Immunodepletion of high abundance proteins coupled on-line with reversed-phase liquid chromatography: A two-dimensional LC sample enrichment and fractionation technique for mammalian proteomics

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ABSTRACT

Proteomic analysis can be hampered by the large concentration distribution of proteins. Immunoaffinity techniques have been applied to selectively remove high abundant proteins (HAP's) from samples prior to analysis. Although immunodepletion of HAP's has been shown to enable greater detection of low abundance proteins, the resulting fractions are often diluted 5–10-fold during the process. Various concentration techniques can be applied; however, many are incompatible with the high salt content of the fractions. To help overcome this limitation, a two-dimensional liquid chromatography (2D-LC) method was developed which couples an IgY immunodepletion column in the first dimension with a large pore C18 analytical column in the second. A protein trap cartridge serves as an injection loop between the columns to facilitate on-line concentration and desalting. Feasibility of this 2D-LC system was demonstrated for mammalian proteomics. Beyond depletion of interfering proteins, this instrumentation provides four advantages which make immunodepletion technology more convenient, including: (1) on-line desalting (2) automatic buffer exchange (3) facile concentration and (4) fractionation by polarity.

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1. Introduction

Multidimensional separations are a common tool in proteomic research because biological sample matrices are immensely complex and often feature a mixture of cells, organelles, proteins, peptides, small organic molecules, salts, and dissolved gases. Although careful extraction procedures can eliminate many interfering species, the complexity of the proteome alone can overwhelm most analytical instrumentation. For instance, a dynamic range of 4–5 orders of magnitude is superb for most analytical instrumentation, yet the proteome spans 12 orders of magnitude [1–5]. In addition, the broad range in molecular weight (MW), isoelectric point (pI), and polarity of proteins complicates separation, identification, and characterization. Due to the high complexity of the sample matrix, a single chromatographic or electrophoretic separation typically does not provide enough resolution for adequate protein characterization [6]. For this reason, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has become a workhorse technique in proteomics because it can separate proteins by two orthogonal modes: pI and MW. However,

2D-PAGE is slow and labor intensive; as a result other multidimensional approaches have been sought [7–11].

Multidimensional protein separations have been practiced for almost 20 years. One of the first methods coupled cation exchange chromatography with size exclusion chromatography to demonstrate feasibility of comprehensive protein separations with enhanced resolution and peak capacity [6]. Since then, many modes of chromatographic and electrophoretic separation have been coupled including: reversed-phase liquid chromatography (RPLC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), affinity chromatography, capillary zone electrophoresis (CZE), capillary isoelectric focusing (cIEF), capillary gel electrophoresis (CGE), capillary electrochromatography (CEC), and micellular electrokinetic chromatography (MEKC) [7,8,11]. Many of these methods utilize electrospray mass spectrometry (ESI-MS) to add an additional third and/or fourth dimension of separation to further isolate species of interest for identification and characterization [7–11].

In some cases, multidimensional separation still does not provide adequate resolution to enable good protein characterization because high abundance proteins (HAP's) can interfere with or mask information from lower abundance species. For this reason, selective depletion of HAP's is becoming an increasingly common method of sample pretreatment. Depletion can be helpful because

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the 12 highest abundance proteins (HAP's) are estimated to comprise less than 0.1% of the total number of proteins in mammalian plasma while they make up more than 96% of total protein by mass [12]. HAP's and their isoforms also span a wide range of polarity, pI, and MW, which further increases the potential for interference. Protein depletion/enrichment methods such as precipitation, solid phase extraction (SPE), ultracentrifugation, molecular weight separation, pI separation, and affinity chromatography have been developed to remove HAP's; but these methods do not necessarily provide specific depletion of interfering proteins [1,2,4,5,13–15]. Alternatively, immunodepletion can provide highly selective depletion of multiple HAP's simultaneously [4,5]. For instance, columns have been developed which can provide specific depletion of up to 21 HAP's and up to 87 mid abundance proteins (MAP's) in a second step [3,12]. In addition, commercial availability of immunodepletion columns for rat, mouse, dog, cattle, human, and plant HAP's has made the technique widely accessible [12,16]. This technology has been applied to improve detection of LAP's for a variety of applications, including: drug toxicity determination, cancer screening, and investigation of inflammatory response [17–19]. Despite these successes, immunodepletion is not suitable for enrichment of all low abundance proteins. Non-specific binding can still occur leading to loss of the protein of interest [20].

Although immunodepletion of HAP's can enable greater detection of low abundance proteins, the resulting fractions are typically diluted 5–10-fold during the process [4,5,21]. Various concentration techniques such as precipitation, lyophilization, or ultrafiltration can be applied; however, these methods tend to be time consuming and inconvenient. Often, a subsequent sample handling step such as SPE or RPLC fractionation is employed to provide concentration, desalting, and further fractionation of the protein samples. This methodology has been demonstrated to enhance mass spectrometry identification of proteins [4,5]. Although the method is amenable to automation, immunodepletion has not been coupled to RPLC because many immunodepletion columns can only be operated at low pressures (<100 psi). In this study, a two-dimensional liquid chromatography (2D-LC) system was developed which couples an IgY immunodepletion column with a large pore C18 analytical column. A protein trap cartridge serves as an injection loop between the columns to facilitate online concentration and desalting, while also providing a disconnect between the immunodepletion column and the higher pressure RPLC column. Feasibility of this 2D-LC system was demonstrated for mammalian proteomics. Samples were automatically depleted of HAP's, desalted, and fractionated by polarity. Facile fraction concentration was demonstrated, and the use of an autosampler and fraction collector enabled automated sample processing. The 2D-LC system was evaluated and downstream proteomic analysis was demonstrated after HAP depletion.

2. Experimental

2.1. Sample preparation

For instrument development and to demonstrate feasibility, 40 μL of hamster serum was diluted to 100 μL with Tris buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4). Samples were loaded into 300 μL plastic microinserts in 2-mL HPLC autosampler vials. The serum was collected from sacrificed male Golden Syrian hamsters (LVG strain, Charles River, Wilmington, MA). The animal study was approved by the Animal Care and Use Committee, Western Regional Research Center, USDA, Albany, CA. Hamster serum was chosen over mouse or rat serum because of its availability from an unrelated study. All chemicals and protein standards (including albumin of rat, mouse, and hamster) were obtained from
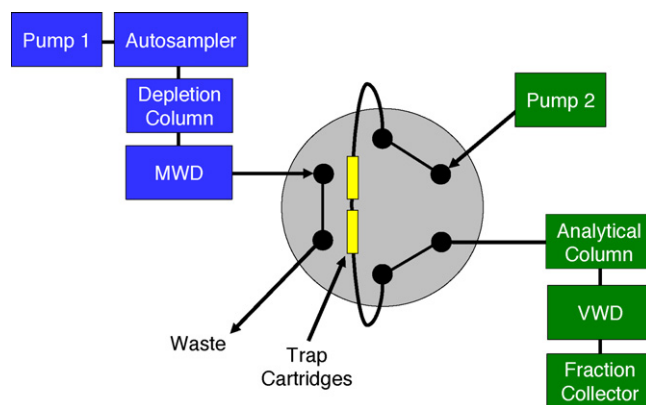

Fig. 1. Instrumental diagram of the 2D-LC HAP depletion instrument. The first dimension (blue) employs an autosampler, quaternary pump, microdegasser (not shown), immunodepletion column, and multi-wavelength detector. It is plumbed to the second dimension (green) with a 6-port valve with protein trap cartridges (yellow) serving as the injection loop. The valve is shown in position 1 in this diagram. The second dimension has a binary pump, microdegasser (not shown), reversed phase analytical column, and variable wavelength detector. Eluent from the second dimension can be collected with a fraction collector. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Sigma–Aldrich unless otherwise stated. Solvents were purchased from J.T. Baker.

2.2. Two-dimensional depletion

Two Agilent (Santa Clara, CA) 1100 HPLC systems were combined to create a two-dimensional LC, and the Chemstation software was updated to manage the new instrumentation. The first dimension consisted of a quaternary pump, microdegasser, autosampler, thermostated column compartment, and multi-wavelength detector (MWD). The second dimension employed a binary pump with its own microdegasser, thermostatted column compartment, and a variable wavelength detector (VWD). A 6-port valve in the second column compartment served to couple the two-dimensions (Fig. 1).

An immunodepletion column kit was purchased from Beckman Coulter (Fullerton, CA) (part# A25404) to selectively deplete albumin, IgG, IgM, α-transferrin, haptoglobin, α1-antitrypsin, and fibrinogen from rat serum. Immunodepletion was performed in the first dimension using three mobile phases: dilution buffer (10 mM Tris, 150 mM NaCl, pH 7.4), stripping buffer (100 mM glycine, pH 2.5), and neutralization buffer (100 mM Tris–HCl, pH 8.0). The buffers were all prepared from $10\times$ concentrates supplied with the column kit. Column temperature was not controlled in this dimension, and detection was performed by UV absorbance at 220 and 280 nm. Injection volumes of 100 μL were used for all samples. The pump program recommended by Beckman was modified to provide a 15 min lag before stripping to enable for separation of the depleted fraction in the second dimension (Table 1).

The 6-port valve coupling the two dimensions was switched to Valve Position 2 to trap protein peaks as they eluted from the first dimension. The trapped proteins were subsequently injected onto the second dimension for reversed-phase separation by switching back to Valve Position 1. Proteins were trapped by two 3 mm × 8 mm, 20 μm, C_4, 4000 Å pore, trap cartridges from Michrom Bioresources, Inc. (Auburn, CA) (part# TR1/25110/00). The valve timing was adjusted to separately trap both the unbound and bound protein bands eluting off of the first dimension and then allow separation in the second (Table 2).

The second dimension employed a Waters (Milford, MA) XBridge™ BEH300, C18, 2.1 mm × 100 mm, 3.5 μm column.

Table 1
Pump program for immunodepletion in the first dimension with a 2 mL format depletion column.

Time (min)	Dilution buffer (%)	Stripping buffer (%)	Neutralization buffer (%)	Flow rate (mL/min)
0.00	100	0	0	0.1
10.00	100	0	0	0.1
10.01	100	0	0	0.2
17.00	100	0	0	0.2
17.01	100	0	0	1.0
36.00	100	0	0	1.0
36.01	0	100	0	1.0
50.00	0	100	0	1.0
50.01	0	0	100	1.0
56.00	0	0	100	1.0
56.01	100	0	0	1.0
62.99	100	0	0	1.0
62.00	100	0	0	0.1
65.00	100	0	0	0.1

Table 2
Timing used to switch the 6-port valve in the second column compartment.

Time (min)	Valve position
0.0	1
12.5	2
19.0	1
39.0	2
45.0	1

Column temperature was controlled at 50 °C with a flow rate of 0.5 mL/min, and detection was performed by UV absorbance at 220 nm. Separation was performed with a steep gradient from mobile phase A (0.05% TFA in 98/2 H_2O/ACN) to mobile phase B (0.05% TFA in 10/90 H_2O/ACN). The gradient program is shown in Table 3. 0.5 mL fractions were collected during each 15 min gradient with a Gilson FC-203B fraction collector (Middleton, WI). Fractions were concentrated by evaporating the solvent under a stream of N_2. The dried fractions were frozen at −20 °C until reconstitution in nuclease free water for downstream analysis.

2.3. System characterization

An Agilent (Santa Clara, CA) Model 2100 Bioanalyzer with the Protein 80 kit (part# 5067–1515) was employed for qualitative comparison between the raw samples and depleted fractions for both mammalian and plant depletions. Kit instructions were followed for sample and chip preparation. Briefly, each fraction was reconstituted in 4 µL of nuclease free water and combined with 2 µL of Protein 80 Sample Buffer. Denaturing conditions were employed, so the sample buffer contained 3.5% β-mercaptoethanol. The resulting mixture was spun at 2000 rpm for 45 s and then heated at 90 °C for 5 min. Condensate was then spun down at 10,000 rpm for 10 s. 84 µL of nuclease free water was then added to the samples, and they were vortexed for 5 s. Protein ladder supplied with the kit was prepared in the same fashion. A new chip was employed for each analysis. Each chip was primed with Gel-Dye Mix, and the reagent reservoirs were loaded appropriately with either 12 µL of Gel-Dye

Table 3
Pump gradient program used to perform the reversed-phase separation in the second dimension.

Time (min)	Mobile phase B (%)	Time (min)	Mobile phase B (%)
0.00	0	46.00	0
20.00	0	47.00	0
21.00	0	61.00	100
36.00	100	62.00	0
37.00	0	65.00	0

Mix or Destaining Solution. 6 µL of each sample and the protein ladder were loaded into the sample reservoirs of the chip, and it was placed immediately in the Bioanalyzer. The Sample Buffer, Gel-Dye Mix, and Destaining Solution were all provided with the Protein 80 kit. The Bioanalyzer software was used to construct "gel images" from the sample electropherograms.

Protein recovery, run-to-run carryover, protein breakthrough, and cross species binding capacity were measured for this system. Recovery and carry over were determined for angiotensin I, ribonuclease A, and phosphorylase B. Prior to depletion, calibration standards were prepared with the model proteins, and a calibration curve was generated using only the second dimension (trap cartridge and RPLC column) of the instrument described above. Next, three, 250 µg/mL model protein standards spiked into serum were depleted in series using the 2D-system. Protein recovery during depletion was quantified using response factors determined for the VWD with the calibration above. In addition, the resulting fractions were collected, dried under a stream of N_2, and reconstituted in 100 µL of nuclease free water. These fractions were analyzed using the same system in 1D-mode to compare recovery after sample handling. Carryover was determined by running a blank depletion with a sample of nuclease free water after the three model protein depletions. Model protein detected in this blank run was carried over from the previous three depletions.

Protein breakthrough of the trap cartridge was determined by comparing the total protein content of the trap waste stream during loading to the total protein content of the flow-through fraction. Hamster serum was depleted thrice following the method described above, and the waste stream from the trap cartridge was collected as the unbound protein fraction flowed through it. For comparison, three more depletions were performed on hamster serum, and the unbound protein band was captured before it entered the trap cartridge. Samples were concentrated with 10 kDa MWCO spin filters, and total protein was measured with a Bradford assay. Finally, binding capacity was determined for rat, mouse, and hamster albumin to demonstrate cross-species applicability of the system. 0.5–6.0 mg/mL samples of albumin were depleted, and the albumin peak in the depletion chromatogram was measured and plotted as a function of concentration to estimate binding capacity.

2.4. Peptide mass fingerprint

Depleted fractions of hamster serum were separated by 1D-PAGE on a Criterion 10.5–14% Tris–HCl gels from BioRad (Hercules, CA; Part# 3459950). Bands of interest were excised and incubated with trypsin at 37 °C overnight. Peptides were extracted from the gels with 50% ACN and 0.5% TFA in 25 mM ammonium bicarbonate buffer. Peptides remaining in the gel were then extracted with

70% ACN and 5.0% formic acid in 25 mM ammonium bicarbonate buffer. The extracts were pooled and dried in a vacufuge. The dried peptides were reconstituted in 18 μL of 0.1% aqueous TFA and loaded onto a Ziptip[TM] (Millipore part# ZTC04S960). Peptides were eluted off of the Ziptip[TM] in four fractions: 10% ACN, 25% ACN, 50% ACN, and 75% ACN. 1 μL of each fraction was deposited on a MALDI plate and allowed to dry. 1 μL of matrix solution (1.0% α-cyano-4-hydroxycinnamic acid, 50% ACN) was spotted on top of the sample spots and allowed to dry. The protein digests were analyzed on a Voyager DE STR MALDI-TOF MS from Applied Biosystems.

All MALDI-TOF MS data were processed manually. The spectra of each spot were summed, smoothed, and *m/z* error corrected. The peptide mass data was searched using the program MASCOT against a nonredundant database (NCBlnr) or Swiss-Prot database located at the Matrix Sciences web site (Internet address: http://www.matrixscience.com) [22]. Typical search parameters were as follows: consideration of up to one incomplete cleavage site per peptide, potential for modification (cysteine oxidation, amidation, and acetylation), peptide mass tolerance of 250 ppm, tryptic peptides, and singly charged monoisotopic peaks.

3. Results and discussion

Immunodepletion is a powerful proteomic tool because it can selectively remove HAP's to enable better detection and identification of lower abundant proteins. In addition, resins produced with polyclonal antibodies can tolerate modest changes in sequence homology, which enables a single column to maintain good depletion efficiency across a number of species [21]. A primary drawback of the technology, however, is the dilution of the proteins into high salt buffers during the depletion process. The dilution of the depleted protein fractions necessitates additional handling steps such as concentration, but concentration techniques are limited to those which can selectively concentrate proteins without increasing the salt content. Ultrafiltration is the recommended process for concentration, but it is time consuming and there is potential for sample loss because of the additional handling steps. Instead of ultrafiltration, protein precipitation, SPE, and off-line RPLC separation have been used [4,5]. To overcome the aforementioned limitations and improve automation of subsequent fractionation techniques, a 2D-LC system was set-up to couple HAP depletion with a reversed-phase analytical column to automate desalting, enable buffer exchange, facilitate concentration, and to fractionate the proteins.

To enable coupling of depletion with RPLC, two Agilent 1100 HPLC's were plumbed together through a 6-port valve in the second column compartment (Fig. 1). Immunodepletion was performed in the first dimension following a method which is similar to that recommended by the manufacturer (Table 1). First 100 μL of sample is injected onto the column, and low flow rates are employed to provide time for HAP binding. The flow rate is then ramped up to sharpen the peak shape of the unbound fraction as it elutes. Next, a low pH buffer is applied to strip bound proteins from the column. After stripping the bound proteins, the column is neutralized and then re-equilibrated with dilution buffer. As the proteins leave the first dimension, they flow through large-pore (4000 Å) C$_4$ cartridges which trap the eluting proteins. The trap cartridges are plumbed as an injection loop in the 6-port valve. After the entire ~5 min wide peak is loaded onto the traps, the valve is switched and flushed with mobile phase A for 1 min to desalt the fraction. Next, an ACN gradient is applied for separation of the proteins in the second dimension. The analytical column is re-equilibrated before elution of the bound protein fractions from the first-dimension column, so that they can be desalted, concentrated, and fractionated as well.

With this method, two separations are performed in the second dimension (one for unbound and one for bound proteins) for every immunodepletion in the first dimension.

The depletion technology utilized in this study requires a few considerations for coupling to an additional dimension. Because of the requirement for three buffers for immunodepletion, an HPLC with ternary or quaternary pump capability is required in the first dimension. In addition, the length of time after elution of the unbound proteins and before application of the stripping buffer was increased by 20 min to facilitate separation and equilibration in the second dimension. A similar delay was not provided for the bound protein peak because the neutralization and equilibration steps in the first dimension can be run in parallel with the reversed-phase separation of the bound proteins. Because the reversed-phase separation is performed in parallel, addition of the second dimension only increases the time required for depletion by a total of 15 min. However, should the reversed-phase separation need lengthened to increase resolution, the first dimension can accommodate this change by increasing the time before stripping the bound proteins, and extending the final equilibration with the dilution buffer after the neutralization step. To preserve the integrity of the column, it is important to not change the length of the stripping or neutralization steps in the first dimension because low and high pH can strip the antibodies from the column over time. A longer second dimension separation can also be used if the bound protein fraction is not of interest. The method can be changed to divert the bound proteins to waste as they elute from the first dimension thus providing up to 45 min for the reversed-phase separation of the unbound proteins.

In addition to buffer considerations, pressure limits of the depletion column were taken into consideration. The commercial depletion column employed for this study is intended for low-pressure applications. Its maximum operating pressure is rated at 100 psi, but high performance analytical columns are typically operated at pressures in excess of 1000 psi. To facilitate 2D separations, pressures of the first and second dimension needed to be decoupled from one another. A trap cartridge was employed for this purpose. The traps used were selected for their large particle size, short path length, and HPLC pressure tolerance. The large particles enabled loading of the protein bands from the first dimension at ~75 psi, while its pressure tolerance enabled the use of a high efficiency analytical column in the second dimension. The trap phase was also selected for its efficient protein binding, compatibility with high salt concentrations, and compatibility with common reversed-phase solvents. Although ACN and TFA were used here, the composition of the second dimension mobile phases can be altered to improve resolution or to accommodate a third dimension. Because the trap desalts the sample, it should be more compatible with other orthogonal techniques such as electrophoresis. An ancillary advantage of desalting is that the bound protein fractions do not require neutralization (an additional handling step with conventional immunodepletion) because they are not eluted in the acidic stripping buffer.

Before plumbing the second dimension column, feasibility of concentration with the trap cartridge was demonstrated. A sample of hamster serum was depleted and the unbound and bound protein fractions were concentrated and desalted on the trap cartridge. The peak width of the protein fractions was decreased from ~6 min to 45 s which decreased the volume of the fraction eightfold without additional concentration steps (Fig. 2). In this automated concentration mode, more protein bands are observed when using the Bioanalyzer (Fig. 3) as expected. Further fractionation with the addition of a separation in the second dimension allows detection of 4-times more protein bands (Fig. 4) than are detected in the depleted (FT) fraction alone (Fig. 3). These proteins are likely mid-abundant proteins (MAP's) due to the limited sensitivity of the
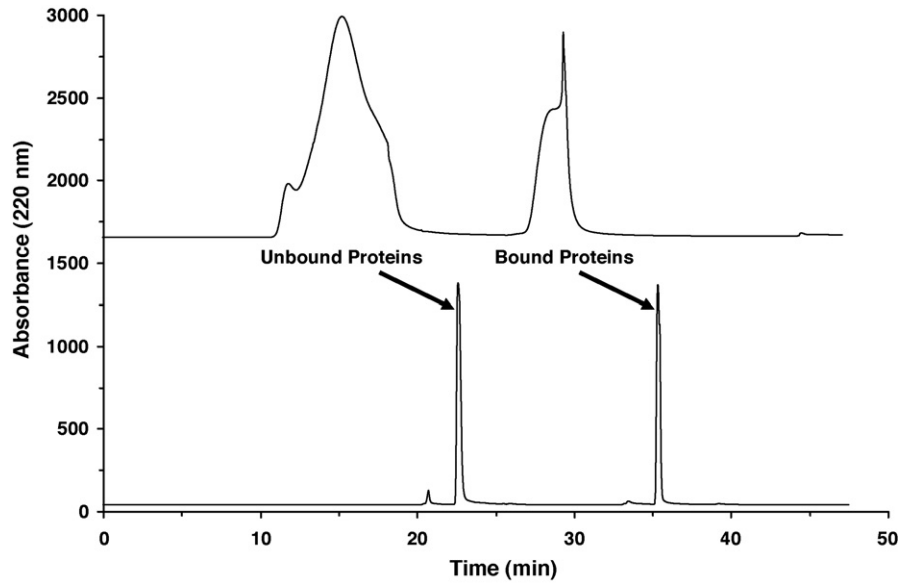
Fig. 2. Chromatograms illustrating the capability of the protein trap to concentrate proteins. The top chromatogram is typical of immunodepletion with the IgY columns used in this study. The HAP's are selectively removed, but the protein bands are broad and dilute. The C$_4$ trap cartridge captures these bands as they elute from the first dimension. They are then held until a low polarity mobile phase releases them from the stationary phase in the trap. The ~6 min wide band of unbound proteins is concentrated into a 45 s band with this method.

Bioanalyzer. Compared to analysis of undepleted serum, the fractionated samples have greater than 10× more discrete bands which are separated and detected (Fig. 5). Although the chromatograms collected with this system show fair separation over the short gradient of the second dimension (Fig. 4), resolution could be improved if necessary by extending the length of the separation as described above.

A potential advantage of this technology is that it is applicable across at least six mammalian species and a number of plant species, including: rat, mouse, human, dog, cattle, spinach, arabidopsis, and canola [21]. Because the binding/stripping chemistries are similar, many of these columns can be used in the first dimension with minor modification to the method as it is described in Tables 1–3. In addition, these columns have been shown to be applicable to species which share HAP's with similar sequence homology. For example, the plant proteomic depletion column was developed for spinach, but it has been shown to work well with arabidopsis, canola, corn, and tobacco [21]. Additionally, the rat HAP depletion column used in this study provided similar binding capacity for rat, mouse, and hamster albumin (~300 μg/mL for each). Mouse and hamster albumin share 89% and 84% sequence homology with rat albumin, respectively. Due to the dearth of information on the hamster proteome, sequence homologies of the other HAP's depleted with this column are unknown.

To measure trap efficiency, effluent from the trap cartridge was collected during protein loading and desalting. Bradford assays were performed on the effluent to measure total untrapped protein. With one trap cartridge, approximately 110 ± 6 μg of protein ($n = 3$) (or 14% of the depleted band) was detected in the effluent.
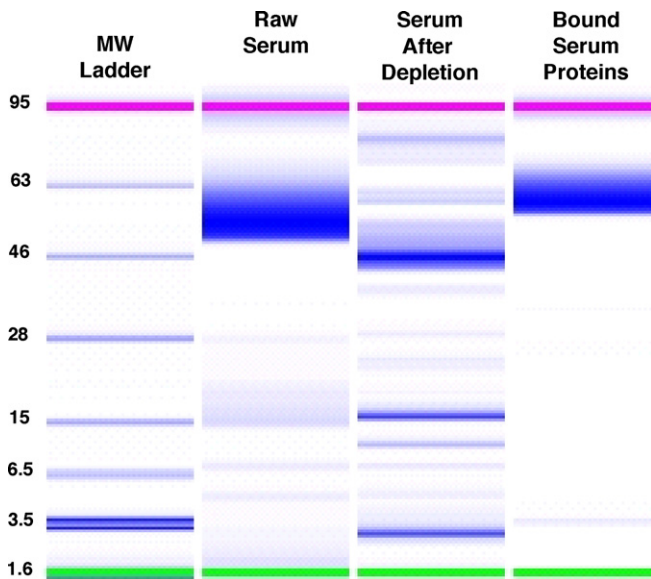

Fig. 3. Gel image comparing raw serum with the focused fractions (unbound and bound) from Fig. 2. This figure illustrates the power of immunodepletion at improving detection of lower abundance proteins, and the protein trap cartridge enables depletion without additional desalting and concentration steps which are usually necessary.
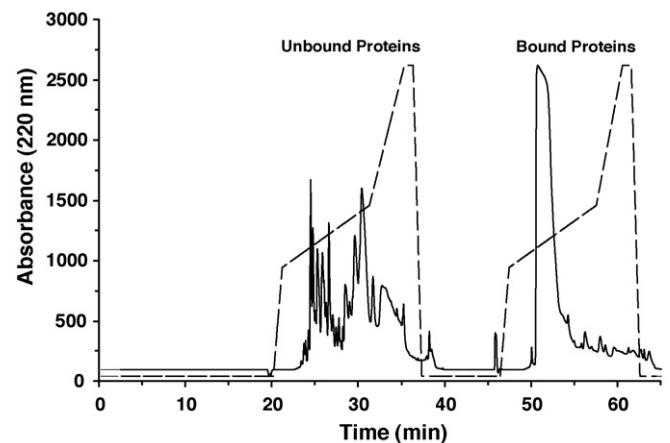

Fig. 4. Chromatogram showing the second dimension separation. With the 2D-LC immunodepletion system, the focused protein bands on the trap cartridge (Fig. 2) are separated by polarity. Fractions of eluent collected from this dimension are enriched in LAP's and do not require extensive sample clean-up steps before downstream proteomic analysis.
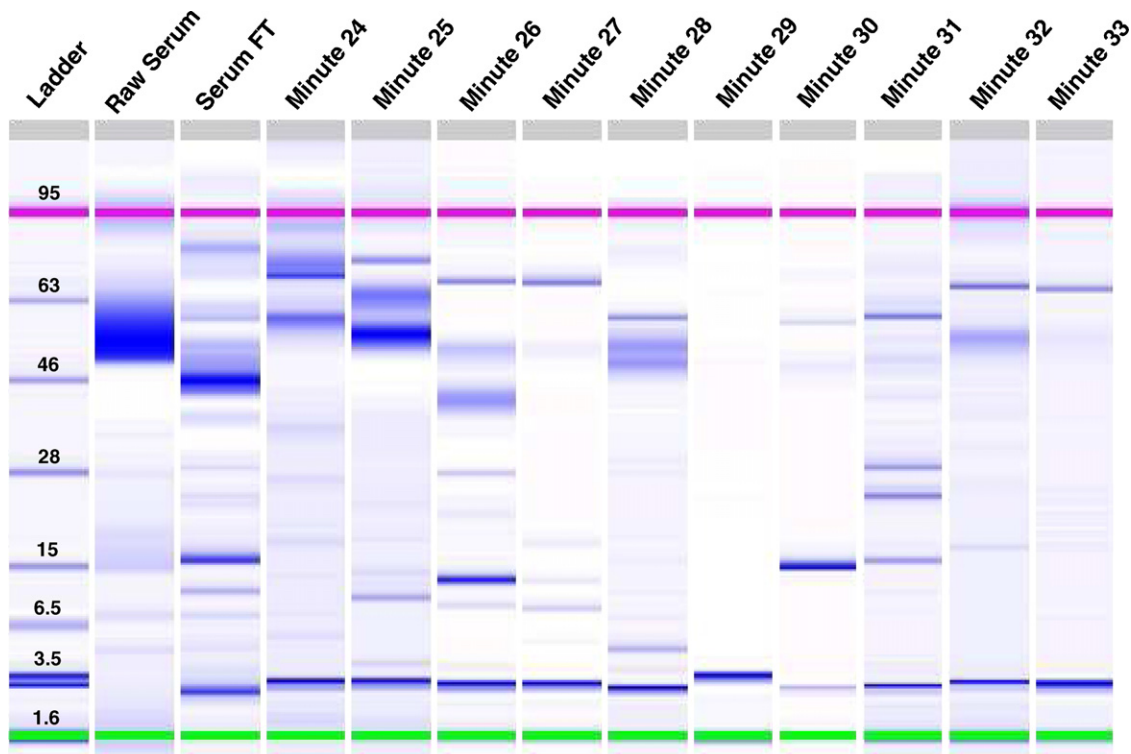
Fig. 5. Gel image of fractions of hamster serum after depletion and separation in the second dimension. After 2D depletion and separation, 10× more protein bands are detected compared to raw serum, and 4× more bands are detected compared to depleted serum alone (lane labeled Plasma FT). It should be noted that some shift in MW in each of the lanes is not uncommon for this chip-based assay.

This loss was thought to result from overloading of the trap cartridge, which is rated to bind approximately 200 µg of protein by the manufacturer. We have found that the actual capacity of these cartridges is higher as more than 800 µg of protein was retained by the trap in the above experiment. To improve capacity, two traps were plumbed in series. With two traps, 1.2 µg of protein was detected in the trap waste stream during loading, which represents approximately 0.1% of the total protein in the depleted fraction. The addition of a second trap causes some peak broadening in the second dimension separation (Fig. 6). Broadening likely results from the additional bed volume of the second trap as well as dead vol-
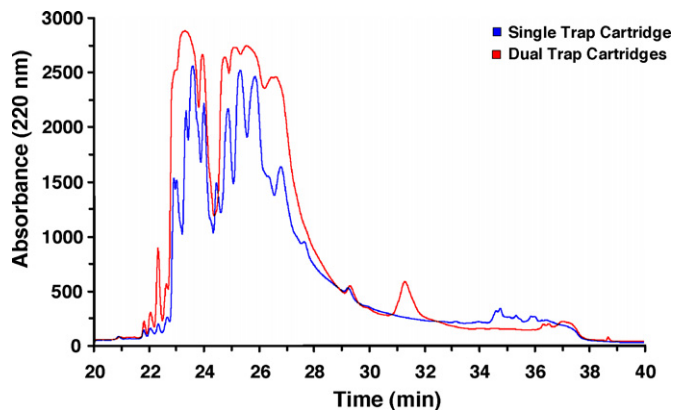

Fig. 6. Overlay of the second dimension separation of the flow-through fraction collected by one versus two trap cartridges. Although the additional cartridge improves trapping efficiency, efficiency of the second dimension separation is sacrificed. The broadening likely does not impact fractionation to a large extent because relatively large fractions (~1 min) were collected for this study. A larger capacity cartridge or less protein loading would likely solve this problem if better resolution is required.

ume of the tubing and unions necessary for connection. A larger capacity trap was unavailable at the time of this study, but it would likely enable better separation efficiency in the second dimension. Although most proteins are captured by the trap cartridges, small, polar proteins and peptides may escape due to their lower capacity factor in polar solvents. These polar molecules would also escape the MWCO filters used in determination of trap binding efficiency. To estimate trapping efficiency of polar peptides, recovery of angiotensin I was quantified. The trap was found to retain greater than 98% of angiotensin I. In addition, RNase A and phosphorylase B (14 and 97 kDa, respectively) recoveries were measured. RNase A shares similar recovery with angiotensin (>97%), but phosphorylase B showed significant loss (~60% recovery). Although the mechanism of loss is unknown, precipitation in the trap cartridge and/or analytical column was a concern. Protein precipitation is known to degrade column stability over time without special regeneration steps or measures to limit precipitation [4]. However, the retention time and detection stability of this method make precipitation unlikely. In a stability experiment, 30 injections of raw serum were performed over two days. Retention times were stable with an R.S.D. of less than 0.1% (15 peaks measured) and an average peak area R.S.D. of less than 10.0% (for the same 15 peaks).

In addition to improved peak capacity and detection through concentration, traditional peptide mass fingerprinting (PMF) can be applied after on-line depletion and fractionation. To demonstrate PMF, fractions of hamster serum were separated by 1D-PAGE, bands of interest were excised and digested, and the tryptic peptides were identified by MALDI-TOF MS. Tentative identifications of albumin, immunoglobulin, and transferrin were made from the bound fractions; and tentative identifications of coagulation factor XIII, ribonuclease, fibrinogen, fibulin, and ferritin were made from the unbound fractions. Although good sequence coverage was attained and the MW of the protein bands in the gel correlated

Table 4

Demonstration of protein identifications by PMF after on-line depletion and RPLC. The fraction time refers to the time shown in Figs. 4 and 5. The presence of albumin precursor in many of the bound fractions demonstrates how much of the separation space is taken by albumin in RPLC. Although good sequence coverage and MW correlations were observed, all of the identified proteins are from rats or mice. The dearth of information on the hamster proteome limits identification by PMF. Here, identifications assume that high sequence homology is shared between hamsters, rats, and mice. Identification of fibrinogen in the unbound fraction may indicate that hamster fibrinogen does not have a strong affinity to the antibodies in the depletion column. However, this would be difficult to test as there is no data on the sequence of hamster fibrinogen nor are standards available for binding studies. The immunoglobulin identified below is likely a fragment of the larger protein as the spot was excised from a region of the gel near 65 kDa.

Fraction (min)	Tentative identification	Peptides matched	Sequence coverage (%)	Known MW (kDa)	Gel MW (kDa)
25	Coagulation factor XIII	6	14.5	76.0	75
26	Ribonuclease precursor	4	30.3	17.7	15
28	Fibrinogen gamma chain precursor	10	31.2	49.4	50
	Fibulin-5 precursor	6	18.1	50.2	
30	Ferritin precursor	7	25.3	27.2	25
50	Serum albumin precursor	15	26.6	68.7	65
	Immunoglobulin super family member	12	7.0	285.6	
	Sero-transferrin precursor	14	21.6	76.7	
51	Sero-transferrin precursor	15	23.2	76.7	75
	Serum albumin precursor	16	28.9	68.7	
52	Serum albumin precursor	14	27.6	68.7	65
53	Serum albumin precursor	10	23.0	68.7	65

with the known MW of each protein (Table 4), all were identified as either from mouse (mus musculus) or rat (rattus norvegicus). These proteins likely share a high degree of sequence conservation with hamster (mesocricetus auratus) proteins, but the dearth of information on the hamster proteome prevented species specific database hits. Unfortunately, rat and mouse sera were unavailable to us for this study. Nevertheless, common PMF procedures such as 1D-PAGE, tryptic digestion, and MALDI-TOF MS appear to be suitable after this automated sample enrichment technique.

4. Conclusions

Feasibility of a 2D-LC HAP immunodepletion system was demonstrated for mammalian proteomics. The instrument provides four advantages which make the depletion technology more convenient, including: (1) on-line desalting (2) automatic buffer exchange (3) facile concentration and (4) further sample fractionation. Although not demonstrated in this manuscript, the advantages listed above may enable coupling of this technology to a third orthogonal technique such as ESI-MS (with on-line digestion) or SEC separation. In addition, the method should be compatible with other immunodepletion columns including those for other species.

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