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A simplified monobuffer multidimensional chromatography for high-throughput proteome fractionation

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Abstract

The complexity of the human serum proteome is attributed to both a large dynamic range of protein abundance, as much as 10 orders of magnitude, and a disproportionate few dozens of proteins representing as much as 99% of the total protein content. These characteristics make it beneficial to use a pre-fractionation step prior to any high-resolution analysis, such as mass spectrometry. The present method describes a unimodal multidimensional chromatography concept to rapidly achieve an effective fractionation of human serum that is directly amenable with surface-enhanced laser desorption/ionization (SELDI)-based mass spectrometry. This method is based on the use of a column composed of a superimposed sequence of sorbents. The assembly is first equilibrated with a single binding buffer and then loaded with the whole crude sample. As the sample crosses the different adsorbent layers proteins within are sequentially trapped according to the complementary properties vis-à-vis of the sorbent. Once the loading and capturing is achieved, the sequence of columns is disassembled and each column, containing different complement of proteins is eluted separately in a single step and under optimal elution conditions. When compared to classical single-chemistry fractionation based on, for example, anion-exchange and pH stepwise elution, the new proposed approach shows much lower protein overlap between fractions, and therefore, greater resolution. This results in a larger number of detectable species, and therefore, reinforces the power of discovery of new biomarkers. A significantly higher sensitivity for low-abundance species was additionally found as evidenced by spiking trials.

Keywords: Human serum proteins; Prefractionation; Chromatography; Mass spectrometry; Surface-enhanced laser desorption/ionization

1. Introduction

The full analysis of proteins constituting a tissue extract or a biological fluid provides a very elegant and powerful method for phenotyping a biological system. When used in a comparative analysis, where the differential titer of a protein or group of proteins are determined between two samples, the differential expression patterns can be used to distinguish between two biological states or phenotypes, such as disease versus normal. However, when dealing with the detection and quantitation of individual peptides or proteins in a complex biological sample, fractionation prior to analysis is generally used to (i) partition away abundant species that may generally interfere with protein detection, (ii) simplify the complex mixture into fractions, containing lower number of entities and (iii) enrich for rare protein species. Fractionation of serum or plasma samples prior to analysis is particularly relevant as both a decrease in the sample complexity and a reduction in the dynamic range of protein abundance can be achieved [1–3]. These two important features enhance the resolving power of detection and increase the sensitivity of analytical techniques, such as two-dimensional electrophoresis and mass spectrometry. The presence of very high-abundance proteins (e.g. albumin from serum) produces large signals with consequent signal overlap (two-dimensional electrophoresis) or signal suppression (mass spectrometry) for other present entities. Classical methodologies of serum fractionation consist of either depleting the proteins that are very abundant (e.g. albumin, transferrin, IgG fraction) from the sample to be analyzed, or

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by fractionating the entire mixture by means of sequential elution steps off a single resin to segregate the abundant proteins in unique partitions. Other methods of fractionating a proteome are well established and include sub-cellular fractionation [1,4–9], isoelectric separation [2,4,10–14], mono-dimensional electrophoresis [2,7,15], molecular sieving [3,4] and liquid chromatography [2,4]. With respect to liquid chromatography, examples of fractionation methods have been reported using ion exchange chromatography [1,16], immobilized metal affinity chromatography (IMAC) for calcium binding protein [17] or separation of phospho-proteins [18], hydrophobic interaction chromatography [17], affinity on immobilized heparin [4] or on immobilized lectins [4,17,19,20].

Two-dimensional liquid chromatography methods have also been proposed for the fractionation of proteomes after global digestion using endoproteases, such as trypsin. These methods generally combine reversed-phase with ion-exchange chromatography [21,22] or, to a lesser extent, with chromatofocusing [23], size-exclusion [24], affinity capture [20], or even a second reversed-phase step [25]. However, multidimensional chromatography as used in proteomics fractionation generally does not exceed two dimensions due to the high number of fractions to manage (pH-adjustment, desalting, re-injection in second dimension) and to analyze.

Most of the previously described pre-fractionation methods suffer from either their low level of separation performance or from a high level of complexity with a large number of fractions to manage requiring the use of sophisticated equipment. The method described in this paper, utilizes several complementary adsorbents to generate high separation performance as compared to similar separation methods, while maintaining a simple and manageable work-flow. The improved separation performance also allows for the enhanced detection of proteins that may normally be masked by abundant protein species. Throughout this paper, we compare side-by-side the separation performance of this unimodal multidimensional chromatography method incorporating selected binding properties and a single elution step, with an anion-exchange method utilizing a stepwise pH elution mode. The separation quality criteria were the final number of peaks of different masses, the fraction-to-fraction overlap and the sensitivity of the two methods regarding the detection of low-abundance non-human proteins that were spiked at femtomolar level in human serum.

2. Experimental

2.1. Chemicals and biologicals

Chromatography sorbents, such as Q Ceramic HyperD F (quaternary amine) and multistaged assembly of sorbents (MultiSelect) were provided Ciphergen Biosystems Inc. (Fremont, CA, USA). 1 M Tris–HCl, pH 8 stock buffer was from Invitrogen (Carlsbad, CA, USA). Human serum was from Intergen (Norcross, GA, USA). Porcine dynorphin A, bovine insulin chain B oxidized, bovine insulin, bovine ubiquitin, horse cytochrome *c*, horse apo-myoglobin, bovine superoxide dismutase (SOD), β-lactoglobulin A, phosphate-buffered saline (PBS), trifluoroacetic acid (TFA), isopropanol (IPA), acetonitrile (ACN), 29% ammonia (NH_4OH) solution, Sigma-Ultra Urea, 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), Trisma base, octyl-glucopyranoside (OGP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium acetate and sodium citrate were from Sigma–Aldrich (St. Louis, MO, USA). Sinapinic acid (SPA) was from Ciphergen Biosystems (Fremont, CA, USA).

2.2. Devices and equipment

For experiments involving high-throughput processing, Silent Screen LoProdyne filter plates were supplied by NUNC (Rochester, NY, USA), while mixing was performed on a MicroMix shaker was from DPC (Los Angeles, CA, USA) and vacuum filtration was performed using a vacuum manifold supplied by Whatman (Clifton, NJ, USA). For experiments involving mini-columns, disposable Wizard mini-columns were from Promega (Madison, WI, USA) and in conjunction with a Minipuls III peristaltic pump was from Gilson (Middleton, WI, USA).

Surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) MS and ProteinChip arrays were from Ciphergen Biosystems.

2.3. Denaturation of human serum proteins

Two milliliters of whole human serum was added of 2.5 mL of a solution composed of 9 M urea and 2% CHAPS for 1 h at room temperature, aliquoted and frozen at −80 °C. At the time of an experiment, 0.4 mL of this denatured serum was added to 36 μL of a 1 M Tris–HCl buffer, pH 9, 100 μL of a 9 M urea-2% CHAPS solution and 364 μL of deionized water to achieve a total human serum protein dilution of 1/5.

2.4. Spiking human serum with non-human polypeptides

A solution constituted of a mixture of porcine dynorphin A (M_r 2147), bovine insulin β-chain oxidized (3495), bovine insulin (5733), ubiquitin (8564), horse cytochrome *c* (12,360), apo-myoglobin (16951), bovine SOD (15600) and β-lactoglobulin A (18363), was prepared in 0.1 M Tris–HCl buffer, pH 8. Final concentration of each protein was 10 μM. This stock solution was diluted and added to native or denatured human serum up to a final concentration of 100, 10 or 1 fmol/μL of each of the polypeptides in serum. The solution thus obtained, was subsequently used for fractionation.

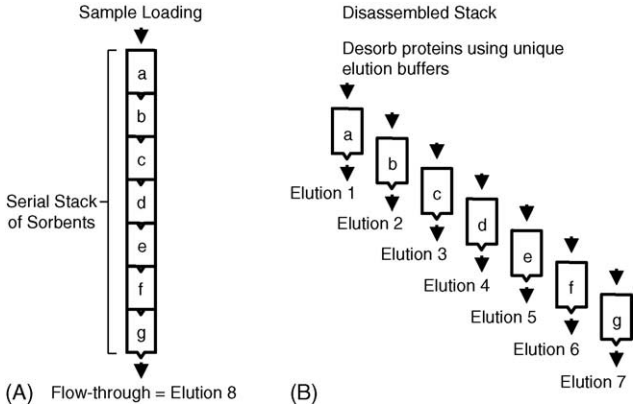

Fig. 1. Schematic representation of a multistaged (MultiSelect) column used in two steps. (A) Column assembled with seven different solid-phase adsorbents (from a to g) and loaded with the human serum sample. FT represents the flow-through fraction or proteins that are not captured by any adsorbent. (B) Desorption phase of captured proteins after columns disassembling yielding fractions 1–7.

2.5. Multistaged fractionation of human serum proteins using stacked mini-columns

MultiSelect staged column was equilibrated in a binding buffer composed of PBS (16 volumes), 1 M Tris–HCl buffer, pH 8 (9 volumes) and deionized water (75 volumes). The flow-rate, driven by a peristaltic pump, was of 0.2 mL/min. Following the equilibration step, 166 μL of sample to fractionate was injected 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 through the series of sorbents. The first 1250 μL of effluent was discarded and the next 1250 μL effluent was collected as the flow-though fraction. Columns sections were then separated each other (see scheme in Fig. 1) and captured proteins desorbed by means of 500 μL following eluents: (i) TFA (0.8 volumes)–water (79.2 volumes)–ACN (6.6 volumes)–IPA (13.4 volumes) and for stages a, d and g and (ii) ammonium hydroxide (8 volumes)–water (72 volumes)–ACN (6.6 volumes)–IPA (13.4 volumes) for stages b, c, e and f. In practice, protein elution was performed by removing the sorbents and gently mixing with elution solutions for 1 h. Supernatants from each sorbent were recovered, frozen per 300 μL aliquots, lyophilized and then re-dissolved in 100 μL of 25 mM Tris–HCl buffer, pH 7.5 before SELDI-MS analysis. A total of eight fractions, including flow-through, was obtained per single sample.

2.6. Anion-exchange fractionation human serum using mini-column format

A disposable Wizard column was filled with 125 μL of Q-HyperD and equilibrated in the above-described binding buffer. Once equilibrated, 140 μL of serum was loaded at a flow rate of 0.01 mL/min. The first 100 μL were discarded before collection of flow-through (250 μL). pH stepwise elution was then performed with the sequence of buffers described above and managed by 250 μL fractions with complete purge of tubings between pH steps.

2.7. Multistaged fractionation of human serum proteins using stacked 96-well filtration plates

An alternative to the multistaged columns described above was the use of 96-well filter plates. Seven 96-well plates stacked each other, each of them dedicated to a single adsorbent, were used as described for MultiSelect column. In this configuration, 96 samples could be fractionated at a time. The equilibration of the sorbents was performed as described above by addition 200 μL per well of the same binding buffer. Plates were then incubated with gentle shaking for 5 min and the supernatant then removed by vacuum filtration. This equilibration step was repeated four times at room temperature. 100 μL of five-fold diluted human serum or other spiked samples was then loaded on each wells of the first 96-well filtration plate.

Incubation time with the sample in the first plate was of 20 min at room temperature. Then, the filtrate obtained under vacuum was directly loaded on the second plate. While this second plate was incubated to have protein adsorbed on the solid phase, the first plate was washed and then proteins adsorbed were desorbed as described for the columns. The same sequence of operations was then applied to the following plates so that samples and wash fractions cross sequentially all the plates. At the end of the process, eight fractions were obtained for each sample. All collected fractions were frozen and lyophilized directly in the receiving plates. Lyophilized fractions were then used as usual for subsequent SELDI-MS analysis.

2.8. Anion-exchange fractionation of human serum using 96-well filtration plates

One filter plate was filled of 90 μL of Q-Ceramic HyperD F per well. Sorbent in each well was equilibrated by addition 200 μL of the binding buffer composed of 1 M urea–0.22% CHAPS, 50 mM Tris–HCl buffer, pH 9. Once equilibrated 100 μL of five-fold diluted sample in 40 mM Tris–HCl buffer, pH 9 was loaded onto each well, followed by an incubation of 45 min under gentle shaking at room temperature. Non-adsorbed proteins were then removed by vacuum filtration and directly collected in a clean 96-well receiving plate. Then 100 μL of a 50 mM Tris–HCl buffer, pH 9 containing 0.1% OGP were added to each well containing the sorbent and incubated for 10 min at room temperature under gentle shaking. The supernatant was then removed by vacuum-filtration and pooled with the previous non-adsorbed fraction. Adsorbed proteins on the anion-exchange resin were then eluted sequentially, using buffers of different pH values, including 50 mM HEPES (pH 7), 100 mM sodium acetate (pH 5 and pH 4), and 50 mM sodium citrate (pH 3). Proteins still adsorbed on the anion exchanger were stripped out by using a hydro-organic solution composed of TFA (0.1 volumes): ACN (16.6

volumes): IPA (33.3 volumes): deionized water (50 volumes). At the end the process, six fractions (non-adsorbed, eluates of pH 7, pH 5, pH 4, pH 3 and hydro-organic eluate) were collected for further SELDI-TOF-MS analysis.

2.9. SELDI-TOF-MS analysis of protein fractions

Each spot of a ProteinChip array was equilibrated two times with 150 μL of the indicated array-specific binding buffer for 5 min. Then, each spot surface was loaded with 30 μL of the sample previously half-diluted in the array-binding buffer. After an incubation period of 30 min under vigorous shaking, each spot was washed two times with 150 μL of the binding buffer for 5 min to eliminate non-adsorbed proteins, followed by a quick rinse with deionized water.

All surfaces were dried and loaded twice with 0.5 μL of a saturated solution of SPA in a mixture of ACN (49.5 volumes)–TFA (0.5 volumes)–deionized water (50 volumes), and dried again. All arrays were then analyzed using a mass spectrometer reader used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector voltage of 2.8 kV. The molecular mass range investigated by mass spectrometer *m*/*z* was from 0 to 300. Focus mass was set at 30 and 7 for high- and low-mass range, respectively. Laser intensity responsible for the desorption/ionization of proteins on the spot surface was set at 200 and 180 units for high- and low-mass range, respectively, and sensitivity of the detector at 9 units.

Validation of all data obtained, including baseline substraction, external calibration using a mixture of known peptide and protein standards and all further data processing, including counting of unique peaks were carried out by using Ciphergen ProteinChip Software 3.2.0. Counting of unique peaks after clustering of the total fractions and/or the different arrays consisted to count only once the peaks of same mass even when detected on more than one fraction or one array. This final number was called the total unique peak count (TUPC), as explained below in more details.

2.10. Calculation of fraction redundancy index (%FRI)

The mass spectral profiles generated from all fractions were clustered within Ciphergen Express software with the following settings; peak detection above S/N thresholds of 7, 4 and 3 for *m*/*z* ranges of 1.5–2.5, 2.5–4 and 4–200, respectively; mass tolerance of 0.3 and 0.6% to achieve the cluster completion within *m*/*z* 1.5–10 and 10–200 windows, respectively. This allowed evaluating the extent of protein presence across multiple fractions, based on the protein mass signature, and calculating the nominal number TUPC of unique peaks (see above section SELDI-TOF-MS analysis). Then, to evaluate the level of redundancy, all fractions were data-processed separately to determine their own number of peaks. These ones were summed to give a total number *N* that includes some fraction-to-fraction redundancy. The fraction redundancy index (%FRI) represents the percentage ($N - \text{TUPC}$)/TUPC of additional peaks due to redundancy. This ratio was calculated on individual or multiple-array basis.

3. Results and discussion

The current method for biomarker discovery involving SELDI-MS is generally associated with pre-fractionation using a 96-well filtration plate format, containing a strong anion exchanger followed by a selective solid-phase extraction on ProteinChip arrays [26]. During the initial sorbent-based fractionation, the sample is denatured and then fractionated using a stepwise elution based on descending pH. The advantage of the 96-well filtration plate approach is its capability to process a large number of samples in very short time with full robotic integration prior mass spectrometry profiling. However, this method suffers from elution tailing of high-abundance proteins, which are consequently, found in more than one fraction. In order to circumvent this problem, a different approach is proposed involving different sorbents packed in discrete mini-columns combined in series. With the serially stacked sorbents a complex sample of human serum was successively fractionated into complementary protein subsets. Once the adsorption operation was achieved, column sections were disconnected and separately eluted with an appropriate solution. Schematically, the process is represented on Fig. 1. High-abundance proteins, such as albumin and IgG were captured by the first two columns as already described [27,28]; however, obtained fractions were considered for further analysis, since a significant number of other protein species are co-adsorbed. Most of serum proteins were adsorbed by the sequence of chromatographic media each of them contributing to capture a set of proteins; nevertheless, a very minor amount of proteins still escaped from the process and was collected in the overall flow-through for analysis.

An immediate benefit of the described approach is the effective separation of protein groups under a limited number of fractions. This is in contrast to the classical separation methods using a single chemistry associated with elution gradients [16,21,22].

Table 1 and Fig. 2 represent typical mass spectrometry data comparing serum fractionated using an anion-exchange column and a multistaged sorbent column. It appears that the number of unique (non-redundant) peaks evidenced by using different ProteinChip array chemistries was always larger with multistaged single elution column than the single-stage column and multiple elution fractions. As indicated in the Table 1, the overall peak count using the single column was of 480 protein peaks, while with the multistaged column it was almost doubling with 940 peaks.

This effect was observed for each type of array, and when adding all the unique peaks found on these four complementary arrays, the same benefit was observed allowing the multiple chemistry fractionation to generate more than two thou-

Table 1
Peak count comparison between multistaged and regular anion exchange fractionations

Type of serum pre-fractionation	Number of fractions	Total Peak count w/o fraction redundancy (sum TUPC arrays)	Total Peak count w/o fraction and array redundancies (overall TUPC)	Fraction redundancy index (%FRI[a])
No fractionation	1	500	270	na[b]
Multistaged fractionation	8	2261 −1404 (1.5–10 kDa) −857 (10–200 kDa)	940 −709 (1.5–10 kDa) −231(10–200 kDa)	0.76
IEX fractionation	6	1129 −597 (1.5–10 kDa) −532 (10–200 kDa)	480 −325 (1.5–10 kDa) −155 (10–200 kDa)	1.80

[a] %FRI was obtained, as described in Section 2.
[b] Non-applicable.

sand peaks (2261), while the classical fractionation could only produce a little more than one thousand (1129). This is a significant gain of information when considering that the separation labor and the number of collected fractions were similar. The larger number of individual species detected was evidently independent from the number of separated fractions (six fractions from ion-exchange column compared to eight fractions from multistaged column); it was rather dependent from a lower level of redundancy, eliminating thus, the risk of peak overlapping and signal suppression. Table 2 summarizes species dispersion throughout sorbent adsorption and ProteinChip array detection system. It is remarkable to see in the multistaged fractionation, that the number of peaks from one stage to another was well equilibrated. Actually, the number of entities ranged between 334 and 835, obtained by the sum of four arrays peak counts with stages d and b, respectively.

An analysis of properties for entity detected by mass spectrometry evidenced that less than 10% (367/3969) of proteins were found in the flow-through (no interaction with all sorbents), while the majority of them had affinity properties for chelated copper (IMAC ProteinChip array, see Fig. 3). As shown in Table 2A, this latter captured the largest number of proteins (almost 40%, 892/2261), essentially located in the first three sorbent stages (54% of all proteins found ((794 + 835 + 533)/3969), which were most of them of relatively low molecular mass. Masses between 1.5 and 10 represented 75% (709/940, see Table 1) of all protein species. With anion-exchange fractionation, the number of polypeptides of the same molecular masses represented about 68% (325/480) of the total, as detailed in Table 1.

These data are interesting when considering that most frequently, the biomarkers of interest are found among small molecules. This indicates that the probability to find new biomarkers using multistaged chromatography is higher [29].

In both cases the largest number of low molecular masses was detected by using IMAC ProteinChip arrays. The analy-

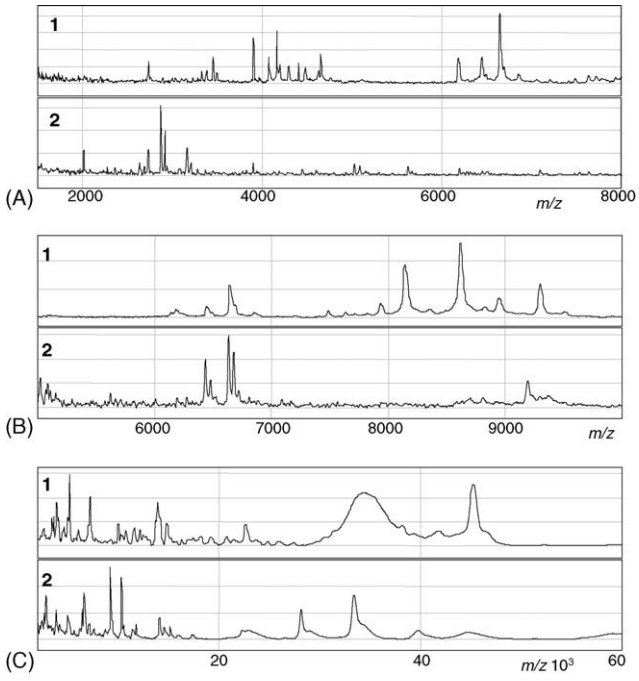

Fig. 2. Comparison of SELDI-TOF-MS spectra from multistaged (1) and anion-exchange (2) fractions. Multistage column fractions were from the first column section (A1 and B1) and from the last column section (C1). They were compared to ion exchange fraction obtained by elution at pH 5. Analysis was performed using different ProteinChip arrays: IMAC30 (A), CM10 (B) and Q10 (C). For more details, see Section 2. *m/z* represent the ratio between the mass and charge.

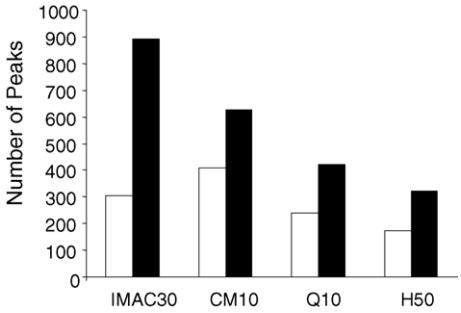

Fig. 3. Total peak count comparison between the two investigated methods. Open bars represent the total genuine peak count from anion-exchange fractionation obtained using different ProteinChip arrays (IMAC30, CM10, Q10 and H50). Black bars represent the peak count from the multistaged fractionation determined under the same analytical conditions.

Table 2
Total peak count distribution in fractions generated by (A) multistaged or (B) anion exchange fractionation and analyzed on different ProteinChip arrays

Fractions	Peak count on specific ProteinChip array chemistries				
	IMAC30	CM10	Q10	H50	Total
(A) Multistaged fractionation					
Fraction FT	155	65	77	70	367
Fraction a	340	193	144	117	794
Fraction b	405	275	86	69	835
Fraction c	268	91	98	76	533
Fraction d	136	83	52	63	334
Fraction e	182	60	68	61	371
Fraction f	147	70	77	65	359
Fraction g	214	60	35	67	376
Total peak count N[a]	1847	897	637	588	3969
Total unique peak count TUPC[a]	892	625	422	322	2261
%FRI = [100 × (N−TUPC)/TUPC]	107	43	51	83	76
(B) Anion exchange fractionation					
Fraction 1 (flow-through)	143	164	109	50	466
Fraction 2 (pH 7)	121	205	72	40	438
Fraction 3 (pH 5)	160	222	156	101	639
Fraction 4 (pH 4)	131	232	138	136	637
Fraction 5 (pH 3)	133	100	95	81	409
Fraction 6 (organic)	138	177	126	129	570
Total peak count N[a]	537	1100	696	537	3159
Total unique peak count TUPC[a]	306	410	239	174	1129
%FRI = [100 × (N−TUPC)/TUPC]	170	168	191	209	180

[a] N and TUPC were obtained, as described in Section 2.

sis of proteins using H50 ProteinChip array indicated that the most hydrophobic species were captured by the first column (a).

A focused analysis of the situation towards high abundant proteins, such as albumin, immunoglobulins G and transferrin showed that a significant reduction of redundancy was also observed between the compared methods (Fig. 4). As shown by the multistaged method IgG, albumin and transferrin were found separately in three different fractions. This is in contrast to the anion-exchange fractionation method where they were most of the time evidenced together and observed over multiple fractions. The reduction of fraction redun-

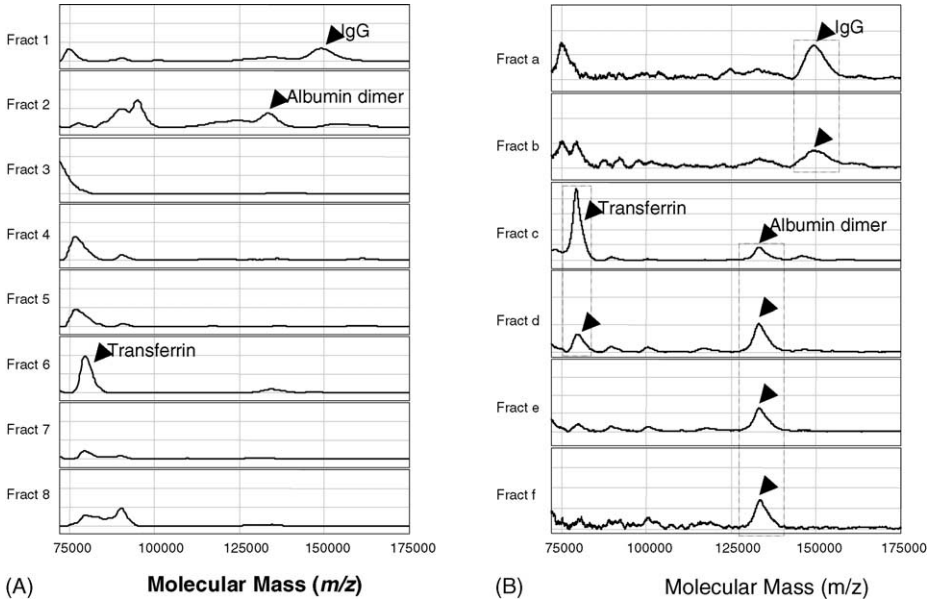

Fig. 4. Distribution of some high-abundance serum proteins throughout multistaged and anion-exchange fractionation. All mass spectrometry analysis was done using an IMAC30 ProteinChip array. Panel (A) represents the analysis of fractions from multistaged column (1–8) and Panel (B) shows results of each individual fraction obtained by pH elution from an anion-exchange column (from a to f). m/z range shown is between 75 and 150 in order to see three major proteins: albumin (by its dimer), transferrin and IgG. m/z represents the mass over the charge.

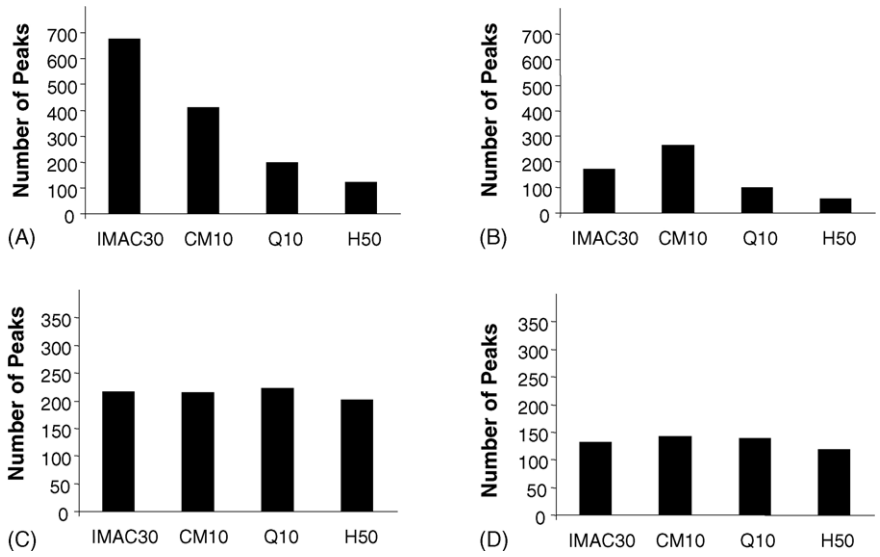

Fig. 5. Peak count comparison relative to mass range between multistaged and anion-exchange fractionations. Panels (A) and (C) represent the peak count of multistaged fractionation obtained using different ProteinChip arrays (IMAC30, CM10, Q10 and H50) representing, respectively, species of *m*/*z* between 1.5 and 10 (A) and 10–200 (C). Panels (B) (*m*/*z* 1.5–10) and (D) (*m*/*z* 10–200) are the corresponding comparative results from fractions obtained from anion-exchange fractionation. In both cases, individual peaks from different separation fractions have been assembled.

dancy for high-abundance proteins is also critical to reduce signal suppression that occurs in most of anion-exchange fractions where abundant proteins are found. This is the reason, why current methods of pre-fractionation frequently recommend, to remove albumin prior analysis of the entire proteome [30–32].

A full exploitation of mass spectrometry data allowed evidencing unique peaks and calculating peak redundancy by the ratio of the number of additional peaks due to fraction-to-fraction redundancy over the total number of unique peaks (TUPC, see definition in Section 2). A decrease in peak redundancy is shown on Table 1 when using the multistaged column (%FRI 0.76) in comparison to the single column fractionation (%FRI 1.8). A single protein found over several fractions, as it is the case when using a pH elution steps on the anion exchanger is most of the time explained by a large peak tailing. This phenomenon is even enhanced with short columns that have a limited number of plates. With 96-well filtration plates the phenomenon is additionally amplified because the ion exchange adsorption and des-

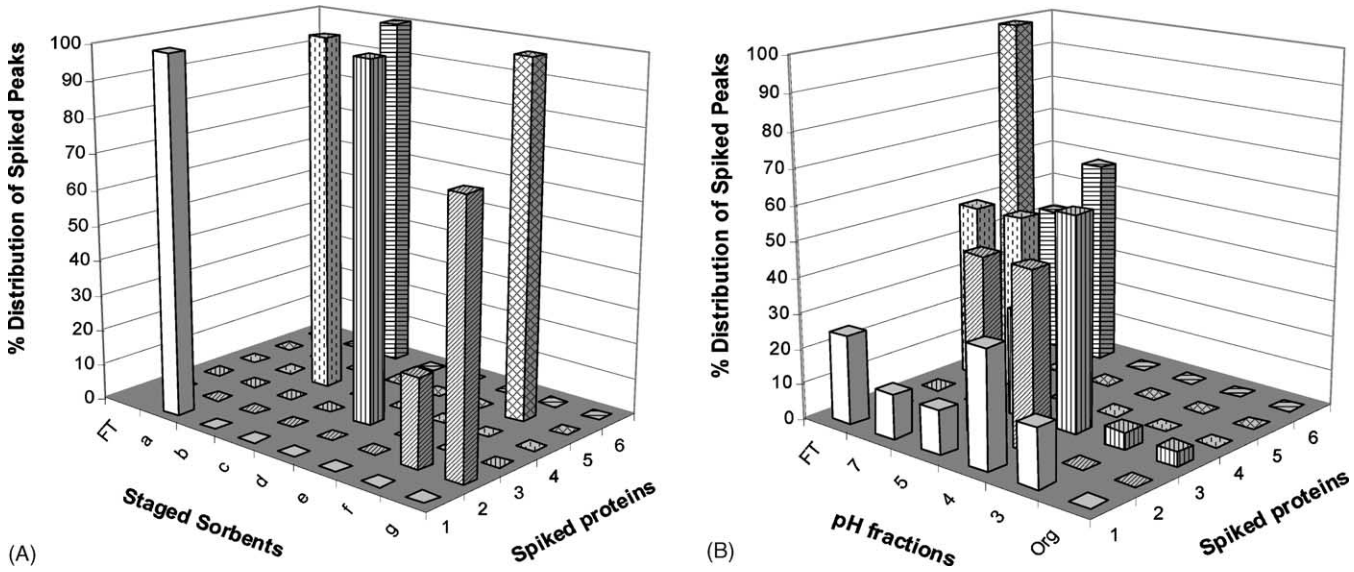

Fig. 6. Distribution of spiked proteins/peptides in human serum throughout fractions obtained by multistaged (A) and anion-exchange fractionation (B). Spiked proteins were porcine dynorphin A (1), bovine insulin chain B oxidized (2), bovine insulin (3), bovine ubiquitin (4), horse cytochrome *c* (5) and horse apomyoglobin (6). The mass spectrometry analysis was performed throughout the fractions of either single stages of elution (from a to g and flow-through FT) or pH eluted fractions (from FT, pH 9–3 and organic elution, org). The presence of each individual spiked peptide was calculated as a percentage of total. For more details, see Section 2.

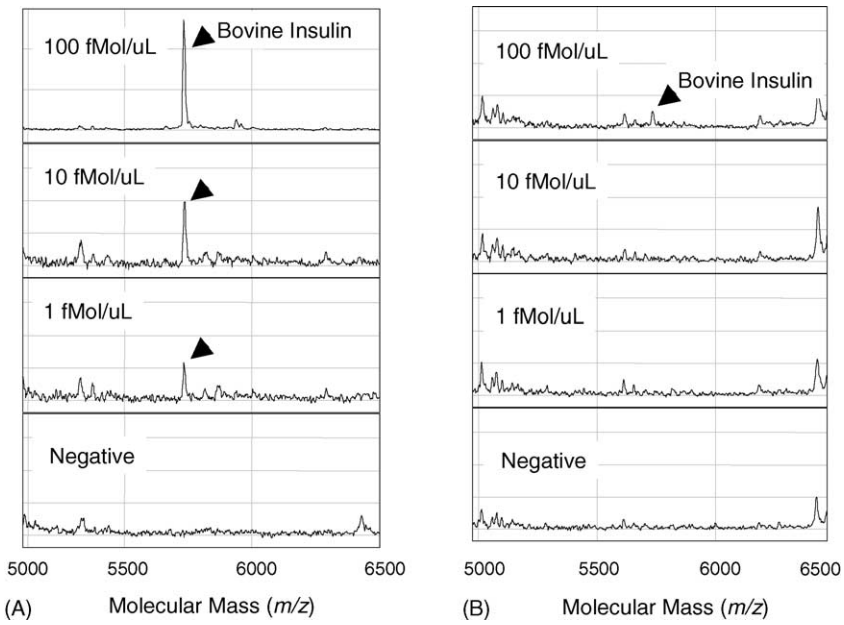

Fig. 7. Detection of bovine insulin spiked in human serum after multistaged (A) and anion-exchange (B) fractionation. Spiking concentrations were 0 (negative control), 1, 10 and 100 fmol/μL. Analytical mass spectrometry determination was performed using CM10 ProteinChip array. *m*/*z* represents the mass over the charge.

orption is produced essentially under a batch mode. A direct consequence of this situation is an unavoidable peak tailing which induces the presence of same species over different fractions. On the contrary, when using a multistaged sorbents addressing different group of proteins, entities are segregated within their location and never cross the neighboring sorbent. In fact, before desorption the different superimposed stages are disassembled and eluted separately in a single step. By nature, this approach does not generate peak tailing, therefore, the spreading of species over different chemistries or fractions is very limited especially when sorbents are used under non-saturated conditions.

Fig. 5 compares the number of mass spectrometry peaks and their distribution between anion-exchange captured fractions followed by pH fractionation and multiple sorbent captured fractions followed by one-step desorption. It appears clearly that the main advantages are in the peak count, on one hand, and in the discovery of a significant larger number of species when using IMAC and carboxymethyl (CM) chips, on the other hand.

Single proteins dispersion over different fractions was investigated by adding trace amount of well-known proteins of identified molecular masses in the human serum prior fractionation. This study was also performed in parallel to compare the performance of the multistaged sorbents versus the single anion exchange. As represented in Fig. 6, all spiked polypeptides detectable on IMAC ProteinChip array were found in different stages of the superimposed sorbent layers. Only bovine insulin chain B (M_r 3495) was found in two fractions, where the essential amount was captured by stage g, while a minor amount was found in column section f. With respect to anion-exchange separation, a large overlapping was found for all spiked species except for horse cytochrome *c* (M_r 12,360). Porcine dynorphin (M_r 2147) was for instance equally spread out through all the anion-exchange fractions. This situation induces a poor detection of species as a result of their dilution over different fractions. In a typical experiment, this phenomenon was clearly identified with the detection of traces of insulin. Fig. 7 shows, in fact, that the detection of insulin by using the described method was much more sensitive than the anion-exchange fractionation. In a comparative experiment, it was easily possible to detect this protein at a concentration of 1 fmol/μL compared to 100 fmol/μL when using anion-exchange fractionation. Insulin was selectively captured by stage d, and therefore, concentrated, explaining thus, the enhanced detection signal. On the contrary when referring to the behavior of insulin on anion exchange (Fig. 6), it was spread over four different fractions. This situation ended up to an easy detection of insulin at only 1 fmol (5.7 pg) per μL of serum. In comparison, the same level of initial spiking was not detectable by using the single anion-exchange fractionation. In practice hundred times higher concentration was necessary to obtain similar mass spectrometry signal intensity.

4. Conclusion

This is the first report that multiple layers of sorbents is used for a pre-fractionation of a protein mixture by discrete groups demonstrating its ability to fractionate human serum into populations of proteins. Such a situation is particularly useful for a complete analysis of a proteome for the purpose of either the discovery of unknown species or to evidence

differences between two proteomes for diagnostic purposes. The technology demonstrated an unequivocal superiority to an anion-exchange pre-fractionation in terms of protein redundancy from fraction-to-fraction with consequent benefit to the peak count and increased sensitivity of detection.

The same principle could be adapted to a large number of other configurations according to the complexity of the situation and the type of proteome. It is anticipated that this method would be extended to the use of resins that are specific for families of proteins, such as categories of glycoproteins or nucleic acid interacting proteins or phosphoproteins by simply superimposing selective adsorbents. This approach is different from the use of antibodies, since these latter are specific for a single protein rendering here the method ineffective for group separation. However, it can be said that antibody columns can easily be used in association with the described method when a single protein is to be removed before the sub-fractionation of the remaining proteome.

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