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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].

There have been three main methods of depleting abundant proteins from serum samples: affinity removal method [1,4,8-10]; membrane filtration method to separate low-mass proteins from high-mass ones [7]; and multidimensional chromatographic fractionation [3,4,6]. But all these methods are expensive, laborious and time-consuming, as depletion of multiple abundant proteins from each plasma sample requires multiple technical steps. Besides, all these studies were mainly concentrated on the depletion of high-abundance proteins, primarily albumin and IgG, with little attention to detection of low-abundance biomarker proteins. We have employed 20% ammonium sulphate precipitation for rapid depletion of abundant proteins from plasma. Fountoulakis and coworkers have earlier reported fractionation of plasma proteins with 50% and 70% ammonium sulphate to reduce concentrations of high-abundance components and enrich lower abundant components in plasma 2D profiles, thereby facilitating the identification of disease markers [11]. Unlike other albumin-depletion studies, we checked the efficiency of our method in detecting differentially regulated plasma proteins in hematological malignancies like B-cell acute lymphoblastic leukemia (B-ALL), acute myeloid leukemia (AML) and in the hemoglobinopathy, HbEβ–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 KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 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 $(NH_4)_2SO_4$ were added to different aliquots for attaining 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, and 55% salt concentrations respectively, and incubated on ice for

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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

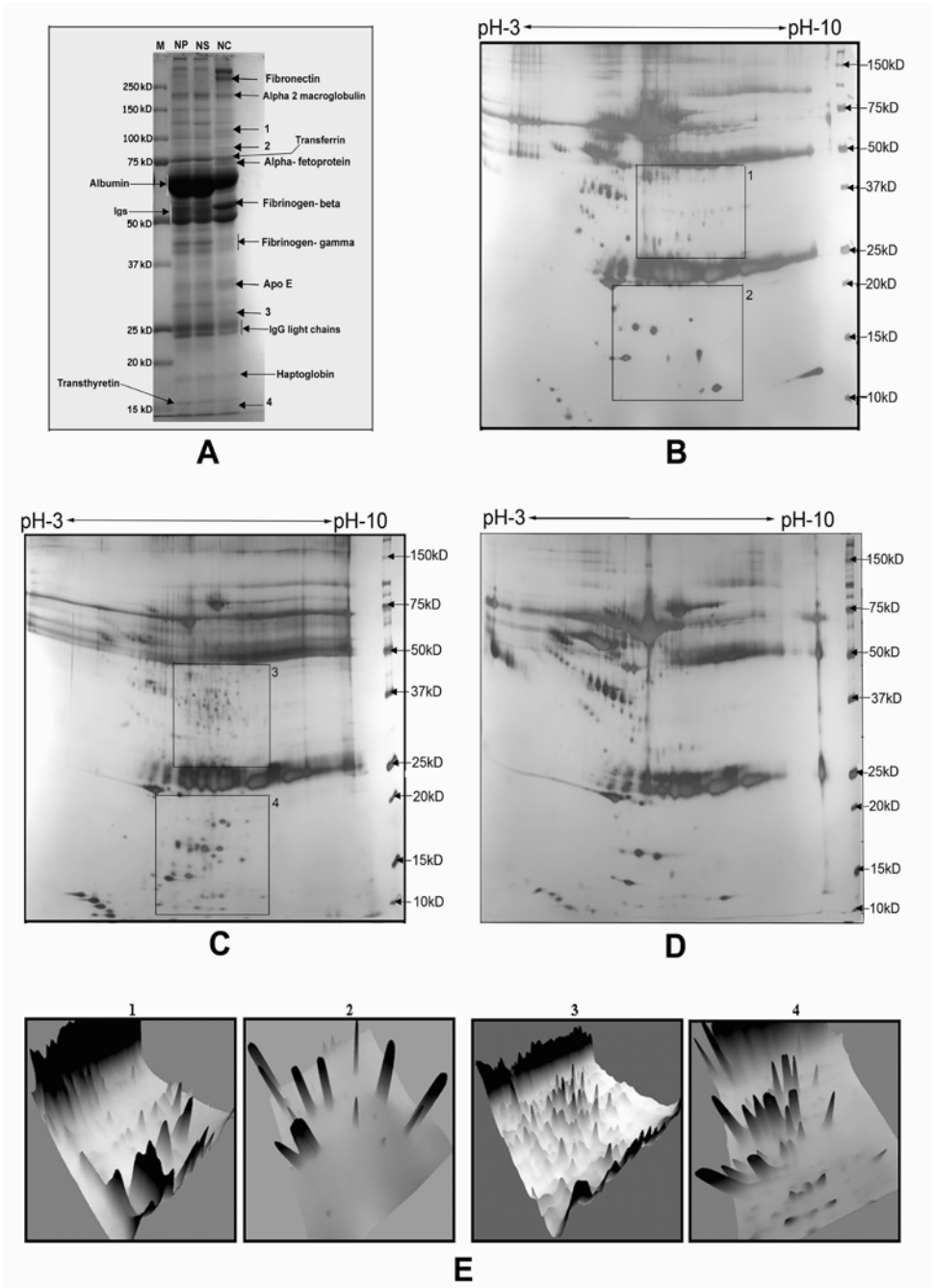

Figure 1: 1A. Coomassie stained 1D SDS-PAGE of raw plasma, the ammonium sulphate precipitate and supernatant. Lane1(from left)- MW marker (M); Lane2- raw normal plasma (NP); Lane 3- supernatant left after 20% $(NH_4)_2SO_4$ precipitation from normal plasma (NS), Lane 4- 20% $(NH_4)_2SO_4$ precipitate/cut from normal plasma (NC).
1B. Silver stained 2D profile of raw normal plasma.
1C. Silver stained 2D profile of 20% $(NH_4)_2SO_4$ precipitate from normal plasma.
1D. Silver stained 2D profile of supernatant left after 20% $(NH_4)_2SO_4$ precipitation.
1E. 3D view of boxed regions in 1B and 1C

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 120000 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 ammoniun 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 Protean 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 $(NH_4)_2SO_4$ precipitations and respective supernatants showed more number of protein bands only in sub-fractions after 20% and 45% $(NH_4)_2SO_4$ precipitations (Supplementary material 2). We've chosen the sub-fraction after 20% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ precipitation and the supernatant after precipitation together revealed that $(NH_4)_2SO_4$ 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

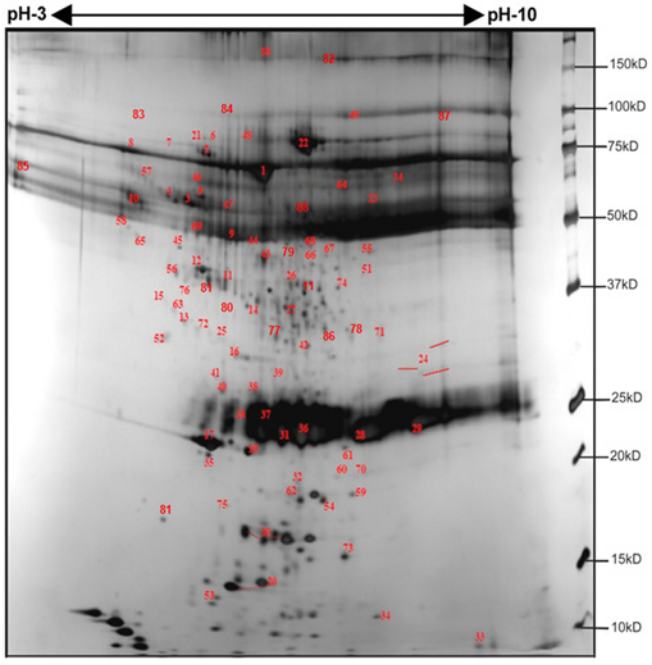

Figure 2: Annotated proteins in the fraction after 20% ammonium sulphate precipitation from normal plasma. Mass spectrometry details appear in Table 1.

doubled from 348 in raw plasma to 617 in the fraction precipitated by 20% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 ($p \leq 0.05$) 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 $(NH_4)_2SO_4$ precipitation. Low-abundance proteins present in amounts five to nine orders of magnitude lower than albumin, like

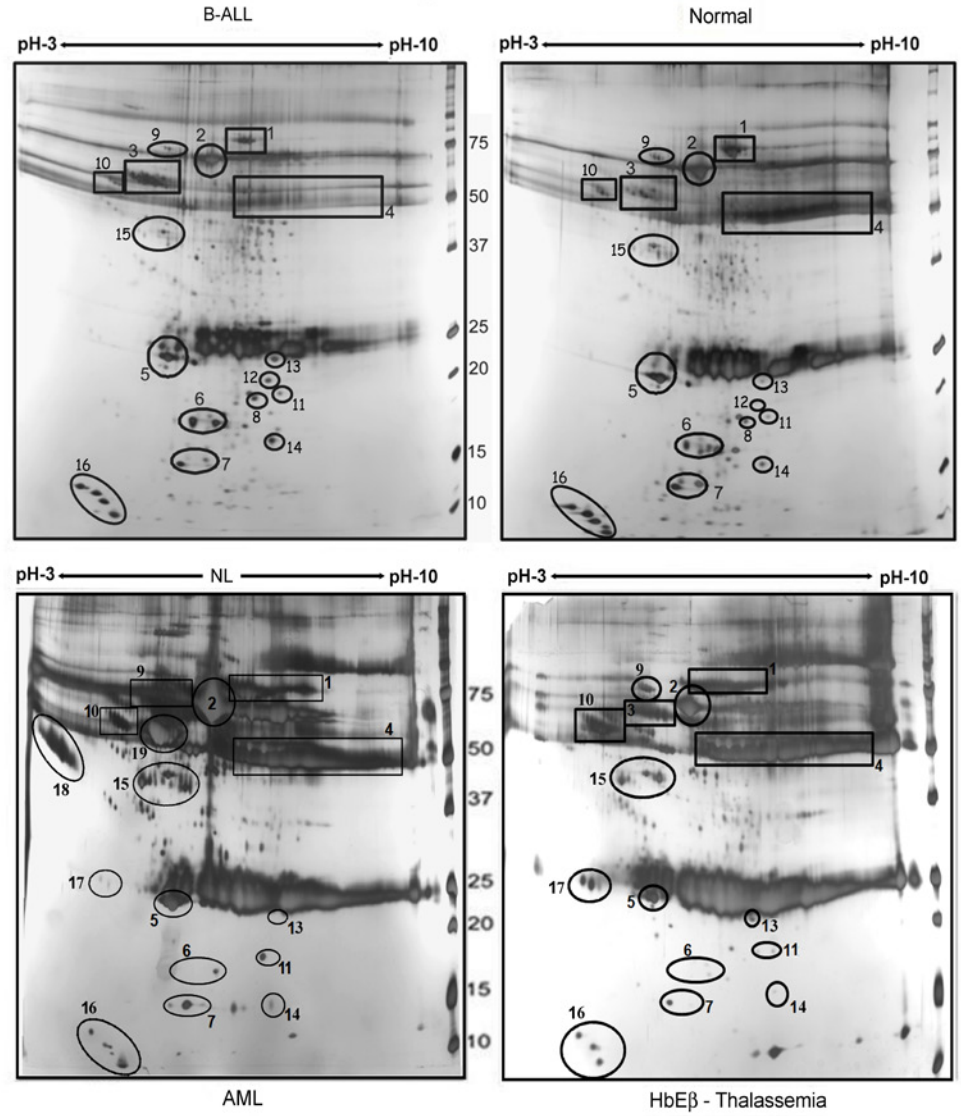

Figure 3: Display of differentially regulated proteins in the blood plasma of patients suffering from B-ALL / AML / HbEβ-thalassemia compared to normal control.

1:Transferrin, 2:Albumin, 3: α1-antitrypsin, 4:IgG heavy chains, 5:Apo-A1, 6:Haptoglobin α-chains, 7:Transthyretin, 8:Retinol binding protein 9:Interferon-β, 10:α1-B glycoprotein, 11:α2-HS glycoprotein, 12:Glutathione-S-transferase, 13:SET domain bifurcated, 14:Adenylate kinase-1, 15:T-cell receptor α-chain, 16:Haptoglobin α-chains, 17:Apo-E, 18:Apo-D, 19: Orosomucoid, 20: Fibrinogen α-chain.

Spot No.	Name of the Protein/Polypeptide	Accession Id.	Mr	pI	Mascot Score	Sequence Coverage	No. of MS/MS matches
1.	Serum Albumin-Human	1BKE	65,993	5.69	191(64)	64%	9
2.	Alpha-1-B-Glycoprotein-Human	Q68CK0_HUMAN	54,220	5.56	160(64)	48%	7
3.	Alpha-1-antitrypsin-Human	AAB59495	46,677	5.43	110(64)	36%	8
4.	Vitronectin precursor-Human	SGHU1V	54,328	5.55	112(64)	23%	5
5.	Kininogen, HMW precursor	KGHUH1	71,900	6.34	115(64)	40%	6
6.	Prothrombin-Human	Q4QZ40_HUMAN	69,920	5.70	202(64)	48%	8
7.	Plasma protease C1 inhibitor (fragment)-Human	Q59EI5_HUMAN	56,695	5.98	116(64)	27%	7
8.	Complement C1 inhibitor precursor-Human	ITHUC1	55,119	6.09	105(64)	29%	5
9.	Vitamin D binding protein-Human	Q53F31_HUMAN	52,916	5.34	221(64)	51%	8
10.	Alpha-2-HS-glycoprotein precursor-Human	WOHU	39,300	5.43	158(64)	40%	5
11.	Haptoglobin precursor-Human	HPHU1	38,427	6.13	242(64)	29%	5
12.	Human apolipoprotein-A-IV	AAA51748	43,358	5.22	494(64)	61%	9
13.	Complement component C3d-Human	1C3D	32,845	6.34	219(64)	44%	6
14.	Haptoglobin precursor	HPHU1	38,427	6.13	75(64)	14%	1
15.	Complement component C3b-Human	S27041	25,280	4.49	49(64)	55%	4
16.	1-microglobulin	HCHU	38,974	5.95	95(64)	35%	5
17.	APOA1 protein (fragment)-Human	CAA00975	28,061	5.27	470(64)	81%	11
18.	Serum amyloid P-Human	YLHUP	25,371	6.10	181(64)	30%	4
19.	Preproapolipoprotein A1-Human	LPHUA1	30,759	5.56	173(64)	59%	7
20.	Transthyretin chain A-Human	2TRYA	13,829	5.35	216(64)	94%	5
21.	Human α fetoprotein	E973181	66,358	5.67	264(64)	49%	6
22.	Transferrin-Human	Q53H26_HUMAN	77,030	6.68	339(64)	51%	8
23.	Fibrinogen β chain-Human	FGHUB	55,892	8.54	592(64)	49%	10
24.	Fibrinogen α chain-Human	FGHUA	69,714	8.23	508(64)	38%	8
25.	Fibrinogen β chain fragment d-Human	1FZAB	35,875	7.66	253(64)	79%	5
26.	Voltage gated Ca channel α23 subunit-Human	Q8IZS8_HUMAN	122,933	5.53	65(64)	29%	0
27.	Fibrinogen α chain extended splice form-Human	D44234	94,914	5.70	204(64)	35%	4
28.	Immunoglobulin κ light chain VLJ region-Human	BAC01677	27,574	7.53	160(64)	33%	3
29.	Immunoglobulin κ chain V-III region (B6)–Human	K3HUB6	11,628	9.34	59(64)	16%	1
30.	Haptoglobin precursor	HPHU2	45,177	6.13	71(64)	53%	5
31.	Immunoglobulin κ chain NIG26 precursor-Human	JEO242	23,504	5.46	203(64)	48%	3
32.	Immunoglobulin λ light chain variable region (fragment)-Human	AAD16673	11,505	5.67	32(64)	25%	1
33.	Hemoglobin α chain (fragment)-Human	Q9BX3_HUMAN	10,703	7.07	87(64)	60%	3
34.	Hemoglobin β chain-Human	2HBSB	15,827	7.26	143(64)	82%	6
35.	Ig light chain VLJ region (fragment)-Human	BAC01701	29,183	8.84	127(64)	39%	4
36.	Anti RhD monoclonal T125 κ light chain precursor-Human	Q5EFE6_HUMAN	25,682	8.70	56(64)	41%	2
37.	Fibrinogen fragment d, chain C-Human	1FZEC	34,457	5.68	64(64)	56%	2
38.	Fibrinogen fragment d, chain B-Human	1FZAB	35,875	7.66	137(64)	53%	5
39.	Fibrinogen fragment d, chain F-Human	1FZEF	34,343	5.68	109(64)	54%	4
40.	Fibrinogen fragment d, chain C-Human	1FZAC	35,144	5.57	91(64)	66%	3
41.	Fibrinogen beta chain precursor	FIBB_HUMAN	55,892	8.54	107(53)	48%	5
42.	Fibrinogen γ A chain precursor-Human	FGHUG	49,465	5.70	313(64)	55%	9
43.	ALB protein (Growth inhibiting protein 20)-Human	Q86YGO_HUMAN	47,330	5.97	170(64)	39%	8

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	Q5TEH4_HUMAN	90,510	7.04	90(64)	40%	6
49.	Fibronectin1-Human	Q60FE4_HUMAN	252,848	5.66	224(64)	26%	13
50.	HUMPIS NID (CDC2-related protein kinase)-Human	AAA60092	35,549	9.02	65(64)	52%	1
51.	Replication licensing factor MCM2-Human	S42228	99,174	5.72	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 DKFZp779N0926-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	39,907	6.32	45(64)	43%	1
63.	AB009303 NID membrane-type matrix metalloproteinase 3	BAA23742	69,451	8.72	58(64)	34%	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	36,132	5.65	284(64)	50%	7
72.	T-cell receptor β-chain precursor	CAA71260	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	Q5ST68_HUMAN	32,378	8.50	146(64)	37%	4
78.	Fibrinogen gamma chain precursor	FIBG_HUMAN	51,479	5.37	226(64)	30%	6
79.	Transthyretin 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	ITIH4_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-acid glycoprotein 1 precursor (Orosomucoid 1)	A1AG1_HUMAN	23,497	4.93	130(53)	31%	3
85.	histidine-rich glycoprotein precursor – human (glycosylation shifts pI)	KGHUGH	59,541	7.09	137(64)	22%	4
86.	Sodium/hydrogen exchanger 2 (NHE-2)	SL9A2_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% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ precipitation, separated on 2D gels. This reflects a significant gain in the dynamic range of plasma proteins visualized in 2-D gels following 20% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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 ($p \leq 0.01$) 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 ($p \leq 0.05$). 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 $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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% $(NH_4)_2SO_4$ 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

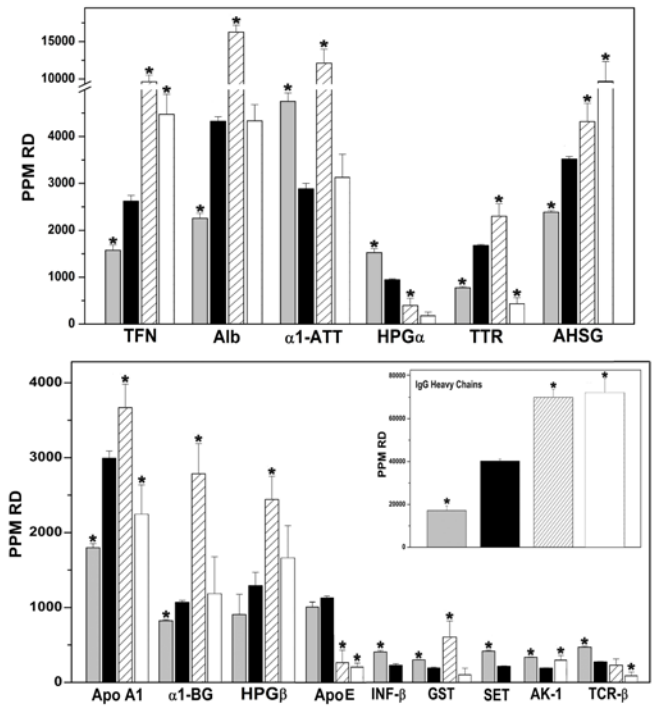

Figure 4: Histogram plots showing change in ppm relative densities (PPM RD) of the 15 differentially-regulated proteins. All data were subjected to unpaired two-tail student's t-test and significant ($p \leq 0.01$) changes from normal samples are marked with asterisk.

Gray Bars: B-ALL (n=8), Black bars: Normal (n=8), Hatched bars: AML (n=3), Hollow bars: HbEβ-thalassemia (n=4), TFN: Transferrin, Alb: Albumin, α1-ATT: α1-Antitrypsin, HPGα: Haptoglobin α-chain, TTR: Transthyretin, AHSG: α2-HS Glycoprotein precursor, Apo A1: Apolipoprotein A1, α1-BG: α1-B Glycoprotein, HPGα: Haptoglobin α-chain, Apo E: Apolipoprotein E, INF-α: Interferon α, GST: Glutathione-S-Transferase, SET: SET Domain Bifurcated, AK-1: Adenylate Kinase 1, TCR-α: T-cell receptor α-chain.

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 $(NH_4)_2SO_4$ 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 $(NH_4)_2SO_4$ 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 transthyretin, 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 emphasize 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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