Fractional Precipitation of Plasma Proteome by Ammonium Sulphate: Case Studies in Leukemia and Thalassemia

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Abstract

Human plasma proteome is a comprehensive source of disease biomarkers. However, the >10 orders-wide dynamic concentration range of its constituent proteins necessitates depletion of abundant proteins from plasma prior to biomarker discovery. Our objective has been to develop a simple method that would deplete the most abundant proteins e.g. albumin and immunoglobulins, effectively facilitating identification of differentially regulated proteins in plasma samples. We employed ammonium sulphate based pre-fractionation of plasma followed by two-dimensional gel electrophoresis (2DGE) for comparison of normal proteins with those from the plasma samples of the patients, after identification of proteins by MALDI-TOF/TOF tandem mass spectrometry. Fractional precipitation of the plasma samples by 20% ammonium sulphate from raw plasma doubled the number of protein spots after 2DGE and led to identification of 87 unique proteins, including several low-abundance proteins. Case studies done with fractional precipitation of the plasma samples of patients suffering from hematological diseases e.g. leukemia and thalassemia indicate the utility of such pre-fractionation in the detection of differentially regulated proteins.

Keywords: Depletion technique; Blood plasma; Differential proteomics; Hematological malignancy; Hemoglobinopathy; 2DGE

Introduction

Blood plasma is a rich source of biochemical products that can indicate physiological or clinical status of a patient [1]. It is the most valuable specimen for protein biomarker determination because it is readily obtainable and contains thousands of protein species secreted from cells and tissues [2,3]. The discovery of protein biomarkers in plasma for diseases is challenging and requires a highly parallel display and quantization strategy for proteins [4-6] like two dimensional gel electrophoresis (2DGE). The protein content of serum however, is dominated by a handful of proteins such as albumin, immunoglobulins (IgG), and lipoproteins present across an extraordinary dynamic range of concentration. This exceeds the analytical capabilities of traditional proteomic methods, making detection of lower abundance serum proteins extremely challenging. Reduction of sample complexity is thus an essential first step in the analysis of plasma proteome [7].

Case Studies in Leukemia and Thalassemia

We could detect differential regulation of several proteins in leukemic and thalassemic plasma samples compared to normal controls which includes many differentially regulated proteins in leukemic plasma samples also identified earlier.

Materials and Methods

Fractionation of plasma proteins using ammonium sulphate

Blood plasma samples of healthy normal volunteers, and B-ALL, AML and HbEβ-thalassemia patients on de novo diagnosis, were collected from R.K. Mission Hospital and Clinical Hematology Service, Kolkata. Clinical details of normal individuals and patients are summarized in Supplementary material 1. Written consent was obtained from all of the participants, and the study was conducted in accordance with the principles of the Helsinki Declaration with the approval of the institutional ethics committee. Complete protease inhibitor cocktail (Roche Diagnostics, Germany) was added whenever plasma was stored at -80°C for later use. Plasma samples were centrifuged at 12000 g, 4°C, for 30 minutes, and the supernatants diluted with PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.4) to protein concentrations ~20 mg/ml. Diluted plasma samples were distributed into 1 ml aliquots. Next, 55, 113, 144, 176, 208, 242, 277, 314, and 351 milligrams of (NH₄)₂SO₄ were added with fractional precipitation of the plasma samples of patients suffering from hematological diseases e.g. leukemia and thalassemia.

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30 minutes with occasional mixing. The solutions were centrifuged at 12000 g, 4°C for 25 minutes, the supernatants taken in fresh tubes and the precipitate dissolved in minimum volume of solubilization buffer (5 mM sodium phosphate, 20 mM KCl, 1 mM EDTA, 0.2 mM DTT, and pH-8.0). The starting plasma, the supernatant and the solubilized ammonium sulphate precipitate, all three were dialysed overnight against 10 mM Tris, 5 mM KCl, pH-7.5, at 4°C.

Two dimensional gel electrophoresis and image analysis

After dialysis, the starting plasma, the supernatant and the solubilized ammonium sulphate precipitate, all three were mixed with equal volume of 2D sample buffer containing 8 M urea, 2% (w/v) CHAPS, 0.05% Bio-lyte 3-10 ampholyte, 20 mM DTT (Bio-Rad, Hercules, CA) and Protease inhibitor (Roche Diagnostics). The

![Figure 1](image-url)
protein concentrations of the samples were estimated using RC DC protein estimation kit (Bio-Rad), and an absolute amount of 1.8 mg for Coomassie staining, or 600 μg for silver staining, or 1.2 mg for SYPRO RUBY staining, was taken in a final volume of 350 μL. 17 cm pH 3-10 IPG strips (Bio-Rad) were passively rehydrated or cup-loaded with the plasma samples. IEF was carried out in a Protean IEF cell (Bio-Rad), stepwise up to 12000 Volt-Hours. Equilibration of the strips post IEF was performed following published protocol [12]. The second dimension was run on 8-16% polyacrylamide gradient gels in a Protean II XL electrophoresis module (Bio-Rad). Gels were stained either with Blue Silver Coomassie [13] or SYPRO-RUBY (Sigma) according to manufacturer’s instructions, or Silver stain according to the method of Rabilloud [14]. Image captures and analyses were done on Versa Doc series 3000 imaging system using PDQuest software (version 7.1, Bio-Rad). Densitometry analysis of the gel spots of interest was performed using the density tool of PDQuest. Spot volume (intensity) of the desired spot(s) was normalized as parts per million (ppm) of the total spot volume using the spots that were present in all gels, to calculate the relative abundance of a spot in a sample.

In-gel tryptic digestion and mass spectrometry

Sequencing grade trypsin was purchased from Promega (Madison, WI). All other reagents were purchased from Pierce (Rockford, USA). The protein spots from Coomassie and SYPRO-RUBY stained 2D gels of normal plasma were excised using a robotic spot-cutter (Bio-Rad). The gel pieces were de-stained with 50% acetonitrile, 25 mM ammonium bicarbonate. Subsequent in-gel tryptic digestion, peptide elution, acquisition of MS and MS/MS spectra and database searches were done following our published protocol [15]. Recrystallized CHCA and 2, 5-DHB (Sigma) were used as matrices. MS of the digested peptides was done in positive reflector mode in a MALDI-TOF/TOF tandem mass spectrometer (Applied Biosystems, AB 4700). Autotryptic and common keratin peaks were validated and subsequently excluded from MS/MS analysis. Twelve most intense peptides from each spot were subjected to MS/MS analysis. Peak lists were prepared from MS and MS/MS data using GPS explorer V3.6 (Applied Biosystems) software and noise reduction and de-isotoping were performed using default settings. Resulting PMF and MS/MS data were searched against human MSDB and Swiss-Prot databases using in-house MASCOT V2.1 (Matrix Science, UK) server and MOWSE score (with p<0.05) was considered to determine significant hits. For homologous proteins having similar MOWSE scores, preference was given to the protein with best match between theoretical and experimental molecular weight and pI. All MS experiments were repeated at least thrice, with spots excised from three separate gels. The database search parameters included one missed cleavage, error tolerance of ± 100 ppm for PMF and ± 1.2 Da for MS/MS ion search and variable modifications like carbamidomethyl cysteine, methionine oxidation, and N-terminal acetylation.

Western immunoblotting

Plasma protein samples (25 μg) were re-suspended in 30 μL SDS-PAGE buffer (2% mercaptoethanol (v/v), 1% SDS, 12% glycerol, 50 mM Tris-HCl and a trace amount of bromophenol blue), heated at 95°C for 5 min, cooled and loaded directly onto 12% gel. 1D-SDS-PAGE was performed in a Mini Protein III-cell (Bio-Rad) using Tris-glycine with 0.1% SDS, following manufacturer’s instructions. Proteins separated on gel were blotted onto PVDF membranes and subsequently blocked with Tris-buffer-saline (TBS), 5% non fat dry milk for 2h at room temperature. Primary antibodies (Abcam) were diluted in TBS/0.1% Tween (TBST) following manufacturer’s protocol. β-Tubulin was used as loading control. Anti-rabbit or anti-mouse HRP-conjugated IgGs were used as secondary antibodies (Abcam). Membranes were washed with TBST and detected by ECL (Pierce) with either the VersaDoc imager (BioRad) or on X-ray film development.

Results

Separation of pre-fractionated plasma proteins using 2DGE

1D-SDS-PAGE profiles of raw plasma, sub-fractions after (NH₄)₂SO₄ precipitations and respective supernatants showed more number of protein bands only in sub-fractions after 20% and 45% (NH₄)₂SO₄ precipitations (Supplementary material 2). We’ve chosen the sub-fraction after 20% (NH₄)₂SO₄ precipitation for further 2DGE analysis, which appeared to precipitate the maximum proportion of lower abundance proteins leaving most of the abundant proteins in solution. From 20 mg protein in raw plasma, 3.5 ± 0.8 mg was obtained in the 20% (NH₄)₂SO₄ precipitate while the supernatant retained the rest of it (15 ± 1.6 mg estimated). Both 1D and 2D profiles of raw diluted plasma, the fraction after 20% (NH₄)₂SO₄ precipitation and the supernatant after precipitation together revealed that (NH₄)₂SO₄ precipitates only a fraction of the whole plasma proteome. The particular fraction contained reduced load of abundant plasma proteins and was enriched with various minor proteins leaving the gel electrophoresis profile of the supernatant almost identical to that of raw plasma (Figure 1). The 1D-SDS-PAGE showed depletion of abundant proteins like albumin and IgG and enrichment/appearance of several low-abundance proteins including a tissue leakage protein, α-fetoprotein in the precipitated fraction (Figure 1A). The high percentage of albumin was found to be depleted (>80%) ensuring resolution of other proteins that were obscured by albumin in 2D gels, and minor proteins that were initially hidden by co-migration with albumin or smears became visible (Figures 1B-1D). The number of spots visible in 2D gels was increased.

![Image](316x91 to 567x361)

Figure 2: Annotated proteins in the fraction after 20% ammonium sulphate precipitation from normal plasma. Mass spectrometry details appear in Table 1.
doubled from 348 in raw plasma to 617 in the fraction precipitated by 20% (NH₄)₂SO₄ with various new spots appearing in the pI region 4.5-6.5 and between 10 kDa and 50 kDa molecular mass (Figure 1E).

We compared the 2DGE profiles of the fraction precipitated by 20% (NH₄)₂SO₄ and the albumin-depleted plasma after treatment with commercially available albumin depletion kit (ProteoPrep Blue Albumin & IgG Depletion kit, Sigma, St.Louis, MO). Supplementary material 3 provides the 2D gel images that shows better performance 20% (NH₄)₂SO₄ to justify the choice of conventional salting-out for enrichment of minor proteins in addition to depletion of abundant proteins from plasma, and preliminary screening of clinical samples. Although the ProteoPrep Blue Albumin and IgG Depletion kit specifically depleted albumin and IgG, the number of spots visible upon albumin-depletion did not increase appreciably as seen from the 2DGE profile.

Identification of plasma proteins by tandem mass spectrometry

Post (NH₄)₂SO₄ precipitation based pre-fractionation, a total of 87 proteins were identified from coomassie and SYPRO-RUBY stained 2D gels of normal plasma, by performing combined searches (MS + MS/MS) against MSDB, NCBI, Swissprot databases, as shown in Figure 2 and elaborated in Table 1. Of these 64 had significant scores (p ≤ 0.05) in the combined searches. Many of the rest 24 protein identifications were supported by either the published SWISS-2D-PAGE map of human plasma (marked with asterisk ‘*’ in Table 1), or ion score ≥ 20 of at least one MS/MS fragment, or other proteomic studies of blood plasma/serum [4,6,7] (marked with ‘**’). The list included many low abundance proteins that were undetectable in normal plasma prior to (NH₄)₂SO₄ precipitation. Low-abundance proteins present in amounts five to nine orders of magnitude lower than albumin, like

![Image](https://example.com/image.png)

**Figure 3:** Display of differentially regulated proteins in the blood plasma of patients suffering from B-ALL / AML / HbE-thalassemia compared to normal control.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Name of the Protein/Polypeptide</th>
<th>Accession Id.</th>
<th>Mr</th>
<th>pI</th>
<th>Mascot Score</th>
<th>Sequence Coverage</th>
<th>No. of MS/MS matches</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Serum Albumin-Human</td>
<td>1BKE</td>
<td>65,993</td>
<td>5.69</td>
<td>191(64)</td>
<td>64%</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Alpha-1-B-Glycoprotein-Human</td>
<td>Q68CK0_HUMAN</td>
<td>54,220</td>
<td>5.56</td>
<td>160(64)</td>
<td>48%</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>Alpha-1-antitrypsin-Human</td>
<td>AAB59495</td>
<td>46,677</td>
<td>5.43</td>
<td>110(64)</td>
<td>36%</td>
<td>8</td>
</tr>
<tr>
<td>4.</td>
<td>Vitronectin precursor-Human</td>
<td>SGHU1V</td>
<td>54,328</td>
<td>5.55</td>
<td>112(64)</td>
<td>23%</td>
<td>5</td>
</tr>
<tr>
<td>5.</td>
<td>Kininogen, HMW precursor</td>
<td>KGHUH1</td>
<td>71,900</td>
<td>6.34</td>
<td>115(64)</td>
<td>40%</td>
<td>6</td>
</tr>
<tr>
<td>6.</td>
<td>Prothrombin-Human</td>
<td>Q4QZ40_HUMAN</td>
<td>69,920</td>
<td>5.70</td>
<td>202(64)</td>
<td>48%</td>
<td>8</td>
</tr>
<tr>
<td>7.</td>
<td>Plasma protease C1 inhibitor (fragment)-Human</td>
<td>Q59E25_HUMAN</td>
<td>56,695</td>
<td>5.98</td>
<td>116(64)</td>
<td>27%</td>
<td>7</td>
</tr>
<tr>
<td>8.</td>
<td>Complement C1 inhibitor precursor-Human</td>
<td>ITHUC1</td>
<td>55,119</td>
<td>6.09</td>
<td>105(64)</td>
<td>29%</td>
<td>5</td>
</tr>
<tr>
<td>9.</td>
<td>Vitamin D binding protein-Human</td>
<td>Q53F31_HUMAN</td>
<td>52,916</td>
<td>5.34</td>
<td>221(64)</td>
<td>51%</td>
<td>8</td>
</tr>
<tr>
<td>10.</td>
<td>Alpha-2-HS-glycoprotein precursor-Human</td>
<td>WOHU</td>
<td>39,300</td>
<td>5.43</td>
<td>158(64)</td>
<td>40%</td>
<td>5</td>
</tr>
<tr>
<td>11.</td>
<td>Haptoglobin precursor-Human</td>
<td>HPHU1</td>
<td>36,427</td>
<td>6.13</td>
<td>242(64)</td>
<td>29%</td>
<td>5</td>
</tr>
<tr>
<td>12.</td>
<td>Human apolipoprotein-A-I</td>
<td>AAA51748</td>
<td>43,358</td>
<td>5.22</td>
<td>494(64)</td>
<td>61%</td>
<td>9</td>
</tr>
<tr>
<td>13.</td>
<td>Complement component C3d-Human</td>
<td>1C3D</td>
<td>32,845</td>
<td>6.34</td>
<td>219(64)</td>
<td>44%</td>
<td>6</td>
</tr>
<tr>
<td>14.</td>
<td>Haptoglobin precursor-Human</td>
<td>HPHU1</td>
<td>36,427</td>
<td>6.13</td>
<td>75(64)</td>
<td>14%</td>
<td>1</td>
</tr>
<tr>
<td>15.</td>
<td>Complement component C3b-Human</td>
<td>S27041</td>
<td>25,280</td>
<td>4.49</td>
<td>49(64)</td>
<td>55%</td>
<td>4</td>
</tr>
<tr>
<td>16.</td>
<td>1-microglobulin</td>
<td>HCHU</td>
<td>38,974</td>
<td>5.95</td>
<td>95(64)</td>
<td>35%</td>
<td>5</td>
</tr>
<tr>
<td>17.</td>
<td>APOA1 protein (fragment)-Human</td>
<td>CAA00975</td>
<td>28,061</td>
<td>5.27</td>
<td>470(64)</td>
<td>81%</td>
<td>11</td>
</tr>
<tr>
<td>18.</td>
<td>Serum amyloid P-Human</td>
<td>YLHUP</td>
<td>25,371</td>
<td>6.10</td>
<td>181(64)</td>
<td>30%</td>
<td>4</td>
</tr>
<tr>
<td>19.</td>
<td>Preproapolipoprotein A-I</td>
<td>LPHUA1</td>
<td>30,759</td>
<td>5.56</td>
<td>173(64)</td>
<td>59%</td>
<td>7</td>
</tr>
<tr>
<td>20.</td>
<td>Transfhyretin chain A-Human</td>
<td>2TRYA</td>
<td>13,829</td>
<td>5.35</td>
<td>216(64)</td>
<td>94%</td>
<td>5</td>
</tr>
<tr>
<td>21.</td>
<td>Human α fetoprotein</td>
<td>E973181</td>
<td>66,358</td>
<td>5.67</td>
<td>264(64)</td>
<td>49%</td>
<td>6</td>
</tr>
<tr>
<td>22.</td>
<td>Transferrin-Human</td>
<td>Q53H26_HUMAN</td>
<td>77,030</td>
<td>6.68</td>
<td>339(64)</td>
<td>51%</td>
<td>8</td>
</tr>
<tr>
<td>23.</td>
<td>Fibrinogen β chain-Human</td>
<td>FGHUH</td>
<td>55,892</td>
<td>8.54</td>
<td>592(64)</td>
<td>49%</td>
<td>10</td>
</tr>
<tr>
<td>24.</td>
<td>Fibrinogen α chain-Human</td>
<td>FGHUA</td>
<td>69,714</td>
<td>8.23</td>
<td>508(64)</td>
<td>38%</td>
<td>8</td>
</tr>
<tr>
<td>25.</td>
<td>Fibrinogen β chain fragment d-Human</td>
<td>1FZAB</td>
<td>35,875</td>
<td>7.66</td>
<td>253(64)</td>
<td>79%</td>
<td>5</td>
</tr>
<tr>
<td>26.</td>
<td>Voltage gated Ca channel q23 subunit-Human</td>
<td>Q81Z8_HUMAN</td>
<td>122,933</td>
<td>5.53</td>
<td>65(64)</td>
<td>29%</td>
<td>0</td>
</tr>
<tr>
<td>27.</td>
<td>Fibrinogen chain extended splice form-Human</td>
<td>D44234</td>
<td>94,914</td>
<td>5.70</td>
<td>204(64)</td>
<td>35%</td>
<td>4</td>
</tr>
<tr>
<td>28.</td>
<td>Immunoglobulin κ light chain VLJ region-Human</td>
<td>BAC01677</td>
<td>27,574</td>
<td>7.53</td>
<td>160(64)</td>
<td>33%</td>
<td>3</td>
</tr>
<tr>
<td>29.</td>
<td>Immunoglobulin κ chain V-III region (B6)-Human</td>
<td>K3HB6</td>
<td>11,628</td>
<td>9.34</td>
<td>59(64)</td>
<td>16%</td>
<td>1</td>
</tr>
<tr>
<td>30.</td>
<td>Haptoglobin precursor-Human</td>
<td>HPHU2</td>
<td>45,177</td>
<td>6.13</td>
<td>71(64)</td>
<td>53%</td>
<td>5</td>
</tr>
<tr>
<td>31.</td>
<td>Immunoglobulin κ chain NIG26 precursor-Human</td>
<td>JEO242</td>
<td>23,504</td>
<td>5.46</td>
<td>203(64)</td>
<td>48%</td>
<td>3</td>
</tr>
<tr>
<td>32.</td>
<td>Immunoglobulin λ light chain variable region (fragment)-Human</td>
<td>AAD16673</td>
<td>11,505</td>
<td>5.67</td>
<td>32(64)</td>
<td>25%</td>
<td>1</td>
</tr>
<tr>
<td>33.</td>
<td>Hemoglobin α chain (fragment)-Human</td>
<td>Q8BX3_HUMAN</td>
<td>10,703</td>
<td>7.07</td>
<td>87(64)</td>
<td>60%</td>
<td>3</td>
</tr>
<tr>
<td>34.</td>
<td>Hemoglobin β chain-Human</td>
<td>2HBSB</td>
<td>15,827</td>
<td>7.26</td>
<td>143(64)</td>
<td>82%</td>
<td>6</td>
</tr>
<tr>
<td>35.</td>
<td>Ig light chain VLJ region (fragment)-Human</td>
<td>BAC01701</td>
<td>29,183</td>
<td>8.84</td>
<td>127(64)</td>
<td>39%</td>
<td>4</td>
</tr>
<tr>
<td>36.</td>
<td>Anti RhD monoclonal T125 κ light chain precursor-Human</td>
<td>Q5EFE6_HUMAN</td>
<td>25,682</td>
<td>8.70</td>
<td>56(64)</td>
<td>41%</td>
<td>2</td>
</tr>
<tr>
<td>37.</td>
<td>Fibrinogen fragment d, chain C-Human</td>
<td>1FZEC</td>
<td>34,457</td>
<td>5.68</td>
<td>64(64)</td>
<td>56%</td>
<td>2</td>
</tr>
<tr>
<td>38.</td>
<td>Fibrinogen fragment d, chain B-Human</td>
<td>1FZAB</td>
<td>35,875</td>
<td>7.66</td>
<td>137(64)</td>
<td>53%</td>
<td>5</td>
</tr>
<tr>
<td>39.</td>
<td>Fibrinogen fragment d, chain F-Human</td>
<td>1FZEF</td>
<td>34,343</td>
<td>5.68</td>
<td>109(64)</td>
<td>54%</td>
<td>4</td>
</tr>
<tr>
<td>40.</td>
<td>Fibrinogen fragment d, chain C-Human</td>
<td>1FZAC</td>
<td>35,144</td>
<td>5.57</td>
<td>91(64)</td>
<td>66%</td>
<td>3</td>
</tr>
<tr>
<td>41.</td>
<td>Fibrinogen beta chain precursor</td>
<td>FIBB_HUMAN</td>
<td>55,892</td>
<td>8.54</td>
<td>107(53)</td>
<td>48%</td>
<td>5</td>
</tr>
<tr>
<td>42.</td>
<td>Fibrinogen γ A chain precursor-Human</td>
<td>FGHUG</td>
<td>49,465</td>
<td>5.70</td>
<td>313(64)</td>
<td>55%</td>
<td>9</td>
</tr>
<tr>
<td>43.</td>
<td>ALB protein (Growth inhibiting protein 20)-Human</td>
<td>Q86YGO_HUMAN</td>
<td>47,330</td>
<td>5.97</td>
<td>170(64)</td>
<td>39%</td>
<td>8</td>
</tr>
</tbody>
</table>
44. Apolipoprotein-A-IV precursor-Human & LPHUA4 & 45,307 & 5.23 & 107(64) & 58% & 5
45. α1-antitrypsin precursor-Human & ITHU & 46,707 & 5.37 & 248(64) & 55% & 8
46. Fibrinogen γ B chain precursor-Human & FGHUGB & 51,479 & 5.37 & 343(64) & 64% & 8
47. Coagulation factor XIII chain b precursor-Human & KFHU13 & 75,442 & 5.97 & 94(64) & 34% & 3
48. Plasminogen-Human & QSTEH4_HUMAN & 90,510 & 7.04 & 90(64) & 40% & 6
49. Fibrinectin 1-Human & Q60FE4_HUMAN & 252,848 & 5.66 & 224(64) & 26% & 13
50. α1-antitrypsin precursor-Human & ITHU & 46,707 & 5.37 & 248(64) & 55% & 8
51. Replication licensing factor MCM2-Human & S42228 & 99,174 & 5.97 & 68(64) & 21% & 0
52. (P02735) Serum amyloid A protein precursor-Human & SAA_HUMAN & 13,524 & 6.28 & 157(64) & 52% & 2
53. (P01574) Interferon beta precursor (IFN-beta) (Fibroblast interferon)-Human & IFNB_HUMAN & 22,279 & 8.93 & 53(53) & 28% & 0
54. (P61011) Signal recognition particle 54 kDa protein (SRP54) & SRP54_HUMAN & 55,668 & 8.87 & 53(53) & 16% & 0
55. Hypothetical protein DKFZp770N0926-Human & Q7Z664_HUMAN & 45,064 & 5.76 & 547(64) & 46% & 6
56. Collagen alpha 1(XI) chain precursor - human & CGHU1E & 181,029 & 5.11 & 53(64) & 21% & 2
57. Nicotinic acetylcholine receptor epsilon chain precursor - human & S34775 & 54,581 & 5.09 & 43(64) & 16% & 1
58. Glutathione S-transferase A2 & GSTA2_HUMAN & 25,531 & 8.54 & 33(64) & 85% & 1
59. SET domain, bifurcated 1-Human & Q5SZD8_HUMAN & 27,685 & 4.85 & 45(64) & 24% & 1
60. Adenylate Kinase 1-Human & Q5T9B7_HUMAN & 23,396 & 8.78 & 29(64) & 29% & 2
61. Interleukin-14 precursor –Human & A48203 & 54,723 & 9.32 & 26(64) & 27% & 1
62. 3' Histone mRNA exonuclease1 & THEX1_HUMAN & 69,907 & 6.32 & 45(64) & 26% & 1
63. AB009303 NID membrane-type matrix metalloproteinase 3 & BAA23742 & 69,907 & 6.32 & 45(64) & 26% & 1
64. Leucine-rich PPR motif-containing protein-Human & Q7Z7A6_HUMAN & 157,805 & 5.81 & 53(64) & 17% & 1
65. BC015875 NID-Human (Selenoprotein P) & AAH15875 & 43,157 & 7.59 & 51(64) & 26% & 1
66. 1-Phosphatidylinositol-4-phosphate 5-kinase-Human & A55967 & 46,163 & 7.70 & 24(64) & 9% & 1
67. HSP63G13 NID (p63 protein)-Human & AAG45609 & 55,652 & 6.41 & 60(64) & 25% & 1
68. (Q9UPY3) Endoribonuclease Dicer-Human & DICER_HUMAN & 217,490 & 5.45 & 47(53) & 11% & 1
69. (P58340) Myeloid leukemia factor 1 MLF1_HUMAN & 30,608 & 9.46 & 37(53) & 34% & 2
70. Matrix metalloprotease MMP-27 & Q9H306_HUMAN & 58,986 & 8.83 & 36(64) & 23% & 2
71. Apolipoprotein E precursor (ApoE) & APOE_HUMAN & 103,294 & 6.41 & 60(64) & 25% & 1
72. T-cell receptor β-chain precursor & CA71260 & 15,318 & 6.07 & 46(64) & 43% & 1
73. Homeobox protein CHX10 & CHX10_HUMAN & 39,386 & 7.11 & 33(53) & 17% & 1
74. Ephrin-A1 precursor & EFNA1_HUMAN & 23,756 & 6.49 & 41(53) & 36% & 1
75. Intestinal alkaline phosphatase precursor PPBI_HUMAN & 56,776 & 5.53 & 36(53) & 18% & 1
76. Ficolin 3 precursor & FCN3_HUMAN & 32,868 & 6.20 & 96(64) & 22% & 4
77. Complement component C4 fragment & QG55F8_HUMAN & 32,378 & 8.50 & 148(64) & 37% & 4
78. Fibrinogen gamma chain precursor & FIBG_HUMAN & 51,479 & 5.37 & 226(64) & 30% & 6
79. Transferritin precursor / Prealbumin (multimer) & TTHY_HUMAN & 15,877 & 5.52 & 214(64) & 81% & 3
80. Adrenocorticotrophic hormone (ACTH) (glycosylation shifts Mr and pI) & CAA00890 & 4,692 & 8.34 & 112(64) & 78% & 1
81. Alpha-2-macroglobulin precursor (Alpha-2-M) & A2MG_HUMAN & 163,175 & 6.00 & 74(53) & 18% & 2
82. Inter-alpha-trypsin inhibitor heavy chain H4 precursor & ITH4_HUMAN & 103,294 & 6.51 & 73(53) & 26% & 3
83. Complement C3 precursor & CO3_HUMAN & 187,046 & 6.02 & 80(53) & 25% & 3
84. Alpha-1-antichymotrypsin precursor (Orosomucoid 1) & A1AG1_HUMAN & 23,497 & 4.93 & 130(53) & 31% & 3
85. histidine-rich glycoprotein 1 precursor – human (glycosylation shifts pI) & KGHUGH & 59,541 & 7.09 & 137(64) & 22% & 4
86. Sodium/hydrogen exchanger 2 (NHE-2) & SLHA2_HUMAN & 91,461 & 9.20 & 62(53) & 13% & 2
87. C4A2 (C4A3) & Q6U2F0_HUMAN & 58,393 & 5.67 & 77(64) & 35% & 6

* - protein identifications supported by the published SWISS-2D-PAGE map of human plasma
** - protein identifications supported by other proteomic studies of blood plasma/serum (Ref. 4, 6, 7)

| Table 1: Protein Identifications from 20% (NH4)2SO4 precipitate of normal plasma by 2DGE-MALDI ToF/ToF Tandem Mass Spectrometry. |
serum amyloid P, vitamin D binding protein, interleukins, interferons, tissue leakage proteins (e.g. α-fetoprotein), ion channels and hormones were detected and identified from the fraction after 20% (NH₄)₂SO₄ precipitation, separated on 2D gels. This reflects a significant gain in the dynamic range of plasma proteins visualized in 2-D gels following 20% (NH₄)₂SO₄ precipitation. We could detect and identify some important blood plasma constituents, like fibrinogen γ-chain and immunoglobulin λ-light chain, that were absent from Anderson and co-workers’ report of an exhaustive list of proteins detected and/or identified in plasma [16]. All the 87 proteins were searched for their molecular function, biological process and localization in the PANTHER classification system database17 indicating the identified proteins to be involved in multiple biological processes like blood coagulation, cargo transport, proteolysis, signal transduction, cell-adhesion, immunity/defense, etc.

**Display of differentially regulated proteins in patient plasma**

The clinical features of the B-ALL, AML, HbEβ-thalassemia patients and the normal controls are summarized in Supplementary material 1. As shown in Figure 3, a comparison between the fraction of raw plasma proteome fraction after 20% (NH₄)₂SO₄ precipitation, obtained from normal, B-ALL, AML and HbEβ-thalassemia samples revealed ~20 differentially regulated proteins. Differences in mean ppm spot volumes between normal controls and patient samples for all protein spots were subjected to unpaired two-tail student’s t-test. Due to the inherent complexity of a 2D gel-based proteomic studies, we have only concentrated on the spots which were significantly different (p ≤ 0.01) between normal and patient plasma sub-proteomes. We observed down-regulation of transferrin, albumin, immunoglobulin heavy chains, apolipoprotein A1 (Apo-A1), transthyretin, α₁-B-glycoprotein, α₂-HS-glycoprotein (AHSG); and up-regulation of α₁-antitrypsin, haptoglobin, interferon-β (INF-β), glutathione-s-transferase (GST), SET domain bifurcated (SETDB), adenylate kinase-1 (AK-1), T-cell receptor-β (TCR-β) in the plasma of B-ALL patients as compared to normal plasma; shown as histogram plots in Figure 4. 2D profiles of samples from AML (hatched bars in Figure 4) and HbEβ-thalassemia (hollow bars in Figure 4) patient plasmas indicated opposite trend of differential regulation of most of these proteins, pointing towards the specificity of the observations.

**Validation by western immunoblotting**

To confirm the results obtained from 2DGE experiments, we quantitated the amounts of four differentially regulated proteins in raw plasma, obtained from a separate set of 3 normal controls and 4 B-ALL patients, using western immunoblotting. Supplementary material 4 shows the immunoblots for 5 proteins with β-tubulin as loading control, and histogram plot of the band intensities. All data were subjected to unpaired two-tail student’s t-test and the changes were found to be significant (p≤0.05). The immunoblots clearly supported results from 2DGE experiments. The four proteins: transferrin, α₁-antitrypsin, Apo-A1 and albumin, were chosen as representatives for proteolysis-modulating, carrier and acute phase proteins exhibiting differential regulation in B-ALL plasma 2D profiles.

**Discussion**

Since proteins differ markedly in their solubility at high ionic strength, salting-out has been the most efficient, time-tested and useful procedure for protein enrichment. The advantage of (NH₄)₂SO₄ is its high water-solubility leading to high ionic strength, and low heat of solvation protecting most proteins from denaturation [17]. This simple inexpensive fractionation of plasma proteins with depletes most of the high-abundance proteins e.g. albumin leading to an increase in low-abundance components, as also observed earlier [11]. As evident in this study, 20% (NH₄)₂SO₄ precipitation led to a representative fraction of the plasma proteome effectively facilitating detection of differentially-regulated protein markers in patient plasma samples with identification of several low-abundance proteins. The composition of the plasma proteome fraction after 20% (NH₄)₂SO₄ precipitation depends primarily on the quantity and solubility of the constituent proteins initially present in the sample, irrespective of the source or nature of the starting material. The fact that (NH₄)₂SO₄ does not differentially deplete plasma proteins from sample to sample has been apparent from the immunoblots of raw undepleted plasma samples, shown in Supplementary material 4. Our investigation of differential regulations in two different-lineage hematological malignancies, i.e. ALL & AML, and an unrelated blood disorder with similar symptoms viz. HbEβ-thalassemia, establishes the specificity of the observed de-regulations with respect to the disease. The specificity of the observations adds an extra line of evidence to the suitability of 20% (NH₄)₂SO₄ precipitation for detection of disease biomarkers in patient plasma samples. Immunoaffinity-based chromatography effectively depletes high-abundance proteins from the plasma, but even these expensive, laborious and time consuming commercially available methods fail to completely remove high-abundance components and suffer from
their own limitations of specificity [18-21]. Our approach has been to use a simple, cost-effective method to obtain plasma fractions with reduced content of abundant proteins and maximum number of well-resolved spots on 2D gels. Although (NH₄)₂SO₄ fractionation does not specifically deplete or remove a particular protein or class of proteins, however, it also does not show preference towards a particular protein mixture, irrespective of sample type (normal or patient). It treats two different types of body fluid samples e.g. plasma & urine, differently, but remains unbiased towards the source e.g. from patients or from normal volunteers. Thus, it could be effectively used for differential proteomics in clinical studies. Use of combinatorial peptide ligand libraries for depletion of abundant proteins and accessing low-abundance biomarkers in clinical proteomics studies of blood plasma [22-24] further supports our notion that any pre-fractionation strategy for plasma could come handy to increased access to disease-markers apart from depletion of high abundance components.

Many of our observations in disease plasma were supported by earlier reports. Haptoglobin up-regulation in AML, CML, and multiple myeloma has also been reported in earlier studies [25-27]. While 2D profiles support the up-regulation of haptoglobin β-chain in AML plasma [26], we emphasize on haptoglobin α-chain that exhibits opposite trends of de-regulation in AML/HbEβ-thalassemia and B-ALL. This further highlights the application of (NH₄)₂SO₄ precipitation for preliminary screening of patient plasma samples. AHSG is reported to be down-regulated in AML, ALL, NHL and multiple myeloma patients [25,26,28]. We observed down-regulation of AHSG in B-ALL plasma in contrast to an up-regulation in AML/HbEβ-thalassemia plasma (Figure 4). Since lymphoblasts fail to mature into antibody-secreting plasma cells in B-ALL, the patients show significant down-regulation of immunoglobulin heavy chains in plasma. In contrast, immunoglobulin heavy chains are up-regulated in AML/HbEβ-thalassemia patient sera [26]. Additionally, Apo-A1 is down-regulated in B-ALL plasma contrary to the observation in AML (Figure 4). Hence IgG and Apo-A1 can serve as important biomarkers of B-ALL. Up-regulation of transferrin, AHSG and AK-1, vs. down-regulation of transferritin, Apo-A1, Apo-E and TCR-β in HbEβ-thalassemia plasma, compared to normal controls, are all preliminary reports that warrant further investigations with increased sample size. As most of the de-regulated proteins participate in multiple physiological processes like proteolysis, cargo-transport and iron homeostasis, their de-regulation might enlighten clinical manifestation of the disease. The western immunoblots qualitatively supported the 2DGE results but showed quantitative discrepancies in the degrees of deregulation of the proteins, most likely attributable to differences in the protein loads and detection limits of the two techniques. Moreover, immunoblot confirmation using a separate set of B-ALL patients and normal controls further emphasizes on the prospects of the reported de-regulations as potential diagnostic and prognostic indicators of the respective diseases, and that the differences do not arise out of the plasma pre-fractionation technique used. In conclusion, 20% ammonium sulphate precipitation shows prospects of accelerating the preliminary screening and detection of disease biomarkers in blood plasma. We further emphasis upon the assets of proteomic studies over single protein detection assays in revealing differential regulation of different classes of proteins, simultaneously in a disease, which might be a step ahead in cutting through the complexity diseases and explaining their pathophysiology and clinical manifestation. This study also reports for the first time a 2DGE based proteomic investigation of B-ALL and HbEβ-thalassemia.

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References


