Efficient and specific removal of albumin from human serum samples

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Abbreviations: 2DE, two-dimensional gel electrophoresis; AFP, alpha fetoprotein; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HSA, human serum albumin; Ig, immunoglobulin.
SUMMARY

Patient serum or plasma is frequently monitored for biochemical markers of disease or physiological status. Many of the rapidly evolving technologies of proteome analysis are being used to find additional clinically informative protein markers. The unusually high abundance of albumin in serum can interfere with the resolution and sensitivity of many proteome profiling techniques. We have used monoclonal antibodies against human serum albumin (HSA) to develop an immunoaffinity resin that is effective in the removal of both full length HSA and many of the HSA fragments present in serum. This resin shows markedly better performance than dye-based resins in terms of both the efficiency and specificity of albumin removal.

Immunoglobulins (Ig) are another class of highly abundant serum protein. When Protein G resin is used together with our immunoaffinity resin, Ig proteins and HSA can be removed in a single step. This strategy could be extended to the removal of any protein for which specific antibodies or affinity reagents are available.
INTRODUCTION

Serum is a rich source of biochemical products that can act as indicators of the physiological or clinical status of a patient. For instance, serum levels of hormones, cholesterol, small molecules, or enzymes and other proteins have all been assayed to provide information regarding conditions as diverse as pregnancy, cardio-vascular or nutritional status, viral disease, or cancer. It is not surprising then, that many efforts to find new biomarkers for disease detection or progression have applied emerging analytical techniques to profile the protein content of human serum.

Serum presents some unique challenges to efforts to characterize its protein content by any single method [for review, see (1)]. Notably, different protein species are present in serum over a very wide range of abundance. A small number of highly abundant proteins such as albumin, immunoglobulins, α-1-antitrypsin, transferrin, and haptoglobins are present in concentrations in the milligram to tens of milligrams per milliliter range and together account for as much as 80% of the total serum protein. A larger number of proteins, including many that are, or could be, diagnostically significant are present at far lower concentrations. Removal of abundant serum proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers.

Over the last several decades, attempts have been made to remove albumin, the single most abundant serum protein, from serum (or plasma) and other body fluids, often by methods based on the high affinity of albumin for certain blue textile dyes (2-4). These methods can be effective in removing albumin, although they suffer from a lack of specificity as many serum proteins in addition to albumin will bind to the dye-based resins. Other methods reported include
a proprietary polypeptide affinity matrix that removes albumin together with IgG, but is now apparently unavailable (5), and a method based on size separation in a centrifugal filtration device that was, perhaps predictably, unsuccessful (6).

In the work reported here, we have investigated the efficacy of an immunoaffinity resin for improving the specificity and efficiency of removing albumin from human serum samples. The use of immunoaffinity resins for the selective removal or purification of proteins from solution is well-established. However, their application to the problem of albumin removal is not entirely straightforward, primarily due to the enormous load of this single protein species in serum and plasma. We show that by using high affinity antibodies heavily loaded on a beaded resin support, albumin and a large number of albumin fragments can be essentially quantitatively removed from human serum samples. Further, when a Protein G affinity resin is used together with the immunoaffinity resin, IgG and HSA are simultaneously removed from the samples. Similar methods could be applied to the removal of additional abundant proteins.

EXPERIMENTAL PROCEDURES

Monoclonal antibody production and purification - Monoclonal antibodies were raised against human serum albumin (HSA) using standard techniques. Briefly, purified human serum albumin (Sigma) was used to immunize BALB/c mice and hybridomas were generated by fusion of spleen cells to SP2/0 myeloma cells. Hybridoma supernatants were tested for reactivity to HSA by ELISA, using the purified HSA preparation as a capture antigen. Hybridomas showing strong reactivity in these initial assays were retested for reactivity to each of the three major
domains of HSA (7). Coding sequence from each of the major domains of HSA (indicated in Figure 1) was amplified by RT-PCR using RNA isolated from HepG2 cells. These sequences were cloned into the vector pET28 (Novagen) for expression as recombinant (His)$_6$-tagged fusion proteins which were then purified on Ni-NTA resin (Qiagen) and used for testing the hybridoma supernatants by ELISA. Hybridomas showing strong reactivity against one of each of the three domains were single-cell cloned, retested by ELISA, and expanded for large scale production of monoclonal antibodies. Two of these antibodies, 12E8H that is reactive against an epitope in domain II, and 2G4A that is reactive against an epitope in domain I, have been used in the experiments described here.

Hybridoma cells were grown in CELLline 1000 flasks (Integra Biosciences) in serum free BD Cell MAb medium (BD Biosciences) and antibodies were purified from culture supernatants by affinity chromatography on HiTrap Protein G columns (Amersham Biosciences). The eluted antibodies were dialysed against PBS and concentrated in Ultrafree-15 centrifugal filter units (Millipore).

Preparation of antibody affinity resin - Antibodies were coupled to NHS-activated Sepharose 4 Fast Flow resin (Amersham Biosciences) at a concentration of 15 mg antibody per ml of resin, following procedures recommended by the manufacturer. After antibody coupling, the resin was washed first with 0.1 M NaCl, 0.1 M glycine, pH 2.8, then with 0.1 M NaCl, 0.1 M glycine, pH 9.0, and finally with 0.01 M Tris, pH 8.0. The resin was equilibrated with TBS/T (0.02 M Tris, 0.15 M NaCl, pH 7.6 with 0.1% Tween-20) prior to use or storage at 4°C.

Removal of HSA from serum samples - a. Antibody affinity resin: Human serum was diluted into one column volume or less of TBS/T and applied to immunoaffinity resin that had been equilibrated in the same buffer. Proteins were allowed to bind while mixing gently for 30-
60 min. at room temperature. Unbound proteins were recovered from the resin by gravity flow in a standard column format (PolyPrep column, BioRad) and then immediately reapplied to the column. This step was repeated so that unbound proteins were collected and immediately reapplied a total of four times. The column was then washed five times, each time with a single column volume of TBS/T, and all washes were combined as the unbound fraction of proteins. Bound proteins were eluted from the column with a total of five washes, each with a single column volume of 0.1 M NaCl, 0.1 M glycine, pH 2.8. Eluted fractions were neutralized by the addition of 0.05 volume of 1 M Tris, pH 8.0, and combined. Proteins from both the unbound and eluted fractions were concentrated by precipitation with 5 volumes of acetone.

b. SwellGel Blue: Albumin was removed from human serum samples using a SwellGel Blue Albumin Removal Kit (Pierce) according to the manufacturer’s instructions. Samples containing 650 µg of total protein (approximately 260 µg albumin) were loaded onto a single removal disc, where each disc is reported to have a binding capacity of >2 mg of albumin.

c. Addition of ProteinG resin to immunoaffinity resin: For the removal of both HSA and IgG in a single column, approximately 0.1 column volume of Protein G immobilized on Sepharose 4B Fast Flow (Sigma) was added to the immunoaffinity resin. Columns were washed, run, and regenerated exactly as for the immunoaffinity resin alone.

2D Gel Electrophoresis - 2DE was carried out as described (8) with minor modifications. Acetone precipitated proteins were redissolved in sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 5 mM tributylphosphine (TBP), and 0.4% ampholytes (Servalyt 3-10). Proteins were focused in an 18 cm, pH 3-10NL IPG strip (Amersham) after rehydration at 50 volts for 14 hours. Gel strips were equilibrated in 6 M urea, 2% SDS, 1.5% DTT, 30% glycerol, 50 mM Tris, pH8.8 for 10 minutes followed by incubation in the same
solution, but replacing DTT with 3% iodoacetamide, for an additional 10 minutes. The gel strips were placed on an 8-18% polyacrylamide gel for resolution in the second dimension. Gels were stained with silver (9) or colloidal Coomassie blue (10) and imaged with a FluorChem 16-bit CCD camera (AlphaInnotech).

For immunoblotting, proteins were transferred to PVDF membrane and the blots were stained with SyproRuby protein blot stain (Bio-Rad). Blots were probed with goat anti-HSA (Fitzgerald Industries) and developed using HRP-conjugated anti-goat antibody (Sigma) and SuperSignal West Pico detection reagents (Pierce).

**Peptide Mass Fingerprint Analysis of Proteins** - Protein spots excised from colloidal Coomassie blue stained 2D gels were destained, reduced and alkylated, and then digested with trypsin (11). Peptides were further treated by elution from ZipTip-C18 reversed phase pipet tips (Millipore). Recovered peptides were prepared for MALDI-TOF mass spectrometry by mixing with alpha-cyano 4-hydroxy cinnamic acid, 1% formic acid in 50% acetonitrile and droplets were allowed to dry on the MALDI sample plate. Peptide mass maps were obtained using a Voyager DE MALDI-TOF mass spectrometer operated in positive ion reflectron mode. Proteins were identified from the peptide mass maps using MASCOT (Matrix Science) to search the non-redundant protein database (12).

**Antibody Array** - The following mouse monoclonal antibodies were used for capture: anti-HSA (Research Diagnostics, Inc.), anti-hepatitis B surface antigen (Fitzgerald Industries), anti-alphafetoprotein (U.S. Biological), and anti-hepatitis B e antigen (Fitzgerald Industries). Antibodies were diluted to a concentration of 0.5 mg/ml in PBS plus 0.02% Tween-20 and spotted onto Hydrogel slides (PerkinElmer) with a MicroGrid TAS (BioRobotics) robotic spotter using MicroSpot 2500 pins. Slides were incubated in a humid chamber overnight and then
washed briefly in TBS/T before blocking for 2 hours in TBS/T plus 1% gelatin (BioRad). Slides were rinsed briefly in TBS/T and then incubated with antigen solution (see below) for 2 hours at room temperature. Slides were washed three times for 20 minutes in TBS/T and then incubated for 2 hours at room temperature with antibodies that had been labeled with Cy3 fluorescent dye (Amersham) according to manufacturer’s instructions. Antibodies used for detection were goat polyclonal anti-human AFP, anti-HBsAg, and anti-HSA (Fitzgerald Industries). Slides were again washed with TBS/T and then imaged using a ScanArray 5000XL scanner (PerkinElmer).

Antigen solution was prepared by adding purified AFP (Fitzgerald Industries) and HBsAg (Chemicon) to human serum at a concentration of 1000 ng/ml each. The spiked serum samples were diluted into TBS/T and fractionated on immunoaffinity resin as described above. Proteins recovered in flow through and eluted fractions were concentrated by acetone precipitation and resuspended in TBS/T. Unfractionated, spiked samples were similarly diluted, acetone precipitated, and redissolved in TBS/T. Antibody microarrays were incubated with the antigen solution at a concentration equivalent to a 1:10 dilution of the starting serum sample, so that AFP and HBsAg would each be present at a maximum of 100 ng/ml in any fraction.

RESULTS

Preparation of monoclonal antibodies and HSA immunoaffinity resin - Monoclonal antibodies to human serum albumin are available from many commercial suppliers. However, albumin is present in human serum at concentrations in the range of 35 to 45 mg per ml and very large quantities of antibody are required for its quantitative removal. Our goal was to have a
renewable source of high affinity monoclonal antibodies reactive against a number of different epitopes in the albumin molecule so that both full length HSA and many HSA fragments could be efficiently removed. Therefore, we decided to develop our own source of monoclonal anti-HSA antibodies.

Hybridomas were prepared from the spleens of mice immunized with full length HSA, as described in Methods. Supernatants from individual hybridoma clones were first tested for reactivity against full length HSA by ELISA. In order to identify hybridomas producing antibodies reactive against different HSA epitopes, clones showing strong reactivity to the full length molecule were further tested against recombinant His-tagged proteins representing each of the three major domains of HSA (see Figure 1). Antibodies against epitopes in two different domains (I and II) were chosen for large scale preparation and purification. These two antibodies were covalently attached to Sepharose resin to prepare an immunoaffinity reagent, as described in Methods.

Efficiency and specificity of HSA removal from serum - To test the efficiency of HSA removal from human serum, 10 µl of serum, containing approximately 700 µg of total protein and an estimated 400 µg of HSA, was incubated with 0.25 ml of the antibody affinity resin in a column format (see Methods). Proteins that did not bind to the column (the flow through fraction) and proteins specifically bound by the resin and then eluted (the eluted fraction) were prepared and examined by 2DE. The abundant HSA protein is evident as a large, poorly resolved and clearly overloaded spot when total serum proteins are displayed in these 2D gels (Fig. 2a). After separation of serum proteins on the affinity column, the flow through fraction shows essentially no remaining HSA (Fig. 2b) while the HSA is recovered in the fraction eluted from the column (Fig. 2c).
The capacity of the resin was tested by attempting removal of HSA from different volumes of serum with a constant volume of resin. As assessed by visual inspection of 1D and 2D gels, the 0.25 ml column was able to remove HSA from volumes of serum up to approximately 12 μl, but became slightly overloaded with 15 μl, or approximately 1.05 mg of total serum protein and 600 μg of HSA (data not shown). No reduction in column capacity has been observed with re-use of the resin up to 8 times (data not shown).

While HSA is by far the most abundant protein seen by 2DE of proteins that bind to and are eluted from the column, many other “spots” are present in the gel (Fig. 2c). A large number of these have been shown to be albumin or albumin-related fragments by two methods. First, eluted proteins separated by 2DE were transferred to PVDF membranes for detection by immunoblotting, using a polyclonal antibody against HSA. Almost all spots visualized by staining of the blot were also detected by reaction with the anti-HSA antibody, as shown in Figure 3. This suggests that proteins (or protein fragments) that bind to the column are doing so by specific interactions with the antibody, rather than by non-specific binding to the resin substrate or to HSA itself.

Further identification of the numerous minor spots found in the eluted fraction was obtained by mass spectrometry. Individual spots excised from the gel were digested with trypsin and analyzed by MALDI-TOF peptide mass fingerprinting. Spots marked with numbers in Figure 4 have been identified as albumin fragments in that peptides produced by trypsin digestion can be mapped to the albumin molecule over a region that is consistent with the size of the polypeptide in the excised spot. The tryptic fragments detected in this analysis are indicated in bold lettering in the albumin sequence shown in Figure 1 and they are also listed in Table 1, together with their occurrence in each of the analyzed spots. In a small number of cases, for
instance in the cluster of spots numbered 31, 33, 34, and 35, peptide mass matches were found over a region that exceeds the expected molecular weight of the fragment excised from the gel. This may be due to incomplete separation of adjacent spots in the gel, resulting in cross contamination of the excised gel plugs. Identification by mass spectrometry was attempted on twenty-three spots and twenty-one of those spots showed a tryptic mass fingerprint that could be matched to albumin (Table 1). One spot was identified as α1-antitrypsin (AT in Figure 4), and this is consistent with its migration in the 2D gel as compared to published data (Swiss 2D PAGE). The data obtained from one spot were insufficient for a protein identification.

Very small amounts of some proteins that are most probably not albumin can sometimes be seen when 2D gel images of eluted proteins are strongly overexposed (not shown). Based on their migration in the gel, these appear to be trace amounts of abundant proteins such as IgG, α1-antitrypsin, and haptoglobins.

**Improvements in the resolution and detection of serum proteins in 2D gels after HSA removal** - Small regions of the 2D gels shown in Figure 2a and b are enlarged in Figure 5 to demonstrate the improvement in the data available from 2D gels, even in the absence of significant overloading of the proteins that remain after albumin depletion. Many of the abundant proteins that were obscured by albumin are now visible (compare resolution in upper left of each image). Less abundant proteins that were lost in the confusion of albumin fragments become distinct and are now available for analysis (see spots indicated by arrows). Minor proteins that were previously hidden by co-migration with albumin fragments or smears can now be resolved (see boxed region and 3D depictions of that region below each image). It is evident that higher protein loads are now possible so that proteins of this and even somewhat lower abundance classes can be detected and analyzed without overwhelming the gel with albumin and
its fragments. We expect that similar improvements will be realized using alternative fractionation technologies, such as liquid chromatography linked to mass spectrometry, for the analysis of serum proteins.

*Albumin removal using a Cibacron Blue-based resin* - Resins used to remove HSA from serum samples have been made from immobilized forms of blue dyes such as Cibacron Blue, that were shown in the early 1970’s (2) to be effective in binding albumin in plasma samples. Since then, attempts have been made to modify the dye to improve the specificity of its binding to albumin, and one such product is marketed as SwellGel Blue (Pierce Endogen). Given the convenience and commercial availability of this, and similar products, it was important to compare its performance with that of our immunoaffinity resin.

Ten μl of serum, containing approximately 400 μg of HSA, was loaded onto SwellGel Blue resin with a capacity of >2 mg of HSA, as stated by the manufacturer. The column was processed according to the manufacturer’s instructions, and proteins in the flow through and eluted fractions were analyzed by 2DE, as before. Results, shown in Figure 6, indicate that a large portion of the HSA was not bound by the resin. Proteins that bound to and were eluted from the resin include albumin, many albumin fragments, and a large number of non-albumin proteins. In particular, significant amounts of relatively abundant serum proteins such as Ig heavy and light chains, apolipoprotein A1, serotransferrin, haptoglobin, and α1-antitrypsin were bound by the column. 1D gel analysis of several sequential wash and elution fractions suggested that this was not the result of incomplete washing during the initial binding steps, but instead represents proteins, in addition to HSA, that bind to the column under the recommended conditions (data not shown). We conclude that immunoaffinity reagents are far superior to the dye-based resin in terms of both the efficiency and specificity of albumin removal.
Potential retention of albumin-related and albumin-binding proteins by the immunoaffinity resin - While the immunoaffinity resin shows a high degree of specificity for binding to HSA, it was of interest to investigate whether proteins structurally similar to albumin, or proteins that can bind to albumin, are also removed by treatment of samples with the resin. Our interest in viral liver disease led us to examine the behavior of two such proteins, alpha fetoprotein (AFP) and hepatitis B virus surface antigen (HBsAg). AFP is a fetal form of albumin with 40% amino acid sequence identity and a highly similar tertiary organization (13, 14). AFP is frequently monitored in patients chronically infected with hepatitis B or C virus, where serum levels greater than 20 ng/ml (usually 100 - 1000 ng/ml or higher) can be indicative of progression to hepatocellular carcinoma. Like albumin, AFP is folded into three major domains that are held together by disulfide bonds at positions conserved between albumin and AFP (14). It was possible that conformation dependent epitopes in AFP would be recognized by antibodies used to make the HSA immunoaffinity resin. HBsAg can be present at concentrations ranging from 1 µg/ml to over 1000 µg/ml in the serum of patients who are chronically infected with HBV (15). HBsAg has been shown to bind to some forms albumin (16) and therefore it could potentially be retained on the column through this interaction.

Human serum was spiked with purified AFP and HBsAg, each at a concentration of 1000 ng/ml of serum. The sample was then treated to remove HSA using the immunoaffinity resin as described above. The total spiked sample (prior to HSA removal), the immunoaffinity column flow through fraction, and proteins eluted after binding to the column were all assayed by an antibody microarray for the presence of HSA, AFP, and HBsAg. The array also contained antibody to hepatitis B virus e antigen (anti-HBeAg) as a negative control. As shown in Figure 7a, this method easily detected HSA, AFP, and HBsAg in the unfractionated, total sample. Both
AFP and HBsAg are present in the flow through fraction (Figure 7b) but are undetectable above background in the eluted fraction (Figures 7c). As expected, HSA is found in the eluted fraction, but is undetectable above background in the flow through fraction (Figures 7b and c). These results provide further evidence of the high degree of specificity that is possible when using an immunoaffinity reagent for the removal of albumin from serum samples.

Although the overall recovery of proteins in the flow through and eluted fractions from our immunoaffinity column is close to 100% (+/-10%), as determined by Bradford assays, we cannot rule out retention of individual low abundance proteins that are not evident in the 2D gel and antibody array analyses we have done to date. Preferential loss of low abundance, non-albumin proteins would need to be checked on an individual basis, if such a loss were suspected.

*Simultaneous removal of HSA and immunoglobulin chains* - Removal of additional abundant proteins can be beneficial in the analysis of serum proteins and therefore we attempted to remove albumin together with the IgG class of immunoglobulins. These proteins can account for ~20 percent of total serum proteins and are present at a concentration of ~8-16 mg/ml (17). To remove IgG, we took advantage of its well known affinity for Protein G. Protein G resin was added to the HSA immunoaffinity resin and serum samples were processed as above. Figure 8 shows a 2D gel of serum where both HSA and IgG have been removed. Very small amounts of IgG heavy chains remain in the flow through fraction and a more specific affinity reagent (e.g. anti-IgG antibody) might be required for their complete removal. Since Protein G binds IgG, but has only a low affinity for other classes of human immunoglobulins, we expect that the light chain proteins that remain in the sample are derived primarily from these other immunoglobulin classes. Again, an affinity reagent with specificity for light chains (e.g. Protein L or anti-light chain antibodies) could be used to achieve a more complete removal, if necessary. It was
possible that Protein G on the resin would interact with the densely loaded anti-HSA immunoaffinity resin when the two were used together in the same column, thereby reducing binding capacity. However, we saw no difference in binding capacity when the two resins were used together in the same column, or sequentially in separate columns (data not shown). Therefore, HSA and most IgG proteins are readily removed from serum samples in a single column.

DISCUSSION

The rapidly evolving of techniques of proteome analysis are making protein profiling of both tissues and body fluids increasingly sensitive and informative. This has important clinical implications and is leading to new strategies for disease detection. Serum has been, and continues to be, a valuable source material for the monitoring of disease markers. However, the serum proteome is dominated by a small number of highly abundant proteins that can obscure detection of other potentially informative proteins. Here we describe a technique for removing albumin, by far the most abundant serum protein, in a way that is efficient and specific enough to be suitable for use with emerging proteome technologies. The method is easily adapted for the simultaneous removal of circulating IgG and could be applied to additional proteins, as well.

Removal of albumin from serum samples is problematic because of its extremely high concentration. A high specificity and high capacity resin is required. While Cibacron Blue and related dyes have been shown to bind albumin with high affinity (2), they also bind many other abundant serum proteins (3). Dye-based resins that have been optimized for albumin binding are
commercially available, but our results suggest that they still lack sufficient specificity. The need to compromise between specificity and capacity may account for the failure of the product we tested to perform well in either area.

Antibodies are an obvious choice as affinity reagents with the specificity required for selective removal of any single protein species. In the case of albumin removal, however, the quantity of antibody needed for essentially complete depletion of albumin, even from microliter quantities of plasma or serum, makes the use of antibodies problematic. To maximize the value of the antibodies used in our resin, we selected individual monoclonal antibodies that each react with high affinity to a unique epitope on the albumin molecule. This not only provides a renewable antibody source, but also is a reagent that can benefit from cooperative binding to different regions of the protein. By using antibodies reactive against different regions of the molecule, we are able to capture a large number of the albumin fragments that are present in serum samples. While our resin was effective when made with only one (not shown) or two of our antibodies, it is possible that further efficiencies could be achieved with additional antibodies.

Several modifications to our procedure that could improve its applicability in different circumstances are possible. For instance, the use of formats such as multi-well plates that are suitable for high throughput will be explored. The use of antibody fragments or single chain antibodies derived from the anti-HSA monoclonals might be beneficial if they can be produced less expensively or loaded onto substrate at higher concentrations.

In the ideal, it would be best to use a non-proteinaceous surface or resin for the specific removal of proteins from any sample. This would eliminate the potential for contaminating the sample with protein leached from the resin, and would likely be a more stable and less expensive
product. To date, however, no affinity resin that can match the specificity and binding capacity of monoclonal antibodies has been discovered. Covalent binding of antibody to the resin has minimized the leaching problem and the ability to re-use the resin helps to keep the cost reasonable for research purposes.

Our research involves the examination of proteins in human serum samples by 2DE and antibody microarrays and both of these techniques benefit from the removal of the bulk of the albumin and IgG. Removal of highly abundant serum proteins improves the detection of less abundant proteins by 2DE, as well as the display of proteins that comigrate and are otherwise obscured by HSA or IgG. The removal of the large and poorly resolved spots created by these highly abundant proteins, and the numerous smaller spots that represent albumin fragments, also helps in computer-assisted gel analysis, particularly with spot delineation, quantitation, and gel to gel matching. We expect that many of the new technologies being developed for protein profiling will benefit in similar ways by the improvements in sensitivity and reduction of background that result from the removal of a small number of very abundant proteins from samples prior to analysis.

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FIGURE LEGENDS

Figure 1. Amino acid sequence of human serum albumin. The sequence of human serum albumin (Swiss-Prot #P02768) is represented showing the three major domains. Tryptic fragments that were detected and used in the identification of albumin-derived polypeptides by mass spectrometry are indicated in bold, underlined type.

Figure 2. 2DE of total human serum proteins and proteins fractionated on anti-HSA immunoaffinity resin. Proteins were resolved by 2DE with separation by isoelectric focusing in pH 3-10NL IPG strips in the first dimension and 8-18% SDS-PAGE in the second dimension. Gels were stained with silver and are shown with the acidic end of the first dimension to the left. a) total human serum proteins with the positions of albumin, IgG heavy chains, and Ig light chains indicated. b) proteins in the flow through fraction; c) proteins eluted from the immunoaffinity column.

Figure 3. Immunoblot analysis of proteins eluted from the anti-HSA immunoaffinity resin. Proteins eluted from an immunoaffinity column were resolved by 2DE, as in Fig. 2, and transferred to PVDF membrane. After transfer, blots were stained with SyproRuby protein blot stain and then probed with polyclonal goat anti-HSA. a) stained blot; b) HSA detection.

Figure 4. Proteins eluted from the anti-HSA column and identified by mass spectrometry. Proteins eluted after binding to an immunoaffinity column were resolved by 2DE and the gel was stained with colloidal Coomassie blue. Protein spots were excised from the gel, digested with
trypsin, and analyzed by MALDI-TOF peptide mass mapping. Spots identified as albumin or albumin fragments are indicated by numbered lines. The spot labeled AT is $\alpha_1$-antitrypsin. Approximate molecular weights are shown in kilodaltons to the left of the gel.

**Figure 5. Improved resolution and detection of proteins after HSA removal.** A close-up view is shown of the gel region including and just below the position of the major albumin spot, as seen in Fig. 2. a) gel with total serum proteins, b) gel with HSA removed. A 3D image of the boxed region is shown below each gel. Note the numerous low abundance spots that can now be visualized for analysis. Arrows in panel (b) point to spots that are obscured in the gel shown in panel (a).

**Figure 6. 2DE of human serum proteins fractionated on SwellGel Blue.** Human serum proteins were fractionated on SwellGel Blue and resolved by 2DE, as in Fig. 2. a) Proteins in the flow through fraction, and b) proteins eluted from SwellGel Blue resin. Some proteins can be identified as follows: 1: serotransferrin, 2: $\alpha_1$-antitrypsin, 3: IgG heavy chains, 4: apolipoprotein A1, 5: Ig light chains, 6: haptoglobins.

**Figure 7. Antibody microarray analysis of AFP and HBsAg binding to HSA immunoaffinity resin.** Capture antibodies were applied to three slides in 16 replicate spots, as labeled to the left. Serum spiked with AFP and HBsAg was fractionated on anti-HSA immunoaffinity resin and the resulting flow through and eluted fractions, as well as total protein prior to fractionation, were incubated on separate slides. Bound antigen was detected with Cy3-labeled antibodies. Slides were scanned at two different laser intensities to account for the very
large amount of HSA relative to AFP and HBsAg. Low intensity scans are shown above the higher intensity scan of each slide, and the negative control Ab spots (anti-HBeAg) are the same spots in each of the two scans. Panel a) slide incubated with total spiked sample prior to fractionation; panel b) slide incubated with flow through fraction proteins; panel c) slide incubated with proteins in eluted fraction.

**Figure 8. Simultaneous removal of albumin and IgG proteins.** Human serum was fractionated on a column containing both anti-HSA and Protein G affinity resins. The flow through fraction was collected and resolved by 2DE, as in Figure 2. The gel was stained with colloidal Coomassie blue.
Table 1: Tryptic peptides used to identify albumin fragments by mass spectrometry.

| Peptide          | start-stop<sup>1</sup> | 45<sup>2</sup> | 43 | 42 | 41 | 39 | 38 | 20 | 25 | 26 | 28 | 35 | 34 | 33 | 31 | 16 | 15 | 18 | 14 | 9  | 7  | 4  |
|------------------|-------------------------|---------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| FKDLGEENFK       | 35-44                   | +             | +  | +  | +  | +  | +  |    |    |    |    |    | +  | +  |    |    |    |    |    |    |    |    |
| LVNEVTEFAK       | 66-75                   | +             | +  | +  |    |    | +  | +  | +  |    |    |    | +  | +  |    |    |    |    |    |    |    |    |
| SLHTLFGDK        | 89-97                   | +             | +  | +  | +  | +  | +  | +  |    |    |    |    |    |    |    | +  | +  |    |    |    |    |    |
| LCTVATLR         | 98-105                  | +             | +  | +  |    |    | +  | +  |    |    |    |    | +  | +  |    |    |    |    |    |    |    |    |
| QEPERNECFLQHK    | 118-130                 | +             | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    | +  | +  |    |    |    |    |    |
| DDNPNLPR         | 131-138                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| KYLYEIA          | 161-168                 | +             | +  | +  |    |    | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| YLYEIA           | 162-168                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| AAFTECCQAADK     | 187-198                 | +             | +  | +  | +  |    | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| LDELRDEGK        | 206-214                 | +             | +  | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| VHTECCHGDLECADDR | 265-281                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| YICENQDSISSK     | 287-298                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| LKECEEPLLEK      | 299-310                 | +             | +  | +  |    | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| RHPDYSVVLRR      | 361-372                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| HPDYSVVLRR       | 362-372                 | +             | +  | +  |    |   | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CCAAADPHECYAK    | 384-396                 | +             | +  | +  |    |    | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| VFDEFKPLVEEPONLIK| 397-413                 | +             | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| QNCELFEQLGLEYK   | 414-426                 | +             | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| FQNALLVR         | 427-434                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| KVPQVSTPVLVEVS   | 438-452                 | +             | +  | +  | +  |    | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| VPQVSTPVLVEVR    | 439-452                 | +             | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CCTESLVNR        | 500-508                 | +             | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| RPCFSALEVDETVPK  | 509-524                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| AVMDDFAAFVEK     | 570-581                 | +             | +  | +  | +  | +  | +  |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

<sup>1</sup> Start and stop positions of tryptic peptides found by MALDI-TOF mass fingerprinting. Amino acid numbering is based on the sequence of human serum albumin precursor (Swiss-Prot #P02768 and Figure 1). Some peptides overlap due to missed trypsin cleavage sites.

<sup>2</sup> Numbers correspond to spots shown in Figure 4.
**pre-pro sequence**

| Domain I          | MKWVTIFSLLFLFSYASRGVFRR |
|-------------------|--------------------------|
| DAHKSEVAHRFKDLGEENFKALVLIAPQYLOCPFEDHVKLVNEVTEFAKTCSVADESAENCDSKLHTLFGLKICTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDMCTAFHDNEETFLKKYLYEIARHPYFYAPELFFFAKRYKAAFTECCQAADKAACLIPKLDLRLDEKGAS |
| Domain II         | SAKQRLKCASLQKFGERAFAKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADRARLYICENQDSISSKLKECCEKPLLEKSHCIAEVENEDMPADLPSLAADFVESKDVCKNYAEEKDVFLGMFLYEYARHPDYSVLLLRLAKTYETTLEKCCAADPHECYAKVFDEFKPLVEEP |
| Domain III        | QNLIKQNCSELFEQLGFQKNALLVRYTKKVPQVSTPTLVEVSRLNLGKVGKCCKHPEAKRMPCAEDYLSVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVKEFNAETFTHADICTLSEKERQIKKQTALVELVHKPKATKEQLKAVMDDFAVFVEKCCADDKETCFAEGKKLVAASQALGL |

Figure 1
Figure 2

a) Albumin

b) IgG heavy

Ig light

Albumin

 Albumin
Figure 3
Figure 6
Figure 7

a) 

- α-HSA
- control Ab

b) 

- control Ab
- α-HBsAg
- α-AFP

c)
