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Technical Note

# Comparison of blood serum peptide enrichment methods by Tricine SDS-PAGE and mass spectrometry

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ABSTRACT

Characterisation of blood serum peptides can provide valuable information on physiological and pathological processes. However, the analysis of raw serum samples by MS results in the identification of a limited number of peptides. In order to improve sensitivity, many peptide enrichment methods have been proposed during the last ten years. Here, we present a comparison of fractionation methods aimed to simplify analysis of small proteins and peptides in blood serum, one of the most promising sources of putative biomarkers. Specifically, three methods based on ultrafiltration, differential precipitation, and peptide ligand libraries (ProteoMiner) were evaluated for the enrichment of peptides and low molecular weight proteins, as demonstrated by Tricine SDS-PAGE and subsequent LC-MS/MS (GeLC-MS/MS). As a result, differential solubilisation (DS) allowed the identification of the highest number of peptides. Moreover, the DS method enabled also the quantitative comparison of samples, producing fundamental information in biomarker discovery approaches.



For years, biofluids such as serum, plasma and urine have played a role of primary importance in the field of biomarker discovery. They are typically considered to be 'rivers' of proteins and peptides [1] which flow through cells or tissues of the organism and, as such, act as a mirror of physiological or pathological conditions. Indeed, the peptidome, which has been described as all the peptides that are expressed in any cell, tissue or fluid at any given time, usually contains hormones, growth factors, neuropeptides, and neurotransmitters that provide valuable information regarding the current state of health or disease and the numerous processes that take place within the body [2]. Each peptide results from the enzymatic processing of large intact protein precursors. Pathologies may involve aberrant processing of a precursor, and those incorrectly processed precursors and peptides produce the corresponding proteome and peptidome, which provide complementary data [3]. In fact, specific endoproteases and exoproteases may cause protein degradation in a disease-dependent process, generating a typical spectral pattern of fragments as demonstrated by Marshall and co-workers [4]. Though blood serum is one of the most informative bodily fluids, containing thousands of different types of proteins and peptides, it represents a challenge to proteomic characterisation. The main problem in the proteomic investigation of blood serum arises from the large dynamic range of its protein concentration (~$10^{10}$). While the overall protein concentration of serum is very high (generally 60–80 mg/ml), only 22 proteins make up 99% of the total protein amount [5], exceeding the dynamic range of current analytical approaches. Moreover, many potential biomarkers are likely present at

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lower protein abundances. Therefore, the remaining 1% of the total serum proteins is of great interest for proteomic studies [2]. At present, the most widely used approach for the study of low molecular weight proteins/peptides in the blood serum proteome is based on the use of mass spectrometry (MS) coupled with different fractionation strategies such as electrophoretic and chromatographic separation techniques. MS analyses provide a dynamic range which is typically lower (nearly 5 orders of magnitude) than the dynamic range of proteins in serum. With the aim of overcoming this limitation, many methods for low-abundance and/or low-molecular weight serum proteins enrichment have been described. Some of these exploited different extraction buffers ranging from aqueous solutions to organic mixtures [6,7], while others illustrated the advantages of ultrafiltration procedures [8], or of the peptide library beads [9]. Other publications compared multiple fractionation methods using heterogeneous systems for evaluations of results, such as SDS-PAGE, MALDI MS, SELDI MS, LC-MS/MS approaches, and so on [2,10–13].

Aim of this study was the comparison of three methods for the reduction of highly abundant proteins and the enrichment of peptides and low molecular weight proteins (below 25 kDa), by using a gel-based approach for evaluation of results. Among all published methods, the ones that appeared to provide the best performance in different applications were selected and evaluated using a univocal experimental pipeline, in order to eliminate biases due to the analytical method. The final comparison of the methods was essentially based on the number of protein and peptide identifications by MS. A gel-based approach for MS analysis was chosen; in fact, GeLC-MS/MS gives the following advantages: information on molecular weight is preserved; protein/peptide yield of the method can be clearly and rapidly assessed by visual examination. On the other hand, direct injection and LC-MS/MS of an undigested sample generally allows the MS fragmentation and consequent identification of the smallest species, while information about low MW proteins is usually lost.

Specifically, DS precipitation as proposed by Kawashima [2], ultrafiltration, and the peptide ligand library treatment with ProteoMiner Beads (Bio-Rad) [14], were evaluated in this work.

Sardinian healthy volunteer sera were collected, homogeneously pooled, and stored at −80 °C until use. The pool was allowed to thaw on ice and then centrifuged at 14,000 ×*g* for 10 min at 4 °C. Total protein concentration, evaluated by the BCA assay (Pierce®, Thermo Scientific), was quantified as 62 mg/mL. According to the Kawashima method [2] with minor modifications, 20 μL of serum samples were diluted in a denaturing and reducing solution (7 M urea, 2 M thiourea, and 20 mM dithiothreitol (DTT)), slowly dropped into ice-cold acetone, and immediately stirred at 4 °C for 1 h, followed by centrifugation at 14,000 ×*g* for 15 min at 4 °C. The precipitates were resuspended in 1% formic acid in 70% acetonitrile (ACN), pH 3.5, and mixed at 4 °C for 1 h, then centrifuged again at 14,000 ×*g* for 15 min at 4 °C. Supernatants, supposed to be enriched in small proteins and peptides, were then lyophilised. In the ultrafiltration method, 20 μL of serum samples were diluted in a denaturing and reducing solution (7 M urea, 2 M thiourea, and 20 mM DTT), transferred to centrifugal filter devices with a molecular mass cut-off of 50 kDa (Microcon YM-50, Millipore), and centrifuged at 8000 ×*g* at 4 °C. The filtrates were further transferred on 30 kDa cut-off membranes (Microcon YM-30, Millipore) and centrifuged again at 8000 ×*g* at 4 °C. The final filtrates were then desalted and concentrated by C18 solid phase extraction (SPE) (Strata C18-E, 55 μm, 760A, 100 mg/mL, Phenomenex), and finally lyophilised. For the ProteoMiner Beads approach, the Protein Enrichment kit was used, and the appropriate bead volume to be settled into columns was calculated according to the manufacturer's instructions. For each technical replicate, two types of elution were carried out, the former being constituted by sequential elutions, and the latter by a single step elution. In both cases, 50 μL of serum were loaded on columns (Compact Reaction Columns CRC, USB) packed with 5 μL of ProteoMiner Beads. After the appropriate binding and washing steps, one aliquot of beads was treated with three sequential steps using: TUC solution (2 M thiourea, 7 M urea, 2% CHAPS), UCA solution (9 M urea, citric acid up to pH 3.3) and HOS, hydro-organic solution (6% (v/v) acetonitrile, 12% (v/v) isopropanol, 10% (v/v) ammonia at 20%, and 72% (v/v) water) [14]; the three eluates were desalted by SPE C18 and then lyophilised. The second sample was eluted by a single step in Laemmli buffer [15]. Fractions arising from all of the methodologies and a small volume of pooled sera (as control) were resuspended in Laemmli buffer and analysed by 16% Tricine SDS-PAGE. In order to actually compare the peptide yield achieved with each tested procedure, sample loading amounts were calculated according to the starting serum volume. Specifically, for DS and ultrafiltration, the whole sample was loaded, while only two fifths of the sample was loaded for the bead elution procedures. Gels were stained with SimplyBlue SafeStain (Invitrogen), and digitised with an ImageScanner III (GE Healthcare). Three replicates were analysed for each sample.

Twelve slices ranging from 25 kDa to 1.7 kDa were cut from DS preparation, ultrafiltration, and Laemmli elution from hexapeptide ligand library beads, and then destained, reduced, carbamidomethylated, and trypsin digested as described previously [16]. Briefly, gel slices were reduced with 10 mM DTT in 50 mM ammonium bicarbonate, pH 8.0, at 56 °C, and then carbamidomethylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate, pH 8.0, at room temperature in the dark. Tryptic digestion was performed at 37 °C overnight using an amount of trypsin between 40 and 80 ng per gel slice, according to the colour intensity of the slice. Peptide mixtures were analysed by LC-MS/MS on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). After loading, peptides were concentrated and desalted at 4 μL/min on a 40 nL enrichment column, with 0.2% formic acid and then fractionated on a C18 reverse-phase (75 μm × 43 mm, Agilent Technologies Chip) at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 3% to 60% in 20 min. ESI parameters were as follows: Capillary voltage 1730 V; dry gas ($N_2$), 5.00 L/min; dry temperature, 325 °C; trap drive, 100; skimmer 30 V; lens 1, −5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion trap mass spectrometer was operated in positive ion mode. Trap ICC smart target was 300,000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation