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Contribution of Protein Fractionation to Depth of Analysis of the Serum and Plasma Proteomes

Vitor Faca,* Sharon J. Pitteri, Lisa Newcomb, Veronika Glukhova, Doug Phanstiel, Alexei Krasnoselsky, Qing Zhang, Jason Struthers, Hong Wang, Jimmy Eng, Matt Fitzgibbon, Martin McIntosh, and Samir Hanash

Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109

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In-depth analysis of the serum and plasma proteomes by mass spectrometry is challenged by the vast dynamic range of protein abundance and substantial complexity. There is merit in reducing complexity through fractionation to facilitate mass spectrometry analysis of low-abundance proteins. However, fractionation reduces throughput and has the potential of diluting individual proteins or inducing their loss. Here, we have investigated the contribution of extensive fractionation of intact proteins to depth of analysis. Pooled serum depleted of abundant proteins was fractionated by an orthogonal two-dimensional system consisting of anion-exchange and reversed-phase chromatography. The resulting protein fractions were aliquotted; one aliquot was analyzed by shotgun LC−MS/MS, and another was further resolved into protein bands in a third dimension using SDS-PAGE. Individual gel bands were excised and subjected to in situ digestion and mass spectrometry. We demonstrate that increased fractionation results in increased depth of analysis based on total number of proteins identified in serum and based on representation in individual fractions of specific proteins identified in gel bands following a third-dimension SDS gel analysis. An intact protein analysis system (IPAS) based on a two-dimensional plasma fractionation schema was implemented that resulted in identification of 1662 proteins with high confidence with representation of protein isoforms that differed in their chromatographic mobility. Further increase in depth of analysis was accomplished by repeat analysis of aliquots from the same set of two-dimensional fractions resulting in overall identification of 2254 proteins. We conclude that substantial depth of analysis of proteins from milliliter quantities of serum or plasma and detection of isoforms are achieved with depletion of abundant proteins followed by two-dimensional protein fractionation and MS analysis of individual fractions.

Keywords: human plasma proteins • human serum proteins • intact protein fractionation • liquid chromatography • LC−MS/MS

Introduction

Human serum and the plasma from which it is derived are complex proteomes that consist of vast assemblies of proteins and complexes that reflect the physiological or pathological state of cells, tissues, and organs. Serum and plasma proteins occur in concentrations ranging from milligrams to picograms, or less, per milliliter.[1] These proteins often occur in multiple isoforms resulting from cleavage or other post-translational modifications as well as from polymorphism and transcriptional and splice variation.[2,3] While such characteristics make this biological fluid one of the most challenging to study, its ready availability makes it suitable for interrogating the state of health of an individual.

Mass spectrometry has evolved sufficiently to detect and identify femtomoles of peptides, but the dynamic range of detection is still a limiting factor for in-depth serum profiling,[4−6] covering less than half of the logarithmic range of abundance of serum proteins that have clinical utility. Two basic strategies are available to increase depth of analysis: (i) removal of high-abundance proteins such as albumin and immunoglobulins that interfere with the detection of less abundant proteins,[7,8] and (ii) fractionation of samples by chromatographic or other means to reduce sample complexity.[2,4,9−14] A recent study[15] has demonstrated a limited depth of analysis with some fractionation or enrichment schemes that were investigated. However, the gains achieved with extensive fractionation have not been fully assessed.

We previously described a gel-based approach to visualize fractionated serum and plasma proteins.[2,9] Initially, with this approach, each serum sample or pool is immunodepleted of abundant proteins and labeled with a different Cy dye followed by mixing and fractionation by liquid isoelectric focusing, reversed-phase chromatography, and SDS-PAGE of individual fractions. Protein bands that exhibit quantitative differences based on Cy dye ratios are excised from gels and their

* Correspondence should be addressed to Vitor Faca. Tel, (206) 667-1943; fax, (206) 667-2537; e-mail, vfaca@fhcrc.org.

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corresponding proteins identified by mass spectrometric analysis. An alternative strategy for protein analysis is to subject chromatographic fractions to LC−MS/MS following isotopic labeling or in a label free approach.[16−18] In this study, we have evaluated the depth of analysis achieved by two-dimensional chromatographic fractionation of immunodepleted serum or plasma followed by LC−MS/MS analysis of individual fractions. We also examined the extent to which proteins identified in gel bands following a third-dimension SDS gel analysis of individual fractions were represented in the protein identifications following digestion and LC−MS/MS analysis of two-dimensional fractions, thus, reducing the need for further fractionation.

Methods

Sample Preparation and Immunodepletion. Two pools of samples were used in the experiments described here. The first consisted of a pool of serum from newly diagnosed subjects with lung cancer and from healthy controls that serves as reference material for ongoing lung cancer studies. A similar pool of plasma from subjects with breast cancer and from healthy controls was prepared that serves as reference material for breast cancer studies. In this study, a volume of 2.4 or 1.2 mL from lung cancer pool or breast cancer pool, respectively, was immunodepleted of the top six most abundant proteins (Albumin, IgG, IgA, Transferrin, Haptoglobin, and α-1-Antitrypsin) using HU-6 columns (4.6 × 100 mm; Agilent, Wilmington, DE)[2,9] prior to further analysis.

Protein Separation. Proteins in the lung cancer serum pool immunodepleted for the top-six proteins, were separated in the first-dimension by anion-exchange chromatography on a Mono-Q 10/100 column (Amersham Biosciences). The buffer system consisted of solvent A, 20 mM Tris in 6% isopropanol and 4 M urea, pH 8.5, and solvent B, 20 mM Tris in 6% isopropanol, 4 M urea, and 1 M NaCl, pH 8.5. The separation was performed at 4.0 mL/min in a gradient of 0−35% solvent B in 44 min; 35−50% solvent B in 3 min; 50−100% solvent B in 5 min, and 100% solvent B for additional 5 min. In total, 12 fractions were collected and run individually by reversed-phase chromatography using a Poros R2 column (4.6 × 50 mm, Applied Biosystems) using TFA/acetonitrile as buffer system (solvent A, 95% H_2O, 5% acetonitrile + 0.1% TFA, and solvent B, 90% acetonitrile, 10% H_2O + 0.1% TFA) at 2.7 mL/min. The gradient used was 5% solvent A until absorbance reached baseline (desalting step) and then 5−50% solvent B in 18 min, 50−80% solvent B in 7 min, and 80−95% solvent B in 2 min. Forty-eight fractions of 900 μL were collected during the run. Then, 200-μL aliquots of some fractions were subjected to mass spectrometry shotgun analysis either individually or after pooling 3 consecutive fractions to determine the yield of protein identifications in relation to extent of fractionation. Additional individual aliquots were subjected to SDS-PAGE (7.5−17% of acrylamide, 16 × 16 cm, 28 wells). Proteins from individual aliquots were dissolved in 20 μL of buffer (120 mM Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 3% DTT, and 0.03 M bromphenol blue) and separated at 65 V overnight at 10 °C. Gels were bonded to the inner plates (18 × 16 cm low fluorescence glass, Amersham Biosciences) using bind-silane as recommended by the manufacturer to permit scanning and automatic spot-picking. Some individual bands detected in gels were excised and subjected to LC−MS/MS analysis following in situ digestion. The two-dimensional fractionation schema was also used for the reference breast cancer pool. After immunodepletion, samples were fractionated by anion-exchange into 12 fractions and subsequently by reversed-phase into 12 fractions, representing a total of 144 fractions that were analyzed individually by shotgun LC−MS/MS.

Mass Spectrometry Analysis. In-solution digestion was performed with lyophilized aliquots from the reversed-phase (second dimension) fractionation step. Proteins in individual fractions were re-suspended in 0.25 M urea containing 50 mM ammonium bicarbonate and 4% acetonitrile and then digested overnight with 200 ng of modified trypsin (Promega). The digestion was interrupted by addition of 5 μL of 10% formic acid solution. For in situ tryptic digestion, gel plugs were extensively washed with 50 mM of ammonium bicarbonate containing 50% acetonitrile, then vacuum-dried and incubated with 20 μL of 20 μg/mL trypsin solution in 50 mM of ammonium bicarbonate. Digestion was carried out overnight at 37 °C. The resulting peptide mixtures were acidified with 5 μL of 1% formic acid. Digests obtained by both in-gel digestion and in-solution digestion were analyzed by a LTQ-FTICR mass spectrometer (Thermo-Finnigan) coupled with a NanoAcquity-nanoflow chromatography system (Waters). The liquid chromatography separation was performed in a 25 cm column (Picofrit 75 μm i.d., New Objectives, packed in-house with MagicC18 resin) using a 90 min linear gradient (60 min for gel spots analysis) from 5 to 40% of acetonitrile in 0.1% formic acid at 250 nL/min for shotgun analysis and 60 min for in-gel digests. Spectra were acquired in a data-dependent mode in m/z range of 400−1800, including selection of the 5 most abundant +2 or +3 ions of each MS spectrum for MS/MS analysis. Mass spectrometer parameters were capillary voltage of 2.0 KV, capillary temperature of 200 °C, resolution of 100 000, and FT target value of 2 000 000.

Acquired data was automatically processed by the Computational Proteomics Analysis System (CPAS)[19] pipeline. This pipeline includes the X!Tandem search algorithm[20] with comet score module plug-in,[21] PeptideProphet[22] peptide validation, and ProteinProphet[23] protein inference tool. The tandem mass spectra were searched against version 3.12 of the human IPI database.[24] All identifications with a PeptideProphet probability greater than 0.2 were submitted to ProteinProphet, and the subsequent protein identifications were filtered at a 5% error rate.

Results

Protein Fractionation and Gel-Band-Derived Protein Identification. Immunoaffinity capture of high-abundance proteins from 2.4 mL (61 mg/mL) of pooled serum removed 83% of the total protein mass. The remaining 25.1 mg of protein was subjected to anion-exchange fractionation into 12 fractions that were further individually separated in a second dimension, based on hydrophobicity, using reversed-phase chromatography (Figure 1a,b). In total, 576 fractions were collected after two-dimensional fractionation, 48 for each of the 12 anion exchange fractions (Figure 1b). Aliquots from individual reversed-phase fractions were subjected to SDS-PAGE (Figure 1c). Additional aliquots were subjected to shotgun LC−MS/MS either individually or after pooling with neighboring fractions to determine the yield of proteins in relation to extent of fractionation.

The SDS gel image of individual fractions exhibited a complex pattern of bands per fraction, demonstrating that even after extensive fractionation, individual reversed-phase fractions still contained a complex mixture of proteins. Densito-

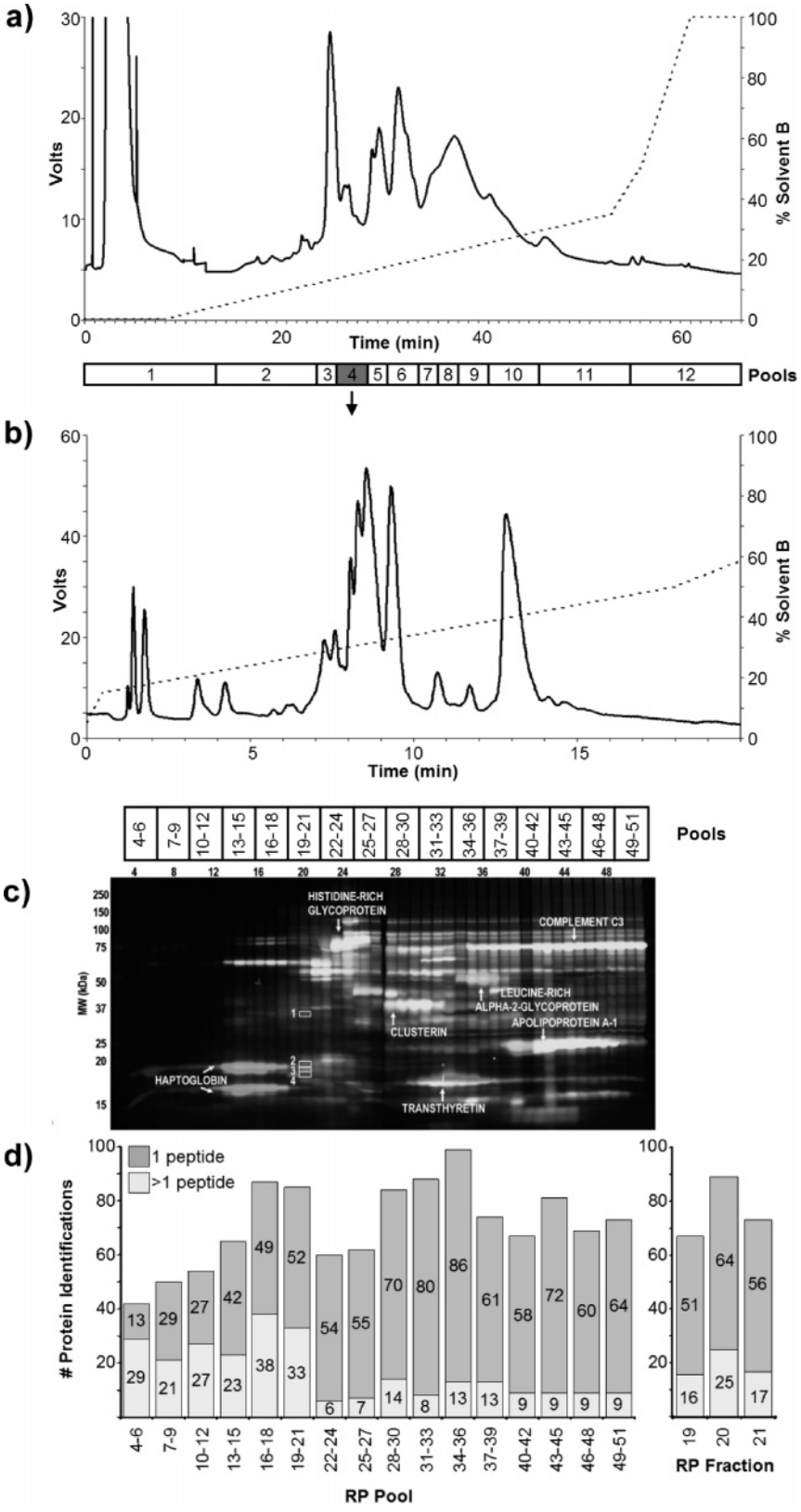

Figure 1. Extensive fractionation of a reference lung cancer serum pool. (a) Anion exchange chromatogram of a serum pool. Individual fractions were collected as indicated by bars on the bottom of the chromatogram. (b) Reversed-phase chromatogram obtained for anion-exchange fraction 4. Reversed-phase fractions were divided and analyzed by LC−MS/MS individually (for as fractions 19−21) or in pools of 3 fractions as indicated by bars at the bottom of the chromatogram. (c) SDS-PAGE gel of individual reversed-phase fractions derived from anion-exchange fraction 4. Arrows point to some abundant proteins identified. Boxes indicate gel bands that were selected for LC−MS/MS identification. (d) Number of protein identifications for each of the fractions or pools analyzed by LC−MS/MS derived from anion-exchange fraction 4.

metric analysis of individual lanes indicated 3 to 4 orders of magnitude of band intensities. A set of bands of varied intensities from SDS gel electrophoresis was subjected to in situ digestion and LC−MS/MS analysis for comparison with LC−MS/MS analysis of the fraction from which they were derived. Bands 1−4 (Figure 1c) yielded a total of 65 unique protein identifications (26 proteins per band on average). The list included proteins with known concentrations ranging from milligram per milliliter (mg/mL) to low nanogram per milliliter (ng/mL) as represented in the latter case by insulin-like growth factor binding protein 2 (15 ng/mL)[25] (see Supplemental Table 1 in Supporting Information). The three reserved-phase fractions whose constituent bands were subjected to MS analysis, were also analyzed by LC−MS/MS following digestion, yielding an average of 76 proteins per fraction and a total of 117 nonredundant proteins for the three fractions (Figure 1d). Comparison of protein identifications indicated that 19 proteins were identified in third-dimension gel bands but failed to be identified by analysis of corresponding second-dimension reversed-phase fractions, indicating greater depth of analysis with addition of a third-dimension SDS gel-based separation. This contrast in depth of analysis between gel bands and whole fractions is further illustrated by peptide coverage in the case of IGFBP2, which was identified in SDS band 1 with 17 unique peptides, but with only 5 peptides from the corresponding second-dimension reversed-phase fraction (Supplemental Table 1 in Supporting Information).

We next compared the yield of identifications based on extent of reversed-phase fractionation. The set of 48 reversed-phase fractions from anion exchange fraction 4 was subjected to digestion and LC−MS/MS analysis either singly or as pools of 3 consecutive fractions. Fractions 19−21 yielded 85 protein identifications when analyzed as a pool, compared to 117 unique protein identifications when analyzed individually (Supplemental Table 1 in Supporting Information). All proteins with 2 or more peptides detected in the pooled LC−MS/MS run were also detected in the LC−MS/MS runs of individual fractions. In terms of protein sequence coverage, an average of 43% more peptides were identified per protein by analysis of individual reversed-phase fractions compared to analysis of pools of three reversed-phase fractions (Figure 2). Proteins identified only by analysis of individual fractions included several that are known to be of low abundance in plasma (<1 μg/mL), namely, ICAM2, IGFALS, L-selectin, and superoxide dismutase (Supplemental Table 1 in Supporting Information).

From the 16 LC−MS/MS runs of pools of 3 fractions, 306 nonredundant proteins were identified based on criteria of one or more peptides and ProteinProphet error rate <5%, with 253 proteins being identified with two or more peptides, representing 71 protein identifications per pool (Figure 1d and Supplemental Table 2 in Supporting Information). These proteins range in known concentration in serum over at least 5 orders of magnitude. Examples are apolipoprotein A-I and epidermal growth factor receptor, which were detected in the same run range in plasma concentration between 1.4 mg/mL and 11 ng/mL, respectively. Many of the proteins were identified in multiple reversed-phase pools, which is expected based on their high abundance and consequently trailing in the reversed-phase fractionation. The redundancy of protein identification between pooled fractions was more prominent for the late fractions, which is consistent with the gel patterns observed for these fractions (Figure 1c).

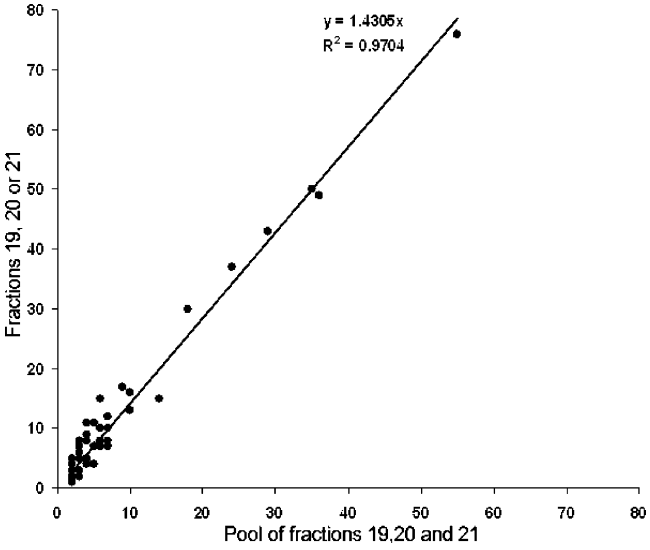

Figure 2. Increased protein sequence coverage with extended fractionation. Reversed-phase fractions that were analyzed individually by LC−MS/MS provided 43% more protein coverage in comparison to pooled fractions. The correlation of number of unique peptides was calculated for all proteins present in both sets of data.

Contribution of Repeat LC−MS/MS Runs to Depth of Analysis. The overall number of protein identifications and protein sequence coverage increased with fractionation and analysis of individual fractions. An alternative approach to increase depth of analysis (i.e., sensitivity of detection and protein sequence coverage) is to implement a more limited fractionation scheme with repeat analysis of each fraction. Data-dependent acquisition with LC−MS/MS analysis involves sampling of the eluate.[6] Even with the fastest and most advanced instruments, the complexity of plasma samples does not permit every single ion to be selected for CID sequencing. Using the same two-dimensional intact protein fractionation schema as described above, we generated 144 fractions (12 anion-exchange × 12 reversed-phase) from our reference pool of normal and breast cancer plasma. LC−MS/MS analysis of these 144 fractions was performed using a 90 min separation gradient. More than 2 million MS2 events were acquired, resulting in high confidence identification of 14 510 peptides. The estimated peptide identification sensitivity was greater than 80% and error rate less than 3% based on PeptideProphet. When combined together into protein groups using ProteinProphet, a total of 1662 protein identifications with less than 5% error rate was obtained. The complete list of peptides and proteins is presented in Supplemental Table 3 in Supporting Information.

An average of 116 proteins were identified per LC−MS/MS run. Figure 3a shows the distribution of protein identifications across all fractions. Chromatographic resolution at the intact-protein fractionation level is evident by the fact that a majority of the proteins were detected in single fractions (Figure 3b). This efficiency at both the peptide and protein levels of fractionation facilitates identification of low-abundance proteins. Several proteins were identified that occur in plasma at concentrations less than 100 ng/mL[25] (Supplemental Table 3 in Supporting Information). Of equal importance is the fact that low-abundance proteins presented good sequence coverage (Supplemental Tables in Supporting Information).

To assess the effect of mass spectrometer sampling rate on plasma protein identification, a technical replication of the LC−

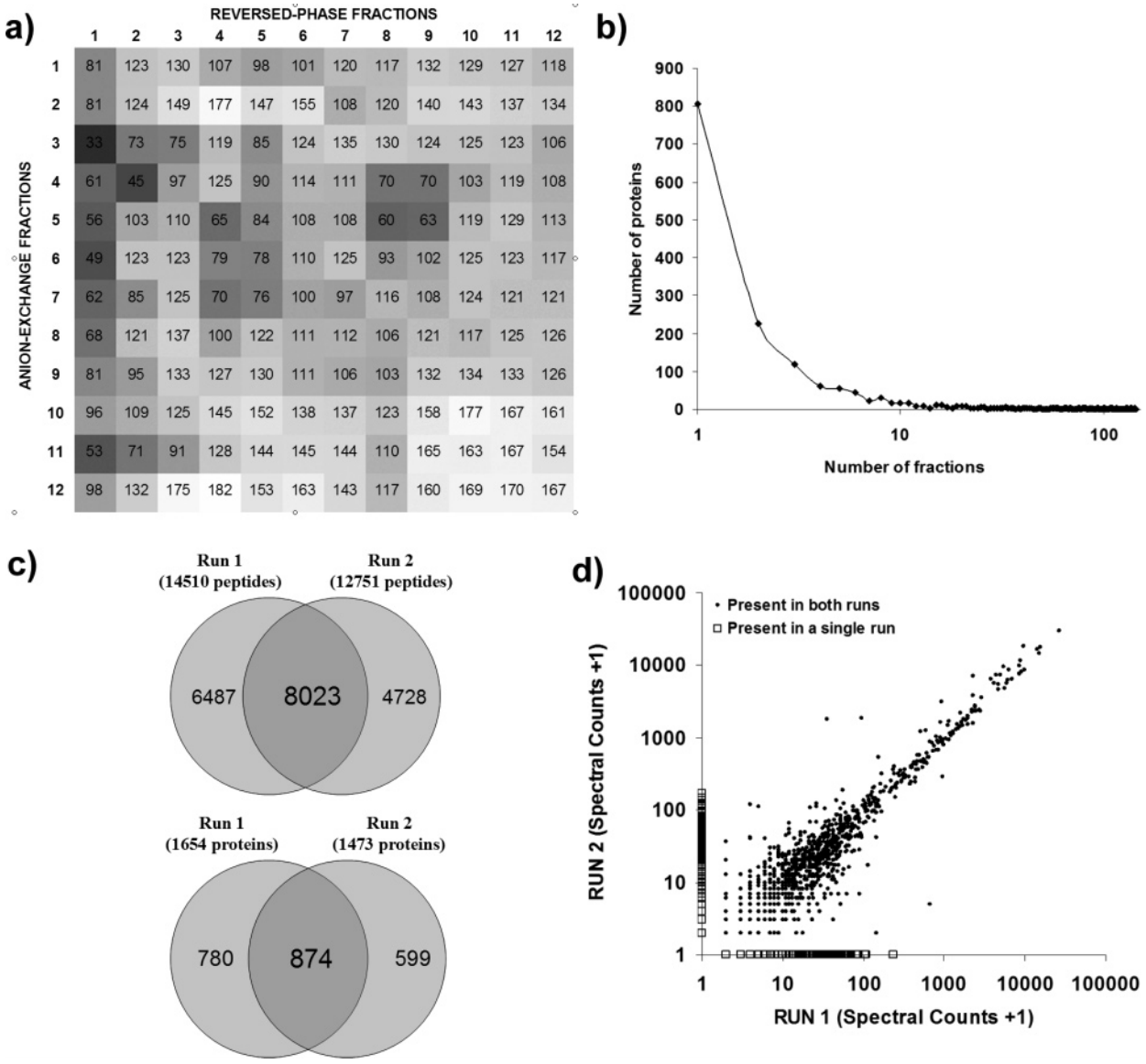

Figure 3. Extensive fractionation and protein identification of a reference breast cancer plasma pool. (a) The sample was fractionated into 144 fractions that were individually analyzed by LC−MS/MS. The number of identifications for each fraction is indicated. On average, 114 proteins were identified per fraction, which resulted in a total of 1654 nonredundant protein identifications for the entire experiment. (b) Most of the proteins were identified in single fractions. (c) Repeat runs of individual fractions yielded 4729 (32%) additional peptides and 599 (36%) additional protein identifications. (d) Proteins identified exclusively in one of the runs mostly represented low-abundance proteins, with spectral counts (number of MS2 events for a given protein) of less than 100.

MS/MS portion of the experiment was performed. Aliquots from the same 144 digested fractions were subjected to LC−MS/MS again under identical conditions (Supplemental Table 4 in Supporting Information). On average, identifications in duplicate runs of the same fractions overlapped by 76%. Overall, of the 19 240 unique peptides identified in duplicate runs, 8023 (42%) were identified in both runs. In total, we identified 2254 unique proteins (Figure 3c). Most proteins identified in only one of the duplicate runs yielded less than 100 spectral counts (Figure 3d) indicative of their low abundance in plasma.

Gene Ontology analysis of this comprehensive data set of 2254 plasma proteins indicated that 32% of proteins were annotated as secreted, 32% as glycoproteins, and 21% as membrane proteins. Peptides for several membrane proteins identified were derived exclusively from the extracellular domain, indicating shedding of extracellular domains into the circulation.

To assess how comprehensive our breast cancer reference data set was, we compared identifications with the Plasma Peptide Atlas[26] (April 2007 Build). The human plasma peptide atlas encompasses identifications from the Human Plasma Proteome Initiative.[1] From the 10 268 unique peptides identified in our data set with PeptideProphet scores above 0.9 (the same minimum score for peptides deposited in the Peptide Atlas), 48% (4908) were also present in Peptide Atlas. The remaining 5360 high-confidence peptides identified in our breast cancer reference data set represent a 20% increment of identified peptides (27 475 peptides) representing 1371 of additional proteins (at FDR <5%) not currently in the Peptide Atlas.

Analysis of Protein Fragments and Protein Isoforms. Fractionation of intact proteins has the potential to separate

protein isoforms and protein cleavage products that have different physicochemical characteristics. Figure 4a illustrates this feature with the separation of a C-terminal fragment of insulin-like growth factor binding protein 2 (IGFBP2) from the intact protein. The short C-terminal peptide eluted early by reversed-phase chromatography, compared to the full-length protein. A slight change in p*I*, resulting in elution in different anion-exchange fractions, was also observed. Several proteolytic enzymes have been identified that cleave insulin-like growth factor binding proteins (IGFBPs). It has been suggested that proteolytic cleavage of IGFBPs is associated with regulation of the proliferative effects of IGFs on their target cells.[27] Likewise, the two-dimensional fractionation schema allowed separation of the active form of transforming growth factor beta 1 (TGFB1) from its associated latency peptide (Figure 4b). Only the active TGFB1 sequence is represented in early anion-exchange fractions. Activation of TGFB1 is achieved by a photolytic cleavage in amino acid 278 by plasmin and other mechanisms.[28]

Elution profiles of particular proteins in the 2-D intact protein fractionation schema, that cannot be explained on the basis of cleavage, are illustrated in Figure 4c for carboxipeptidase b2 which was detected in 3 distinct clusters of fractions. Although, carboxypeptidase b2 can be activated by thrombin/thrombomodulin through cleavage of its N-terminal peptide (23−114),[24] there is no evidence that the clusters observed are due to cleavage. This protein has several known glycosylation sites, and differences in glycosylation may account for the chromatographic behavior observed.

Discussion

We provide evidence that extent of fractionation of intact proteins in serum or plasma has a substantial impact on overall number of protein identifications and protein sequence coverage (i.e., depth of analysis) by LC−MS/MS and on the potential to identify low-abundance proteins and protein isoforms. A total of 1662 proteins was identified from 144 plasma fractions analyzed once each by LC−MS/MS runs, and a total of 2254 proteins was identified in duplicate runs of each fraction. Our findings stand in contrast with a recent publication[15] which evaluated individually different fractionation techniques that yielded at most 252 unique protein identifications. In our study, we explored two approaches to increase depth of analysis. One consisted of vastly increasing the number of fractions analyzed, and another consisted of duplicate runs of a more limited number of fractions generated. It is evident that both approaches increase depth of analysis. Duplicate runs in our study yielded 599 additional protein identifications compared with single runs (overall increase of 35% from 1662 to 2254). The additional number of identifications greatly exceeds the estimated number of false positives (5%) propagated by duplicate analysis. Duplicate runs help overcome the sampling rate limitation of MS data acquisition and, therefore, increase the probability that a low-abundance ion is selected for MS/MS analysis. Ideally, the total number of fractions should be based on the resolution of the chromatography method utilized. As shown here, a 3-fold increase in fraction number increased the number of identifications by 38% and protein sequence coverage by 43% based on the analysis of a limited number of fractions.

In this study, the detection limit achieved with 144 fractions is estimated to be close to 1 ng/mL. This concentration corresponds to that of prostate specific antigen (PSA) in normal plasma. Achieving such depth of analysis is of particular

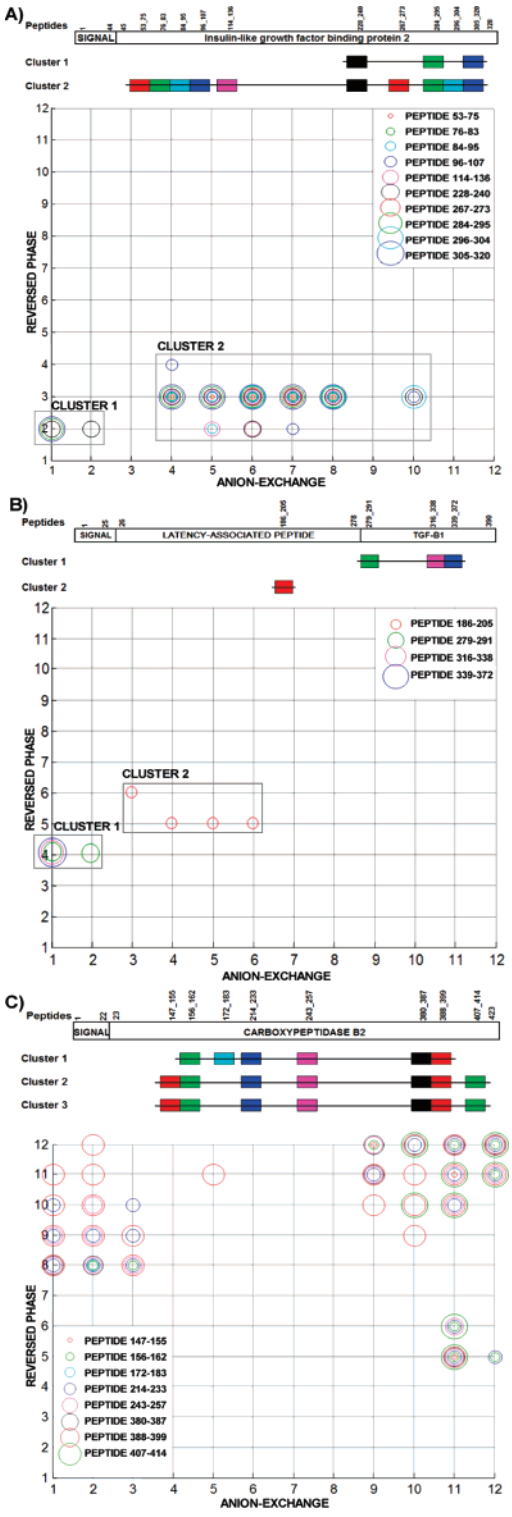

Figure 4. Identification of protein isoforms/fragments using intact protein fractionation. (a) Insulin-like growth factor binding protein 2 peptides were identified in two different clusters in the two-dimensional fractionation schema. Alignment of these peptides indicated the occurrence of two forms, an intact form and a C-terminal fragment. (b) Likewise, protein fractionation separated the mature form of transforming growth factor beta 1 from its latency-associated peptide. (c) Three different clusters of carboxypeptidase b2 were observed that likely result from post-translational modification. The colored circles in the legend indicate the peptide sequence position and match with the colored boxes in the peptide alignments on top of each panel.

importance for biomarker discovery as most disease-specific biomarkers represent low-abundance proteins in a range that encompasses PSA. These proteins largely represent secreted or intracellular proteins that "leak" into the blood stream from cell death or other mechanisms.

Another important feature of intact protein fractionation is the potential to detect protein cleavage products and isoforms. Protein cleavage may result from the action of circulating plasma proteases or through the tissue environment in which extracellular proteases cleave surface proteins resulting in their release into extracellular fluids. The IPAS approach utilized in this study allows isoforms to be distinguished as illustrated for insulin-like growth factor binding protein 2, transforming growth factor beta 1, and carboxypeptidase b2.

In conclusion, the use of an extensive intact protein fractionation schema together with shotgun LC−MS/MS analysis of individual fractions has allowed comprehensive profiling of serum and plasma. Extensive fractionation was found to be critical for the identification of low-abundance proteins as a result of "de-complexing" the preparation subjected to mass spectrometry. This also permits a more detailed investigation of the particular protein forms identified and their post-translational modifications that may be associated with disease, a feature that is not readily achievable with protein digest-based fractionation approaches.

Supporting Information Available: Tables listing the correlation of protein identifications between fractions and subfractions obtained for the reference lung cancer sample (supplemental Table 1), protein identifications obtained for the 16 LC−MS/MS runs corresponding to the reversed-phase pools of the reference lung cancer sample (supplemental Table 2), protein identifications obtained for the 144 LC−MS/MS runs corresponding to the reversed-phase fractions of the reference breast cancer sample (supplemental Table 3), and protein identifications of the 144 LC−MS/MS duplicate runs corresponding to the reversed-phase fractions of the reference breast cancer sample (supplemental Table 4). This material is available free of charge via the Internet at http://pubs.acs.org.

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