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Short communication

Quantitative 2-D gel electrophoresis-based expression proteomics of albumin and IgG immunodepleted plasma

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Abstract

Proteomic analysis of plasma is challenging because of its large dynamic range, which prevents the detection of low abundance proteins. Immunodepletion of high abundance proteins, such as albumin and IgG, has emerged as a favored technology to overcome this problem; however its suitability in quantitative expression proteomics has not yet been adequately addressed. In this study, albumin and IgG immunodepletion was evaluated by ELISAs and the reproducibility of depletion was tested with 2-DGE. Depletion of plasma resulted in removal of $62 \pm 1.2\%$ of the total protein, $93 \pm 1.4\%$ of the albumin (0.43 μg/μL, residual), and $94 \pm 1.5\%$ of the IgG (0.21 μg/μL, residual). These results were confirmed by immunoblotting. Computerized image analysis of 2-D gels using Progenesis SameSpots software revealed an enhancement in the number of visible spots (675–1325), with $10 \pm 6\%$ inter-gel variability in spot density. LC–ESI-MS/MS identification of newly resolved protein spots further validated the procedure. An innovative application of the software employed led to identification of 11 proteins lost non-specifically during depletion. This study demonstrates the effectiveness of immunodepletion of albumin and IgG in quantitative 2-DGE-based differential analysis of plasma proteins.

Keywords: Plasma proteome; Albumin; IgG; Immunodepletion; ELISA; 2-DGE software; Expression proteomics

1. Introduction

Differential profiles of plasma proteins can illustrate changes due to altered metabolism or disease development. Consequently, these proteins are highly relevant both in diagnosis and in therapeutics [1,2]. Our specific interest is in analyzing maternal plasma by 2-D gel electrophoresis (2-DGE) to uncover potential markers of fetal growth restriction [3]. However, proteomic analysis of plasma is challenging due to the plasma proteome's large dynamic range and the presence of highly abundant proteins, such as albumin and IgG [2]. Their presence, and that of other high abundance proteins, is a major ongoing technical impediment to detecting less abundant proteins [4–12].

Immunoaffinity depletion of albumin and IgGs, or as many as 20 abundant proteins from plasma, is a preferred method of removal due to the relative specificity of the procedure over others [10,13]. Concomitant loss of non-target proteins through protein–protein interactions has thus far proven inevitable [6,14], even though the addition of acetonitrile (ACN) appears to mitigate this loss [15]. Improved post-depletion resolution in subsequent 2-DGE or in mass spectrometry (MS)-based analysis has been demonstrated [10,16]. In expression proteomics, however, these challenges are further compounded by the need to obtain quantitative data with high reproducibility. Although, the utility of the depletion process for improving spot resolution analysis of 2-DGE has been demonstrated [4,10,12,15,17,18], its compatibility in quantitative 2-DGE expression proteomics has been largely unaddressed.

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With albumin and IgG representing as much as 85% of plasma protein, any variability in their depletion substantially affects the remaining protein loaded on the gels. Densitometric quantitation of 2-DGE is ultimately no more reliable than the consistency in sample loading. The most critical aspects are therefore to establish and minimize the non-specific loss of proteins at the depletion step, as well as attain high efficiency and reproducibility with the subsequent quantitative analyses.

Proceeding with conventional immunodepletion using a commercial kit, this study evaluates the outcome of depletion and its reproducibility using 2-DGE followed by computerized image analysis. It furthermore identifies some proteins removed non-specifically during depletion.

2. Materials and methods

2.1. Samples, reagents and equipment

A single blood sample was taken with informed consent and approval as described previously [3]. Electrophoretic equipment and reagents were from Bio-Rad (Hercules, CA, USA) and all procedures were according to manufacturer's instructions unless otherwise specified. Protein measurements were made by Bradford assay.

2.2. Albumin and IgG depletion

Depletion of albumin and IgG from whole plasma was performed using the Qproteome Albumin/IgG Depletion Kit (Qiagen, Valencia, CA, USA). Buffer 2 (250 mM Tris–HCl, 4% (w/v) CHAPS and 200 mM urea, pH 7.5) was used with 5% ACN added for maximal recovery [15]. Additional washes (2×0.25 mL and 1×0.5 mL) were essential for optimal recovery of the protein in the depleted fraction. The albumin and IgG bound to the resin were eluted using 3×0.5 mL washes with 20 mM glycine buffer (pH 1.5) in a total volume of 1.5 mL. An adjustment for pH (neutral) was made with ~0.05 mL of 1.5 M Tris–HCl buffer (pH 8.8) added to the final sample.

2.3. Immunoassays

1-D gels were transferred to PVDF membrane for Western blotting as described previously [3]. Blocking was with 0.5% gelatin for albumin or 2% polyvinyl pyrrolidone (PVP) for IgG. Immunoblots were developed using primary goat (anti-human albumin) and rabbit (anti-human IgG) polyclonal antibodies (Bethyl Laboratories, Montgomery, TX, USA) (1:15,000), and HRP conjugated goat anti-rabbit or mouse anti-goat IgG secondary antibodies (1:8000). Western lighting enhanced chemiluminescence (ECL) Reagent plus (PerkinElmer) and Kodak XOMAT LS films were used.

Albumin and IgG quantification was performed employing respective ELISA kits for human albumin and IgG according to manufacturer's instructions (Bethyl Labs). Samples in 96-well microplates were quantified using a MultiSkan Ascent (Thermo Electron Corp. Waltham, MA, USA).

2.4. 2-DGE

Nanosep Omega 3K MWCO Centricons (PALL) were used to desalt and concentrate protein prior to 2-DGE. Samples (300 μg protein) were reconstituted in rehydration buffer (0.45 mL), and rehydrated actively onto 24 cm ReadyStrip™ immobilized pH gradient (IPG) strips (pH 3-10NL). Triplicate gels of a sample depleted three separate times were analyzed for reproducibility. The gels were stained with SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) protein stain.

2.5. 2-DGE image processing and software analysis

2-DGE images were captured using a Fluorochem 8800 imaging system (Alpha Innotech Corp. San Leandro, CA, USA), with consistent exposure time in the linear range. Images were analyzed using Progenesis SameSpots software (Nonlinear Dynamics, Durham, NC, USA). All gels were warped to a single whole plasma gel template. The data analysis for densitometric aspects of the study was typical for expression proteomics. Matching was automatic but verified manually: artifacts, or spots that could not be confidently verified as true matches, were disregarded rather than manually edited, and misalignments were corrected by manual warping when appropriate.

2.6. LC–ESI-MS/MS analysis

Excised spots were digested in-gel, using a Waters MassPREP automated digester (Waters, Milford, MA, USA). Samples were analyzed by LC–ESI-MS/MS using a 2000 QTrap coupled to a LC Packings Ultimate HPLC system equipped with a 100 μm × 15 cm CapRod RP-HPLC column (Merck, Whitehouse Station, NJ, USA) operated at a flow rate of 1 μL/min as previously described [19]. A linear gradient from 5 to 50% B over 30 min was used (A, 5% ACN and 0.5% formic acid; B, 90% ACN and 0.5% formic acid). The LC system was interfaced to the mass spectrometer via a nanoflow source equipped with a 15 μm internal diameter spray tip (New Objective, Woburn, MA, USA). The resulting tandem MS data was searched against NCBI protein sequence database. All identified peptides during Mascot (Matrix Science, London, UK) or Peaks (Bioinformatics Solutions Inc., Waterloo, ON, Canada) searching were verified by manual interpretation of the data.

3. Results

3.1. Evaluation of immunoaffinity depletion levels

To visualize depletion prior to 2-DGE, 1-D immunoblot analysis of bound, depleted, and whole plasma fractions was performed for albumin and IgG, respectively (Fig. 1A and B). As expected, albumin was highly abundant in both whole plasma (Fig. 1A, lane wp), and the bound fraction (Fig. 1A, lane b). Albumin was not detected in the depleted plasma (Fig. 1A, lane d). Intense bands corresponding to IgG light and heavy chain proteins were prominent in both whole plasma (Fig. 1B, lane, wp) and the bound fraction (Fig. 1B, lane b). The correspond-

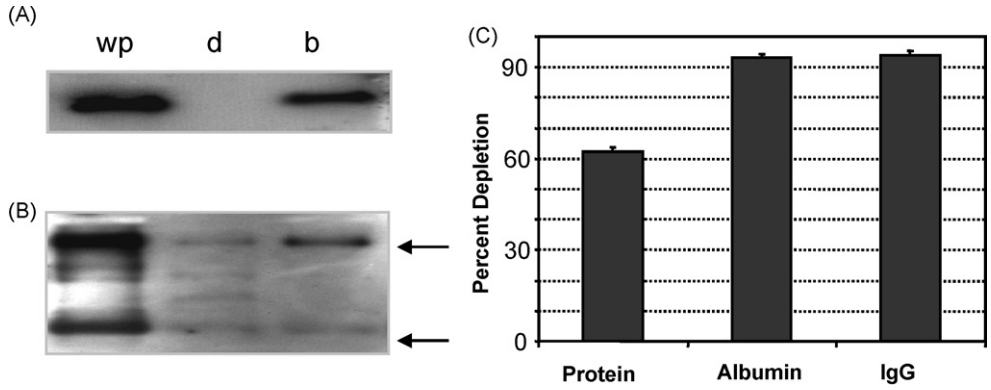

Fig. 1. Western blots for albumin (A) and IgG (B) are shown with whole plasma (wp), depleted (d), and bound fraction (b). To determine relative quantities of albumin and IgG contents in fractionated samples, proportionate volumes were loaded so that each lane has 0.01% of its fraction's total volume for albumin (A) and 0.03% for IgG (B). Arrows show heavy and light chains of IgG. (C) Residual quantities of total protein (Bradford), albumin and IgG (ELISAs) of a sample after depletion of plasma in the presence of 5% acetonitrile ($n = 6$) are shown as percent depletion. Error bars are SD.

ing light and heavy chain bands were, however, greatly reduced in the depleted plasma (Fig. 1B, lane d), suggesting significant removal of IgG overall. Fig. 1C is an assessment of the level of albumin and IgG depletion by ELISA ($n = 6$); $38 \pm 1.2\%$ of total protein was recovered, however $93 \pm 1.4\%$ of albumin and $94 \pm 1.5\%$ of IgG was removed (Fig. 1C).

3.2. Improvement in 2-DGE spot resolution

2-D gels of the whole, the depleted, and the bound fractions of plasma are shown in Fig. 2A–C, respectively. Spots from smears of albumin and IgG were discounted (Table 1, parenthesis) as they do not represent true resolution of proteins. There was a considerable increase in the number of spots resolved in all six areas on the depleted gel (Fig. 2B), and overall the number of spots nearly doubled (675–1325) (Table 1), thereby increasing the potential to identify new proteins. The alkaline region (area c), had considerably higher gains than other high M_r regions (a and b). The enhancement was accounted for partly by the significant amounts of albumin and the majority of IgG that would otherwise be present, and occlude other spots in this field. The greatest improvement in spot resolution was in the lower M_r regions (d–f). This is largely attributed to the relative enrichment of proteins after the removal of the high abundance proteins.

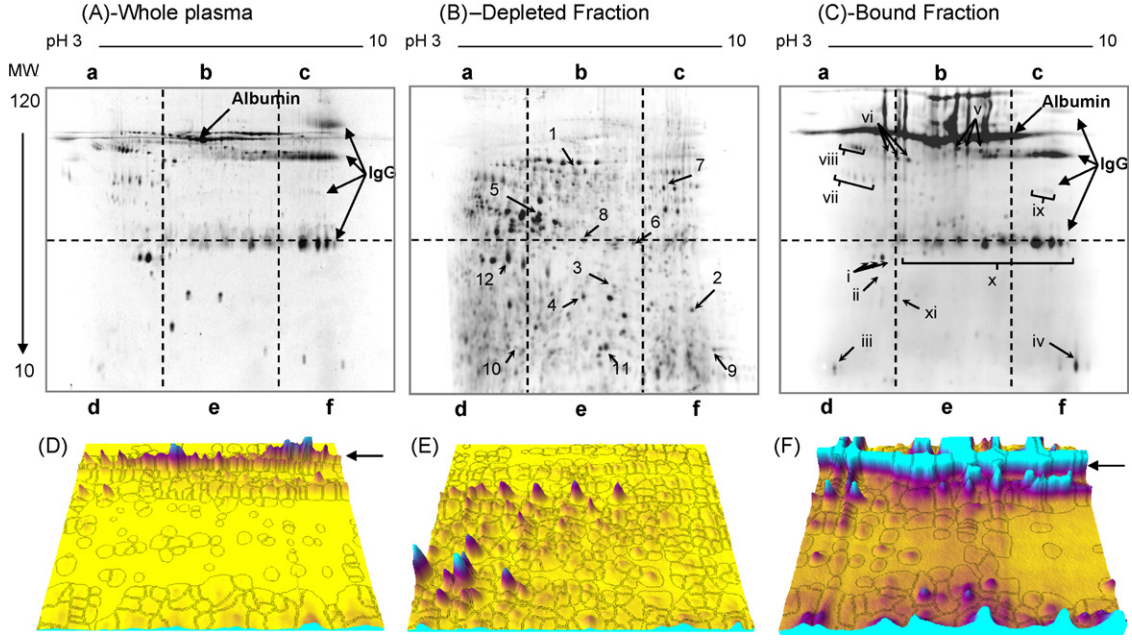

Fig. 2. 2-D gels (24 cm) equally loaded with protein (300 μg) and warped to near perfect alignment. Whole plasma (A), depleted (B), and bound (C) fraction images were divided in six (a–f) and their spots counted (Table 1). Spots identified by LC–ESI-MS/MS are labeled in the depleted fraction (B) (Table 2). Proteins identified based on comparison to the Swiss-2-D PAGE plasma protein map by matching through the whole plasma gel are labeled in the bound fraction (C). Accession numbers are in brackets. i, Apolipoprotein A–I (P02647); ii, plasma retinol-binding protein (P02753); iii, apolipoprotein C-II (P02655); iv, hemoglobin subunit beta (P68871); v, fibrinogen beta chain (P02675); vi, fibrinogen gamma chain (P02679); vii, haptoglobin (P00738); viii, alpha-1-antitrypsin (P01009); ix, immunoglobulin heavy chain gamma (intermediate segment) (P99008); x, immunoglobulin light chain (P99007), and xi, haptoglobin (P00738). (D–F) Area b from each (A), (B), and (C), respectively, rendered in 3-D for spot density. Arrows in (D) and (F) point to smeared albumin.

Table 1
Comparison of the number of spots detected on 2-D gels in regions a–f from whole plasma, depleted, and bound fractions (Fig. 2A–C)

	a	b	c	d	e	f	Total
Whole	175 (57/0)	227 (75/0)	93 (65/44)	53	76	51	675
Depleted	212	254	233	197	225	204	1325
Increase from whole to depleted	37	27	140	144	149	153	650
Bound	42 (57/0)	55 (185/0)	30 (78/59)	30	50	36	243

Maximizing resolution of spots in whole plasma typically involves tolerating the overloading of abundant proteins, albumin and IgG. Spots identified by the software that resulted from smears due to albumin or IgG (Fig. 2 arrows) were inconsistent from gel to gel and therefore not compatible with expression analysis. Accordingly, spots from smears are listed in brackets (albumin/IgG) but otherwise excluded from analysis.

3.3. Relative protein enrichment

The relative enrichment is most apparent in three-dimensional images. Area b of Fig. 2A–C is shown in Fig. 2 D–F, respectively, with the third dimension as density, which is proportional to the amount of protein for the spot. The area in the depleted fraction (Fig. 2E) clearly shows an increase in the low level proteins compared to the same area in whole plasma (Fig. 2D). As calculated from the total protein and ELISA data (Fig. 1C), an increase of 7.3-fold in relative abundance of residual proteins was expected due to enrichment. When matched spots, corresponding to proteins present on both gels, were compared for density, it was established that the spot density on average increased 8 ± 3-fold (Table 2, matched), which was consistent with the theoretical expectation. However, the moderately high variance suggests that some proteins were preferentially retained. These proteins were subsequently identified by LC–ESI-MS/MS along with others (Fig. 2B, numbered) (Table 2).

3.4. Non-targeted protein loss

Large smears of albumin and IgG were clearly present in the bound fraction (Fig. 2C and F, arrows) compared to whole plasma (Fig. 2A and D, arrows), and especially the depleted fraction (Fig. 2B and E). However, other protein spots were visible in the depleted fraction contrary to expectation (Fig. 2C). By comparing the gel to the Swiss-2-D PAGE human plasma protein map by matching through our whole plasma gel (Fig. 2A), we were able to set probable identities for many of the visible protein spots in the depleted fraction (Fig. 2C, numerals).

The major spots identified were typically high abundance proteins (Fig. 2C), none of which are known to directly bind albumin for transport. Of the proteins identified, i, iv–viii, x, and xi, are among the ten most abundant proteins in plasma [20]. Only three proteins were not among the top ten, including hemoglobin (iv), which may circulate bound to haptoglobin (vii). Some proteins (i, xi, and xii) were identified in both the bound and the depleted fractions, indicating that their

Table 2
Identification of spots excised from an albumin and IgG depleted gel (Fig. 2B) by LC–ESI-MS/MS

2-D spot #	Migration		Database	ID	*M* score	Protein	Fold volume change
	p*I*	MW (kDa)					
Matched spots							
1	5.7	50	NCBInr	gi|45708661	134	α2 Macroglobulin	5.6
			NCBInr	gi|119626068	63	Albumin, isoform CRA_e	
2	8.5	17	NCBInr	gi|119599289	54	Ceruloplasmin (ferroxidase), isoform CRA c	22.8
3	6.3	19	NCBInr	gi|119625312	108	Fibrinogen γ, isoform CRA_c	12.9
4	5.9	18	NCBInr	gi|119625326	127	Fibrinogen γ, isoform CRA_o	1.8
5	5.6	35	NCBInr	gi|577055	56	Fibrinogen γ, chain fragment	7.3
6	6.4	26	NCBInr	gi|10121059	44	IgG1 Fc fragement γ receptor III complex	−1.8
7	7.0	42	NCBInr	gi|118137965	99	Chain B, complement C3b	4.3
8	5.8	27	NCBInr	gi|119625335	118	Fibrinogen β chain, isoform CRA_a	4.0
			NCBInr	gi|119625336	118	Fibrinogen β chain, isoform CRA_b	
Unmatched spots							
9	9.3	12	NCBInr	gi|119625338	51	Fibrinogen β chain, isoform CRA_d	–
10	5.4	12	NCBInr	gi|119579598	50	Haptoglobin, isoform CRA_a	–
11	6.2	13	NCBInr	gi|33337724	90	FWP007	–
12	5.2	23	NCBInr	gi|119625320	169	Fibrinogen γ, CRA_j	–

Matched spots are those that could be matched to the whole plasma 2-D gel (Fig. 2A). The spots were quantified by optical densitometry using SameSpots software. Spot volumes of the depleted gel are expressed here as fold change from corresponding matches on the whole plasma gel (Fig. 2A). See Fig. 2B for the labeled spots corresponding to the spot numbers.

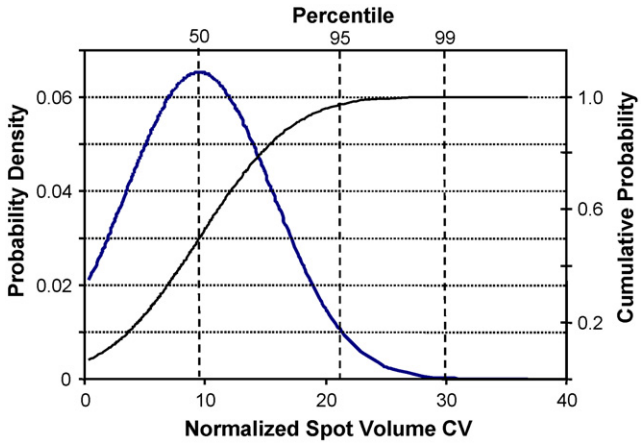

Fig. 3. Probability distribution for the coefficient of variation of the normalized volume of matched spots from three replicate (depleted fraction) gels (Fig. 2B). The mean coefficient of variation is 10% (SD 6.1) for spots verified as matched. The 95th and 99th percentiles are marked. Of manually verified matching spots; 95% of spots had a coefficient of variation <21% and 99% had a coefficient of variability <30%.

elution from the bound fraction was incomplete. Hemoglobin (iv) appeared relatively enriched in the bound compared to both depleted and whole fractions, indicating its preferential retention. The data therefore suggests that although some proteins lost may couple with albumin and in this manner may be distinctively retained through protein–protein interactions [14], there was largely non-specific loss of proteins.

3.5. 2-DGE quantitative reproducibility

To analyze the quantitative spot reproducibility, three replicates of the depleted fraction (Fig. 2B) were produced and analyzed by 2-DGE. The distribution of the variation between matching spots is plotted in Fig. 3. The mean variability was $10 \pm 6\%$, while 95% of matching spots had a variability of less than 21% and the probability of the variability exceeding 35% was <0.0001.

4. Discussion

Immunoaffinity depletion prior to 2-DGE has been shown to be a powerful fractionation technique in the identification of new proteins [21]. In this study, we were able to demonstrate a much improved number of useful identifiable spots ($n = 1325$) resolved on a single 24 cm 2-D gel. As reported by others [4,10,12,15,17,18], the achievement of high resolution on 2-DGE was attributable to a substantial improvement in dynamic range post-depletion, but additionally to the combined use of 24 cm 2-D gels over the more commonly used 18 cm gels.

This study demonstrates the quantitative feasibility of depletion in combination with high resolution 2-DGE. Evaluation of depletion for the MARS LC column (Agilent, Santa Clara, CA, USA) was reported to eliminate >99% for albumin and IgG, measured using the same ELISA kits [6]. In our study, we found considerably less complete depletion, although neither protein was prominent on 2-D gels. A possible explanation may be the use of less sensitive antibody in the Qproteome kit, or the lower sensitivity or binding capacity of the spin column-based depletion compared to the LC technique, which is inherently more efficient. Reproducibility as measured by 2-DGE spot variance (Fig. 3) was nevertheless better than reported for 2-DGE without fractionation, albeit with modest differences in methodology [22].

Multiple protein affinity columns may achieve even greater improvements in resolution, for as many as 20 of the most concentrated proteins are depleted, which accounts for >99% of plasma protein. The corollary of higher levels of depletion however, is that much greater sample volumes are required to yield several hundred micrograms of depleted protein required for large-format 2-DGE applications. A substantial amount of pooling of depleted fractions or a very high capacity LC affinity column would be required.

The flexibility in spot alignment enabled comparison of the depleted and bound fractions of whole plasma, and tentative spot assignment using 2-D maps rather than spot excision and MS analysis. This facilitated the identification of proteins lost during depletion and the estimation of the relative protein enrichment post-depletion at the 2-D gel level.

Using this method, we were able to confidently interpret the protein loss inherent to depletion. Many proteins were lost that may not have been due to specific interactions with albumin or the depletion antibodies. Variations in the number of washes during the depletion process or the composition of the buffers have a strong influence on the proportion of non-targeted proteins retained on the resin [15]. However, for maximal recovery, conditions were currently implemented as per Huang et al. [15], as well as our own optimizations. Despite removal of proteins that were identifiable, and undoubtedly others that were not, the depleted sample exhibited dramatic improvement in the number of spots resolved (Table 1) and reproducibility in spot density (Fig. 3).

Albumin and IgG removal improves 2-DGE spot resolution. However, the application of immunodepletion of plasma samples to quantitative densitometric 2-DGE analysis further demonstrates its reproducibility. Proteins in the resulting 2-D gels were identifiable by LC–ESI-MS/MS despite the low density of some specific spots (Fig. 2B spots 1, 3 and 10). Together, these aspects of the study illustrate that quantitative 2-DGE of depleted plasma is an effective expedient to identifying plasma proteome expression changes in clinical investigations.

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