Applied Biosystems

Affinity Depletion Cartridges for Removal of Human Serum Albumin and Immunoglobulins from Human Serum

Overview:

One of the major difficulties in analyzing the proteome of human serum is the dynamic range of the concentrations of the proteins present in the sample. Human serum albumin (HSA) constitutes 57-71% of total serum protein and gamma-immunoglobulin (IgG) ranges from 8-26%. Removal of these two proteins alone clears about 75% of the total protein present in serum, thereby allowing the enhanced detection of the remaining proteins that are present in far lower concentration. To address this problem, a new POROS® Anti-HSA support has been developed using an antibody ligand that has been optimized to specifically bind human serum albumin. Using the 0.2mL cartridge device, we have shown that the media will completely bind the albumin in a 10-70uL sample of human serum that has been diluted 1:10 with an albumin concentration of 3.5mgs/mL. In combination with the Protein G cartridge, both albumin and IgG can be effectively removed in one step. Immobilization of this immunoaffinity ligand was developed on our POROS Perfusion chromatography® media, which is proven robust and rigid support capable of rapid protein separations. The cartridges can be cleaned and re-used up to one hundred cycles.

Here we describe the characterization of Anti-HSA antibody and Protein G affinity chromatography cartridges, which effectively remove HSA and IgG from human serum, enabling detection and analysis of the less abundant proteins. These cartridges have been characterized with respect to their percentage of removal of these abundant serum proteins as well as non-specific binding and cross-reactivity.

Key features:

- Highly specific, immunoaffinity ligand, exhibiting little to zero non-specific binding
- Proven POROS® Anti-HSA support for high throughput processing and cleanability
- Long lifetime and reusability
- Process samples via high throughput Multi Dimensional Liquid Chromatography system such as VISION® Workstation as well as processing single samples in a manual mode using unique cartridge format.
- Use in conjunction with 2D gels, ICAT™ reagent technology, LC/MS or MALDI_TOF MS analysis

Experimental Conditions:

In this work, a sample of human serum was passed over both a Protein G and Anti-HSA cartridge. The flow through (serum proteins) and eluted fractions (albumin and IgG) were analyzed by 1 dimensional and 2 dimensional SDS PAGE gel as well as a commercially available ELISA assay, in order to quanitate the removal of HSA and IgG from the sample, and examine the level of non specific binding. Bands from the 1D Gel were further analyzed using peptide mass fingerprinting analysis on the VOYAGER™ Workstation in order to identify visualized bands that did not correspond to the intact molecular weight of HSA. Comparisons of binding capacities of albumin from different species was also compared to determine cross reactivity.

70 uL samples of human serum diluted 1:10 (7uL undiluted serum) with phosphate buffered saline (PBS) was first passed through a 4mmDx15mmL (0.2mL) Protein G cartridge and then the flow-through fraction was diluted to 400uL. Then 100 uL of the diluted protein G flow-through fraction was applied to a 4mmDx15mmL (0.2mL) Anti-HSA

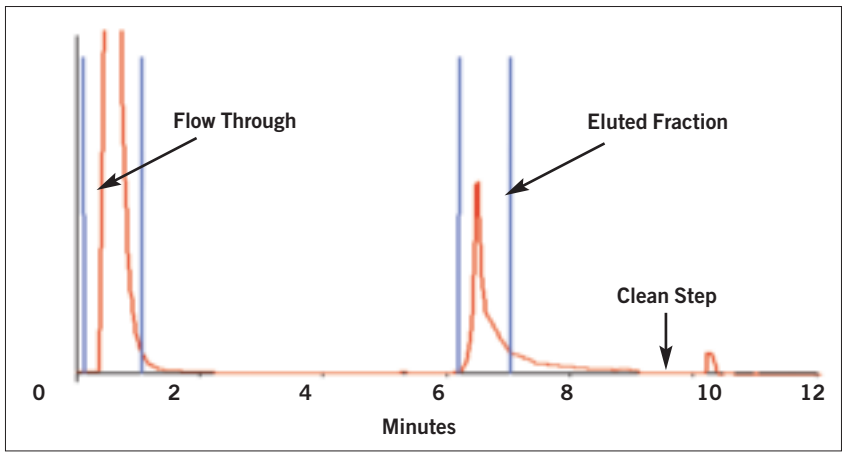

Figure 1. Chromatograph of affinity subtraction using POROS Anti-HSA cartridge

cartridge at a flow rate of 0.5mL/min. Elution of each cartridge was done using 3 mL of 12mM HCL at 1mL/min and the cartridge was then cleaned with a 5 mL step of 1M NaCl. All chromatographic steps were carried out using the Vision™ Workstation and the peak flow-through and eluted fractions were collected for further analysis.

Protein concentration for each fraction was determined using Bradford assay. Equal amounts of protein (6.5ug) from each fraction were analyzed by SDS-PAGE and the proteins were visualized by colloidial Coomassie stain. 2-D gel analysis was performed using 200 ug human serum, before and after affinity depletion by both cartridges and the proteins visualized by silver stain. MALDI-TOF peptide mass fingerprinting (PMF) was performed on bands from 1D Gel that did not correspond to the intact molecular weight of human Albumin using Voyager DE™ STR Biospectrometry™ Workstation (ABI) from in-gel digested peptides using 10ug/mL modified bovine trypsin in 25mM ammonium bicarbonate.

Anti-HSA and anti-human IgG ELISA kits (Bethyl Laboratory) were run according to the manufacture protocol. The flow-through fractions used for the ELISA were pooled from multiple runs (six runs from two Anti-HSA cartridges from the same lot and four runs from two Protein G cartridges from the same lot).

Results and Discussion:

Figure 1 shows the chromatograph of affinity depletion of HSA from human serum using the POROS® Anti-HSA antibody affinity cartridge operated on the Vision™ Workstation. The sample was previously run over a POROS protein G cartridge in order to remove IgG from the solution. The separations can be performed separately or in tandem in minutes using an auto-

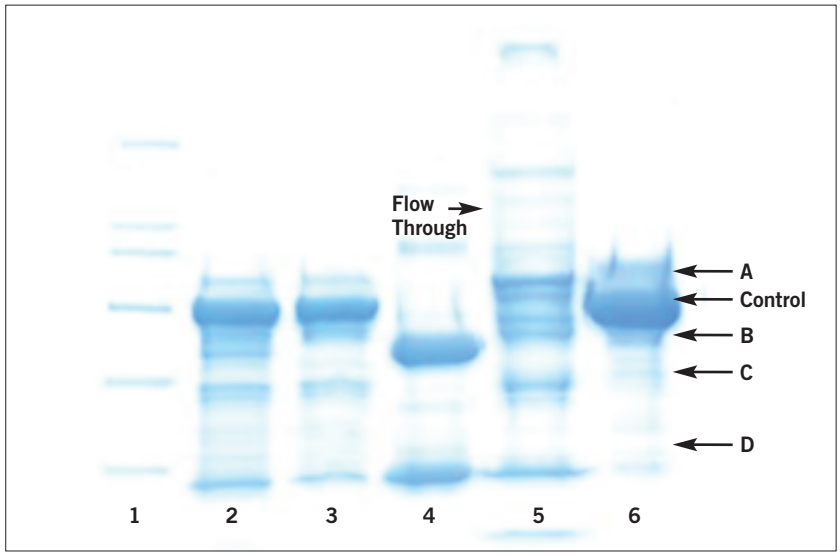

Figure 2. 4-20% SDS PAGE gel stained with coomassie blue. All lanes loaded with 6.5ug total protein.
Lane 1 Molecular weight markers,
Lane 2 Serum sample diluted 1:10 with Phosphate buffered saline,
Lane 3 Flow through of POROS Protein G cartridge,
Lane 4 Eluted fraction of POROS Protein G cartridge
Lane 5 Flow through of POROS Protein G and POROS Anti-HSA cartridges
Lane 6 Eluted fraction of POROS Anti-HSA cartridge

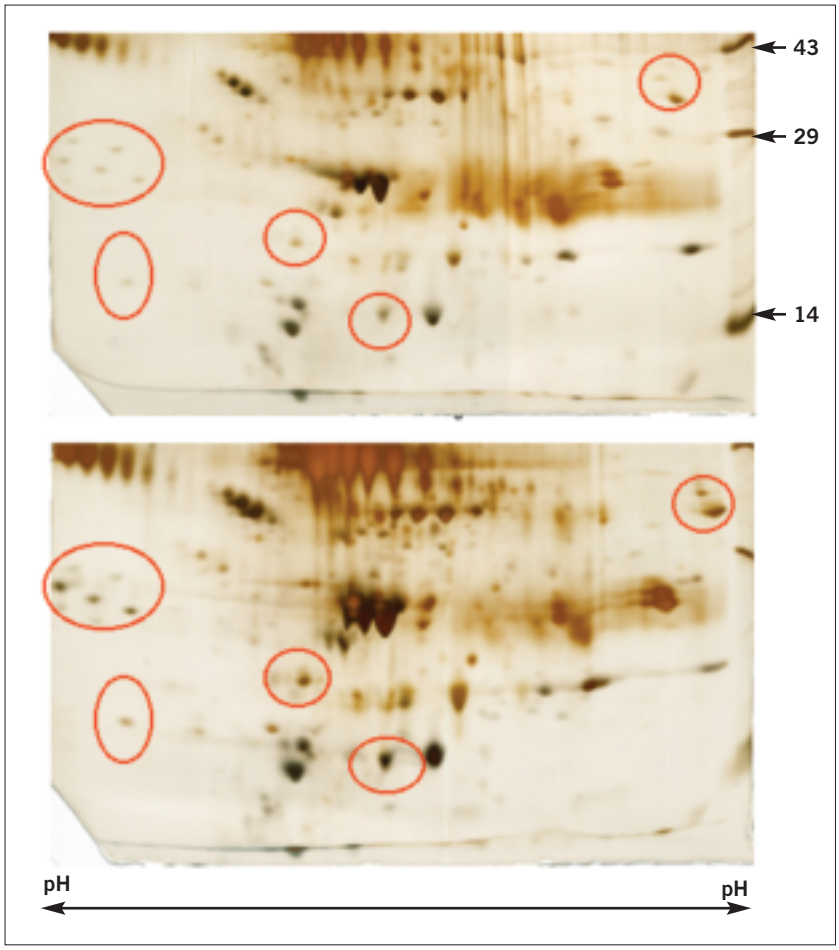

Figure 3. Expanded section of 2-Dimensional Silver stained gels. Top gel is serum sample before removal of IgG and HSA. Bottom gel is serum sample after processing on POROS Protein G and Anti-HSA cartridges. Circled spots represent increase in concentration of some low abundant proteins

mated Multidimensional Workstation such as the VISION® Workstation as well as being performed manually using a syringe inlet adapter.

Figure 2 shows SDS-PAGE analysis of serum sample (lane2) the flow-through and eluted fractions and the enrichment of low intensity bands in the protein G and Anti-HSA FT fraction (lane 5). The predominant proteins in the eluted fractions (lanes 4 and 6) are IgG heavy chain (55kDa) and light chain (25kDa) and HSA (66kDa) respectively. The goal of depleting these major serum proteins is to increase the relative concentration of the low abundance proteins that are typically masked by these major proteins in the sample. Figure 3 shows an expanded region of a 2 dimensional gel. The enrichment of low intensity protein spots before and after the affinity depletion. In many cases, protein spots that were barely visible in the unprocessed sample became much more intense upon depletion of the albumin and IgG.

To further characterize the proteins eluting from the Anti-HSA cartridge, peptide mass fingerprinting was performed on low intensity bands that did not correspond to the intact molecular weight of albumin. Figure 4 shows the mass spectra performed on peptide digests from the bands with corresponding protein identification using SwissProt. Most of these bands were identified as human serum albumin and are most likely proteolytic fragments of albumin in the sample since most migrated at a lower molecular weight than expected for intact HSA.

Removal of albumin and IgG were quantified using commercially available ELISA assays. Figure 5 shows results of measuring the removal of IgG and albumin from the flow. The

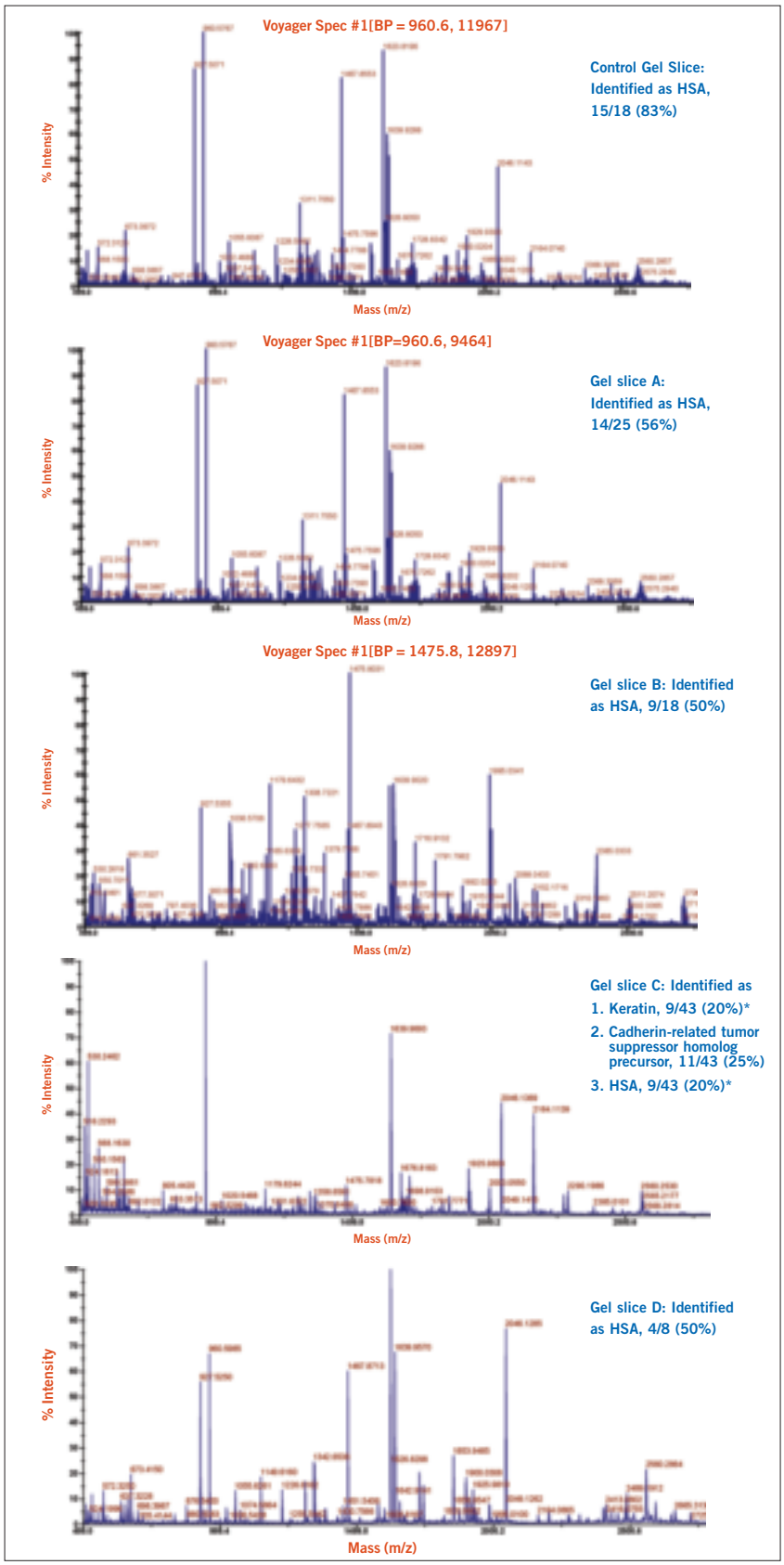

Figure 4. Peptide mass fingerprinting data on gel slices identified in figure 2 as gel slices A, Control, B, C and D. Only slice C contained proteins other than HSA. The kerratin identified was most likely due to handling of the gel since this is not a protein normally found in Serum

removal of both IgG and HSA was shown to be greater than 99%.

The cross reactivity of the Anti-HSA antibody was also examined to determine binding capacity for various species of albumin. Table 1 shows the capacity measurements made on the media using purified forms of albumin from various species. Although albumin from other species bind with some specificity, this antibody demonstrates the highest specificity for Human Serum Albumin.

Conclusions:

POROS® Protein G and Anti-HSA cartridges can quickly and efficiently remove both IgG and HSA, which are the two most abundant proteins in human serum. Using this immunoaffinity technique greater than 99% of both proteins can be removed from serum with little to zero non-specific binding of other proteins in the sample. Using a convenient cartridge format allows for samples to be processed manually or automated with an LC system to increase throughput.

The cartridges can be used as an effective sample prep device and are compatible with all types of down stream separation or analysis techniques, such as ICAT® reagents, 2D gels, MDLC, LC/MS or MALDI-TOF MS.

Table 1

Species	mg Bound per mL media	mMoles Bound per mL media
Bovine	0.24	0.004
Goat	0.01	0.000
Human	2.04	0.031
Mouse	0.70	0.011
Porcine	0.52	0.008
Rabbit	0.31	0.005
Rat	0.75	0.011
Sheep	0.01	0.000

Table 1. Cross reactivity of Anti-HSA media for albumin from various species. Capacity measurements are based on 10% breakthrough capacity measured on a 2.1mmDx100mmL column.

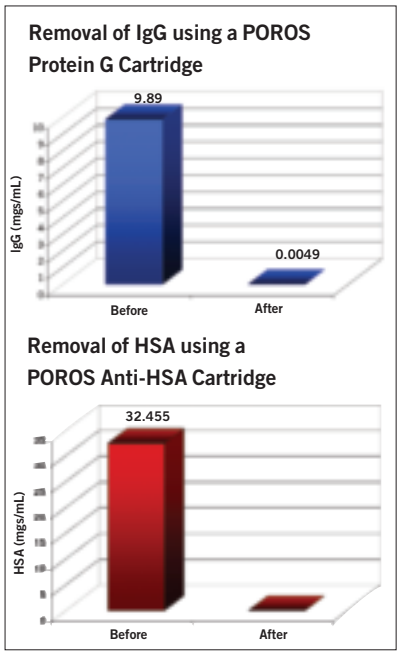

Figure 5. Results from measurement of IgG and HSA in the serum sample using an ELISA assay, before and after affinity subtraction using the POROS Protein and Anti-HSA cartridges.

Recommendation Table

Cartridge/Column Size		0.2mL	2.0mL	4.4mL*
Load:	10% diluted serum	30-70uL	300-750uL	600-1600uL
	Flow rate(mLs/min)	0.5	1.2	1.8
Capture the load effluent in up to 5, 0.5cartridge volume fractions. Fractions containing protein (measured by U.V absorbance at 280nm) can be pooled for further proteomics analysis				
Wash:			15-30 cartridge volumes	
	Flow rate (mLs/min)	1	2.4	3.5
Elution:			5-10 cartridge volumes	
	Flow rate (mLs/min)	1	2.4	3.5
Cleaning:			5-10 cartridge columns	
	Flow rate (mLs/min)	1	2.4	3.5

*** 4.4 mL size is packed in a 7.5mmDx100mmL PEEK column format**

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