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Validation of an Easy Acetonitrile Fractionation for the Simplification of Protein Samples Prior to Proteomics Analyses

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Abstract

In most proteomics analyses and in particular for the "off-gel" approaches, based essentially on chromatography, the complexity of the proteome should be reduced; otherwise identifications can be hindered, especially if the mass spectrometry analysis is not conducted using state-of-the-art instrumentation. Even if the method used is a bottom-up proteomics, it appears mandatory to pre-fractionate the proteins in order to reduce the complexity. We report here the development and validation of a pre-fractionation based on the differential solubilisation of proteins using increasing concentrations of acetonitrile (ACN). This "ACN fractionation" was applied to the study of the Triton X-100 soluble sub-proteome of brain capillary endothelial cells (BCEC) with re-induced blood-brain barrier (BBB) functions.

Keywords

Proteomics, Acetonitrile, Fractionation

Subject Area: **Biochemistry**

1. Introduction

In proteomics analyses, the sample preparation is the most important step in order to obtain the right, reliable and reproducible result. For global proteomics approaches, ideally the preparation should allow solubilisation of all the proteins in a biological sample, without any chemical modification. But it is also essential to eliminate all the interfering non-protein compounds, incompatible with subsequent analytical methods [1]. No universal pro-

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tocols exist for the sample preparation although several protocols are developed depending on the biological sample and the objectives of the study [2]. The separation step can be carried out directly on proteins or on the set of peptides derived from the enzymatic digestion of the corresponding proteins. The separation of proteins or peptides can be considered in two ways: "in-gel", based on electrophoresis and, "off-gel", based essentially on chromatography. In most cases and in particular for the "off-gel" approaches, the complexity of the samples should be reduced. The mandatory pre-fractionation of proteins can be achieved by electrophoresis, chromatography, differential solubility or aqueous two-phase system (for review [1]). In a previous report [3], a precipitation with acetonitrile (ACN) was used for the depletion of high abundance serum proteins prior to nano-LC-MS/MS analysis. Taking advantage of this observation, we developed and validated an easy multi-step fractionation based on the differential solubilisation of proteins using increasing concentrations of acetonitrile (ACN). This "ACN fractionation" was applied to the study of the Triton X-100 soluble sub-proteome of brain capillary endothelial cells (BCEC) with re-induced blood-brain barrier (BBB) functions [4].

2. Experimental Design

The bovine BCEC were solo- or co-cultured with primary cultures of new-born rat cerebral cortex mixed glial cells to re-induce BBB functions [5]. The endothelial cells (8×10^5 cells) were harvested by collagenase treatment (*Clostridium histolyticum*, Sigma, Lyon, France), lysed and partitioned in Triton X-100 soluble and insoluble fractions [6]. The Triton-soluble proteins from BCEC are extremely heterogeneous. To prevent this phenomenon, the samples are fractionated by solubilisation with increasing concentrations of acetonitrile (ACN) namely 0%, 25%, 50%, 75% and 100% ACN (**Figure 1**).

The proteins in the obtained fractions were either directly subjected to two-dimensional electrophoresis (2-DE) or, after trypsin digestion, to nano-LC before analysis by MALDI-TOF-MS/MS.

For nano-LC, 20 µL of the sample were digested for 16 h with 10 µL of trypsin solution, 20 ng/µL in 40 mM NH_4CO_3. Following the tryptic digestion, nano-separations were performed on an U3000 nano HPLC system (Dionex-LC-Packings, Sunnyvale, CA, USA). After a conventional pre-concentration step (C18 cartridge, 300 µm, 1 mm), the peptide samples were separated on a Pepmap C18 column (75 µm, 15 cm) using an ACN/water gradient from 5% to 12% over 20 min, 12% to 50% over 140 min and 50% to 70% over 15 min and, lastly, and a wash with 70% of ACN for 15 min. The flow was set to 300 nL/min and 380 fractions were automatically collected every 30 s on an AnchorChip™ MALDI target using a Proteineer™ FC fraction collector (BrukerDaltonik). 2 µL of α-cyano-4-hydroxycinnamic acid matrix (0.3 mg/mL in acetone:ethanol: 0.1% TFA acidified water, 3:6:1 v/v/v) were added during the collection process. The MS and MS/MS mass measurements were performed off-line using the Ultraflex™ II TOF/TOF mass spectrometer, as described in [7].

For 2-DE, after resolubilization in an isoelectrofocusing buffer (7M urea, 2M thiourea, 4% (v/v) CHAPS and 2% (v/v) ampholytes), 300 µg of cytosolic proteins were subjected to 2-DE on 24-cm length pH 4 - 7 IPG strips at 100,000 V and in the 10 - 100 kDa molecular mass range. The IPG strips were passively and actively rehydrated for 7 h and 9 h at 50 V. The pre-focusing and focusing procedures were carried out at 50 mA/strip in 4 steps: 200 V for 1 h, a gradient up to 1000 V for 1 h, a gradient up to 10000 V for 6 h and, lastly, 10000 V for 4.5 h. The IPG strips were wiped up and successively equilibrated for 15 min with gentle shaking in 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 93 mM TRIS-HCl pH 8.8 buffers supplemented with 20 mM DTT and 100 mMiodoacetamide and a trace of BPB, respectively. The equilibrated strips were sealed on the top of the second-dimension duracrylamide/bis-acrylamide gel (12% T, 2.6% C) with 0.5% (w/v) low-melting point agarose (Biorad, Marnes-la-Coquette, France) in SDS running buffer. Migration as a function of molecular weight was performed in the EttanDALTsix electrophoresis unit (Amersham Bioscience) at 16 mA/gel for 30 min and then at 32 mA/gel until the tracking dye reached the anodic end. The proteins were stained with silver nitrate [8] for image acquisition (with a freshly calibrated Umax scanner (Amersham Biosciences, Orsay, France) at 300 dpi using Labscan 3.0 software) and with colloidal Coomassie Brilliant Blue for protein identification by MALDI-TOF/TOF mass spectrometry. Protein identifications from 2-DE gels were performed using a Proteineer™ workflow from BrukerDaltonik GmbH (Bremen, Germany). Colloidal Coomassie-blue-stained spots were excised from gels with a spot picker (the Proteineer™spII) equipped with a 2 mm needle and placed into 96-well microtitre plates. In-gel digestion and sample preparation for MALDI analysis were performed according to the manufacturer's instructions using a digester/spotter robot (the Proteineer™dp) and tryptic digest kits (the DP 384 standard kit from BrukerDaltonik). Briefly, after destaining of the gel plugs with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate, protein spots were digested [8]. Peptide were

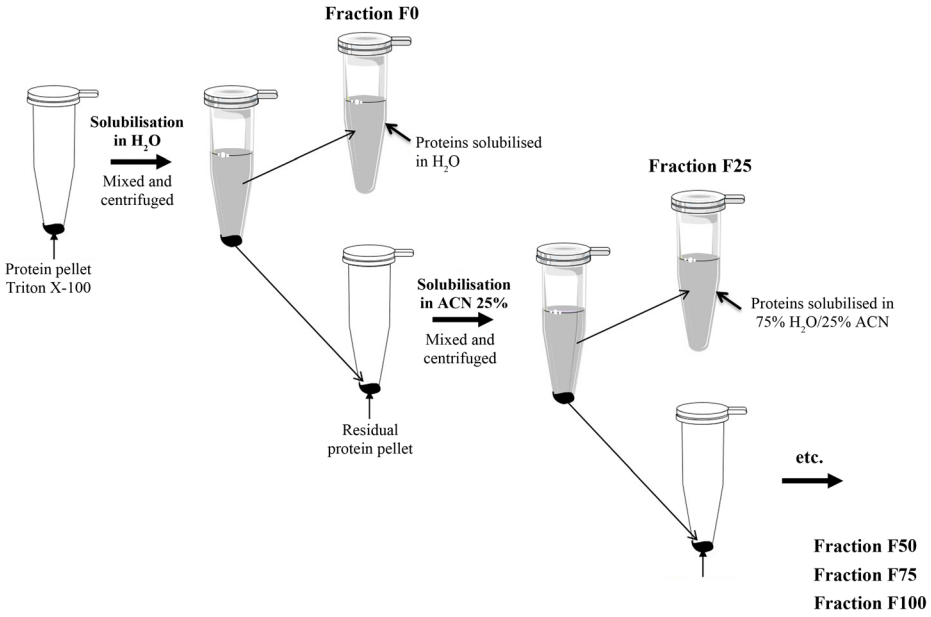

Figure 1. Schematic representation of the ACN fractionation. A protein extract, in the example given the protein pellet after Triton X-100 extract from BCEC, is separated into 5 fractions, namely F0, F25, F50, F75 and F100, using a step gradient of ACN, 0%, 25%, 50%, 75% and 100% respectively. Illustration realised with *Servier Medical Art* (http://smart.servier.fr/servier-medical-art).

extracted with acetonitrile: 0.1% TFA-acidified water (1:1) and then mixed with an α-cyano-4-hydroxycinnamic acid matrix (0.3 mg/ml in acetone: ethanol, 3:6 v/v) on the MALDI target plate (AnchorChip™, BrukerDaltonics). The MS and MS/MS mass measurements were performed off-line using the Ultraflex™ II TOF/TOF mass spectrometer, as described in [7].

3. Results and Discussion

We initially used the "ACN fractionation" in order to complete the data collected by 2-dimensional electrophoresis (2-DE) in view of elaborating a reference proteome of Triton X-100-soluble species from bovine BCEC with limited BBB functions [4]. A total of 215 protein spots (corresponding to 130 distinct proteins) were identified by 2-DE. As it was shown in this preliminary study, when the total protein extract was directly subjected to in-solution digestion followed by nano-LC, only a few peptides yielded a fragmentation pattern suitable for identification. The few identified proteins correspond to the most abundant proteins in the cytoplasm, namely actin, vimentin, vinculin, tubulin and myosin or heat shock proteins. When the "ACN fractionation" was used prior to tryptic digestion, the number of identifications was significantly increased, over 350 proteins were identified. Each individual fraction displays actually a reduced protein heterogeneity that allows improved nano liquid chromatography (nano-LC) separations and ensures as many protein identifications as possible in each fraction. An average of 138 proteins were identified from F0, 235 from F25, 180 from F50, 185 from F75 and only 9 from F100 (not further investigated). Among them 41, 64, 22 and 35 proteins were specifically identified in F0, F25, F50 and F75 respectively. Overall 363 proteins were identified by using the 'ACN fractionation' prior to nano-LC. Together with the protein identifications obtained by 2-DE, the reference proteome of the bovine BCEC was completed to a total of 432 with only 15% proteins common to the two techniques.

We subsequently applied the "ACN fractionation" in a semi-quantitative differential proteomics approach to reveal proteins over-expressed in bovine BCEC after the re-induction of BBB properties, compared to the cells with limited BBB functions. This allowed the identification of 447 distinct proteins. Among them, only 11 were specific for the BCEC with re-induced BBB and 39 with limited BBB properties [9]. Those proteins were identified in at least two out of three nano-LC MALDI-TOF/TOF-MS analyses and at least one peptide sequenced. The rationale of the current study is based on two facts, first 1D-PAGE presented in the previous study showed changes in the protein expression. Second, nano-LC and 2DE methods are complementary [4]. The data obtained

by 2-DE from the different fractions of BCEC proteins, with either limited or re-induced BBB functions, are presented in **Figure 2**. The amount of proteins loaded into the gels varied from 80 µg for F0 to 10 µg for F75. There were no significant differences in protein content between homologous fractions (e.g. F25 from limited/F25 from re-induced BBB). The obtained profiles validated the ACN fractionation: when the 2DE-gel images obtained from homologous fractions were compared using the Progenesis Same Spots software, clear differences were observed in each case in the number of differentially expressed proteins (**Table 1**). In this comparison we first aligned the gel images and then counted the spots, comparing the number of spots between conditions.

The results were compared to those previously obtained by nano-LC of peptides (**Table 1**). In this case the results display the number of proteins identified with a minimum of two peptides and using the nwCompare software [10]. Interestingly the distribution of proteins over expressed in one condition varied from fraction to fraction confirming that the two approaches—nano-LC and 2-DE—are complementary.

Indeed most of the differences on the 2-DE gel images are shown between fraction F0 and the others, fewer differences are detected between F25, F50 and F75. We can notice that images of F25, F50 and F75 fractions, in both cell types, may almost be superimposed suggesting that an important part of the proteins are identical for the three fractions, which is confirmed by nano-LC—more than 90%—of the identified proteins were identical in those fractions. The increase in the complexity of the data analysis due to the spreading of proteins over the several fractions is counterbalanced by the use of dedicated software (nwCompare [10]). This redundancy could be

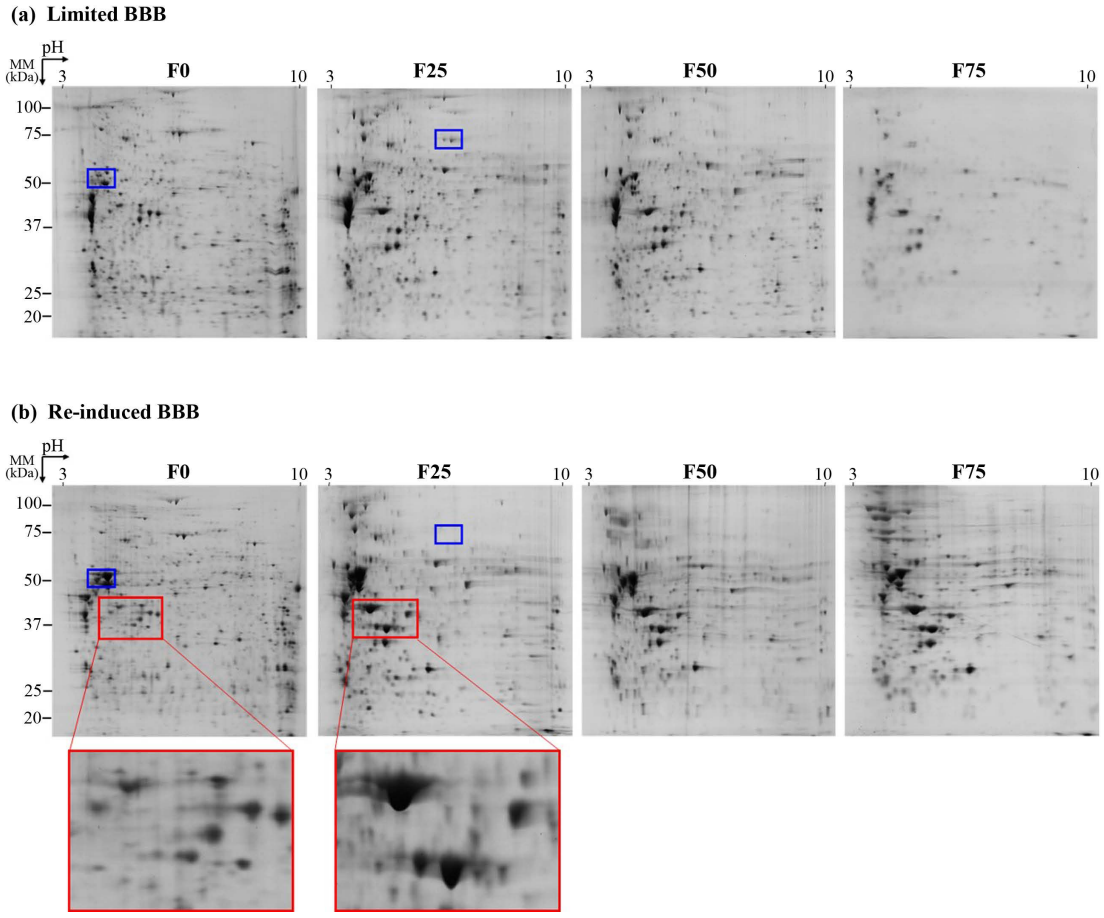

Figure 2. Qualitative analysis of the ACN fractionation by 2-DE. Gel images of one of the 2-DE (10%, pH 3 - 10) data-sets, obtained with the fractions issued from BCEC with either limited or re-induced BBB functions. Proteins were revealed by silver nitrate. Red boxes present the enlargement of one zone to compare between two fractions from one type of cells. Blue boxes show an example of proteins differentially expressed between the two cell types.

Table 1. Proteins differentially expressed in endothelial cells with Limited or Re-induced BBB properties.

Fraction	Obtained by 2-DE of proteins	Quantitative fold change	Overexpressed in limited BBB (%)	Overexpressed in re-induced BBB (%)	Obtained by 1D-LC of peptides	Overexpressed in limited BBB (%)	Overexpressed in re-induced BBB (%)
F0	130	5.5 to 1.7	46.90	53.10	192	53.10	46.90
F25	77	7.5 to 1.7	62.30	37.70	38	55.30	44.70
F50	49	5.3 to 1.7	79.60	20.40	73	79.60	20.40
F75	19	3.9 to 1.7	36.80	63.20	29	65.50	34.50

explained by the particular solvent properties of ACN when combined to water. Both liquids are miscible and colourless but, within an ACN mole fraction range between 0.15 - 0.3 and 0.7, are postulated to separate into discrete regions of liquid acetonitrile and liquid water and that the part of free ACN increases over a 75% ACN concentration [11]. The particular solvent properties of ACN were previously used for the design of a method for the depletion of high abundance serum proteins prior to nano-LC-MS/MS analysis [3]. The used conditions, 56% (v/v) ACN, permitted the complete removal of proteins of mass over 75 kDa and most of the albumin present in serum. In our hands, no significant difference occurs between the different fractions concerning high molecular weight proteins.

4. Conclusion

In most proteomics analyses and in particular for the "off-gel" approaches, the complexity of the proteome should be reduced; otherwise identifications can be hindered, especially if the mass spectrometry analysis is not conducted using up-to-date instrumentation. We report here the development and validation of an easy fractionation based on the differential solubilisation of proteins using increasing concentrations of acetonitrile (ACN). Each resulting fraction displays actually a reduced protein heterogeneity that allows improved nano-liquid chromatography (nano-LC) separations and ensures as many protein identifications as possible in each fraction. This "ACN fractionation" was successfully applied to the study of the Triton X-100 soluble sub-proteome of brain capillary endothelial cells (BCEC) with re-induced blood-brain barrier (BBB) functions. Such easy and low cost method of sample simplification can thus be employed in all sorts of research projects where the complexity of the sample is an issue.

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