Rembert Pieper
Qin Su
Christine L. Gatlin
Shih-Ting Huang
N. Leigh Anderson*
Sandra Steiner

Large Scale Biology Corporation, Germantown, Maryland, USA

Multi-component immunoaffinity subtraction chromatography: An innovative step towards a comprehensive survey of the human plasma proteome

In order to discover novel protein markers indicative of disease processes or drug effects, the proteomics technology platform most commonly used consists of high resolution protein separation by two-dimensional electrophoresis (2-DE), mass spectrometric identification of proteins from stained gel spots and a bioinformatic data analysis process supported by statistics. This approach has been more successful in profiling proteins and their disease- or treatment-related quantitative changes in tissue homogenates than in plasma samples. Plasma protein display and quantitation suffer from several disadvantages: very high abundance of a few proteins; high heterogeneity of many proteins resulting in long charge trains; crowding of 2-DE separated protein spots in the molecular mass range between 45–80 kD and in the isoelectric point range between 4.5 and 6. Therefore, proteomic technologies are needed that address these problems and particularly allow accurate quantitation of a larger number of less abundant proteins in plasma and other body fluids. The immunoaffinity-based protein subtraction chromatography (IASC) described here removes multiple proteins present in plasma and serum in high concentrations effectively and reproducibly. Applying IASC as an upfront plasma sample preparation process for 2-DE, the protein spot pattern observed in gels changes dramatically and at least 350 additional lower abundance proteins are visualized. Affinity-purified polyclonal antibodies (pAbs) are the immunoaffinity reagents used to specifically remove the abundant proteins such as albumin, immunoglobulin G, immunoglobulin A, transferrin, haptoglobin, α-1-antitrypsin, hemopexin, transthyretin, α-2-HS glycoprotein, α-1-acid glycoprotein, α-2-macroglobulin and fibrinogen from human plasma samples. To render the immunoaffinity subtraction procedure recyclable, the pAbs are immobilized and cross-linked on chromatographic matrices. Antibody-coupled matrices specific for one protein each can be pooled to form mixed-bed IASC columns. We show that up to ten affinity-bound plasma proteins with similar solubility characteristics are eluted from a mixed-bed column in one step. This facilitates automated chromatographic processing of plasma samples in high throughput, which is desirable in proteomic disease marker discovery projects.

Keywords: Antibody immobilization / Clinical diagnostics / Immunoaffinity chromatography / Plasma proteome / Protein marker discovery / Serum PRO 0366

1 Introduction

There is currently a major resurgence of interest in the human blood plasma proteome because of the central role played by plasma in clinical diagnostics. In addition, proteomes of other body fluids such as cerebrospinal fluid (CSF) and urine are of interest for clinical research in selected disease areas. There is evidence that many disease processes are associated with quantitative and functional changes of proteins in body fluids. Twenty-five years ago, 2-DE was developed as a technique for the parallel display of many proteins from complex biological samples [1–4] and adapted to generate a protein map for plasma [5]. Since then, the information available on the protein composition in serum and plasma

Correspondence: Dr. Rembert Pieper, Large Scale Biology Corporation, 20451 Goldenrod Lane, Germantown, MD 20876, USA
E-mail: rembert.pieper@lsbc.com
Fax: +1-301-354-1300

Abbreviations: CB, Coomassie Blue R-250; **IASC**, immunoaffinity subtraction chromatography; **Ig**, immunoglobulin; **pAbs**, polyclonal antibodies

* Present address: Plasma Proteome Institute, P.O. Box 21466, Washington D.C. 20009, USA

 0173-0835/03/0404–422 $17.50+.50/0

has increased, in part due to improved resolution of proteins in 2-DE gels [6, 7] and also because of rapid advances in mass spectrometry technologies. The well established MS techniques of peptide mass fingerprinting and peptide sequencing have been instrumental for identifying numerous proteins and polypeptides in plasma [6–9]. Likewise, many proteins present in CSF, urine and other body fluids have been mapped by 2-DE followed by identification and further characterization using MS methods [10–14]. Excluding the cellular components of the blood, differential display comparing body fluid samples from normal and diseased donors is only meaningful on the proteome level and not at the transcript level. Proteomics tools have been applied to identify disease-associated quantitative protein changes in urine and plasma [15–18]. It is generally expected that exploration of plasma and other body fluids at higher sensitivities surveying as large a number of proteins as possible will yield a variety of new protein markers for disease processes and drug effects, leading in turn to major advances in patient monitoring and treatment. In light of the limitations to detect numerous low abundance proteins using standard 2-DE [19], there is a search for alternative and additional technologies: microscale solution IEF and narrow range pH gradients [7, 20–22]; high-reslution chromatography [23–25]; surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) combined with extensive bioinformatic pattern analysis [26, 27]. While these new technologies may contribute to speedier biomarker discovery in plasma, they also have distinct limitations. Sample throughput can be low due to difficult technical automation. The resolution and reproducibility of protein quantitation may not be sufficient in the range above 30 kDa, where most plasma proteins of known function are found. Eighty-five percent of the total plasma protein consists of five to ten very abundant proteins. A technology, which specifically removes them, would lead to the relative enrichment of less abundant proteins without the need of further fractionation. This problem is in theory easily solved by protein subtraction methods based on a variety of affinity separation techniques [28–34] and the concept of negative immunoaffinity chromatography introduced by Wheatley [35]. However, practical problems with the labor-intensiveness and cost of conventional approaches as well as with unsatisfactory protein-ligand interaction specificities [36] have proved to be major barriers to high-throughput surveys of plasma samples. We present a systematic approach to generate multicomponent immunoaffinity subtraction chromatography (IASC) matrices and apply it to quantitative global protein surveys in body fluid samples.

2 Materials and methods

2.1 Materials and reagents

The Integral®-100Q and Biocad-Vision™ workstations (Applied Biosystems, Foster City, CA, USA) were used for all chromatographic procedures. The following human proteins were available in purities between 95 and 99%: from Calbiochem (San Diego, CA, USA) albumin, transferrin, α-2-macroglobulin, α-1-antitrypsin; from Sigma (St. Louis, MO, USA) transthyretin, haptoglobin, fibrinogen, immunoglobulin (Ig) A, IgM; from Biodesign International (Saco, ME, USA) apolipoprotein A1, α-2-HS glycoprotein, α-1-antichymotrypsin, α-1-acid glycoprotein; from Enzyme Research (South Bend, IN, USA) antithrombin III. One mg of hemopexin was purified to 95% homogeneity from 10 mL human serum using IASC to remove the five most abundant serum proteins before selective enrichment of hemopexin *via* metal chelate affinity chromatography as described in [37]. Polyclonal antisera and IgG-enriched fractions of antisera were obtained from various manufacturers. Sigma: IgG fraction of anti-albumin (rabbit), IgG fraction of anti-haptoglobin (rabbit), IgG fraction of anti-transferrin (goat), IgG fraction of anti-transthyretin (rabbit), IgG fraction of anti-α-1-antitrypsin (rabbit), IgG fraction of anti-IgM μ-chain (rabbit). Kent Laboratories (Bellingham, WA, USA): antiserum to α-2-macroglobulin (goat), antiserum to α-1-acid glycoprotein (goat), antiserum to IgA α-chain (goat), antiserum to hemopexin (goat), antiserum to fibrinogen (goat), antiserum to apolipoprotein A1 (goat). Strategic Biosolutions (Ramona, CA, USA): antiserum to α-1-antichymotrypsin, IgG fraction of anti-α-2-HS glycoprotein (goat), IgG fraction of anti-antithrombin III (goat). The pre-activated chromatography resin POROS® AL, the protein A-derivatized resin POROS® A 20 and the protein G-derivatized resin POROS G 20 were purchased from Applied Biosystems. The BioSilect® SEC 250–5 (7.8 × 300 mm) column was from Bio-Rad Laboratories (Hercules, CA, USA). Human serum and plasma samples were obtained from consenting donors.

2.2 Generation of affinity chromatography resin matrices for antibody purification

The POROS AL resin has amine-reactive aldehyde groups. Covalent coupling of proteins to POROS AL was conducted similar to the resin manufacturer's instructions: 1–10 mg of dry protein was dissolved in buffer A (25 mM sodium phosphate, 0.5 M sodium sulfate, pH 7.2) at a concentration of 2–15 mg/mL. Sodium cyanoborohydride (5 mg/mL) was dissolved before adding the POROS AL resin (300 mg/mL settled resin volume). The batch was incubated at room temperature for 30 min, before the sodium sulfate concentration was increased

stepwise (0.1 M) with buffer B (25 mM sodium phosphate, 1.5 M sodium sulfate, pH 7.2) every 30 min. The salting-out conditions facilitated rapid covalent coupling of proteins to the resin. At a final molarity of 1.1 M sodium sulfate, the coupling solution was replaced with 0.2 M Tris-HCl, pH 7.6 and 250 mg/mL sodium cyanoborohydride. Residual aldehyde groups were thus deactivated through reductive amination. The resin was washed with 1 M sodium chloride.

2.3 Purification of antibodies and their immobilization on POROS A or G columns

A multi-step chromatographic procedure (Fig. 1A) was set up to purify and immobilize polyclonal antibodies (pAbs) using three columns equilibrated in 25 mM sodium phosphate, 150 mM sodium chloride, 0.01% sodium azide, pH 7.2 (buffer C). Aliquots of an antiserum or its IgG fraction containing pAbs specific for the protein antigen, which was immobilized as described in Section 2.2, were loaded onto the antigen column. Affinity-bound antibodies were eluted with one of the following two acidic solutions: elution buffer D (5% acetic acid, 150 mM NaCl, pH 2.1) or elution buffer E (0.5 M glycine, 150 mM NaCl, 0.25% CHAPS, pH 2.1). The Bio-Silect SEC 250–5 column in position 2 separated antibodies from the elution reagents. A POROS G column (for goat antisera) or a POROS A column (for rabbit antisera) mounted in position 3 affinity-trapped IgG-type antibodies.

2.4 Covalent cross-linking of antibodies to POROS A and G columns

Antibodies bound to 0.8 or 1.7 mL POROS A and G columns were subjected to covalent immobilization with a procedure described in [38] and by the resin manufacturer including slight modifications: a 10 mL solution of two chemical cross-linking reagents, 15 mM dimethylpimelimidate (DMP) and 15 mM dimethylsuberimidate (DMS), was freshly prepared in ice-cold 0.2 M triethanolamine, pH 8.4. The solution was applied in repetitive chromatographic cycles. In each cycle, 2 mL of the cross-linking solution were injected at a flow rate of 0.6 mL/min followed by brief column re-equilibration in buffer C. This antibody-coupling process was repeated once with a fresh solution of DMS and DMP. Remaining amine-reactive groups were quenched running ten column volumes of 150 mM monoethanolamine, pH 9.0 through the column. Non-covalently bound antibodies were desorbed eluting with two column volumes of buffer D.

2.5 Determination of the specificities and capacities of antibody-coupled columns

To evaluate antigen-binding capacities, the following chromatographic method was used: (1) stepwise loading of purified protein antigen onto the antibody-coupled column (0.5–1 mg in each of several steps); (2) applying buf-

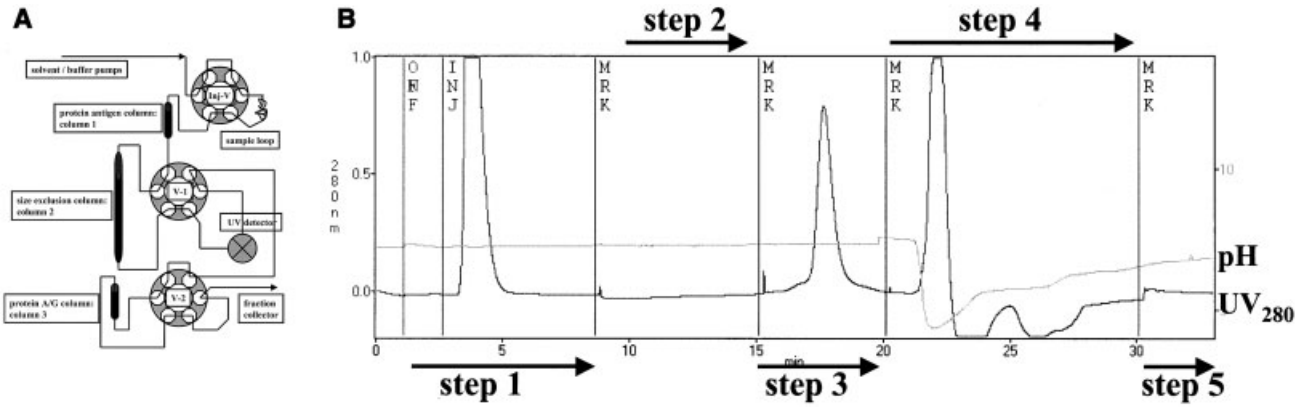

Figure 1. A. Three-column chromatographic configuration for the affinity purification and immobilization of antibodies from antiserum. The configuration is shown with all three columns switched in-line: V-1 (valve 1), V-2 (valve 2), Inj-V (injection valve). B. Chromatogram for the antibody purification and immobilization experiment. Using the configuration depicted in Fig. 1A, a method was run as follows: antiserum was loaded onto the antigen column at a flow rate of 1 mL/min buffer C (step 1); 1 mL acidic eluant D was injected at the start of step 2 switching the size exclusion column in-line with the antigen column; all three columns were switched in-line to immobilize eluting antibodies on column 3 (step 3); this protein A/G column was bypassed to re-equilibrate the size exclusion column monitoring the pH and UV_{280} traces (step 4); buffer C was run through column 3 to monitor protein A/G saturation with antibodies (step 5). Monitoring the UV_{280} trace, proteins of the antiserum not bound by column 1 were detected in step 1. The antibody fraction was detected in step 3 and eluant D reagents eluting from the size exclusion column in step 4.

fer C (4–8 mL depending on the column size) after each loading step; (3) monitoring the UV_{280} trace to detect the protein flowing through the column after the event of antibody resin saturation. The presence of protein in the collected UV_{280} peak fractions was verified by SDS-PAGE. For a few resins, this procedure was not applied due to the lack of sufficient available antigen (*e.g.* hemopexin, antithrombin-III, α-2-HS glycoprotein). In these cases, serum containing the antigen in a known concentration was cumulatively loaded onto the antibody column in buffer C. To visualize the lack of antigen binding upon antibody resin saturation, the column-flowthrough serum fractions were prepared for and analyzed in 2-DE gels. Spot location-based detection of the investigated protein antigen in the 2-DE gel was facilitated by additional removal of albumin and IgG from the same serum samples. In order to evaluate the specificity of antigen binding, a human plasma or serum aliquot was loaded onto the antibody-coupled column. Here, the affinity-bound protein fraction was recovered eluting with either eluant D, E, F (5% acetic acid, 150 mM sodium chloride, 3 M urea, pH 2.1) or G (0.5 M glycine, 150 mM sodium chloride, 3 M urea, 0.25% CHAPS, pH 2.1). The eluate containing the affinity-bound protein(s) was prepared for 2-DE and checked, as to which protein spots were present.

2.6 Pooling of antibody-coupled resins into mixed-bed IASC resins and columns

Antibody-coupled columns quality-controlled as described in Section 2.5 were unpacked and suspended in buffer C. To prepare a mixed-bed column, three questions needed to be answered: (1) the antigen-binding capacities *per* resin volume; (2) which elution buffers were effective to accomplish complete elution of each of the affinity-bound proteins; (3) the relative abundance of all proteins in serum and plasma to be subtracted from a sample. Antibody-coupled resins were combined only, if the respective protein antigens could be solubilized with the same eluant. We took into account the approximate molar abundance ratios of the target proteins in plasma and serum in order to optimally utilize available binding sites for all antigens. *Ergo*, antibody-coupled resin suspensions were combined in volume ratios proportional to the concentrations of protein antigens in these body fluids and inversely proportional to their protein-binding capacities. For example, a later described 7.8 mL mixed-bed IASC column was composed of the following antibody-coupled resins: anti-albumin (3.8 mL), anti-transferrin (0.9 mL), anti-haptoglobin (1 mL), anti-α-1-antitrypsin (0.6 mL), anti-α-1-acid glycoprotein (0.3 mL), anti-α-2-HS glycoprotein (0.35 mL), anti-hemopexin (0.5 mL), anti-transthyretin (0.2 mL), anti-antithrombin-III (0.2 mL). About half of the IASC resin matrix consisted of POROS A derivatized with anti-albumin antibodies, because albumin is the most abundant protein in serum.

2.7 Recyclability of antibody-coupled columns

The eluants mentioned in Section 2.5 were evaluated to determine the recyclable use of antibody-coupled columns. In addition, elution buffer volumes required to completely solubilize and elute the affinity-bound proteins were examined in the range 0.5–2 mL eluant/mL column volume. Recyclable use, which is centered on sustainable antigen-binding of the immunoaffinity column during repeated use, was evaluated loading a capacity-reaching amount of purified protein onto the investigated column, eluting it and repeating this two-step cycle 10–25 times. The column-flowthrough fractions were run on SDS-PAGE gels to detect reduced protein binding. For mixed-bed IASC columns with specificity for multiple serum proteins, recyclable use was investigated applying serum instead of pure protein samples and analyzing for the sustained removal of multiple proteins *via* 2-DE.

2.8 Preparation of protein fractions for 2-DE and gel image analysis

Protein samples eluted with acidic buffers were neutralized with 3 M Tris-HCl, pH 9.0 before transfer into Ultrafree®-4 centrifugal filter units (Millipore, Billerica, MA, USA) with a molecular mass cutoff of 5 kDa. Fractions collected in buffer C were directly transferred to the filter units. They were spun at 3500 rpm and proteins were concentrated, desalted and equilibrated in buffer H (25 mM ammonium bicarbonate, 0.5 mM sodium EDTA, and 0.5 mM benzamidine). Concentrates of 200–400 μL were pipetted into microvials, lyophilized for 15–24 h and solubilized in an IEF buffer (2% CHAPS, 9 M urea, 62.5 mM DTT and 2% pH 8–10.5 carrier ampholytes). 2-DE was performed in Large Scale Biology's ProGEx™ system. The 8–15% T gradient slab gels were stained with Coomassie Blue R-250 (CB) or silver following standard procedures. Gel images were digitized followed by protein spot pattern analysis using Kepler® software as reported previously [39].

2.9 Sample preparation for MS and MALDI-TOF analysis

Protein spots were manually cut from CB stained 2-DE gels. Standard procedures were used for tryptic digestion and peptide extraction [39]. A small volume of the peptide extract was spotted onto a Bruker 600 μm

AnchorChip MALDI target with α-cyano-4-hydroxycinnamic acid matrix using standard MALDI-spotting procedures. MALDI targets were automatically run on a Biflex or Autoflex mass spectrometer equipped with delayed ion extraction (Bruker Daltronics, Bremen, Germany). All mass spectra represented signal averaging of 120 laser shots. The performance of the mass spectrometers yielded sufficient mass resolution to produce isotopic multiplets for each ion species below *m/z* 3000. For peptide mapping analysis, spectra were registered, analyzed and searched using an in house-modified application of Radars (Harvard Biosciences, Holliston, MA, USA). Mascot (Matrix Science, London, UK) and ProFound (Harvard Biosciences) were the database search engines employed.

3 Results and discussion

3.1 Antibody purification and immobilization

The purification of pAbs from antisera and their immobilization on protein A- and protein G-derivatized columns was accomplished in an automated chromatographic procedure as described in Sections 2.3 and 2.4. It could be conveniently monitored in real time following the UV_{280} trace during the chromatography (Fig. 1B). pAbs with the desired affinities were temporarily trapped on a protein antigen column, eluted and further purified by size fractionation and, selecting the 150 kDa range of IgG antibodies, immobilized on a protein A or G column. Protein A and G share a high affinity for the constant region of IgG and bind such antibodies strongly. Running a series of chromatographic cycles, antibodies accumulated on and finally saturated the POROS A or G column (*ca*. 6 mg IgG/mL for POROS G and *ca*. 9 mg IgG/mL for POROS A). A separate cross-linking procedure established covalent amidine bonds between the antibodies and either protein A or G molecules with coupling efficiencies found to be reproducibly higher than 80%. With the exception of IgG, for all the other 15 plasma proteins listed in Table 1 antibody-coupled columns were generated.

3.2 Variations in the binding capacities of antibody-coupled columns for different plasma proteins

Initial antigen-binding capacities of antibody-coupled columns were quickly determined, whereas the evaluation of recyclable antigen-binding capacities was more

Table 1. Protein abundance in serum and data on the elution and protein-binding capacities of antibody columns

Protein name	Relative abundance in serum (average, %)[a)]	Elution buffer D	Elution buffer E	Elution buffer G	pAb column[b)] capacity (mg/mL)
albumin	54	+	+	n.d.	POROS A: 3.5
immunoglobulin G	17	+	+	n.d.	POROS A: 10
transferrin	3.3	○	+	n.d.	POROS G: 2.6
haptoglobin	3.0	+	+	n.d.	POROS A: 3.2
α-1-antitrypsin	3.8	+	+	n.d.	POROS A: 2.9
α-2-macroglobulin	3.6	−	−	+	POROS G: 1.2
immunoglobulin A	3.5	−	○	+	n.d.
immunoglobulin M	2.0	−	−	_	n.d.
α-1-acid glycoprotein	1.3	○	+	n.d.	POROS G: 1.5
hemopexin	1.1	+	+	n.d.	POROS G: 2
α-2-HS glycoprotein	0.8	○	+	n.d.	POROS G: 1.8
α-1-antichymotrypsin	0.6	−	○	n.d.	n.d.
transthyretin	0.3	+	+	n.d.	POROS A: 3
apolipoprotein-A1	3.0	−	−	−	POROS G: <1
antithrombin III	0.3	+	+	n.d.	POROS G: 2
fibrinogen	n.r.	−	n.d.	+	POROS G: 1.7

Elution buffer D (5% acetic acid, 150 mM sodium chloride, pH 2.1); elution buffer E (0.5 M glycine, 150 mM sodium chloride, 0.25% CHAPS, pH 2.1); elution buffer G (eluant E plus 3 M urea).
(+) good, a protein-binding capacity loss of < 10% between 2nd and 10th binding/elution cycle; (○) (medium); (−) ineffective; (n.d.) not determined; (n.r.) not reported;
a) polyclonal antibody column capacity in mg protein/mL resin; POROS A/G: immobilization matrices
b) Approximate weight-based protein abundance values in normal serum as listed in *The Plasma Proteins*, Putnam, F. R. (Ed.) vol. IV, Academic Press, Orlando, USA 1984.

complex, required a lot of protein antigen and was found to be elution buffer-dependent. Initial binding capacities of POROS G-based antibody-coupled resins were typically found to be 15–35% lower than those of POROS A based resins. Calculating with a 1:2 binding ratio (antibody/protein ligand) and the measured amounts of matrix-immobilized antibodies, the observed capacities for serum proteins were generally lower than the theoretical values. Examples are listed in Table 1. Binding capacities of separately generated antibody columns specific for the same antigen were reproducible, if no variations occurred in: (1) the purity of the protein immobilized on the affinity column for antibody purification; (2) the species in which the antiserum was generated; and (3) the choice of the antibody-coupling matrix. For example, three POROS A-based anti-albumin columns were determined to have a binding capacity of 3.5 mg albumin/mL resin each (± 8%). Similarly reproducible results were obtained for three POROS A-based anti-haptoglobin columns with a capacity of 3.2 mg haptoglobin/mL resin each (± 10%) and for three POROS G-based anti-transferrin columns with a capacity of 2.6 mg transferrin/mL resin each (± 12%).

After the first cycle elution of an antigen, binding capacities dropped 10–25%. This so-called first cycle effect was reported previously [33]. It is probably caused by high-avidity binding of the antigen to a fraction of the immobilized antibodies. This renders the antigen elution from its affinity-bound state more difficult. In our experiments, antigen-binding capacity losses beyond the first cycle effect were low for antibody coupled columns, whose antigens, such as albumin, transferrin and haptoglobin, displayed good solubility in acidic buffers. Lower recyclable antigen-binding capacities (1–2 mg antigen/mL resin) were observed for plasma proteins of high M_r and for components of large plasma protein complexes such as α-2-macroglobulin, fibrinogen, IgA, IgM and apolipoprotein A1. As previously argued [40], a reason for this could be the large size of the antigen-antibody complexes formed. Not all immobilized antibodies can engage in complex formation with such protein antigens simultaneously due to steric hindrance. Interestingly, we observed that the elution of high M_r plasma proteins from IASC columns was ineffective using eluants D or E. Low solubility and precipitation of such proteins in acidic solutions was likely involved in causing the reduction of antigen-binding capacities.

Superior column recycling was observed, when 3 M urea was added to the elution buffers (eluants F and G). The effectiveness of acidified 2–4 M urea as a solubilizing reagent for affinity-bound proteins was reported before [41, 42]. For example, a POROS G-based anti-fibrinogen

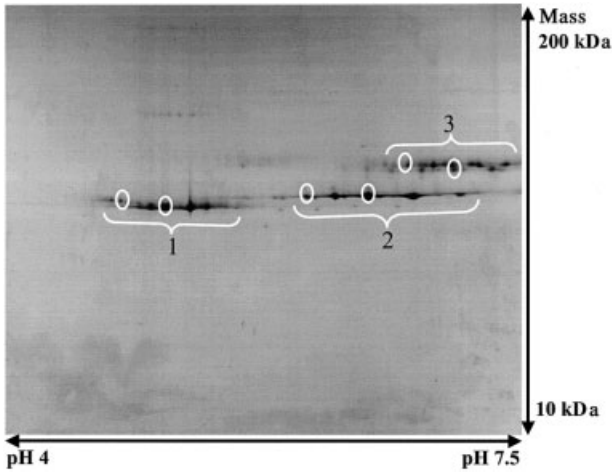

Figure 2. Coomassie Blue stained 2-DE gel of the plasma protein fraction affinity-bound by a POROS G based antifibrinogen column. In the first dimension, proteins were focused in an IEF tube gel applying 25 000 Vh. This gel was stacked on an 8–15%T second dimension slab gel and proteins were resolved in a range between 200–10 kDa over 1300 Vh. The circled spots were identified by MALDI-MS: in spot train 1, fibrinogen-γ; in spot train 2, fibrinogen-β; in spot train 3, fibrinogen-α.

matrix was measured to have an initial antigen-binding capacity of 2.4 mg fibrinogen/mL resin. The recyclable binding capacity using elution buffer F was 1.7 mg/mL over 25 elution cycles, whereas it dropped to less than 0.5 mg/mL using elution buffer D. The CB-stained gel image in Fig. 2 demonstrates the binding specificity of this column for fibrinogen chains α, β and γ. Table 1 presents an overview of eluants applied to evaluate the recyclable elution of proteins from antibody-coupled columns. Similar recyclability data were obtained, when more complex antigen sources (serum, plasma) were loaded onto antibody-coupled columns specific for albumin and the mixed-bed IASC column described in Section 2.6.

3.3 2-DE gel analysis of immunoaffinity-subtracted serum and plasma samples

Due to the distinct elution conditions required for various proteins in their affinity-bound states, not all antibody-coupled resins were pooled into one IASC column. We adapted the Scout-component of the Biocad-Vision workstation to allow in-line plumbing of a series of four columns (Fig. 3A). A 50 μL human serum sample was typically applied and, as illustrated in Fig. 3B, fractionated: the liquid chromatography run included the elution with a neutral pH buffer in step A (column-flowthrough) and four separate IASC column elution steps (B to E). Figure 3C

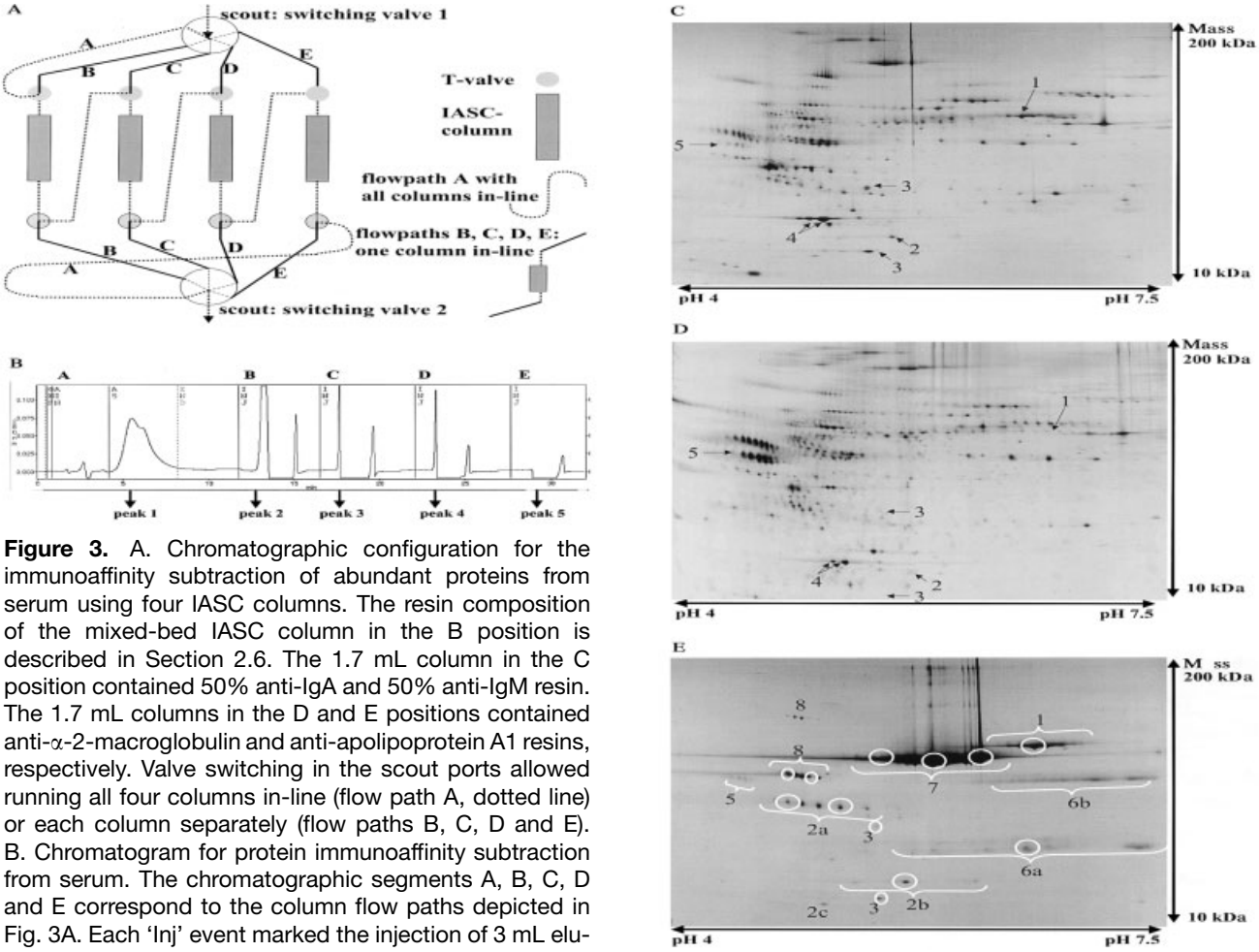

Figure 3. A. Chromatographic configuration for the immunoaffinity subtraction of abundant proteins from serum using four IASC columns. The resin composition of the mixed-bed IASC column in the B position is described in Section 2.6. The 1.7 mL column in the C position contained 50% anti-IgA and 50% anti-IgM resin. The 1.7 mL columns in the D and E positions contained anti-α-2-macroglobulin and anti-apolipoprotein A1 resins, respectively. Valve switching in the scout ports allowed running all four columns in-line (flow path A, dotted line) or each column separately (flow paths B, C, D and E). B. Chromatogram for protein immunoaffinity subtraction from serum. The chromatographic segments A, B, C, D and E correspond to the column flow paths depicted in Fig. 3A. Each 'Inj' event marked the injection of 3 mL elution buffer E or G from a loop into one column. At a buffer C flow rate of 1.5 mL/min (25 mM sodium phosphate, 150 mM sodium chloride, pH 7.2), a serum fraction not binding to the columns (peak 1) was monitored at UV_{280} and collected. The flow rate was increased to 2.5 mL/min during the following four individual column elution steps. Two UV_{280} peaks appeared during each elution step (B to E). One represents affinity-bound protein as indicated by the arrow, the following one has no protein and might be the result of pH and conductivity changes occurring when the column eluant is replaced by buffer C. C. Serum protein fraction after immunoaffinity subtraction of 13 proteins visualized in a CB-stained 2-DE gel. The 2-DE gel run parameters are described in the legend of Fig. 2. 130 μg protein, corresponding to a concentrated aliquot of the peak 1 fraction in Fig. 3B, was applied to the IEF gel. *Ca.* 85% of the total serum protein was removed so that the remaining less abundant proteins were enriched as visible in this protein pattern. Arrows for five protein spots or spot areas are denoted as follows. 1, transferrin; 2, major spot of haptoglobin α-2 chain; 3, transthyretin monomer and tetramer; 4, major spots of apolipoprotein A1; 5, spot train of α-2-HS glycoprotein. In this particular experiment, α-2-HS glycoprotein was depleted, whereas varying amounts of the other four proteins were not retained by the IASC columns and detected in the gel. The denoted protein spots allow positional comparisons to the gel images in Figs. 3D, 4 and 5. D. Serum protein fraction after immunoaffinity subtraction of 12 proteins visualized in a silver-stained 2-DE gel. The 2-DE run parameters are described in the legend of Fig. 2. 100 μg protein derived from the column-flowthrough of an experiment similar to the one described in Fig. 3A, B was loaded on the IEF gel. Specifically, a less often recycled apolipoprotein A1-specific antibody column and a slightly altered resin composition for the mixed-bed IASC column were used: 3% higher amounts of anti-transferrin, anti-haptoglobin and anti-transthyretin resins, no anti-α-2-HS glycoprotein resin. The spots and spot trains are indicated by arrows and numbered as in Fig. 3C. The apolipoprotein A1 spots were less intense compared to the gel in Fig. 3C. Transferrin, haptoglobin and transthyretin were depleted, but not α-2-HS glycoprotein. E. CB-stained 2-DE gel of an IASC affinity-captured and subsequently eluted protein fraction. The 2-DE run parameters are described in the legend of Fig. 2. 100 μg protein (*ca.* 1/7 of the protein eluted in the B segment, peak 2, Fig. 3B) was concentrated and analyzed by 2-DE. The circled protein spots were identified by MALDI-MS. In spot train 1, transferrin; 2a and b, haptoglobin β and α-2 chain, respectively; 3, transthyretin; 6a, Igκ light chain; 7, albumin; 8, α-1-antitrypsin. The 2-DE gel positions for most of the unidentified spots appeared to match the known spot locations for the following proteins and subunits. In spot 2c, haptoglobin α-1; 5, α-2-HS glycoprotein; 6b, Igγ heavy chain.

shows the protein pattern of the flowthrough fraction in a 2-DE gel image. Eight proteins – albumin, IgG, IgA, α-1-antitrypsin, α-1-acid glycoprotein, α-2-HS glycoprotein, hemopexin and antithrombin III – were completely removed. Five proteins – transferrin, haptoglobin, transthyretin, IgM and α-2-macroglobulin – were subtracted to a significant extent, between 75 and 95%, as approximated from densitometric spot comparisons in 2-DE gels derived from serum with and without subtraction. The protein pattern of the 2-DE gel image in Fig. 3D also represents an IASC column-flowthrough fraction. Due to the altered resin composition of the mixed-bed IASC column, the proteins transferrin, haptoglobin and transthyretin were removed completely from a 50 μL serum sample. The gel in Fig. 3C is CB-stained, whereas the gel in figure 3D is silver-stained. In both experiments, an amount of 400–450 μg protein was recovered from the serum fraction not retained by the IASC columns. Applying an aliquot of 130 μg protein to 2-DE and staining the gel with CB, 620–670 serum protein spots were detected following image digitization (see Fig. 3C). Approximately 950 spots were detected in the silver-stained image of an equivalent fraction loading a 100 μg serum protein aliquot onto a 2-DE gel (Fig. 3D). In comparison, only *ca*. 220 protein spots were visualized in a CB-stained 2-DE gel applying 160 μg protein derived from whole serum (Fig. 4). No spot data are available for silver stained 2-DE gels applying whole serum. For all experiments, the same serum sample pooled from three normal healthy blood donors was applied. The apparent differences in 2-DE spot numbers, spot shapes and intensities, which result from distinct antibody matrix compositions in the mixed-bed IASC columns and, more significantly, from the applied gel-staining dyes, clearly demonstrate that variations in chromatographic, electrophoretic and gel-staining processes must be avoided in differential serum protein display studies.

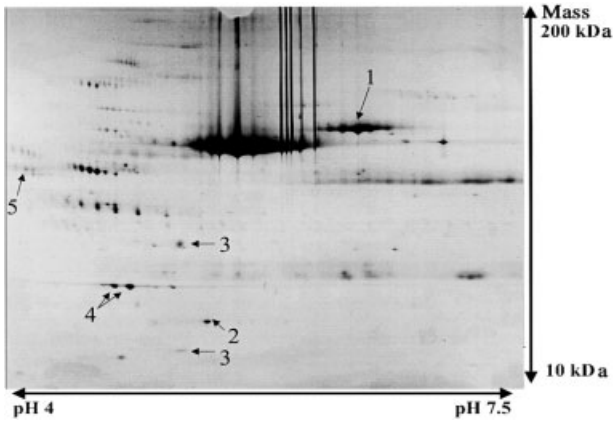

Figure 4. CB stained 2-DE protein pattern of whole serum. 2 μL serum equivalent to *ca.* 160 μg protein were diluted with 2-DE solubilization buffer and directly applied to 2-DE. The gel run parameters are described in the legend of Fig. 2. The indicated spots and spot trains are numbered as described in Fig. 3C.

In order to demonstrate the specificity of the protein subtraction by a mixed-bed IASC column, the affinity-bound protein fraction subsequently acid-eluted (peak 2, Fig. 3B) was collected. *Ca*. 750 μg protein was recovered in the 2-DE sample, a total amount lower than that theoretically present in 50 μL of human serum with respect to the following ten subtracted proteins: albumin (2.4 mg), IgG (0.8 mg), transferrin (0.2 mg), haptoglobin (0.2 mg), α-1-antitrypsin (0.2 mg), α-1-acid glycoprotein (< 0.1 mg), α-2-HS glycoprotein (< 0.1 mg), hemopexin (< 0.1 mg), transthyretin (< 0.1 mg) and antithrombin III (< 0.1 mg). The losses were likely caused by protein denaturation, precipitation and adsorption, while the sample was processed.

As evident in the gel image of Fig. 3E, many of the immunosubtracted serum proteins were displayed in their characteristic charge trains. Several of the protein spots were analyzed by MALDI-MS. The proteins expected to be present based on the antibody resin composition were identified by MALDI-MS. Moreover, all of the major CB-stained protein spots matched 2-DE positional locations known for the ten subtracted proteins based on their M_r, p*I* and charge trains. Other antibody-coupled columns described in Fig. 3 removed α-2-macroglobulin and IgM partially and all of the IgA present in 50 μL serum. Those with specificity for apolipoprotein A1 were found to be difficult to recycle, as elution of this lipoprotein after binding to an antibody matrix with all tested eluants was largely unsuccessful (see spots Fig. 3C and D). We also observed an unspecific loss of *ca*. 30% serum protein calculated from the ratio of recovered protein in the column-flowthrough fraction (peak 1, Fig. 3B, 400–450 μg) and the theoretical amount assuming that 85% of the serum protein was affinity-removed. This loss is apparently not linked to unspecific binding of serum proteins to immobilized antibodies (see gel, Fig. 3E). Rather, the lack of complete protein recovery appears to be caused by the LC process in general, because such protein losses have been observed as well applying serum to other hydrophilic chromatographic matrices.

The usefulness of the IASC procedure for proteomic applications is evident comparing the protein patterns in the 2-DE gels of whole serum (Fig. 4) and of immunoaffinity-subtracted serum. With the absence of several highly abundant proteins, numerous proteins of lower abundance in serum are made visible and amenable to quantitation and MS identification (MS data are not reported here). High concentrations of one single protein, such as albumin, in a 2-DE sample often lead to gel breaks and

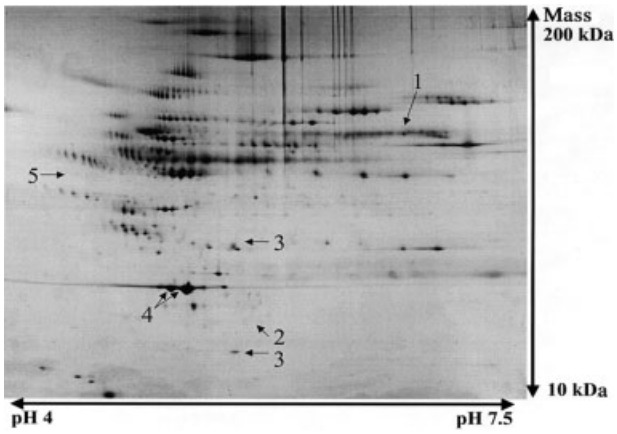

Figure 5. Plasma protein fraction after immunoaffinity subtraction of 10 proteins visualized in a CB stained 2-DE gel. The 2-DE gel run parameters are described in the legend of Fig. 2. 150 μg protein derived from an aliquot of a plasma fraction not retained by the mixed-bed IASC column described in Section 2.6 and 1.7 mL antibody-coupled columns specific for α-2-macrogloblin and fibrinogen was prepared for 2-DE. The indicated spots and spot trains are numbered as described in Fig. 3C.

aggravate spot streaking during IEF. Thus, its removal increased the success rate of well resolved first dimension IEF tube gel runs used in our ProGEx system. The immunoaffinity subtraction process was also applied to plasma removing highly abundant fibrinogen in addition to aforementioned serum proteins. Fibrinogen is nearly absent in serum. Therefore, anti-fibrinogen antibody-coupled resins were not pooled into IASC mixed-bed resins. To avoid fibrin formation in plasma during chromatography, 1 mM EDTA was added to buffer C. Applying the IASC procedure to plasma samples, 350–400 additional lower abundance protein spots in a CB-stained 2-DE gel were detected compared to whole plasma (Fig. 5).

3.4 Reproducibility of sample preparation for human serum using IASC

In order to differentially display proteins across many samples, high-throughput and reproducibility of sample preparations are of critical importance. The chromatographic columns used for IASC have POROS® bead matrices and were run in perfusion chromatography mode at high flow rates [43]. An immunoaffinity subtraction cycle for a method with one IASC column was completed in less than 15 min. The use of an autosampler increased the sample throughput and allowed processing up to 20 plasma samples *per* day *per* chromatography station.

The reproducibility of IASC was explored at different stages of serum sample preparation: (1) monitoring the integrated peak areas of the UV_{280} traces, which represent the IASC-unretained protein fractions; (2) measuring the protein concentration prior to protein sample lyophilization for 2-DE; (3) comparing the protein spot intensities observed for an array of 2-DE gel spots among two sample groups, one group consisting of gels derived from separately IASC-prepared serum samples, the other group consisting of gels derived from one serum sample pooled after immunoaffinity subtraction. The latter comparison factors in the variations of protein spot quantities solely based on the 2-DE gel performance. The reproducibility of IASC serum sample preparation was excellent looking at the coefficient of variation (CV) of UV_{280} peak areas. Applying 50 μL of serum and generating flow-through fractions for 12 IASC method runs, the CV for protein peak areas was calculated to be 3.5% (average over three experiments). The same samples and set of three experiments were applied to measure protein amounts after concentration and exchange into buffer H using the colorimetric bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA). With a value of 10.5%, the CV was slightly higher at this sample preparation step. Finally, a comprehensive experiment to calculate CVs was carried out on the level of protein spots separated by 2-DE. Serum fractions from both groups were solubilized in 2-DE buffer in 1:0.8 volume ratios (whole serum sample *vs*. 2-DE concentrate). Approximately 130 μg protein were loaded onto each gel. Following 2-DE, CB stained protein spots were quantitated and 50 well resolved spots were chosen to calculate the CVs comparing a set of 12 gels for each of the two study groups. The arithmetic mean of the 50 CVs for each of the groups was determined. It was 17% for the pooled serum sample group and 27% for the separately prepared serum sample group. Not surprisingly, some gel-to-gel variability was observed on the level of 2-DE protein spot quantitation (measured as an average CV value of 17%). Separate sample processing of serum implemented by IASC and prior to 2-DE (consisting of desalting, lyophilization, solubilization) added an additional 10% on average to the CVs for the selected protein spot groups. In a proteomic study, where the purpose is to differentially display serum or plasma proteins, significant protein spot abundance changes can typically be identified, if CV values are smaller than 30%.

4 Concluding remarks

Human plasma is the most important readily accessible clinical sample source for the investigation of disease processes and drug effects. While many assays are avail-

able to evaluate protein function and abundance for individual plasma protein targets, technologies for fast differential plasma protein surveys still are in a developmental phase. Technologies, which permit reproducible and parallel protein quantitation, are particularly needed to profile disease-associated changes in body fluid proteomes. Information on proteins of lower abundance in plasma is critical, because the highly abundant proteins and their roles in disease processes have been characterized already. We have developed an elegant quantitative strategy to accomplish the selective profiling of the less abundant proteins in serum and plasma. It is based on immunoaffinity subtraction of highly abundant plasma proteins and used in combination with 2-DE. Compared to recycable immunoaffinity methods and strategies published previously [31–35, 38, 41, 42], we have elevated the methodology from an analytical to a preparative scale, demonstrated the feasibility of generating high capacity multi-component pooled IASC columns, and set the stage to use this approach in combination with 2-DE for global profiling of lower abundance proteins in plasma and serum. At least ten of the most abundant proteins could be removed specifically, equivalent to *ca*. 85% of the total plasma proteome. Lower abundance proteins were recovered with good chromatographic sample-to-sample reproducibility. We have also applied the technology successfully to subtract major proteins from CSF and urine (not reported here).

The future challenges will include extending this subtraction technology to the 25–50 most abundant proteins in plasma and decreasing sample-to-sample variability, mostly introduced after IASC, when plasma protein samples are prepared for 2-DE. This will permit the detection of smaller quantitative protein abundance changes. Sources for purified plasma proteins and high quality polyclonal and monoclonal antibodies are currently limited. Alternative affinity reagents to antibodies have been evaluated such as matrix-immobilized triazine dyes (Cibachrom Blue) with affinity to albumin. Their specificity, however, is not satisfactory, as several other proteins in plasma are partially removed making reproducible quantitative plasma protein surveys difficult [36]. Our IASC approach also holds promise for protein profiling in body fluids omitting 2-DE technologies. It can easily be combined with other chromatographic protein fractionation methods and LC-MS/MS to detect and analyze proteins in plasma at even lower concentrations. The immunoaffinity subtraction technology in-house developed is a powerful addition to our ProGEx™ proteomics platform and strengthens our capability to discover clinically relevant protein markers in body fluids.

Received September 22, 2002

5 References

[1] O'Farrell, P. H., *J. Biol. Chem.* 1975, *250*, 4007–4021.

[2] Klose, J., *Humangenetik* 1975, *26*, 231–243.

[3] Scheele, A. G., *J. Biol. Chem.* 1975, *250*, 5375–5385.

[4] Anderson, N. G., Anderson, N. L., *Anal. Biochem.* 1978, *85*, 331–354.

[5] Anderson, N. L., Anderson, N. G., *Proc. Natl. Acad. Sci. USA* 1977, *74*, 5421–5425.

[6] Sanchez, J. C., Appel, R. D., Golaz, O., Pasquali, C. *et al.*, *Electrophoresis* 1995, *16*, 1131–1151.

[7] Herbert, H., Righetti, P. G., *Electrophoresis* 2000, *21*, 3639–3648.

[8] Richter, R., Schulz-Knappe, P., Schrader, M., Standker, L. *et al.*, *J. Chromatogr. B. Biomed. Sci. Appl.* 1999, *726*, 25–35.

[9] Herbert, B. R., Sanchez, J. C., Bini, L., In: Wilkens, M. R. *et al.* (Eds.), *Proteome Research*: *New Frontiers in Functional Genomics*, Springer Verlag, New York 1997, pp. 13–30.

[10] Anderson, N. G. Anderson, N. L., Tollaksen, S. L., *Clin. Chem.* 1979, *25*, 1199–1210.

[11] Spahr, C. S., Davis, M. T., McGinley, M. D., Robinson, J. B. *et al.*, *Proteomics* 2001, *1*, 93–107.

[12] Goldman, D., Merril, C. R., Ebert, M. H., *Clin. Chem.* 1980, *26*, 1317–1322.

[13] Yun, M., Wu, W., Hood, L., Harrington, M., *Electrophoresis* 1992, *13*, 1002–1013.

[14] Davidsson, P., Paulson, L., Hesse, C., Blennow, K., Nilsson, C. L., *Proteomics* 2001, *1*, 444–452.

[15] Doherty, N. S., Littman, B. H., Reilly, K., Swindell, A. C. *et al.*, *Electrophoresis* 1998, *19*, 355–363.

[16] Celis, J. E., Ostergaard, M., Rasmussen, H. H., http://biobase.dk/cgi-bin/celis.

[17] Rasmussen, H. H., Orntoft, T. F., Wolf, H., Celis, J. E., *J. Urol.* 1996, *155*, 2113–2119.

[18] Steel, L. F., Mattu, T. S., Mehta, A., Hebestreit, H. *et al.*, *Dis. Markers* 2001, *17*, 179–189.

[19] Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., Aebershold, R., *Proc. Natl. Acad. Sci. USA* 2000, *97*, 9390–9395.

[20] Zuo, X., Speicher, D. W., *Proteomics* 2002, *2*, 58–68.

[21] Goerg, A., Obermaier, C., Boguth, G., Weiss, W., *Electrophoresis* 1999, *4–5*, 712–717.

[22] Westbrook, J. A., Yan, J. X., Wait, R., Welson, S. Y., Dunn, M. J., *Electrophoresis* 2001, *22*, 2865–2871.

[23] Wall, D. B., Kachman, M. T., Gong, S., Hinderer, R. *et al.*, *Anal. Chem.* 2000, *72*, 1099–1111.

[24] Butt, A., Davison, M. D., Smith, G. J., Young, J. A. *et al.*, *Proteomics* 2001, *1*, 42–53.

[25] Badock, V., Steinhusen, U., Bommert, K., Otto, A., *Electrophoresis* 2001, *22*, 2856–2864.

[26] Petricoin, E. F., Ardekani, A. M., Hitt, B. A., Levine, P. J. *et al.*, *Lancet* 2002, *359*, 572–577.

[27] Verma, M., Wright, G. L., Hanash, S. M., Gopal-Srivastava, R., Srivastava, S., *Ann. NY Acad. Sci.* 2001, *945*, 103–115.

[28] Phillips, T. M., Dickens, B. F., *Affinity and Immunoaffinity Purification Techniques*, Eaton Publishing, BioTechniques Books Div, Natick, MA 2000.

[29] Winzor, D. J., *J. Biochem. Biophys. Methods* 2001, *49*, 99–121.

[30] Gersten, D. M., Marchalonis, J. J., *J. Immunol. Methods* 1978, *24*, 305–309.

[31] Schneider, C., Newman, R. A., Sutherland, D. R., Asser, A., Greaves, M. F., *J. Biol. Chem.* 1982, *257*, 10766–10769.

[32] Nakamura, K, Hahimoto, T., Kato, Y., *J. Chromatogr.* 1990, *510*, 101–113.

[33] Kim, H. O., Durance, T. D., Li-Chan, E. C., *Anal. Biochem.* 1999, *268*, 383–397.

[34] Phillips, T. M., Krum, J. M., *J. Chromatogr. B. Biomed. Sci. Appl.* 1998, *715*, 55–63.

[35] Wheatley, J. B., *J. Chromatogr.* 1992, *603*, 273–278.

[36] Lollo, B. A., Harvey, S., Liao, J., Stevens, A. C. *et al.*, *Electrophoresis* 1999, *20*, 854–859.

[37] Mantovaara, T., Pertoft, H., Porath, J., *Biotechnol. Appl. Biochem.* 1991, *13*, 371–379.

[38] Sisson, T. H., Castor, C. W., *J. Immunol. Methods* 1990, *127*, 215–220.

[39] Steiner, S., Gatlin, C. L., Lennon, J. L., McGrath, A. M. *et al.*, *Toxicol. Lett.* 2001, *120*, 369–377.

[40] Wheatley, J. B., *J. Chromatogr.* 1991, *548*, 243–253.

[41] McConnell, J. P., Anderson, D. J., *J. Chromatogr.* 1993, *615*, 63–75.

[42] Fu, A., Morris, J. C., Ford, G. C., Sreekumaran Nair, K., *Anal. Biochem.* 1997, *247*, 228–236.

[43] Afeyan, N. B., Gordon, N. F., Mazsaroff, I., Varady, L. *et al.*, *J. Chromatogr.* 1990, *519*, 1–29.