Quantitation of Serum and Plasma Proteins After Enrichment of Low-Abundance Proteins With the ProteoMiner™ Protein Enrichment System

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Introduction

Biomarker discovery is an area of intense interest well suited for the application of protein analytical technologies. Biomarkers have been described as "proteins that undergo a change in concentration or state in association with a biological process or disease" (Adkins et al. 2002), and their utility as indicators of disease status or response to therapy has long been established in clinical research. Biomarker-related proteomics studies have focused on their identification, which would facilitate distinction between different states of a given disease due to their differential expression. Sensitive and reproducible methods are required to identify these quantitative differences in expression leading to the discovery of new biomarkers.

Typically, changes in protein concentration for a given disease can be measured in tissue samples or body fluids. Plasma and serum are used most often because they are easily and routinely obtained from patients and since blood is in contact with the whole organism, proteins leaked from all tissues should be present. This offers both an opportunity and a challenge: The Human Plasma Proteome Project has thus far identified the expression products of 3,778 unique genes and a total of 7,518 proteins and isoforms in plasma (Muthusamy et al. 2005; www.plasmaproteomedatabase.org). The proteins present in serum and plasma span a concentration range of 11 orders of magnitude, with the 20 most abundant proteins representing 97–99% of the total protein mass (Anderson and Anderson 2002).

No single analytical method is capable of resolving all plasma or serum proteins, and no detection method can cover more than 4 or 5 orders of magnitude. Due to limitations in resolving power and the high abundance of a relatively small number of proteins, most analytical schemes for serum and plasma involve the depletion of high-abundance proteins to reduce both the complexity and dynamic range of samples. Prior to the availability of ProteoMiner protein enrichment kits, immunodepletion, the selective binding to specific antibodies against the proteins of highest abundance, was commonly used to remove the most abundant 6, 12, or 20 serum and plasma proteins (Linke et al. 2007, Luque-Garcia and Neubert 2007, Porter et al. 2006). This approach involves binding selected antibodies to a chromatographic support. When serum or plasma proteins are incubated with the support, the high-abundance proteins are retained and the low-abundance proteins are eluted. Although this approach works well, it has the disadvantages of relatively high cost and low sample capacity (Luque-Garcia and Neubert 2007). In addition, immunodepletion can result in the loss of less abundant proteins that bind to the proteins being removed, as well as more than 100-fold dilution of the original sample, necessitating an additional sample concentration step (Linke et al. 2007).

ProteoMiner technology overcomes most, if not all, of the disadvantages of immunodepletion, while effectively depleting high-abundance proteins (Antonioli et al. 2007, Sennels et al. 2007). Combinatorial synthesis creates a diverse library of hexapeptides that act as binders for proteins. The hexapeptides are bound to beads that are packed into mini spin columns. The bead population has such diversity that a binding partner should exist for most, if not all, proteins in a sample. High-abundance proteins quickly saturate the binding partners for which they have the highest affinity, and excess proteins are washed away. In contrast, the low-abundance proteins are concentrated on the ligands for which they have the highest affinity. After incubation and elution, the concentration of low-abundance proteins is greatly increased, while the concentration of high-abundance proteins is lowered.

Unlike immunodepletion, in which the capacity of the bound antibodies limits the sample amount to typically less than 100 µl, large sample volumes of 1 ml or more can be used with ProteoMiner spin columns, thereby increasing the amount of low-abundance proteins that can be applied to the column. The increase in the concentration of low-abundance proteins and concomitant decrease in the concentration of high-abundance proteins is not dependent on sample type as with the immunodepletion methods. Additionally, the sample is recovered in a small volume, and no additional concentration steps are required for most downstream applications.

For this approach to be viable in a biomarker discovery program, the enrichment method must preserve the quantitative information for the low- to medium-abundance proteins. To demonstrate linearity of quantitation in response to increasing amounts of low-abundance proteins, serum amyloid A protein (SAA) was added at different concentrations to serum and plasma samples and analyzed using 2-D gel electrophoresis, surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, and western blot analysis. Our results show that this novel enrichment method does indeed preserve the ability to quantitate low- and medium-abundance proteins in serum and plasma and is therefore suitable for biomarker discovery.

Methods

Sample Enrichment for Low-Abundance Proteins

Serum or plasma samples (1 ml, Bioreclamation, Inc.) were spiked with 0, 1, 5, 10, 20, 40, 80, and 160 ng/µl SAA one set of these samples was used as a control while the other set was processed with the ProteoMiner large-capacity protein enrichment kit (Bio-Rad Laboratories, Inc), which contains 10 columns, each with 100 µl bead volume, as well as all of the buffers required for binding, washing, and eluting. After removing the storage solution by centrifugation, the beads were washed with deionized water followed by phosphate buffered saline (PBS). The human serum or plasma samples were then applied to the column and, to ensure effective binding, the samples were slowly rotated with the ProteoMiner beads for 2 hr prior to washing away unbound proteins with PBS buffer. To elute the bound proteins, the beads were treated 3 times with 100 µl of an acidic urea/CHAPS buffer. Protein quantitation was performed using the Quick Start Bradford assay (Bio-Rad). Two sets of samples were produced. One set was subjected to the ProteoMiner treatment described. The other was not and was used as a control set.

Two-Dimensional Electrophoresis

For 2-D gel electrophoresis experiments, 100 µg of protein was loaded onto an 11 cm ReadyStrip™ IPG strip, pH 5–8 (Bio-Rad). Isoelectric focusing was performed using a PROTEAN® IEF cell (Bio-Rad) at 250 V for 30 min followed by 8,000 V until 45,000 volt-hours were reached. After transfer of the IPG strip onto Criterion™ Tris-HCl 8-16% linear gradient gels (Bio-Rad), the second dimension was run for 1 hr at 200 V prior to staining with Flamingo™ fluorescent gel stain (Bio-Rad). Gels were imaged using the Molecular Imager® PharosFX™ system and analyzed with PDQuest™ 2-D analysis software, version 8.0 (Bio-Rad). Selected spots were excised with the EXQuest™ spot cutter (Bio-Rad) and digested with trypsin prior to protein identification using an Agilent 1100 nanoflow liquid chromatography (LC) system (Agilent Technologies, Inc.) coupled to an LTQ ion-trap mass spectrometer (Thermo Fisher Scientific).

SELDI Mass Spectrometry Analysis

For this study, a ProteinChip® SELDI system, Enterprise Edition, and ProteinChip CM10 (carboxymethyl weak cation exchange) arrays were used (Bio-Rad). The arrays were equilibrated twice with 5 µl 100 mM sodium acetate, pH 4, buffer. After equilibration, the liquid was removed and 0.5 µl ProteoMiner bead-treated serum or plasma sample was mixed with 4.5 µl 100 mM sodium acetate, pH 4, buffer and spotted onto the array. After a 30 min incubation with shaking, each spot was washed three times with 5 µl binding buffer for 5 min, followed by a rinse with deionized water. After air drying, sinapinic acid dissolved in an acetonitrile:trifluoroacetic acid:water mixture (49.5:0.5:50) was added twice in 1 µl increments and allowed to air dry. All arrays were analyzed with an ion acceleration potential of 20 kV and a detector voltage of 2.8 kV. Data processing steps included baseline subtraction and external calibration using a mixture of known peptide and protein calibrants. Peak detection (with signal-to-noise set to >3) and peak clustering were performed automatically using ProteinChip data manager software, version 3.2 (Bio-Rad).

Western Blot Analysis

20 µg of SAA-spiked human serum sample was then separated on 18% Tris-HCl Criterion precast gels as described above. Each gel was blotted onto nitrocellulose membrane (Bio-Rad). 50 ng of pure SAA was loaded in a separate lane on each gel as a positive control. The membrane blot was incubated for 2 hr in blocking buffer (20 mM Tris, 150 mM NaCl, 5% BSA, and 0.1% Tween 20). Monoclonal anti-SAA antibody 585 (GeneTex, Inc.) was prepared in the blocking buffer and applied to the membranes at room temperature for 1 hr. After three rinses in TBST buffer (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20), the membranes were incubated with HRP-conjugated goat anti-mouse antibody (Bio-Rad) for 1 hr at room temperature. After three more rinses in the TBST buffer, the membranes were incubated with HRP chemiluminescent reagents (Bio-Rad) and imaged using a Molecular Imager ChemiDoc™ XRS system (Bio-Rad).

Results and Discussion

We chose SAA, an 11.8 kD protein, as a model compound. SAA is normally present in serum at a concentration of 1–5 ng/µl, but in cases of infection, acute phase response, and inflammation, levels can increase to as much as 688 ng/µl (Berendes et al. 1997).

A series of plasma and serum samples were spiked with 0, 1, 5, 10, 20, 40, 80, and 160 ng/µl SAA. 2-D gel electrophoresis analysis of plasma samples revealed the known isoforms of SAA (Figure 1A) with measured pIs of 7.22 and 7.40, which were quantitated using image analysis. Analysis of a series of control samples (not enriched using the ProteoMiner beads) illustrated that increasing concentrations of added SAA can be visualized as increasing spot densities on a 2-D gel with a minimum detection level of about 10 ng/µl (Figure 1B). However, after ProteoMiner bead processing of identically spiked samples, the minimum detection level of SAA dropped to about 1 ng/µl due to the enrichment effects of the ProteoMiner technology (Figure 1C). More importantly, increases in the concentration of the SAA correlating to initial starting amount can be detected after processing with the ProteoMiner kit (Figure 1C), thereby preserving the quantitative information of the spiked SAA.

SELDI analysis is tailored to finding biomarker candidates in plasma or serum samples, preferably for proteins in the mass range of 3–25 kD (Biomarker Discovery Workflow Brochure, bulletin #5460, (Weinberger et al. 2002)). Analysis of samples containing SAA again demonstrated that increasing levels of SAA (11.8 kD peak) could be detected and quantitated after treatment with the ProteoMiner kit, with a minimum detection level of about 1 ng/µl. In contrast, the minimum detection level with untreated samples was about 5 ng/µl (Figure 2).

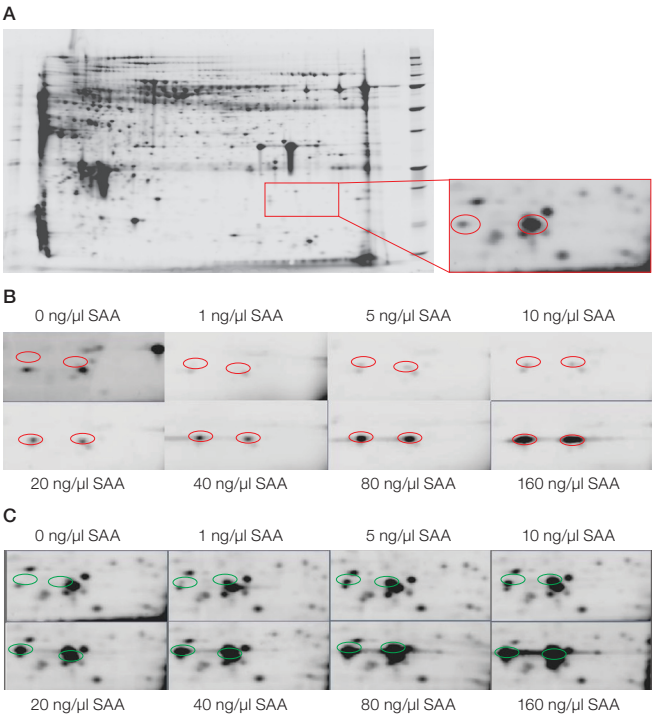

Fig. 1. 2-D gel electrophoresis analysis of control and SAA-spiked plasma processed with the ProteoMiner protein enrichment kit. **A,** plasma sample was spiked with 72 µg SAA and processed with the ProteoMiner kit. 100 µg protein from the elution fractions was analyzed by 2-D gel electrophoresis using a pH 5–8 IPG strip. Insert shows enlarged area with two SAA isoform spots (pI of isoforms 6.0 to 8.0, MW 11.8 kD). **B,** panels of 100 µg of control SAA-spiked plasma proteins analyzed by 2-D gel electrophoresis. **C,** panels of 100 µg of SAA-spiked plasma protein samples processed with the ProteoMiner kit and analyzed by 2-D gel electrophoresis. Plasma samples in (B) and (C) were spiked with 0, 1, 5, 10, 20, 40, 80, and 160 ng/µl SAA. The SAA isoforms circled in green were further identified by tandem MS analysis (data not shown).

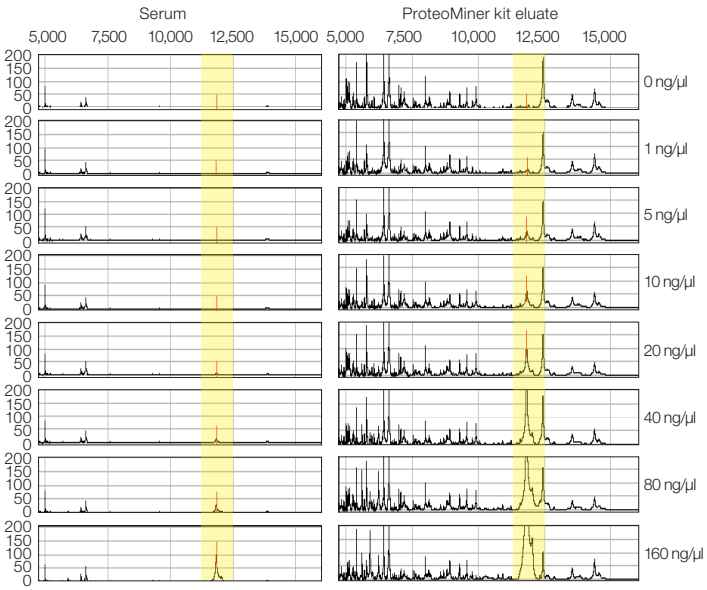

Fig. 2. SELDI analysis of SAA spiked serum treated with ProteoMiner beads. Serum samples were spiked with 0, 1, 5, 10, 20, 40, 80, and 160 ng/µl SAA. Both control (left panels) and ProteoMiner kit-treated (right panels) are shown. 0.5 µl of control and ProteoMiner eluate were analyzed with a ProteinChip CM10 array.

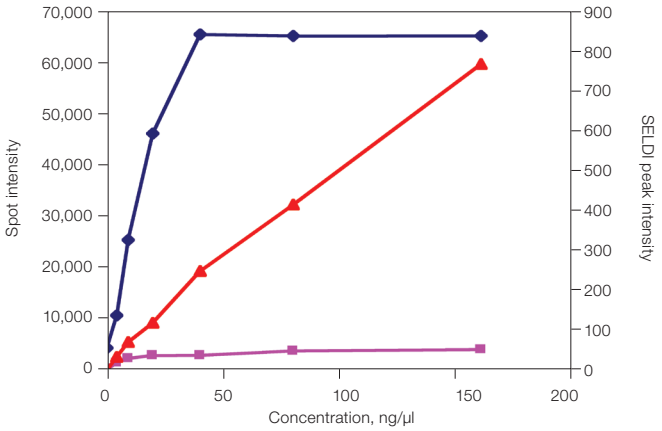

Fig. 3. Linear response range for protein quantitation by three different methods. The concentration of SAA added to human plasma samples is plotted versus spot quantitation on gel (◆) or western blot (■) and SELDI-TOF-MS quantitation (▲). Spots were quantitated on 2-D gels using the Molecular Imager PharosFX system and PDQuest 2-D analysis software, and on western blots using the Molecular Imager ChemiDoc XRS system. 2-D gel analysis is only capable of detecting changes in protein concentrations up to 40 ng/µl, and western blots can only provide information on concentrations up to 20 ng/µl.

Quantitation of the SAA protein in samples treated with ProteoMiner beads was a function of the linear range of the detection method (in the range where protein concentration differences can be detected). SELDI analysis provided the broadest linear response, from 0 to 160 ng/µl. 2-D gel electrophoresis provides a linear response from 0 to 40 ng/µl, and western blots of one-dimensional gels (data not shown) gave a linear response only from 0 to 20 ng/µl SAA (Figure 3).

Conclusions

In summary, pretreatment of plasma and serum samples with the Proteominer protein enrichment kit has been shown to provide enrichment and quantitative detection of varying levels of an added protein. SAA as a model compound exhibited a linear response versus the amount added to the plasma, demonstrating that the quantitative information for low- to medium-abundance proteins is preserved after ProteoMiner processing. Quantitation has been demonstrated with three different methods, including 2-D gel electrophoresis, SELDI, and western blots. Because treatment with the ProteoMiner kits does not preclude the ability to quantitate low- to medium-abundance proteins in serum or plasma, inclusion of this method in a biomarker discovery workflow is not only possible, it is advantageous.

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