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Reducing protein concentration range of biological samples using solid-phase ligand libraries☆

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Abstract

The discovery of specific polypeptides of diagnostic relevance from a biological liquid is complicated by the overall vast number and the large concentration range of all polypeptides/proteins in the sample. Depletion or fractionation methodologies have been used for selectively removing abundant proteins; however, they failed to significantly enrich trace proteins. Here we expand upon a new method that allows the reduction of the protein concentration range within a complex mixture, like neat serum, through the simultaneous dilution of high abundance proteins and the concentration of low abundance ones in a single, simple step. This methodology utilizes solid-phase ligand libraries of large diversity. With a controlled sample-to-ligand ratio it is possible to modulate the relative concentration of proteins such that a large number of peptides or proteins that are normally not detectable by classical analytical methods become, easily detectable. Application of this method for reducing the dynamic range of unfractionated serum is specifically described along with treatment of other biological extracts. Analytical surface enhanced laser desorption/ionization mass spectrometry (SELDI-MS) technology and mono- and two-dimensional electrophoresis (1-DE and 2-DE) demonstrate the increase in the number of proteins detected. Examples linking this approach with additional fractionation methods demonstrate a further increase in the number of detectable species using either the so-called "top down" or "bottom up" approaches for proteomics analysis. By enabling the detection of a greater proportion of polypeptides/proteins within a sample, this method may contribute significantly towards the discovery of new biomarkers of diagnostic relevance.

Keywords: Proteomics; Ligand library; Selective capture; Fractionation; Mass spectrometry; Electrophoresis; Dynamic range

1. Introduction

In proteomic investigations, major obstacles to resolve are around the discovery of specific, disease-related peptide/protein species that are present in trace amounts among a large background of non-relevant and/or abundant proteins. The situation is particularly complex in higher eukaryote organisms due to the large number of genes and gene splice-variants that encode proteins, as well as the number and extent of post-translational modifications (PTM) such as cleavage, phosphorylation, glycosylation, lipidation, etc. that can impart unique functions on a particular gene product, depending on the nature of the PTM. To this complexity one has to further consider that proteins within the sample can be present over a large concentration dynamic range. For example, in human serum it is estimated that the dynamic range of protein concentration is in excess of 10 orders of magnitude [1], and that the 50 most abundant proteins represent about 99% of the total amount of protein mass but only less than 0.1% in number [2]. This situation renders the discovery of peptides/proteins of diagnostic or therapeutic importance challenging; as a consequence, sample preparation strategies must be specifically conceived and/or optimized to complement the chosen method of detection.

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Common methods used for proteome analysis include mono- and two-dimensional electrophoresis (1-DE, 2-DE), and mass spectrometry (MS); however, these methods have limitations due to the complex composition of samples [3]. In electrophoresis, for example, the protein bands/spot corresponding to the high abundance proteins can exhibit deformation and/or smearing during the electrophoretic separation due to protein overloading and thus obscure the detection of other proteins of similar mass and/or p*I*. Additionally, protein species below about 5 kDa or at the extreme p*I* ranges of the selected 2-DE gels are either lost during the separation or not resolved, and low abundance proteins of any mass and suitable p*I* range may not be visible because they fall below the sensitivity of the staining method. MS, both electrospray and laser desorption methods, can also suffer in analytical performance due to the nature and complexity of biological samples. To overcome many of these drawbacks, sample treatment strategies have been developed, ranging from simple clean-up methods to more complicated strategies of high-load 2-DE [4,5], prefractionation processes [6–9], abundant protein depletion [10–12], global digestion followed by MS analysis [13] and multidimensional chromatography followed by gel electrophoresis or MS analysis [14]. However, depending on the method used, specific cautions must be considered with respect to limited resin capacity, loss of low-abundance proteins during high-abundance protein depletion [15] or protein precipitation during sample treatment, inefficiencies in sample digestion and protein losses due to choices made in chromatographic separation modes.

In spite of these efforts, the ability to detect low abundant species still remains a critical challenge in deciphering complex proteomes and correlating proteome changes with metabolic events for diagnostic and therapeutic purposes. Recently the principle of a novel sample preparation approach that can decrease the protein concentration dynamic range without depletion has been described as it applies to a variety of proteomes [16,17]. This method is based on the selective adsorption of proteins on a solid phase combinatorial ligand library under capacity-limited binding conditions. In this paper the principle is briefly described, with additional emphasis on physicochemical parameters that are optimal for the detection of the maximum number of proteins from very complex mixtures.

2. Basis of the use of ligand libraries for the reduction of protein concentration differences

Solid phase affinity adsorption is a well-known chromatographic process for selectively capturing and concentrating a given protein. Its intrinsic limitation is the binding capacity of the sorbent; when the saturation is reached the excess of the protein in question cannot bind and is subsequently discarded in the flowthrough. Starting from this simple mechanism one can extend the phenomenon to a large number of different affinity ligands for a large number of different proteins. If such a diversity of affinity ligands is mixed together to form an affinity-ligand pool, and contacted with a diverse protein mixture, each unique affinity ligand beads within the pool will bind and concentrate its specific protein up to the point of ligand saturation and independently of all other affinity ligands and proteins. When the relative concentration from each species within the protein mixture forms a large dynamic range such that the high abundance proteins exceed the capacity and the low abundance proteins are below the capacity of their respective specific affinity ligand, the high abundance proteins will rapidly saturate their corresponding beads while low abundance ones will continue to adsorb as long as the sample is available. After removal of all proteins that are not bound, the composition of proteins retained by the beads will be defined by the presence of their specific affinity ligands, and the relative concentration of each retained protein species will be defined by the capacity and saturation degree of each of the affinity ligands and the relative starting concentration of each protein species.

This principle has been described using solid-phase peptide ligand libraries [16,17]. The library is generated using classical combinatorial synthesis methods, and is capable of producing tremendous ligand diversity where theoretically there is a ligand for every peptide and protein present in the starting material. For example, if the combinatorial synthesis for generating hexapeptide ligands is made using 20 amino acids, the total amount of ligands obtained is theoretically of 64 million, a number much larger than the expected number of different protein in biological samples. The use of such a highly diverse combinatorial library of affinity ligands under the described capacity limiting conditions results in a compression of the dynamic range of protein concentration (dilution of high-abundance proteins and concentration of low abundance proteins), while retaining representatives of all proteins within the mixture. Retained proteins can then be eluted in bulk or selectively from the affinity library using buffer modifiers such as ionic strength, pH, chaotropic agents or organic solvents with subsequent analysis by any number of analytical methods.

3. Materials and methods

3.1. Chemicals and biologicals

The solid-phase combinatorial hexapeptide library (Protein Equalizer™ beads) was supplied by Ciphergen Biosystems Inc., Fremont, CA; it was made using a previously described "split, couple and recombine" method [18,19]. By incorporating 20 different amino acids in the synthesis, the theoretical number of different ligand structures was 20^6 or 64 million. Each bead of 65 μm average diameter carried about 50 pmol of hexapeptide. Urea, thiourea, tributylphosphine (TBP), glycine, sodium and lithium dodecyl sulfate (SDS and LDS), and 3-[3-cholamidopropyl dimethylammonio]-1-propansulfonate (CHAPS) were obtained from Fluka Chemie (Buchs, Switzerland). Ethanol, methanol, glycerol, sodium hydroxide, hydrochloric acid, acetone, and acetic acid were from Merck (Darmstadt, Germany). Bromophenol blue, agarose and carrier ampholytes (Pharmalyte) were from Pharmacia-LKB (Uppsala, Sweden). Linear Immobiline dry strips (pH gradient 3–10, 7 cm long) were from Bio-Rad Laboratories (Hercules, CA, USA). Protein molecular weight standards as well as frozen human serum were from Sigma Chemicals, St. Louis, MO.

ProteinChip® Arrays with different chromatographic surfaces as well as Q-HyperD anion exchange resin were from Ciphergen Biosystems Inc., Fremont CA and Biosepra-Pall, Cergy-Pontoise, France, respectively.

3.2. Binding capacity determinations

Total protein binding was measured using human serum proteins. 0.5 mL of ligand library beads were packed in a 6 mm diameter column and equilibrated either in PBS (10 mM phosphate, pH 7.4 containing 0.15 M sodium chloride) or in 25 mM phosphate, pH 7.0 depending on the sample to be loaded. The column was washed extensively with equilibrium buffer, and then oversaturated with serum proteins. Additionally, the influence of pH on bead binding capacity was evaluated using 25 mM acetate buffer, pH 5.0 (including 150 mM NaCl) and 25 mM Tris–HCl buffer, pH 9.0 (including 150 mM NaCl).

Columns were then washed extensively with the equilibration buffer and the wash continuously monitored by UV absorbance (214 nm) until the absorbance returned to baseline. Finally proteins were desorbed using a solution of 9 M urea–2% CHAPS (adjusted to pH 3.8 using concentrated acetic acid). The total protein concentration of the eluted proteins was quantified by a BCA™ kit (Pierce, USA) and used for the determination of the binding capacity of the beads.

3.3. Elution studies

An essential part of the method involves recovering the captured proteins from the bead library; thus several elution strategies were evaluated. The main criteria for the selection were the elution buffers ability to effectively desorb the captured proteins and its compatibility with subsequent protein detection techniques. Glass columns of 6 mm diameter were packed with 0.5 mL of the combinatorial bead library, equilibrated in PBS, and then contacted with 5 mL human serum in PBS. After sufficient washing to remove unbound protein, several different elution buffers were evaluated, including: 9 M urea, pH 3.8 (pH adjusted with acetic acid); 9 M urea, pH 11.0 (pH adjusted with ammonia); 9 M urea–2% CHAPS, pH 3.8 (pH adjusted with acetic acid); 9 M urea–2% CHAPS, pH 11.0 (pH adjusted with ammonia); 2.2 M thiourea–7.7 M urea–4% CHAPS (TUC buffer); 6 M guanidine–HCl, pH 6; and a water solution containing 33.3% isopropyl alcohol–16.7% acetonitrile–0.5% trifluoroacetic acid.

3.4. Sample processing with combinatorial ligand library

Human serum was filtered through a 0.8 μm filter and then mixed in batch mode with a given volume of solid phase ligand library that had been equilibrated with a buffer of appropriate pH and ionic strength according to the type of experiment and incubated for 2 h. The ratio of the volume of solid phase ligand library to the volume of serum sample was also evaluated and ranged from 1:1 to 1:150. A human urinary protein sample was also processed with the same ligand library. Briefly after urine concentration, proteins were diafiltered and lyophilized; then 150 mg of the crude mixture was solubilized in 42 mL of neutral phosphate buffer and processed using 1 mL of peptide ligand beads. Proteins captured by the solid phase ligand library were either desorbed all together using 6 M guanidine-HCl, pH 6 or sequentially using a combination of buffers, including 2.2 M thiourea–7.7 M urea–4% CHAPS (TUC), followed by 9 M urea–2% CHAPS, pH 3.8, then subjected to electrophoresis and MS analysis.

3.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis of protein fractions was performed by using 10-well pre-cast 1 mm thick 16% Bis–Tris polyacrylamide gel plates (Invitrogen, Carlsbad, CA). Samples of appropriate protein concentration were diluted two-fold in sample buffer. Thirty microlitres of diluted sample was loaded per lane and electrophoresis migration was performed at 150 V for 90 min. Coomassie staining and de-staining were achieved using the method described by the supplier of reagents (Invitrogen, Carlsbad, CA).

3.6. Two-dimensional electrophoresis

Desalted proteins by precipitation in a cold mixture of acetone and methanol (v/v ratio of 8:1) were solubilized in the 2-DE sample buffer, containing 7 M urea–2 M thiourea–3% CHAPS–0.5% Pharmalyte pH 3–10 and bromophenol blue. Volumes of 150 μL of the obtained protein solution (control sample), or of the fractions obtained via solid phase ligand library, were then used to rehydrate 7 cm long IPG 3–10 strips for 4 h. Isoelectric focusing was carried out with a low initial voltage and then by applying a voltage gradient up to 5000 V, with a limiting current of 50 μA/strip. The total product time × voltage applied was 25,000 V h for each strip, and the temperature was set at 20 °C. For the second dimension, the IPG strips were equilibrated for 26 min in a solution of 6 M urea–2% SDS–20% glycerol–375 mM Tris–HCl, pH 8.8, under gentle agitation. The IPG strips were then cemented with 0.5% agarose on a 12% SDS-PAGE in the cathode buffer (192 mM glycine, 0.1% SDS and Tris to pH 8.3). The anodic buffer was a solution of 375 mM Tris–HCl, pH 8.8. The electrophoretic run was performed by setting a current of 5 mA for each gel slab for 1 h, then 10 mA/gel for 1 h and finally 20 mA/gel until the end of the run. During the entire run the temperature was set at 11 °C. At the end of the run the gels were stained overnight with colloidal Coomassie Blue G 250. Destaining was performed in 5% acetic acid for 2 h.

3.7. Fractionation by anion exchange chromatography

A disposable Wizard column was filled with 125 μL of Q-HyperD anion exchange resin and equilibrated in 50 mM Tris–HCl pH 9.0 containing 1 M urea and 0.22% CHAPS (loading buffer). Once equilibrated, 50 μL of serum was diluted into 90 μL column equilibrium buffer, and loaded onto the column at a flow rate of 0.01 mL/min. The first 100 μL were discarded

before collection of flow-through (250 μL). Elution of protein fractions was obtained by lowering the pH in a stepwise mode. The sequence of buffers used was as follows: 50 mM HEPES, pH 7; 50 mM acetate, pH 5; 50 mM acetate, pH 4; 50 mM citrate, pH 3 and finally by a hydro-organic solution containing 33.3% isopropyl alcohol, 16.7% acetonitrile, 50% 0.5% trifluoroacetic acid.

3.8. SELDI MS analysis

Each spot of a ProteinChip® Array (Ciphergen Biosystems Inc., Fremont, CA; named as biochip throughout the text) was equilibrated twice with 5 μL of the indicated array-specific binding buffer for 5 min according to the manufacturers instructions. Each spot was then loaded with 6 μL of the sample previously six-fold diluted in the array binding buffer. After an incubation period of 30 min with constant shaking, the sample was carefully removed and each spot was washed three times with 5 μL of the binding buffer for 5 min for eliminating non-adsorbed proteins, followed by a quick rinse with deionized water.

All surfaces were air-dried followed by two applications of a saturated solution of sinapinic acid in a mixture of 50% acetonitrile in water containing 0.5% aqueous trifluoroacetic acid; 1 μL of this solution per application with air drying in between. Biochips were then analyzed by laser desorption time-of-flight mass spectrometry using a linear reader (Ciphergen Biosystems, Fremont, USA) in positive ion mode (SELDI-TOF-MS), with an ion acceleration potential of 20 kV and a detector voltage of 2.8 kV. The molecular weight range investigated by MS was from 1 to 300 kDa. Time-lag focusing was optimized at either 5 or 70 kDa for low and high mass ranges, respectively.

Processing of data obtained included baseline subtraction and external calibration using a mixture of known peptide and protein calibrants. Peak detection (s/n > 3) and peak clustering was performed automatically using Ciphergen ProteinChip® Software 3.2. Biochips used throughout this study were CM10 (cation exchange), Q10 (anion exchange), H50 (hydrophobic surface) and IMAC30 (metal ion chelating surface) loaded with Cu^{2+} ions.

4. Results and discussion

The described solid phase ligand library has previously been characterized as a whole [19,20] and due to the method of synthesis, the ligand density is expected to be consistent from bead to bead. Analytical determinations indicated that the ligand density is close to 50–60 μmol of ligand/mL of beads. This is logical when considering that the linker density present on the beads prior to library synthesis was approximately 80 μmol/mL of swollen beads. The bulk bead library was also characterized by a pH titration curve and closely resembles that of a protein mixture in solution; with expected extreme p*K* inflexion points and a large central buffering zone (data not shown).

The binding capacity for serum proteins, measured after incubation with a large excess of serum in a column binding mode, was determined to be 12.5 mg protein/mL beads in physiological buffer (PBS). This binding capacity appeared, however, dependent on the ionic strength and pH of the binding buffer. For example, the binding capacity for human serum proteins increased to 19 mg protein/mL beads in neutral phosphate buffer in the absence of sodium chloride. This phenomenon is probably due to the presence of ionic charges from the ligand library and could be classified as non specific; it was thus decided for all following experiments to use a neutral buffer containing a physiological concentration of sodium chloride (PBS). Using buffers of similar ionic strength (all buffers containing 150 mM NaCl), the binding capacity/mL beads appeared to be negatively correlated with pH; at pH 5.0, 7.4 and 9.0 the binding capacity for serum protein was determined to be 18.6, 12.6 and 9.0 mg/mL, respectively. The modification of the binding capacity may be due to the variation of the affinity constant as a function of pH, as one would predict and repeatedly reported in affinity chromatography techniques.

SDS-PAGE analysis of eluates as shown in Fig. 1 reveal that protein pattern is mostly the same but some species present at different concentrations as the pH of incubation is changed.

Protein harvesting from the ligand library is an important aspect of the described technology. To generate a comprehensive analysis of the processed sample and preserve the differential protein concentrations between multiple samples, it was essential to develop highly efficient desorption methods. In this respect, a variety of elution solutions have been evaluated to maximize protein recovery from the library. The most effective elution methods included 9 M urea containing 2% CHAPS, pH 3.5 and 6 M guanidine–HCl, pH 6. These elution methods were subsequently used in all experiments, except where specifically indicated.

The premise of this work involves a technology that simultaneously dilutes high-abundance proteins through a solid phase saturation phenomenon and enriches low-abundance proteins through solid phase concentration. When a complete representation of the ligand library diversity is contacted with a sample, the ability to concentrate low-abundance proteins will be necessarily dependent upon the overall volume of sample contacted with the beads. As this volume increases, more low-abundance species are available to concentrate onto their specific hexapeptide ligand and at a critical sample:bead ratio, a quantity will be present that exceeds the lower limit of detection of the selected analytical method. Experiments done to demonstrate this property involved contacting a fixed volume of bead library (1 mL) with increasing volumes of human serum through the ranges of 1:1, 10:1, 50:1 and 150:1 sample:bead volumes. After subsequent elution and detection by SELDI-MS, a difference in protein abundance is demonstrated as the sample:bead ratio increased (Fig. 2). Contrary to regular MS probes, ProteinChip arrays have chromatographic-like interacting surfaces on which proteins are adsorbed proportionally to the loading up to the saturation of the binding capacity. In the present set of experiments protein loading was below the saturation and therefore the signal proportional to the protein loaded and captured. Using a same level of detection intensity a signal was detected proportional to the sample:bead ratio. With a serum:bead ratio of 150:1, the greatest number of peaks and the greatest peak intensities were observed. This result was also confirmed by using SDS-PAGE (data not

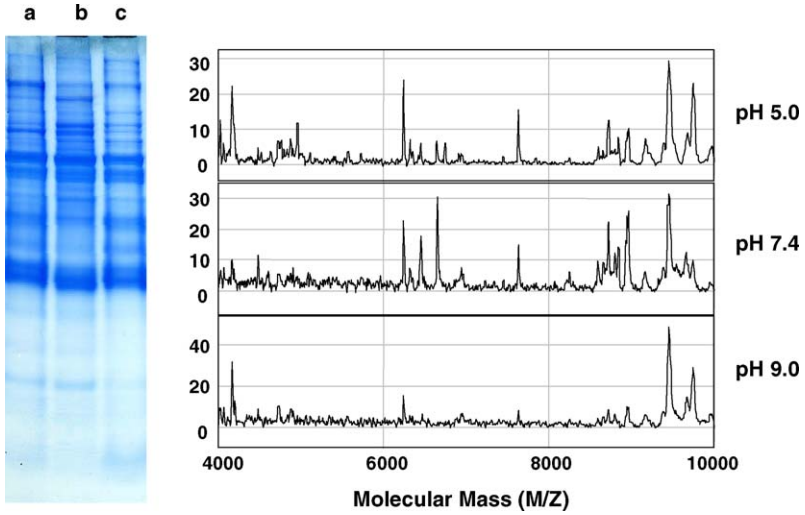

Fig. 1. Analysis of serum proteins recovered from ligand library beads following an incubation at pH 5.0, 7.4 and 9.0. On the left are SDS-PAGE results under reduced conditions; lanes "a", "b" and "c" are respectively proteins recovered after pH 5.0, 7.4 and 9.0. On the right are SELDI MS data of the same samples when profiled using a ProteinChip Array with a quaternary amino polymer coated on the surface (Q10 Array).

shown). The increase of specific low abundance protein concentration has been unambiguously demonstrated by ELISA assays for two low abundance serum proteins: TGFα and VEGF (data not shown). Their detectability was increased respectively of at least a factor of 5 and a factor of 70 while high abundance proteins like IgG decreased in their concentration of at least 10 times.

Due to the molecular complexity of hexapeptides, the ability of a protein to dock to a given ligand is dependent on the complementarity between the secondary or tertiary structure of the protein epitope and the ligand, involving ionic interactions, hydrogen bonding and hydrophobic associations. Disrupting these interactions through the use of denaturants and/or ion, pH or hydrophobic modifiers represents the most direct method of effective recovery of the captured proteins from the bead library [17].

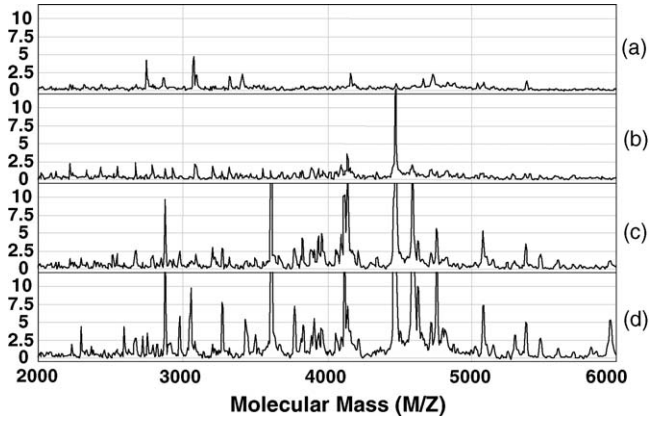

Fig. 2. Effect of 'sample volume' to 'beads volume' ratio on the detection of proteins from human serum. The volume of ligand library was fixed at 1 mL while the serum volume was varied across 1, 10, 50 and 150 mL ("a" to "d", respectively). After protein capture and elution, analysis was performed using ProteinChip Arrays coated with IMAC-Cu^{2+}. The same amount of protein was loaded on the chip surface. Detection was made using the same sensitivity for all samples.

Hence desorption of protein from these libraries can be accomplished in either a single or a sequential process using solutions capable to target one or more types of interaction. Sequential elutions may represent some advantages over a single elution when considering the subsequent analytical detection method. In general, the theoretical complexity of the captured proteome is not different from what it was in the original sample. However, because more of the protein members are within the lower limit of detection of the analytical method, the 'practical' complexity does increase and the separation efficiency of the analytical method must be sufficiently capable. Although single-step elutions can be sufficiently effective for simple samples, when complex biological extracts such as serum are processed, the reduction of sample complexity using conventional secondary fractionation methods shows benefits. This is unambiguously the case with serum proteins eluted from the bead library and secondarily fractionated via an anion exchange chromatographic column (Fig. 3). After binding onto the column, the sample was fractionated using a step-wise pH gradient and collected fractions then analyzed by SELDI-MS using a complementary cation exchanger (CM10) biochip. Comparing the processed and fractionated serum sample with native fractionated sample under similar conditions the total number of detectable proteins significantly increased (from 714 to 1029 non-redundant proteins of mass between 2000 and 150,000 Da).

Alternatively, sample fractionation can be accomplished directly from the bead library by the use of a series of sequential elution buffers. As described elsewhere [17], an increase of ionic strength in a first elution buffer, followed by an increase in hydrophobicity in a second elution buffer yields two large unique populations of proteins. A number of possible combinations of elution buffers can be used to desorb proteins sequentially from the bead library, thus simplifying the protein composition within each of the selected elution buffers.

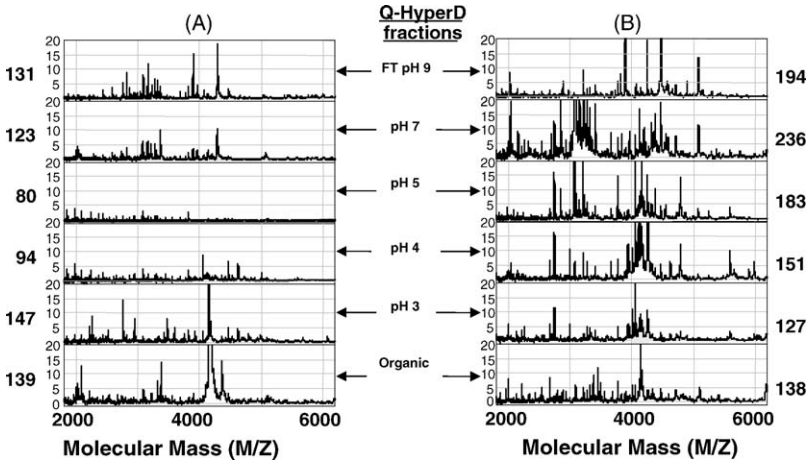

Fig. 3. SELDI-MS analysis of neat serum fractionated by anion exchange chromatography on Q-HyperD (panel A) and serum proteins first enriched for using the Protein Equalizer bead libraries followed by anion exchange chromatography (panel B). After binding of sample, either neat serum or serum processed using the bead library, to the Q-HyperD chromatographic resin at pH 9, proteins were subsequently desorbed by lowering the pH in a stepwise manner from pH 9 to pH3, and finally remaining proteins stripped out by a hydro-organic eluent mixture. Protein analysis was performed using a weak cation exchange (CM) ProteinChip Array. Numbers at the extreme left and right represent the peak count for each fraction.

As it has been described previously [21] the elution sequence involving thiourea–urea–CHAPS buffer followed by acidic urea solution is used here for serum proteins. Sequentially eluted fractions of processed serum on the ligand library analyzed by two-dimensional electrophoresis (see Fig. 4) demonstrate that the number of detectable spots is considerably increased as comparing to either non-treated sample or treated sample with a single elution methodology. The number of protein spots accounted from native serum was 115 while after treatment with bead ligand library 305 proteins were found in the first eluate and 252 in the second eluate. An additional important observation is the low level of redundancy between fractions eluted sequentially, suggesting that the mechanisms of extraction between the two methods are complementary.

A similar strategy has been adopted for the analysis of urine proteins. First urine proteins have been concentrated and then treated with the ligand library. Fig. 5 represents the chromatographic elution profile of urine proteins first captured by the beads, and then eluted sequentially using thiourea–urea–CHAPS buffer followed by urea acidified with acetic acid. Protein desorption for each of the elution methods was considered complete when the UV trace returned to baseline. As with serum proteins, the urine protein composition within each of the two recovered samples was generally unique from one-and-another, indicating the mechanism of elution for each of the methods was complementary. In addition to the typical electrophoresis and SELDI-MS analytical methods of protein detection, aliquots of eluted protein have now also been subjected to Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) [22]. This demonstrated for the first time that the number of proteins directly identifiable from a biological sample after enrichment using the ligand library increased from about 130 proteins to

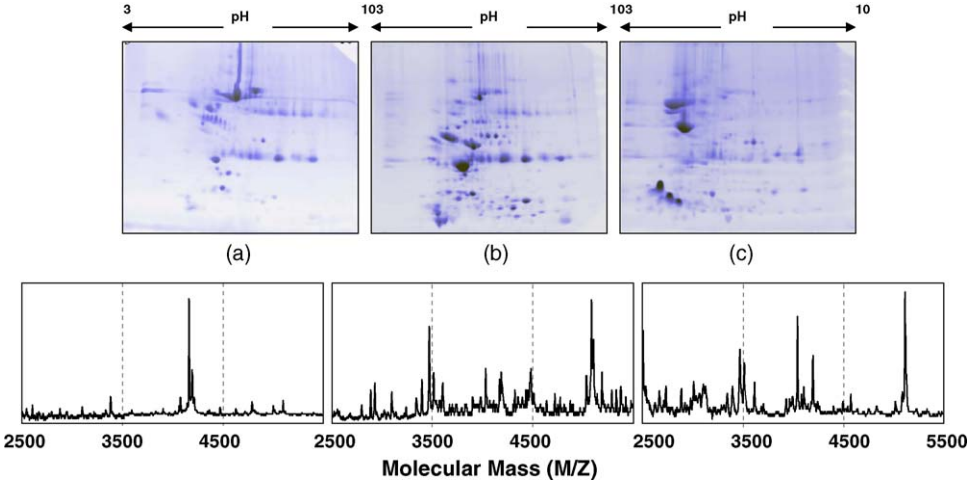

Fig. 4. Analysis of serum proteins by two-dimensional electrophoresis (upper panel) and SELDI MS (lower panel) before (“a”) and after sequential elution from ligand library beads (“b” and “c”) using respectively thiourea–urea–CHAPS and acidic urea desorption agents. 2-DE analysis shows the differences in the composition of proteins of relatively large masses while SELDI MS relates to low masses up to 7 kDa. This latter analysis was done using a cationic (Q10) ProteinChip Array. 2-DE analysis addresses medium and large molecular masses; MS analysis was focused on low molecular mass species not visible in 2-DE.

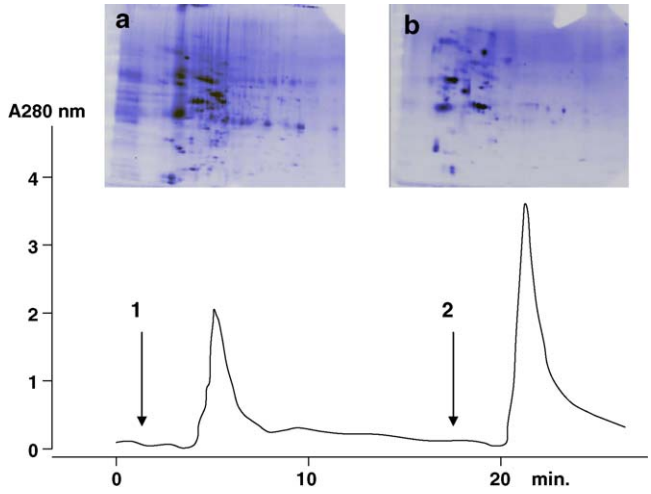

Fig. 5. Elution profile of captured urine proteins using ligand library beads. Protein desorption was made using a sequence of two eluents: TUC buffer (arrow "1") and 9 M urea acidified to pH 3.5 with acetic acid (arrow "2"). Desorbed fractions were analyzed by 2-DE, SELDI MS and separated proteins analyzed by FT-ICR [21]. Inserts "a" and "b" represents, respectively, 2-DE analysis of elution 1 and elution 2.

more than 412 proteins [22]. In both cases the numbers referred to unique gene products, not to isoforms of a more limited number of true species, as customarily seen in 2-DE maps, where the number of spots detected can be related to only 1/6 to 1/10 of unique gene products.

The described method of sample treatment for increasing the detection of low abundance proteins by means of the overloading principle well-known in chromatography, associated with an extremely large number of affinity-like ligands is now of being extensively applied to a variety of complex samples. Beyond the above described applications, this method has now been demonstrated to be useful in cell extracts such as *E. coli*, cytosol, or cell supernatants such as culture of lymphocytes [17] and other cells such as Guinea pig macrophages.

One of the most important differentiating aspects of the described method is its ability to concomitantly reduce the concentration of high abundance species while concentrating proteins that are present in trace amount. This feature is clearly evidenced when the volume of sample is progressively increased until the saturation of all beaded ligands. Currently the first aspect – reduction of high abundance proteins – is only partially resolved using the so-called depletion methods [10,12,15,23,24]; in fact, depletion of abundant species frequently results in a removal of associated proteins, thus eliminating them from subsequent analysis. The use of highly specific antibodies has been proposed [25]; however, the resulting solid-phase sorbents have a limited binding capacity and their preparation is contingent upon the availability of antibodies against the targeted protein. Immunosorbents suffer additionally from their high specificity, often resulting in a reagent designed for one single type of sample, e.g. human serum, but not suitable for another sample type, e.g. mouse or rat sera.

The second aspect of the described approach – the concentration of low abundance proteins – is not resolved by the depletion methods. In practice, the sample dilution resulting from the depletion operation can even aggravate the situation by lowering the concentration of analytes below the limit of detection of the analytical method used. Enrichment of low-abundance species by other means such as concentration, precipitation or lyophilization, is possible; nevertheless they are time-consuming and during these processes a number proteins may be lost. When comparing the described method and depletion procedures using various solid phase materials [22] it appears that the number of species found by either MS and/or SDS-PAGE were always larger (data not shown).

As a summary it is believed that the process described herein as a mean to detect low abundance proteins from very complex mixtures is particularly attractive for its effectiveness and simplicity (single easy step). The identified limits of this process, as reported earlier [22], include situations where (i) the ligand library diversity is not always sufficient to comprise ligands for all proteins present within the sample, or (ii) when the dissociation constant has a value above the initial concentration of the proteins within the sample, or (iii) when an insufficient volume of the sample is available as to provide enough analyte to concentrate beyond the lower limit of detection.

The described method appears as an appropriate tool for reaching a portion of proteome that is not easily visible by standard methods and allows making more complete protein repertoires starting from a large variety of biological fluids or extracts. In association or not with fractionation methods it allows a better and/or earlier detection of possible biomarkers of pathological relevance; investigations in this domain are in progress.

5. Conclusions

The described method of reduction of protein concentration difference in biological extracts is very straightforward, rapid, and applicable to a wide variety of starting materials. Hence it significantly improves sample handling and thus facilitating the construction of protein repertoires and novel biomarker detection.

The use of a combinatorial library in the reduction of protein concentration difference may represent a significant supplement to current fractionation methods and is clearly usable as a valuable alternative to depletion approaches, including situations when the biological extracts are of non human origin. The progressive increase in detectable species when using larger sample:bead ratios suggests that very rare proteins, for example proteins predicted by gene or mRNA sequence but previously undetected are now able to be detected [17,21].

Used under the simplest of conditions (adsorption followed by a single elution) or with sequential desorption or in association with other fractionating methods, this technique should represent a powerful mean for new discoveries in proteomic investigations. It will also be a complement to the detection of protein interaction when partners are of very low abundance.

The described approach, with or without additional fractionation methods, will significantly improve the early detection of biomarkers of biological and pathological relevance that are currently unavailable.

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