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ORIGINAL PAPER

Proteomic Analysis of Human Plasma Proteins by Two-Dimensional Gel Electrophoresis and by Antibody Arrays Following Depletion of High-Abundance Proteins

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Abstract Detecting proteins that are present at lower levels in human plasma, for the identification of potential disease biomarkers, is complicated by a few highly abundant proteins. One promising strategy is the removal of these abundant proteins interfering with the analysis of plasma content by proteomic techniques. This study compared three affinity-based methods to remove the most abundant proteins in human plasma. Two of them, based on antibodies, which depletes the six or the 12 most abundant proteins, demonstrated the highest efficiency in enriching less abundant plasma proteins. Comparison of two anticoagulant treatments for plasma preparation, EDTA and CTAD, showed that this treatment influenced the patterns of lower-abundance proteins visible on 2-dimensional (2-D) gels. Several staining procedures including two fluorescent dyes, Sypro Ruby and Deep Purple, were also compared with a very sensitive silver staining method for the visualization of lower-abundance proteins on 2-D gels. Furthermore, treatments of lower-abundance plasma proteins with hydroxyethyl disulfide enhanced protein sharpness and resolution. The purpose of all these systematic comparisons was to select the most reliable methods in different steps of plasma preparation and handling as well as in analysis of proteins by 2-D gels to obtain highly reproducible patterns of lower-abundance plasma proteins. Importantly, the lower-abundance plasma proteins enriched by these optimized conditions were further analyzed by antibody microarrays allowing the identification of 61 proteins using 350 antibodies directed against signalling proteins suggesting that this proteomic strategy is a valuable approach for detecting potential plasma biomarkers.

Keywords Plasma · Two-dimensional gels · Antibody microarrays

Abbreviations

BSA	Bovine serum albumin
CTAD	Citrate, theophylline, adenosine, and dipyridamole
IgGs	Immunoglobulins
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
SB3-10	*N*-Decyl-*N*,-*N*-Dimethyl-3-Ammonio-1-propane-sulfonate
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBS-T	TBS containing 0.1% Tween 20
tPA	Tissue plasminogen activator

Introduction

Human plasma is easily collected and it contains large amounts of proteins including many proteins originating

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from vascularized tissues. Plasma or serum analyses may thus provide relevant information regarding these tissues. For example, some of the proteins released by tissues could be from the cells that undergo cell death, whereas other proteins could be secreted by tumour cells. Notably, these released proteins could be characterized in terms of their presence or absence or by changes in their concentrations between healthy and diseased states. Consequently, a systematic characterization of proteins in human plasma in health and in diseased states has a considerable potential for identifying possible biomarkers for disease diagnosis, for the development of new therapeutic products and for monitoring responses to drug treatments.

The most abundant plasma protein, albumin, is present at about 40 mg/ml but several other proteins are highly abundant including immunoglobulins (IgGs), transferrin and fibrinogen. The combined quantities of 22 of the most abundant proteins comprise about 98% of the protein content of serum [1]. Conversely, it is estimated that over 10,000 different proteins are present in plasma and most of them exist in very low quantities [2]. Furthermore, serum proteins exhibit large dynamic range concentrations, somewhere between 10 and 15 orders of magnitude [1, 3]. Both the great number of proteins and the extended range of their concentrations result in a great challenge for the identification of potential biomarkers.

Since, protein complexity is elevated in plasma, fractionation approaches are required to simplify the protein composition. Removal of the most abundant proteins is a preliminary and critical step for characterization of the plasma proteome. Accordingly, proteomic strategies could be made more reliable by studying plasma subproteomes rather than using intact plasma. In order to reach this goal, a number of methods have been proposed for the removal of the most abundant proteins prior to proteomic analysis of plasma. Removal of albumin, IgGs and other abundant plasma proteins could be accomplished by methods such as salt or ethanol precipitation [4, 5], affinity methods using a dye such as Cibacron Blue for albumin depletion or by using Protein A for IgG removal [6, 7]. Although these depletion methods have shown good efficiency for albumin and IgG depletion, they often do not completely extract the targeted proteins or alternatively they non-specifically bind some other plasma proteins. Recently, immunoaffinity methods have been developed to improve both the efficiency and the specificity for depletion of several abundant proteins in addition to albumin and IgGs in plasma or serum [7–14]. These chromatographic affinity methods enable specific depletion of 6–9 of the most abundant plasma proteins. Recently, it has been reported that 12 of the most abundant plasma proteins could be depleted by immunoglobulin yolk (IgY) antibodies [15].

These studies have demonstrated that depletion methods based on antibody affinities may permit the detection of lower-abundance plasma proteins. For instance, silver staining of two-dimensional (2-D) gels of plasma which had been depleted of the six most abundant proteins allowed detection of tissue plasminogen activator (tPA) whose concentration is estimated at about 10–60 ng/ml [12]. This tPA concentration is about six orders of magnitude lower than that of albumin indicating that depletion of the most abundant plasma proteins could be sufficient for the identification of potential biomarkers among lower-abundance proteins. Furthermore, these results show that although several analytical methods can be employed to separate plasma proteins, 2-D gels remain a powerful method for plasma characterization following removal of the most abundant plasma proteins.

In this study, fractionation of human plasma was first used to compare three affinity methods, including two immunoaffinity methods, for the removal the most abundant proteins. Then, different approaches for improving the quality and reliability of the plasma protein patterns obtained in 2-D gels were compared by characterizing the effects of hydroxyethyl disulfide (DeStreak™ reagent) on protein resolution and sharpness and by employing several different staining procedures for visualizing proteins on gels. Furthermore, two anticoagulant treatments, (a mixture of citrate, theophylline, adenosine, and dipyridamole (CTAD) and EDTA), were used in the collection of human blood and their effects on the patterns of plasma proteins observed in 2-D gels were compared. The result of all these comparisons was to select the most reproducible methods for 2-D gel characterization of lower-abundance plasma proteins. Finally, several signalling proteins were analyzed in fractionated plasma by antibody microarrays to evaluate whether this approach was valuable to detect potential biomarkers.

Materials and Methods

Materials

Acrylamide, Tris and urea were purchased from Roche Diagnostics (Laval, QC, Canada). Bis-acrylamide was from Rose Scientific (Mississauga, ON, Canada). Glycine was from Fisher (Ville-St-Laurent, QC, Canada). Sodium chloride, glycerol, dithiothreitol (DTT), iodoacetamide, thiourea, Chaps, *N*-Decyl-*N*,-*N*-Dimethyl-3-Ammonio-1-propane-sulfonate (SB3-10) and ammonium sulfate were from Sigma-Aldrich (Oakville, ON, Canada). 2-Mercaptoethanol was obtained from GE Healthcare (Baie-d'Urfé, QC, Canada). Sodium dodecyl sulfate, Bromophenol Blue, Coomassie Brilliant Blue (CBB) G-250, Temed and

ammonium persulfate were purchased from Bio-Rad (Mississauga, ON, Canada). Methanol (ACS grade), acetic acid, formic acid, silver nitrate, sodium sulfite, sodium carbonate and BD Vacutainer EDTA and CTAD tubes were from VWR International (Ville Mont-Royal, QC, Canada).

Collection of Human Plasma Samples

Venous blood samples were obtained from healthy volunteers. Blood samples used for the experiments were not pooled. However, the blood samples from the same person served to analyze plasma proteins in each set of conditions to reduce differences when comparing methods. Plasma samples were prepared using CTAD and EDTA as anticoagulant reagents. For preparation of CTAD plasma, blood samples were collected into 4.5 ml BD Vacutainer CTAD tubes which were then centrifuged at 2,000*g* for 10 min at 4°C. Supernatants were filtered using Millipore 0.45 μm polyvinylidene difluoride (PVDF) filter units (Fisher, Ville St-Laurent, QC, Canada) and isolated plasma samples were stored at –80°C until further analysis. For the preparation of EDTA plasma, blood samples were collected into 6.0 ml BD Vacutainer EDTA tubes. These samples were centrifuged for 10 min at 2,000*g* at 4°C and the supernatants obtained were centrifuged a second time. The isolated plasma samples were stored at –80°C until used.

Depletion of Highly Abundant Plasma Proteins

Plasma samples were thawed at 4°C on ice before chromatographic analysis. Three different types of depletion columns were used in this study following the manufacturer's protocols. Steps involved in the ProteoExtract Albumin/IgG Removal Kit were performed by gravity-flow, while other fractionation kits were done with spin columns.

When using the ProteoExtract Albumin/IgG Removal Kit (Calbiochem, La Jolla, CA, USA), column were equilibrated with 850 μl of ProteoExtract Albumin/IgG Binding Buffer. For depletion of albumin and IgGs, 30 μl of plasma was diluted 12-fold with the Binding Buffer before loading onto columns. Columns were then washed twice with 600 μl of Binding Buffer. Flow-through and washes were pooled, constituting the low-abundance plasma protein fraction. This fraction was concentrated and washed with water using Millipore spin concentrators, 5 kDa MWCO, 4 ml (Fisher).

For each purification with the Multiple Affinity Removal System from Agilent Technologies (Wilmington, DE, USA), 10 μl of plasma was diluted 20-fold in the manufacturer's buffer A and filtered through a 0.22 μm spin filter. Columns were equilibrated with 4 ml of buffer A and then plasma samples were centrifuged through the columns at 100*g* for 1.5 min. Next, columns were washed twice with 400 μl of buffer A by centrifugation at 100*g* for 2.5 min. Flow-through and washes were pooled constituting the low-abundance plasma protein fraction. High-abundance proteins (albumin, IgG, IgA, transferrin, haptoglobin and antitrypsin) bound to the columns were eluted using 2 ml of manufacturer's buffer B before re-equilibration with buffer A. Low and high-abundance protein fractions from three runs were combined and then both of the resulting samples were concentrated and washed with water using the protein spin concentrator, (5 kDa MWCO, 4 ml) from the kit, to a final volume of 70 μl.

For each run using a Proteome Lab IgY-12 Spin Column from Beckman Coulter (Mississauga, ON, Canada), 10 μl of plasma was diluted 50-fold in manufacturer's dilution buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Diluted plasma samples were added to the beads, mixed with them and the columns were incubated 15 min on a serological shaker at room temperature. Columns were centrifuged for 30 s at 400*g* at 18°C as was done for all subsequent centrifugations. Columns were washed with 500 μl of dilution buffer and the flow-through was combined with the washes to obtain the maximal yield of low-abundance plasma proteins. Columns were washed twice with 500 μl of dilution buffer to remove all non-specifically bound proteins. High-abundance plasma proteins were stripped off from the beads by adding 500 μl of manufacturer's stripping buffer (0.1 M Glycine-HCl, pH 2.5). Columns were incubated 3 min on a serological shaker at room temperature and centrifuged to collect high-abundance proteins which were neutralized with 50 μl of 10 × manufacturer's neutralization buffer (1 M Tris-HCl, pH 8.0). A second stripping step was performed and the eluted proteins were combined with those collected during the first elution step, constituting the high-abundance plasma proteins (albumin, IgG, α1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α1-acid glycoprotein, α2-macroglobulin, HDL apolipoproteins A-I and A-II and fibrinogen). Columns were then neutralized by adding 600 μl of 1 × neutralization buffer for 5 min at room temperature on a serological shaker then centrifuged. Next 500 μl of dilution buffer was added to the columns which were then ready for the next fractionation. For storage, 0.02% of sodium azide was added to the dilution buffer and columns were rinsed with dilution buffer before the next use. Low and high-abundance plasma protein fractions from three runs were combined and then concentrated using Millipore spin concentrators (5 kDa MWCO, 0.5 ml) (Fisher) to a final volume of 10–12 μl.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Total protein quantification was performed with the Pierce Micro BCA Protein Assay (Fisher) according to the manufacturer's instructions with the kit's bovine serum albumin (BSA) as a standard. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with a Mini-Protean II apparatus from Bio-Rad, according to the method of Laemmli [16]. Protein samples (50 μg/well) were suspended to a final concentration of 1 mg/ml in a sample buffer composed of 62.5 mmol/l Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.00625% Bromophenol blue. The samples were heated to 95°C for 5 min then loaded onto 10% acrylamide/bis-acrylamide (29.2:0.8) gels. Electrophoresis was carried out at a constant voltage of 100 V.

Two-Dimensional Gel Electrophoresis

Total protein quantification was performed with the Pierce Micro BCA Protein Assay as described above. Protein samples were diluted in rehydration buffer containing 5 M urea, 2 M thiourea, 2% Chaps, 2% SB3-10, 0.3% DTT, 1% of appropriate IPG buffers (GE Healthcare) and usually 1.2% of DeStreak™ reagent (GE Healthcare) in a final volume of 350 μl. IPG strips (18 cm) from GE Healthcare were passively rehydrated overnight in Ettan IPGphor strip holders at room temperature. Electrode pads were placed between the IPG strips and each strip holder electrode just prior to the isoelectrofocalisation which was carried out with an Ettan IPGphor isoelectric focusing system (GE Healthcare) in four steps at 18°C: 200 V for 3 h, 500 V for 1 h, 1,000 V for 1 h and 8,000 V for 13 h. After the first dimensional separation of proteins, proteins in the IPG strips were reduced in equilibration buffer (50 mM Tris–HCl, pH 6.8, 6 M urea, 30% glycerol and 2% SDS) containing 1% DTT for 15 min at room temperature. Then proteins were alkylated in the same buffer but containing 2.5% iodoacetamide and bromophenol blue for 15 min at room temperature. The second separation was performed with an Ettan DALTsix electrophoresis system (GE Healthcare). IPG strips were placed at the top of a 10% SDS-PAGE gel and overlaid with 0.5% agarose in running buffer (50 mM Tris-HCl, pH 6.8, 1.44% glycine and 0.1% SDS). The electrophoresis was performed at 25 mA/gel for 1 h and then at 60 mA/gel until the tracking dye reached the bottom of the gels.

Gel Staining

On a routine basis, gels were stained with colloidal CBB G-250. After incubation in 30% methanol and 10% acetic acid, gels were stained overnight in 0.1% CBB G-250, 34% methanol, 0.5% acetic acid and 17 % ammonium sulphate with slight agitation. Gels were then destained in water. Silver staining was performed with a method kindly provided by Dr. Sam Dougaparsad from Beckman Coulter. This procedure employed slight modifications of Shevchenko's method [17]. Gels were fixed in 50% methanol, 5% acetic acid and 0.02% formic acid for 1 h then were washed in 50% methanol three times for 10 min and kept overnight in water at room temperature. For sensitization, gels were incubated in 0.02% sodium sulfite for 1 min at room temperature and rinsed three times in water for 10 s. After that, gels were incubated in 0.2% silver nitrate and 0.03% formic acid for 20 min at 4°C in darkness and rinsed 3 times in water for 10 s. Gels were then developed by incubating in 3% sodium carbonate, 0.02% formic acid and 0.0004% sodium sulfite. When the protein staining reached the desired intensity, the developing step was stopped by rinsing gels in 50% methanol and 5% acetic acid three times for 10 s before storage in 2% acetic acid. For Sypro Ruby staining, gels were fixed 1 h or longer in 10% methanol and 7% acetic acid before staining overnight at room temperature with gentle agitation in Sypro Ruby protein gel stain from Bio-Rad. The next day, gels were rinsed in 10% methanol and 7% acetic acid for 1 h then washed in water. In some cases, 2-D gels were stained with Deep Purple Total Protein Stain (GE Healthcare). In this procedure, gels were fixed overnight after electrophoresis in 7.5% acetic acid and 10% ethanol at room temperature. Gels were washed with 35 mM sodium hydrogen carbonate and 300 mM sodium carbonate at room temperature for 30 min with gentle agitation and then stained with Deep Purple Total Protein Stain for 1 h at room temperature in darkness. Gels were washed twice in 7.5% acetic acid for 15 min at room temperature in darkness. For imaging, Coomassie and silver stained gels were scanned using an ImageScanner II (GE Healthcare) while those stained with fluorescent dyes Sypro Ruby or Deep Purple were scanned with a Typhoon Scanner (GE Healthcare).

Antibody Microarrays

The Antibody Microarrays services from Kinexus Bioinformatics Corporation (Vancouver, BC, Canada) were used. Briefly, this consisted of individual antibodies for signalling proteins spotted in duplicate with two slides per experiment. Preparation of plasma samples from two donors and dilution of proteins at the appropriate concentration were done as requested by Kinexus Bioinformatics Corporation. Subsequent protein spotting and hybridization onto the arrays as well as scanning, imaging and quantitative analysis of the enhanced chemiluminescence signal

of the detected signalling proteins in plasma samples were performed by Kinexus Bioinformatics Corporation. Only signals showing strength twice above the background in both sets of plasma samples were considered.

Western Blot Analysis

Electrophoresis was performed as described in SDS-PAGE gels using appropriate acrylamide concentrations. After electrophoresis, proteins were transferred electrophoretically to a 0.45 μm pore size BioTrace PVDF membrane (Pall Life Sciences, Pensacola, FL) using a Mini Trans-Blot Cell (Bio-Rad). Transfer was carried out for 90 min at a constant voltage of 100 V at 4°C with transfer buffer composed of 96 mM glycine, 10 mM Tris, 20% methanol and 0.01% SDS. Hydrophobic or non-specific sites were blocked overnight at 4°C with 3% BSA in Tris-buffered saline (TBS), pH 7.5, containing 0.1% Tween 20 (TBS-T). Membranes were washed three times for 15 min in TBS-T, at room temperature, before incubation with appropriate antibodies diluted in 3% BSA in TBS-T for 1 h. The antibody against CD45 from BD Biosciences (Franklin Lakes, NJ), was diluted 1/500, the anti-Flt-4 from Santa Cruz Biotechnology (Santa Cruz, CA), was diluted 1/200 and the anti-caspase-2, from BD Biosciences was diluted 1/1,000. PVDF membranes were washed 4 times for 15 min in TBS-T and then incubated for 1 h with the secondary antibodies (Jackson Immunoresearch, Hornby, ON, Canada) which were horseradish peroxidase-conjugated and diluted 1/10,000 for anti-mouse and 1/40,000 for anti-rabbit, in TBS-T. Membranes were washed 4 times for 15 min before detection with Western Lightning Chemiluminescence Reagent Plus from Perkin Elmer (Boston, MA) according to the manufacturer's instructions.

Results

Comparative Analysis by 2-D Gel Electrophoresis of Various Affinity Procedures for Removing the Most Abundant Plasma Proteins

In this study, samples of crude plasma were subjected to different affinity techniques to enrich proteins of lower-abundance following the removal of the most abundant proteins. The various depletion columns tested were Calbiochem ProteoExtract Removal kit which removes albumin and IgGs, the Agilent Multiple Affinity Removal System which can deplete the six most abundant proteins and Beckman-Coulter ProteomeLab IgY Partitioning System (IgY-12) which extracts the 12 most abundant proteins. Since the latter procedure removes more abundant proteins than the other affinity methods, it was likely the most efficient one for depleting plasma of its abundant proteins. Thus, initial analyses of 2-D gel protein patterns from crude and fractionated plasma were performed with this method. Preliminary experiments served to determine how much plasma proteins (varying between 50 and 500 μg) could be separated by the 2-D gel system to maximize the detection of lower-abundance proteins. The best conditions for the resolution of proteins were obtained with 180 μg of proteins (data not shown).

When gel strips with a wide immobilized pH gradient (between 3 and 10) were used in the first dimension to obtain an overview of total plasma protein distribution, the results indicated marked differences in the intensity and the number of detected proteins in the depleted plasma fraction (109 spots) relative to the crude (72 spots) and abundant protein (58 spots) fractions (Fig. 1). Furthermore, 2-D gel analysis of plasma proteins under these conditions showed that most proteins were migrated in the acidic part of the gels (Fig. 1). Narrow pH range strips with pH values between 4 and 7 were then used to generate a more detailed view of the acidic region. As expected, the higher protein resolution with narrow pH range strips facilitated the detection of greater numbers of proteins in depleted (234 spots) crude (159 spots) and abundant (115 spots) plasma fractions (Fig. 2). Importantly, this improved resolution permitted detection of more proteins in the lower-abundance fraction relative to crude plasma (see the ellipses in Fig. 2).

The 2-D patterns of lower-abundance plasma proteins separated over the pH range between 4 and 7 were then compared following removal of abundant proteins with the three depletion systems. The protein patterns visualized by colloidal Coomassie staining in the 66–200 kDa range cleared varied between the three methods (see the ellipses in Fig. 3). The number of detected spot proteins was 319 with the ProteomeLab IgY Partitioning System, greater than the 258 spots seen with Multiple Affinity Removal System (Fig. 3). In the case of the Calbiochem ProteoExtract Removal kit, 387 spots were observed (Fig. 3). The column with the IgY antibodies was the best for the removal of abundant proteins as illustrated by the proteins found around 25, 60 and 80 kDa (see boxes in Fig. 3). This explained why the number of spots was lower with the ProteomeLab IgY Partitioning System than with the Calbiochem ProteoExtract Removal kit. Thus, the ProteomeLab IgY Partitioning System was an efficient depletion step allowing enhanced detection of lower-abundance proteins in human plasma.

The reproducibility of chromatographic runs and the 2-D protein patterns is critical when comparing different samples. In order to evaluate the reliability of the developed method for enrichment of plasma proteins in lower-

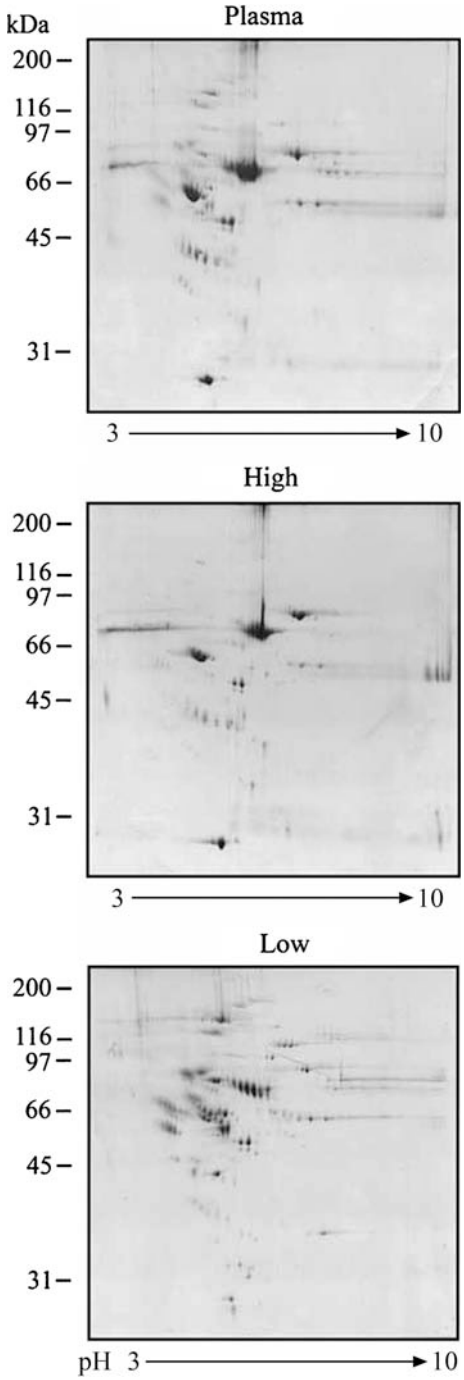


Fig. 1 Human plasma protein partitioning analysis by 2-D gel with wide pI range. Plasma proteins were fractionated using IgY-12 affinity column. Same amounts of proteins (180 μg) from crude plasma (Plasma), from the fraction containing high-abundance plasma proteins (High) and from the fraction enriched in low-abundance plasma proteins (Low) were focused in the first dimension according to their pIs over a pH range from 3 to 10 and then were separated in the second dimension according to their molecular weights by 10% SDS-PAGE. Gels were stained with colloidal CBB. These data are representative of three independent experiments

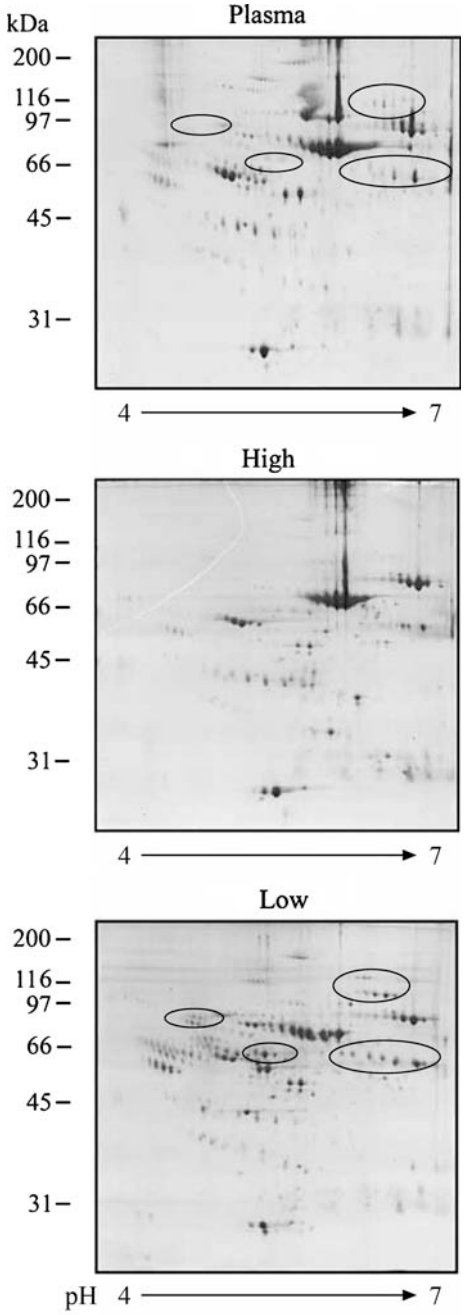
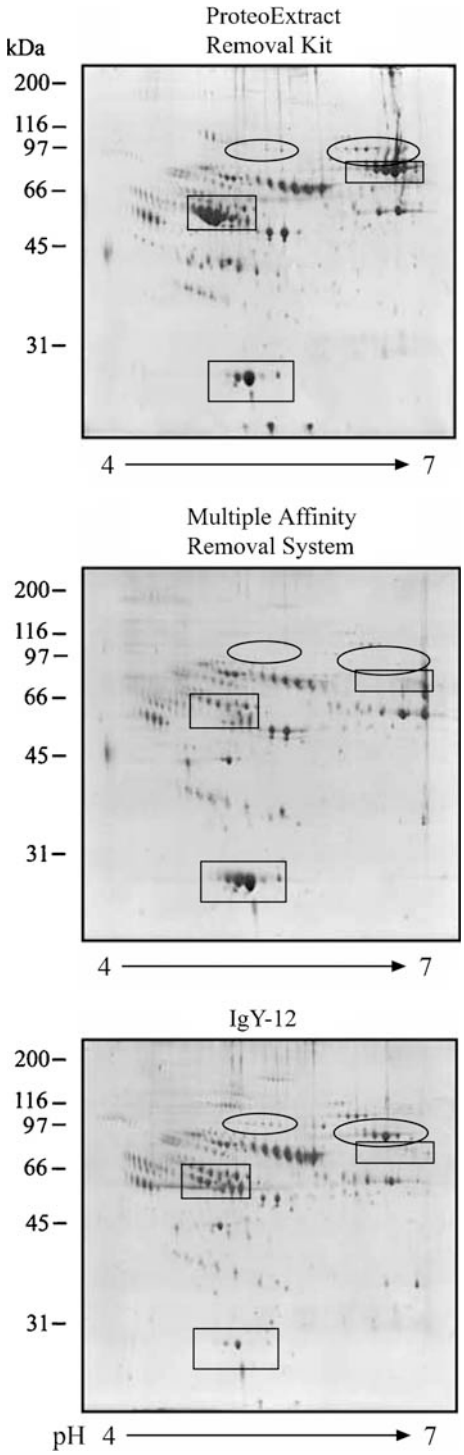


Fig. 2 Human plasma protein partitioning analysis by 2-D gel with narrow pI range. Plasma proteins were fractionated using IgY-12 affinity column. Same amounts of proteins (180 μg) from crude plasma (Plasma), from the fraction containing high-abundance plasma proteins (High) and from the fraction enriched in low-abundance plasma proteins (Low) were focused in the first dimension according to their pIs over a pH range from 4 to 7 and then were separated in the second dimension according to their molecular weights by 10% SDS-PAGE. Gels were stained with colloidal CBB. These data are representative of three independent experiments. Ellipses are examples of regions showing marked differences in protein numbers



◀**Fig. 3** 2-D gel electrophoresis of human plasma proteins following removal of high-abundance proteins by various affinity columns. The most abundant proteins in plasma samples (30 µl) were depleted by using different affinity columns: Calbiochem ProteoExtract Removal kit, Agilent Multiple Affinity Removal System and Beckman-Coulter ProteomeLab IgY Partitioning System (IgY-12). Unbound proteins in the flowthrough fractions were focused in the first dimension according to their pIs over a pH range from 4 to 7 and then separated in the second dimension by 10% SDS-PAGE. Gels were stained with colloidal CBB to reveal enriched plasma proteins. These data are representative of three independent experiments. Ellipses are regions showing marked differences in protein numbers. Boxes are examples of regions with different efficiencies in the depletion of proteins

abundance fraction with the ProteomeLab IgY Partitioning System followed by 2-D gel analysis, three independent experiments were performed. For the affinity chromatography, 2,117 µg, 2,520 µg and 2,655 µg of crude plasma was loaded onto the columns in each run. The collected proteins in the flow through were 633 µg, 984 µg and 1,298 µg while the eluted proteins corresponding to those present in the lower-abundance fractions were 192 µg, 195 µg and 215 µg. Thus, for plasma proteins in lower-abundance fraction, the mean recovery ± S.D. was 8.3 ± 0.7% and the %CV was within 8.4%. For the 2-D gels performed following these chromatographic runs, the numbers of stained spots with colloidal Coomassie were 319, 325 and 366 respectively. So, the mean ± S.D. of detected spots was 337 ± 33 and a %CV within 10. These data clearly showed that both the chromatographic step and the 2-D gel procedure were consistent and reproducible for the enrichment of plasma proteins of lower-abundance.

Comparison of 2-D Gel Patterns of Human Plasma Proteins Obtained with Two Anticoagulant Methods

Blood contains multiple types of cells including erythrocytes, platelets, leukocytes and others which can release some of their proteins upon clotting activation. Clotting is an easy way to remove some of these cells. However, clotting also induces the depletion of the proteins involved in blood coagulation as fibrinogen and of several other types of proteins as well [18]. These two alterations in plasma protein contents could modify the measured composition of serum. In order to overcome these problems, we, therefore, compared two anticoagulation methods, EDTA and CTAD (a mixture of citrate, theophylline, adenosine, and dipyridamole), for the preparation of plasma prior to protein characterization by 2-D gels. A greater number of spots was detected in lower-abundance protein fractions when using EDTA (255 spots) rather than CTAD (209 spots) (Fig. 4). Although similar 2-D gel patterns were obtained when crude plasma and highly abundant proteins were prepared with the EDTA and CTAD methods (Fig. 4), the protein patterns generated by these methods showed differences in the intensity of certain proteins present in the lower-abundance plasma protein fractions (see the ellipses in Fig. 4). When most proteins were found in equivalent amounts for both

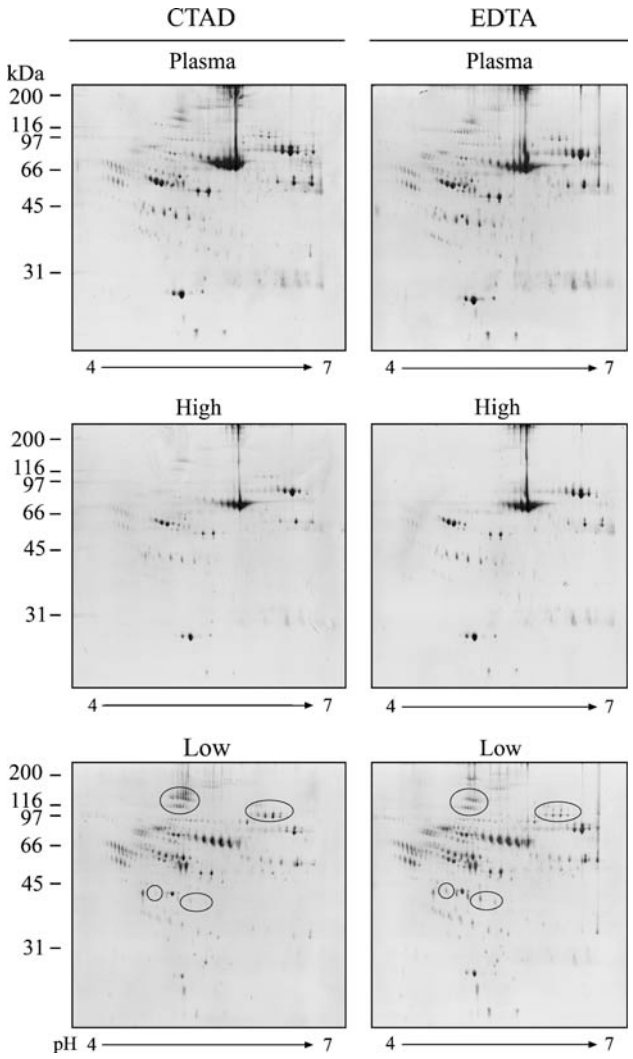


Fig. 4 Comparison of 2-D gel patterns of human plasma proteins prepared with two anticoagulant methods. Venous blood was collected into Vacutainer tubes containing EDTA or CTAD as anticoagulants. Plasma proteins were fractionated using IgY-12 affinity columns to extract high-abundance plasma proteins. Same amounts of proteins (180 µg) from crude plasma (Plasma), high-abundance plasma proteins (High) or low-abundance plasma proteins (Low) were focused according to their pIs over a pH range from 4 to 7 and then separated according to their molecular weights by 10% SDS-PAGE. Proteins were stained with colloidal CBB. These data are representative of three independent experiments. Ellipses are examples of regions showing differences in protein numbers or protein intensities

methods, some were more detectable in plasma prepared in the presence of CTAD and others were more present in samples obtained with EDTA (Fig. 4). Thus the anticoagulant used, CTAD or EDTA, to prepare plasma appeared to influence the relative abundance of certain plasma proteins isolated by immunoaffinity after removal of the 12 major proteins.

The Reagent Hydroxyethyl Disulfide Improves Protein Resolution During 2-D Gel Electrophoresis of Human Plasma Depleted of High-Abundance Proteins

Hydroxyethyl disulfide (DeStreak™) prevents the formation of disulfide bridges [19] by oxidizing protein cysteinyl groups. The advantage of using hydroxyethyl disulfide over reducing agents is that the latter become depleted in the alkaline pH range resulting in the reformation of inter- and intra-molecular disulphide bridges [19]. Thus, hydroxyethyl disulfide is usually added to the solubilization buffer to reduce horizontal streaking of basic proteins in alkaline pH gradients in the IEF dimension. The ability of hydroxyethyl disulfide to stabilize protein cysteinyl groups suggested that it could also improve the reproducibility of 2-D gels of plasma proteins by decreasing the formation of protein trains or by giving sharper protein spots. In order to test these possibilities, the lower-abundance proteins obtained following plasma depletion of major proteins by fractionation with IgY-12 affinity column were loaded onto IPG strips with rehydratation solutions containing or lacking 1.2% DeStreak™. Interestingly, protein staining with colloidal CBB revealed 167 spots in the absence of DeStreak™ but 204 spots in its presence (Fig. 5). Furthermore, the appearance of protein spots was sharper in the presence than in the absence of DeStreak™ (Fig. 5). These observations indicated that DeStreak™ improved the reliability and the quality of 2-D gel patterns of lower-abundance plasma proteins by modifying the thiol groups present in proteins.

Comparison of Different Methods for Staining 2-D Gels of Human Plasma Depleted of High-Abundance Proteins

Visualization of lower-abundance plasma proteins is a key step for the mapping of protein patterns. Several protein stains were tested for their ability to detect lower-abundance plasma proteins collected in the flow through from IgY-12 affinity columns and analyzed by 2-D gels. Some gels were stained with colloidal CBB G-250 since this stain is routinely used in proteomic studies for its convenience. Two fluorescent protein gel stains, Sypro Ruby and Deep Purple, were also compared. Similar protein numbers were detected with colloidal CBB G-250 (318) Sypro Ruby (298) and Deep Purple (273). Generally, Deep Purple showed more intense coloration of protein spots than Sypro Ruby and colloidal CBB (Fig. 6). However, some proteins recognized by both colloidal CBB and Sypro Ruby were poorly detected by Deep Purple (see boxes in Fig. 6). Silver staining is commonly used to visualize proteins, because its detection limit is in the low nanogram range. A

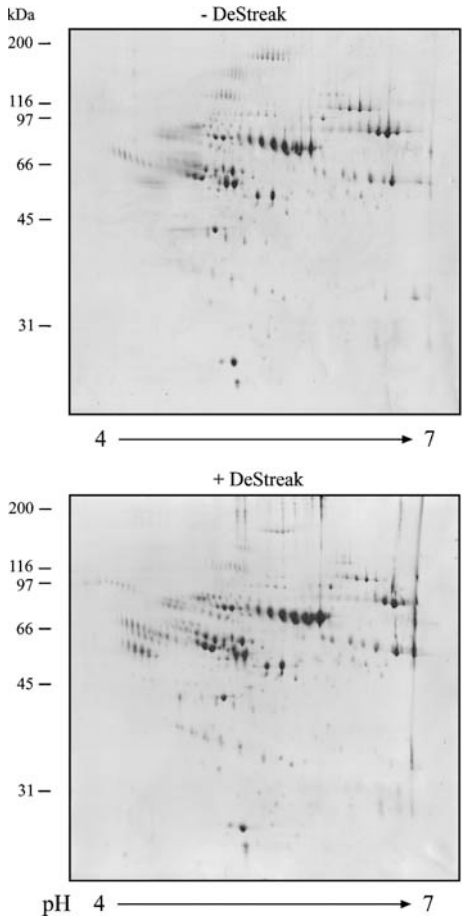


Fig. 5 Effect of DeStreak™ reagent on protein resolution during 2-D gel electrophoresis of human plasma depleted of high-abundance proteins. Low-abundance proteins obtained from plasma (30 µl) by depletion of the major proteins present in plasma by fractionation with IgY-12 affinity columns were loaded onto IPG strips with a pH range from 4 to 7 in the absence (–DeStreak) or presence (+DeStreak) of 1.2% DeStreak™ rehydration solution. Second dimensional separation was performed with 10% SDS-PAGE. Proteins were visualized by staining with colloidal CBB. These data are representative of three independent experiments

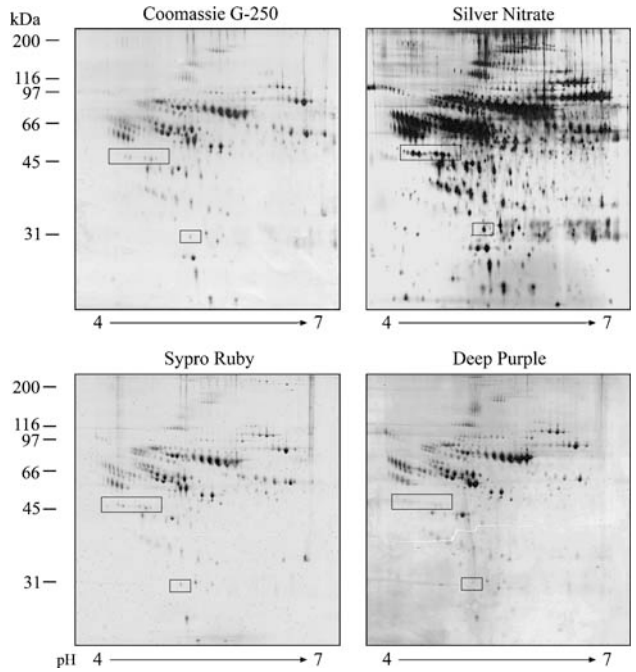


Fig. 6 Comparison of different staining methods to reveal 2-D gel protein patterns of human plasma depleted of high-abundance proteins. Plasma (30 µl) was fractionated with IgY-12 affinity columns to deplete high-abundance proteins. Proteins not bound to the column were then analyzed by 2-D gels using focalisation according to their pI over a pH range from 4 to 7 and by separation according to their molecular weight on 10% SDS-PAGE. Gels were then stained by colloidal CBB, silver nitrate, Sypro Ruby and Deep Purple as described in the materials and methods section. These data are representative of three independent experiments. Boxes are examples of regions showing differences in protein numbers or protein intensities

major concern about silver staining is that this technique is often considered to covalently modify proteins which could interfere with subsequent protein analysis. Here, a sensitive silver staining procedure compatible with mass spectrometry [17] served to detect plasma proteins in the lower-abundance fraction. This method was clearly much more sensitive than colloidal CBB, Sypro Ruby and Deep Purple since it stained 811 protein spots (Fig. 6). In particular, several proteins with molecular weights lower than 45 kDa were only detected by the silver staining method (Fig. 6). Thus, this silver staining procedure could be useful for detecting proteins in trace amounts while fluorescent staining with Deep Purple could be more appropriate and reliable for quantifying plasma proteins.

Antibody Microarrays as a Tool to Detect Proteins in Human Plasma Depleted of High-Abundance Proteins

Protein microarrays, and in particular antibody microarrays, are new technologies in the field of proteomics. The patterns generated by antibody microarray data can be used to create proteomic maps reflecting changes in proteome composition [20]. Furthermore, the experimental features of antibody microarrays have the advantages of performing the simultaneous analysis of several hundreds of proteins in parallel. Here, the Kinex™ antibody microarray services of the company Kinexus were used to investigate the protein epitopes present in plasma following the depletion of the 12 most abundant proteins. These microarrays served to monitor the interaction of lower-abundance plasma proteins with over 350 antibodies. Arrays were performed in duplicate with plasma from two subjects. Only proteins showing signal intensity greater than two-fold above the noise for both subjects were considered (Table 1). Under these conditions, antibodies positively reacted against 61 lower-abundance plasma proteins representing about 17%

Table 1 Proteins detected by antibody microarrays following immunoaffinity depletion of the 12 most abundant proteins in human plasma.

Proteins	Full protein name	Signal/noise ratios
Aurora 3	Aurora 3 (gamma) protein-serine kinase	2.3
Axl	Axl proto-oncogene-encoded protein-tyrosine kinase	2.1
CaMK1d	Calcium/calmodulin-dep. protein-serine kinase 1 delta	2.3
CaMK1g	Calcium/calmodulin-dep. protein-serine kinase 1 gamma	2.7
CAMK2b	Calcium/calmodulin-dep. protein-serine kinase 2 beta	2.5
CAMK2d	Calcium/calmodulin-dep. protein-serine kinase 2 delta	2.8
CAMK2g	Calcium/calmodulin-dep. protein-serine kinase 2 gamma	2.4
CAS	Cellular apoptosis susceptibility protein (CSE1L)	2.4
CASP2	Pro-caspase 2 (ICH1 protease)	2.1
CASP5	Caspase 5 (ICH3 protease, ICE(rel)-III)	3.0
CD45	Leukocyte common antigen CD45 receptor-tyrosine phosphatase (LCA, T200)	2.4
Cdc34	Cell division cycle 34 (ubiquitin-conjugating ligase)	2.4
CDK1	Cyclin-dependent protein-serine kinase 1 (cdc2)	2.5
CDK2	Cyclin-dependent protein-serine kinase 2	2.5
CDK5	Cyclin-dependent protein-serine kinase 5	2.1
CDK8	Cyclin-dependent protein-serine kinase 8	3.1
CK1e	Casein protein-serine kinase 1 epsilon	2.2
Csk	C-terminus of Src tyrosine kinase	2.4
DFF45	DNA fragmentation factor alpha (ICAD)	2.1
DGKZ	Diacylglycerol kinase zeta	2.7
DNAPK	DNA-activated protein-serine kinase	2.5
DRAK2	DAP kinase-related apoptosis-inducing protein-serine kinase 2 (STK17B)	2.3
Erk4	Extracellular regulated protein-serine kinase 4	2.1
FGFR1	Fibroblast growth factor receptor-tyrosine kinase 1	2.1
FGFR2	Fibroblast growth factor receptor-tyrosine kinase 2	2.2
FLT4	Vascular endothelial growth factor receptor-protein-tyrosine kinase 3 (VEGFR3)	2.5
Grp78	Glucose regulated protein 78	2.0
HO2	Heme oxygenase 2	2.0
HSF4	Heat shock transcription factor 4	2.5
HspBP1	Hsp70 binding protein 1	2.2
IRAK4	Interleukin 1 receptor-associated kinase 4	2.4
JAK1	Janus protein-tyrosine kinase 1	2.6
JIK	STE20-like protein-serine kinase	2.2
LOK	Lymphocyte-oriented protein-serine kinase	2.7
MKP2	MAP kinase phosphatase 2 (VH2)	2.5
MST2	Mammalian STE20-like protein-serine kinase 2	2.3
Nek4	NIMA (never-in-mitosis)-related protein-serine kinase 4	3.0
p18 INK4c	p18 INK4c cyclin-dependent kinase inhibitor	2.1
PP5C	Protein-serine phosphatase 5—catalytic subunit (PPT)	2.2
PACSIN1	Protein kinase C + casein kinase substrate in neurons protein 1	2.1
PAK5	p21-activated serine kinase 5 (Serine/threonine-protein kinase PAK 7)	2.1
PAK6	p21-activated serine kinase 6	2.2
PCK2	Phosphoenolpyruvate carboxykinase	2.3
PKA	cAMP-dependent protein-serine kinase	2.1
PKBg (Akt3)	Protein-serine kinase B gamma (Akt3)	2.1
PKCa	Protein-serine kinase C alpha	2.5
PRKWNK4	Putative protein-serine kinase WNK4	2.1
PRP4K	Protein-serine kinase PRP4 homolog	2.5

Table 1 continued

Proteins	Full protein name	Signal/noise ratios
PTP1C	Protein-tyrosine phosphatase 1C	2.2
PTP1D	Protein-tyrosine phosphatase 1D	2.5
RPTPa	Protein-tyrosine phosphatase, receptor type, A	2.3
RPTPb	Protein-tyrosine phosphatase, receptor-type, Z polypeptide 1	2.3
SGK3	Serum/glucocorticoid regulated kinase 3	2.7
SIRPa1	Signal regulatory protein substrate of PTP1D phosphatase	2.5
SLK	STE20-like protein-serine kinase	2.8
STI1	Stress induced phosphoprotein 1 (Hsc70/Hsp90 organizing protein (Hop))	2.2
STK33	FLJ35932 protein-serine kinase	2.0
TAK1	TGF-beta-activated protein-serine kinase 1	2.4
TEK	Angiopoietin-1 receptor-tyrosine kinase (TIE2)	2.6
TrkB	BNDF/NT3/4/5 receptor-tyrosine kinase	2.0
XIAP	X-linked inhibitor of apoptosis protein (baculoviral IAP repeat-containing 4)	2.1

Microarrays consisted of antibodies for signalling proteins spotted in duplicate with two slides per sample. Only chemiluminescence signals showing strength twice above the background in plasma samples from the two donors were considered to calculate the means. The signal/noise ratios were divided into three groups: 2.0–2.3, 2.4–2.7 and 2.8–3.1

of tested antibodies. The signal/noise ratios were divided into three groups: those in the range between 2.0 and 2.3 included 32 proteins, those with a ratio between 2.4 and 2.7 comprised 24 proteins and finally a small group of five proteins that possessed ratios between 2.8 and 3.1 (Table 1).

Antibody microarrays generate false positive data due to cross-reactivity generated by non-specific protein binding or by formation of protein complexes. Consequently, Western blot analysis was carried out against some of the proteins recognized by the antibodies in order to validate the microarray results. The potential of the antibody microarray technology was investigated by probing the

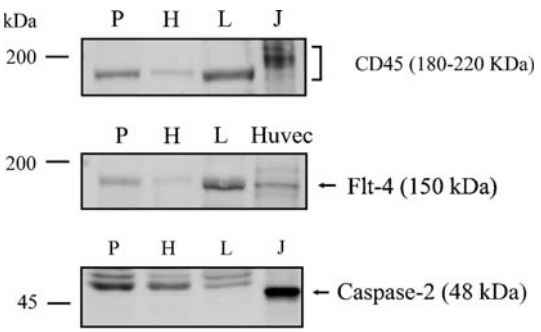


Fig. 7 Immunodetection of proteins found by antibody microarrays in human plasma depleted of high-abundance proteins. Plasma proteins were fractionated using IgY-12 columns. Then, 50 μg of proteins from crude plasma (P) or from low (L) and high (H) abundance plasma protein fractions were analyzed by SDS-PAGE. Immunodetection with CD45, Flt-4 and Caspase-2 antibodies was performed as described in the materials and methods section. Lysates from Jurkat cells (J) and from Huvec cells (Huvec) were also loaded and separated by SDS-PAGE as positive controls. These data are representative of two independent experiments

presence of pro-caspase 2 (2.1-fold over background), CD45 (2.4-fold) and Flt-4 (2.5-fold). Interestingly, the receptor tyrosine phosphatase CD45 and the vascular endothelial growth factor receptor-protein-tyrosine kinase-3 Flt-4 were recognized by their antibodies by Western blotting (Fig. 7). As expected, CD45 and Flt-4 were enriched in the plasma fraction depleted of abundant proteins and, conversely, they were reduced in the fraction containing the abundant plasma proteins relative to crude plasma (Fig. 7). Furthermore, Flt-4 comigrated with the 150 kDa positive control present in the lysates of Huvec endothelial cells (Fig. 7). The migration of CD45 in plasma was slightly faster than that seen in the control, the lysates of Jurkat cells (Fig. 7). This could be explained by a difference in the glycosylation state of the extracellular N-terminal region of CD45 which is responsible for the protein isoforms [21]. On the other hand, none protein co-migrating with the pro-caspase 2 was detectable in any plasma fractions but some non-specific proteins were recognized by the antibody (Fig. 7). This latter observation underlines the importance of validating antibody microarray data by alternative methods. The results with CD45 and Flt-4 for which the signal/noise ratios were of moderate levels, clearly demonstrate that antibody microarrays could be applied to plasma samples depleted of high-abundance proteins.

Discussion

Since, human plasma can be easily obtained, several laboratories have developed various proteomic strategies for

the purpose of using plasma proteins as a source for possible disease-related biomarkers [7–15, 22]. However, the great variety of protein types present, as well as the extended dynamic range of protein concentrations greatly hinders the identification of plasma biomarkers. One promising approach is to fractionate plasma proteins into two categories: one corresponding to the depleted abundant proteins and another which is enriched in less abundant plasma proteins facilitating their detection and identification. The fraction containing the depleted abundant proteins could also be informative since certain proteins remain associated to albumin, for instance, which is a carrier for several other proteins. Thus, it is possible that some minor proteins remain bound to the affinity columns essayed in this study and were lacking when low abundant proteins were analyzed by 2-D gels. However, the main focus of proteomic approaches is usually based on the identification of proteins found in the less abundant fraction.

Typically, most approaches for protein separation are based on affinity capture resulting in binding and removing of abundant plasma proteins. This is the case for methods using Cibacon Blue for albumin removal [6, 7]. Recently, an affinity method based on the selective adsorption of proteins on a solid phase combinatorial ligand library has been applied to plasma with promising results [23]. However, immunoaffinity capture using antibodies is a classical and a reliable method for the depletion of abundant proteins and thus the enrichment of lower-abundant proteins in plasma [7–15]. Thus the goal of this study was to compare systematically different protocols in plasma handling and protein analysis by 2-D gels. These investigations were integrated into a strategy that also compared commercially available columns to enrich plasma proteins of lower-abundance by using three commercially available columns including two immunoaffinity columns able to remove 6 and 12 of the more abundant plasma proteins. Another finding of our work was applying antibody microarrays to the lower-abundance plasma proteins enriched by these optimized conditions. This allows the identification of 61 proteins using 350 antibodies directed against signalling proteins suggesting that this proteomic strategy is a valuable approach for detecting potential plasma biomarkers released into blood by damaged tissues or cells in different diseases.

The ProteomeLab IgY Partitioning System, which removes the 12 most abundant proteins was found to be most efficient for the enrichment of lower-abundance proteins as judged by the number of spots detected following 2-D gel analysis. Additionally, these two steps in plasma proteins fractionation by the affinity column followed by 2-D gel analysis of enriched proteins of lower-abundance are reproducible. This conclusion is based on our data showing that mean recovery for lower-abundance plasma proteins is 8.3 ± 0.7% and %CV is within 8.4% while the number of detected spots by collidal Coomassie staining is 337 ± 33 and %CV within 10. This conclusion agrees with recent studies demonstrating that immunoaffinity depletion is an efficient approach for enriching lower-abundance proteins in plasma [7–15]. The increase in spot number present in lower-abundance proteins in plasma following the depletion of numerous high-abundant proteins could arise for various reasons. Indeed, it could be due to the detection of new proteins in trace amounts but also from degradation products or enrichment of post-translationally modified proteins for instance.

The contribution of individual factors influencing either the detection of lower-abundance plasma proteins or the formation of 2-D gel patterns, such as the presence of anticoagulants when plasma samples are prepared, the effect of cysteinyl modification with hydroxyethyl disulfide on protein spot sharpness and different protein staining methods, has been further analyzed. Thus, the quality of plasma preparation is a key step in plasma proteomics and evaluating the effect of different anticoagulants such as EDTA and CTAD is essential. Our data present some interesting facts since fewer spots were detected in lower-abundance protein fractions when using CTAD (209 spots) instead of EDTA (255 spots) to prepare plasma. These observations suggest that CTAD could reduce artifacts associated with platelet activation. However, it has been reported that the optimal method for preparing plasma depends also on the subsequent technical analysis to be performed [24]. On the other hand, cysteinyl modification using specific oxidation by hydroxyethyl disulfide improves the appearance of protein spots for an acidic pH gradient ranging from 4 to 7 although this modification was initially developed to ameliorate protein migration in alkaline pH ranges [19]. Importantly, cysteinyl modification by this method during the first dimension has been reported to remain compatible with mass spectrometry for protein identification [25] which is crucial for the identification of plasma biomarkers.

Each of the staining methods tested for revealing lower-abundance proteins has different advantages. Colloidal CBB G-250 that detected 318 protein spots is quite simple and highly reproducible. Experiments with fluorescent dyes showed that both Deep Purple and Sypro Ruby have similar capacity for detecting lower-abundance plasma proteins following 2-D gels, 273 and 298 protein spots, respectively. The interest of comparing different dyes to detect plasma proteins separated by 2-D gels was to determine whether alternative stains currently available possess enough advantages to replace typical stains such as colloidal Coomassie and fluorescent Sypro Ruby. The great advantage of fluorescent dyes such as Sypro Ruby and

Deep Purple is their very wide linear dynamic ranges which are essential for protein quantification [26]. Our results indicate that Deep Purple shows a detection limit similar to that of Sypro Ruby. But in contrast to Sypro Ruby, Deep Purple does not induce the formation of speckles on the gel and as a natural compound it is easier to dispose of. In addition, the lower cost of Deep Purple is another reason to prefer it to the popular Sypro Ruby dye. The silver staining method used gives the best results in terms of visualizing the greatest number of lower-abundance plasma proteins (811 spots). This method is an acidic version of the silver staining methods. In this particular method, the glutaraldehyde is omitted since this reagent is known to covalently modify proteins and it is not crucial for obtaining considerable sensitivity [17]. Thus, this staining method is compatible with other analytical methods for the identification of lower-abundance plasma proteins. Due to its high sensitivity and simplicity, this silver staining procedure would be very useful for visualizing plasma proteins and could replace classical staining with colloidal Coomassie on a routine basis. Furthermore, the capacity of this silver staining method to detect plasma proteins in trace amounts that are not seen with fluorescent dyes is another critical advantage for the identification of potential biomarkers. Nevertheless, protein staining with the fluorescent dye Deep Purple would be more reliable for the quantification of lower-abundance plasma proteins.

The depletion of higher-abundance proteins by immunoaffinity as described here, followed by antibody microarray analysis, provide a novel strategy for the identification of specific biomarkers or protein patterns in the lower-abundance plasma protein fraction. Out of 350 antibodies tested using microarrays, 61 (17%) of these showed signal intensity greater than 2-fold above the background when incubated with lower-abundance plasma proteins from two subjects. Among the 61 recognized proteins by the microarrays, the majority, 52%, presented a signal/noise ratio between 2.0 and 2.3, while 39% had a value between 2.4 and 2.7 and 8% possessed a ratio between 2.8 and 3.1. The small number of lower-abundance plasma proteins that were recognized by the antibody microarrays could reflect the composition of antibodies on the arrays. These have been manufactured to detect the proteins in signalling pathways involved in signal transduction, cell cycle control and apoptosis. The presence of such proteins in the lower-abundance plasma protein fraction can be seen by the detection of receptor tyrosine kinases (FGFR1, FGFR2, Flt-4), different isoforms of calcium/calmodulin-dependent protein-serine kinase 1, several members of cyclin-dependent protein-serine kinases (CDK1, CDK2, CDK5 and CDK8), two protein-serine phosphatases (MST2 and PP5C) and protein-tyrosine phosphatases (PTP1D, RPTPa and RPTPb). The detection of kinases and phosphatases in the lower-abundance proteins suggests that this plasma fraction could be an excellent source of biomarkers released by tissues or cells for various diseases including different cancers where many signalling pathways are altered.

Of the low-abundance plasma proteins that were detected by antibody microarrays, three were studied further by Western blot analysis. Two of them, the receptor tyrosine phosphatase CD45 and the vascular endothelial growth factor receptor-protein-tyrosine kinase-3 Flt-4, show a clear enrichment in the lower-abundance protein fraction while decreasing in the fraction containing the abundant plasma proteins. This demonstrates the ability of the antibody microarrays to recognize certain proteins in the lower-abundance protein fraction. Importantly, antibody microarrays are rapidly performed and could be further employed to characterize the changes in the intensity of biomarkers or groups of biomarkers. Together, the proteomic approaches investigated in this study where the lower-abundance plasma proteins are enriched by immunoaffinity depletion of abundant proteins followed by 2-D gel analysis or by antibody microarrays could provide new opportunities for the discovery of biomarkers and for the understanding of molecular mechanisms in diseases.

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