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Modulating the protein content of complex proteomes using acetonitrile

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ABSTRACT

In this work we present acetonitrile as a tool to modulate the dynamic range of the proteome of complex samples. Different concentrations of acetonitrile ranging from 15% v/v to 65% v/v were used to modulate the protein content of serum samples from healthy people and patients with lymphoma and myeloma. We show that the proteome above 70 kDa is pelleted as a function of the concentration of acetonitrile and that profiling with PCA or Clustering is only possible using the supernatants obtained for concentrations of acetonitrile higher than 45% v/v or the pellets for concentrations of acetonitrile of 35% and 45%. The differentiation and classification of the three groups of sera samples (healthy, lymphoma and myeloma) were possible using acetonitrile at 55% v/v concentration. This work opens new avenues for the application of acetonitrile as a cost-effective tool in proteomics applications.

1. Introduction

Plasma and serum are complex proteomes interrogated by researchers and physicians for diagnostics and prognostic purposes because both are composed of thousands of proteins that contain vital information about the health status of individuals. Variations in proteins and their concentrations can be linked to diseases or to the organism's response to drugs [1–4]. One of the challenges of studying complex proteomes is the range in proteins concentration, which for serum and plasma is higher than ten orders of magnitude [1–4]. Some proteins, called high-abundance proteins, HAP, are present at levels of gram per litter and they constitute the vast majority of the total protein mass. In fact as little as 12 proteins constitute around 95% of the total mass of proteins in plasma and serum [5]. On the contrary, some proteins are present at very low levels when compared with the HAP, and they can be present temporarily as a result of a disease or a therapy. These low-abundance proteins, LAP, carrying great diagnostic potential, are often obscured by the presence of high-abundance serum proteins because the dynamic range of concentrations surpasses the capabilities of existing separation and analysis techniques. Therefore, a large number of standard approaches for reducing the complexity of plasma and serum proteomes have been proposed and they can be assigned into three approaches; immunodepletion, affinity enrichment, and fractionation [1]. There are different commercial approaches to compress the dynamic range of the proteins by depleting the high-abundance ones. Thus, The ProteoPrep 20 Plasma immunodepletion kit, from Sigma-Aldrich [6], has been developed for the removal of the 20 high-abundance proteins and the Proteominer, from Bio-Rad, is based on treating complex protein samples with a library of hexapeptides bound to chromatographic supports [7]. Protein equalization for biomarker discovery has been performed with success in sera samples and peritoneal liquid using dithiothreitol by our team [8,9]. Furthermore, protein depletion with acetonitrile was also proven to be very useful [10]. In the latter case, however, an exhaustive study on the effects of the ACN concentration in the process of depletion has not been done yet, to the best of our knowledge. In this work we demonstrated that acetonitrile works as a modulator of the protein content of sera samples and that this property can be used for reducing the complexity of the serum proteome. As proof of concept, matrix-assisted laser desorption ionization mass spectrometry-based profiling of ACN-depleted sera samples of healthy volunteers and myeloma and lymphoma patients is presented.

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2. Material and methods

2.1. Reagents

All reagents used were HPLC grade or electrophoresis grade. Albumin, from bovine serum (BSA), (N, N, N, N′-tetramethylethylenediamine (TMED), glycine, β-mercaptoethanol, glycerol 86–88%, Bradford reagent, coomassie blue G-250, tris–base, DL-dithiothreitol (DTT), iodoacetamide (IAA), acrylamide/bis- acrylamide 30% solution (37.5:1) were purchased from Sigma-Aldrich (Basel, Switzerland). Formic acid, ammonium bicarbonate (Ambic), ammonium persulfate and α-cyano-4-hydroxycinnamic acid were purchased from Fluka (Basel, Switzerland). Hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), methanol and acetonitrile were purchased from Panreac (Barcelona, Spain). 4× Laemmli SDS sample buffer from Alpha Aesar containing 250 mM Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 8% beta-mercaptoethanol, and 0.02% bromophenol blue (Karlsruhe, Germany) and 10× Tris/Tricine/SDS Running Buffer from Bio-Rad (CA, USA). Pierce™ Trypsin Protease, MS Grade and trifluoroacetic acid (TFA) were purchased from ThermoFisher Scientific. Peptide Calibration Standard II from Bruker (Bremen, Germany) was used as a mass calibration standard for MALDI-TOF-MS measurements.

2.2. Apparatus

Protein digestion was done in safe-lock tubes of 0.5 mL from Eppendorf (Hamburg, Germany). A vacuum concentrator centrifuge model UNIVAPO 150 ECH Speed Vac and a vacuum pump model UNIJET II (Munich, Germany) were used for sample drying and sample pre-concentration. A mini incubator from Labnet (New Jersey, USA) was used for protein reduction. Centrifuge MPW-350 from MPW Med. Instruments (Warsaw, Poland), vortex models ELMI CM70M-09 SkyLine (Southern California, USA), and Prism™ R Refrigerated Microcentrifuge, VX-200 Lab Vortexer Vortex Mixer, AccuBlock™ Digital Dry Baths from Labnet (New Jersey, USA), were used throughout the sample treatment. CLARIOstar® High Performance Monochromator Multimode from BMG LABTECH (Germany). was used for Bradford assays. PowerPac™ Basic from Bio-Rad (CA, USA) was used as a current supplier for SDS-PAGE protein separation. Gel images were obtained using a ProPic II (Digilab-Genomic Solutions, USA). Acquisition of mass spectrometry data was done in an Ultraflex II MALDI-TOF/TOF instrument from Bruker Daltonics.

2.3. Human serum samples

Human sera samples of lymphoma and multiple myeloma were purchased from Patricell Ltd (BioCity Nottingham, UK). Data of such samples is presented in Table 1. The healthy volunteers were informed about the project, and their consent was requested in written form. The local ethics committee approved the procedure.

Table 1
Data of sera samples.

Patient	Diagnosis	Age	Gender	Medication	Stage / other comments
HS1	Healthy control	58	Female	–	–
HS2	Healthy control	63	Male	–	–
HS3	Healthy control	60	Female	–	–
HS4	Healthy control	57	Male	–	–
LA	Lymphoma	90	Female	–	Pretreatment
LD	Lymphoma	53	Female	–	Pretreatment
LE	Lymphoma	83	Female	–	Pretreatment
MMA	M. Myeloma	57	Male	Revlimid, velcade	–
MMB	M. Myeloma	57	Male	Revlimid, velcade	–
MMC	M. Myeloma	57	Male	Revlimid, velcade	Stable
MMD	M. Myeloma	57	Male	Revlimid, velcade	Stable
MME	M. Myeloma / High Colesterol	57	Male	Aredia, revlimid	Stable

2.4. Sample treatment

2.4.1. Protein precipitation with ACN

Protein depletion with ACN was performed according to the protocol described by Kay et al. with minor modifications [9,11]. In brief, samples (20 µL) were mixed with ACN and water to a total volume of 149 µL, making the following concentrations of ACN (%, v/v): 15, 25, 35, 45, 55 and 65. Each sample was sonicated for 10 min in an ultrasonic bath (100% amplitude, 35 kHz, 50 W). Then, the samples were vortexed 30 s and then sonicated for 10 min again. Once the precipitate was formed, the samples were pelleted by centrifugation at 12,000 × *g* for 10 min at 18 °C. Then, the supernatants were collected to new tubes and then pellets and supernatants were evaporated to dryness in a vacuum centrifuge. After evaporation, samples were resuspended in 30 µL of urea 6 M prepared in ammonium bicarbonate 12.5 mM, vortexed and sonicated with an ultrasonic probe during 10 s (50% amplitude, 50 W). Then samples were quantified using the Bradford protein assay. Once all the supernatants and pellets were quantified, aliquots were taken from each sample, in order to obtain a final volume of 20 µL with a protein concentration of 1 µg/µL.

2.4.2. Protein reduction and alkylation

To 20 µL of the samples, 2 µL 110 mM DTT were added to reduce disulfide bonds. The samples were then vortexed and incubated (Labnet incubator) during 45 min at 37 °C. The resulting cysteines were then blocked with 2 µL IAA 400 mM, the samples were once again vortexed and incubated for 30 min at room temperature in the dark. The samples were diluted to a final volume of 100 µL with AmBic 12.5 mM with 2% (v/v) of ACN.

2.4.3. Protein digestion and clean-up

To the above solutions trypsin was added in a proportion 1:50. Protein digestion lasted for 12 h at 37 °C. Samples were then vacuum evaporated and then resuspended in 20 µL of TFA 0.1% (v/v), vortexed and sonicated for 10 min using an ultrasonic bath (100% amplitude, 35 kHz, 50 W). Before MS analysis, peptides were desalted using reverse phase C18 Zip-tips. The micro column was activated using first two up and down cycles with 20 µL of 100% (v/v) ACN and then two up and down cycles using 20 µL of 0.1% (v/v) TFA. Then peptides were retained by passing each sample three times through the micro-column. Then the micro-columns were further desalted using two up and down cycles with 20 µL of 0.1% (v/v) TFA. Then, peptides were extracted from the micro-column using a 90% (v/v) / 0.1% (v/v) ACN/TFA solution in two up and down cycles. The samples were evaporated to dryness using a vacuum centrifuge.

2.5. 1-D gel electrophoresis

The protein content of sample obtained in 2.4.1 were further analysed by 1D-SDS-PAGE using a 1 mm thickness separating gel containing 12% (w/v) acrylamide/bis-acrylamide (37.5:1) and a staking gel containing 4% acrylamide/bis-acrylamide (37.5:1). The pellets and supernatants were resuspended in 50 µL of 1× sample buffer, vortexed for 1 min and heated at 100 °C for 5 min, and finally samples were centrifuged at 7,000 × *g* for 30 s. Then 3 µL of the samples were loaded on the SDS-PAGE gel and proteins were separated at 200 V and 400 mA until the blue line of bromophenol blue was around 0.5 cm from the bottom of the gel.