
3 Separations in Proteomics: Use of Camelid Antibody Fragments in the Depletion and Enrichment of Human Plasma Proteins for Proteomics Applications

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3.1 INTRODUCTION

When the bulk of the human genome was deciphered in 2000, the scientific community quickly realized that we could draw few meaningful conclusions from the vast amount of data generated by the project. The event triggered the dawn of proteomics—the study of how proteins interact with each other and other molecules in metabolic pathways. The three billion nucleic acid base pairs identified in the human genome are believed to make up roughly 40,000 genes, which after full cotranslational and posttranslational modifications are believed to total in the millions of proteins. Unlike DNA and RNA, protein activity is based on molecular structure. While there have been countless attempts to predict protein activity with supercomputers, these efforts have produced little useful results due to the complex structure of proteins.

The single largest proteomics market is in the field of differential proteomics, where samples of serum from diseased and nondiseased populations are compared and contrasted to search for differences in protein levels. These proteins become diagnostic markers of disease, targets for clinical therapeutic intervention, or therapeutics themselves. More than half the human plasma proteome comprises housekeeping proteins such as human serum albumin (HSA) and immune gamma globulins (IgGs). These proteins have no relevance to disease state and mask the presence of important proteins. As the analytical tools for proteomic analysis offer increasingly improved sensitivity, the ability to differentiate important proteins from housekeeping proteins is become increasingly important and difficult. Ian Humphrey Smith of the human Proteome Organization said in 2001 “. . . solving this problem will require designing affinity reagents, or molecules that capture certain classes of proteins, to screen out the high abundance proteins found in cells before analysis . . .”

Scientists have struggled with technologies such as size-exclusion chromatography and isoelectric focusing to deplete samples of HSA and IgGs, yet these techniques are not specific to the unwanted molecules, and important proteins are removed along with the depleted fractions. Some attempts have been made at standard products based on dye-based ligands and conventional bioprocessing media such as Protein-A, but the poor specificities, low capture capacities, and high costs have stalled these products in the market. We have developed a technology based on affinity chromatography that uses naturally occurring camelid single chain antibody (VHH) fragments as ligands. The application areas for this technology are numerous (e.g., the purification of proteins from different sources).^{1,2} The camelid single domain antibodies can solve the problems in proteomics by providing high-affinity, high-specificity binders that can remove over 95% of these proteins. Unlike antibody reagents, VHH fragments can be easily manufactured and are very stable. Unlike

conventional affinity reagents such as blue dye and Protein-A, these molecules offer much better specificity and higher capacities. Unlike antibody fragments such as Fabs and scFvs, VHHs are designed by nature and do not suffer the same stability and hydrophobic agglomeration problems. Finally, unlike peptides, VHHs offer multiple binding sites for multiple epitope recognition and better capacity, and they are far less expensive.

3.2 HEAVY-CHAIN ANTIBODIES FROM THE CAMELID FAMILY

The serum of animals of the camelid family contains a unique type of antibodies devoid of light chain. This type of antibodies was discovered in 1993 at the University of Brussels.³ Only a fraction of the immune repertoire of the animals of the camelid family comprises the heavy chain antibodies. The other fraction are normal classical antibodies. The heavy chains of these so-called heavy-chain antibodies bind their antigen by one single domain, the variable domain of the heavy immunoglobulin chain, referred to as VHH.^{4,5} With a molecular weight of approximately 12 kDa, the VHH domain is the smallest known intact binding fragment derived from a functional immunoglobulin (Figure 3.1). VHHs show homology with the variable domain of heavy chains of the human VHIII family. The VHHs obtained from an immunized camel, dromedary, or llama have a number of advantages compared to the Fab, Fv, or scFv fragments derived from other mammals. One major advantage is that only one domain is cloned and expressed to generate an active binding fragment. This makes effective production in microorganisms such as *Saccharomyces cerevisiae*^{6,7} possible. The BAC has chosen to work with llamas to obtain its VHH fragments because they are easy to keep and to handle.

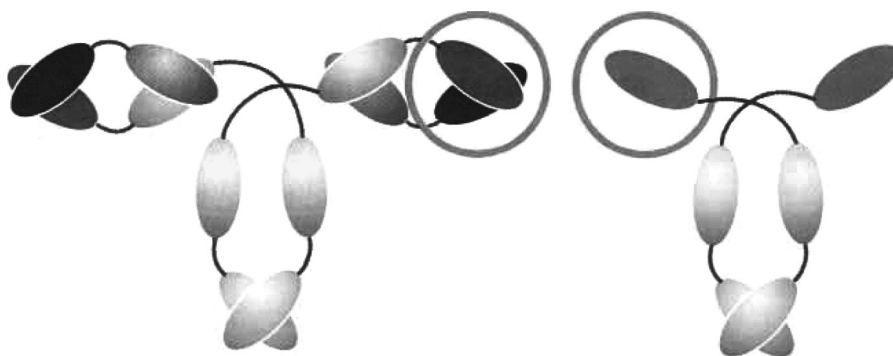


FIGURE 3.1 The difference between classical and single domain camelid antibodies. The difference between classical antibodies (left) and heavy chain antibodies (right). The binding domain is encircled in yellow.

3.3 ROADMAP TO LLAMA ANTIBODIES

The route to acquire the best antibodies for the task, such as in finding highly specific binders to abundant and irrelevant proteins in serum, is always the same and can be divided in three stages. The first stage is the immunization of the llama and the construction of the library. In this stage the immune repertoire of the llama after immunization is copied to a large microbial library. The second stage is the screening stage. In this stage we screen the library for several antibodies that can perform the task we want. For instance, in this stage, we design in the specificity of the VHH and the binding and elution conditions under which we would like to use the VHH as ligand in affinity chromatography. The third stage is the small-scale chromatography stage. In this stage we produce the ligand through microbial production and immobilize the VHH to a solid support. The affinity matrix constructed this way is used to test chromatographic parameters such as dynamic capacity and binding and elution conditions.

In the remainder of this chapter, we describe the process of finding VHH fragments against human serum albumin and human IgG. These VHH fragments were subsequently used to test the hypothesis that heavy-chain antibody fragments can be used as affinity ligands in sample preparation depletion for proteomics applications and to determine the benefits of this technology compared to other methods.

3.3.1 Stage 1: Immunization of Llamas and Creation of the VHH Library

Two llamas were immunized using standard procedures.⁷ One llama was immunized with HSA, and the other llama was immunized with human Fc. Immune response of the llamas to these antigens was checked at regular intervals, using a small serum sample from the immunized animal. When the immune response reached a plateau, a larger blood sample was taken from the llama. From the peripheral blood lymphocytes, the mRNA was isolated. Using polymerase chain reaction (PCR) techniques, the VHH encoding fragments were amplified.⁷ The DNA was cloned to the yeast *Saccharomyces cerevisiae*,⁷ creating a VHH library. The size of both libraries was 1×10^9 .

3.3.2 Stage 2: Screening for Antigen-Binding VHH Fragments

Screening for VHH fragments that could bind to the target of interest was done in the following way. The library was plated out on agar plates in a dilution high enough that single colonies were expected. Colonies were transferred to a non-protein binding 96-well plate. The colonies were grown in these plates and induced to produce VHH. The VHH-containing supernatant was used to check if the produced VHH could bind to the antigen using an enzyme-linked immunosorbent assay (ELISA). The positive clones from this ELISA were produced at small scale in shake flasks. For quick screening purposes, the VHH was purified from the supernatant using a Superdex 75 gel filtration column after removal of the biomass.

3.3.3 Stage 2: Screening for the Ultimate Ligand

The selected VHH fragments were screened for several parameters to see if they could be used as ligands. In this case the parameters tested were affinity and specificity. Testing was performed using surface plasmon resonance (SPR). This technology can be used to accurately measure the antibody antigen interaction. For the anti-HSA and anti-human IgG ligands that were chosen for the experiments described in this chapter, the affinity was in the nanomolar range, as can be seen in Table 3.1. One of the important aspects of an anti human IgG ligand is that the ligand binds to all subclasses of human IgG. Table 3.2 shows binding of the anti human IgG ligand to all subclasses of IgG as determined by SPR on a BiaCore 3000. Also crossreactivity of the HSA ligand to serum albumin from other species was tested. As can be seen from the results (Figure 3.5) there is no cross-reactivity of the HSA ligand to rat and rabbit serum albumin; however the HSA ligand does bind to mouse serum albumin. For the human IgG ligand, the crossreactivity to IgGs from other species was tested. As can be seen from the results (Figure 3.6) no cross-reactivity to mouse, bovine, or goat IgG was found.

3.3.4 Stage 3: Ligand Production

Ligands are produced by BAC's proprietary host system *Saccharomyces cerevisiae*. The DNA sequence encoding the ligand is integrated in the genome of the yeast. Subsequently the ligand is produced by fed-batch fermentation.^{6,7} After fermentation,

TABLE 3.1 Affinity Constants for the Anti-Human IgG and Anti-HSA Ligands

Affinity Constants for the Anti-Human IgG Ligand	
Ka (1/Ms)	3.2E + 05
Kd (1/s)	3.7E - 03
KA (1/M)	8.6E + 08
KD (M)	1.2E - 09
Affinity Constants for the Anti-Human Serum Albumin Ligand	
Ka (1/Ms)	7.1E + 05
Kd (1/s)	5.0E - 03
KA (1/M)	1.4E + 08
KD (M)	7.1E - 09

TABLE 3.2 Subclass Specificity

	Response			
	IgG1	IgG2	IgG3	IgG4
hIgG binder	118.3	71.1	37.8	39.2
Control	6.1	7	5.3	8.3

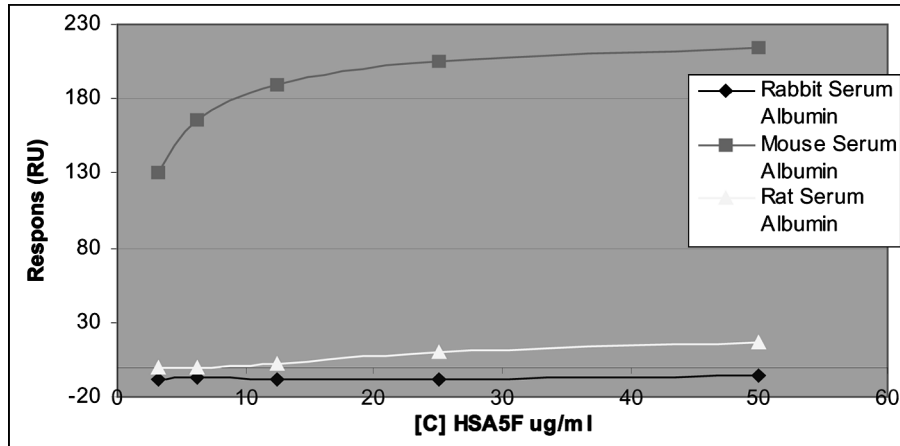


FIGURE 3.5 On a BiaCore 3000, the crossreactivity of the HSA ligand to serum albumin from other sources is tested. As can be seen, there is no cross-reactivity of the HSA ligand to rat and rabbit serum albumin; however, the HSA ligand does bind to mouse serum albumin.

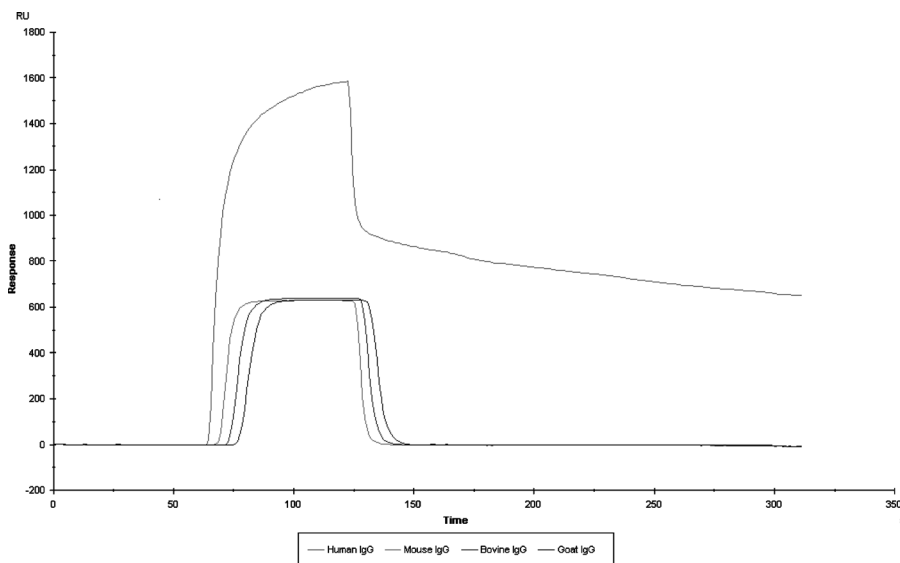


FIGURE 3.6 The cross-reactivity of the human IgG ligand to IgGs from other species was tested on a BiaCore 3000. As can be seen from the results, no cross-reactivity to mouse, bovine, or goat IgG was found.

the biomass is removed by microfiltration. The cell-free material is concentrated by means of ultrafiltration. The ligand is then purified using ion-exchange chromatography. This whole downstream processing route results in low-colored product, and the ligand has a protein purity of higher than 95%, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

3.3.5 Stage 3: Coupling to Matrices

The ligand can be coupled to different matrices using several coupling chemistries. In the examples shown, ligand was coupled to a matrix using *N*-hydroxysuccinimide (NHS) coupling chemistry. Ligands containing primary amino groups couple directly to the active ester of NHS to form a chemically very stable amide linkage.⁸ Base matrix sepharose (Amersham Biosciences) was used because it is known for its low nonspecific binding. The coupling procedure used is as follows.

After purification, the anti human IgG ligands were dialyzed to NHS coupling buffer, 0.1 M HEPES, pH 8.0. The following procedure was used for coupling of the ligands to NHS sepharose. Prior to coupling of the ligand to NHS, the matrix was washed with cold demineralized water acidified with acetic acid to pH 3. Then the matrix was washed twice with NHS-coupling buffer. The washed matrix was mixed with the ligand solution and left overnight at 4°C head over head or 1 hour at room temperature. Subsequently the gel material was filtered over a sintered glass filter, and the nonreacted groups of the gel material were blocked with Tris (0.1 M, pH 8.0) for 1 hour at room temperature. The coupled medium was washed using alternate low and high pH (3 × 10 column volumes phosphate-buffered saline (PBS) pH 2 and 3 × 10 column volumes PBS pH 7.4). The coupled gel material was now ready to use. Using the nonbound fraction, the coupling efficiency was determined by looking at the protein pattern on SDS-PAGE of the coupling solution before and after coupling.

The dynamic capacity of the affinity matrices was determined on an AKTA explorer 100 (Amersham Biosciences). Column volume that was used for these tests was 400 μl. Conditions for testing were the following: equilibration buffer PBS, pH 7.4 (Roche), flow 150 cm/hr; elution buffer PBS with an adjusted pH to 2.1. The low pH in the elution ensures full elution of the proteins of interest from the affinity column. The eluted fractions are immediately neutralized with 2 M Tris buffer. As sample for these experiments, pure HSA (Sigma) and pure human IgG (Sigma) was used. The dynamic capacity was determined using peak integration of the elution peak. For the HSA affinity matrix, typical dynamic capacities fell in the range of 8 to 10 milligrams HSA per milliliter affinity matrix of a settled matrix bed. For the human IgG affinity matrix typical dynamic capacities fall in the range of 13 to 15 milligrams human IgG per milliliter of affinity matrix of a settled matrix bed.

3.3.6 Stage 3: Depletion of HSA and Human IgG from Human Plasma

The affinity matrix was tested using an Akta explorer 100 for efficiency of the depletion and nonspecific binding of the affinity matrix. Onto a small affinity column

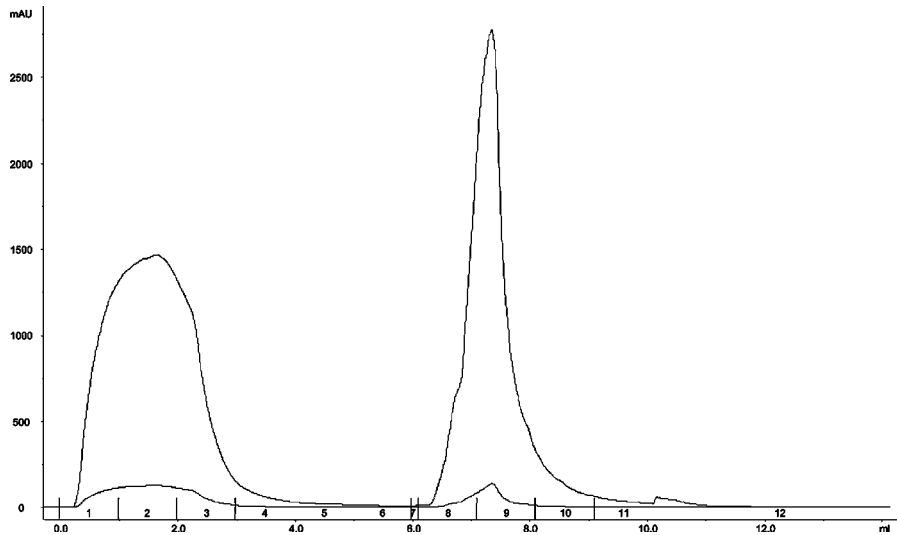


FIGURE 3.2 Depletion of a HSA from human plasma. The red line shows the optical density at 280 nm, the blue line is the optical density at 214 nm. At time point 0, a sample of human plasma is injected onto the column. The HSA-depleted fraction is the flow through of the column (fractions 1, 2, and 3). After washing out of the flow through, the column is eluted, the depleted HSA is collected (fractions 8, 9, and 10), and the eluted fractions are immediately neutralized with 2 M Tris buffer.

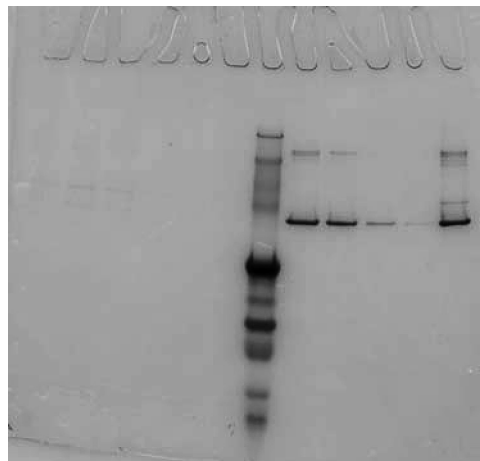


FIGURE 3.3 From left to right: 1, fraction 1 flow through; 2, fraction 2 flow through; 3, fraction 3 flow through; 4, fraction 4 flow through; 5, fraction 5 flow through; 6, fraction 6 flow through; 7, marker; 8, fraction 8 elution; 9, fraction 9 elution; 10, fraction 10 elution; 11, fraction 11 elution; 12, starting material—diluted human plasma. The flow-through and elution fractions are the fractions of the chromatography experiment of Figure 3.2.

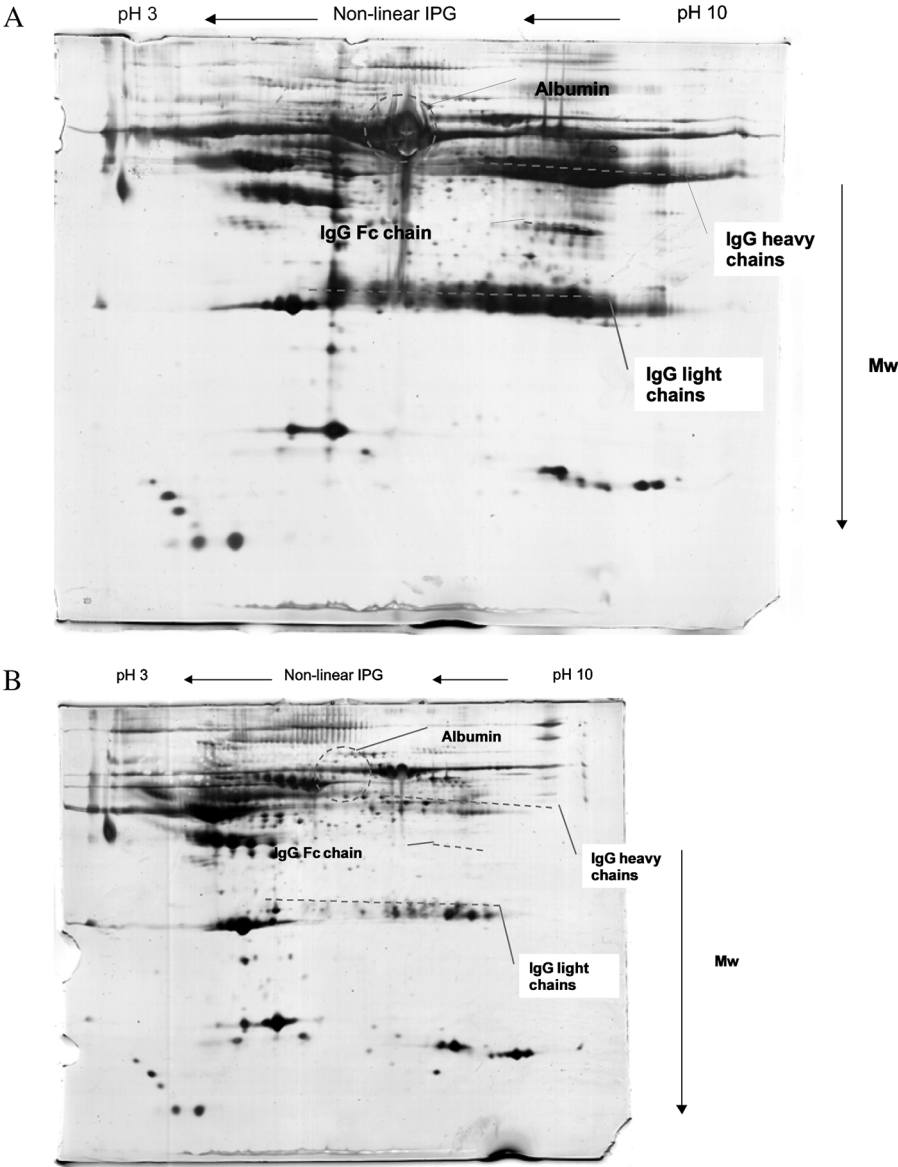


FIGURE 3.4

TABLE 3.3 The Abundant Proteins in Human Plasma
Increased Relevant Protein Load on Gels as f(depletion efficiency)

Plasma Protein	mg/ml	mg/0.1ml	Depleted at 95.0%	Cumulative Removed	Remaining	Protein Load Multiplier Increase
Albumin	40.0	4.00	3.80	3.80	4.80	1.8
IgG	11.0	1.10	1.05	4.85	3.76	2.3
Macroglobulin	3.0	0.30	0.29	5.13	3.47	2.5
Transferrin	2.8	0.28	0.27	5.40	3.20	2.7
AAT	2.4	0.24	0.23	5.62	2.98	2.9
IgA	2.4	0.24	0.23	5.85	2.75	3.1
C3	1.7	0.17	0.16	6.01	2.59	3.3
Haptoglobin	1.1	0.11	0.10	6.11	2.49	3.5
LDL	1.1	0.11	0.10	6.21	2.39	3.6
IgM	1.1	0.11	0.10	6.31	2.29	3.8
Alpha-a Acid Glycoprotein	1.0	0.10	0.10	6.41	2.19	3.9
ACT	0.4	0.04	0.04	6.44	2.16	4.0
Ceruloplasmin	0.3	0.03	0.03	6.47	2.13	4.0
C4	0.3	0.03	0.03	6.50	2.10	4.1
C1 Inhibitor	0.2	0.02	0.02	6.52	2.08	4.1
Others (from study)	—	1.74	0.00	6.52	2.08	4.1
Total Protein Load	—	8.60	6.52	—	—	—

(400 μ l) a sample of human plasma was loaded, and the same chromatographic procedure as described previously was used. The flow-through fractions and elution fractions (Figure 3.2) were collected and used for SDS-PAGE analyses. SDS-PAGE was performed on NOVEX Tris glycine 4–20% gels according to the supplier's protocol. Figure 3.3 shows the starting material, flow through (depleted fractions), and the eluted fractions.

Using a combined approach and thus depleting a sample of human plasma from HSA and human IgG, a 2D gel electrophoresis experiment was performed. Figure 3.4A and 3.4B show the nondepleted and depleted 2D gels of human plasma. The 2D gel electrophoresis was performed as described by Klooster et al.

3.4 CONCLUSIONS

BAC's affinity anti-HSA and anti-human IgG ligands can be used for the depletion of HAS and human IgG from plasma before proteomics analysis. The removal of the target molecules is very efficient, more than 95% can easily be achieved. Non specific binding of other proteins to either the ligand or the matrix was found. The anti HSA ligand can also be used for the depletion of mouse serum albumin.

BAC's proprietary VHH discovery, development and manufacturing capabilities provide a fully integrated supply of custom affinity ligands for proteomics depletions and enrichment applications that can be immobilized on virtually any matrix support material. Collaborators have adopted VHHs as an attractive alternative to antibodies, peptides and small molecule dyes due to their specificity, stability and ease of consistent supply. At the moment BAC ligands have been chosen by several analytical kit suppliers for use in new sample preparation for proteomics kits.

Currently, by using affinity chromatography, the two most abundant proteins can be depleted from human serum using these two ligands. After depletion, the total amount of protein that can be loaded onto a 2D gel can be increased by a factor of 2.3 (Table 3.3). At this increased protein level, however, other proteins become problematic. Table 3.3 shows the next targets that require specific depletion. These include macroglobulin, transferrin, IgA, C3, haptoglobin, α -a acid glycoprotein, fibrinogen, and IgM. The BAC has been working on these targets for some time now, and we are currently looking at the efficiency of the removal of these proteins from human serum. These results will be published in 2004 by Rinse Klooster.

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