Comprehensive Analysis of Low Molecular Weight Serum Proteome Enrichment for Mass Spectrometric Studies

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Cite This: ACS Omega 2020, 5, 28877−28888

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ABSTRACT: Rationale: The low molecular weight (LMW) proteins present in circulating body fluids, such as serum and plasma, hold biological significance as possible biomarkers. A major obstacle in mass spectrometry-based proteomics of serum is the presence of abundant high molecular weight proteins which mask the identification and quantitation of lower molecular weight proteins. Traditional methods involve the use of affinity resins to remove high molecular weight proteins, such as albumin and immunoglobulin G, with concomitant loss of lower molecular weight proteins. Considering the importance of depleting high molecular proteins, this paper compares an affinity resin, a gel-filter, and an acetonitrile (ACN) precipitation method to achieve successful removal of high molecular weight proteins and recovery of lower molecular weight proteins. Methods: Serum enrichment was carried out by multiple methods such as with the commercially available serum protein mini kit, ACN precipitation, and a gel filter method. Mass spectrometry analysis of the enriched serum obtained by ACN precipitation and gel filter method was performed for global proteome profiling. Quantitative mass spectrometry using isobaric tags for relative and absolute quantitation (iTRAQ) for ACN-precipitated enriched serum was also carried out. Results: The gel filter method, though allowing for the resolution and identification of LMW proteins, was better suited for global proteome analysis and not preferred for quantitative proteomic experiments. In contrast, enrichment by the ACN precipitation method allowed for the reproducible identification and quantitation of LMW proteins having molecular weight ≥ 4 kDa. Conclusions: Using only chilled ACN and centrifugation, most of the highly abundant proteins were successfully removed from the serum, while recovering a significant portion of the LMW proteome. A more rapid protocol, which is compatible with iTRAQ labeling, to achieve improved results has been elucidated, thus allowing for better screening and identification of potential biomarkers.

1. INTRODUCTION

Serum is the component of blood that lacks fibrinogen, prothrombin, and other clotting factors. Different proteins, peptides, nutrients, electrolytes, and organic wastes are present in abundance. Since every organ of the body remains in contact with blood, physiological and pathological events such as tissue lesions, organ dysfunctions, and infections can alter the metabolite and protein composition of blood serum, thus increasing its utility as an important diagnostic metabolite and protein composition of blood serum, thus lesions, organ dysfunctions, and infections can alter the serum to clot, followed by centrifugation to collect the clear supernatant. Protein serum biomarkers such as prostate specific antigen for prostate cancer and cancer antigen-125 for ovarian cancer have been used in the practice of clinical oncology. However, serum biomarkers have achieved only a modest success rate in cancer research due to the immense complexity of disease and the dynamic range of the proteins present in serum. Serum has a concentration range spanning at least 10 orders of magnitude and reported to have 60−80 mg/mL of protein. Of noteworthy importance are the low molecular weight (LMW) proteins which, though less abundant, hold biological significance as possible biomarkers. The proteins belonging to the LMW region of the serum proteome are usually either intact small proteins which have been actively secreted by the cells or small fragments of larger proteins generated by cleavage, degradation, or other cellular processes. The LMW proteins have short half-lives and get speedily cleared from the blood stream. Hence, most of these proteins bind to a larger carrier protein with a longer half-life, such as albumin, which ensures that these proteins still remain within the detectable range of a mass spectrometer.

Proteomics deals with the study of systematic separation, identification, and characterization of all the proteins present in a biological sample such as tissue, serum, plasma and so forth. The significant evolution of quantitative mass spectrometry
MS-based technologies has made proteomics a powerful tool for biomarker discovery studies. Liquid chromatography coupled with tandem MS (LC–MS/MS) for qualitative and quantitative identification of proteins has been routinely used for protein profiling. The proteomic analysis of circulating body fluids has significant potential in the diagnosis, monitoring, and prognosis of a disease.

The major obstacle in MS-based serum and plasma protein profiling is the abundant presence of high molecular weight (HMW) proteins such as albumin and immunoglobulins that hinder the identification of the LMW proteins. MS profiling of serum or plasma requires the depletion of these highly abundant proteins which in turn enrich the LMW proteome. The most commonly used method for the depletion of highly abundant proteins involves the use of affinity columns, which usually ensures the removal of about 75% of the total proteins to facilitate the identification of the less abundant proteins. However, this method is has its drawbacks because of concomitant loss of the LMW proteins bound to HMW proteins. Furthermore, fractionation strategies prior to MS runs decreases the amount of LMW proteins identified. Therefore, it is evident that in order to utilize the treasure-trove of information of the LMW proteome, one must ensure the recovery of the LMW proteome and concomitant removal of abundant HMW proteins. This can be achieved successfully by breaking the hydrophobic interactions between LMW proteins and the carrier proteins. Therefore, a comparative study of different serum depletion protocols such as Bio-Rad affinity columns, acetonitrile (ACN) precipitation, and a gel filter method has been evaluated for enrichment of the LMW proteome.

2. RESULTS

2.1. Serum Enrichment Using Bio-Rad Aurum Serum Protein Mini Kit. Serum depletion using the Bio-Rad Aurum serum protein mini kit was performed. The significant removal of albumin and immunoglobulin G (IgG) was observed, as indicated by the lower band intensity at 66 kDa (Figure 1a, lane 3) compared to the corresponding bands of the crude serum sample (Figure 1a, lane 1). However, a concomitant loss of the LMW proteins was also observed, as indicated by the faint bands present in the region below 28 kDa (Figure 1a, lane 4). Isoelectric focusing (IEF) and two dimensional (2D) gel electrophoresis of the depleted serum sample (Figure 1a, lane 3) showed the significant absence of LMW proteins indicated by the absence of protein spots in the gel (Figure 1b).

2.2. Serum Enrichment Using a Gel Filter Method Followed by Nano LC–MS/MS IDA Runs. A method developed by Chen, et al. employs the use of different concentrations of acrylamide–bisacrylamide to achieve different porosity of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels in order to trap the HMW proteins, while allowing LMW proteins to resolve properly. The gel-filter method is composed of a stacking gel, funnel gel, blocking gel, and resolving gel. The funnel gel serves as a “buffer” area which prevents the spreading and smearing of the proteins when they enter into the high percentage blocking gel. However, due to short length of the funnel gel and its relatively lower percentage, the separation of the proteins does not take place in this region. The proteins further enter into blocking gel, which has a very high concentration of acrylamide–bisacrylamide. In the blocking gel, HMW proteins are retained due to the back pressure generated by extremely high concentration of the gel. Because the blocking gel is as short as the funnel gel, the resolution of the LMW proteins does not take place and are allowed to pass freely into the lower percentage resolving gel. The concentration of the resolving gel can be adjusted to achieve the degree of resolution required. In the present study, optimized composition of the gel-filter method is as follows: stacking gel, 5%; funnel gel, 10%; blocking gel, 20%; and resolving gel, 15%. Using this four layer gel, successful enrichment of the LMW proteome in the resolving gel was achieved (Figure 2).

The LMW protein bands were excised from the resolving gel and processed for in-gel reduction, alkylation, and trypsin digestion followed by peptide extraction. The peptides were subjected to nano LC (nLC)–MS/MS IDA runs. The raw spectra was analyzed by PeakView software, version 1.2 (AB SCIEX) and ProteinPilot software, version 4.5 (AB SCIEX). 120–130 proteins with 95% confidence from multiple runs could be identified (Table S1). Most of the proteins identified (≥73%) are LMW proteins (≤65 kDa).

2.3. Serum Enrichment under Partly Denaturing Conditions Using ACN Precipitation. The addition of ACN was found to disrupt the binding of the LMW proteins to...
the carrier proteins such as albumin. Multiple strategies were applied to standardize the optimum protocol for depleting albumin, IgG, and other HMW proteins from serum, while ensuring good recovery of the LMW proteins. 10 mg of serum (from a total protein concentration of 60−70 mg/mL) was directly precipitated and enriched using chilled ACN with serum/ACN 1:1 and 1:2 ratios. It was observed that both the ratios allowed for efficient removal of the HMW proteins, as was evident by the proteins bands observed in the precipitate (Figure 3a). In addition, a significant amount of the LMW proteins were recovered in the supernatant, and their presence was confirmed in the 2D gel (Figure 3b). A serum/ACN 1:1 ratio was used for all subsequent experiments as better results were observed. Furthermore, consistently reproducible results were obtained which indicates the efficacy of the method for serum enrichment (Figure 3c).

2.4. MS Analysis of Serum Enriched by ACN Precipitation. SDS-PAGE gels indicate a successful enrichment of serum by ACN precipitation (Figure 3). These enriched fractions were subjected to MS analysis to determine the number of proteins identified.

In the first approach, in-solution reduction, alkylation, and trypsin digestion of 20 μg of the enriched serum were carried out. The peptides were then cleaned-up using C18 columns, followed by nLC−MS/MS IDA run in the mass spectrometer. Data analysis was performed with PeakView software, version 1.2 (AB SCIEX) and ProteinPilot software, version 4.5 (AB SCIEX), and molecular weights of the proteins identified were computed using ProtParam Expasy software. The biological class of the proteins was determined using PANTHER analysis (Figure 4). Using this approach, 50−80 proteins with 95% confidence were identified in each run. Though albumin was still identified in each of these runs, other LMW proteins were also identified apolipoprotein C-I being identified as the protein with the LMW of 9.33 kDa (Table 1).

In the second approach, four aliquots of the enriched serum were labeled using the isobaric tags for relative and absolute quantitation (iTRAQ) 4-plex label set and fractioned by strong cation exchange (SCX). SCX fractions were collected at 50, 100, 150, 200, 250, 300, 400, and 500 mM ammonium formate concentrations. nLC−MS/MS IDA runs were performed for each of these fractions in the mass spectrometer, and data was analyzed using PeakView and ProteinPilot software. Using this approach, 180−250 proteins with 95% confidence were consistently identified in multiple runs. Hence, a higher number of LMW proteins were identified using this approach than the unlabeled and unfractionated approach (Table 2, Figure 5). Of the total number of proteins identified, about 73% of the proteins belonged to the LMW region (≤65 kDa). The smallest protein identified was hepatocyte growth factor activator, having a molecular weight of 3.95 kDa.
DISCUSSION

The significant evolution of MS based techniques has made proteomics a powerful tool for proteome profiling and biomarker discovery for translational research. Most of the studies involve the use of LC−MS/MS for the quantitative identification of proteins. Different proteomics biomarker discovery groups have exploited the treasure trove of information found in serum using MS-based serum proteomics. One of the major drawbacks of using serum as the initial material for MS studies lies in its sample preparation. Crude serum cannot be injected directly into an LC−MS/MS system as the high concentration of proteins in the serum would most certainly block and damage the LC column. Moreover, the presence of HMW proteins in the serum hinders the detection of the LMW proteome. The LMW proteome of the serum is composed of small intact proteins and the fragments of some larger proteins, possibly due to degradation and cleavage. It is, therefore, rational to assume that this LMW fraction of the proteome represents all the classes of proteins present in the serum. Hence, the study of the LMW proteome of the serum can provide a reasonably complete picture of the ongoing physiological and pathological events of the body.

Most of the approaches to deplete the HMW proteins of the serum involve the use of affinity chromatography columns, immunoaffinity methods, precipitation with organic solvents, and ultracentrifugation. Affinity chromatography systems usually comprise two or more kinds of resins which have

| sr. no | protein     | MW (kDa) | sr. no | protein     | MW (kDa) |
|--------|-------------|----------|--------|-------------|----------|
| 1      | ALBU_HUMAN  | 69.36668 | 32     | APOF_HUMAN  | 35.3994  |
| 2      | APOA1_HUMAN | 30.77783 | 33     | HRG_HUMAN   | 59.5783  |
| 3      | A1AT_HUMAN  | 46.73655 | 34     | FIBA_HUMAN  | 94.9730  |
| 4      | TRFE_HUMAN  | 77.06389 | 35     | APOE_HUMAN  | 36.1540  |
| 5      | APOA4_HUMAN | 45.39906 | 36     | K2C5_HUMAN  | 62.3783  |
| 6      | K2C1_HUMAN  | 66.03873 | 37     | VTN_C_HUMAN | 54.3055  |
| 7      | APOA2_HUMAN | 11.17502 | 38     | KNG1_HUMAN  | 71.9573  |
| 8      | K1C10_HUMAN | 58.82709 | 39     | AFAM_HUMAN  | 69.0691  |
| 9      | FETUA_HUMAN | 39.34074 | 40     | APOM_HUMAN  | 21.2532  |
| 10     | K22E_HUMAN  | 65.43289 | 41     | A1AG2_HUMAN | 23.6026  |
| 11     | A1BG_HUMAN  | 54.25352 | 42     | CERU_HUMAN  | 122.205  |
| 12     | ANGT_HUMAN  | 53.1542  | 43     | CLUS_HUMAN  | 52.4945  |
| 13     | A2GL_HUMAN  | 38.17791 | 44     | SAA1_HUMAN  | 13.5320  |
| 14     | YTD8_HUMAN  | 52.91754 | 45     | FA5_HUMAN   | 251.703  |
| 15     | APOC3_HUMAN | 10.85231 | 46     | CO3_HUMAN   | 187.148  |
| 16     | APOD_HUMAN  | 21.27555 | 47     | GP1BA_HUMAN | 71.5400  |
| 17     | K1C9_HUMAN  | 62.06432 | 48     | IGG1_HUMAN  | 49.3288  |
| 18     | HBB_HUMAN   | 15.99841 | 49     | SHBG_HUMAN  | 43.7792  |
| 19     | APOC1_HUMAN | 9.33193  | 50     | K1C14_HUMAN | 51.5614  |
| 20     | LUM_HUMAN   | 38.42902 | 51     | ITIH2_HUMAN | 106.463  |
| 21     | TTHY_HUMAN  | 15.88703 | 52     | CD44_HUMAN  | 81.5376  |
| 22     | HEMO_HUMAN  | 51.67637 | 53     | IGLC3_HUMAN | 11.2655  |
| 23     | RET4_HUMAN  | 23.01001 | 54     | F13A_HUMAN  | 83.2673  |
| 24     | ITIH4_HUMAN | 103.35743 | 55     | B2MG_HUMAN  | 13.7145  |
| 25     | LCAT_HUMAN  | 49.57792 | 56     | TETN_HUMAN  | 22.5368  |
| 26     | APOC2_HUMAN | 11.28387 | 57     | CCL7_HUMAN  | 13.8942  |
| 27     | CO4B_HUMAN  | 192.75146 | 58     | A2AP_HUMAN  | 54.5657  |
| 28     | HBA_HUMAN   | 15.25755 | 59     | RABE1_HUMAN | 99.2904  |
| 29     | PGRP2_HUMAN | 62.21702 | 60     | KPRP_HUMAN  | 64.1357  |
| 30     | A1AG1_HUMAN | 23.51156 | 61     | PF4_V_HUMAN | 11.5526  |
| 31     | THR8_HUMAN  | 70.03687 |        |              |          |
Table 2. Proteins Identified with More than 95% Confidence of nLC–MS/MS-iTRAQ Analysis of Serum Enriched Using ACN Precipitation, Followed by iTRAQ Labeling

| sr. no. | protein (HUMAN) | MW (kDa) | sr. no. | protein (HUMAN) | MW (kDa) |
|---------|----------------|----------|---------|----------------|----------|
| 1       | ALBU_HUMAN     | 66.4722  | 115     | APOD_HUMAN     | 19.3038  |
| 2       | A1AT_HUMAN     | 44.3245  | 116     | ITIH1_HUMAN    | 71.4150  |
| 3       | APOA1_HUMAN    | 28.9616  | 117     | HPT_HUMAN      | 43.3490  |
| 4       | TTHY_HUMAN     | 13.7614  | 118     | TETN_HUMAN     | 20.1389  |
| 5       | APOA4_HUMAN    | 43.4023  | 119     | ZA2G_HUMAN     | 32.1449  |
| 6       | TRF_E_HUMAN    | 75.1956  | 120     | APOF_HUMAN     | 17.4245  |
| 7       | APOA2_HUMAN    | 9.30365  | 121     | HBA_HUMAN      | 15.1263  |
| 8       | APOB_HUMAN     | 52.8583  | 122     | LUM_HUMAN      | 36.6609  |
| 9       | ITIH2_HUMAN    | 72.4523  | 123     | PON1_HUMAN     | 39.6002  |
| 10      | FETU_A_HUMAN   | 30.2380  | 124     | APOC3_HUMAN    | 8.76467  |
| 11      | THR_HUMAN      | 65.3082  | 125     | CD14_HUMAN     | 37.2147  |
| 12      | CO3_HUMAN      | 184.9513 | 126     | CFAB_HUMAN     | 83.0008  |
| 13      | AIBG_HUMAN     | 51.9216  | 127     | TSP1_HUMAN     | 127.4953 |
| 14      | CO4A_HUMAN     | 71.6789  | 128     | HEMO_HUMAN     | 49.2954  |
| 15      | KNG1_HUMAN     | 69.8967  | 129     | CO2_HUMAN      | 81.0851  |
| 16      | VTB_HUMAN      | 51.1973  | 130     | FINC_HUMAN     | 269.2587 |
| 17      | FIBA_HUMAN     | 1.5367   | 131     | ANT3_HUMAN     | 49.0391  |
| 18      | APOE_HUMAN     | 34.23668 | 132     | LG3P_HUMAN     | 63.2765  |
| 19      | A2GL_HUMAN     | 34.34641 | 133     | ALS_HUMAN      | 63.2465  |
| 20      | K2C1_HUMAN     | 65.9075  | 134     | CNDP1_HUMAN    | 53.8775  |
| 21      | ITIH4_HUMAN    | 70.5891  | 135     | K22E_HUMAN     | 65.4328  |
| 22      | K1C10_HUMAN    | 58.82709 | 136     | FA5_HUMAN      | 248.6869 |
| 23      | A2AP_HUMAN     | 50.45085 | 137     | AACT_HUMAN     | 45.2658  |
| 24      | A2MG_HUMAN     | 160.8099 | 138     | APOH_HUMAN     | 36.2546  |
| 25      | ANGT_HUMAN     | 49.76111 | 139     | CO5_HUMAN      | 73.2918  |
| 26      | CO6_HUMAN      | 102.4122 | 140     | B2MG_HUMAN     | 11.7311  |
| 27      | HRG_HUMAN      | 57.6599  | 141     | FA12_HUMAN     | 39.6471  |
| 28      | CERU_HUMAN     | 120.0855 | 142     | AFAM_HUMAN     | 66.57702 |
| 29      | SAA1_HUMAN     | 11.6827  | 143     | IGLL5_HUMAN    | 19.2787  |
| 30      | RET4_HUMAN     | 21.0716  | 144     | CBPN_HUMAN     | 50.0343  |
| 31      | APOM_HUMAN     | 21.25329 | 145     | PLF4_HUMAN     | 7.76918  |
| 32      | PGRP2_HUMAN    | 59.98032 | 146     | CFAD_HUMAN     | 24.40478 |
| 33      | APOC2_HUMAN    | 8.91492  | 147     | PLMN_HUMAN     | 88.4326  |
| 34      | ECM1_HUMAN     | 58.81187 | 148     | IBP2_HUMAN     | 31.44688 |
| 35      | AMBP_HUMAN     | 20.84674 | 149     | SAA2_HUMAN     | 11.64775 |
| 36      | CO9_HUMAN      | 60.97868 | 150     | A1AG2_HUMAN    | 21.65119 |
| 37      | K1C9_HUMAN     | 62.06432 | 151     | LCAT_HUMAN     | 47.08389 |
| 38      | CLUS_HUMAN     | 50.06256 | 152     | GELS_HUMAN     | 82.9591  |
| 39      | APOC1_HUMAN    | 6.63058  | 153     | APO1_HUMAN     | 41.1268  |
| 40      | A1AG1_HUMAN    | 21.56012 | 154     | CYTC_HUMAN     | 13.34714 |
| 41      | SHBG_HUMAN     | 40.46817 | 155     | CXC17_HUMAN    | 10.26583 |
| 42      | VTNC_HUMAN     | 52.27796 | 156     | IBP3_HUMAN     | 28.74962 |
| 43      | HBB_HUMAN      | 15.86722 | 157     | APOC4_HUMAN    | 11.48718 |
| 44      | VASN_HUMAN     | 69.35664 | 158     | IBP5_HUMAN     | 28.57278 |
| 45      | SBSN_HUMAN     | 58.01877 | 159     | DCD_HUMAN      | 9.25929  |
| 46      | KV205_HUMAN    | 10.9221  | 160     | SEPP1_HUMAN    | 41.23226 |
| 47      | CPN2_HUMAN     | 58.22677 | 161     | KV308_HUMAN    | 10.2714  |
| 48      | F13A_HUMAN     | 79.24488 | 162     | MA1A1_HUMAN    | 72.96853 |
| 49      | SPRC_HUMAN     | 32.69772 | 163     | TRY1_HUMAN     | 24.11424 |
| 50      | CIR_HUMAN      | 78.21316 | 164     | TLN1_HUMAN     | 269.7671 |
| 51      | IBP4_HUMAN     | 25.9745  | 165     | CBRP2_HUMAN    | 35.80054 |
| 52      | HBD_HUMAN      | 15.92429 | 166     | IGF2_HUMAN     | 7.47546  |
| 53      | SAA4_HUMAN     | 12.80324 | 167     | KV108_HUMAN    | 10.22932 |
| 54      | KV313_HUMAN    | 10.3024  | 168     | NUCB1_HUMAN    | 51.14578 |
| 55      | HGFA_HUMAN     | 3.95363  | 169     | VCA1M_HUMAN    | 78.74504 |
| 56      | ZP1_HUMAN      | 48.46211 | 170     | CAH1_HUMAN     | 28.73902 |
| 57      | VWF_HUMAN      | 81.3497  | 171     | CROCC_HUMAN    | 228.5233 |
| 58      | PSPB_HUMAN     | 8.70988  | 172     | FBX47_HUMAN    | 51.96781 |
| 59      | ISLR_HUMAN     | 43.90877 | 173     | MYO1C_HUMAN    | 121.6817 |
| 60      | FBLN3_HUMAN    | 52.7651  | 174     | CO6A3_HUMAN    | 340.8079 |

https://dx.doi.org/10.1021/acsomega.0c04568
ACS Omega 2020, 5, 28877–28888
an affinity toward albumin, IgG, and some of the other HMW proteins present in serum.12 These resins are commercially available as pre-packed columns and show variable success in depleting the HMW proteins, as occasionally the resins have been shown to be susceptible to non-specific protein binding.12

Chen, et al. elucidated a modified TCA/acetone precipitation method for serum pre-fractionation to remove highly abundant proteins.11 It was observed that albumin could be successfully removed by adding two or four volumes of 5−10% TCA/acetone, while ensuring the recovery of the LMW proteins.12

| sr. no. | protein        | MW (kDa) | sr. no. | protein        | MW (kDa) |
|---------|----------------|----------|---------|----------------|----------|
| 61      | IGF1_HUMAN     | 7.65474  | 175     | PHLD_HUMAN     | 89.8136  |
| 62      | ICAM2_HUMAN    | 28.0603  | 176     | TIGD1_HUMAN    | 67.29913 |
| 63      | FIBB_HUMAN     | 1.56961  | 177     | SPR1_HUMAN     | 73.5687  |
| 64      | FHR4_HUMAN     | 63.26719 | 178     | P2P_HUMAN      | 161.0565 |
| 65      | FHR2_HUMAN     | 28.73837 | 179     | SPEP1_HUMAN    | 388.2777 |
| 66      | KIC14_HUMAN    | 51.56147 | 180     | SLAMP_HUMAN    | 95.1983  |
| 67      | DAG1_HUMAN     | 67.78623 | 181     | RAD51_HUMAN    | 36.83499 |
| 68      | II16_HUMAN     | 46.7206  | 182     | FI3B_HUMAN     | 73.2068  |
| 69      | ICAM1_HUMAN    | 55.21507 | 183     | RHG28_HUMAN    | 82.05962 |
| 70      | IC1_HUMAN      | 52.84336 | 184     | LMAN2_HUMAN    | 35.51504 |
| 71      | HEP2_HUMAN     | 54.98002 | 185     | CD44_HUMAN     | 79.21082 |
| 72      | PRG4_HUMAN     | 148.3157 | 186     | DEF3_HUMAN     | 6.35037  |
| 73      | ATL4_HUMAN     | 113.6746 | 187     | CD248_HUMAN    | 79.11005 |
| 74      | SRGN_HUMAN     | 14.71208 | 188     | KNG1_HUMAN     | 69.8967  |
| 75      | COMB_HUMAN     | 71.67889 | 189     | HPT_HUMAN      | 39.02957 |
| 76      | C1R_L_HUMAN    | 49.45048 | 190     | KLKB1_HUMAN    | 41.42437 |
| 77      | LV302_HUMAN    | 10.66157 | 191     | PON3_HUMAN     | 39.60749 |
| 78      | SODM_HUMAN     | 22.20414 | 192     | GRP75_HUMAN    | 68.759   |
| 79      | MENT_HUMAN     | 34.5126  | 193     | KV122_HUMAN    | 10.2023  |
| 80      | SAP3_HUMAN     | 17.58935 | 194     | KV119_HUMAN    | 10.14628 |
| 81      | FA9_HUMAN      | 46.57823 | 195     | NID1_HUMAN     | 133.4575 |
| 82      | KV121_HUMAN    | 10.40944 | 196     | TTHY_HUMAN     | 13.76141 |
| 83      | SG3A1_HUMAN    | 8.22472  | 197     | B4GT1_HUMAN    | 43.92027 |
| 84      | PTGDS5_HUMAN   | 18.69903 | 198     | MIE_HUMAN      | 31.73439 |
| 85      | APMAP_HUMAN    | 46.34917 | 199     | LCP2_HUMAN     | 60.18819 |
| 86      | K2C5_HUMAN     | 62.37834 | 200     | PRDX2_HUMAN    | 21.76073 |
| 87      | MGTS5A_HUMAN   | 84.54263 | 201     | S10A7_HUMAN    | 11.33978 |
| 88      | COFA1_HUMAN    | 138.7656 | 202     | LV403_HUMAN    | 9.98689  |
| 89      | TRHY_HUMAN     | 253.9253 | 203     | SHC1_HUMAN     | 62.8221  |
| 90      | CMGA_HUMAN     | 48.91826 | 204     | GHTM_HUMAN     | 32.08109 |
| 91      | PDL11_HUMAN    | 35.94052 | 205     | FKB1A_HUMAN    | 11.81951 |
| 92      | F221A_HUMAN    | 33.0826  | 206     | KTDAP_HUMAN    | 8.76889  |
| 93      | SYNE1_HUMAN    | 796.4424 | 207     | HV323_HUMAN    | 10.44567 |
| 94      | LTBP1_HUMAN    | 184.3762 | 208     | LV102_HUMAN    | 10.29918 |
| 95      | THYG_HUMAN     | 302.7277 | 209     | PDCD6_HUMAN    | 21.73729 |
| 96      | FA8H3_HUMAN    | 127.1233 | 210     | PRORY_HUMAN    | 19.97636 |
| 97      | INHBC_HUMAN    | 12.5344  | 211     | B4GA1_HUMAN    | 47.11906 |
| 98      | TFP1_HUMAN     | 31.9503  | 212     | FCG3A_HUMAN    | 27.32489 |
| 99      | VNN3_HUMAN     | 50.53724 | 213     | CRAC1_HUMAN    | 68.43407 |
| 100     | TSK_HUMAN      | 36.11526 | 214     | HEG1_HUMAN     | 144.4067 |
| 101     | RCN1_HUMAN     | 35.86721 | 215     | INHBE_HUMAN    | 12.46516 |
| 102     | RS27A_HUMAN    | 8.56484  | 216     | PRAP1_HUMAN    | 14.98591 |
| 103     | DKK3_HUMAN     | 36.28462 | 217     | CLU_HUMAN      | 146.698 |
| 104     | CPXM2_HUMAN    | 83.53717 | 218     | IGHG1_HUMAN    | 41.28696 |
| 105     | ASGR2_HUMAN    | 35.09221 | 219     | LV405_HUMAN    | 10.21415 |
| 106     | QS0X1_HUMAN    | 79.5781  | 220     |                |          |
| 107     | TRML1_HUMAN    | 31.12755 | 221     |                |          |
| 108     | GPX3_HUMAN     | 23.46374 | 222     |                |          |
| 109     | TIMP1_HUMAN    | 20.70883 | 223     |                |          |
| 110     | CUTA_HUMAN     | 15.95245 | 224     |                |          |
| 111     | CC126_HUMAN    | 12.64244 | 225     |                |          |
| 112     | CSN5_HUMAN     | 37.44762 | 226     |                |          |
| 113     | SPIN4_HUMAN    | 28.6599  | 227     |                |          |
| 114     | DERM_HUMAN     | 21.96428 | 228     |                |          |
proteins in the precipitate. LC–MS/MS analysis of the precipitate obtained after TCA/acetone precipitation revealed the presence of 81 unique proteins, including many LMW proteins.11

The effect of ACN precipitation was first evaluated by Merrell et al. by adding two volumes of ACN to one volume of serum and capillary LC–MS (cLC–MS) comparison of the untreated and treated serum.13 MS spectra of untreated serum indicated the representation of proteins having molecular weights of 60−70 kDa. The MS spectra of ACN precipitated serum revealed the presence of a more number of molecular species lying in the lower m/z region. As most of the regulatory molecules, that hold potential as biomarkers, have been shown to be small molecular weight proteins, this method of serum enrichment bears merit for further study.13

Another study by Kay et al. further demonstrated the efficiency of ACN precipitation in enriching the LMW proteins of serum.14 Serum was diluted in a 1:2 ratio with water and precipitated using ACN, followed by two cycles of sonication of 10 min each. The supernatant obtained after centrifugation was subjected to nLC–MS/MS and multiple reaction monitoring (MRM) studies were set up for 57 proteins.14,15 Of these 57 proteins, 29 proteins were successfully detected using MRM, indicating the success of the method in depleting albumin from serum. Therefore, an important study to compare two different serum enrichment protocols and evaluate their ability to identify and quantify the LMW region of the serum proteome using MS was carried out here. For the purpose of the current study, the LMW proteome was defined as the proteins that were recovered after the removal of albumin, lipoproteins, transferrin, and immunoglobulins and having molecular weight ≤65 kDa. A rapid, reproducible, and cost-effective method of serum enrichment by ACN precipitation, which can be successfully coupled with quantitative proteomic experiments, was also formulated in the present study.

Initial attempts to enrich serum using the commercially available serum protein kit were not completely successful as many proteins of the LMW region were not recovered successfully. Most of these proteins remained bound to carrier proteins such as albumin and were removed while passing through the resin (Figure 1a). Depletion of serum with higher dilutions was also carried out, but no appreciable difference in the LMW proteome was observed.

A novel gel-based method to carry out selective enrichment of the LMW proteome of serum has already been reported earlier by Chen, et al.9 This gel-filter assembly was used to achieve successful enrichment of the LMW proteome. 120−130 proteins were identified from the bands of the resolving gel with 95% confidence, from multiple runs (Table S1). Most of the proteins identified lie in the LMW region, and some HMW proteins were also identified, probably due to fragments of these proteins entering the resolving gel. Cell adhesion proteins, extracellular matrix proteins, calcium-binding proteins, and so forth were successfully identified using the gel filter method, indicating that the masking effect of the HMW proteins was overcome successfully. The sample preparation steps for this method did not require much processing, thus ensuring minimum protein loss. The gel profiles were highly reproducible even with varying concentrations of initial serum loaded (Figure S1). As only the protein bands present in the resolving gel were processed further, the drawback of the method lies in the inability to subject the extracted peptides to quantitative proteomic studies. Of the total amount of protein loaded onto the gel, the amount of protein entering into and separating in the resolving gel could not be accurately quantified. All quantitative proteomic studies require an equal amount of the initial sample, thus rendering this method ineffective for such studies. In addition, in gel reduction, alkylation, trypsin digestion, and peptide extraction following enrichment is a tedious and time-consuming procedure that requires extreme care during handling. This increases the chances of protein/peptide loss, further affecting the accurate quantitation of proteins and reproducibility of the MS results. In the gel-filter method, proteins with molecular weights less than 5 kDa may have been lost if the gel ran for too long. Hence, it was necessary to monitor the progress of the stained protein marker while running the gel to ensure maximum recovery of the LMW proteins. The gel-filter method, however, bears excellent merit for global qualitative proteomic studies of the LMW serum proteome. As the sample has been fractionated while running the gel, a high degree of resolution could be achieved by nLC–MS/MS studies, allowing the user to identify even the smaller size proteins with a high degree of confidence.

Due to the shortcoming of the gel-filter method in quantitative proteomics, a gel-free protocol of serum enrichment was preferred. Using only chilled ACN and centrifugation, most of the highly abundant proteins were successfully removed from the serum, while recovering a significant portion of the LMW proteome. In contrast to the kit-based depletion strategy, a significantly higher number of proteins were visualized on the silver stained 2D gel below the 25 kDa region (Figure 3b).

Through nLC–MS/MS analysis of the enriched serum fraction, 50−80 proteins were identified, with almost 75% of the proteins having a molecular weight below 65 kDa. The

Figure 5. PANTHER analysis of proteins identified by nLC–MS/MS-iTRAQ of serum enriched using ACN precipitation, followed by iTRAQ labeling.
The smallest protein identified was apolipoprotein C-I, having a molecular weight of 9.33 kDa. Though the number of proteins identified in the enriched fraction was less than expected, identification of some proteins from the LMW proteome of serum (Table 1, Figure 4) belonging to various biological classes was possible. This shows that ACN precipitation...
successfully freed the smaller proteins from their carrier proteins and allowed selective precipitation of the HMW proteins. The selective precipitation of HMW proteins is not completely efficient as proteins such as some apolipoproteins, complement proteins, coagulation factors, and so forth having molecular weights >100 kDa were still present in the enriched fraction. The presence of these HMW proteins, however, did not mask the identification of the LMW proteins.

In an effort to increase the number of proteins identified and check the compatibility of the enrichment protocol with quantitative proteomics approaches, the enriched fractions were processed for iTRAQ labeling and SCX fractionation. The labeling and further sample fractionation drastically increased the number of proteins identified with more than 95% confidence. As compared to the unlabeled approach, a higher number of proteins were identified. Of these, almost 75% of the proteins belonged to the LMW proteome region, having molecular weights \( \leq 65 \) kDa, indicating the sensitivity of the enrichment protocol (Table 2, Figure 5). Hepatocyte growth factor activator was the smallest protein identified with a molecular weight 3.95 kDa. In addition, proteins such as IGF-1 (7.65 kDa) and IGF-2 (7.45 kDa), which are present in sub-1 \( \mu \)g/mL concentrations in serum and plasma, were also successfully identified using this approach. Identified proteins also included calcium-binding proteins, cell adhesion molecules, extracellular matrix proteins, G-protein activity modulator proteins, transmembrane signal receptors, and so forth, and these may hold potential to function as biomarkers.

Proteins belonging to these families were not identified in the previous approach of ACN precipitation, followed by nLC–MS/MS. This indicates that proteins with much LMW could be successfully recovered and identified using ACN precipitation (Table 2). Clean MS/MS spectra obtained for each run indicated that the enrichment protocol did not interfere with the labeling technique and subsequent MS (Figure 6).

The lower number of proteins that were identified using only ACN precipitation could be attributed to the fact that the identification of smaller proteins, having molecular weights \( \leq 5 \) kDa, was masked by those higher in abundance and molecular weights. Additionally, these smaller proteins may have been lost during sample clean-up by the C18 tips, whereas the additional step of SCX ensures better recovery and resolution of small proteins. SCX also helped reduce the sample complexity and increase the resolution obtained during MS/MS. Albumin was identified in such a less amount that it did not seem to mask the detection of the LMW proteins present.

### 4. CONCLUSIONS

Both the gel filter method and ACN precipitation method ensured recovery of a significant portion of the LMW proteome. Due to the shortcomings of the gel-filter method in quantitative proteomics, ACN precipitation for serum enrichment was preferred. Using only chilled ACN and centrifugation, most of the highly abundant proteins were successfully removed from the serum, while recovering a significant portion of the LMW proteome, which could be reproducibly used for high-throughput quantitative proteomics experiments. The results were comparable to those produced by Merrell et al. and Kay et al., in the number of proteins identified by cLC and nLC. A more rapid protocol, which is compatible with iTRAQ labeling, to achieve improved results has been elucidated. To our conclusion, serum enrichment by ACN precipitation followed by quantitative proteomics, allows for successful identification and quantitation of LMW proteins which may hold potential as biomarkers. This method is much more cost-effective than the commercially available affinity methods and may lower the cost of biomarker discovery studies.

### 5. MATERIALS AND METHODS

#### 5.1. Serum Samples

Blood samples were collected from patients undergoing treatment for cancer in an ongoing clinical trial study at ACTREC-TMC, Kharghar, Navi Mumbai. The blood samples were collected in red capped 12.5 mL vacutainers during routine blood test evaluation that was carried out prior to starting any treatment. The blood was allowed to clot at 37 °C for 15–30 min. The vacutainers containing the clotted blood were spun at 4500 rpm at 4 °C for 10 min in a Rota 4R centrifuge. This allows separation of the serum from the clotted blood in the form of a clear yellow supernatant, which is collected into a fresh Eppendorf tube. Delipidation of the freshly collected serum was achieved by centrifugation at 13,000 rpm for 30 min in an Eppendorf 5415R centrifuge. The lipids present formed a whitish layer floating at the top of the tube, which was carefully decanted using a micropipette tip. To preserve the protein content of the delipidated serum for an extended period of time, protease inhibitor (Sigma P2714) was added to each tube and mixed thoroughly. The delipidated serum was stored at -20 °C for a period of 3–4 years.

#### 5.2. Protein Quantitation Using the Bradford Assay

Protein quantitation was carried out in 96 well plates (HIMEDIA ELISA plates, medium binding, EP6-10X10NO) using bovine serum albumin (Sigma A7906) standards: 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mg/mL. The serum samples were diluted in the ratio 1:10 using Milli-Q water. 200 \( \mu \)L of Bradford reagent (Sigma B6916) was added to each well, along with 5 \( \mu \)L of standard and protein sample. The plate was incubated in the dark for 10 min. Readings were taken on an Epoch 2 Microplate spectrophotometer (BioTek) at UV wavelength \( (\lambda) \) 595 nm.

#### 5.3. Serum Depletion Using Bio-Rad Aurum Serum Protein Mini Kit

The resin in the Micro Bio-Spin column of the Aurum serum protein mini kit comprises of a mixture of Affi-Gel Blue and Affi-Gel protein A. Affi-Gel Blue has the ability to bind to albumin, and Affi-Gel protein A has affinity toward IgG molecules, thus effectively removing both the proteins from serum. Serum depletion was carried out as per the Bio-Rad protocol. In brief, the resin was washed twice with the binding buffer provided in the kit, after which the column was blocked to allow for application of the serum sample. Slight changes in the protocol included diluting the crude serum in the ratios of 1:1, 1:10, 1:50, 1:100, and 1:1000 with the binding buffer and loading onto the affinity column. The sample was allowed to bind on the resin for 15–20 min, with intermittent gentle mixing. The unbound fraction of the sample was removed by centrifugation at 10,000g for 20 s and collected in a fresh collection tube. Furthermore, two elutions of the bound fraction were collected by passing 200 \( \mu \)L of binding buffer through the column and centrifugation at 10,000g for 20 s. The combined fractions contained the albumin and IgG depleted serum sample. The bound albumin and IgG were recovered from the affinity resin column by eluting with 500 \( \mu \)L of Laemml sample buffer.

Quantitation of undepleted serum, depleted serum, and fraction bound to the resin was performed using the Bradford method.

### Articles

- HEMEDIA ELISA plates, medium binding, EP6-10X10NO
- Bovine serum albumin (Sigma A7906)
- Milli-Q water
- Bradford reagent (Sigma B6916)
- Bio-Rad Aurum Serum Protein Mini Kit
- Micro Bio-Spin column
- Affi-Gel Blue
- Affi-Gel protein A
- Albumin
- IgG
- Binding buffer
- Centrifugation at 10,000g for 20 s
assay. Equal amounts of each fraction and a protein marker were loaded onto a 15% SDS-PAGE gel. The gel was run at a constant voltage of 100 V until the dye front reached the bottom of the gel. The gel was stained using Coomassie stain and then left overnight in destaining solution containing 50% methanol, 40% water, and 10% glacial acetic acid to visualize the protein bands.

5.4. IEF, 2D Gel Electrophoresis, and Silver Staining. Overnight acetone precipitation (protein/acetone ratio of 1:4) was carried out for 200 μg protein from a total concentration of 1–2 μg/μL enriched serum at −20 °C. Next day, the sample was centrifuged at 13,000 rpm for 20 min at 4 °C. The protein pellet was then resuspended in 300 μL rehydration buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.1% bromophenol blue, and 0.2% amphotelyte. 17 cm IPG gel strips having pH range 3–10 were procured from Bio-Rad (ReadyStrip IPG strips, 1632007). Active rehydration of the IPG strips was carried out at 50 V for 16 h at 20 °C. IEF was carried out with the following conditions: 250 V for 30 min (linear), 600 V for 30 min (linear), 10,000 V for 2 h 30 min (linear), and 30,000 V h (rapid). IPG strip was equilibrated with equilibration buffer I (6 M urea, 1 M Tris pH 8.8, SDS, glycerol and DTT) and equilibration buffer II (6 M urea, 1 M Tris pH 8.8, SDS, glycerol and IAA) for 10 min each. The IPG strip was then overlaid on a 15% SDS-PAGE gel using 1% Tris pH 8.8, SDS, glycerol and IAA for 10 min each. The IPG strip was again washed with fresh IPG gels. A single IPG strip was used per gel. Each layer was allowed to solidify thoroughly in the Bio-Rad gel assembly casket before adding the next layer. Once the IPG gel was again washed with freshMilli-Q water, before placing it for 1 h in a dry bath. The tubes were cooled down, and excess solution was removed. Alkylation was activated by freshly prepared 55 mM IAA (iodoacetamide, Sigma A1149) in 100 mM ammonium bicarbonate, in dark at room temperature for 1 h. The excess solution was removed, and the gel pieces were washed thrice with 100 mM ammonium bicarbonate and 50% ACN. The pieces were then rehydrated using 100% ACN and treated for trypsin digestion. Lyophilized trypsin was obtained from Sigma (Trypsin Singles, Proteomics grade, T7755), and reconstituted trypsin was added to each tube, depending on the amount of the gel pieces. Once the trypsin was completely absorbed, the rest of the volume was made up with 25 mM ammonium bicarbonate to use in-gel reduction, alkylation, and trypsin digestion before being subjected to MS.

5.6. In-Gel Reduction, Alkylation, Trypsin Digestion, and Extraction of Peptides. The LMW protein bands were excised out from the resolving gel and chopped into smaller pieces using a sterile blade. The gel pieces were transferred into a fresh, un-autoclaved Eppendorf tube and vortexed for 15 min in Milli-Q water. The tubes were centrifuged to settle down gel pieces, and the excess water was decanted carefully in order to avoid loss of gel pieces. The gel pieces were washed twice with Milli-Q water. Next, 1 mL of destaining solution comprising 50% methanol, 40% water, and 10% glacial acetic acid was added to each tube and vortexed for 15 min. The destaining solution was removed when it turned blue. This destaining procedure was repeated until all the stain was removed from all the gel pieces. The gel pieces were then washed thrice with 100 mM ammonium bicarbonate (Sigma A6141) and 50% ACN (Sigma A7906). The pieces were then shrunk with 100% ACN for reduction with 10 mM DTT (dithiothreitol, HIMEDIA MB070) in 100 mM ammonium bicarbonate. Reduction was carried out at 60 °C for 1 h in a dry bath. The tubes were cooled down, and excess solution was removed. Alkylation was carried out with freshly prepared 55 mM IAA (iodoacetamide, Sigma A1149) in 100 mM ammonium bicarbonate, in dark at room temperature for 1 h. The excess solution was removed, and the gel pieces were washed thrice with 100 mM ammonium bicarbonate and 50% ACN. The pieces were then rehydrated using 100% ACN and treated for trypsin digestion. Lyophilized trypsin was obtained from Sigma (Trypsin Singles, Proteomics grade, T7755), and reconstituted trypsin was added to each tube, depending on the amount of the gel pieces. Once the trypsin was completely absorbed, the rest of the volume was made up with 25 mM ammonium bicarbonate to ensure that the gel pieces did not dry out. The samples were then incubated at 37 °C overnight. The next day, the excess solution was removed from the tubes and transferred to a fresh tube. Extraction of the peptides was carried out by incubating the gel pieces with 0.1% TFA in 50% ACN for 15 min, and excess solution was collected in the same tube into which the tryptic solution were transferred. Extraction was carried out thrice, and a final extraction was carried out by incubating for 5 min in 100% ACN. The pooled fractions were completely dried in a SpeedVac (Labconco CentriVap Console) and stored at −20 °C till injection into the mass spectrometer. Prior to injection, the dried peptides were reconstituted in 20 μL of 0.1% formic acid.

5.7. Sample Preparation for Mass Spectrometer Injection. Sample clean-up was carried out using Pierce C18 spin tips. The columns were activated using solution A (0.1% formic acid in 80% ACN). The peptides, reconstituted in solution B (0.1% formic acid in H2O), were loaded on to the column after washing thoroughly to remove traces of ACN. Once the sample was passed through the column, it was washed thrice with solution B. Elution was carried out by passing solution A through the column thrice. The elutions were pooled, dried in the SpeedVac, and stored at −20 °C for further use. Prior to injection, the dried peptides were reconstituted in 20 μL of solution B.

5.8. Serum Enrichment under Partly Denaturing Conditions Using ACN Precipitation. Serum enrichment
was carried out under partly denaturing conditions by adding different concentrations of ACN. 10 mg of serum from a total protein concentration of 60–70 mg/mL was directly precipitated using chilled ACN, with serum/ACN, 1:1 and 1:2 ratios. The samples were incubated on ice for an hour, with intermittent gentle vortexing, and then centrifuged at 13,000 rpm for 30–40 min. The supernatant was transferred to a fresh Eppendorf tube and centrifuged at 13,000 rpm for 10 min to remove the final traces of the precipitate and get a clear supernatant. This supernatant was completely dried in a SpeedVac and re-suspended in 100 μL Milli-Q water. This is now the enriched serum fraction. This enriched fraction was quantified using the Bradford assay, and a protein concentration of 60 μg/mL was determined. This enriched fraction was stored at −20 °C. To visualize the effectiveness of the enrichment by ACN precipitation, 20 μg of enriched serum was mixed with SDS loading dye and loaded onto a 15% SDS-PAGE gel. The gel was run at a constant voltage of 100 V until the dye front reached the bottom of the gel. Coomassie staining and destaining were performed to visualize the protein bands.

5.9. In-Solution Reduction, Alkylation, and Trypsin Digestion of Enriched Serum. 20 μg of enriched serum was denatured with 6 M urea freshly prepared in 50 mM TrisCl, pH 8.0. This denatured serum was incubated for 1 h at room temperature in buffer containing 200 mM DTT in 50 mM TrisCl, pH 8.0 for reduction. 200 mM IAA prepared in 50 mM TrisCl, pH 8.0 was further added and incubated for 1 h at room temperature in the dark for alkylation. Unreacted IAA was quenched by adding 200 mM DTT. Urea concentration was reduced to ~0.6 M by the addition of 1 mM CaCl2. Trypsin was added in 1:20 (trypsin/protein) ratio and incubated at 37 °C overnight. The next day, the samples were dried in SpeedVac and further re-suspended in 20–30 μL 0.1% formic acid and subjected to C18 spin tip clean-up.

5.10. iTRAQ Labeling and SCX. Two sets of patients were made, with four random patients in each set. Four aliquots, 20 μg each, of enriched serum were designated as patient 1, 2, 3, and 4 and were subjected in-solution reduction, alkylation, and trypsin digestion. The peptides thus generated were labeled using 4-plex iTRAQ labels obtained from AB SCIEX (SCIEX 4352135). Each set of peptides were labeled with one of the iTRAQ labels individually. viz. peptides of patient 1 were labeled with iTRAQ label 114, patient 2 with 115, patient 3 with 116, and patient 4 with 117. The labeled peptides were then mixed in a single tube, and SCX was carried out to fractionate the sample and remove unlabeled peptides, if any. The cation exchange cartridge was obtained from AB-SCIEX (ICAT Cartridge—Cation Exchange-4326695). The cartridge (200 μL, 4.0 mm x 15 mm) is packed with POROS 50 HS, with particle size 50 μm. SCX was carried out as per the manufacturer’s instructions with some modifications. The labeled peptides were reconstituted in approximately 2.5 mL of loading buffer containing 8 mM ammonium formate, pH 3.0. Furthermore, the SCX column was conditioned with 2 mL loading buffer. The re-suspended labeled peptides were loaded onto the SCX column at a flow-rate of 1 drop/s. This allows the binding of labeled peptides to the SCX column. The column was washed with 2 mL loading buffer. Fractions were collected at 50, 100, 150, 200, 250, 300, 400, and 500 mM ammonium formate concentrations. Each fraction was dried out completely and washed thrice with 0.1% formic acid and stored at −20 °C. Similarly, enriched serum samples from the second set were designated as patient 5, 6, 7, and 8. Peptides were generated, labeled with 4-plex iTRAQ, labels, and fractioned using SCX. MS analysis was carried out in biological and technical duplicates for both these sets.

5.11. Nano Liquid Chromatography Tandem Mass Spectrometry. nLC—MS/MS IDA runs were carried out in an ESI QTOF 5600 mass spectrometer coupled to a nanoLC column pre-packed with ChromXp C18 (3 μm, 120 AA) beads. 6 μL of the sample was injected into the column at a flow rate of 0.3 μL/min, and a 146 min run was carried out using a gradient flow of solution C (0.1% formic acid in H2O) and solution D (0.1% formic acid in 80% ACN). The gradient is as follows—initiate with 95% solution C and 5% solution D, 90% solution C and 10% solution D at 12 min, 70% solution C and 30% solution D at 92 min, 50% solution C and 50% solution D at 112 min, 20% solution C and 80% solution D at 113 min, and finally 95% solution C and 5% solution D at 127 min and 146 min. Data was acquired with 2.2 kV ion spray voltage, 25 psi curtain gas, and 20 psi nebulizer gas. MS runs were operated with a resolving power of 30,000whm. The Q1 selection range was set at 350–1250 m/z, and IDA scans were acquired in 100 ms, with 20–50 product ion scans collected for precursor ions exceeding a threshold for 100 counts per second. Data was acquired for product ions with a +2 to +5 charge-state. The MS runs were performed in biological and technical duplicates, and the data presented is representative of these runs.

5.12. Data Analysis and Software. All MS raw data was processed using PeakView software, version 1.2 (AB SCIEX), and protein identification and quantitation were carried out with ProteinPilot software, version 4.5 (AB SCIEX). The Paragon method parameters were set at 10%, which corresponds with p-value 0.05, as the threshold for protein detection. Proteins identified with 95% confidence and 1% global FDR were considered for further analysis. Molecular weights of the proteins identified were computed using ProtParam Expasy (https://web.expasy.org/protparam/) software. The biological class of the proteins was determined using PANTHER (http://www.pantherdb.org/) analysis, a comprehensive classification system that covers 131 complete genomes, organized into gene families and sub-families.16 The UNIPROT accession numbers were submitted as a list, and functional classification was carried out for Homo sapiens. The results were viewed in the form of a pie-chart.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04568.

Four-layer SDS gel loaded with different volumes of serum: lane 1: protein marker; lane 2: 5 μL; lane 3: 10 μL; lane 4: 15 μL; lane 5: 20 μL; lane 6: 30 μL; lane 7: 40 μL and proteins identified from MS analysis of proteins extracted from the gel filter method, with their corresponding molecular weights (PDF)

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Author Contributions

L.D. performed the experiments, L.D., V.M., and A.K.V. designed the experiments, and L.D. and A.K.V. wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Funding for this study was supported by the Terry Fox Foundation and Annual Scientific Fund from ACTREC-TMC. L.D. is thankful to University Grants Commission (UGC) for fellowship. The authors thank the Proteomics facility at ACTREC for providing necessary support to this study.

■ ABBREVIATIONS

HMW, high molecular weight; LMW, low molecular weight; LC−MS/MS, liquid chromatography−mass spectrometry; iTRAQ, isobaric tags for relative and absolute quantitation; SCX, strong-cation-exchange; MRM, multiple reaction monitoring; ACN, acetonitrile

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