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STAGE-diging: A novel in-gel digestion processing for proteomics samples

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ABSTRACT

Proteomics based on high-resolution mass spectrometry has become a powerful tool for the analysis of protein abundance, modifications and interactions. New generation mass spectrometers and UPLC are able to cover approximately an entire cell proteome in one run, but sample preparation, in terms of time and sample recovery is still a critical step. Here we present a modification of the in-gel digestion method, called STAGE-diging. This approach was compared with the well-established procedures for sample preparation, both on high and low complexity samples, on quantitative SILAC-based experiments and on two different mass spectrometers. The results show that STAGE-diging reduces sample handling, decreases the analysis time and improves protein identification and quantification. Moreover, shorter instrument time allows performing multiple replicates that produce wider proteome coverage and more accurate quantitation.

Significance: In our work we detailed the set-up of a novel in-gel digestion processing for proteomics samples, called STAGE-diging. This new method can be applied to samples of different complexity both for qualitative and quantitative proteomics studies. We proved that STAGE-diging streamlines sample preparation as it is easy to use, reduces sample handling and improves protein identification and quantification all with a decreased analysis time. All these benefits make this new method appealing for laboratories handling a large number of samples, where time and reproducibility play a substantial role.

1. Introduction

In the last twenty years, proteomics evolved enormously in the fields of analysis of protein abundance, detection of post translational modifications and protein-protein interactions with the help of high-resolution new generation mass spectrometers combined with Ultra Performance Liquid Chromatography that allowed to cover approximately an entire cell proteome in a single run, with high reproducibility [1,2].

Asynchronously, the digestion of proteins into peptides, that is the prerequisite of MS-based proteomics bottom-up approach, is still a common and critical step, in terms of time and sample recovery. While it is quite recent the proposal of new protocols for in solution digestion of protein samples (FASP, filter-aided sample preparation, cleanup and digestion in commercial spin filters or on-column proteolytic digestion of samples) [3–5] and cell lysate in a single encapsulated volume [6], the method for in gel digestion, initially described more than two decades ago by Rosenfeld et colleagues and modified in 1996 by Shevchenko and coworkers, has not substantially changed in the years. [7–10].

Advantages of in-gel digestion that makes it preferable to other sample preparations are: i) it is a simple and cost-effective procedure for sample pre-fractionation; ii) it has the ability to remove contaminants and detergents that can interfere with digestion and MS analysis; iii) it provides visual quality control of the samples in terms of complexity and abundance; iv) it is a highly efficient denaturation method and can be applied to a large variety of sample types [11]. On the other hand, disadvantages of the technique that makes it relatively low throughput and have been overlooked for large screens and quantitative assays are: i) it is a rather laborious process with numerous steps of washing and incubation, that are still operator dependent; ii) it has a lower enzymatic efficiency (~20%) relative to that in solution and suffers from low efficiency in peptides recovery from the gel matrix in comparison with other techniques, typically used when cleaning from detergents is required, that incorporate proteins into polyacrylamide gel matrix without electrophoresis, [12–14]; iii) multiple handling procedures predispose samples to stochastic mistakes and contaminations. Therefore, in this work, we present a faster and highly reproducible adaptation of the in-gel digestion method called STAGE-diging where an entire gel lane is processed in a single, enclosed stop-and-go extraction tips (StageTip) [15]. This procedure can be applied both on high and low complexity samples and in proteomics qualitative and quantitative studies.

2. Experimental procedures

2.1. STAGE-diging procedure

The digestion workflow was adapted from the method described by Shevchenko et al. and Rappsilber et al. with minor changes but with the

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substantial difference that the entire protocol occurs in a p1000 tip (Gilson or similar) filled at the orifice with a double C18 Empore Disk (3M, Minneapolis, MN) plug. Briefly, after Coomassie blue or Silver [16] staining, half on an entire lane was carefully cut into ~1 mm^3 cubes and transferred into the STAGE-diging tip. These gel cubes were dehydrated with 100% acetonitrile (ACN) and rehydrated in 100 mM NH_4HCO_3 twice before being dehydrated by the addition of ACN. To ensure that the gel pieces do not create a sticky surface on the C18, all the solutions were added with a gel-loader tip. The removal of solutions was accomplished by centrifugation at 1800 rpm using the commercial tip box as holder. Otherwise the solutions were forced through the double C18 plug by pushing with a syringe. Reduction of protein disulfide bonds was carried out with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 and subsequent alkylation was performed with 55 mM iodoacetamide (IAA in complete darkness) in 100 mM NH_4HCO_3, at room temperature for 30 min. Both DTT and IAA were removed by centrifugation or by syringe as previously described. The gel pieces were rehydrated and dehydrated with 100 mM NH_4HCO_3 and ACN respectively prior to digestion. Gel pieces were rehydrated with 40 μL of Trypsin (12.5 ng/μL in 100 mM NH_4HCO_3, after few minutes 60 μL of NH_4HCO_3 were added and samples were incubated at 37 °C o/n in a commercial tip box filled by water on the bottom to ensure that buffer will not evaporate. The digestion solution was then forced through the double plug with a syringe and the flow through was collected. Samples were acidified with 100 μL of formic acid (FA) 0.1%, forced with the syringe and collected as flow-through. In this way the desalting of peptides occurs. Peptides were eluted twice by adding 100 μL of a solution composed of 80% ACN, 0.1% FA, an extra step of extraction with 100% ACN was performed and then all the eluates were dried in a Speed-Vac and resuspended in 20 μL of solvent A (2 % ACN, 0.1% formic acid). 2 and 5 μL were injected for each technical replicate on the Q-Exactive and the LTQ-FT mass spectrometers respectively.

2.2. Protein digestion and peptides preparation with the Standard Procedure

The second half of the lane was divided into different number of slices and trypsinized as previously described by Shevchencko et al. Peptides were desalted as described by Rappsilber et al., dried in a Speed-Vac and resuspended in 20 μL of solvent A (2 % ACN, 0.1% FA). 2 μL and 5 μL were injected for 2 technical replicates on both mass spectrometers.

2.3. LC separation and mass spectrometry analysis

Mass spectrometry analysis was performed by LC–MS/MS on a Fourier transformed-LTQ mass spectrometer (FT-LTQ, Thermo Electron, San Jose, CA) equipped with an Agilent chromatographic separation system 1100 (Agilent Technologies, Waldbronn, Germany) where the LC system was connected to a 15 cm fused-silica emitter of 75 μm inner diameter (New Objective, Inc. Woburn, MA USA), and on a quadrupole Orbitrap Q-Exactive mass spectrometer (Thermo Scientific) coupled with an UHPLC Easy-nLC 1000 (Thermo Scientific) with a 25 cm fused-silica emitter of 75 μm inner diameter. Both columns were packed in-house with ReproSil-Pur C18-AQ beads (Dr. Maisch Gmbh, Ammerbuch, Germany), 3 μm of diameter for the nano-LC and 1.9 for the UPLC using a high-pressure bomb loader (Proxeon, Odense, Denmark). Peptides separation was achieved with different gradients on each instrument.

2.3.1. For the in gel digestion with Standard Procedure

i) A linear gradient from 100% solvent A (5 % ACN, 0.1% formic acid) to 20% solvent B (ACN, 0.1% formic acid) over 33 min, from 20 to 60% in 1 min and from 60% to 80% solvent B in 2 min at a constant flow rate of 0.3 μL/min on Agilent 1100 coupled to LTQ-FT for both less and more complex samples with a single run time of 57 min.

ii) A linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 40% solvent B (80% acetonitrile, 0.1% formic acid) over 30 min and from 40% to 100% solvent B in 2 min at a constant flow rate of 0.25 μL/min for the UHPLC coupled to Q-Exactive for less and more complex samples with a single run time of 33 min.

2.3.2. For the STAGE-diging protocol

i) A linear gradient from 100% solvent A (5 % ACN, 0.1% formic acid) to 20% solvent B (ACN, 0.1% formic acid) over 107 min, from 20 to 60% in 3 min and from 20% to 80% solvent B in 5 min at a constant flow rate of 0.3 μL/min on Agilent 1100 coupled to LTQ-FT for less complex samples with a single run time of 135 min.

ii) A linear gradient from 100% solvent A (5 % ACN, 0.1% formic acid) to 20% solvent B (ACN, 0.1% formic acid) over 161 min, from 20 to 60% in 4 min and from 20% to 80% solvent B in 7 min at a constant flow rate of 0.3 μL/min on Agilent 1100 coupled to LTQ-FT for more complex samples with a single run time of 190 min

iii) A linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 40% solvent B (80% acetonitrile, 0.1% formic acid) over 55 min and from 40% to 100% solvent B in 5 min at a constant flow rate of 0.25 μL/min for the UHPLC coupled to Q-Exactive for less complex samples, with a single run time of 60 min.

iv) A linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 40% solvent B (80% acetonitrile, 0.1% formic acid) over 85 min and from 40% to 100% solvent B in 5 min at a constant flow rate of 0.25 μL/min for the UHPLC coupled to Q-Exactive for more complex samples with a single run time of 90 min.

v) A linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 40% solvent B (80% acetonitrile, 0.1% formic acid) over 115 min and from 40% to 100% solvent B in 5 min at a constant flow rate of 0.25 μL/min for the UHPLC coupled to Q-Exactive for SILAC STAGE-diging samples, with a single run time of 120 min

MS data were acquired using a data-dependent top 5 and top 12 methods for LTQ-FT (CID fragmentation) and top 10 and top 12 for Q-Exactive analysis (HCD fragmentation). For the LTQ FT survey full scan MS spectra (350–1650 Th) were acquired in the FT with 1e6 resolution, AGC target 5e5 while MS/MS spectra, in the ion trap, were limited to one scan per precursor ion followed by 1 min of exclusion, AGC target 2e5; normalized collision energy 35 and isolation width 2.0 m/z. In the Q-Exactive, survey full scan MS spectra (300–1750 Th) were acquired in the Orbitrap with 70000 resolution, AGC target 1e6, IT 120 ms. For HCD spectra resolution was set to 35000, AGC target 1e5, IT 120 ms; normalized collision energy 25% and isolation width 3.0 m/z. This acquisition method was applied both for methodological development and for SILAC analysis.

SILAC STAGE-diging samples were acquired on a Q-Exactive mass spectrometer with 70000 resolution in the Orbitrap for MS survey scan, AGC target 3e6, IT 60 ms. For HCD MS/MS spectra resolution was set to 17500, AGC target 1e5, IT 60ms; normalized collision energy 25% and isolation width 2.6 m/z.

2.4. Protein identification and quantification

For protein identification the raw data were processed using Proteome Discoverer (version 1.4.0.288, Thermo Fischer Scientific). MS^2 spectra were searched with Mascot engine against uniprot_human_201503 database (89909 sequences; 35686673 residues), with the following parameters: enzyme Trypsin, maximum missed cleavage 2, fixed modification carbamidomethylation (C), variable modification oxidation (M) and protein N-terminal acetylation, peptide tolerance 10 ppm, MS/MS tolerance 0.5 Da for the FT data and 20 mmu for the Orbitrap. Peptide Spectral Matches (PSM) were filtered using percolator based on q-values at a 0.01 FDR (high confidence). Proteins were considered identified with 2 unique high confident peptides [17].