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New Approaches for Biomarker Discovery: The Search for Liver Fibrosis Markers in Hepatitis C Patients

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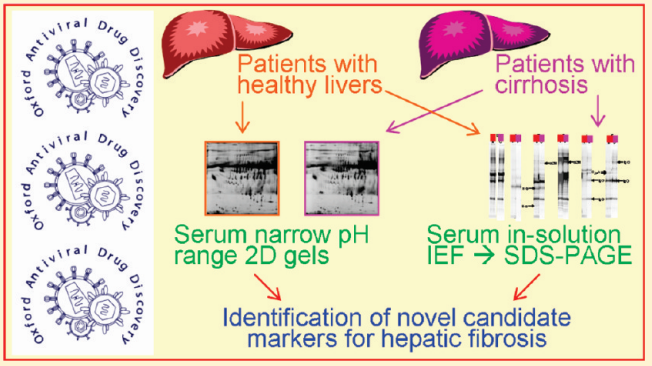
Supporting Information

ABSTRACT: Despite many shortcomings, liver biopsy is regarded as the gold standard for assessing liver fibrosis. A less invasive and equally or more reliable approach would constitute a major advancement in the field. Proteomics can aid discovery of novel serological markers and these proteins can be measured in patient blood. A major challenge of discovering biomarkers in serum is the presence of highly abundant serum proteins, which restricts the levels of total protein loaded onto gels and limits the detection of low abundance features. To overcome this problem, we used two-dimensional gel electrophoresis (2-DE) over a narrow pH 3−5.6 range since this lies outside the range of highly abundant albumin, transferrin and immunoglobulins. In addition, we used in-solution isoelectric focusing followed by SDS-PAGE to find biomarkers in hepatitis C induced liver cirrhosis. Using the pH 3−5.6 range for 2-DE, we achieved improved representation of low abundance features and enhanced separation. We found in-solution isoelectric focusing to be beneficial for analyzing basic, high molecular weight proteins. Using this method, the beta chains of both complement C3 and C4 were found to decrease in serum from hepatitis C patients with cirrhosis, a change not observed previously by 2-DE. We present two proteomics approaches that can aid in the discovery of clinical biomarkers in various diseases and discuss how these approaches have helped to identify 23 novel biomarkers for hepatic fibrosis.



KEYWORDS: hepatitis C, fibrosis, two dimensional gel electrophoresis, narrow range immobilized pH gradient, in-solution isoelectric focusing

INTRODUCTION

Over 170 million people, approximately 3% of the world's population, are infected with the hepatitis C virus (HCV).[1] Infection with this virus can result in hepatic fibrosis which can progress to cirrhosis and hepatocellular carcinoma. Liver biopsy is the gold standard for the diagnosis and prognosis of hepatic fibrosis,[2] but this approach is costly, invasive, painful, and unreliable if the scarring in the liver is not homogeneous or biopsies under 10 mm are analyzed.[2,3] Current serum biomarkers only eliminate the need for biopsies in 26% of cases.[4] More reliable noninvasive markers to replace liver biopsy would benefit patients and practitioners alike.

Blood proteins whose expression levels change with increasing fibrosis could be used as less invasive biomarkers which are easily obtainable. Such novel serological markers could be identified using proteomics to separate serum or plasma and identify the differentially expressed proteins. However, the wide dynamic range of protein concentrations which span over 10 orders of magnitude in serum and plasma, poses a significant problem for proteomic analysis. Highly abundant proteins, especially albumin, immunoglobulins and transferrin, restrict protein load on gels for electrophoresis and limit the detection of low abundance proteins.[5] Despite this we have previously used two-dimensional gel electrophoresis (2-DE) over the wide pH 3−10 range to identify several novel candidate serum biomarkers for liver fibrosis.[6] To address the problem posed by highly abundant proteins, here we use 2-DE over a narrow pH 3−5.6 range since this lies outside the range of highly abundant albumin, transferrin and immunoglobulins. This enables the loading of four times more protein than in our previous fibrosis marker study, and considerably enhances representation of low abundance features. We present significantly improved gel-based separation of the acidic proteome and have identified low abundant features which

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were below the detection threshold in the study using the wide pH 3−10 range. We also examine the basic serum proteome for fibrosis markers using in-solution isoelectric focusing (IEF) followed by SDS-PAGE and discuss the benefit of this approach over 2-DE for basic high molecular weight proteins. Using this approach we have identified the beta chains of both complement C3 and C4 as candidate fibrosis markers which were not observed previously by 2-DE.

The two proteomic approaches shown in this study can aid clinical biomarker discovery not only for hepatic fibrosis but many other diseases.

EXPERIMENTAL SECTION

2-DE

One and 2 mg of pooled normal human serum (Sigma, Dorset, UK) were separated using 18 cm pH 3−10, pH 5−6, pH 3−5.6 nonlinear (NL) and pH 6−11 immobilized pH gradient (IPG) strips (GE Healthcare, Bucks, U.K.). Samples were made up to 375 μL in IEF rehydration buffer (5 M urea, 2 M thiourea, 2 mM tributyl phosphine, 65 mM DTT, 4% (w/v) CHAPS, 150 mM nondetergent sulfobetaine 256 (NDSB-256) and 0.0012% (w/v) bromophenol blue). 2-DE was performed as described earlier for the pH 3−10 gels.[6] Gels covering the other pH ranges were also run in the same way except with 1.8% (v/v) pH 5−6, pH 3−6 and pH 6−11 ampholytes (SERVALYT, SERVA, Heidelberg, Germany) for the pH 5−6 NL, pH 3−5.6 and pH 6−11 strips, respectively. Samples were left overnight to rehydrate 18 cm pH 3−5.6 NL IPG DryStrips (GE Healthcare, Bucks, U.K.). IEF was carried out for 75 kVh at 17 °C. Strips were incubated in equilibration solution (4 M urea, 2 M thiourea, 50 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 2% (w/v) SDS, 130 mM DTT, 0.002% (w/v) bromophenol blue) for 15 min. Proteins were separated by 9−16% (w/v) SDS-PAGE gradient gels using 20 mA per gel for 1 h, followed by 40 mA per gel for 4 h at 10 °C. Following electrophoresis, gels were fixed in 40% (v/v) ethanol and 10% (v/v) acetic acid and stained with the fluorescent dye OGT 1238.[7] Although this dye is proprietary, it is similar to other commercial dyes such as Sypro Ruby or Sypro Orange which can be used as alternatives. Gels were scanned using an Apollo II linear fluorescence scanner (Oxford Glycosciences, Abingdon, U.K.) to obtain 16-bit images at 200 μm resolution. This Apollo II scanner is custom built but any imager which can image fluorescently stained gels, such as the Fuji LAS range of cameras, could be used as alternatives. Features were detected using a custom version of the Melanie II software (Oxford Glycosciences, Abingdon, UK).[7] Although customized, the software was only used to curate spots and determine the number of spots per gel which can be done with any commercially available software such as Melanie 7. Artifacts were removed and features were manually edited if spot splitting was required. Serum (500 μg) was also resolved by 2-DE using pH 3−10 NL IPG strips as previously described.[6]

In-Gel Digestion and Peptide Extraction

Differentially expressed bands on the SDS-PAGE gels were assigned for mass spectrometric analysis. The bands on the SDS-PAGE gels were excised manually and dried in a SpeedVac. In-gel trypsin digestion was carried out manually according to the protocol of Shevchenko and co-workers.[8] Digested samples were lyophilized and dissolved in 0.1% (v/v) formic acid prior to mass spectrometric analysis.

Mass Spectrometric Analysis

Tryptic peptides were analyzed using a Q-TOF 1 mass spectrometer coupled to a CapLC (Waters, Hertfordshire, U.K.). Peptides were concentrated and desalted on a 300 μm I.D./5 mm C18 precolumn and resolved on a 75 μm I.D./25 cm C18 PepMap analytical column (LC packings, Sunnyvale, CA) with a 45 min 5−95% (v/v) acetonitrile gradient containing 0.1% (v/v) formic acid at a flow rate of 200 nL/min. Spectra were acquired in positive mode. MS to MS/MS switching was controlled in an automatic data-dependent fashion with a 1 s survey scan followed by three 1 s MS/MS scans. Ions selected for MS/MS were excluded from further fragmentation for 2 min. Raw MS/MS spectra were smoothed and centered using ProteinLynx Global server 2.1.5, spectra were not deisotoped. Processed peak list (.pkl) files were searched against the Swiss-Prot database (release 54.4) using MASCOT (Matrix Science, London, U.K.). Searches were restricted to the human (17 565 sequences) and virus (11 132 sequences) taxonomies. Carbamidomethyl cysteine was defined as a fixed modification and oxidized methionine as a variable modification. Data were searched allowing 0.5 Da error to accommodate calibration drift and up to 2 missed tryptic cleavage sites. All data were checked for consistent error distribution and all positive identifications were checked manually.

In-Solution IEF

Healthy control and cirrhotic blood samples were collected in Serum Separator Tubes (BD, Oxford, U.K.). Patients were age and sex matched (males in 30s). The patients were recruited from the John Radcliffe Hospital, Oxford, U.K. and cirrhosis was determined using the Ishak scoring method as previously described.[9] These scores along with the ages and gender are displayed in Supporting Table 1, Supporting Information. Collection of patient samples for this study was approved by the Central Oxford Research Ethics Committee (No. 98.137) and consent was obtained from each patient. Sera were diluted in IEF rehydration buffer with 1.8% (v/v) pH 3−10 carrier ampholytes and 0.002% (w/v) bromophenol blue to a final protein concentration of 298.5 μg/mL. In-solution IEF was performed as previously described[10,11] using an IEF fractionator (Invitrogen, Paisley, U.K.) with the following pH ranges in each fractionation chamber: pH 3−4.6; pH 4.6−5.4; pH 5.4−6.2; pH 6.2−7; pH 7−10. The anode buffer was prepared with 7 M urea, 2 M thiourea and Novex IEF Anode buffer pH 3.0 (Invitrogen). The cathode buffer was prepared with 7 M urea, 2 M thiourea and Novex IEF Cathode buffer pH 10.4 (Invitrogen). Anode and cathode buffers (17.5 mL each) were loaded into the respective electrode reservoirs of the IEF fractionator. 670 μL of the diluted serum samples were added to the five fraction chambers. Fractionation was performed using 100 V for 20 min, 200 V for 80 min and 600 V for 80 min at 2 mA and 2 W. Detergents and salts were removed from the samples by chloroform−methanol precipitation as previously described for in-solution IEF[12] before being resolved by 4−12% (w/v) NuPAGE Bis-Tris-HCl SDS-PAGE (Invitrogen) according to the manufacturer's recommendation. The intensity of gel bands for healthy control were compared to cirrhotic serum using Advanced Image Data Analyzer software (Raytest, Straubenhardt, Germany).

RESULTS

2-DE Optimization

Narrow range IPG strips with the ranges pH 6−11, pH 5−6 and pH 3−5.6 along with wide range pH 3−10 IPG strips were

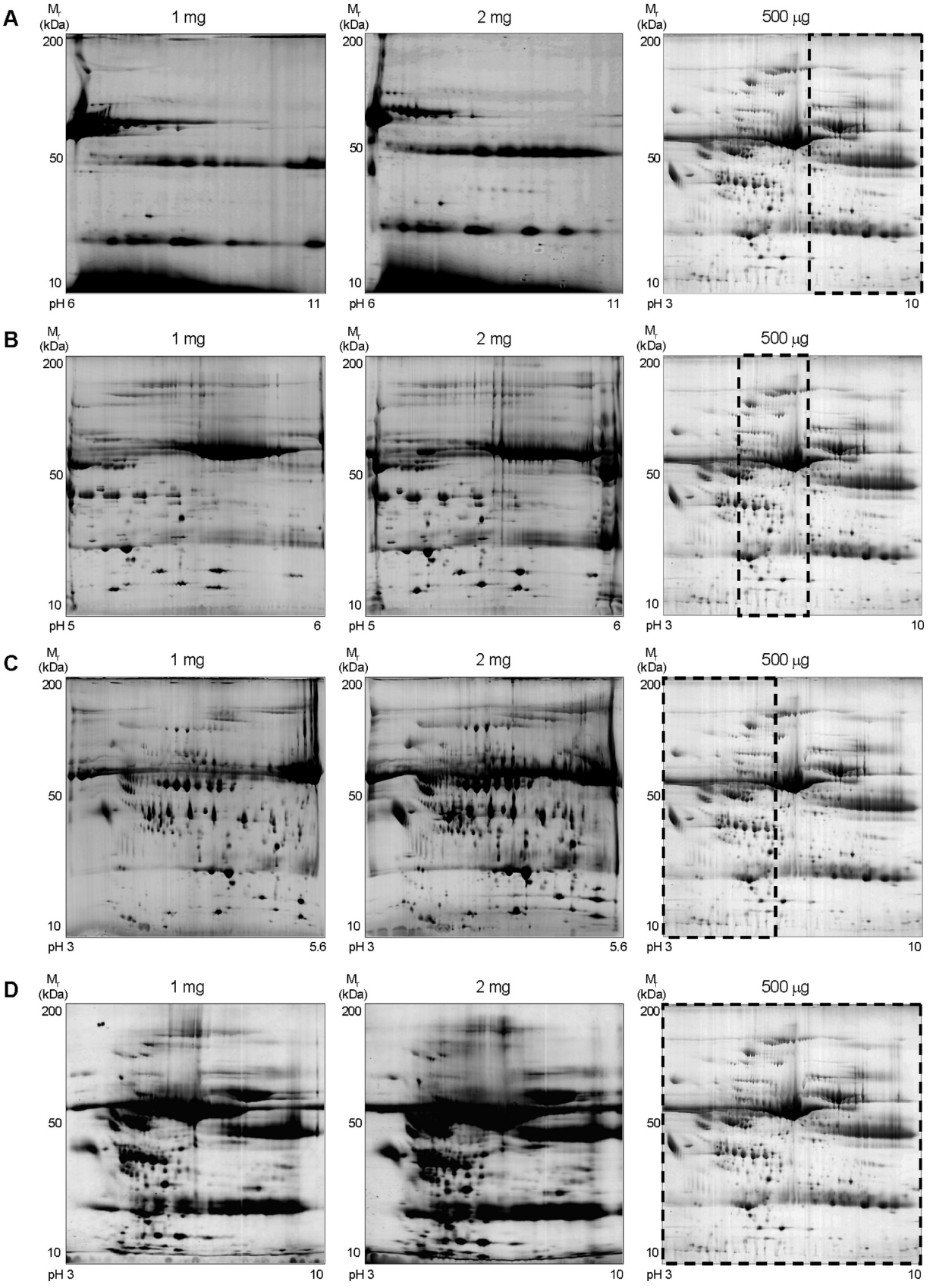

Figure 1. Optimisation of 2-DE separation of human serum proteins using narrow range IPG strips. Gels were run with (A) pH 6−11, (B) pH 5−6, and (C) pH 3−5.6 NL IPG strips. In each case, 1 mg was separated using a narrow range IPG strip (left), 2 mg was separated using the same narrow range IPG strip (middle) and 500 μg separated using pH 3−10 NL IPG strips with the narrow pH range investigated highlighted within a dashed box (right).

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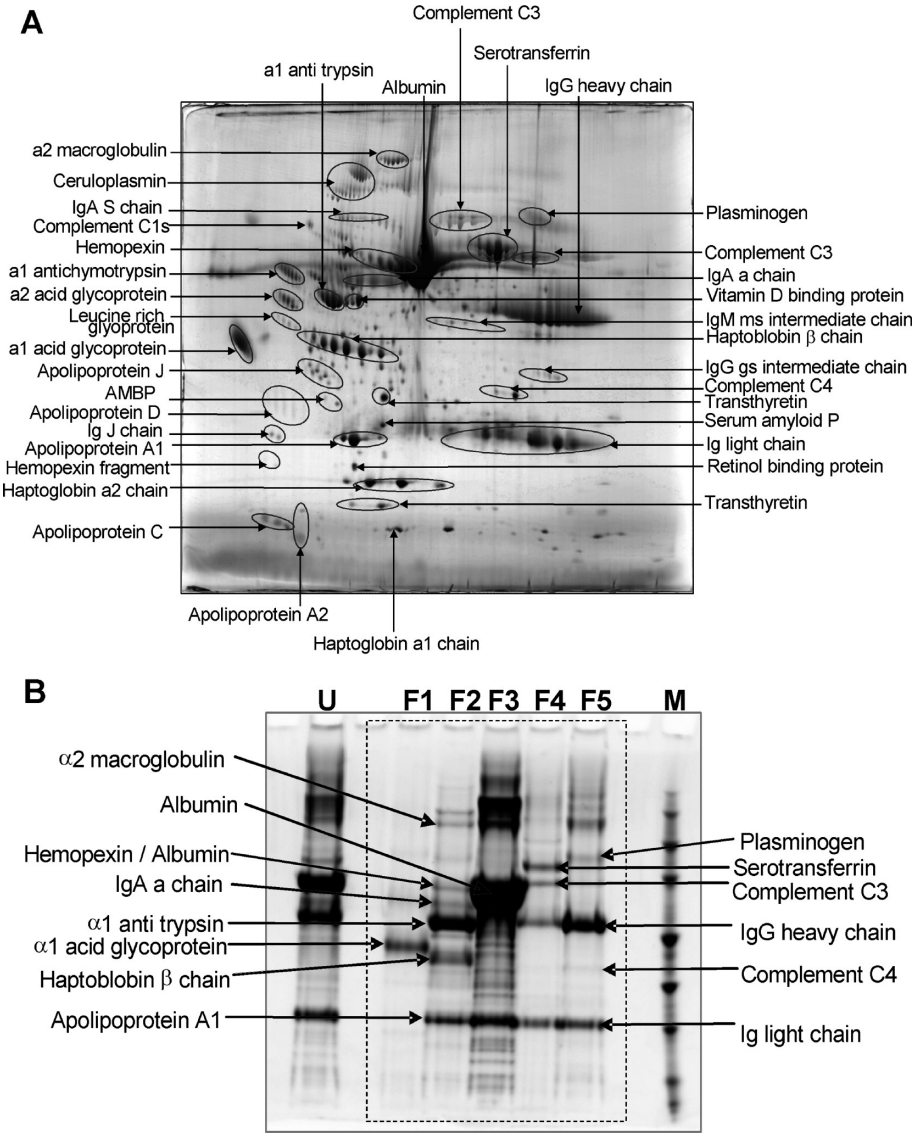

Figure 2. Serum banding pattern for the IEF fractionator fractions run by SDS-PAGE shows a similar profile to the spots seen by 2-DE. (A) Serum was separated by 9−16% 2-DE with pH 3−10 NL IPG strips using a load of 500 μg. The gel was calibrated with landmarks of known pI and molecular weight. (B) Serum was separated using in-solution IEF into five fractions (F1 = pH 3−4.6; F2 = pH 4.6−5.4; F3 = pH 5.4−6.2; F4 = pH 6.2−7; F5 = pH 7−10). Each fraction was run by SDS-PAGE alongside unfractionated serum (U). *M* = Molecular weight markers (225, 150, 100, 75, 50, 35, 25, 15, 10 kDa). The five fractions from the IEF fractionator span pH 3 to pH 10 and therefore the banding pattern seen by SDS-PAGE is similar to the spot profile seen using a pH 3−10 2-DE gel.

used to separate 1 and 2 mg of normal human serum. These gels were compared with 500 μg of normal human serum loaded on pH 3−10 strips, as used in our previous study,[6] (Figure 1). The pH 3−5.6 range with a load of 2 mg showed the best separation with almost twice as many features as a pH 3−10 gel across the same pH 3−5.6 range (Supporting Figure 1, Supporting Information). A spot number count on the Melanie II software showed that the pH 3−5.6 range with a 2 mg load had 551 features whereas the pH 3−10 gel with a 500 μg load had only 289 features across the same pH 3−5.6 range. The total spot number count on the 500 μg pH 3−10 gel was 523, less than the total feature count on the pH 3−5.6 gel. Spots which were well separated in the 500 μg pH 3−10 gel were merged together in the pH 3−10 gels with 1 and 2 mg of serum.

In-Solution IEF

Healthy control and cirrhotic serum samples were separated by in-solution IEF into five different pH ranges using an IEF fractionator. Figure 2 shows an SDS-PAGE gel with the five fractions from the IEF fractionator where the band profile seen by SDS-PAGE resembles that of a pH 3−10 2-DE gel since the fractions range from pH 3 to 10. Figure 3 shows each fraction resolved by SDS-PAGE highlighting the differentially expressed bands between the healthy control and cirrhotic samples. As with our previous 2-DE analysis,[6] the following changes were observed in cirrhotic serum with respect to healthy control serum: a decrease in haptoglobin, an increase in alpha 2 macroglobulin, and an increase in IgG (both heavy and light chains). Consistent with the 2-DE results, other fragments of complement C3 and C4 were found to be more abundant in the serum of controls than cirrhotic patients. In the pH 7−10 fraction, differentially expressed bands at approximately 75 kDa on the gel contained peptides derived from complement C3 and C4, which in both cases span their β-chains (Supporting Figure 2, Supporting

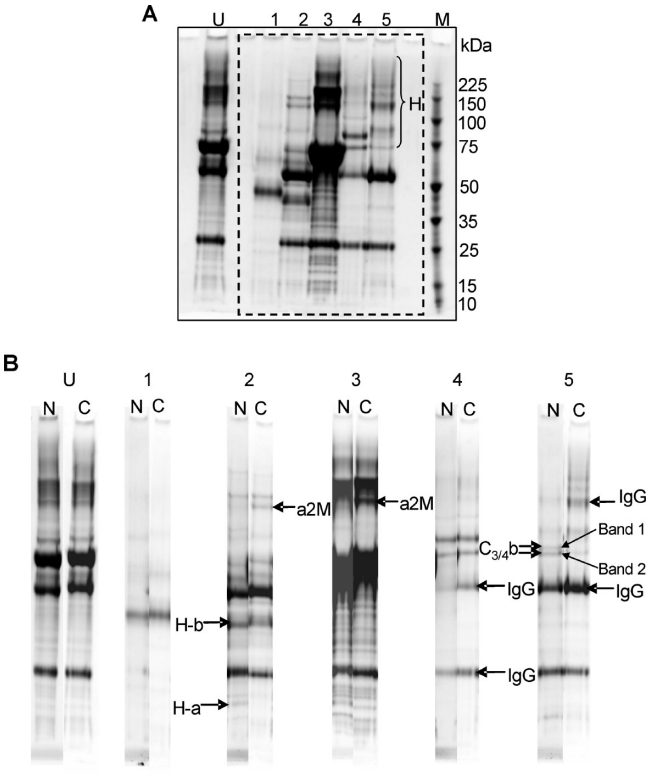

Figure 3. In-solution IEF combined with SDS-PAGE allows improved representation of high molecular weight basic proteins. Normal controls (N) and cirrhotic (C) serum samples were fractionated by in-solution IEF. The resulting five fractions from the IEF fractionator were then separated by 4−12% (w/v) SDS-PAGE alongside unfractionated serum. A) A typical profile observed for human serum. The dashed box region indicates the serum profile that is comparable to a pH 3−10 2-DE gel (see Figure 3). *H* = high molecular weight basic proteins that are not as well represented by 2-DE. B) Differential analysis of the SDS-PAGE lanes comparing controls with cirrhotic serum for each of the fractions. *U* = Unfractionated serum; 1 = pH 3−4.6; 2 = pH 4.6−5.4; 3 = pH 5.4−6.2; 4 = pH 6.2−7; 5 = pH 7−10; *M* = Molecular weight markers; H-*b* = Haptoglobin β-chain; H-*a* = Haptoglobin α-chain; a2M = α2 macroglobin; C3/4b = β-chains of complement C3 and C4. Bands 1 and 2 show the bands containing the complement proteins and the number of MS/MS peptide matches, percentage sequence coverage and protein score for these bands are shown in Table 1.

Information). The protein score, sequence coverage and peptides identified for these complement proteins are shown in Table 1. In-solution IEF and SDS-PAGE were repeated for additional samples followed by gel band densitometry (Supporting Figure 3A, Supporting Information). This data shows that the higher molecular weight band, which contains complement C3 beta but predominantly C4 beta (Table 1), is clearly expressed to a higher extent in controls compared to cirrhosis when analyzing multiple samples (Supporting Figure 3B, upper panel). The lower molecular weight band containing complement C4 beta and predominately C3 beta (Table 1) is seen in both controls and cirrhosis but expressed to a higher extent in controls (Supporting Figure 3B, lower panel). To validate these candidate cirrhosis markers, unfractionated control and cirrhosis samples were blotted for complement C3 beta and C4 beta (Supporting Figure 3C). Blot band densitometry showed a clear decrease for both complement C3 beta and C4 beta, the results being more consistent for complement C4 beta with lower standard deviation (Supporting Figure 3D). The in-solution IEF fractions from control serum were also separated by 2-DE to confirm fractionation (Supporting Figure 4, Supporting Information). The results show that the IEF fractionator does not give a clear-cut fractionation but is able to reproducibly enrich proteins in the pH range of the fraction.

DISCUSSION

Individuals infected with HCV can develop hepatic fibrosis which can progress to liver cirrhosis and hepatocellular carcinoma. Currently the most reliable way of assessing liver fibrosis is by biopsy and so there is an urgent need for less invasive serological biomarkers. In this study we use two novel proteomics approaches which can aid biomarker discovery for all diseases including liver fibrosis. The use of in-solution IEF reveals the beta chains of both complement C3 and C4 to be decreased in cirrhosis.

A major difficulty for discovering novel biomarkers in serum and plasma is the presence of highly abundant proteins which limits the detection of low abundant features and restricts the amount of total protein loaded onto gels.[5] Both albumin and IgG alone make up more than 75% of the total plasma/serum protein content.[13] To overcome this obstacle in finding biomarkers several groups have tried prefractionation strategies to deplete high abundance proteins from samples prior to electrophoresis and thus improve the representation of low abundant proteins. Antibody-based immunoprecipitation methods appear to be most suitable for removal of highly abundant proteins and we have successfully performed this in the past to identify a novel biomarker for uveal melanoma by removing twelve of the more abundant serum proteins.[14] We achieved this using chicken IgY antibodies since they offer broader host antigen binding and cleaner capture than IgG methods due to the greater evolutionary distance between chickens and mammals. However, immunoprecipitation is expensive due to the vast amount of antibody required to deplete these highly abundant proteins. Less expensive options for the removal of albumin include using Cibacron Blue-based prefractionation approaches. These dye-affinity methods have been compared alongside immunoprecipitation and are less efficient and less specific causing unwanted removal of a large number of nonalbumin proteins,[15] possibly including potential biomarkers. Unlike the large amount of protein used in this study (2 mg), it is very challenging to load similar high levels of protein post depletion for multiple samples due to the low recovery rates during the removal of highly abundant proteins as well as losses during concentration.

Serum protein loads of 1 and 2 mg were investigated, the latter being the maximum load recommended by the manufacturer for preparative IEF using 18 cm IPG strips. For the narrow pH ranges investigated, Figure 1 shows improved representation of low abundant features for 2 mg serum compared to 1 mg serum. In the case for pH 3−10 gels, features which were well separated using a load of 500 μg were merged together when using the higher 1 and 2 mg loads due to overloading indicating that this wide pH range is unsuitable for high protein loads. The pH 6−11 range showed no improvement over our previous pH 3−10 gels[6] in this alkaline region possibly due to the presence of highly abundant IgG and transferrin. Also, basic proteins are poorly represented by 2-DE due to their reduced solubility. The reducing agent we used in this study, dithiothreitol, becomes

Table 1. Differentially Expressed Complement Proteins Identified in Serum Samples of Controls versus Cirrhotic Patients[a]

band	protein name	no. of peptides	peptides	sequence coverage (%)	protein score
1	Complement C4	6	LLLFSPSVVHLGVPLSVGVQLQDVPR	5.21	277.29
			HLVPGAPFLLQALVR		
			TTNIQGINLLFSSR		
			GHLFLQTDQPIYNPGQR		
			RGHLFLQTDQPIYNPGQR		
			AVGSGATFSHYYYMILSR		
	Complement C3	4	EPGQDLVVLPLSITTDFIPSFR	4.50	205.37
			TELRPGETLNVNFLLR		
			IPIEDGSGEVVLSR		
			TMQALPYSTVGNSNNYLHLSVLR		
2	Complement C3	5	TMQALPYSTVGNSNNYLHLSVLR	4.69	304.64
			IPIEDGSGEVVLSR		
			QVREPGQDLVVLPLSITTDFIPSFR		
			TELRPGETLNVNFLLR		
			EPGQDLVVLPLSITTDFIPSFR		
	Complement C4	2	LLLFSPSVVHLGVPLSVGVQLQDVPR	2.35	100.36
			HLVPGAPFLLQALVR		

[a] Bands 1 and 2 shown in Figure 3 were decreased in cirrhosis. The number of MS/MS peptide matches, percentage sequence coverage and protein score were determined by the Mascot Daemon search engine.

negatively charged during IEF and migrates toward the anode thereby decreasing the concentration of dithiothreitol in the basic end of the strip. These unreduced proteins have decreased solubility resulting in streaking in the basic region of the 2-DE gel.[16] This particularly affects basic proteins which have a high molecular weight, since they have difficulty in entering the second dimension gel matrix from the IEF strip.[17]

The pH 5−6 range, although covering the main isoform of albumin, did display improved separation of proteins but with only a few additional features not previously seen in the wide range pH 3−10 analysis.[6] All of these additional features had already been observed in the pH 3−5.6 gels, therefore this pH range did not warrant complete analysis.

The use of narrow range IPG strips in a range outside the isoelectric points of the main isoforms of the most abundant plasma/serum proteins would enable greater protein loads thereby increasing the representation of low abundant features. The three most abundant plasma/serum proteins are albumin, IgG and transferrin. Serum was separated using a wide pH 3−10 NL IPG strip (Figure 1) and the isoelectric point ranges of the main isoforms of albumin, IgG and transferrin were determined using calibrated landmarks as pH 5.6−5.9, 6.3−8.5 and 6.2−6.5, respectively, which is consistent with previously reported 2-DE data.[18] The pH range of these three most abundant plasma/serum proteins were above pH 5.6 which may explain why the pH 3−5.6 NL range chosen for analysis led to superior separation and highest feature number.

Some albumin "bled over" into the pH 3−5.6 range when using a serum load of 2 mg, although this was no worse than what we observed in our previous study.[6] We judged the gain in low abundance features with the higher protein load to outweigh this problem and the load of 2 mg was chosen for determining fibrosis biomarkers using these narrow range IPG strips. The pH 3−5.6 range with a load of 2 mg appeared to be the best narrow pH range for biomarker discovery. This pH range allowed four times more protein to be loaded (2 mg) than in our previous study[6] (500 μg), which allowed visualization of several new low abundant features. Also the narrow pH range helped to increase the separation of the acidic plasma/serum proteome. The separation achieved in this pH range appeared to be better than previous large scale 2-DE studies for identifying the human serum proteome[19] showing new features that were previously not observed by 2-DE. Therefore not only is this pH range better for biomarker discovery but it also may reveal new serum/plasma proteins that were previously not detected by 2-DE. To prove that this pH range was suitable for biomarker discovery, plasma samples from healthy control individuals and patients with HCV induced cirrhosis were compared using 2-DE over this range to identify novel biomarker candidates for hepatic fibrosis in hepatitis C patient.[20] Using this pH 3−5.6 range 21 novel candidate fibrosis biomarkers were identified which were not seen in our previous study using the pH 3−10 range. This confirms that the pH 3−5.6 range helps in the discovery of clinical biomarkers for hepatic fibrosis and would be advantageous in determining novel serological markers for other diseases.

The pH 3−5.6 range used in this study only covers the acidic proteome and therefore any biomarkers present in the alkaline region would be missed. The pH 6−11 range was investigated (Figure 1) but this range showed no improvement to the basic area compared to our previous study[6] and high molecular weight basic proteins are poorly resolved by 2-DE. These difficulties with basic and high molecular weight proteins are not encountered with SDS-PAGE, a technique with lower resolution. To attain a higher resolution while taking advantage of the benefits of SDS-PAGE, we decided to fractionate serum samples by pH prior to electrophoresis. Although the combination of in-solution IEF and SDS-PAGE has previously been reported,[21] we indicate for the first time that this approach is beneficial for analyzing basic, high molecular weight proteins. SDS-PAGE analysis of unfractionated control and cirrhotic serum appeared to show no difference in differential band analysis due to the low resolution separation of this technique. However, by combining the in-solution IEF method with the SDS-PAGE approach, the proteins were separated to an extent which allowed to discern differences.

Changes in complement C3 and C4 were observed in the high molecular weight basic fraction (pH 7−10). To confirm the reproducibility of this data, in-solution IEF and SDS-PAGE were repeated for additional samples followed by gel band densitometry (Supporting Figure 3A, Supporting Information) which showed that the changes in both bands containing C3 and C4 beta were consistent. These additional samples were not matched to any category such as age or sex (Supporting Table 1, Supporting Information). The point and advantage of this kind of marker finding exercise is that biomarkers should not be dependent on these categories and so we aimed to eliminate any group specific hits from the outset. We have previously identified 21 markers for liver fibrosis which were not dependent on age or sex.[20] The changes in complement C3 and C4 beta were not observed by 2-DE analysis due to the problem with reduced solubility of basic proteins, demonstrating an advantage of the combined in-solution IEF and SDS-PAGE approach over solely gel-based technologies. The theoretical pI of the β-chain for C4 is pH 8.7, which is consistent with the pH 7−10 range for this fraction. The theoretical pI of the β-chain for C3 was determined to be pH 6.8 which is marginally outside the range of this fraction but this was expected since we found that the IEF fractionator enriches proteins rather than providing a clear-cut fractionation for the pH range; however, as proteins are enriched reproducibly this poses no problem. The differentially expressed band immediately below the 75 kDa also contained sequences within the β-chains of both C3 and C4 but these were fragments since the band was at a lower molecular weight. This band was seen in both control and cirrhosis samples but expressed to a higher extent in control samples, whereas the higher molecular weight band which was differentially expressed appeared only in control samples (Figure 3 and Supporting Figure 3A, Supporting Information). This lower molecular weight band contained predominantly complement C3 beta (Table 1) and showed a clear but less consistent change among multiple samples when validated by Western blot (Supporting Figure 3C and D). The more clearly changing higher molecular weight band contained predominantly complement C4 beta (Table 1) and showed a clear and consistent change among multiple samples when validated by Western blot (Supporting Figure 3C and D). This suggests that complement C4 beta may be a better biomarker for cirrhosis than complement C3 beta and would need to be validated using a larger number of samples.

CONCLUSION

This study shows how two different proteomic approaches can aid in the discovery of disease biomarkers. To our knowledge this is the first time the pH 3−5.6 range has been used to separate serum by 2-DE and we have shown that this pH range is useful for discovering novel biomarkers in diseases. We have also shown that the use of in-solution IEF followed by SDS-PAGE improves the separation of the basic proteome thereby helping to identify disease biomarkers in the basic region of plasma and serum proteomes. Using this approach we show that the beta chains of complement C3 and C4 decrease in serum from hepatitis C patients with cirrhosis, a change not observed previously by 2-DE.

ASSOCIATED CONTENT

Supporting Information

Images of a pH 3−10 and pH 3−5.6 gel with spot numbers, complement C3 and C4 peptide sequence information, in-solution IEF and SDS-PAGE of multiple samples, complement C3 and C4 beta Western blots, 2-DE of the in-solution IEF fractions and clinical details of all samples used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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