Journal of Chromatography B, 879 (2011) 3645–3648

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Short communication

Depletion of highly abundant proteins in blood plasma by ammonium sulfate precipitation for 2D-PAGE analysis

Andrea Mahn*, Maritza Ismail

Department of Chemical Engineering, University of Santiago of Chile, Av. Libertador Bernardo O'Higgins 3363, Santiago, Chile

ARTICLE INFO

Article history:
Received 22 July 2011
Accepted 12 September 2011
Available online 18 September 2011

Keywords:
Ammonium sulfate precipitation
Two-dimensional gel electrophoresis
Plasma proteomics
Protein depletion

ABSTRACT

Ammonium sulfate precipitation (ASP) was explored as a method for depleting some highly abundant proteins from blood plasma, in order to reduce the dynamic range of protein concentration and to improve the detection of low abundance proteins by 2D-PAGE. 40% ammonium sulfate saturation was chosen since it allowed depleting 39% albumin and 82% α-1-antitrypsin. ASP-depletion showed high reproducibility in 2D-PAGE analysis (4.2% variation in relative abundance of albumin), similar to that offered by commercial affinity-depletion columns. Besides, it allowed detecting 59 spots per gel, very close to the number of spots detected in immuno-affinity-depleted plasma. Thus, ASP at 40% saturation is a reliable depletion method that may help in proteomic analysis of blood plasma. Finally, ASP-depletion seems to be complementary to hydrophobic interaction chromatography (HIC)-depletion, and therefore an ASP-step followed by a HIC-step could probably deplete the most highly abundant plasma proteins, thus improving the detection of low abundance proteins by 2D-PAGE.

1. Introduction

Blood plasma or serum is an extremely rich source of biochemical markers related to health/disease or physiological status of an individual. Proteomics is a fast growing field, relevant in the task of discovering such biomarkers. However, the use of proteomic technology is somewhat limited because of the presence of a few highly abundant proteins in blood plasma, mainly albumin, immunoglobulins, α-1-antitrypsin, haptoglobin and fibrinogen [1], which amount up to 90% total protein concentration in blood plasma. As a consequence, the wide dynamic range of protein concentration makes it difficult to detect low-abundance proteins, which are more likely to be possible biomarkers that can be detected through the conventional proteomic methods, namely mass spectrometry and two-dimensional gel electrophoresis (2D-PAGE) [2]. In order to overcome this limitation, several strategies have been used to partially deplete the highly abundant proteins from blood plasma. Immuno-affinity depletion is the most popular method, since it offers high reproducibility and it enhances the number of visible spots [3,4]. However, this depletion method has some drawbacks, mainly the occurrence of non-specific interactions that may lead to the loss of some low abundance proteins and the relatively high cost [5,6]. Alternative methods have been proposed and used with relative success, such as ion exchange chromatography [7–10], thiophillic chromatography [11], and affinity chromatography (dye-based, protein A and G, lectins, peptides or inorganic ligands) [12]. Recently, Mahn et al. [13] proposed the use of hydrophobic interaction chromatography (HIC) to deplete some highly abundant proteins from plasma. They found that the main abundant proteins exhibit a similar hydrophobicity, and then they could be removed in a single HIC step. Based on this fact, the use of other separation methods that exploit protein hydrophobicity could probably be useful for depleting the highly abundant proteins from blood plasma.

In this work the use of ammonium sulfate precipitation (ASP) is explored as a method for depleting some highly abundant proteins from blood plasma, in order to reduce the dynamic range of protein concentration in the sample and to improve the detection of low-abundance proteins by 2D-PAGE. Precipitation methods are commonly used as a cleaning step before 2D-PAGE. Jiang et al. [14] reported a method based on ammonium sulfate precipitation for sample preparation prior 2D-PAGE, which was effective in increasing protein concentration. Chen et al. [15] reported an acetone/TCA method for albumin removal from serum. ASP has not been explored for depleting highly abundant proteins other than albumin from blood plasma samples so far.

2. Experimental

2.1. Plasma samples

Six Wistar rats, 21-days old, were fed a Torula yeast-based diet (Dyets Inc., Bethlehem, USA) during 10 weeks. Animals were maintained at 20 °C, 12 h/12 h day/night cycles in stainless steel

* Corresponding author. Tel.: +56 2 7181833; fax: +56 2 6817135.
E-mail addresses: amahn_2000@yahoo.es, andrea.mahn@usach.cl (A. Mahn).

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doi:10.1016/j.jchromb.2011.09.024

cages with free access to deionised water and the diet. After the breeding period, blood from each one of the rats was collected by cardiac puncture, using standard protocols [16]. Blood plasma was obtained using the methodology previously reported [13], and were kept at −80 °C until analysis.

2.2. Ammonium sulfate precipitation

A 60 μL aliquot of blood plasma was diluted to 150 μL with PBS and shaken during 10 min. Protein concentration in the diluted plasma solution was determined through absorbance at 280 nm. The plasma solution was mixed with the corresponding ammonium sulfate mass to give the desired saturation percentage in 1.5 mL centrifuge tubes, according to Bollag [17]. The tubes were shaken during 10 min and then left at room temperature for 30 min. After that, tubes were centrifuged at 7000 × *g* for 30 min, the supernatant was discarded and the precipitate was analyzed. Since ammonium sulfate interferes with electrophoresis, the protein-salt precipitate was dissolved in 1 mL PBS and subjected to dialysis against 1 L PBS in 0.2 μm dialysis bags (Spectra/Por MWCO 3500). After 24 h stirring at room temperature, the sample solution was recovered from the dialysis bag and subjected to acetone precipitation. Pellet was let air drying.

2.3. Polyacrylamide gel electrophoresis

Proteins in the depleted and untreated plasma samples were analyzed by PAGE using a standard protocol [17] and 2D-PAGE using the method of O'Farrell [18] adapted as previously reported [13]. PAGE was performed in a mini-chamber; each gel had 10 wells, and in each well 20 μL protein solution was loaded. The gels were run at 120 V during 10 min and after that at 180 V until the advancing front reached the button of the gel. For 2D-PAGE, 300 μg of protein were resuspended in 50 μL of lysis buffer (9.5 M urea, 2% Triton X-100, 1.6% ampholytes 4–7 range, 0.4% ampholytes 3–10 range, and 5% β-mercaptoethanol), incubated at room temperature for 15 min and loaded onto lab-made first dimension gels (115 mm height and 3 mm internal diameter capillary tubes). A 4.0–7.0 pH gradient was used. Gel prefocusing was carried out according to the following program: 200 V for 15 min, 300 V for 15 min and 400 V for 15 min. Isoelectric focusing (IEF) was performed at 400 V for 20 h, to complete 8000 Vh. After IEF, the gels were extruded from the glass capillary tubes and equilibrated immediately in 2 mL of equilibration solution (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 0.0625 M Tris–HCl pH 6.8) for 10 min. Vertical SDS-PAGE was run with lab-made homogeneous acrylamide gel (11.5% acrylamide; 180 mm height and 120 mm wide), at a constant voltage of 50 V during 16 h. PAGE and 2D-PAGE gels were soaked in a solution of 25% methanol and 7.5% acetic acid for 30 minutes, stained in Coomassie Brilliant Blue R-250 for 12 h (0.1% Coomassie Blue R-250, 25% methanol, 7.5% acetic acid) and destained in a solution of 25% methanol and 7.5% acetic acid. The analyses were made in quadruplicate. All chemicals were analytical grade and were purchased form Sigma Co. (St. Louis, MO, USA).

2.4. Image analysis

Image acquisition was performed with an ImageScanner II device (GE Healthcare, Uppsala, Sweden). Intensity calibration was carried out using an intensity step wedge prior to the image capture. The TotalLab™ v2.01 software (Nonlinear Dynamics Ltd., NC, USA) was used for image analysis. Spots were automatically detected and matched. Each spot volume was determined densitometrically and processed by background subtraction. Spot volumes of all gels were normalized by dividing the raw quantity of each spot by the total quantity of all valid spots in that gel. Finally, gel images were manually annotated.

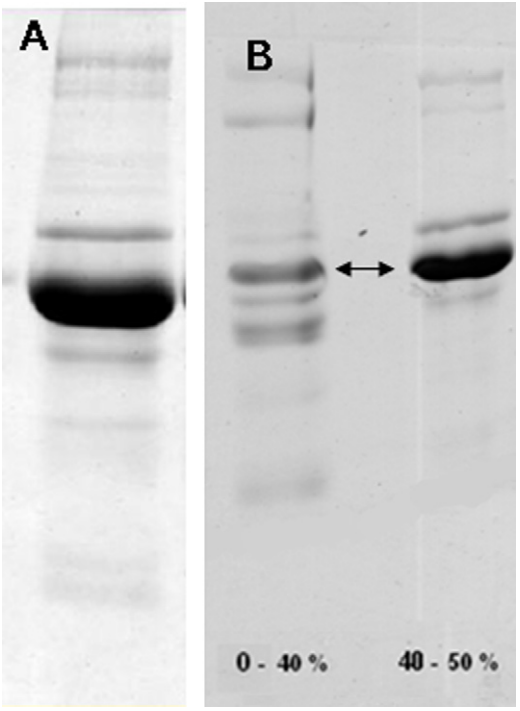

Fig. 1. SDS-PAGE images of intact blood plasma (A) and protein fractions that precipitate at 0–40% and 40–50% saturation (B). Albumin band is highlighted with an arrow.

2.5. Statistical analyses

Statistically significant differences between the depletion methods and with respect to the untreated plasma were determined by a Student's *t* test at a 95% confidence level. Statgraphics™ Plus 5.1 (Statistical Graphics Corp., USA, 2000) was used in all the statistical analyses.

3. Results and discussion

First, different saturation percentages of ammonium sulfate, beginning at 10% up to 80% in 10% steps, were used to screen the plasma protein precipitation fractions. The fractions were analyzed by SDS-PAGE (1D). Fig. 1 shows a 1D gel of intact blood plasma (Fig. 1A) and the protein fractions that precipitate at 0–40% and 40–50% saturation (Fig. 1B). Here the albumin band is highlighted with an arrow. Most of the albumin precipitated in the 40–50% fraction, while in the 0–40% fraction a much smaller albumin band was observed. Besides, in the 0–40% fraction it was possible to detect a larger number of bands (16 bands) as compared to the 40–50% fraction (8 bands). In the not treated plasma gel it was possible to detect 13 protein bands. At saturation percentages higher than 50% no bands were detected (data not shown). Based on these results, 40% was chosen as the saturation level that allowed the highest albumin depletion.

Fig. 2 shows representative 2D-PAGE images of not treated plasma (A) and the plasma fraction subjected to ammonium sulfate precipitation at 0–40% saturation (B). The gel zones where the highly abundant proteins are located are highlighted in both gel images. These zones were identified based on the 2D-PAGE images reported by Mahn et al. [13]. From Fig. 2 it is clear that the relative abundance of albumin (zone 3) and α-1-antitrypsin (zone 1) in the ASP-depleted plasma was considerably reduced

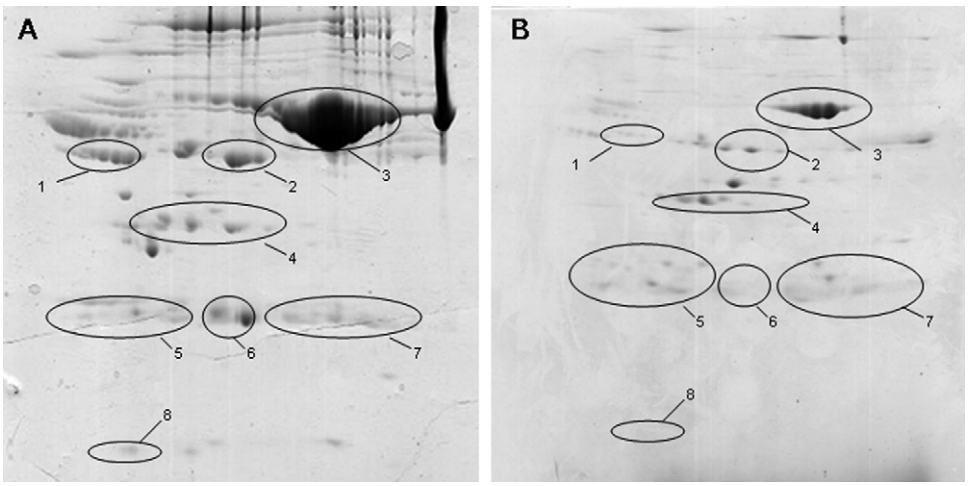

Fig. 2. Representative 2D-PAGE images of not treated plasma (A) and the plasma fraction subjected to ammonium sulfate precipitation at 0–40% saturation (B). Zone 1: α-1-antitrypsin; zone 2: fibrinogen; zone 3: albumin; zones 4 and 8: haptoglobin; zones 5 and 7: immunoglobulin; zone 6: apolipoprotein.

Table 1
Relative abundance of the highly abundant proteins using ammonium sulfate precipitation (ASP).

Protein	Untreated	ASP depleted	
	$V_n \pm SD$[a]	$V_n \pm SD$[a]	p-Value[b]
Albumin	7064 ± 266	4353 ± 183	0.0000
Immunoglobulin	215 ± 17	663 ± 108	0.0009
α-1-Antitrypsin	344 ± 65	61 ± 20	0.0003
Haptoglobin	94 ± 13	941 ± 158	0.0002
Fibrinogen	180 ± 4	477 ± 103	0.0045

[a] The relative abundance of the proteins was expressed as normalized spot volume (V_n). The reported values correspond to the average of three experiments. SD is the standard deviation.
[b] Statistically significant differences were determined by a Student's t test with a 95% confidence level.

in comparison with the not treated plasma. On the contrary, the relative abundance of apolipoprotein (zone 6) apparently increased in the ASP-depleted plasma. Haptoglobin (zones 4 and 8) and immunoglobulin (zones 5 and 7) were affected in a minor way.

Table 1 presents the relative abundance of the highly abundant proteins in the not treated and in the ASP-depleted plasma, as well as a statistical comparison between them. Statistically significant differences were detected between the relative abundance of the five highly abundant proteins considered (p-value < 0.05 in all cases). The relative abundances of albumin and α-1-antitrypsin were significantly lower in the ASP-depleted plasma compared to the not treated plasma, while that of immunoglobulin, haptoglobin and fibrinogen were higher. The increase in the relative abundance of these proteins responds to the lower solubility exhibited by albumin and α-1-antitrypsin compared with immunoglobulin, haptoglobin and fibrinogen. As a consequence, a higher mass of albumin and α-1-antitrypsin was depleted by ASP, and therefore a higher relative concentration of immunoglobulin, haptoglobin and fibrinogen was loaded in the 2D-PAGE gel. It has to be noted that, according to the protocol used in this work, the protein load in 2D-PAGE is limited to 300 μg per gel, and consequently depletion of one protein results in a decrease in the relative abundance of that protein but in an increase in the relative abundance of all the other proteins that remained in the sample. ASP depleted 39% albumin and 82% α-1-antitrypsin initially found in the not treated plasma. Then ASP at 40% saturation allows partially depleting some highly abundant proteins from blood plasma.

Table 2 shows a comparison of the performance of different separation techniques used for albumin depletion from blood plasma samples. The relative abundance of albumin obtained in ASP-depleted plasma was 30% lower than that obtained in HIC-depleted plasma, and was comparable to that obtained in immuno-affinity-depleted plasma (18% higher). In both immuno-affinity and ASP-depleted plasma the relative abundance of albumin was significantly lower (p-value = 0.000) than that found in not treated plasma (50% and 39% lower, respectively). Accordingly, ammonium sulfate precipitation at 40% saturation is useful for depleting albumin from plasma samples for 2D-PAGE analysis, showing a depletion percentage similar to that immuno-affinity yield. The number of spots that could be detected in 2D-PAGE of ASP-depleted plasma was significantly lower than that detected in the not treated plasma; however this number (59 spots) was very similar to that obtained in immuno-affinity-depleted plasma (61 spots). Besides, the reproducibility of ASP-depletion (4.2% variation of the relative abundance of albumin spot in 2D-PAGE) was very

Table 2
Comparison of the performance of the different separation techniques used for albumin depletion in blood plasma samples. A Student's t test at a 95% confidence level was used. p-Values are reported.

Treatment	Albumin relative abundance[b]	Nr detected spots[c]	Comparison with non-treated plasma (p-value)	
			Albumin abundance	Nr spots
No treatment	7064 ± 266	113 ± 21	–	–
Immuno-affinity[a]	3568 ± 117	61 ± 16	0.0000	0.0269
HIC[a]	6187 ± 780	110 ± 30	0.1391	0.8940
ASP	4353 ± 183	59 ± 5	0.0000	0.0124

[a] From Ref. [13].
[b] The relative abundance of albumin was expressed as normalized spot volume ($V_n \pm SD$). The reported values correspond to the average of three experiments. SD is the standard deviation.
[c] The reported values correspond to the average of three experiments (average ± SD). SD is the standard deviation.

close to that obtained in the not treated plasma (3.8%) and in the immuno-affinity-depleted plasma (3.3%), but it was considerably lower than that exhibited by HIC (12.6%). Thus, ASP-depletion exhibits a performance similar to that offered by immuno-affinity-depletion, which is the most popular and reliable plasma depletion method.

Mahn et al. [13] reported that HIC allowed depleting much of the immunoglobulin from plasma, but it was not as effective in albumin depletion. Since ASP allows depleting 39% albumin and 82% α-1-antitrypsin, both HIC and ASP could be combined to attain the depletion of the most abundant proteins in blood plasma prior proteomic analysis.

4. Conclusions

Ammonium sulfate precipitation at 40% saturation allowed partially depleting albumin and α-1-antitrypsin from blood plasma. It allowed detecting 59 valid spots in 2D-PAGE with a high reproducibility, resulting in 4.2% variation of the relative abundance of albumin spot. As a consequence, ASP-depletion exhibits a performance similar to that offered by commercial immuno-affinity columns for albumin depletion. Additionally, ASP-depletion seems to be complementary to HIC-depletion, and therefore a combination of both, namely an ASP-step followed by a HIC-step, could probably deplete the most highly abundant proteins found in blood plasma. This strategy would represent a simple and economic way to improve the detection of low abundance proteins by 2D-PAGE.

Acknowledgements

This work was partially supported by Department of Scientific and Technological Investigation (DICYT) of University of Santiago of Chile and FONDECYT Programme.

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