

Chapter 2

Solid-Phase Fractionation Strategies Applied to Proteomics Investigations

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Abstract

Methods for protein fractionation in the proteomics investigation field are relatively numerous. They apply to the prefractionation of the sample to obtain less complex protein mixtures for an easier analysis; they are also used as a means to evidence specific proteins or protein classes otherwise impossible to detect. They involve depletion of high-abundance proteins suppressing the signal of dilute species; they are also capable to enhance the detectability of low-abundance species while concomitantly decreasing the concentration of abundant proteins such as albumin in serum and hemoglobin in red blood cell lysates. Fractionation of proteomes is also used for the isolation of targeted species that are selected for their different expression under certain pathological conditions and that are detected by mass spectrometry. Two unconventional methods of large interest in proteomics due to the low level of protein redundancy between fractions are also reported.

All these methods are reviewed and detailed method given to allow specialists of proteomics investigation to access selected separation methods generally dispersed on different technical reviews or books.

Key words: Protein fractionation, Chromatography, Depletion, Concentration range, Identification, Proteomics

1. Introduction

The very large complexity of proteomes in terms of protein diversity and concentration differences is not any longer to be demonstrated. If we limit the analysis to human proteome, already in 2002, Anderson underlined the very large diversity and an extremely wide dynamic concentration range (1). With the expression of a large number of genes, splicing proteins and stitching different portions together, then cutting out amino acids sequences from the melted pieces and making a number of posttranslational modifications, the capability of living cells to make protein species is extremely large.

In this very intricate situation, it is clear that an obvious way to analyze the content of a proteome is the fractionation into discrete groups for individual analysis. A difficulty that is encountered is the extremely large difference in concentration of individual proteins that can range up to 12 and even more orders of magnitude (1) with extreme examples where one protein dominates very largely, such as hemoglobin in red blood cells representing about 98.5% of total protein mass. As a consequence of such differences in protein concentration, fractionation represents a hard task. This is the reason why sample pretreatments such as removal of proteins of high abundance (2–4) prior fractionation or reduction of the dynamic concentration range (5–8) are proposed methods that encounter a great success.

Fractionation gives access to many more proteins, including some dilute ones as a consequence of the concentration effect due to solid-phase adsorption. A number of reviews have been published on the theme of protein prefractionation using a variety of approaches; a recent presentation of prefractionation methods has been made by Herbert et al. (9). Prefractionation tools comprise not only all possible chromatographic and electrophoretic methods (especially those based on isoelectric focusing) but also organelle prefractionation (10–12).

In spite of numerous investigational approaches and hundreds of published reports, major limitations still exist to getting access to whole proteomes. Highly hydrophobic proteins such as membrane proteins cannot be properly solubilized and consequently are not easily analyzed and/or identified. Strongly alkaline proteins are also poorly represented using classical two-dimensional electrophoresis (13). Moreover, posttranslational modifications and especially glycosylations are still very difficult to analyze.

The centrifugal cell-fractionation method is well-ingrained since its initial development (14). Sub-cellular organelles are isolated in reasonably pure form such as mitochondria, nuclei, lysosomes, microbodies, synaptosomes, and the like. This is the most direct and efficient method for enrichment of the desired protein fractions as they are part of specific substructures of the cells. This fractionation technique is, however, challenged by other techniques such as electrical and solid-phase based separations methods.

Preparative fractionation methods based on differential migration under an electrical field cover a wide range of technologies, the most popular being free-flow fractionation (15), multicompartiment isoelectric focusing (16, 17) and the so-called Off-Gel fractionation (18). While electrophoretic methodologies, in all of their variegated aspects, may represent a high potential especially for their efficiency, they are dependent on the use of samples with preestablished low ionic strength and, for pI-based separation, they require an added carrier ampholine that is problematic for further mass spectrometry analytical determinations.

This document focuses in particular on solid-phase-based separation techniques involving in particular beaded material such as a variety of chromatographic fractionation methods. Various types of protein chromatography are currently used in proteome investigation; they are based on ion-exchange effect (19, 20), (cation and anion exchange), hydrophobic interactions (21), chromatofocusing (22), molecular sieving (23), and a variety of affinity or affinity-like interactions. Chromatographic fractionation gives access to many more proteins, including some dilute ones as a consequence of the concentration effect due to solid-phase adsorption. A number of reviews have been published on the theme of protein prefractionation using a variety of chromatographic approaches (24–28); an extensive description of prefractionation methods has been made by Herbert et al. (9) and more recently by Jmeian et al. (29). However, chromatographic approaches much in vogue today regard the capture of classes of proteomes based on their structure or function, such as the specific seizure of the glycoproteome via lectin columns (30–33) or the capture of the phosphoproteome via chelated metal ions (Fe^{3+} , Ga^{3+} , Ti^{4+} , Zr^{4+}) on solid phases (34).

2. Materials

2.1. Sample Pretreatment

2.1.1. High-Abundance Protein Depletion

1. Immunosorbents capable of removing 6 or 12 different high-abundance proteins from human serum are available from various vendors (e.g., Agilent, Palo Alto, CA, Sigma-Aldrich, Saint Louis, MO or Beckman Coulter Fullerton CA) with the capacity to treat 20–25 μL of human serum per run.
2. Current buffers used in biochemistry are needed, including the following:
 - (a) 10 mM Tris-HCl, 150 mM sodium chloride, pH 7.4 for dilution and washing.
 - (b) 100 mM glycine-HCl, pH 2.5 for stripping.
 - (c) 100 mM Tris-HCl, pH 8 for neutralization.

2.1.2. Reduction of Dynamic Protein Concentration Range

Three main ingredients are necessary for this approach. Elution can be performed as a single step or in multiple steps to further fractionate the eluate.

1. The solid-phase library (ProteoMiner beads, Bio-Rad, Hercules, CA), a washing buffer (phosphate-buffered saline, PBS).
2. Elution buffers
 - (a) For single-step elution: 9 M urea containing 5% acetic acid and 2% CHAPS.

(b) For multistep elution, several elution buffers are used sequentially. Two main options are available:

- Option 1:
 - 1 M sodium chloride in 20 mM Hepes.
 - 200 mM glycine-HCl pH 2.4.
 - 60% ethylene glycol in water.
 - Hydro-organic mixture composed of 33.3% isopropanol, 16.7% acetonitrile and 0.1% TFA in DI water.
- Option 2:
 - 1 M sodium chloride.
 - 7 M urea, 2 M thiourea, 2% CHAPS.
 - 9 M urea containing 50 mM citric acid and 2% CHAPS.

3. Protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO).

*2.1.3. Classical
Chromatography
Separation Using
Fractionated Elution*

1. Solid-phase media such as ion exchangers, gel-filtration media, hydrophobic resins and affinity-based sorbents can be obtained from current manufacturers of chromatographic materials such as Bio-Rad Laboratories (Hercules, CA), General Electric (Uppsala, Sweden), or Biosepra (Cergy, France).
2. Chromatographic columns of various dimensions are obtained from solid-phase media suppliers.
3. Buffers used for protein adsorption are made of Tris-HCl, sodium acetate-acetic acid, phosphates, or “good” buffers. The pH and ionic strength are dependent from the type of chromatographic separations:
 - (a) For cation exchange buffer, pH is between 4 and 6 and ionic strength is relatively low (10–25 mM).
 - (b) For anion exchange buffer, pH is between 7.5 and 9 and ionic strength is relatively low (10–25 mM).
 - (c) For gel filtration, the buffer is generally neutral and ionic strength corresponding to 100 mM sodium chloride.
 - (d) For hydrophobic chromatography, high concentrations of lyotropic salts (such as 0.8 M ammonium sulfate) are added to the adsorption buffer at neutral pH.
 - (e) Affinity-based separations are most generally started with physiological buffers.
4. Protein elution (except for gel filtration that is an isocratic chromatography) is obtained by:
 - (a) For ion exchangers: fractionated desorption by increasing the ionic strength of buffer.
 - (b) For hydrophobic separations: decreasing the concentration of lyotropic salts.

- (c) For affinity chromatography, elute using either displacements with specific or nonspecific agents or by simply using acidic pH (200 mM glycine-HCl, pH 2.4 or 100–200 mM acetic acid).

2.1.4. Fractionation with Stacked Sorbents and Single Buffer

1. Chromatography media can be obtained from specialized manufacturers such as Bio-Rad Laboratories (Hercules, CA), General Electric (Uppsala, Sweden), Biosepra, (Cergy, France), or Sigma-Aldrich (Saint Louis, MO).
2. Columns are selected from minicolumns that can be superimposed or any other practical device having systems connectable each other such as luer-lock capabilities between the upper column and the lower column.
3. The first buffer used is generally a Tris-HCl buffer. Elution buffer is any kind of stripping solution that is compatible with downstream analytical methods. Good eluting agents include the following:
 - (a) 9 M urea containing 2% CHAPS and 2% ammonium hydroxide or
 - (b) A mixture composed of 20% ammonium hydroxide (8 volumes)–distilled water (72 volumes)–acetonitrile (6.6 volumes) and isopropanol (13.4 volumes).

2.2. *pI* Separation Groups by Solid-Phase Buffers

1. Solid-State Buffers are made as described by Fortis et al. (35).
2. Ion exchangers are selected from those proposed by current producers such as Bio-Rad Laboratories (Hercules CA), General Electric (Uppsala, Sweden), or Biosepra (Cergy, France).
3. Columns are selected from minicolumns that can be superimposed or any other practical device having systems connectable each other such as luer-lock capabilities between the upper column and the lower column. Small fixed columns are from Promega (Madison, WI) or Omnifit (Cambridge, UK).
4. Urea.
5. Potassium chloride.
6. Sodium chloride.
7. Tris (hydroxymethyl) aminomethane.
8. Sodium acetate.
9. Acetic acid.

3. Methods

3.1. Sample Pretreatment

Since it is unlikely that improved sensitivity instruments and methods could go deep enough to detect trace proteins, the pretreatment of biological samples represents a possible way to improve detectability

of very dilute gene products. At present, it is unanimously admitted that the detectability of trace proteins is mostly hindered by both the presence of high-abundance proteins and the too low concentration of a number of species. To resolve this issue, several approaches have been proposed, but two of them are dominating:

- Removing high-abundance species that hide minor species, such as via so-called depletion approaches.
- Reducing the dynamic concentration range by reducing the concentration of high-abundance species while concomitantly and proportionally concentrating low-abundance proteins.

Detailed descriptions of these two methods are given below. These methods could be either used singularly or in association.

3.1.1. High-Abundance Protein Depletion Methods

The term “depletion” is used to indicate the selective removal of one or several proteins from a complex mixture by means of a selective solid-phase media as an immunoabsorbent. Most generally, proteins that are removed include albumin, immunoglobulins, and transferrin from serum or plasma (36), hemoglobin from red blood cell lysates (37), actin from cell extracts (38), and ribulose-bisphosphate-carboxylase-oxygenase from leaf extracts (39). The removal of these high-abundance proteins allows evidencing the presence of other species by two-dimensional electrophoresis as well as by mass spectrometry due to elimination of suppression effects in both cases.

Various depletion strategies have been proposed over the past few years involving various sorbents for the removal of undesired proteins and to analyze the resulting depleted sample directly by 2-DE and mass spectrometry (40). Dye ligands, or other natural proteinaceous ligands such as protein A and G, lectins, and synthetic ligands are among described techniques. Nevertheless, the most used methods involve antibodies against proteins targeted for removal.

In spite of better visibility of hidden proteins, specific drawbacks limit the use of depletion methods:

- Codepletion (total or partial) of associated species due to non-specific binding or to protein–protein interaction (41).
- Limited binding capacity of immunosorbents with consequently very small volumes of biological extract treated.
- Dilution of the initial extract rendering it even more difficult to detect already diluted species (42).

Method

1. Dilute 20–25 μL of serum with an equal volume of 10 mM Tris–HCl, 150 mM sodium chloride, pH 7.4
2. Centrifuge the diluted sample at $2,500\times g$ to remove possible small particles in suspension.

3. Inject the sample into the immunosorbent column according to the instructions provided by the supplier.
4. Collect the column effluent at the outlet of the column.
5. Wash the column with approximately 3–5 volumes of washing buffer (per supplier instructions).
6. Repeat steps 1–5 if necessary to generate sufficient sample. Often several depletion cycles are required to generate sufficient yield for downstream applications.
7. Regenerate the column by injecting about three volumes of stripping buffer followed by about 10 volumes of neutralization buffer.

The collected protein sample is always diluted by both the passage through the column and the washing step. Depending on supplier recommendations, the dilution factor can range from 5- to more than 150-fold. With most diluted treated samples, a concentration step is required prior to downstream analysis. Available processes to concentrate the sample include dialysis followed by lyophilization, ultrafiltration, and protein precipitation. Although all of them are routinely used, they all suffer from several drawbacks such as losses during dialysis, nonspecific binding on filtration membranes, and incomplete precipitation of low mass proteins. In addition to concentration, other adjustments may be required depending on the downstream analysis method to be used (see Note 1). Depleted samples are often subjected to additional purification steps to further reduce sample complexity (see Note 2).

Table 1 reports experimental data on several depletion methods.

3.1.2. Reduction of Dynamic Protein Concentration Range

As opposed to depletion, this process consists of “compressing” the dynamic range of protein concentrations by simultaneously diluting the high-abundance species and concentrating trace proteins. The reduction of dynamic protein concentration range is based on the use of a solid-phase mixture of an extremely large number of specific sorbents, each of them carrying a given hexapeptide ligand (5). Proteins contacted with such a mixture of affinity media are captured by their respective partner ligand under physiological conditions, until saturation is reached. Proteins present at a high concentration rapidly saturate their partner bead ligands so that the vast majority of the same protein remains unbound. Conversely, low-abundance proteins concentrate on their ligand up to saturation as long as the load is sufficient. On the basis of the saturation-overloading chromatographic principle, a solid-phase ligand library enriches for trace proteins while concomitantly reducing the concentration of abundant species. Desorption of captured species is operated by classical eluting agents used in affinity chromatography as summarized in Table 2; sodium dodecyl

Table 1
Main characteristics of high-abundance protein depletion methods

Removed proteins	Affinity ligands	Loading conditions	Dilution factor ^a
Human serum albumin	Cibacron Blue	20 mM phosphate buffer pH 7.0	20
Human serum albumin and IgG	Cibacron Blue and protein A	10 mM Tris-HCl, pH 7.5 + 25 mM NaCl	10
Serum immunoglobulins G	Protein G	Phosphate buffered saline	5
Human serum albumin	Antibodies	Phosphate buffered saline	180
Human serum albumin and IgG	Antibodies	Phosphate buffered saline	35
Humans serum albumin and IgG	Specific ligands	Phosphate buffered saline	30
Six abundant human serum proteins	Antibodies	10 mM Phosphate pH 7 + 0.5 M NaCl	60

^aDilution obtained using the recommended protocol from the vendor

Table 2
Protein desorbing solutions for the use with peptide libraries

Possible elution solution	Applicability	Mechanisms, effects and compatibility
5–10% sodium dodecyl sulfate	Single elution, denaturing	Desorbs all proteins. The method is compatible with SDS-PAGE, but not with isoelectric focusing migrations and mass spectrometry
6 M guanidine-HCl, pH 6	Single elution, denaturing	Dissociates all types of interactions; proteins are fully denatured. Proteins need to be precipitated for further analytical methods, except for RPC separation
9 M urea, 2% CHAPS, citric acid to pH 3.0–3.5	Single elution, partial denaturation	Dissociates most interactions except very strong hydrophobic associations. Fully compatible with isoelectric focusing, 2DE and SELDI (if neutralized and fivefold diluted). Otherwise proteins need to be precipitated for further analytical methods
9 M urea, 2.4% CHAPS, ammonia to pH 11	Single elution, partial denaturation	Dissociates most interactions except very strong hydrophobic associations. Fully compatible with isoelectric focusing, 2DE and SELDI (if neutralized and fivefold diluted). Otherwise proteins need to be precipitated for further analytical methods. The eluate requires neutralization

(continued)

Table 2
(continued)

Possible elution solution	Applicability	Mechanisms, effects and compatibility
0.5–1 M sodium chloride	Fractionated elution	Desorbs proteins that are captured by a dominant ion exchange effect. Compatible with certain types of liquid chromatography such as HIC, RPC, IMAC
200 mM glycine–HCl, pH 2.4	Fractionated elution	Desorbing solution for the dissociation of biological affinity interactions (e.g., Ag–Ab). Proteins need to be precipitated for further analytical methods, except for SELDI and RPC analysis. The eluate requires neutralization
50% ethylene glycol in water	Fractionated elution	Dissociates mild hydrophobic interactions. Compatible with most analytical methods except HIC and RPC
Acetonitrile (6.6)–isopropanol (13.3)–trifluoroacetic acid (0.8)–water(79.3)	Fractionated elution	Dissociates strong hydrophobic interactions. Compatible with most analytical methods except HIC and RPC, unless solvents are evaporated. The eluate requires neutralization
7 M urea–2 M thiourea–4% CHAPS	Fractionated elution	Dissociates mixed modes, hydrophobic associations and hydrogen bonding. Fully compatible with isoelectric focusing, 2DE and SELDI.

sulfate solution for electrophoresis (e.g., Laemli buffer) could be used for an exhaustive protein desorption (43).

An example of human serum treated with ProteoMiner is illustrated on Fig. 1 where elution was performed in two sequential steps. Analyses of eluates were performed using two-dimensional electrophoresis and SELDI MS.

Since this treatment is based on thermodynamic principles, its performance depends on the combinations of dissociation constants and the concentration of very dilute proteins. Although the large diversity of peptide ligands from the library covers all necessary affinity interactions to capture the proteins present in the sample, its reproducibility is ensured by a rigorous control of environmental pH, ionic strength and temperature. In the case of comparative profiling, all different operations must be maintained rigorously identical and the ratio between the bead volume and the sample volume are to be calculated so that the beads are overloaded (44).

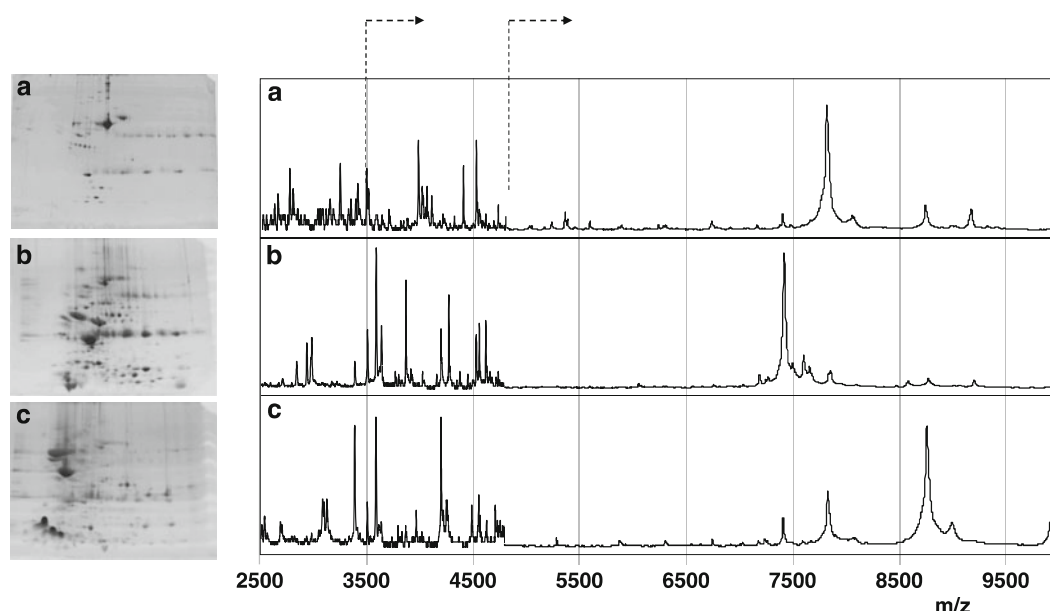


Fig. 1. Analytical determinations of human serum treated with ProteoMiner. *Left*: two-dimensional electrophoresis; *right*: SELDI MS. (a) Initial untreated human serum; (b) bead library eluate using 7 M urea containing 2 M thiourea and 2% CHAPS; (c) eluate using 9 M urea containing 2% CHAPS and 50 mM citric acid. Two-dimensional electrophoresis was performed using a pH gradient between 3 and 10. SELDI MS analysis was performed using an IMAC chip; the mass range shown is from 2.5 to 10 kDa.

Method

1. Wash repeatedly 100 μ L of a hexapeptide ligand library (e.g., ProteoMiner) with PBS either in a spin column or in a small chromatographic packed column.
2. Load the clear protein extract (e.g., 1 mL human serum) and incubate for 60 min at room temperature under gentle shaking or using a downward column flow. In case of other protein extracts, the amount of proteins to be injected should be at least 30–50 mg total. In case of proteases are suspected to be present, add appropriate inhibitors such as protease inhibitor cocktail.
3. Wash out the excess of proteins from the hexapeptide ligand library by either centrifugation or column washing depending on the mode of protein contact.
 - (a) For spin columns, the excess of proteins is removed by centrifugation at about $1,250 \times g$ followed by at least three washings with ten bead volumes of the initial physiological buffer. In between centrifugations the bead slurry is shaken gently for 10 min.
 - (b) When using a chromatographic column, pump through 10 column volumes of PBS to ensure that all protein excess is eliminated (baseline signal at 280 nm).

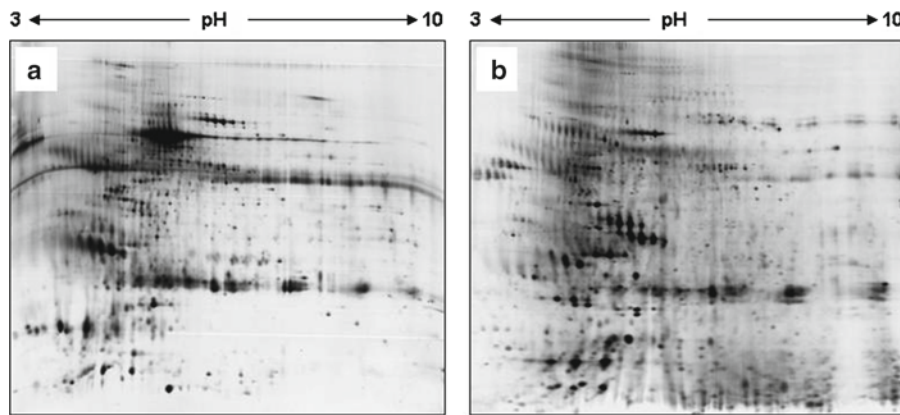


Fig. 2. Two-dimensional electrophoresis of urinary proteome before (a) and after (b) a treatment with ProteoMiner to reveal low-abundance proteins. Protein desorption was performed using 10% sodium dodecyl sulfate. Courtesy of Dr G. Candiano, Gaslini Institute, Genova (44).

4. Elute proteins captured by the hexapeptide ligand library beads, either by one single operation or by a sequence of elution steps. A single elution is performed using the urea-CHAPS-acetic or citric acid solution. Desorb captured proteins using three successive steps with 1 volume of elution solution.
 - a. Between elution steps, shake the suspension for 10 min at room temperature.
 - b. Centrifuge at $1,250 \times g$.

The collected pooled eluates contain captured proteins from the initial sample and can be directly analyzed by SELDI-TOF MS following dilution into the appropriate binding buffer. Multi-step elution schemes, which fractionate bound proteins using three to four sequential elution buffers, have also been used routinely for SELDI analysis. This method is performed as described above except that a washing sequence with different eluting agents is applied sequentially. A variety of elution conditions can be used depending on the purpose and are summarized in Table 2 (see Notes 3 and 4). When collected fractions are acidic or alkaline they must be immediately neutralized. Eluted fractions are used for proteomics studies. Figure 2 shows a comparative example of the urinary proteome before and after treatment with a combinatorial hexapeptide ligand library.

3.2. Fractionation Approaches with Solid-Phase Media

Chromatographic separation of proteins from complex mixtures is the result of interactions with solid phases followed by sequential specific or nonspecific desorption. A large number of protein–solid phase interactions are known in the field of proteomics investigations (29). They are based on physicochemical properties of proteins

to separate or on biochemical recognition. In addition to the different mechanisms of action, various modes of exploitations are also possible such as packed beds, suspension separation, or even fluidized bed fractionations. Multiple columns with complementary properties are used to enhance the capability of chromatography to separate and isolate gene products. The field of liquid chromatography of proteins is extremely large and it is beyond the scope of this chapter to review all practical possibilities. Instead, we will describe protein separations methods that have been used successfully in the SELDI-TOF proteomics workflow. The main driving force of these methods is to find modes of separations that are the most powerful to decrease protein redundancy between a reduced number of collected fractions. Reduced redundancy of proteins in collected fractions is a way to reduce the number of fractions necessary to maintain a high level of analytical resolution and hence the power of discovery. Reducing the number of fractions required is of utmost importance, especially when a very large number of samples has to be analyzed comparatively with a reasonable workload. Other objectives include selecting conditions that yield the best complementary behavior between solid phases, the minimum dilution and the absence of polypeptide losses due to, for instance, nonspecific binding on solid phases.

3.2.1. Classical Chromatography Separation Using Fractionated Elution

Chromatography is the most popular way to fractionate protein mixtures with the objective to simplify the analysis of components in proteomics investigations. A number of reviews have focused on liquid chromatography for protein fractionation exploiting various physicochemical interaction principles (29, 45–48). The process of liquid chromatography could be described as an adsorption of proteins on a solid-phase media followed by a fractionated elution of proteins using progressively more stringent desorbing agents. Many fractionation strategies use one or more of three generic separation methods: (1) ion exchange chromatography, which separates proteins based on protein charges, (2) gel filtration, which separates based on differences in protein masses and (3) hydrophobic associations between lipophilic sites of proteins with hydrocarbon chemical functions attached on the surface of chromatography sorbents. Outside these generic fractionation possibilities, there are more selective adsorptions addressing groups of proteins or even single proteins.

Within the proteomics world, Fountoulakis' group has already in 1998 extensively developed and described chromatography approaches as ways to simplify complex proteomes (49). In a typical example, affinity chromatography on a heparin affinity sorbent was used as a prefractionation step for enriching certain protein fractions.

Hydrophobic, cationic and anionic proteins are easily fractionated using classical liquid chromatography methods. On the contrary,

affinity chromatography (50) can be used to enrich for classes of proteins based on specific interaction properties, such as glycoproteins using lectins of different specificities (30, 33, 51), or phosphoproteins via metal chelating resins (21), affinity for zirconium or titanium oxide (52, 53) or proteins interacting with heparin (54) just to mention a few.

While easy to implement and quite well known, one of major drawbacks of liquid chromatography, especially when performed in a 96-well filtration plate format with a large number of samples and low number of fractions, is protein tailing. This results in the presence of individual proteins in several fractions along with a significant dilution of proteins within each fraction. Another limitation is the dilution effect of collected proteins.

As many different combinations of samples and chromatography types are currently used, this paragraph limits its scope to the most popular protein fractionation of human serum by anion exchange chromatography for proteomics analysis (19).

Method

1. Fill a spin column (or a well of a 96-well filtration plate) with 125 mL of anion exchange solid-phase media (e.g., Q-HyperD).
2. Equilibrate column in binding buffer (composed of 1 M urea-0.22% CHAPS, 50 mM Tris-HCl, pH 9).
3. Separately, mix 70 μ L of human serum with an equal volume of a solution composed of 9 M urea and 2% CHAPS and gently stir for 60 min at room temperature.
4. Once the column is equilibrated, add the diluted serum to the solid phase and incubate for 30 min.
5. Collect the flow-through fraction including a wash with 140 μ L of U9 (250 μ L).
6. Elute captured proteins in a stepwise manner with buffers of decreasing pH:
 - (a) 50 mM HEPES, pH 7. Use 140 μ L eluting solution each step.
 - (b) 100 mM sodium acetate pH 5.
 - (c) 100 mM sodium acetate pH 4.
 - (d) 50 mM sodium citrate pH 3.
 - (e) Finally, the solid phase is stripped using a mixture constituted of 33.3% isopropanol, 16.6% acetonitrile, 0.1% trifluoroacetic acid in DI water).

In practice, in each step the solid-phase media added with the elution solution, is incubated for 30 min and then the supernatant is collected by centrifugation or filtration.

7. The six protein fractions obtained are compatible with further proteomics analysis using SDS-PAGE, two-dimensional electrophoresis or SELDI-MS.

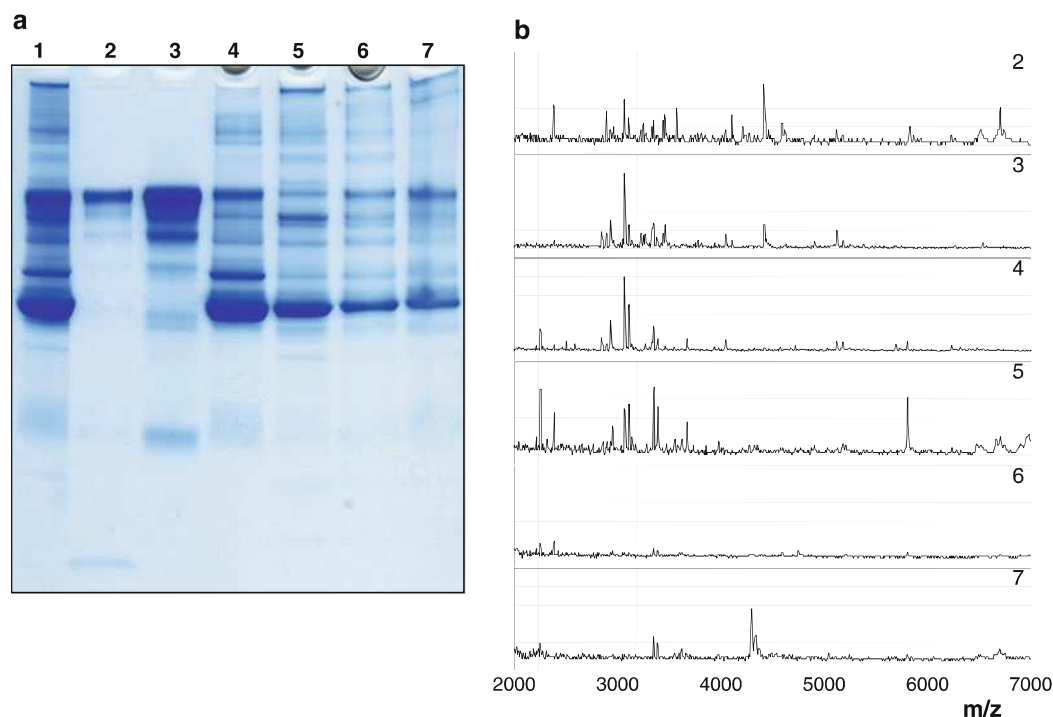


Fig. 3. Analysis of human serum polypeptide species separated by anion exchange chromatography by SDS-PAGE (high masses “a”) and by SELDI MS (low masses “b”). (a) SDS-PAGE analysis of human serum fractions obtained from Q-HyperD fractionation in view of protein profiling with SELDI MS. The fractionation was performed using a stepwise decrease of pH and after having treated the initial serum proteins with urea. *Lane 1*: initial human serum; *Lane 2*: flowthrough fraction pH 9.0; *Lane 3*: elution at pH 7.0; *Lane 4*: elution at pH 5.0; *Lane 5*: elution at pH 4.0; *Lane 6*: elution at pH 3.0; *Lane 7*: elution with a hydro-organic mixture. (For details see Subheading 3.1.2). (b) SELDI MS associated with CM10 ProteinChip arrays of fractions obtained as indicated above. m/z =mass over charge.

Figure 3 shows a typical analytical result after SDS-PAGE and SELDI MS on CM10 ProteinChip array.

3.2.2. Fractionation with Stacked Sorbents and Single Buffer

A novel method for protein fractionation from complex mixtures is the use of superimposed chromatographic sorbents as a cascade with a single loading buffer and a single elution solution to recover captured proteins (55). This system is composed of a set of solid-phase media serially connected in a stacked mode, equilibrated in the same binding buffer. The protein sample is applied at the top and it crosses all the solid-phase layers, each of them capable of capturing a subset of proteins, so that, when the sample reaches the bottom-positioned media, most if not all proteins are removed. Once the protein adsorption phase is completed, the sequence of stacked beds is washed with the equilibration buffer until it reaches baseline (as measured at 280 nm). Then each stacked section is dissociated and proteins desorbed independently from each sectional column. In essence, this resembles a series of depletion columns, each of them in charge of removing a given group of proteins.

Based on the selection of orthogonal solid-phase chemistries and their sequence, bound proteins are different in each of the capturing blocks. Naturally the process should be used in such a way so that sectional columns are not overloaded with proteins that are supposed to be captured, so as to minimize or eliminate the level of protein redundancy (the presence of a same protein in different fractions) between fractions. When superimposing solid-phase layers, media are assembled so that the top comprises the most selective material, and the least selective media is positioned at the bottom of the sequence.

It has been reported that for a same limited number of fractions (e.g., five or six), the total number of species observed by mass spectrometry was more than doubled compared to a classical elution chromatography (56).

Such an approach, when coupled with high throughput capability, appears to be useful for discovering protein species with diagnostic relevance. Only a limited number of fractions is generated and the separation process can be operated very rapidly even with a large number of samples. Interestingly, species that are relatively diluted are captured only by one column section and are consequently concentrated and therefore better detected in comparison to classical methods.

The selection of solid-phase media can be randomized or can be adapted to well-defined fractionations. For instance, for glycoprotein fractionation, lectin media could be used (concanavalin A, the least selective would be positioned in the last position). Chelating media loaded with different metal ions could also be used or different hydrophobic solid-phase media where the least hydrophobic will be positioned first.

Figure 4 shows the separation of species captured by a cascade of chelated metal ions immobilized on IMAC resins, as analyzed by SELDI MS.

Although this approach is quite different from the use of Immunosorbents for “depletion” of high-abundance species (see Subheading 2.1.1), a cascade of immunosorbents could also be envisioned.

Detailed methods for fractionation of serum using stacked sorbents are described below. These methods can be modified for other sample types and binding specificities.

Method

1. In order to prevent protein–protein interactions and formation of aggregates, partially denature serum from the sample prior to loading on the resin. Add an equal volume of a solution composed of 9 M urea and 2% CHAPS to the human serum sample and incubate for 60 min at room temperature.
2. Adjust the denatured serum solution, adding 36 μ L of a 1 M Tris–HCl buffer pH 9, 100 μ L of a 9 M urea-2% CHAPS solution and 364 μ L of deionized water for every 0.4 mL of initial protein solution.

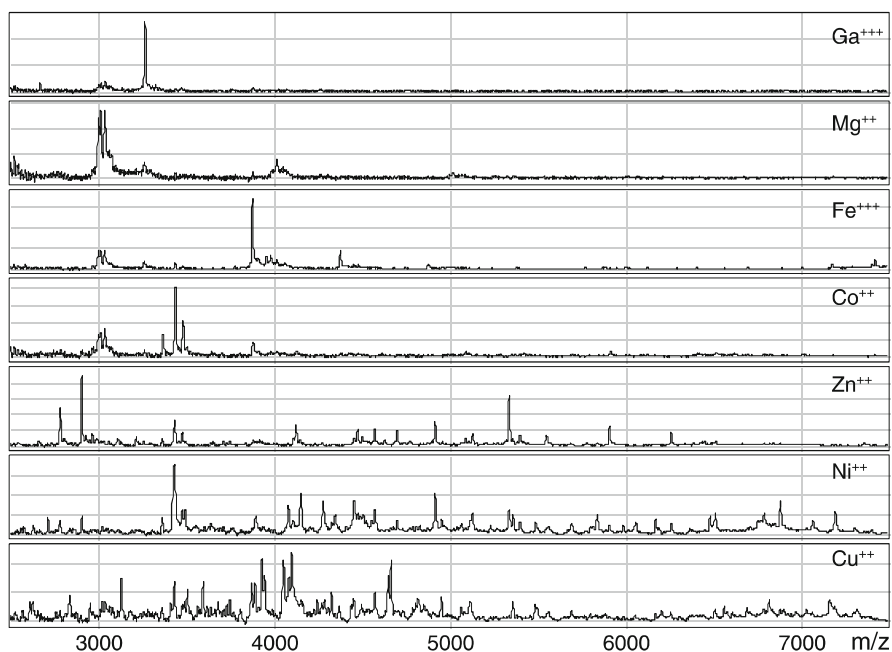


Fig. 4. SELDI MS analysis of serum protein fractions obtained from a cascade of IMAC columns loaded with metal ions as indicated in the *boxes* (from Ga^{3+} to Cu^{2+}). Loading was performed in physiological buffer and elution was obtained by decreasing the pH to 4.0. Each fraction was analyzed using a CM10 ProteinChip array. The visualized mass window is between 2,500 and 7,500 Da.

3. Superimpose columns in the following order from the top to the bottom: Immobilized Protein A, Affigel Cibacron Blue, Heparin-agarose, MEP-HyperCel, Green-5 agarose and PPA-HyperCel. Volumes of each solid-phase media should be 150 μL . Volumes of Protein A and Cibacron Blue resins may need to be adjusted depending on the sample type and resin capacity (see Note 5).
4. Equilibrate superimposed media columns with a binding buffer composed of 16 volumes of phosphate buffered saline, 9 volumes of 1 M Tris-HCl buffer, pH 8 and 75 volumes of distilled water. The operation is performed using a peristaltic pump at a flow-rate of 0.2 mL/min.
5. Following the equilibration step, inject 160 μL of the denatured serum sample to be fractionated at the top of the column at a flow rate of 0.01 mL/min followed by the binding buffer at the same flow rate to push the sample throughout the series of sorbents.
6. The first 1,250 μL of effluent is discarded and the next 1,250 μL effluent is collected as the flow-through fraction.
7. Separate columns and elute adsorbed proteins using 500 μL of desorbing solution (see Note 6).

Many variants of this general method are possible especially when using other solid-phase media. An example is given in Fig. 3 where the purpose was to separate proteins having affinity for different metal ions.

3.3. *pI* Separation Groups by Solid-State Buffers

A specialized variant of fractionation with stacked sorbents is the separation of proteins according to their isoelectric point using so-called “Solid-State Buffers” (SSB) associated with ion exchangers (35). This approach contrasts with current methods for separation by isoelectric point since it uses chromatographic-like processes compared to electrically driven separation. Advantages of this approach include the ability to perform separation rapidly without ampholine carriers and to use sample volumes that can be of undefined volume and concentration.

Stacked columns are mixed beds of cation or anion exchange media mixed in equal volumes with SSB capable of spontaneously generating predetermined pHs (35, 56). Solid-State Buffers are discrete beads of amphoteric polymers inducing a predetermined environmental pH in the presence of ionizable molecules such as proteins. SSB-IEX mixtures are packed in distinct columns under a cascade configuration as described in the previous section. When the packed media blend comprises an anion exchange resin, the top sectional column contains the SSB of the lowest pH value and the bottom sectional column contains the SSB with the highest pH value. On the contrary, when a cation exchanger is mixed with SSBs, an inverse pH sequence is to be configured. A sample of the protein extract solution to fractionate is introduced at the top of the first column. Proteins and small ions present in the solution enter in contact with the SSB, which generates a well-defined environmental pH, influencing thus the net charge of proteins present. Those having a net charge complementary to the ion exchange resin are captured and the others are progressively pushed in the second sectional column. In this second mixed media environment, another SSB induces a different environmental pH generating consequently a different net charge of upcoming proteins from the first sectional column. Here also proteins of complementary charges are attracted by the ion exchanger while proteins with antagonistic charges are repelled and pushed into the following sectional column and so on. The process is then repeated a number of times equivalent to the number of sectional columns. Once the injection of sample and washings are complete, sectional columns are dissociated and adsorbed proteins from each mixed bed are eluted and collected separately by increasing ionic strength or changing pH or both. The collected fractions comprise proteins of pI ranges depending on the pH induced by the local SSB used.

To avoid the effects of ion interferences and prevent protein–protein interactions, the protein sample is solubilized in low ionic strength solutions containing dissociating agents such as urea or is

diluted two to five times with a similar solution. It has been recently demonstrated that human serum proteome is better fractionated using Solid-State Buffers compared to classical anion exchange chromatography (57).

3.3.1. Method

1. Prepare SSB-IEX blends by mixing equal volumes of SSB with either a Q anion exchanger or a CM ion exchanger. For optimal performance acidic proteins should be separated using media blends comprised of cation exchangers and alkaline proteins with anion exchanger blends.
 - (a) The pHs induced of selected SSBs are 4.6, 5.4, 6.2, 7.0, 8.5, and 9.3.
 - (b) Wash each mixture with a liquid buffer of a pH identical to the SSB and then exhaustively rinse with water or 2 mM potassium chloride containing 4 M urea, to eliminate all components of the buffers.
2. Pack the conditioned bead mixtures into distinct small columns (e.g., 6 mm diameter and 3 cm long) and connect to each other according in an appropriate order.
 - (a) When using an anion exchanger, connect columns in order of increasing pH.
 - (b) When using a cation exchanger, connect columns in order of decreasing pH.
3. Inject the protein sample (previously dissolved in 4 M urea containing 2–5 mM potassium chloride) into the first column and wash with 5–10 column volumes of urea-KCl solution to remove all noncaptured proteins (see Note 7).
4. Disconnect columns and elute bound proteins separately using a solution of 1 M sodium chloride or a change of pH to ensure proper dissociation of proteins from the ion exchanger.
5. Collected proteins are ready for proteomics studies (see Note 8). In case of incompatibility of pH and/or ionic strength, proteins can be desalted according to currently available methods.

Figure 5 illustrates an example of serum protein separation within the acidic region.

4. Notes

1. Protein samples obtained using depletion processes are equilibrated with the initial buffer (see details on Table 1). Samples are generally at neutral pH and the buffer is often PBS. Under these conditions the sample is neither directly compatible with

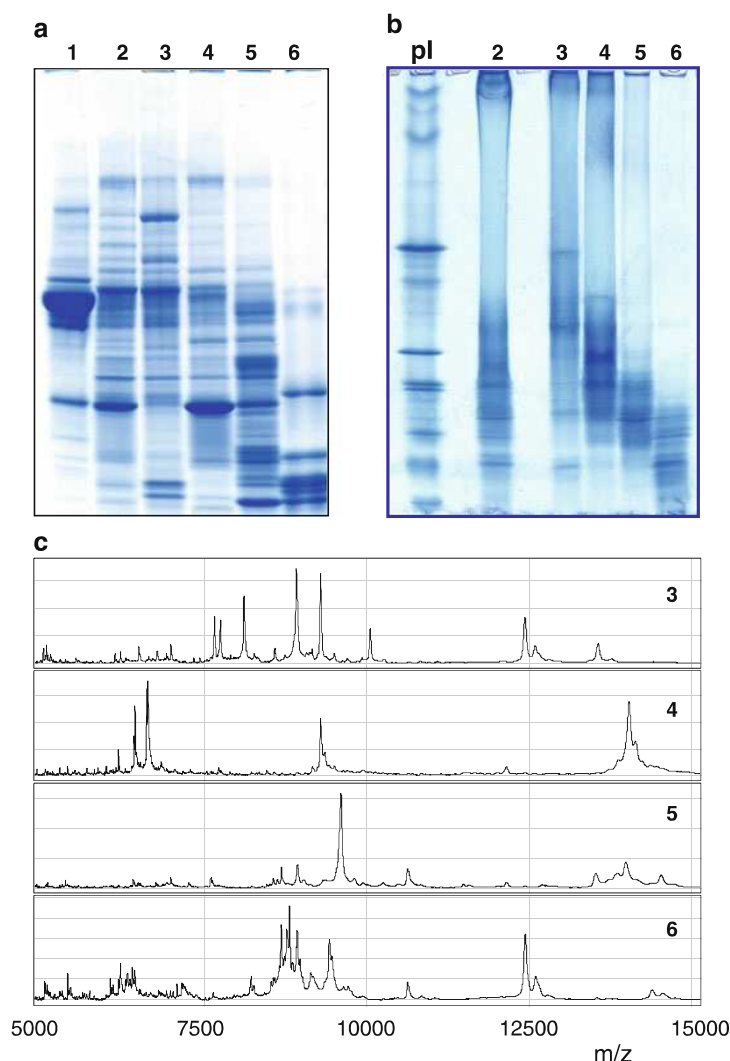


Fig. 5. Fractionation of human serum by isoelectric groups of proteins using Solid-State Buffers. Human serum was first treated with ProteoMiner to enhance the presence of low-abundance proteins and reduce the concentration of high-abundance ones. The fractionation was performed using SSB of pH 4.6, 5.4 and 6.2 associated with a CM cation exchange media. (a) SDS-PAGE analysis of produced fractions. (b) IEF analysis of same fractions. (c) SELDI MS associated with CM10 ProteinChip array for the mass analysis of separated fractions. *Lane 1*: Initial human serum; *Lane 2*: Serum treated with ProteoMiner; *Lane 3*: Flowthrough fraction or proteins of $pI > 6.2$; *Lane 4*: Protein fraction of pI between 5.4 and 6.2; *Lane 5*: Protein fraction of pI between 4.6 and 5.4; *Lane 6*: Protein fraction of pI below 4.6. pI : markers of isoelectric point. Proteins from each fraction are complementary each other.

analytical two-dimensional electrophoresis nor with direct MALDI mass spectrometry. It can, however, be used for LC-MS/MS or SELDI-MS analysis after simple dilution and chip washing.

2. Several options are available if further fractionation is desired to simplify the protein content. Samples can be used for ion exchange fractionation after diluting at least tenfold and adjusting pH to 4.5–6.0 for cation exchange chromatography or 7.5–9.0 for anion exchange chromatography. Fractionation by isoelectric focusing requires a complete desalting.
3. Although there are several methods to desorb proteins en masse, an interesting way to quantitatively collect all captured proteins is to use 5–10% SDS in water. However, the presence of SDS may or may not be compatible with analytical methods to follow, so that in some cases the elimination of SDS is necessary (for more details see ref. 43). When the following analysis is a determination of a biological activity or an interaction with antibodies such as immunoblots or ELISA-based assays, non-denaturing solutions should be tried.
4. As opposed to one single elution, there is the possibility to desorb proteins sequentially to further separate proteins based on their physicochemical characteristics and thus simplify the following analysis (5).
5. The most critical point is to avoid overloading each individual solid-phase media. Therefore an excess of resin is recommended even when the binding capacity is known. In the case of serum, where albumin and IgG dominate, it is advisable to use a relatively large volume of Cibacron resin (about three times compared to other resins) and to select Protein A media of high binding capacity.
6. These chromatographic media are theoretically reusable after proper cleaning performed according to the recommendations of vendors; however, it is advised not to reuse the resins or to limit the number of reutilizations to prevent binding capacity losses and thus a possible overloading situation as well as cycle to cycle carryover.
7. Although less critical than in isoelectric focusing, the initial ionic strength of the protein extract to fractionate may be important for a proper fractionation without saturation of ion exchange resin. If the ionic strength of the initial protein solution is considered high, it is advised to dilute with water or better with a solution of 4 M urea even if this results into a large volume of sample to treat. The method in fact is perfectly adapted to large volumes of dilute proteins without interfering with the final fractionation results.
8. Obtained pI cuts may not be extremely precise at the expected pH value. This is not critical because the primary goal is to obtain fractions that contain complementary proteins with minimal redundancy.

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