Biochemical and Biophysical Research Communications 455 (2014) 84–89

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

High abundant protein removal from rodent blood for biomarker discovery

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ARTICLE INFO

Article history:
Received 9 September 2014
Available online 24 October 2014

Keywords:
Proteomics
Plasma
Affinity chromatography
MARS
2-D DIGE
Albumin

ABSTRACT

In order to realize the goal of stratified and/or personalized medicine in the clinic, significant advances in the field of biomarker discovery are necessary. Adding to the abundance of nucleic acid biomarkers being characterized, additional protein biomarkers will be needed to satisfy diverse clinical needs. An appropriate source for finding these biomarkers is within blood, as it contains tissue leakage factors as well as additional proteins that reside in blood that can be linked to the presence of disease. Unfortunately, high abundant proteins and complexity of the blood proteome present significant challenges for the discovery of protein biomarkers from blood. Animal models often enable the discovery of biomarkers that can later be translated to humans. Therefore, determining appropriate sample preparation of proteomic samples in rodent models is an important research goal. Here, we examined both mouse and rat blood samples (including both serum and plasma), for appropriate high abundant protein removal techniques for subsequent gel-based proteomic experiments. We assessed four methods of albumin removal: antibody-based affinity chromatography (MARS), Cibacron® Blue-based affinity depletion (SwellGel® Blue Albumin Removal Kit), protein-based affinity depletion (ProteaPrep Albumin Depletion Kit) and TCA/acetone precipitation. Albumin removal was quantified for each method and SDS–PAGE and 2-DE gels were used to quantify the number of protein spots obtained following albumin removal. Our results suggest that while all four approaches can effectively remove high abundant proteins, antibody-based affinity chromatography is superior to the other three methods.

1. Introduction

Difficulties in sample preparation currently limit the discovery of protein biomarkers from biofluids, in particular blood plasma and serum. One of the biggest challenges in the study of blood plasma involves the broad concentration range of its protein constituents. In humans, there is approximately a 10^9 order of magnitude from most to least abundant proteins [1]. In addition, few high abundant proteins dominate the plasma, making biomarker discovery of lower abundance proteins even more difficult. For example, twenty-two proteins comprise over 90% of the total protein mass in human serum and albumin alone accounts for over 50%. These dominant species prevent the detection of lower-abundance proteins that may be of greater interest as putative biomarkers [2]. Therefore, a successful system of proteomic sample preparation to remove these high abundant proteins is needed to examine lower abundant proteins of interest and to reduce the complexity for improved biomarker discovery. Researchers have developed successful ways to remove these proteins, but these methods vary in the efficiency and mechanism for removing targeted highly abundant proteins [3–7].

Putative protein biomarkers discovered after the removal of high abundant proteins may serve to detect diseases earlier with higher accuracy, but may prove to be challenging for subsequent validation in humans. Therefore, animal models are necessary to validate these biomarkers and for the discovery of additional biomarkers. Initial 2-DE proteome maps of mouse and rat produced species specific patterns and showed serum proteins can vary substantially [8–11]. However, these samples have a similar wide dynamic range in protein concentrations as seen in human

Abbreviations: TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2-DE, two dimensional electrophoresis; 2-D DIGE, two-dimensional difference gel electrophoresis; MARS, Multiple Affinity Removal system; PBS, phosphate buffered saline.

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http://dx.doi.org/10.1016/j.bbrc.2014.09.137
0006-291X/

samples and therefore face some of the same technological challenges. Since the same high abundant proteins are found in blood of animals, their removal from these models is also necessary. There are many ways to accomplish high abundant protein removal for rodent blood including hydrophobic interactions [12], ammonium sulfate precipitation [13], ion exchange [10], antibody-based affinity chromatography [14,15], and TCA/acetone precipitation [16], and these approaches have been used to enable discovery of putative biomarkers [15,17–20]. In one of these studies, plasma protein biomarkers found in a mouse model of pancreatic cancer were used to translate to human protein orthologs, providing putative early detection markers applicable to human cancer [15]. These studies have focused on a single technique and have not directly compared removal methods to each other using the same samples. Moreover, each study has not compared these techniques for both serum and plasma obtained from both mice and rats.

In this study, four different methods for high abundant protein removal were compared using rat serum/plasma and mouse serum/plasma. SDS–PAGE was used to compare the extent of albumin removal between these methods. Further characterization using 2-D DIGE was done to assess the improvement in total protein spots after removal of high abundant proteins by each of the four different methods.

2. Materials and methods

2.1. Sample collection

Rodent blood was collected under IACUC protocols for (DH) and (PL). For mice, whole blood was collected by ocular bleed. For rats, blood was collected from the saphenous vein on the inside of the thigh using a 21 gauge needle. Serum was allowed to clot at room temperature for 2–5 h followed by centrifugation at 5000×*g* for 10 min. The supernatant was collected and stored at −80 °C in fresh tube. For plasma, blood was collected into BD 0.5 ml microtainer tubes containing Potassium EDTA (Becton Dickinson, Franklin Lakes, NJ). Blood was centrifuged at 15,000×*g* for 10 min to separate the plasma from the red blood cells. Plasma was collected, aliquoted and stored at −80 °C until analysis.

2.2. High abundant protein removal

Depletion of high abundant proteins was carried out according to the manufacturer's instructions with minor modifications as detailed below. TCA/acetone was carried out similarly as previously published [16]. TCA was dissolved in water to make a 20% solution and this solution was diluted 1:1 with the protein sample on ice for 30 min. Following incubation the proteins were centrifuged and the protein pellet was washed 2× with ice-cold acetone. The ProteaPrep procedure was carried out as described in the manufacturer's protocol (Protea). Protein samples were diluted in sample buffer 1:4 and then loaded into pre-packed columns containing a proprietary dry powder that facilitated non-antibody, affinity-based serum albumin removal. The capture ligand is a recombinant protein that claims to be more specific than an antibody-based system with stronger binding constants. For SwellGel® Blue Albumin (Pierce), 40 µl samples of plasma or serum were diluted into 160 µl of bind/wash buffer. Albumin binding incubations were done for 2 min (twice). Incubations were washed 3 times with 200 µl. The flow through and washes were pooled as the albumin removed sample. For antibody-affinity chromatography using the MARS MS-3 (Agilent Technologies), rodent plasma or serum was diluted five times in Buffer A (40 µl sample and 160 µl of buffer, 200 µl total volume) and centrifuged through a 0.22 micron spin filter tube (Millipore) at 16,000×*g* for 5 min to remove particulates. Then, plasma or serum was processed using 4.6 × 50 mm Multiple Affinity Removal Column Mouse-3 (Agilent Technologies), which specifically removes albumin, IgG, and transferrin. A low abundant protein fraction was collected for each sample. Fractions were concentrated by precipitating with an equal volume of 20% TCA solution and incubated at 4 °C for 30 min. Precipitate was spun down and washed twice with cold 100% acetone, allowed to air dry and then resuspended in DIGE labeling buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5). Protein quantification was performed using Precision Red Advanced Protein Assay Reagent (Cytoskeleton).

2.3. SDS–PAGE

Crude and high abundant protein depleted plasma or serum samples (5 µg) were mixed with 5× sample loading buffer (0.2 M Tris pH 6.8, 20% glycerol, 10% SDS, 5% BME), boiled for 10 min at 100 °C and resolved on a 4–20% Tris–Glycine gel (Invitrogen). Gels were stained for total protein using Sypro Ruby Protein Gel Stain (Invitrogen, S-12000) and visualized using the BioChemi system (UVP BioImaging Systems).

2.4. 2-D DIGE

Crude and high abundant protein depleted plasma and serum samples were separated in two dimensions using the GE Life Sciences Ettan DIGE system protocol. Briefly, each sample (50 µg) was minimally labeled with 1 µl of 200 pM Cy2, Cy3 or Cy5 for 30 min. Labeling reactions were stopped by the addition of 1 µl of 1 mM lysine. The samples were pooled together and added to rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1.2% DeStreak, 1% pharmalytes). A final volume of 450 µl sample was loaded onto 24 cm pH 3–10NL Immobiline DryStrips (GE Life Sciences) and focused by active overnight rehydration, followed by isoelectric focusing for a total of 62,500 Vhrs. Strips were equilibrated in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS) for 15 min with 10 mg/ml DTT, then 15 min in fresh buffer with 25 mg/ml 15 min with IAA, then applied to DIGE gels (GE Life Sciences) for 2nd dimension separation. The resulting CyDye labeled protein gels were scanned using 100 micron resolution on Typhoon 9410 (GE Life Sciences).

2.5. Image analysis

Data analysis was carried out using DeCyder 2-D 7.0 software (GE Life Sciences). Spot detection and abundance quantification was performed using the differential in-gel analysis (DIA) module of DeCyder. Densitometry, using ImageJ processing program (available free online at rsb.info.nih.gov/ij/), was performed on selected albumin bands to determine the percent removed.

3. Results

Four different methods were tested for their ability to remove albumin from both rodent blood samples. Both rat and mouse samples of plasma and serum were used. SDS–PAGE and 2-DE were used to evaluate the overall improvements in proteomic sample preparation following high abundant protein removal. Table 1 shows the recovery of the total protein following these different methods. Most of the protein remains in the high abundant fraction, but this table shows that the total protein obtained from these different methods does not vary substantially. Therefore, none of these methods reduce total protein recovery more than another.