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Journal of Chromatography B, 849 (2007) 273–281
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Rat plasma proteomics: Effects of abundant protein depletion on proteomic analysis☆

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Received 11 July 2006; accepted 20 November 2006
Available online 22 December 2006

Abstract

The proteomic analysis of plasma and serum samples represents a formidable challenge due to the presence of a few highly abundant proteins such as albumin and immunoglobulins. Detection of low abundance protein biomarkers requires therefore either the specific depletion of high abundance proteins with immunoaffinity columns and/or optimized protein fractionation methods based on charge, size or hydrophobicity. Here we describe the depletion of seven abundant rat plasma proteins with an immunoaffinity column with coupled antibodies directed against albumin, IgG, transferrin, IgM, haptoglobin, fibrinogen and α1-anti-trypsin. The IgY-R7-LC2 (Beckman Coulter) column showed high specificity for the targeted proteins and was able to efficiently remove most of the albumin, IgG and transferrin from rat plasma samples as judged by Western blot analysis. Depleted rat plasma protein samples were analyzed by SELDI-TOF MS, 2 D SDS-PAGE and 2D-LC and compared to non-depleted plasma samples as well as to the abundant protein fraction that was eluted from the immunoaffinity column. Analysis of the depleted plasma protein fraction revealed improved signal to noise ratios, regardless of which proteomic method was applied. However, only a small number of new proteins were observed in the depleted protein fraction. Immunoaffinity depletion of abundant plasma proteins results in the significant dilution of the original sample which complicates subsequent analysis. Most proteomic approaches require specialized sample preparation procedures during which significant losses of less abundant proteins and potential biomarkers can occur. Even though abundant protein depletion reduces the dynamic range of the plasma proteome by about 2–3 orders of magnitude, the difference between medium-abundant and low abundant plasma proteins is still in the range of 7–8 orders of magnitude and beyond the dynamic range of current proteomic technologies. Thus, exploring the plasma proteome in greater detail remains a daunting task.

Keywords: Plasma; Depletion; Immunoaffinity; SELDI-TOF MS; 2D SDS-PAGE; 2-Dimensional chromatography; Chromatofocusing; Reversed phase; Proteomics

1. Introduction

Rapidly advancing technologies in the areas of mass spectrometry and liquid chromatography have made it possible in recent years to investigate ever larger portions of the proteome that is expressed in a particular cell or organ. In contrast to transcriptomics, proteomic profiling directly analyzes the molecules that carry out biological functions. The new field of nutritional proteomics tries to identify peptides and proteins whose expression responds to changes in diet or nutritional status [1]. Such peptides and proteins could serve as potential biomarkers that may enable nutrition scientists to study the complex interactions between physiology and diet at multiple levels of regulation [2]. Of special interest to the nutrition research community are accurate biomarkers that can support diet-based interventions that focus on bioactive food components as potential chemopreventive agents in diseases such as cancer, atherosclerosis, asthma or diabetes [3,4].

Plasma, serum and urine are routinely used for the detection and quantitation of proteins associated with both health and disease because they are easily obtained and can be repetitively sampled. There is great interest in the plasma proteome as a source for the discovery of new biomarkers because it is thought that it contains subsets of other tissue proteomes as well as hepatically secreted plasma proteins. However, plasma and serum represent a considerable challenge because several high-abundance proteins such as albumin, immunoglobulins, haptoglobin, antitrypsin and transferrin typically constitute greater than 90% of

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

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doi:10.1016/j.jchromb.2006.11.051

total protein mass [5]. These dominant proteins can prevent the detection of lower-abundance proteins that are of special interest for biomarker discovery. Limitations in sample capacity of current methods such as 2D gel electrophoresis and liquid chromatography make it necessary to reduce sample complexity prior to proteomic analysis. Different approaches for the depletion of highly abundant proteins such as albumin and IgGs from plasma samples have been described. Among these are chemical-based extraction methods [6], electrophoretically-driven fractionation [7,8] and various chromatography methods [9,10]. In recent years, liquid chromatography has become the preferred method for the depletion of abundant proteins from plasma and serum samples. A variety of stationary phases have been tested for this purpose, including Cibacron blue F3GA [11], a chlorotriazine dye which has a high affinity for albumin, Proteins A or G for depletion of immunoglobulins [12,13] and specific antibody-based affinity columns [14]. Dye-based kits have the advantage that they are relatively inexpensive and that they can be scaled up easily to bind significant amounts of albumin. However, non-specific binding, especially of those proteins that contain dinucleotide binding domains, to the Cibacron blue stationary phase or to albumin itself has been observed and can result in the loss of potential biomarkers [15–18]. Protein A and G stationary phases have long been used in immunoprecipitation studies and can be used for the removal of immunoglobulins from a variety of species but suffer from the drawback that they do not bind all immunoglobulin subgroups [19]. Recent efforts have focused on individual, immobilized monoclonal and polyclonal antibodies [11,20,21]. Monoclonal antibodies offer high specificity, but may not recognize and bind to all forms of the targeted protein, including proteolytic fragments and post-translationally modified antigens. In contrast, polyclonal antibodies are generally able to recognize and bind multiple epitopes of a protein and, as a result, give a more complete removal of abundant proteins. Several different immunoaffinity depletion columns are now commercially available that contain multiple, high-specificity, immobilized polyclonal antibodies to deplete up to 20 proteins from human plasma or serum samples in a single purification step. A recent development has been the application of immunoglobulin yolk (IgY) antibodies in the construction of immunoaffinity columns for the depletion of abundant plasma proteins [20]. IgYs have unique molecular structures and biochemical features and offer the advantage of high avidity and less cross-reactivity with heterologous human proteins. In contrast to IgGs, the Fc region of polyclonal, avian IgYs does not bind to bacterial or mammalian Fc receptors, mammalian IgG and IgM, or human proteins such as rheumatoid factor and complement, thereby significantly reducing non-specific interactions to IgY [22].

The majority of current biomarker discovery efforts utilize human plasma and serum as sample source. A major challenge is the intrinsic person-to-person variability in these sample sets which complicates both discovery and subsequent validation of new biomarkers. Rodents including mice and rats have long been used in research areas such as cancer, nutrition and toxicology and, unlike humans, represent highly controllable experimental model systems. This has led to a renewed interest in characterizing the plasma and serum proteomes of mice and rats in greater detail [23,24]. However, these samples have a similar wide dynamic range in protein concentrations as seen in human samples and therefore face some of the same technological challenges. A possible solution to this problem are two different immunoaffinity columns that have recently become commercially available. The multiple affinity removal system (MARS) from Agilent can remove albumin, IgG and transferrin from mouse plasma whereas the IgY-R7-LC2 column from Beckman depletes albumin, IgG, IgM, transferrin, haptoglobin, fibrinogen and α1-anti-trypsin from rat plasma samples. Using the latter column, we tested the hypothesis that depletion of these seven high abundance proteins from rat plasma will improve the detection low abundance proteins. Here we report the first results of our study in which we tested the specificity and efficiency of the microbead, IgY-based anti-rat immunoaffinity LC column. The chromatographic depletion step was monitored by 1D SDS-PAGE. The capacity of the immunoaffinity column for abundant protein removal was assessed by Western blots against rat albumin, IgG and transferrin. We then analyzed neat, depleted and eluted plasma samples by SELDI-TOF MS, 2-dimensional SDS-PAGE, and 2-dimensional liquid chromatography to detect peptides and proteins that were previously masked by high abundance proteins.

2. Materials and methods

2.1. Reagents

The prepacked IgY-R7-LC2 affinity column was obtained from GenWay Biotech (San Diego, CA). However, the column is now commercially available from Beckmal Coluter. All chemicals, sample preparation kits, isoelectric focusing strips, second-dimension SDS-PAGE gels and stains used in 2-DE were from Bio-Rad (Hercules, CA). The PF 2D chemistry kit with the chromatofocusing column, the reversed phase column, start and elution buffers was purchased from Beckman/Coulter (Fullerton, CA). HPLC-grade water was purchased from J.T. Baker (Phillipsburg, NJ). HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ). Trifluoroacetic acid, the ProteoMass Peptide and Protein MALDI MS Calibration kit, sinapinnic acid (SA) and α-cyano-4-hydroxycinnamic acid (CHCA) and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Hydrophobic H50 SELDI-TOF MS protein arrays and gold-coated MALDI-TOF MS sample plates were from Ciphergen (Freemont, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL). Fasted Sprague–Dawley rat plasma with EDTA as anticoagulant was from Innovative Research, Inc. (Southfield, MI). Polyclonal antibodies against rat albumin, rat transferrin and rat IgG were from Immunology Consultants Laboratory (Newberg, OR).

2.2. Partitioning of rat plasma with the IgY-R7-LC2 affinity column

Rat plasma partitioning with the IgY-R7-LC2 affinity column (6.4 mm × 63 mm) was carried out according to the manufac-

turer's protocol on an AKTA FPLC chromatography system (GE Healthcare, Piscataway, NJ). Briefly, 50 μl rat plasma were diluted with 200 μl Tris-buffered saline (TBS, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl) and filtered through a 0.45 μm spin filter in a table-top centrifuge (60 s at 9000 × *g* at RT). Two hundred μl of diluted plasma sample were loaded onto the affinity column at 0.1 ml/min. Depleted plasma samples were initially collected at 0.1 ml/min for 10 min and then for an additional 7 min at 0.2 ml/min. The column was washed for 5 min at 1 ml/min. Bound plasma proteins were eluted with 14 ml of a 0.1 M glycine solution (pH 2.5) before the column was neutralized with 6 ml of 0.1 M Tris/HCl (pH 8.0) and reequilibrated with TBS. Elution was monitored at 280 nm. Plasma fractions were pooled and concentrated with the ReadyPrep™ 2-D Cleanup Kit from Biorad according to the included protocol.

2.3. SDS-PAGE and Western blot analysis

SDS-PAGE analysis was carried out with the Tris/Tricine buffer system according to Schagger and Von Jagow [25]. Proteins were separated under reducing conditions on 10% SDS-PAGE mini gels (10 cm × 7 cm) and visualized by colloidal Coomassie Blue G-250 or silver staining according to standard protocols. For Western blot analysis, SDS-PAGE separated proteins were transferred electrophoretically onto a PVDF membrane using the Towbin transfer buffer system (25 mM Tris, 192 mM Glycine, pH 8.3, 20% methanol). Membranes were blocked with 5% non-fat dry milk in TTBS (0.05% Tween-20, 50 mM Tris/HCl, pH 7.6, 150 mM NaCl). Primary rabbit anti-rat antibodies were diluted 1:1000 in TTBS. Secondary anti-rabbit HRP conjugates were diluted 1:10,000 in TTBS. Chemiluminescent detection was carried out with the SuperSignal West Dura Extended Duration Substrate kit (Pierce) according to the manufacturer's protocol.

2.4. Two-dimensional liquid chromatography of partitioned plasma samples

Two-dimensional liquid chromatography separations of neat, depleted and eluted rat plasma samples were carried out on the ProteomeLab™ PF 2D system from Beckman/Coulter (Fullerton, CA). The system was modified with a 5 ml stainless steel sample loop. Precipitated samples were first disolved in membrane lysis buffer (5 M urea, 2 M thiourea, 50 mM Tris/HCl, pH 7.8, 2% (w/v) octylglucoside, 2.5% SB 3–10, 5 mM TCEP, 1 mM protease inhibitor cocktail) and then buffer exchanged against PF-2D start buffer on a PD-10 desalting column (GE Healthcare). A total of 3.5 mg protein in start buffer were injected into the first-dimension. First-dimension chromatofocusing was carried out at ambient temperature at a flow rate of 0.2 ml/min. Before each run, the column was equilibrated with start buffer (pH 8.5) for about 30 column volumes until a stable baseline value of pH 8.3–8.5 was achieved. Forty-five minutes after sample injection the elution was started by switching to the elution buffer (pH 4.0). Proteins were eluted in order of decreasing isoelectric point. At the end of the elution, the column was washed with 10 column volumes of 1 M NaCl, followed by 10 column volumes of water. Fractions were collected into deepwell plates at 0.2 pH intervals and stored at 10 °C in the autosampler prior to injection into the 2nd-dimension, reversed phase column.

Second-dimension separations were performed by reversed phase chromatography at 50 °C and a flow rate of 0.75 ml/min. Solvent A was 0.1% (v/v) TFA in water. Solvent B was 0.08% (v/v) TFA in acetonitrile. Two hundred μl from each 1st-dimension pH fraction in the range pH 8.0 to 4.0 were injected. Peptides and proteins were eluted with a gradient of 0–100% B in 30 min, followed by a 5 min wash at 100% solvent B. Fraction were collected every 30 s starting at 6.3 min and ending at 24.3 min. Before each run, the column was reequilibrated with 10 column volumes of solvent A.

2.5. Two-dimensional gel electrophoresis

Two hundred μg of precipitated samples were disolved in 185 μl sample rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% (w/v) 3/10 ampholytes, 0.002% Bromophenol Blue) and incubated for 16 h with a ReadyStrip™ IPG strip (pH 3–10, 11 cm). Following rehydration, IPG strips were focused for a total of 35,000 V-hr at 20 °C in a PROTEAN IEF cell (Biorad, Hercules, CA). Prior to 2nd-dimension SDS-PAGE, focused IPG strips were first reduced with DTT in 2nd-dimension equilibration buffer (6 M urea, 0.375 M Tris/HCl, pH 8.8, 2% SDS, 20% glycerol and 2% (w/v) DTT) and then alkylated with iodoacetamide (6 M urea, 0.375 M Tris/HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide) for 10 min each at room temperature. Second-dimension separations were carried out on 4–12% Bis-Tris gradient gels in a MES buffer system. Two-dimensional gels were stained with colloidall Coomassie Blue, scaned and analyzed with the Imagemaster 2D Platinum 6.0 software package.

2.6. SELDI-TOF and MALDI-TOF mass spectrometric analysis

Twenty μg of neat and partitioned rat plasma samples per spot were incubated on activated hydrophobic H50 protein microarrays for 1 h with shaking at room temperature. Following binding, spots were washed three times with binding buffer (10% ACN, 0.1% TFA) and two times with water. Arrays were allowed to air dry for 10 min before the additon of MALDI matrix. Second-dimension PF 2D fractions were evaporated to dryness in a SpeeedVac and reconstituted with 20 μl of 50% (v/v) ACN and 0.1% (v/v) TFA in water. One μl of sample was spotted on a gold chip, mixed with MALDI matrix and allowed to air-dry at room temperature. Low molecular mass spectra (*m/z*, 1000–25,000) of peptides and proteins were aquired with a saturated solution of CHCA in 50% (v/v) ACN and 0.1% (v/v) TFA in water (MALDI matrix I). Mass spectra of proteins larger than *m/z* 25,000 were aquired with a saturated solution of SA in 50% (v/v) ACN and 0.1% (v/v) TFA in water (MALDI matrix II). Each mass spectrum shown represents the sum of a total of 150 laser shots collected at 30 different position within each spot. MALDI-TOF and SELDI-TOF mass spectra were aquired on a ProteinChip Biomarker System (PBS II) mass spec-

trometry instrument (Ciphergen, Freemnot, CA) in the linear ion mode using a nitrogen laser. Mass spectra were collected in the positive ion mode at an accelerating voltage of 20 kV and a delay time of 600 ns. Before each analysis, the PBS II instrument was calibrated with a mixture of proteins of known molecular weights. For the range of *m/z* 1000–25,000 the oxidized beta-chain of insulin (av. *m/z*: 3495.89), insulin (av. *m/z*: 5734.51 Da), equine cytochrome c (av. *m/z*: 12,361.96 Da) and apomyglobin (av. *m/z*: 16,952.27 Da) were used as mass standards. For proteins larger than *m/z* 25,000 apomyoglobin (av. *m/z*: 16,952.27 Da), aldolase (av. *m/z*: 39,212.28 Da) and bovine serum albumin (av. *m/z*: 66,430.09 Da) were used as mass standards. Protein arrays were read both at low and high intensity laser energies, depending on the molecular weight range under investigation. Instrument control and initial data analysis was carried out with the Biomarker 3.1 software. The Biomarker Wizard application was used for SELDI-TOF MS-based protein expression profiling of isolated second-dimension fractions. Only peaks with an S/N ratio greater than 5 were reported.

3. Results and discussion

The IGY-R7-LC2 depletion column from Beckman Coulter has been designed to remove seven abundant proteins from rat plasma including albumin, IgG, transferrin, fibrinogen, haptoglobin, IgM and α1-antitrypsin. In humans, these seven proteins contribute approximately 80% to the total protein mass found in plasma [26]. While the composition of the rat plasma proteome has not been reported in great detail yet, blood chemistry profiles have consistently shown that two proteins alone, namely albumin and IgG, make up about 80–85% of the total protein in plasma of various strains of rats [27]. Depletion of just these two proteins from rat plasma should result in a 4- to 6-fold increase in relative protein concentration of medium- and low-abundance proteins. As a result, the detection, identification and quantification of medium- and low-abundance rat plasma proteins by proteomic methods should be more easily achieved and aid in the characterization of the important rat plasma proteome.

The IgY-R7-LC2 column has a capacity of 40 μl rat plasma per injection, which corresponds to about 2.0–2.4 mg total plasma protein in male Sprague–Dawley rats. Fig. 1A shows the typical chromatogram of 40 μl of rat plasma, diluted 5-fold in TBS, injected into the LC2 immunoaffinity depletion column. Medium- and low abundant proteins are recovered in the column flow-through fractions whereas high abundant, bound plasma proteins are stripped and eluted from the column with an acidic glycine buffer. No loss in protein binding capacity has been observed during 40 injections performed so far in this laboratory. Integration of the two peak areas in the chromatogram showed that about 55–65% of the injected protein were recovered in the depleted protein fraction, with the remainder being found in the eluted fraction. In the context of what is known about the composition of rat plasma and the amount of albumin and IgG typically present, this was an unexpected result. A possible explanation for this observation is the fact that proteins differ in their content of aromatic side chains and that detection at a wavelength of 280 nm is therefore not truly quantitative. However, the peak integration results were confirmed by BCA protein assay that also found more than half of the injected protein in the depleted, flow through fraction of the immunoaffinity column. Immunoaffinity depletion of rat plasma was monitored by SDS-PAGE (Fig. 1B) and Western blot analysis (Fig. 1C). Fig. 1B clearly shows that removal of high abundance proteins lead to the appearance of several proteins that were not detectable in

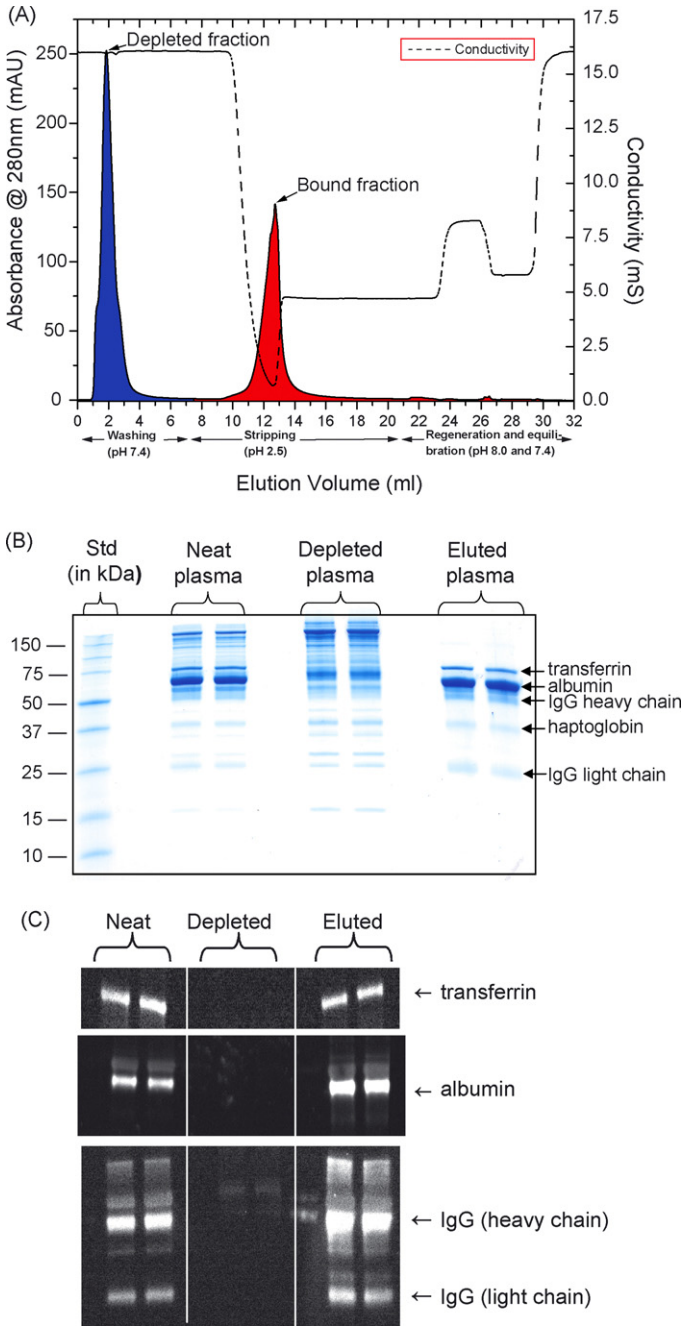

Fig. 1. Rat plasma partitioning with the IgY-R7-LC2 column. (A) UV elution profile of rat plasma partitioning monitored at 280 nm on an AKTA FLPC unit. Depleted fraction is marked by blue area; eluted (high abundance protein) fraction is marked by red area. (B) One-dimensional SDS-PAGE analysis of neat (non-depleted), depleted and eluted rat plasma protein fractions. Ten μg protein per lane were separated under reducing conditions on a 10% Tris–Tricine gel and stained by colloidal Coomassie Blue. (C) Western blot analysis of 10 μg protein of neat (non-depleted), depleted and eluted rat plasma protein.

the non-depleted plasma samples. Fig. 1B also gives a measure of the specificity of the rat protein depletion column because unspecifically bound proteins were not detetected in the eluted protein fraction by 1D SDS-PAGE analysis. The performance of the immunoaffinity column and the depletion protocol is also demonstrated by Fig. 1C which shows that albumin, IgG and transferrin were undetectable by Western blot analysis in the depleted, flow-through fraction. These results suggest that considerably less protein should be recovered in the flow-through fraction than is actually found. The reasons for this discrepancy are not clear and are under current investigation. A disadvantage of chromatographic immunoaffinity depletion step is the more than 100-fold dilution of the original plasma sample. As a consequence, a protein concentration step has to be incorporated into the sample preparation protocol prior to proteomic analysis. Methods such as trichloroacetic acid/acetone precipitation [19] or centrifugal filter devices (Centricon-YM3, Millipore) have been applied successfully in our laboratory with protein recoveries of about 75–85%.

SELDI-TOF mass spectrometry is a novel approach to protein biomarker discovery that combines specially designed protein chip arrays with surface ionization mass spectrometry [28]. SELDI-TOF mass spectrometry in the linear ion modus is especially suited to detect changes in relative peak intensities of

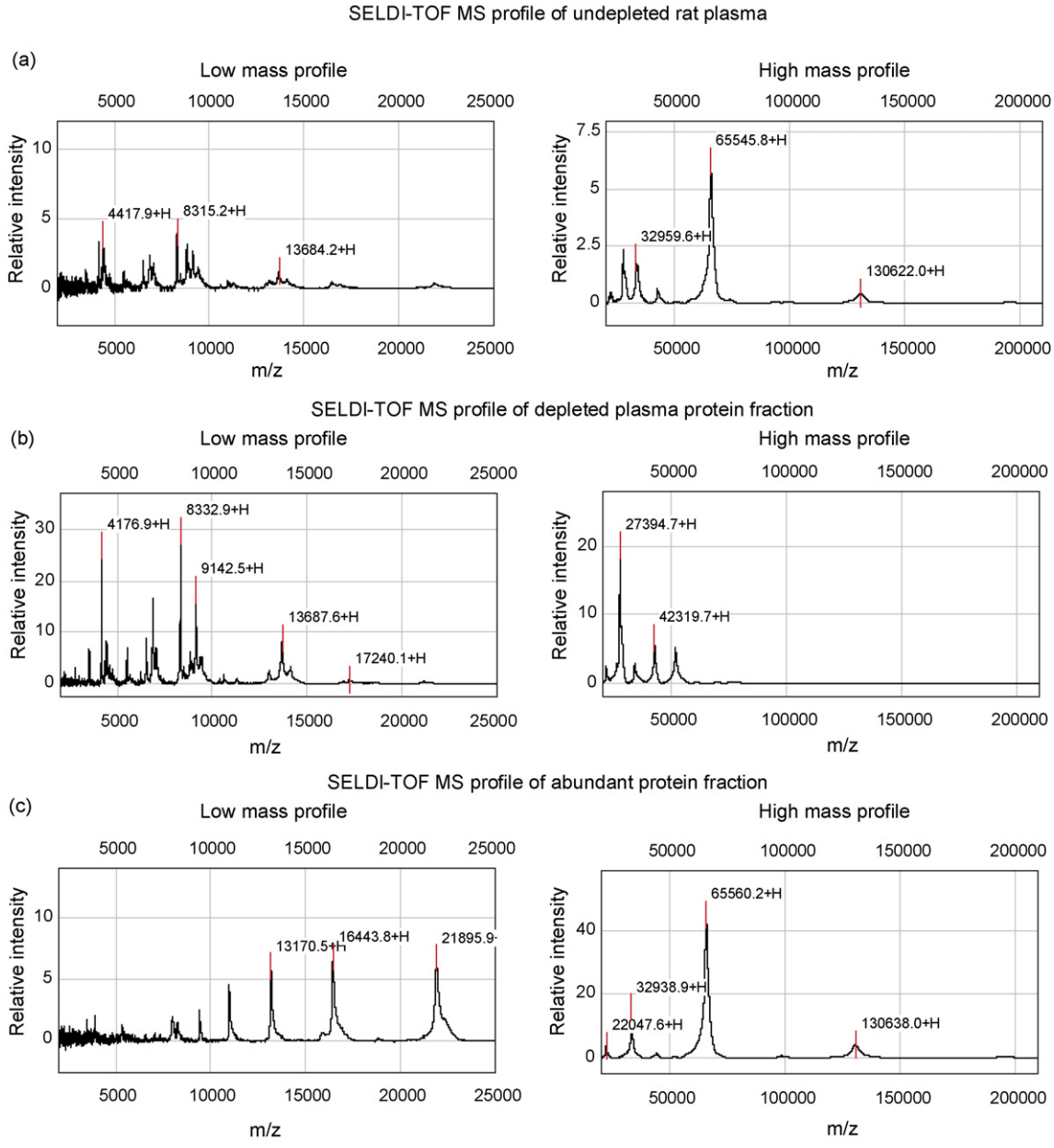

Fig. 2. SELDI-TOF MS profiles of rat plasma fractions. Twenty μg of non-depleted, depleted and eluted rat plasma proteins were incubated in a bioprocessor on a H50 hydrophobic protein array and subsequently analyzed by SELDI-TOF MS. Low mass spectra (*m/z* 2000–25,000) were acquired with α-hydroxycinnamic acid as UV-absorbing matrix. High mass spectra (*m/z* 20,000–200,000) were acquired with sinnapinic acid UV-absorbing matrix: (a) SELDI-TOF MS profile of undepleted rat plasma; (b) SELDI-TOF MS profile of depleted plasma protein fraction; (c) SELDI-TOF MS profile of abundant protein fraction.

peptides and small proteins with mass to charge ratios (*m/z*) between 2000 and 15,000, a mass range that is not readily accessible by 2D SDS-PAGE. Many SELDI-TOF MS studies published to date focus on the detection of diagnostic or prognostic biomarkers of disease in plasma or serum samples [29]. In most studies, diluted but non-depleted plasma or serum samples were spotted on different protein chip arrays and analyzed by time-of-flight mass spectrometry. However, the limited protein binding capacity of the protein chip arrays have led to the observation that only a small number of high and medium abundant plasma proteins can actually be detected by this approach. We have previously reported that dye ligand chromatography with Cibacron Blue can remove some of the albumin present in rat plasma and increase the number of peptide and protein peaks that can be observed in the SELDI-TOF MS profiles [30]. Better results were obtained by anion exchange fractionation of rat plasma samples with Q Ceramic Hyper F as stationary phase. The anion exchange fractionation approach, however, has not been widely applied in SELDI-TOF MS biomarker discovery research because it is time consuming, difficult to standardize and because, most importantly of all, it increases the number of samples that need to be analyzed 5- to 7-fold [30]. As an alternative approach, we have applied samples from the depleted, flow-through fraction and the eluted fraction directly to the spots of a hydrophobic H50 protein array. The buffer solutions of the depletion protocol are compatible with the surface chemistry of the revered-phase H50 protein array, so that samples could be directly spotted with no further adjustments. Equal amounts of non-depleted, depleted and eluted plasma protein were applied and analyzed by SELDI-TOF MS (Fig. 2a–c). The SELDI-TOF MS profiles of the non-depleted and the eluted plasma samples are dominated by singly (∼66 kDa) and multiply-charged (∼33 kDa and 22 kDa) albumin species as well as gas-phase complexes of albumin dimers (∼131 kDa) and trimers (∼198 kDa). The H50 SELDI-TOF MS profile of the depleted fraction again demonstrates that albumin was depleted from rat plasma to undetectable levels (Fig. 2b). However, only between 6 and 8 new protein peaks (at a S/N ratio of 5) were repeatedly detected in the depleted fraction in the mass range *m/z* 20,000–250,000. A preliminary identification of the most intense proteins detected in Fig. 2b and c was attempted by searching the Swiss Prot database for known rat serum or plasma proteins with the measured, intact protein mass. The most prominent peaks in Fig. 2b most likely correspond to rat Apo A1 (Swiss Prot Access. No.: P04639, measured *m/z* = 27,394.7; calculated mass 23,794.8) and rat Apo-AIV (Swiss Prot Access. No.: P02651, measured *m/z* = 42,319.7; calculated mass: 42428). Similar result was obtained for the low molecular mass range *m/z* 1000–25,000. Only six to nine new peptides and small proteins were detected in the depleted fraction that were not initially observed in the non-depleted rat plasma sample. A similar Swiss Prot database search, as described above, showed that the cluster of peaks in the range *m/z* 7000–9500 can most likely be attributed to different glycoforms of Apo AII (Swiss Prot Acces. No. P04638, calculated mass = 8956), Apo CI (Swiss Prot Access. No.: P19939, calculated mass = 7187) and Apo CIII (Swiss Prot Access. No.: P06759, calculated mass = 8997). While this approach only slightly increased the number of detectable peaks in the depleted sample, a more pronounced effect was observed in regard to signal intensities of peptides and proteins of the low molecular weight, SELDI-TOF mass spectrum. Signal to noise ratios improved on average 5- to 7-fold, indicating improved binding kinetics of less abundant proteins in the absence of albumin and other high abundant proteins. Despite the incorporation of the immunoaffinity depletion step into the sample preparation protocol for SELDI-TOF MS analysis, the results indicate that the number of detectable proteins and peptides did not significantly increase. This is probably due to the limited binding capacity of the protein array spots and the large concentration differences that still exists between the remaining classical plasma proteins and low-abundance peptides and proteins that are the focus of biomarker discovery research [26].

As mentioned above, SELDI-TOF MS is particularly useful for detection of low mass proteins not readily accessible by SDS-PAGE. However, it is not particularly sensitive for high mass proteins whose resolution and detection are better suited to gel electrophoresis. Thus, non-depleted, depleted and eluted plasma protein samples were also analyzed by medium format, two-dimensional gel electrophoresis on linear pH 3–10 IPG strips and 4–12% gradient SDS-PAGE gels (Fig. 3a–c). Gels were scanned after staining with colloidal Coomassie Blue and analyzed with the Imagemaster Platinum 6.0 Software package (GE Healthcare). Most notably again is the absence of the albumin in the depleted protein fraction (Fig. 3b) and the appearance of several protein spots that were previously masked by the presence of albumin. A total of about 180 protein spots were detected in the non-depleted samples, compared to about 190 protein spots in the depleted rat plasma sample. In the eluted, abundant protein fraction 120 protein spots were detected by the software, most of which are due to the different post-translational modifications of the seven depleted proteins. Several spots are due to proteins bound non-specifically to either the column or to albumin. Because the depletion protocol was carried out under non-denaturing buffer conditions, proteins bound to albumin did not dissociate from albumin during sample preparation and coeluted with the abundant protein fraction. Most of the detected protein spots in the non-depleted and depleted plasma samples could be matched to each other with the Imagemaster software, indicating that only a few proteins were unmasked by the removal of albumin in our experiments. Overall, the numbers of detected spots by this staining protocol are comparable to a recent study in which nine abundant proteins were removed from human serum samples by multi-component immunoaffinity subtraction chromatography. The same authors report up to 950 spots in a depleted human plasma protein fraction when a more sensitive silver staining protocol was used for visualization of 2D SDS-PAGE gels [31]. There is some disagreement about the effectiveness of immunoaffinity depletion and combination with 2D SDS-PAGE analysis. Very high numbers of protein spots were detected by 2D SDS-PAGE analysis when highly sensitive, fluorescent protein dyes were used in the staining protocols. Chromy et al. (2004) report the detection of 850 protein spots in crude serum compared to 1500 protein spots in

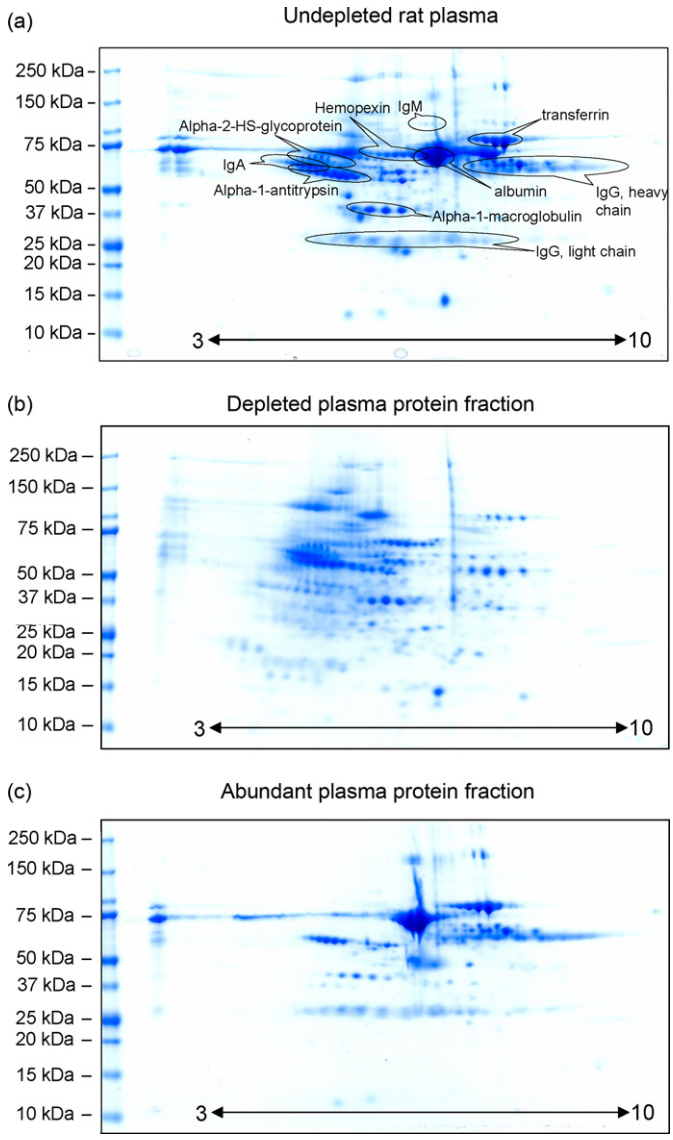

Fig. 3. 2D-SDS-PAGE comparison of fractionated rat plasma samples. Two hundred μg of protein samples were applied to a non-linear pH 3–10 IPG strip in the first-dimension and separated on a 4–12% SDS-PAGE gel in the second-dimension. Proteins were visualized by staining with colloidal Coomassie Blue: (a) Undepleted rat plasma; (b) depleted plasma protein fraction; (c) abundant plasma protein fraction. Annotations are based on visual comparison with rat serum 2D-SDS-PAGE reference gels published by the rat serum protein study group at http://linux.farma.unimi.it/RSPSG/2D/index.html.

depleted serum after the removal of six highly abundant human plasma proteins by DIGE analysis [32]. It is however not clear whether these additional spots are new proteins or new, minor protein isoforms of more abundant proteins. These results nevertheless indicate that depletion of highly abundant proteins is not sufficient by itself to detect low abundance peptide and protein biomarkers but that highly sensitive visualization or detection techniques have to be employed as well.

Despite the availability of premade IPG strips and precast SDS-PAGE gels, 2D SDS-PAGE analysis remains a time consuming technique which is difficult to standardize and automate. In addition, separated proteins are embedded in a gel matrix and not readily available for further analysis. As an alternative, Beckman Coulter has recently introduced the PF-2D protein fractionation system. This separation system is based on a 2-dimensional liquid chromatography approach. In the 1st-dimension peptides and proteins are separated according to isolelectric point on a chromatofocusing column. In the 2nd-dimension, fractionated samples from the 1st-dimension are injected onto a non-porous, reversed-phase colunm and separated according to hydrophobicity. Each 2D-LC separation can generate several hundred fractions in a MALDI-TOF MS compatible buffer system. The PF-2D system has a sample capacity of about 3.5 mg protein per experiment. Data from the 1st- and 2nd-dimension separation can be organized into 2-dimensional protein expression maps and used for label-free, differential protein expression profiling experiments [33]. We have previously reported on the application of this approach toward the analysis of non-depleted rat plasma samples and used the ProteoVue maps of vitamin A-sufficient and vitamin A-deficient rats for differential protein expression profiling [34]. Here we tested whether abundant plasma protein depletion prior to fractionation with ProteomeLab PF-2D system can uncover low abundance peptides and proteins. In order to utilize the large sample capacity of the PF-2D system, depleted and eluted plasma fractions from several runs were pooled and precipitated by a TCA/acetone procedure. Precipitated samples were resolubilized in a reducing lysis buffer and buffer-exchanged against the 1st-dimension start buffer before injection. About 3 mg non-depleted, depleted and eluted plasma protein were injected and separated by 2D-LC. The protein and peptide composition of separated fractions was analyzed by MALDI-TOF MS in linear ion modus. Mass maps were generated by plotting detected peptide and protein masses as a function of p*I*. Fig. 4 shows both the mass maps and the ProteoVue maps of each injected sample. The comparison of the ProteoVue maps in Fig. 4a and b illustrates that the removal of abundant plasma proteins leads to improvements in the signal to noise ratio of several proteins that were previously detected in the non-depleted plasma sample as well (white arrows). Fractions in the p*I* range ~4.00–6.74 were analyzed by MALDI-TOF MS. About 240 peptides and proteins were detected in the non-depleted samples, compared to about 275 peptides and proteins in the depleted samples and about 90 peptides and proteins in the eluted samples. In addition, we were able to detect minor amounts of rat albumin at about ~66 kDa in multiple fractions of the depleted plasma sample (Fig. 4b), indicating that removal of albumin was not quite as complete as the Western blot analysis suggested. Similar to our previous results with SELDI-TOF MS and 2D SDS-PAGE, we were not able to detect significantly higher numbers of unique peptides and proteins in the depleted plasma protein fractions. We speculate that the multi-step sample preparation protocol (precipitation, resolubilization and buffer exchange) necessary for injection into the 1st-dimension resulted in a significant loss of low abundance peptides and proteins. Our experiments show that additional enrichment steps will be required to dig deeper into the rat plasma proteome. A recent paper by Tang et al. suggested a 4-dimensional separation strategy based on abundant protein depletion, isoelectric focusing in solution, 1D

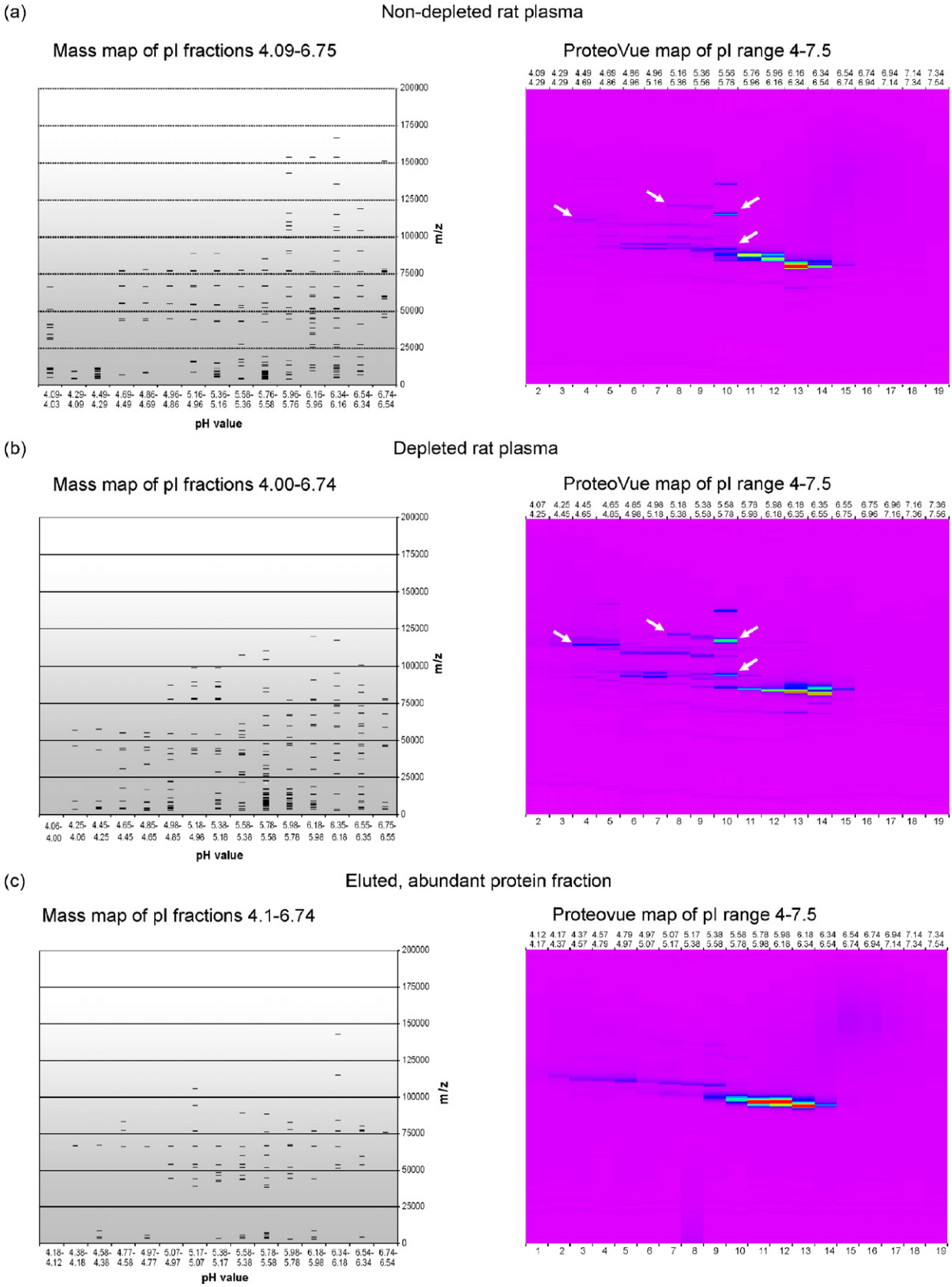

Fig. 4. Mass maps and ProteoVue maps of fractionated rat plasma samples. Three mg of neat (non-depleted), depleted and eluted protein were separated in the 1st-dimension by chromatofocusing and reversed-phase chromatography in the 2nd-dimension. Fractionated samples were spotted on the surface of a gold MALDI-TOF chip and analyzed by MALDI-TOF MS. Mass maps were derived by plotting detected protein masses (black bars) as function of p*I*. Only singly charged proteins with S/N > 5 are included in the map: (a) Non-depleted rat plasma; (b) depleted rat plasma; (c) eluted, abundant protein fraction.

SDS-PAGE followed by nano-capillary LC-MS/MS analysis of in-gel tryptic digests. Using this strategy, refered to as protein array pixelation, the authors were able to detect approximately 3000 non-redundant proteins, differing in abundance by up to 9 orders of magnitude. However, the analysis time of about 12 days per sample will make it challenging to apply this method to high throughput screening of samples for biomarker discovery [35].

4. Conclusion

Here we describe the combination of abundant protein depletion with various common proteomic analysis approaches. The rat plasma immunoaffinity depletion column from Beckman Coulter was effective in removing several highly abundant proteins from rat plasma. However, the protein concentration range in the depleted rat plasma protein sample was still several orders of magnitude larger than what could be effectively resolved by SELDI-TOF MS, 2D SDS-PAGE and 2D liquid chromatography. As a consequence, the number of additional proteins observed in our analysis was rather small. Similar results have been observed by other research groups comparing different abundant protein depletion strategies in human plasma/serum samples [34]. Three- and four-dimensional fractionation strategies have been published that allow on one side a deeper exploration of the plasma proteome, but, on the other side, become increasingly time intensive and less adaptable to high throughput screening. Two new immunoaffinity columns have been recently introduced which can remove either 12 (Beckman Coulter) or 20 (Sigma–Aldrich) of the most abundant plasma proteins which comprise 95–99% of total plasma protein content. These columns may become useful tools in simplifying the plasma proteome for a more effective and fruitful biomarker discovery.

Acknowledgements

The authors gratefully acknowledge the support of this research by the US Department of Agriculture and the National Institutes of Health (DK044498 and HL 049879).

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