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Short communication

Depletion of high-abundance proteins from serum by immunoaffinity chromatography: A MALDI-FT-MS study☆

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Abstract

Immunodepletion of high-abundance proteins from serum is a widely used initial step in biomarker discovery studies. In the present work we have investigated the reproducibility of the depletion step by comparing 250 serum samples from prostate cancer patients. All samples were depleted on a single immunoaffinity column over a time period of 6 weeks with automated peak detection and fraction collection. Reproducibility in terms of surface area of the depleted serum protein peak at 280 nm was below 7% relative standard deviation (R.S.D.) and the collected volume of the relevant fraction was 0.97 mL (4.5% R.S.D.). Proteins in the depleted serum fraction were subsequently digested with trypsin and analyzed by MALDI-FT-MS. The degree of the depletion of albumin, transferrin and alpha-1-antitrypsin was determined by comparing the intensity of peptide peaks before and after depletion of 11 samples taken at regular time intervals from amongst the 250 depleted, randomized samples. As a positive control we evaluated peaks of apolipoprotein A1 (the most abundant serum protein remaining after depleteion) showing a clear increase in intensity of these peaks in the depleted samples. From this study we conclude that the depletion of the 250 serum samples was complete and reproducible over a period of 6 weeks.

Keywords: Biomarker discovery; Fourier transform mass spectrometry; Immunoaffinity chromatography; Prostate cancer; Proteomics

1. Introduction

Human body fluids carry information about the status of an organism that may help in recognizing physiological misbalances. Analyzing proteins in body fluids faces a number of challenges, the most difficult being the enormous dynamic range of protein concentration and the presence of a few high-abundance proteins that overshadow those of lower abundance. A number of depletion strategies have therefore been developed that attempt to remove these high-abundance proteins in a specific manner allowing to gain one to two orders of magnitude in dynamic range [1–14]. These methods ought to be highly reproducible and most ideally complete. Depletion using immobilized antibody immunoaffinity columns are generally superior in efficacy and selectivity when compared to dye affinity ligands like Cibacron Blue. One concern is, however, that such columns are less robust and that some antibodies loose their depletion efficacy over time giving rise to reduced reproducibility [3].

In an ongoing effort to find novel biomarkers for prostate cancer that allow discrimination between aggressive, metastatic disease and more slowly developing tumors, we are analyzing a large serum sample set from multiple clinical centres across Europe (P-Mark consortium) by MALDI-FT-MS [15]. As a first step in this analysis the collected sera are depleted using a multiple affinity removal column containing six antibodies against albumin, IgG, IgA, transferrin, haptoglobin, and alpha-1-antitrypsin. In order to assess the reproducibility of the depletion procedure 11 samples were taken from the 250 depleted samples and compared to full serum samples. Depleting all samples took

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about 6 weeks and was performed on a single immunoaffinity depletion column.

2. Experimental

2.1. Serum collection

Serum samples were obtained via the European Study of Screening of Prostate Cancer (ERSPC) and the P-Mark consortium (www.p-mark.org/) and provided by the Erasmus Medical Centre Rotterdam and the University of Sheffield. A diagnostic set of 250 prostate carcinoma samples was defined and randomized. Nine aliquots of one control serum sample were randomized amongst the 250 other serum samples to assess the methodological variability.

2.2. Depletion

Depletion was performed on a Multiple Affinity Removal System immunoaffinity column (4.6 mm × 50 mm; Agilent article number: 5185–5984) according to the recommendations of the manufacturer. Twenty-five 25 μL serum were diluted to 125 μL with loading buffer and spin-filtered (0.22 μm) for 20 min at 13,000 rpm and 4 °C. Seventy-five microlitre of each sample, equivalent to 15 μL original serum, were injected from an autosampler cooled to 4 °C. Depletion was performed at room temperature on an AKTA FPLC system (GE-Healthcare) using the following program: 10 min at 100% eluent A at 0.25 mL/min; 3.5 min at 100% eluent B at 1 mL/min; 5.5 min at 100% eluent A at 1 mL/min. Fraction collection was started automatically when a threshold of 25 mAU at 280 nm was exceeded and the collected fractions (Fig. 1) were snap frozen in liquid nitrogen within 5 min after collection and stored at −80 °C.

2.3. Total protein determination

Total protein concentration in the depleted fractions was determined using the Micro BCA™ Protein Assay (Pierce Biotechnology). The assay was calibrated with Bovine Serum Albumin (BSA) and a blank was performed using water. Absorbance was measured on a Fluostar Optima plate reader (BMG Labtech).

2.4. Trypsin digestion

Two microlitre of a trypsin solution (0.1 μg/μL in 3 mM Tris/HCL, pH 8; gold grade trypsin (Promega)) were added to 20 μL of depleted serum. Samples were incubated overnight at 37 °C. For full serum 20 μL of 0.2% Rapigest (Waters) in 50 mM ammoniumbicarbonate were added to 20 μL of serum. The sample was incubated for 2 min at 37 °C. Two μL of 0.1 μg/μL trypsin were added to each well and the sample was incubated at 37 °C. After overnight incubation, 2 μL of 500 mM HCl was added in order to obtain a final concentration of 30–50 mM HCl (pH < 2). Subsequently, the sample was incubated again for 45 min. Subsequently, the samples were desalted using a reverse-phase ZipTip™ procedure (Millipore) according to the manufacturer's protocol and measured directly in the MALDI-FT-MS.

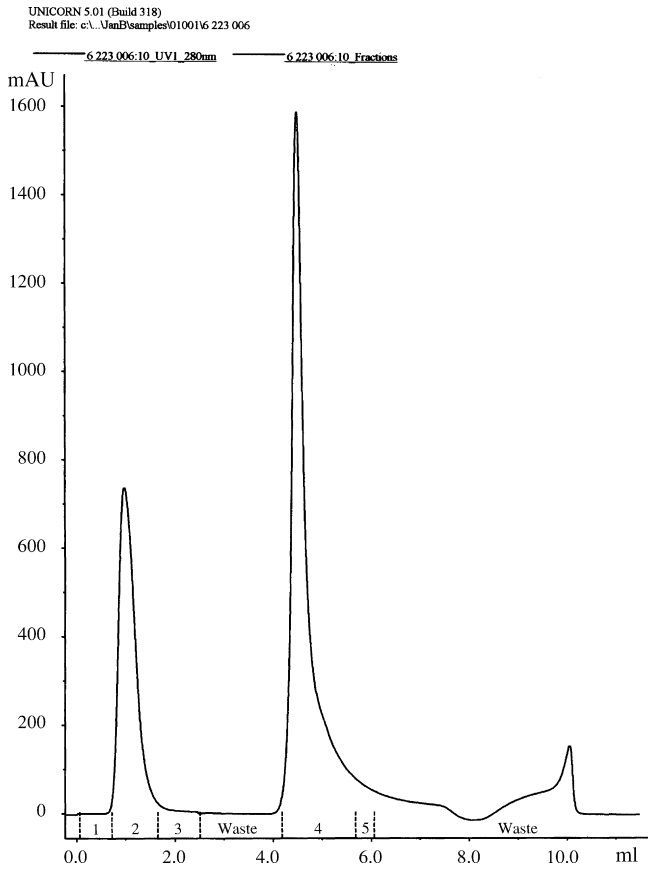

Fig. 1. Immunoaffinity depletion of the six most abundant proteins from serum of a prostate cancer patient. Fraction 2 contains the depleted serum while the bound, high-abundance protein fraction is collected in fraction 4.

2.5. MALDI-FT-MS

First 0.5 μL of 10 mg dihydroxy-benzoic acid (DHB) matrix (Bruker Daltonics) dissolved in 1 mL 0.1% TFA water were spotted onto an anchorchip target plate (600/384 anchorchip with transponder plate; Bruker Daltonis), followed by the direct mixing with 0.5 μL desalted peptide solution. The mixture was allowed to dry at ambient temperature. The measurements were performed by MALDI-FT-MS (Apex Q 9.4 Tesla equipped with a combi-source, Bruker Daltonics). For each measurement 100 scans were summed up and for each scan ions generated by 10 laser shots were accumulated in a mass range of 800–4000 *m/z*. Spectra were manually examined and included in our analysis when no salt adducts were observed for the most intense signals.

2.6. Data processing and analysis

FT-MS spectra were processed with a Gaussian filter and two zero fillings. A standard peptide calibration mix (Bruker Daltonics, Leipzig, Germany) that contained angiotensin I and II, substance P, bombesin, renin substrate, ACTH clip 1–17, ACTH clip 18–39 and somatostatin 28 was used for exter-

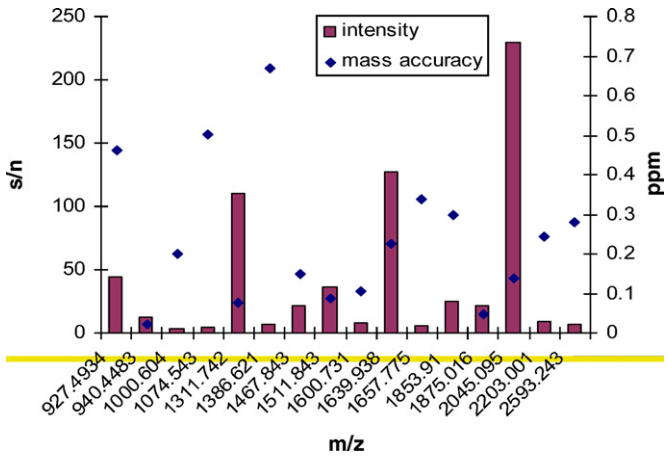

Fig. 2. Mass accuracies of the albumin-derived peaks in full serum calibrated against the five apolipoprotein A1 peaks are shown. On the *x*-axis the masses of the peaks are displayed, on the left *y*-axis the signal-to-noise ratio (bars) and on the right *y*-axis the mass accuracies (squares).

nal calibration. To obtain better mass accuracies, an additional post-acquisition internal calibration step in DataAnalysis v3.4, built 169 software (Bruker Daltonics, USA) was performed. The most abundant signals were sequenced by MALDI-TOF/TOF (Ultraflex, Bruker Daltonics, Germany) and the calculated masses derived therefrom were used for the internal calibration, i.e. 1031.51897, 1226.54364, 1612.78531, 1932.93373, 2202.11890. These five peptides correspond to apolipoprotein A1. Next, other apolipoprotein A1 derived peaks (e.g. 1235.68814) were used to assess the accuracy of the measurements. Peak picking was performed with the data analysis (version 3.4 build 169) software package using the Snap 2 algorithm (Bruker Daltonics). A database search was performed on the resulting peak list of full serum digests with the Mascot search engine (Matrix Science) against the MSDB human database using a 5 ppm mass tolerance. The list of resulting proteins was compared with the six depleted proteins. The peptides of the six depleted proteins were used in a manual comparison with a set of 11 depleted samples randomly selected from the 250 samples. The 11 samples were chosen over the entire time range of 6 weeks necessary to deplete the 250 serum samples. The spectrum of a full serum sample was compared with the mass spectra of the 11 depleted samples. As a reference control we performed the same comparison for five tryptic peptides of apolipoprotein A1, a protein that is not depleted.

3. Results

3.1. Reproducibility of depletion: analytical versus biological variation

The analytical variation of the depletion step was assessed by randomizing 9 aliquots of the same control human serum among

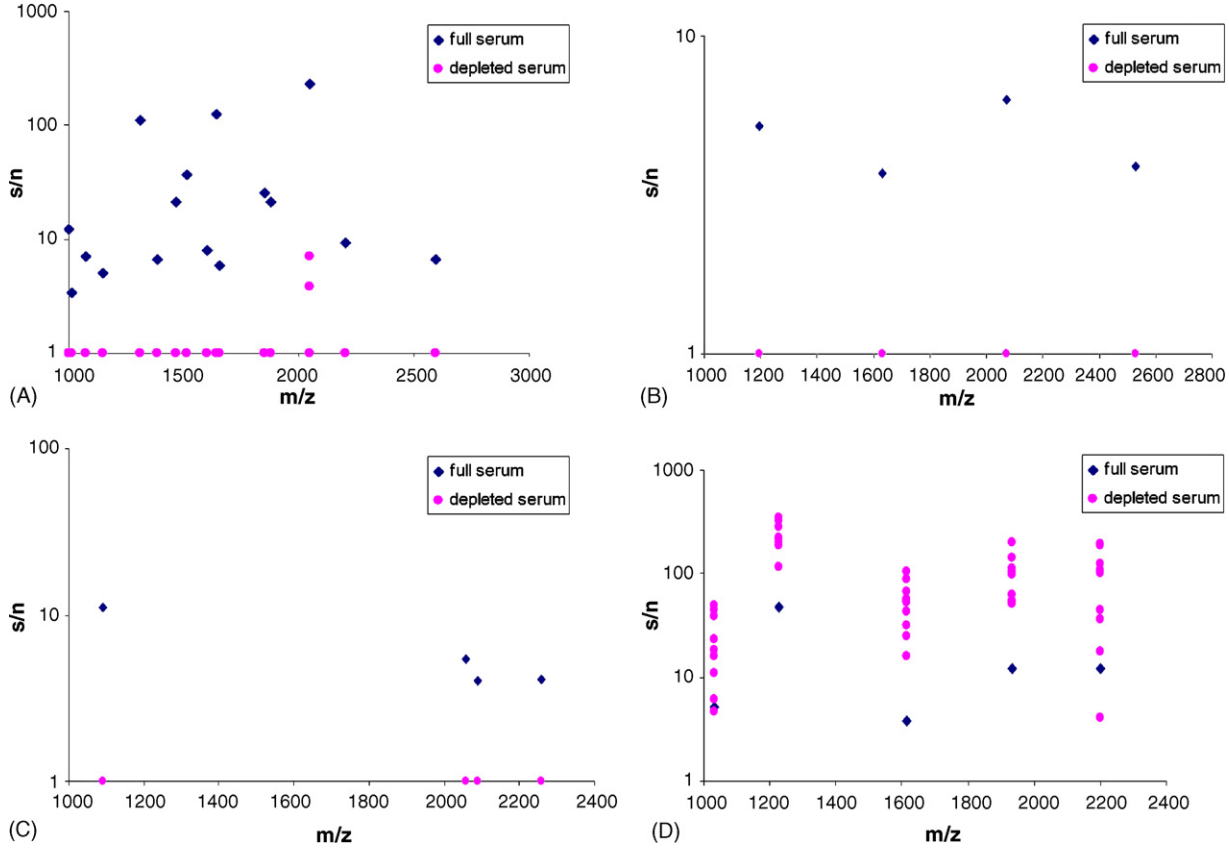

Fig. 3. The depletion efficacy for 11 serum samples taken at different time from a set of 250 serum samples. Albumin (panel A), transferrin (panel B), and alpha-1-antitrypsin (panel C). As a reference control apolipoprotein A1 was included (panel D). The signal-to-noise for the full serum sample is represented by circles and for the depleted samples by squares. The squares at signal-to-noise 1 can overlap and represent more samples.

the 250 prostate cancer serum samples. This resulted in an R.S.D. of 6.7% (average median = 272.0 ± 18.2 absorbance units) with respect to the surface area of the peak of depleted serum and an R.S.D. of 4.4% with respect to the collected fraction volume based on automatic peak detection. Biological variation was, as expected, significantly larger, since it reflects also the different serum protein concentrations in the patient/control samples. The R.S.D. for the surface area of the depleted serum was 17.8% (329.2 ± 58.5 absorbance units) and 10.0% for the volume of the collected fraction based on automatic peak detection. The reason for the larger variation in collected fraction volume is likely due to the variable peak area and the fixed peak detection threshold of 25 mAU at 280 nm. However, an R.S.D. of ±10.0% is well within the range for proteomics studies [2]. Total protein concentration measured with the BCA assay and BSA as calibration standard showed that the depleted serum fraction contained 12.5 mg/mL ± 8.4% (R.S.D.) protein. These results show that reproducible depletion results have been obtained over a time period of approximately 6 weeks using a single immunoaffinity depletion column treating more than 250 samples.

3.2. Percentage of remaining high-abundance proteins assessed by MALDI-FT-MS

In order to assess the efficacy of the immunoaffinity depletion step, the depleted protein fraction was digested with trypsin and analyzed by MALDI-FT-MS. The mass accuracy of the albumin derived peaks in full serum calibrated against five apolipoprotein A1 peaks was ±0.25 ppm and proved independent of peak intensity and mass over the range 800–4000 *m/z* (Fig. 2), which is crucial when comparing signals of widely varying intensities.

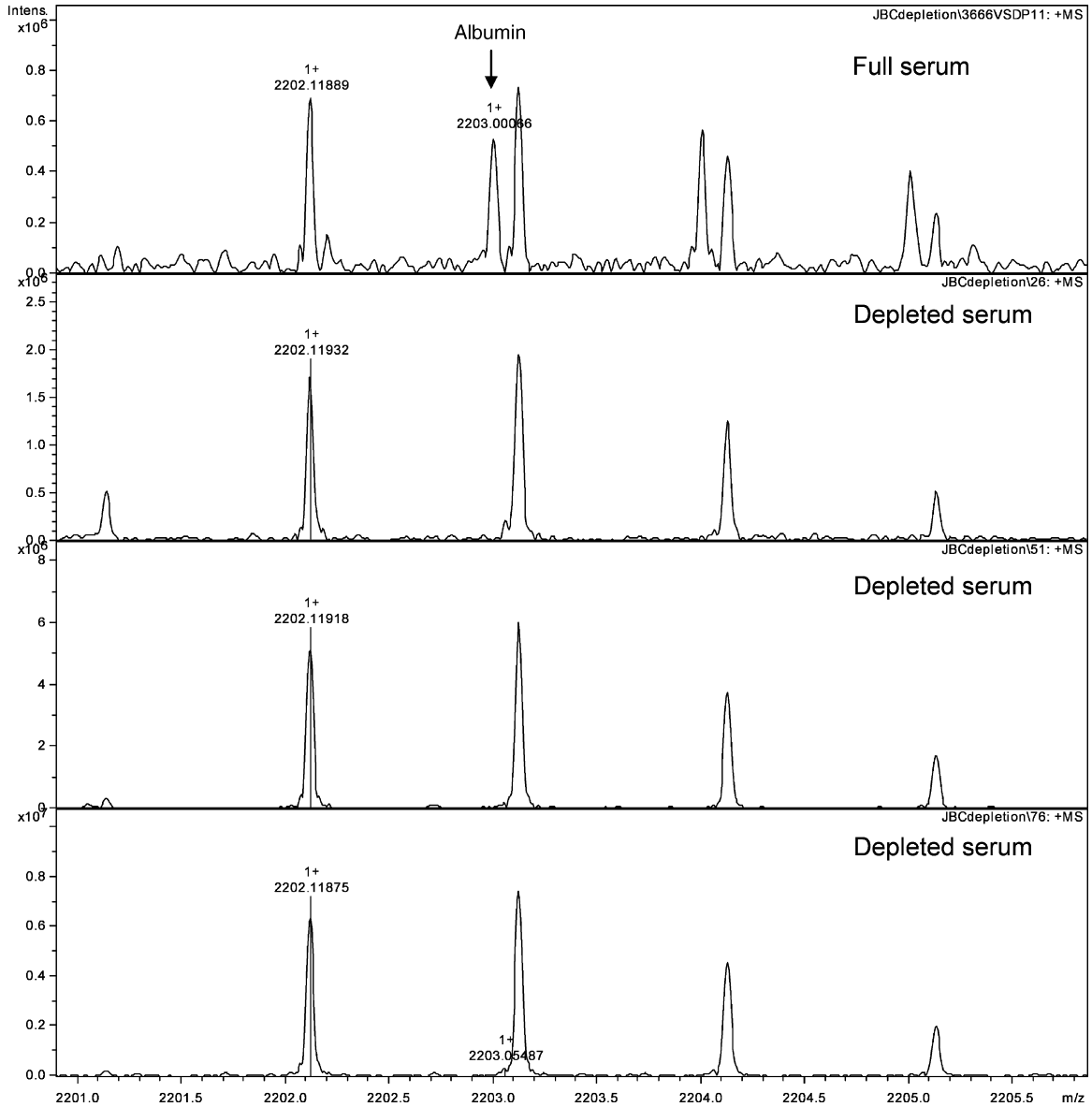

Fig. 4. Mass spectra of a full serum sample and three representative depleted serum samples. It is clear that the indicated albumin peptide is completely absent in the depleted samples, in contrast to a peptide of apolipoprotein A1 (2202.11890 Da) that is still present in the depleted samples and is significantly enriched. In time the depletion efficacy remained constant for 250 samples.

In non-depleted serum we detected 26 peptide masses at a signal-to-noise ratio higher than 4 that corresponded to peptides derived from three of the six most abundant proteins (18 from albumin, 4 from alpha-1-antitrypsin and 4 from transferrin). These 26 peptides were used as indicators of the presence of remaining high-abundance proteins after depletion and checked manually in 11 randomly selected depleted serum samples. Only 1 albumin-derived peptide was detectable in 2 out of the 11 samples indicating efficient depletion. The signal-to-noise ratio of this peptide peak was $\leq$4 in depleted serum as compared to over 100 in non-depleted serum (Fig. 3). The five tryptic peptides of apolipoprotein A1, a non-depleted protein, showed on average a seven-fold increase in signal-to-noise ratio (Fig. 3D) as a result of depletion. Fig. 4 shows the example of one albumin-derived peptide (2203.00066 Da) that was completely absent in the spectra of depleted samples in contrast to the apolipoprotein A1-derived peptide (2202.11890 Da).

4. Conclusions

The present work shows that depletion of the six most abundant proteins from serum by immunoaffinity chromatography is reproducible and efficient. Two hundred-fifty serum samples from prostate cancer patients and healthy controls were depleted over a timeframe of about 6 weeks using a single immunoaffinity column. Analytical variability was assessed by repetitive depletion of 9 identical control serum samples that were randomized in the set of 250 serum samples. Analytical variability in terms of protein concentration in the depleted fraction was 8.4% R.S.D. and variability of the volume of the automatically collected fraction containing depleted serum was below 4.5%. Not surprisingly, biological variation between the 250 different serum samples from prostate cancer patients and controls was significantly higher resulting in 17.8% R.S.D. for the peak area and 10.0% for the fraction volume.

In order to assess the efficacy of depleting the six most abundant serum proteins over time, depleted serum was trypsin-digested and analyzed by MALDI-FT-MS. Based on the high mass accuracy of this instrument, it was possible to assign individual tryptic peptide fragments to the six most abundant proteins (Fig. 2). It is concluded that complete removal of these abundant proteins depleted by the immunoaffinity column is achieved with the exception of one of the most intensive albumin peaks, which were detectable in 2 of the 11 randomly picked samples. The limited number of detectable peptides of high abundant proteins indicates that they are removed to a level that is very close to the detection limit of the MALDI-FT-MS instrument (sub-femtomol sensitivity). For apolipoprotein A1-derived peptides an increased intensity in MS spectra was obtained in the depleted samples. In addition significantly more peaks were observed in the mass spectra of the depleted serum samples when compared with full serum. There was no indication of a decreased depletion efficiency over this 6 week time period, since the observed albumin-derived peptide was detected in two different samples that were not at the end of the sample series.

We are presently analyzing all of the 250 serum samples from prostate cancer patients in search for biomarkers that can discriminate aggressive metastatic from more benign forms of this disease.

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