Up to date sample preparation of proteins for mass spectrometric analysis

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Abstract
For the identification of proteins from biological samples by mass spectrometry there are two scenarios. Proteins are pre-fractionated in some way or are analysed as a complex mixture (shotgun). Shotgun proteomics recently became more popular, because of technological developments on the mass spectrometer side which now allows the identification of several thousand proteins from a complex biological matrix at one go. However, in many cases it is still useful to separate proteins first in a gel. But not only mass spectrometer technology made progress. Recently, new protocols and techniques which make the analysis of starting material in the low microgram range possible, and also simplify the whole procedure, were developed. In this small review detailed protocols will be described which also allow inexperienced beginners to get good results.

Keywords
Fractionation of peptides, in solution digest, in-gel digest, OASIS, shotgun proteomics

Introduction
Mass spectrometry became a valuable tool in biological analysis of proteins. During recent years the sensitivity of the instruments improved dramatically and this development is still ongoing. We are close being able to analyse whole proteomes of low microgram range in a single shot (shotgun analysis). The sample preparation becomes more and more important. It should be quick, relatively easy to perform, reproducible and with no, or minimal, loss of sample. Also recently protein sample preparation for mass spectrometry has made great progress towards the easy handling of small samples. Multistep procedures are usually not suited for starting material in the low microgram range. The loss of material during sample preparation is substantial. Therefore one step procedures are preferred. Inexperienced scientific staff are facing the problem of old protocols in the literature, incomplete handling procedures or protocols are scattered throughout several publications making it difficult to achieve a working protocol. The lack of important information or little ‘tricks’ is the most common reason for irreproducible experiments. In this small review we will disclose all the details and following the protocols will produce good results instantly. We are using these protocols daily, but also in training courses for scientists and scientific staff.

In gel digestion (Eckerskorn & Lottspeich, 1989; Rosenfeld et al., 1992) of silver stained gels (Kerenyi & Gallyas, 1973) using the OASIS® HLB μElution Plate (Franz & Li, 2012)

Protein separation in gels is still commonly used. For maximum sensitivity it is important to use a mass spectrometry compatible silver staining method (Chevallet et al., 2008). Manual de-staining of bands, reduction of cysteine bridges and alkylation of sulfides are labour intensive. Therefore we start with a manual high-throughput method for in gel digestion which will work for 1D gel bands as well as for 2D gel spots picked by a robot directly into the OASIS® HLB μElution Plate. The investment in the whole OASIS set up is only in the range of 5–10 chromatographic columns used for the mass spectrometric analysis.

The OASIS platform allows the use of an eight-channel pipette to add solutions, and with the help of the positive pressure stand (Waters, Milford, MA, USA) the solutions are then simultaneously removed. Thus, one can make a manual digest and de-salting of 96 samples in the same time and of the same quality that one could typically digest only 12 samples manually using standard 0.5 mL tubes. The samples were then de-stained, reduced, alkylated, digested, and the peptides extracted and desalted in the OASIS® HLB μElution Plate using the following protocol.

Materials
- 100 mM ammonium bicarbonate (Bio Ultra >99%, Fluka, Steinheim, Germany) in Milli-Q® water pH 8.0.
• Acetonitrile (ULC-MS grade, Biosolve, Valkenswaard, The Netherlands).
• 0.1% trifluoroacetic acid in the case of MALDI preparation (TFA, for LC-MS >99%, Fluka, Steinheim, Germany).
• 0.1% formic acid for following LC-MS (ULC/MS grade, 99%, Biosolve, Valkenswaard, The Netherlands).
• 10 mM DL-dithiothreitol (>99%, Sigma-Aldrich, Steinheim, Germany) in 100 mM ammonium bicarbonate.
• 30 mM Potassium ferricyanide K3[Fe(CN)6].
• 100 mM sodium thiosulphate.
• 55 mM iodoacetamide (IAA) (>99%, Sigma, Steinheim, Germany) in 100 mM ammonium bicarbonate.
• Digestion buffer: 7.4 ng/μL trypsin (Gold mass spectrometry grade, Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate.
• Extraction solution MALDI: acetonitrile:water:TFA = 49.95:49.95:0.1%.
• Extraction solution LC-MS: acetonitrile:water:FA = 47.5:47.5:5.5%.
• OASIS® HLB μElution Plate 30 μm, positive pressure-96 stand (Waters, Milford, MA, USA).

Methods

In-gel digestion by OASIS® HLB μElution Plate (Franz, 2012)

(1) Use a spot cutter to excise the gel plugs from the 2D-gel directly into the OASIS® MTP well or alternatively cut a band by scalpel directly from the gel, place it at the upper rim of an OASIS® MTP well and cut it into three 1 mm pieces. Then push them down into the well by pipette tip. For 96 wells it will manually take about 1 h.
(2) Add 70 μL acetonitrile (ACN) and push the gel pieces down into the solution if necessary.
(3) Remove the liquid from the excision process into the waste plate (24 × 10 mL MTP plate) using the positive pressure stand (PPS, if not otherwise stated 15 PSI of nitrogen pressure was always used).
(4) Leave samples covered at room temperature overnight or if available in an incubator at 37 °C.
(5) Using the PPS, remove all liquid into a waste plate.

Destaining (Kerenyi & Gallyas, 1973) of mass spectrometry compatible silver gel (Chevallet et al., 2008; Franz & Li, 2012)

(1) Rehydrate gel pieces with 50 μL potassium ferricyanide and incubate 10 min at 37 °C.
(2) Then add 50 μL sodium thiosulphate and incubate 20 min at 37 °C.
(3) After 15 min remove the liquid.
(4) Then add 50 μL water and after 10 min 50 μL acetonitrile.
(5) Remove all the liquid after 15 min.
(6) Add 70 μL ammonium bicarbonate and wait for 10 min.
(7) Remove all the liquid after 15 min.
(8) Add acetonitrile and wait for 10–15 min, if it runs through add again after 5 min.
(9) Remove ACN by PPS.

Reduction

(1) Rehydrate gel pieces in 100 μL 10 mM DTT in 100 mM ammonium bicarbonate and incubate for 1 h at room temperature to reduce the cysteine bridges in the protein.
(2) Remove excess liquid using the PPS.
(3) Incubate the gel pieces with 100 μL ACN and wait for 10–15 min until the gel pieces have dehydrated, if ACN runs through add again.
(4) Remove all liquid with the PPS.

Alkylation

(1) Swell gel pieces with 100 μL 55 mM IAA in 100 mM ammonium bicarbonate and incubate for 20 min at room temperature in the dark.
(2) Remove IAA solution with the PPS and wash gel pieces with 100 μL of 100 mM ammonium bicarbonate for 15 min.
(3) Remove ammonium bicarbonate with the PPS, add 100 μL ACN and wait 10 min for the gel pieces to dehydrate and shrink, if ACN runs through add again after 5 min.

Application of trypsin

(1) Remove all liquid with the PPS, then rehydrate the gel pieces in 33 μL digestion buffer at room temperature.
(2) After 20 min remove remaining buffer with the PPS.
(3) Add 70 μL of the same buffer (50 mM ammonium bicarbonate), but prepared without trypsin, to cover the gel pieces and keep them wet during enzymatic digestion.
(4) Leave samples covered at room temperature overnight or if available in an incubator at 37 °C.

Extraction of peptides

(1) With the PPS remove the digest solution into the waste plate (peptides will bind to the HLB column).
(2) Wash the OASIS® HLB column with 70 μL 0.1% FA.
(3) Exchange the waste plate for the collection plate (0.5 mL/well).
(4) Add 50 μL of extraction solution (47.5% ACN/47.5% Water/5% FA) to Oasis plate and wait 20 min, then remove the extraction solution into collection plate with the PPS.
(5) Add 50 μL of acetonitrile to OASIS plate and wait 10 min, then remove the extraction solution into collection plate with the PPS.
(6) Completely dry eluted peptides in the collection plate using a vacuum centrifuge at 45 °C for 60 min.
(7) Freeze the dried extracts (peptides) at −20 °C or −80 °C (for storage >1 month) and/or
(8) Re-dissolve the peptides in 7 μL 0.1% formic acid for ESI-MS or in 5 μL 0.1% TFA for MALDI-MS.

One step in solution protein solubilization, reduction, alkylation and in solution digest (Kulak et al., 2014)

Shotgun approaches with very little starting material in the low microgram range can only be successful if sample loss is
minimal and the solubilization of proteins is at the highest. Sample loss can be minimized by reducing the steps involved in the whole procedure. Usually for solubilizing proteins detergents are used, which are problematic in mass spectrometric analysis and involve a lot of steps to remove them. Here we describe a very detailed protocol based on the latest Guanidinium method (Kulak et al., 2014) with a C. elegans sample as example.

**One-step C. elegans sample preparation with Guanidinium chloride buffer**

**Lysis buffer:** 6 M GdmCl (5.73 g/10 mL)
- 10 mM TCEP (Tris(2-carboxyethyl)phosphine) (28.7 mg/10 mL)
- 40 mM CA (chloroacetamide) (37.6 mg/10 mL)
- 100 mM Tris (122 mg/10 mL) pH 8.5

**Trypsin:** Trypsin Gold, LC MS grade, Promega

**Empore™ C18-SD for desalting**
- (3 M, 66871-U); capacity: 150 μg

**Bioruptor plus Sonicator:** Diagenode, Seraing (Ougrée), Belgium

**Protocol:**

It is important that the buffer is always fresh! First dissolve 5.73 g of GdmCl in 10 mL of Milli-Q water, then weight the other chemicals into a separate container and dissolve them completely with 3000 μL of the just prepared GdmCl solution and mix it with the rest of the GdmCl solution. As a rough estimation 100 μL of ‘‘worm pellet’’ corresponds to ~1.8 mg peptide.

**Worm lysis:**

1. Centrifuge the worm ‘‘wet mass’’ at 5000 × g for 5 min, remove the water.
2. Add 100 μL of lysis buffer to about 30 μL of ‘‘worm pellet’’.
3. Heat it in a Thermomixer at 95 °C for 10 min at 600 rpm.
4. Sonicate with Bioruptor plus at: 30 s sonication, 30 s break, 10 cycles, position: high performance in an ice bath.
5. Heat again in the Thermomixer at 95 °C for 10 min at 600 rpm.
6. Sonicate again with Bioruptor plus: 30 s sonication, 30 s break, 10 cycles, position: high performance.
7. For C. elegans in dauer state repeat steps 5 and 6.
8. Centrifuge at 20 000 × g for 10 min.
9. Keep the supernatant.
10. Take 4 μL of the protein solution, dilute at least 10 times with 20 mM Tris/10% ACN (240 mg Tris/10 ml Acetonitrile/90 ml Milli-Q® water) to reduce the concentration of GdmCl to lower than 0.6 M.
11. Check the protein concentration at A280/260 with NanoDrop2000 (Thermo Fisher Scientific, USA) (Desjardins et al., 2009).

**Protein digestion**

1. Digest the diluted protein solution at 37 °C overnight.
   - Trypsin:protein = 1:50 (for SILAC sample: LysC).
2. Centrifuge the digest at 15 000 × g for 10 min. Retain the supernatant.
3. For MS analysis the peptides have to be cleaned by STAGE (Rappsilber, 2003) or ZIP-Tip® (Millipore, Billerica, MA, USA).

With this one-step sample processing we could identify around 4500 worm proteins with our standard 240 min gradient on a Q Exactive plus™ instrument (Thermo).

If a maximum of proteins should be identified an easy to perform pre-fractionation routine can be done.

**Pre-fractionation of protein digests with Styrene Divinyl Benzene (SDB-RPS) (Kulak et al., 2014)**

The SDB membrane containing tips are made in the same way as the STAGE tips (Rappsilber et al., 2003) with a 1.5 mm inner diameter syringe. Also three layers of the SDB membrane are used.

**SDB:** 47 mm styrene divinyl benzene (SDB-RPS) from 3 M (Supelco, Bellefonte, PA, USA)

**Buffer 1:** 60 mM ammonium formate, 20% ACN, 0.5% formic acid
- 37.8 mg ammonium formate, 8 mL water, 2 mL ACN, 50 μL Formic acid

**Buffer 2:** 100 mM ammonium formate, 35% ACN, 0.5% formic acid
- 63 mg ammonium formate, 6.5 mL water, 3.5 mL ACN, 50 μL Formic acid

**Buffer 3:** 150 mM ammonium formate, 50% ACN, 0.5% formic acid
- 94.5 mg ammonium formate, 5 mL water, 5 mL ACN, 50 μL Formic acid

**Buffer 4:** 5% ammonium hydroxide, 80% ACN
- 1.85 mL ammonium hydroxide, 8 mL ACN, 150 μL H2O

**Procedure:**

1. The dried digest is re-suspended with 80 μL of 1% TFA in Milli Q® water.
2. Equilibrate the SDB tips with 200 μL of 0.2% TFA.
3. Load 80 μL of the digest to the SDB tips and remove the liquid by centrifugation at 3000 × g for 2 min.
4. Add 200 μL of 0.2% TFA for washing and centrifugate at 3000 × g for 2 min.
5. Eluted the peptides with 80 μL of Buffer 1, Buffer 2, buffer 3 and Buffer 4 sequentially, and collect eluates separately in 0.5 mL Eppendorf tubes.
6. Dry the 4 fractions in a vacuum centrifuge at 45 °C for about 25 min.
7. Re-suspend the fractions with 20 μL 0.1% FA.
8. Check the concentration with NanoDrop at A280/260.

With the combination of the four fractions (4 × 150 min gradient duration) we were able to identify about 5600 worm proteins with a Q Exactive plus™ and a 50 cm reversed phase column.

**Conclusion**

The technical development of mass spectrometers is very fast and also the sample preparation is continuously adapting to
the needs of less starting material and more sensitivity. In this small review we show two good working protocols in every detail. With the detailed protocols beginners or advanced users will be quickly successful in the technically challenging analysis of complex proteomes.

Declaration of interest

The authors report no declarations of interest.

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