and precise quantification of proteins [3,10].

Recently, the use of ultrasonic energy, UE, in ^{18}O-based workflows for protein quantification [11,12], as a tool to speed the sample treatment pipeline has been reported. Thus, when UE is used, digestion of complex proteomes, such as plasma or serum, can be done in less than 5 min [10,12]. However, the use of UE seems to speed only the mechanisms involved in the protein digestion, being negligible its influence in the efficiency or in the speed of the ^{18}O incorporation [6–12]. In other words, UE can only speed the protein digestion but not the ^{18}O labelling of peptides.Up to date, the use of UE in the digestion of proteins or complex proteomes has been described using trypsin immobilized in solid supports or trypsin in solution. The use of immobilized trypsin brings a number of benefits, such as (i) the possibility to reuse the enzyme,and (ii) the possibility to use higher amounts of enzyme without compromising the subsequent mass spectrometric analysis with the presence of interfering peptides [10,12]. Research dealing with UE and immobilized trypsin was developed using an ultrasonic probe to deliver the ultrasonic energy into the sample. Current research focused towards the application of ultrasonic energy in proteomics, has addressed the convenience to use indirect high intensity ultrasonic energy, IHI-IUE, through cup-horns instead of ultrasonic probes, as a better way to speed digestion of proteins [5]. IHI-UE allows to treat more samples at once, it avoids sample contamination and cross-contamination caused by the tip of the ultrasonic probe, it allows sample cooling and dangerous samples are treated sealed [10]. Recently, however, we have verified that IHI-UE is not an appropriate way to speed protein cleavage when immobilized trypsin is used. Unexpectedly, we have observed that the beds tend to form aggregates when they

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are submitted to IHI-UE, leading to a decrease in the digestion efficiency (see videos of supplementary material, VSM1 and VSM2).

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.talanta.2012.12.022.

In the present academic paper a complex proteome, human serum, was digested with trypsin immobilized in beds or with trypsin in solution. Both procedures were accelerated with UE provided by either a cup-horn or an ultrasonic probe. The efficiency of both methods of digestion was compared using ^{18}O isotopic labelling. The results clearly demonstrated that UE provided by a cup-horn in combination with immobilized trypsin has a worst performance than a cup-horn in combination with trypsin in solution.

2. Results and discussion

2.1. Materials and methods

2.1.1. Reagents

DL-dithiothreitol (DTT, $\geq$99%), iodoacetamide (IAA, $\geq$99%), trifluoroacetic acid (TFA, 99% FOR LC–MS), acetonitrile (ACN, LC–MS CHROMASOLV), water (LC–MS CHROMASOLV) and ammonium bicarbonate ($\geq$99%) were purchased from Sigma (Steinheim, Germany), and were used for protein precipitation, reduction and alkylation. Trypsin (sequencing grade) from Roche (Mannheim, Germany) and Mag-Trypsin (5% suspension) from Clontech (Mountain View, USA) were used for protein digestion. NuTips large 10–200 μl, C-18 for sample desalting were purchased from Glygen (Columbia, USA). Water- ^{18}O (97 atom % ^{18}O) from Sigma-Aldrich (Steinheim, Germany). α-Cyano-4-hydroxycinnamic acid puriss for MALDI-MS from Fluka (Steinheim, Germany) was used as MALDI matrix. Calibration 1, 4700 Proteomics Analyzer Calibration Mixture from Applied Biosystems was used as mass calibration standard for MALDI-TOF/TOF-MS measurements.

2.2. Sera samples

The human sera samples were obtained from anonymous donors in the Complejo Hospitalario Universitario de A Coruña, Spain. Osteoarthritis (OA) patients and controls were characterized radiographically. All patients signed the informed consent. The study was approved by the local ethics committee of Galicia (Spain).

Serum samples of OA and Normal patients were stored at −80 °C until further processing.

2.3. Sequential protein depletion

Sequential protein depletion was done according to the protocol previously developed in our laboratory [13]. The sera were subjected to a sequential depletion protocol involving two precipitation steps. Protein depletion was first perform with DTT according to the protocol described by Warder et al. [14] and the second depletion was perform with ACN according to the protocol described by Kay et al. [15] with minor modifications. In brief, 2.2 μl of 500 mM DTT were added to 20 μl of serum and vortexed shortly. Then the sample was incubated at room temperature for 1 h until a viscous white precipitate was observed, then the sample was centrifugued at 14000*g* for 2 × 20 min. The supernatant was transferred into a clean LoBind tube and was further depleted with 57% (*v*/*v*) ACN, vortexed to mix and sonicated 10 min in an ultrasonic bath. Then the sample was vortexed again briefly and sonicated 10 min. The protein precipitate was then pelleted by centrifugation at 14000*g* for 10 min, The pellet was discharged and the supernatant was transferred into a clean LoBind tube and evaporated to dryness in a vacuum concentrator centrifuge Savant SPD121P SpeedVac (Thermo, Waltham, USA) and stored at −20 °C.

2.4. Serum digestion in homogeneous phase (trypsin in solution)

Ultrasonic in solution digestion was done according to the ultrafast proteolytic digestion protocol previously developed in our laboratory [4]. The pellet was re-suspended in 20 μl of ammonium bicarbonate (AmBi) 25 mM and then 10 μl of ACN were added. The sample was then vortexed and sonicated 1 min (50% amplitude) in the sonicator (SONOPULS HD 2200 with cup-horn BB6 accessory, Bandelin). Protein cysteine residues were reduced with 2 μl DTT 110 mM, and then the sample was vortexed for a short time and then sonicated again for 1 min (50% amplitude) in the sonicator. The resulting cysteines were then blocked with 2 μl IAA 600 mM and then the sample was vortexed for a short duration and then sonicated again for 1 min (50% amplitude) in the sonicator. Afterwards, 10 μl of DTT 110 mM were added for IAA inactivation. Then, the sample was diluted to a final volume of 200 μl with AmBic 12.5 mM. Afterwards, tryspin was added with ratio 1:20 (*w*/*w*) trypsin/protein, as recommended by the manufacturer. The digestion was performed in the sonicator operating at 50% amplitude for 2.5 min. Then, 2 μl of formic acid 50% *v*/*v* were added to stop the enzyme activity. Samples were acidified with 1 μl of pure TFA and desalting was then performed using NuTip™ pipette tips.

2.5. Serum digestion in heterogeneous phase (immobilized trypsin)

The pellet was resuspended in 20 μl of ammonium bicarbonate (AmBi) 25 mM and then 10 μl of ACN were added, the sample was vortexed and sonicated 1 min (50% ultrasonication amplitude) in the sonicator (SONOPULS HD 2200 with cup-horn BB6 accessory, from Bandelin, USA). Protein cysteine residues were reduced with the addition of 2 μl DTT 110 mM, vortexed and sonicated again 1 min (50% amplitude) in the sonicator. The resulting cysteines were then blocked with 2 μl IAA 600 mM and vortexed and sonicated again 1 min (50% amplitude) in the sonicator. 10 μl of DTT 110 mM were added for IAA inactivation. The sample was diluted to a final volume of 200 μl with AmBi 12.5 mM and then was evaporated to dryness in a vacuum concentrator centrifuge. Prior to use, 200 μl of Mag-trypsin (5% suspension) were washed with H_2O-LCMS three times. Then, the sample was reconstituted in this volume of clean immobilized trypsin and was digested in the sonicator with cup-horn at 50% of amplitude for 2.5 min. The supernatant was recovered employing a magnet for retaining the Mag-trypsin beds. The digested sample was acidified with 1 μl of pure TFA and was subsequently NuTip™ desalted. The cleaned sample was evaporated to dryness and label with ^{18}O as described in Section 2.7.

2.6. Digestion with immobilized trypsin assisted with ultrasonic probe

The depleted serum was reduced, alkylated and digested with the same protocol as described in Section 2.5, but the digestion was assisted with direct ultrasounds (Ultrasonic robe DRH-UP50H, Dr. Hielscher, 0.5 mm tip) at 50% of amplitude for 2.5 min. The supernatant was recovered employing a magnet for retaining the Mag-trypsin beds. The digested sample was acidified with 1 μl of pure TFA and was subsequently NuTip™ desalted. The cleaned sample was evaporated to dryness and label with ^{18}O as described in Section 2.7.