

ABSTRACT

YANG, HAIYOU. Fc-Binding Hexamer Peptide Ligands for Immunoglobulin Purification. (Under the direction of Dr. Ruben G. Carbonell.)

Antibodies and their fragments have found a wide array of applications as pharmaceutical compounds, in addition to their common usage in the purification and localization of proteins. Antibody-based therapeutics accounts for roughly 20% of the therapeutic products in development in the USA, with the majority being of the immunoglobulin G (IgG) isotype. For IgG purification, affinity chromatography has been greatly used where *Staphylococcus aureus* Protein A and *Streptococcus* Protein G are the most common affinity ligands for IgG. However, the drawbacks associated with these two proteins have given rise to the searching for alternative affinity ligands for antibody purification. We therefore set our research goal as to find a small peptide that can bind hIgG through its Fc portion and can be used in purification of antibodies and Fc-fusion proteins. Small peptides are interesting for their advantages of being more stable, less immunogenic, less expensive and milder in elution than protein ligands.

Peptides were searched by a radiolabeled-screening technique in a combinatorial linear hexamer peptide library built on solid phase Toyopearl AF-Amino resins. The screening identified a family of ligands with homogenous composition of His + aromatic group + positively charge group having the ability to match Protein A in binding human IgG (hIgG) through its Fc portion. The selectivity to the Fc portion is comparable to Protein A.

The HWRGWV ligand of the Fc-binding peptide family has been investigated in many aspects and exhibits some interesting tributes. It has broad binding spectrum. It can bind all subclasses of hIgG, human IgD, IgE, IgM, and to a less extent human secretory IgA. It also displays the ability to retain chicken and several mammalian IgGs. Deglycosylation of hIgG has no influence on its binding to the HWRGWV ligand and the ligand does not compete with Protein A or Protein G in binding hIgG. It is suggested by the mass spectrometry data that HWRGWV binds to the pFc portion of hIgG and interacts with the amino acids SNGQPEN in the loop Ser383 – Asn389 by specific interactions. The selectivity of the HWRGWV resin to Fc over Fab is affected by its

peptide density which also influences the equilibrium constant for hIgG. Increasing density increases both the association constant which is in the order of 10^5 M^{-1} and the binding of the Fab fragment. A ligand density of around 0.1 meq/g was determined to have both the specificity and appropriate affinity to Fc.

HWRGWV demonstrates the ability to purify IgG. It can isolate hIgG from mammalian cell culture media containing 10% fetal calf serum (cMEM) with more than 95% of both purity and yield. The ligand was also used to isolate hIgG from Cohn II+III paste and a yield of 82% and purity of 73% were obtained in one step. The bound IgG can be recovered using phosphate buffer at pH 4 and its binding capacity for hIgG is 130 mg/g-dry-resin. Acetylation of the N-terminal amine does not pose any influence on either the static binding or the dynamic isolation of hIgG. Temperature has no significant influence on hIgG isolation from cMEM. Increasing peptide density improve the yield but with a compensation of the purity to a similar degree. Feed hIgG concentration in the range of 0.5 – 10 mg/mL affects the recovery yield where the yield is favored with higher IgG concentration. The separation of hIgG from cMEM by HWRGWV is comparable to Protein A at an initial hIgG concentration of 10 mg/mL and to A2P agarose gel at both 10 and 0.5 mg/mL, but using a milder pH 4 elution condition.

HWRGWV was immobilized on Sepharose CL-4B. HWRGWV-modified Sepharose CL-4B can isolate hIgG from cMEM with similar purity to and a lower yield than on Toyopearl AF-Amino under the conditions optimized on the latter matrix. Our experiments also show that 2% AcOH is not strong enough to completely remove the bound proteins on HWRGWV. A better column regeneration and sanitation procedure is needed for longer column lifetime.

This work demonstrates the possibility to use a peptide as short as six amino acids to mimic Protein A in IgG isolation by binding through the Fc portion.

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DEDICATION

献给我的父母

和胡祖寿

To my parents,

and Zushou Hu

BIOGRAPHY

Haiou Yang was born on July 29th, 1973, in a family of six, living in Hongqi, a small village at the edge of a big desert in the Xingjiang province, north-west of China. She spent most of her childhood in the farm fields and classrooms located close to her home. In August 1991, she was enrolled in the Department of Chemistry at Sichuan University, located in Central China, for her BS degree in Chemistry. After graduation, she worked as a chemist at the Institute of Chemical Materials in Sichuan. Four years later, the author went to Tsinghua University in Beijing for further education in the Department of Chemical Engineering, obtaining her Masters degree in 2002 under the direction of Dr. Jichu Yang. In the same year, Haiou Yang joined the Department of Chemical and Biomolecular Engineering at North Carolina State University to pursue her PhD degree, where she became a part of Dr. Ruben G. Carbonell's Bioseparations group.

Ms. Yang met her husband Zushou Hu at Tsinghua University and got married after they both graduated from their Masters programs. They have two children, Yang Forest Hu and Hairui Harris Hu.

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Chapter 1. Introduction

1.1 Motivation

Proteins are difficult to isolate and protein purification is technically demanding, given their complex physico-chemical properties, and possibility of unfolding and denaturation. Virtually every step in a protein purification process has the potential to alter protein structure and reduce biological activity. An efficient purification scheme needs to result in high activity and high yield of isolated proteins. Precipitation, filtration, and chromatography are commonly used techniques for protein purification. There are various modes for column chromatography of proteins, including ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), size exclusion (SEC), and affinity chromatography (AC). Among these methods, purification of proteins using affinity chromatography allows the possibility of obtaining several fold purification with high recovery in fewer steps.

Antibodies are a common, but highly important class of proteins. The unique specificity that antibodies can display for a target antigen makes them invaluable tools in diagnostics, structural analysis, histology, and immunotherapy. For instance, purified immunoglobulins have been used to treat patients with inadequate immunoglobulin levels. This and related applications have, in turn, given rise to an increased need for medical-grade immunoglobulins and better methods for their isolation from various sources. Antibody-based therapeutics account for roughly 20% of the therapeutic products in development in the US (1). The majority of these are of the immunoglobulin G (IgG) isotype, which can effectively activate complement- and antibody-dependent cellular cytotoxicity pathways. As a result of this growth in the commercial use of antibodies there is great interest in establishing more efficient, robust and low cost antibody purification processes.

IgG is usually isolated from pooled blood plasma, or produced in hybridoma cell culture as antigen-specific monoclonal antibodies (MAbs), or generated in polyclonal antisera. The crude source of IgG can be subjected to a combination of different processes. Usually, the purification scheme encompasses one or more affinity chromatography steps. *Staphylococcus aureus* Protein A and *Streptococcus* Protein G are the most commonly used ligands in the affinity purification of IgG. However, their high cost, low stability, the

possible presence of contaminants, and potential antibody activity loss associated with harsh column elution conditions (pH 3), make it important to find less expensive and more robust ligands for IgG purification. Furthermore, Protein A cannot be utilized for affinity purification of IgG from human plasma that is meant for intravenous use since it does not bind human IgG3, neither can it be employed for chicken and some other mammalian IgG purification because of its lack of affinity. Despite these drawbacks, Protein A is still the most widely used affinity ligand for antibody purification due to not only its high affinity to antibodies but also its specificity to the Fc portion of the antibodies. Binding through the Fc portion of an antibody allows the ligand to be a universal binder for antibodies and Fc-fusion proteins. Binding through the Fc portion also frees the Fab domains for antigen binding, which is desirable when using the antibody for immunoassays and diagnostic applications. Therefore, it would be desirable to find a ligand that mimics Protein A binding by recognizing the Fc portion of IgG.

The attributes of small peptides as potential ligands for protein purification have been gaining great attention (2-7) due to their unique advantages of being more stable, less immunogenic, and less expensive than protein ligands. A small peptide ligand may also exhibit less strong binding to the target protein than antibodies or other proteins, like Protein A, allowing for milder elution conditions to preserve the activity of target proteins. Solid phase one-bead-one-peptide (OBOP) combinatorial libraries have been successfully employed to search for affinity ligands for large proteins (8-13). The “couple-divide-recombine” technique (14, 15) makes it possible to rapidly synthesize millions of compounds for a library and the high-throughput screening technique enables the screening of the library for a desirable ligand. A good ligand is expected to have high specificity, large capacity, and appropriate affinity to the target protein. The dissociation constant (K_d) values for affinity chromatography resins are usually within the range of 10^{-4} - 10^{-8} M (16) and K_d values of 10^{-5} - 10^{-7} M have usually found with short peptides as affinity ligand for proteins (12, 17, 18).

1.2 Goals

The primary goal of this project was to find a small ligand that can mimic Protein A binding IgG through its Fc portion and eventually use this ligand as a Protein A replacement

in IgG purification to avoid the disadvantages associated with the large protein ligand. Human IgG (hIgG) was employed as the model antibody since hIgG or humanized IgG has been of great interest in therapeutics with less immunogenic effects in patients. The ligand was obtained by screening a hexamer OBOP library using radiolabeled screen process. The identified ligand was then packed into a chromatographic column to examine its ability to purify hIgG from mammalian cell culture media (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth. The ligand was also characterized for its retention capacity, binding affinity, and effects of peptide density etc on binding pure IgG in PBS and IgG in complex mixtures. The possibility to immobilize the ligand on other solid support and to isolate hIgG from human plasma Cohn paste II+III was investigated for broader application. The storage stability of the peptide, its interaction mechanism and binding site on hIgG was explored to better understand the forces in a small peptide-large protein pair system.

1.3 Overviews of this thesis

This dissertation focuses on antibody purification using small peptides as the affinity ligands and the interactions between antibody and the ligands.

Chapter 2 describes the structure and properties of antibodies, especially human immunoglobulin (hIgG), reviews the current purification methods for IgG antibody, and the current stage of research on peptide ligands for IgG isolation. The advantages and disadvantages of each separation method are discussed.

Chapter 3 presents the library screening process used to discover the ligands that bind hIgG specifically through its Fc portion. A family of linear hexamer ligand with the amino acid composition of histidine + aromatic groups + positively charged groups has been identified. Specifically, these ligands are HWRGWV, HFRRHL, and HYFKFD. The binding spectrum of the ligand HWRGWV is displayed and its potential to separate IgG from mammalian cell culture media (cMEM) is primarily demonstrated, where cMEM contains 10% fetal calf serum and 5% tryptose phosphate broth.

The ligands identified are further characterized in chapter 4 by isothermal adsorption measurement and protein complex separation in a column format. The investigation on the best lead HWRGWV is shown for the influence of the peptide density, temperature, binding

conditions etc. on its specificity to Fc and its capability to chromatographically isolate hIgG from cMEM.

Intravenous immunoglobulin G (IVIG) is now the leading plasma product in the therapeutic market. The possible application of HWRGWV to isolate hIgG from Cohn paste II+III has been examined in chapter 5. This chapter also includes the immobilization of the peptide ligand HWRGWV to commonly used Sepharose CL-4B beads for IgG purification from cMEM.

Chapter 6 examines the storage stability of the Fc-binding ligands, HWRGWV, HFRRHL, and HYFKFD, over the time after the usage of the ligands for isolation.

The interactions between a peptide as small as a hexamer and a large protein as big as an antibody has never been systematically studied in literature. In chapter 7 of this thesis, the possible binding site of small peptide HWRGWV on Fc fragment of hIgG has been examined and the possible interaction mechanism between hIgG and HWRGWV is discussed.

All above results are summarized in chapter 8 with recommended future work.

Additional information about the peptide HWRGWV are included in appendices. Appendix 1 compares the HWRGWV resins of different batches and appendix 2 is about the thermodynamics of interactions between hIgG and the HWRGWV ligand.

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Chapter 2. Literature Review

2.1 Immunoglobulins

2.1.1 Overview

Immunoglobulins (Igs, also referred to as antibodies or gamma-globulins) constitute a very important class of proteins that are present in blood, milk, saliva, and other body fluids of human, mammals, birds and fish functioning as protective agents against challenging substances. The challenging substances are called antigens which can be bacteria, viruses, and toxins. Igs are glycoproteins produced by white blood cells, each having a special antigen-binding pocket that is sensitive to specific parts of an antigen protein. The binding process can elicit a variety of secondary effector functions which depend on constant region determinants on COOH-termini of antibodies. The secondary functions generally inactivate or remove the infectious agents and their products.

Antibodies can be generated mainly by two approaches. The first is as the conventional serum product of an immunized animal, namely, polyclonal antiserum; the second uses the hybridoma technique by fusing a non-immunoglobulin-producing B-lymphocyte cancer cell with a single antibody-producing spleen cell to generate monoclonal antibodies (mAbs). MAbs produced in one culture are homogeneous in specificity, affinity, and isotype; each mAb product being specific to a single epitope of antigens. In contrast, polyclonal antibodies in an antiserum are the product of many responding clones of cells and are, as a consequence, heterogeneous at many levels: in the classes and subclasses of the antibody produced, their specificity, titer, and affinity. In one antiserum there may be antibodies to many discrete antigens, or to discrete epitopes on a single antigen. Polyclonal antisera often have higher level of IgG than mAbs - up to 10% of the total IgG content is specific antibody. Both polyclonal and monoclonal antibodies have been generally used for identification, measurement, purification of biological molecules, and for therapeutic applications (*1*).

2.1.2 Structure and isotypes of human immunoglobulins

The human immunoglobulins (2), account for approximately 20% of all proteins in human plasma, and can be divided into five classes or isotypes: IgG, IgA, IgM, IgD, and IgE. They are different in size, configurations, functions, and distributions (Table 2-1). IgG is the most abundant class of Ig in the body, constituting approximately 75% of the total immunoglobulins at an average concentration of 12 mg/ml in adult blood. IgG dominates in secondary immune responses, and also is the most commonly seen myeloma protein. IgA is predominant in secretory Igs in polymeric form; in contrast, it is present in serum mainly as a monomer at an amount of approximately 15% of total serum Igs. It blocks uptake of antigens. IgM is the dominant antibody produced in primary immune responses, and is physically much larger than the other immunoglobulins. IgD is generally found with IgM on the surface of human B lymphocytes but its principal function is as yet unknown. IgE is responsible for autoimmune responses, such as allergies and diseases like arthritis etc.

As a family, human Igs share a basic structural unit that comprises two identical heavy (H) chains and two identical light (L) chains (e.g. L_2H_2) connected together by disulfide bonds (Figure 2-1). Each polypeptide chain is constituted of several domains that are formed by antiparallel strands (3) and have about 100 amino acid residues. Starting from the amino termini, the L chains include the V_L and C_L domains while the H chains encompass the V_H , C_{H1} , hinge, C_{H2} , and C_{H3} regions. The “V” label indicates a variable region that exhibits striking differences in amino acid composition within the same class of immunoglobulins, while the “C” label denotes a constant region where the amino acid composition is relatively invariant within the same class of immunoglobulins. The V regions of both heavy and light chains are responsible for antigen binding by forming a pocket with six loops. The C region is engaged in a variety of effector functions. Heavy chains are categorized into five classes, γ , α , μ , δ , ϵ , respectively corresponding to IgG, IgA, IgM, IgD, and IgE. They are distinguished by the amino acid sequences in C regions and by the number of domains. Light chains are classified into two types, κ and λ , according to the amino acids in their C regions. J chain, the key to facilitate the polymerization of IgA and IgM, is an elongated 15 kDa glycoprotein containing high quantity of glutamic acid and aspartic acid. In all classes the interchain disulfide bonds associated with the hinge region of Igs are outside exposed to bulk solvent.

The intrachain disulfide bonds associated with the domains on the other hand are buried inside the globular molecules and hence are resistant to proteolytic enzymes.

IgG has a Y-shaped structure (Figure 2-1) with the molecular weights of H and L chains being 50 and 25 kDa, respectively. It exhibits a compact globule fold with dimensions of approximately $235 \times 44 \times 44$ Å (4). The domains of IgG are formed by antiparallel strands arranged into two β -pleated sheets. The sheets are tightly packed and connected by conserved disulfide bonds, which maintains the stability of the domains (3).

When human IgG (hIgG) is partially cleaved with the proteinase papain, the molecule is cut at the top of the hinge region (towards the amino-terminal) to give two Fab (antigen binding) and one crystallizable Fc fragments (Figure 2-1). Two Fab arms when linked by the hinge region comprises the structure of the $F(ab')_2$ fragment which can be produced by limited pepsin digestion of intact IgG. The conserved Fc region has carbohydrates at the N-glycosylation site Asparagine 297 and the glycosylation has many effector functions (binding complement and binding to cell receptors) expressed by antibodies. Disulfide bonds form bridges between chains in hinge region, which confer flexibility to the IgG molecule. The flexibility of the IgG increases with the longer hinge regions. The extensive flexibility of the antibody molecules permits antibodies to adapt to a vast array of antigen shapes and sizes, while retaining a covalent link between the Fab domain and the conserved Fc region. Glycosylation has little, if any, effect on antigen binding. However, deglycosylation of IgG compromises the recognition by all three cellular Fc receptors (Fc γ Rs) and decreases the thermal stability of antibodies (5). Other activities, e.g. Protein A and rheumatoid factor binding are unaffected by glycosylation (6). Fc γ Rs are known to bind to the hinge proximal region of the Fc portion.

Human IgG consists of four subclasses, IgG1, 2, 3, and 4 (7) (Table 2-2). Among these subclasses, IgG1 is the most dominant protein, making up anywhere between 43 to 75% of all the hIgG (8). A major structural difference between them is the length of the hinge region and the numbers of disulfide bonds at this region. The four subclasses show more than 95% homology in the amino acid sequences of the Fc regions, however, each has a unique profile of biological activities (Table 2-2), such as complement activation and Fc γ R engagement, as well the different binding properties to Protein A (Table 2-3).

2.1.3 IgG-binding proteins: Protein A, G, and L

Protein A (9) is a cell wall component of the bacterium *Staphylococcus aureus*. It has an elongated structure consisting of five highly homologous (share 65%-90% amino acid sequence identity) IgG-binding domains, namely A, B, C, D, and E domains, as well as one cell wall associated region. The IgG-binding domains are roughly cylindrical (26 x 16 Å) with individual molecular weight and pI of about 6.6 kDa and 5.1, respectively. The flexible random coil sequences that link the domains provide a fair degree of steric mobility but are very susceptible to proteolytic cleavage, even though the binding domains themselves are protease resistant (10). The molecular weight of intact Protein A by theoretical calculation is 54 kDa while by SDS-PAGE estimate averages range from 42-45 kDa due to its resistance to unfolding in SDS (11). The primary binding site for Protein A is on the Fc portion of IgG at the junctures of the C_{H2} and C_{H3} domains (Figure 2-2) (12). The affinity of Protein A for human IgG1 is about 10^8 M^{-1} (13), whereas variable affinity for Igs from different species and subclasses are well known for Protein A (Table 2-3). ¹³C-NMR analysis (14) suggests that the ionization of His-310 in the Fc fragment at around pH 5.0 plays a major role in the dissociation of the protein A and IgG complex, although the bound IgG cannot be eluted by buffers of pH above 4 (15).

Streptococcal Protein G binds tightly to the Fc region without interfering with the antigen binding sites (Figure 2-2). It has three highly homologous IgG-binding domains and also sites for albumin and cell-surface binding (16). Protein G exhibits an affinity for IgG in the range of $10^8 - 10^{10} \text{ M}^{-1}$ (17, 18), depending on species and subclasses (Table 2-3). It has greater affinity than Protein A for most mammalian IgGs (19), especially for certain subclasses including human IgG3, mouse IgG1 and rat IgG2a etc. However, unlike Protein A, Protein G does not bind to human IgM, IgD, and IgA.

Even though the Fc binding sites for Protein G and Protein A overlap extensively), the modes of interaction are quite different in both cases. In addition, there is very low the sequence homology between Protein A and Protein G (20, 21). The protein G-Fc complex mainly involves charged and polar contacts: 12 polar or charged interactions with Fc and no hydrophobic contacts. Protein A and Fc contacts are stabilized mainly through nonspecific hydrophobic interactions and fewer polar interactions.

The Fc-binding domains of both Protein A (22-25) and Protein G (26) are known to also bind to the Fab portion of IgG. The binding affinities are about $10^5 - 10^6 \text{ M}^{-1}$, weaker by two to three orders magnitude than their affinity for IgG-Fc portions. The binding sites for Fab on Protein A are different than those for Fc, therefore, it is possible to engineer Protein A to eliminate the Fab-binding sites for better specificity.

Engineered Protein A and Protein G products have been intensively studied and most of them are commercialized. Recombinant Protein A, Protein G, and the chimeric Protein A/G, are mostly engineered to exclude the cell wall associated region (Pierce, Rockford, IL). It is also possible to alter the C-terminus region to facilitate the purification of the protein by insertion of polyhistidyl sequences (Biovision Research Products, Mountain View, CA) or to support the directional coupling of the ligand to solid phase supports by incorporation of cysteine (GenScript Corp., Piscataway, NJ). Chimeric Protein A/G contains five Ig-binding regions of protein A fusion with three Ig-binding region of protein G to retain the binding capacities of both the parental constituents (27). One single domain of Protein A and protein G have been engineered to reduce the binding strength hence the elution harshness (28, 29) or to improve the stability (30-32) or to increase the specificity of the ligand (15).

Protein L (33) is a multidomain *Peptostreptococcus magnus* protein that, as opposed to Protein A and Protein G, binds to the immunoglobulin kappa light chains (Figure 2-2) without interfering with the antigen-binding sites. It binds a wider range of Ig classes and subclasses than Protein A and Protein G. Protein L binds to all classes of Ig, as well as single chain variable fragments (Scfv) and Fab fragments.

2.1.4 Application of IgG-binding bacterial proteins in antibody purification

Protein A and Protein G affinity chromatography, including their recombinant derivatives, are very popular and widespread methods for isolation and purification of immunoglobulins, particularly for isolation of monoclonal antibodies (34), mainly due to the ease of use and the high selectivity and high purity obtained. Binding through the Fc moiety of IgG results in the preference for Protein A and Protein G chromatography among other ligands since it allows process harmonization for multi-product manufacturing, such as different antibodies and Fc-fusion proteins.

However, it is recognized that Protein A and Protein G pose several problems to the user even though some have been improved when using engineered Protein A (35). The issues related to Protein A affinity ligand include: very high cost, variable binding efficiency of different monoclonal antibodies (particularly mouse IgG₁), leakage of immunogenic Protein A/G into the product, loss of antibody activity associated with harsh column elution and wash conditions, low stability of the matrix in typical cleaning solutions, e.g. 1 M sodium hydroxide and expensive ligand disposal. Each of these drawbacks has its specific consequence in the individual application, ranging from insignificant to very serious and prohibitive consequences. Protein A resin is over 30 times more expensive than some ion exchange resins, and may account for more than 35% of the total recovery raw material costs at large scale (36). The low pH exerted on antibodies reduces the product stability and/or activity and causes aggregation that can compromise drug safety. It was also found that cholesterol and phospholipids were co-eluted with IgG when Protein G was used as the affinity ligand (37). In addition, Protein A is not a good option for therapeutic human IgG purification since Protein A has no affinity to hIgG3. These drawbacks drive the research for alteration or alternatives to Protein A or for non-Protein A chromatography purification schemes.

Protein L, unlike Protein A and G, is much less developed and employed in antibody purification although it showed comparable binding properties of humanized r-IgG1- κ to Protein A and Protein G (38). Protein L seems to be more attractive in scFv fragments purification (39, 40). With the development of more new antibody fragment products containing Fab or F(ab')₂ instead of Fc, Protein L may become more popular. At the same time, it should be kept in mind that Protein L, also a protein from bacteria, suffers from the same drawbacks as Protein A.

2.2 Purification of Immunoglobulins

2.2.1 Overview

Igs can be isolated from pooled normal plasma or polyclonal antisera, as well produced in hybridoma cell culture as antigen-specific monoclonal antibodies (mAbs). Isolation of a particular protein from human plasma is a complex process that involves separating it from

hundreds of other proteins. During purification of pharmaceutical mAbs, impurities including host cell proteins, DNA, antibody variants, small molecules and potential contaminants including endotoxins and viral particles must be removed. To purify Igs, the crude source has to be subjected to a combination of different processes, such as precipitation, filtration, and the most popular, chromatography. Generally, there exists a very broad range of different methods available for isolation of immunoglobulins giving a very broad range of final purities, yields and cost of the product. Corresponding to the original source and the final usage of antibodies, there are preferred procedures, including capture, purification, and polishing three steps.

Traditional methods for the isolation of immunoglobulins are based on selective reversible precipitation of the protein fraction comprising the immunoglobulins while leaving other groups of proteins in solution. Typical precipitation agents are ethanol, polyethylene glycol, lyotropic (anti-chaotropic) salts such as ammonium sulfate, potassium phosphate, and caprylic acid. The Cohn–Oncley method (41, 42) using cold ethanol precipitation is still commonly employed today by the various fractionation companies who manufacture intravenous IgG (IVIg) from human plasma due to the large volume of processed plasma and to the huge amount of proteins to be treated per lot (e.g. >1000 kg). Typically, these precipitation methods give very impure products with poor reproducibility, at the same time being time consuming and laborious. They have to be coupled with other purification methods, commonly chromatography, to achieve sufficient purity of IgG from human serum (43, 44).

Ion exchange chromatography (IEC) is a well known method of protein fractionation frequently used for isolation of immunoglobulins. IEC separates proteins based on differences in the surface charge of the molecules. Usually in industry a cation exchange chromatography step (CEX) is accompanied by an anion exchange (AEX) step since they complementally bind proteins with different charges (45). IEC has also been employed in antibody fragment purification (46). Newly developed IEC resins have been reported to exhibit protein capacities as high as 180 mg/ml media (46) or to be capable of direct binding of antibodies from media of physiological ionic strength (150-200 mM NaCl) (47). However, the isoelectric point variation in antibodies makes it necessary to explore and determine the

binding to an IEC resin on an individual basis. As a consequence, the contaminant spectrum with which an antibody is associated on resins is usually different from one binding condition to another, involving numerous iterations since the lack of harmonization in antibody purification process, i.e. more time and cost consuming in downstream development. When IEC is the capturing step, salt concentration in feedstocks may need to be reduced by dialysis/diafiltration which adds more steps/costs to the process, or by dilution which may adversely affect the recovery. Despite its weaknesses, IEC is still broadly adopted in antibody manufacturing thanks to the low cost of resins.

Hydrophobic chromatography (HIC) is also a method widely described for isolation of immunoglobulins. HIC differentiates proteins according to the hydrophobic groups available to interact with immobilized ligand such as phenyl and butyl in the presence of water. Since antibodies have a stronger hydrophobicity than the majority of their contaminants, HIC is an effective technique for purification. However, there are some limitations in using HIC: the possible denaturation of antibodies on excessively hydrophobic supports; the insolubility of Igs at the salt concentration required to support retention on weakly hydrophobic media; and the necessity to add a lyotropic salt (i.g. ammonium sulfate) to the raw material which increases processing cost to the large scale user.

Thiophilic adsorption chromatography (TAC) was introduced by J. Porath in 1985 (48) and commercialized as “T-gel” (49) and its alternative “Avid-AL” (50, 51). The incorporation of the sulfur, oxygen, or nitrogen groups in otherwise hydrophobic chemical ligands was believed to be a structural necessity to obtain the specificity for antibodies (52). TAC, sometimes described as affinity chromatography, is an analogue of HIC with milder salt concentration required in loading (less than 0.5M ammonium sulfate). However, it also non-specifically binds other proteins such as albumin, α -2-macroglobulin (53), and α -amylase II (54) etc. Nevertheless, TAC has been successfully employed in lab research to purify monoclonal (55), polyclonal (56), and antibody fragments (57, 58). Based on TAC, mixed mode chromatography methods including MEP HyperCel and FastMabs as discussed in detail later have been developed to reduce the salt concentration in binding.

Size exclusion chromatography (SEC) is primarily limited to polishing in antibody purification due to the high cost resulting from its low capacity and long separation times (59).

It is easy to see from the above discussion that chromatography is one of the most powerful methods in protein, especially antibody, purification in modern industry. Every common chromatography mode has been used in immunoglobulin G (IgG) processing. Among them, purification of antibodies using affinity chromatography (AC) allows the possibility of obtaining several fold purification with high recovery in fewer steps (60). Half the marketed therapeutic antibodies incorporate affinity ligands, usually bacterial wall proteins (Protein A or Protein G), in their production process (45).

2.2.2 Protein A alternatives for antibody purification

Being aware of the drawbacks associated with Protein A/G, the attributes of small peptides as potential ligands for large scale affinity purification are of great interest (61-65) due to their advantages of being more stable, less immunogenic, and less expensive than protein ligands. The use of high throughput screening techniques, sometimes combined with molecular modeling, has generated new and powerful small ligands for potential Protein A replacement.

Roque et. al. (66) summarized that approximately a dozen synthetic ligands including non-peptidic and peptidic ligands have been discovered for antibody purifications. Some of them have been extensively studied and are already commercialized, such as synthetic ligand MAbsorbent A2P (Prometic BioSciences Inc., Wayne, NJ) derived from artificial Protein A 22/8 (67), hydrophobic charge induction chromatography (HCIC) ligand MEP Hypercel (Life Technologies, Rockville, MD) based on TAC (52), and Kaptiv-GY (Interchim, Montlucon, France) based on Protein A mimetic peptide (RTY)₄K₂KG (68). Meanwhile, the search for new ligands to be used in antibody purification is still highly active in both industry and academia (64, 69).

Hydrophobic charge induction ligand MEP (4-mercapto ethyl pyridine) (Figure 2-3), immobilized on a cellulose matrix, is reported to adsorb IgG by hydrophobic interactions while the desorption is achieved by electrostatic charge repulsion (61, 62). MEP is a pH-

sensitive weak binder with a binding capacity for hIgG of 25-33 mg/ml. Proteins or aggregates can be separated by finely tuning pH and salt concentrations in adsorption and desorption buffers. HCIC showed good concentration ability when the feedstock solution was from protein-free cell culture supernatant containing 0.0474 mg/ml IgG, yielding the purity and recovery of 44% and 75%, respectively (70). A purity of 98% was reported when IgG concentration in feedstock was 0.1 mg/ml (71). When dealing with cell culture supernatant containing 5% fetal calf serum (FCS) at an IgG concentration of 0.1 mg/ml, the purity and recovery of hIgG were found to be 76% and 69% respectively (71). Using this ligand, the washing salt concentration and elution pH needed to be experimentally decided for each antibody in order to separate it from other contaminant proteins.

Another mixed mode chromatographic ligand FastMabs (Figure 2-3) from Upchurch Chromatography A/S (Copenhagen, Denmark) is composed of an aromatic or hetero-aromatic core to which different hydrophilic or ionic substituents, typically carboxylic, sulphonic, phenolic or amino groups are attached (72, 73). The binding is strongly pH-dependent but largely independent of ionic strength in the raw material. This ligand showed the ability to purify IgG and IgM from medium containing 5% FCS (74).

Artificial Protein A ligand 22/8 (65, 67) was the basis for MAbsorbent A2P (Figure 2-3), mimicking the IgG binding site of Protein A using triazine framework substitutions. This ligand was optimized to purify polyclonal IgG from hyper-immunized ovine serum with both the yield and purity greater than 95% (67). Ligand A2P has such a high affinity to some antibodies and Fc-fusion proteins that bound proteins have to be eluted at a pH lower than 2.5 (75).

Protein A mimetic peptide TG19318 (63, 64) led to the commercialized ligand Kaptive-GY for antibody purification. Kaptive-GY is a tripeptide tetramer (Arg-Thr-Tyr)₄-Lys₂-Lys-Gly with all amino acids in D configuration to obtain high resistance to proteolytic digestion (68). It was reported to isolate hIgG from human serum with both purity and yield of about 90% (64), but no literature has been identified for this ligand to be used in purification of IgG from cell culture media. This ligand was also used to capture IgM, IgA, IgE, and IgY (chicken IgG) from cell culture supernatant, antisera, or ascetic fluids under almost identical chromatographic conditions with purities and recoveries of above 90% and 80%, respectively

(76-79). In all these separations, antibodies were loaded at pH 7.0 and eluted with pH 3.5 buffers.

Bak and Thomas (80) compared these small ligands (MEP HyperCel, MAbSorbent A1P and A2P, FastMabsA, and Kaptiv-GY) with several modified Protein A ligands in capturing of polyclonal rabbit antibodies from clarified antiserum. They concluded that even though MEP HyperCel was the best among the small ligands, it was not yet comparable with modified Protein A. The same conclusion was also drawn in another independent study by Ghose et al. (75) that the small ligands need to be improved to match the selectivity and purification factors of Protein A.

Among all identified small ligands, including some specifically designed to mimic Protein A, no ligands have been found to behave like Protein A in their specificity to the Fc fragment of antibodies. Binding of Fc fragments renders the ligand a universal binder for antibodies and Fc-fusion proteins, which will possibly diminish the optimization process for a new antibody. Binding through the Fc portion also frees the Fab domains for antigen binding, which will be desirable when using the antibody for immunoassays and diagnostic applications.

2.2.3 Future of antibody purification

Manufacturers are exploring multiple ways of streamlining product recovery and purification processes. Strategies include decreasing the number of steps, avoiding complex steps and reducing raw materials costs. Three-step non-Protein A purification processes have been screened and have demonstrated comparable purity and yield to the traditional 3-step process including Protein A followed by CEX and then AEX chromatography in purification of mAb from serum free media (36). Membrane chromatography could be used in the polishing step with faster processing time and as much as 95% less buffer usage than for conventional packed-bed chromatography (81). However, it was recognized that membranes were not likely to be clearly advantageous in the bind and elution mode (82).

In addition, alternative formats for recovery and purification unit operations from the nutraceutical and industrial enzyme industries are being reconsidered including expanded bed chromatography (73, 83), magnetic separation (84) (85), membrane chromatography and

non-chromatographic methods such as flocculation, precipitation, crystallization and aqueous two-phase systems. Low et. al. (34) reviewed the potential methods for future antibody production. With the emergence of antibody production in transgenic plants and animals and in bacterial media, these methods may become more suitable and applicable (86, 87).

2.3 Peptides as affinity chromatography ligands

Combinatorial peptide libraries can be created on solid supports through the “split synthesis” approach (88, 89) or phage display technique (90), which have been used in drug discovery (91) and in affinity ligand search (92). The major advantage of this method is the capability of rapid synthesis and screening of millions of compounds in a short time (93). Wang et al. (94) reviewed the library generation and screening techniques, together with screening results, for protein purification. Peptidic affinity ligands are low in toxicity and immunogenicity, high in sterilizability and stability, moderate in affinity and hence mild in elution. With these reasons, many researchers have been searching in peptidic libraries for antibody purification ligands.

Except the Protein A mimetic peptide TG19318 mentioned above, there are some other peptide ligands in literature discovered for antibody purification. A tetrapeptide library was screened using an immunoaffinity method (95) and ligand APAR was selected to isolate anti-Granulocyte Macrophase-Colony Stimulating Factor (GM-CSF) from 1:40 diluted ascitis. A strong binding was observed ($K_a = 9.4 \times 10^{-8}$ M) and bound mAb was not able to be completely eluted by 0.1M glycine/HCl or by a pH 2.8 solution. An electrophoretically pure (single band) mAb product was extracted by 5M LiCl solution with a yield of 95%. However, the dynamic capacity of the APAR modified Sepharose gel was only 3.9 mg-mAb/ml-gel, which might be the reason that the ligand was not further reported by the authors. Phage displayed library was employed for finding a ligand to bind the constant region of humanized anti-Tac IgG1 mAb (HAT) by using the pFc' fragment as the target (96). The peptide EPIHRSTLTALL was identified with 42% homology with Protein A and displayed the ability to bind HAT. Unfortunately, no further information was found in the literature to better judge this ligand's performance and binding domain for antibodies. A 22-mer peptide ligand, AEGEFINVPMMVDGITMGDPAK or M[46-2], was reported (97) by the same

group that discovered TG19318. This ligand was for purification of the murine anti-human tumor-associated tenascin-C (Tn-C) mAb from 10 times concentrated protein free cell culture media. Its shorter form, PMMVDGITMG, displayed a similar binding and elution profile as M[46-2] but with better enzymatic and base-acid resistance. M[46-2] was found to recognize only the Fab fragment of the mAb, making it a good potential ligand for specific anti-Tn-C antibody separation. A cyclic dimeric tripeptide library was screened against monoclonal anti-TNF receptor I antibody (7H3) and the ligand $(\text{NH}_2\text{-CFHH})_2\text{-KG-OH}$ was identified to be able to bind both the Fc and Fab fragments of several G class antibodies (64). The ligand showed the potential to purify 7H3 from cell culture supernatant and IgG from human blood plasma with a purity of over 90% and a yield of 67-90%. This ligand was claimed by the authors to be more stable than Kaptiv-GY due to its “synthetic” nature, a questionable statement since the two ligands are similar in their scaffold structure. Feng *et al* (69) screened 200 compounds, some containing amino acids, that represent the active site of Protein A by using program docking to find a ligand that could bind the Fc fragment of IgG. Their best ligand, N-benzyloxycarbonyl-L-tyrosine (N-cbz-L-Tyr) was found to be able to isolate hIgG from human blood plasma. However, it was also reported that this ligand bound HSA even stronger than IgG. In addition, it was not yet clear if the ligand just interact with the Fc portion of hIgG.

Combinatorial library technology was also employed in the discovery of the linear epitope PDTRPAP by epitope mapping using antibodies raised against carcinoma-associated MUC1 mucin (98). The epitope was then used as an affinity ligand to purify anti-MUC1 mucin antibody, C595, from hybridoma supernatant (99) and C595 diabody fragment (dbFv) from *Escherichia coli* lysates (100). Single SDS-PAGE band antibody products were eluted using linear gradient of 3M sodium thiocyanate (NaSCN) in PBS. Similarly, mimotope ligand GLFYD was identified to purify mAb4155, a murine IgG1 antibody against estriol-3-glucuronide (101). Obviously, both epitope and mimotope affinity chromatography target the variable region of antibodies.

It is clearly shown that all these peptide ligands, if tested against the IgG fragments, bound the Fab fragment regardless of its affinity to Fc, even though some were originally aimed to solely capture Fc fragments. This seems to suggest that it is hard to find a real

Protein A replacement to be highly specific to Fc for antibodies and proteins fused with Fc, two protein forms commonly utilized in therapeutics. The objective of this thesis is to identify and characterize such a ligand using a solid phase combinatorial peptide library.

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Table 2-1 Properties of immunoglobulin classes*

	IgG	IgA	IgM	IgD	IgE
Concentration in serum (mg/ml)	12	3	1	0.1	0.001
% total Ig in adult serum	77.8-71.8	12-18	10	0.2	0.004
Mass (kDa)	150	180-500	950	175	200
Sedimentation coefficient(s)	7	7,10,13	18-20	7	8
Light chains	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Heavy chains	γ	α	μ	δ	ϵ
Chain structure	L_2H_2	L_nH_n	$L_{10}H_{10}$	L_2H_2	L_2H_2
Tail piece	No	Yes	Yes	Yes	No
J chain	No	Yes/No	Yes	No	No
Carbohydrate average %	3-4	8-10	12-15	18	18
Number of oligosaccharides	1	2-3	5	NA	5

* data from (2)

Table 2-2 Structural and binding differences in human IgG subclasses*

Heavy chain type	IgG1	IgG2	IgG3	IgG4
Molecular mass, kDa	146	146	170	146
Amino acids in hinge region	15	12	62	12
Inter-heavy chain disulfide bonds (in hinge region)	2	4	11	2
Susceptibility to proteolytic enzymes	++	+/-	+++	+
Number of allotypes	4	1	13	0
Mean adult serum level, mg/ml	6.98	3.8	0.51	0.56
Proportion of total IgG, %	43-75	16-48	1.7-7.5	0.8-1.7
pI range mean (SD)**	8.6(0.4)	7.4(0.6)	8.3(0.7)	7.2(0.8)
Fc γ RI***	+++	-	+++	++
Fc γ RII***	+	-	+	-
Fc γ RIII***	+	-	+	-
Complement activation classical path**	++	+	+++	-

* Data from (102)

** From Leffell et al. (2).

*** From Hulett and Hogarth (103)

Table 2-3 Protein A and Protein G specificity to antibodies from different species and classes

Species/ Subclass	Protein A	Protein G
Monoclonal		
Human		
IgG 1	++++	++++
IgG2	++++	++++
IgG3	—	++++
IgG 4	++++	++++
Mouse		
IgG 1	+	++++
IgG 2a	++++	++++
IgG 2b	+++	+++
IgG 3	++	+++
Rat		
IgG 1	—	+
IgG 2a	—	++++
IgG 2b	—	++
IgG 2c	+	++
Polyclonal		
Rabbit	++++	+++
Cow	++	++++
Horse	++	++++
Goat	—	++
Guinea pig	++++	++
Sheep	+/-	++
Pig	+++	+++
Rat	+/-	++
Mouse	++	++
Chicken	-	+
Human IgG	++++	++++
Human IgM	Variable	+
Human IgD	-	+
Human IgA	variable	+
Human IgE		-

* Data from (102) — (weak or no binding) ++++ (Strong binding)

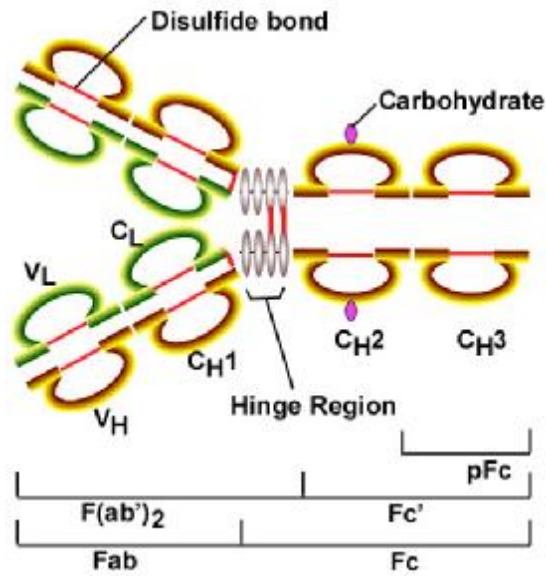


Figure 2-1 Structural diagram of human IgG1

Green line: light chain; brown line: heavy chain. Each “C” like structure denotes one of the IgG domains. The enzymatic fragments Fab, Fc, F(ab')₂, Fc' and pFc are indicated below the diagram.

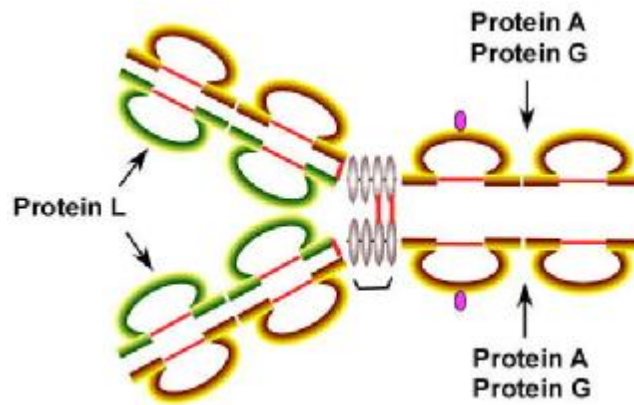


Figure 2-2 Binding sites for Protein G, A, and L on IgG

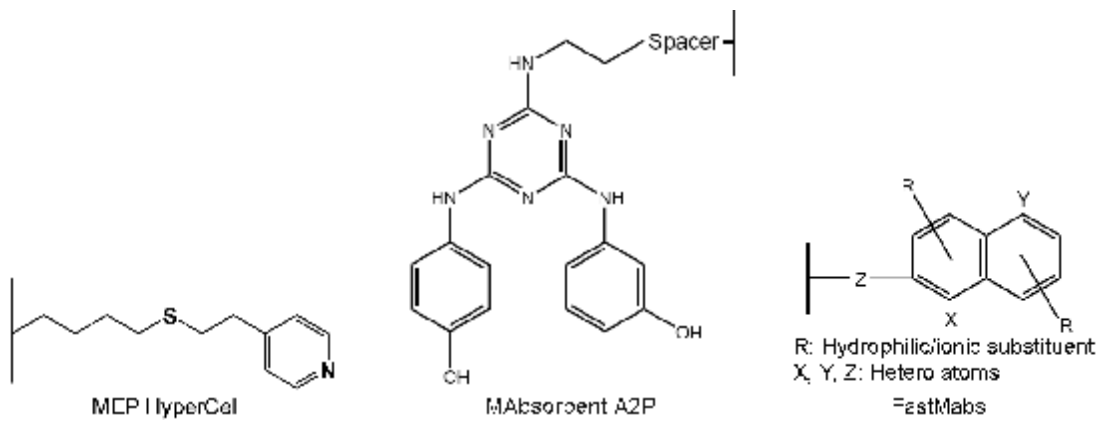


Figure 2-3 Ligand structures of potential Protein A replacement on market

Chapter 3. Hexamer Peptide Affinity Resins that Bind the Fc Region of Human Immunoglobulin G

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3.1 Introduction

Antibodies and their fragments have found a wide array of applications as pharmaceutical compounds, in addition to their more common usage in the purification and localization of proteins (1). The high affinity and specificity of antibodies have enabled very diverse clinical functions as imaging reagents, and as immuno-modulatory agents, enhancing host response against pathogens and neoplasms or suppressing rejection in clinical transplantation (2). Antibody-based therapeutics account for roughly 20% of the therapeutic products in development in the USA (3). The majority of these are of the immunoglobulin G (IgG) isotype, which can effectively activate complement- and antibody-dependent cellular cytotoxicity pathways. As a result of this growth in the commercial use of antibodies there is great interest in establishing more efficient, robust and low cost processes to purify human IgG (HIgG).

HIgG is usually isolated from pooled human plasma, produced in hybridoma cell culture as antigen-specific monoclonal antibodies (MAbs), or produced as polyclonal antisera. The crude source of HIgG can be subjected to a combination of different processes, such as precipitation (4, 5), ion-exchange chromatography (IEC) (6), hydrophobic interaction chromatography (HIC) (7) or affinity chromatography (AC) (8-10) for purification of HIgG. ImClone Systems, for instance, integrates IEC and Protein A affinity chromatography in the manufacture of Erbitux®, a monoclonal antibody that targets the epidermal growth factor receptor on the surface of normal and tumor cells (11).

Among these methods, purification of HIgG using affinity chromatography allows the possibility of obtaining several fold purification with high recovery in fewer steps. *Staphylococcus aureus* Protein A and *Streptococcus* Protein G are the most commonly used ligands in the affinity purification of HIgG. However, their high cost, low stability, the possible presence of contaminants, and antibody activity loss associated with harsh column elution and wash conditions, make it important to find less expensive and more robust ligands for IgG purification. Synthetic small ligands may overcome some of these disadvantages and have been studied by many research groups. A list of small ligands for HIgG, some available commercially, is shown in Table 3-1.

The attributes of small peptides as potential ligands for large scale affinity purification are of great interest (12-16) due to their unique advantages of being more stable, less immunogenic, and less expensive than protein ligands. The “couple-divide-recombine” technique (17, 18) has been applied in previous work to create solid phase one-bead-one-peptide (OBOP) combinatorial libraries on solid chromatographic supports. The major advantage of the OBOP libraries is the capability of rapid synthesis and screening of millions of compounds. OBOP libraries with different peptide lengths and structures have been used successfully in the search for some of the small molecular weight ligands to purify antibodies. Some of these are also included in Table 3-1.

This work describes the identification and characterization of a family of linear hexameric peptide ligands that can bind HIgG through its Fc fragment and is able to isolate HIgG from mammalian cell culture media. Model ligand HWRGWV of this family exhibits some specificity to the IgG isotype over other human immunoglobulins and shows the same binding ability for HIgG and for immunoglobulins from other species. This is the first known reported instance in which a small ligand on a chromatographic support is shown to bind specifically through the Fc fragment of HIgG, a property normally associated with large ligands such as Protein A and Protein G.

3.2 Materials and Methods

3.2.1 Peptide library

Peptide libraries were synthesized directly on Toyopearl AF-Amino-650 EC (particle size 100 – 300 μm) or M (particle size 65 μm) resins (Tosoh Bioscience, Inc., Montgomeryville, PA) with 18 of the 20 naturally occurring amino acids (except cysteine and methionine) using fluorenylmethyloxycarbonyl (Fmoc) chemistry (19). In this paper, “large bead” and “small bead” libraries refer to the libraries built on EC and M resins, respectively. The peptides were attached to the amino groups at the end of an undisclosed hydrophilic spacer arm which is part of the structure of the commercial resin used. The base resin from Tosoh Bioscience is a hydroxylated polymethacrylate amino resin with a pore size of 1000 \AA and a surface area of 30 m^2/g . Libraries with six (hexameric library), three (trimeric library) and one (monomeric library) amino acid residues were synthesized, all at a

peptide density of 400 μ moles/g-dry-resin. Peptides International (Louisville, KY) synthesized all libraries using the “couple-divide-recombine” strategy (17) for combinatorial solid-phase libraries. In the case of the hexameric small bead library, the aliquots were not mixed after the final coupling round, which allowed for the use of a biased library that excluded histidine as the final amino acid residue.

3.2.2 Radiolabeling of intact HIgG and Fc fragment of HIgG

Intact HIgG and its Fc fragment from human blood serum (Bethyl Laboratories, Inc. Montgomery, TX) were labeled by reductive methylation (20) utilizing sodium cyanoborohydride (NaCNBH_3) (Sigma, St. Louis, MO) and ^{14}C -formaldehyde (H^{14}CHO) (PerkinElmer, Wellesley, MA). The reaction was performed at 4°C overnight. Following the reaction, the labeled protein was separated from unreacted ^{14}C -formaldehyde using an EconoPac 10DG Desalting Column (BioRad, Hercules, CA). The radioactivity of each fraction was determined on a Packard 1500 Tri-Carb Liquid Scintillation Analyzer (Meridan, CT) in CytoScint ES scintillation liquid from ICN (Mesa, CA). The protein concentration in each fraction was assayed by Micro BCA assay (Pierce, Rockford, IL) using a Shimadzu UV-160 UV-Vis Recording Spectrophotometer (Kyoto, Japan) and bovine serum albumin (BSA) as the standard. The protein-rich peak was collected and stored at 4°C .

3.2.3 Effect of radiolabeling of proteins on binding to control resins

In order to determine the impact of radiolabeling the Fc fragment on its binding to the control resins, an excess of Fc and ^{14}C -Fc was applied to Protein A Sepharose CL-4B resin (Amersham Biosciences, Piscataway, NJ) and MAbsorbent A2P resin (ProMetic Biosciences, Burtonsville, MD). The ratio of target protein to resin was 32mg/ml resin when using Protein A (capacity: 20 mg HIgG/ml gel) and 79 mg/ml resin when using A2P (capacity: 40 mg HIgG/ml gel). The resin and the protein solution in phosphate buffer (PB) at pH 7.0 were incubated for 2 hours at room temperature, and the amount of unbound protein in the supernatants was determined by absorbance at 280 nm, from which the bound Fc was calculated based on the weight of resins. The extinction coefficient of Fc ($E_{280}^{0.1\%}$) was 1.4 (21).

3.2.4 Primary screening

Twenty-milligram aliquots of the library beads were washed with 20% methanol and equilibrated overnight at 4°C with the binding buffer, phosphate buffered saline (PBS) (Sigma, St. Louis, MO) containing 10 mM phosphate buffer, 2.0 mM KCl and 138 mM NaCl, pH 7.4. The beads were incubated with 1% (w/v) casein (Pierce, Rockford, IL) for 1 hour followed by 1-hour incubation with cMEM, a complete mammalian cell culture medium. cMEM was formed by combining Minimum Essential Medium (MEM) with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB). ¹⁴C-labelled protein (¹⁴C-Fc or ¹⁴C-HIgG) was then added to the bead solution slurry to form a protein concentration of 1 μM and incubated for 2 hours at room temperature under gentle agitation. Following the incubation with ¹⁴C-protein, the beads were transferred to a Poly-Prep chromatography column (BioRad, Hercules, CA) and washed with pH 5 acetate buffer containing 0.05% (w/v) Tween 20 (Sigma, St. Louis, MO) and between 0.5 to 2 M NaCl (Sigma, St. Louis, MO) until the radioactivity of the flow-through reached background levels. Protein A Sepharose CL-4B beads and MAbsorbent A2P beads were used as positive controls for IgG binding and acetylated Toyopearl AF Amino 650M resin was the negative control.

Washed beads were suspended in 1% low melting agarose (BioRad, Hercules, CA) at 45°C and poured onto a 160-180 mm GelBond film (Bio Whittaker Molecular Applications, Rockland, ME) to form an approximate density of 10 mg beads/gel casting. The gels were allowed to dry overnight inside a fume hood, followed by exposure of a Kodak Biomax MR autoradiography film (Fisher, Atlanta, GA) to the air-dried agarose gel for 7 days. A second piece of film was incubated for 10 days with the gels for confirmation of the positive signals. When using the large bead hexameric library, beads displaying positive signals were excised from the gels using a scalpel and washed in 6M guanidine (Sigma, St. Louis, MO) followed by an extensive wash with PBS. The selected beads were then re-screened against Fc, following the same procedure as above. Positive beads identified during the screening of small bead libraries were not re-screened. All beads selected were sequenced by Edman degradation (Protein Chemistry Lab, Iowa State University, Ames, IA).

3.2.5 Secondary screening

To reduce the possibility of non-specific binding, larger batches of resins using the peptide ligands identified in primary screening were synthesized on Toyopearl AF-Amino-650 M resin at a substitution of 100 $\mu\text{mol/g-dry-resin}$ for secondary and tertiary screening, instead of the 400 $\mu\text{mol/g-dry-resin}$ used in the libraries. Twenty milligrams of re-synthesized resins carrying the leads identified in primary screening were washed thoroughly and equilibrated with PBS after being swollen with 20% methanol for one hour. The beads were then incubated with 400 μl of 1 μM ^{14}C -IgG in PBS (non-competitive mode) or in cMEM (competitive mode) in a 0.5 ml centrifuge filter with a 0.45 μm Durapore membrane (Millipore, Milford, MA) for two hours. After incubation, the particles were washed with PBS buffer for 15 min, followed by sequentially washing for one hour each with 400 μl of 1 M NaCl in PBS and 2% acetic acid (AcOH). The supernatant at each wash, as well as the resins after final wash, were collected for radioactivity counting. The percentage of bound and unbound protein was then calculated by mass balance. The ligands that bound more than 30% percent HIgG under competitive condition were selected for further study.

3.2.6 Tertiary screening

Peptide resins were dry packed into 3 cm long \times 0.21 cm (ID) columns (0.1 ml, Upchurch, Oak Harbor, WA) and tested on a Waters 626 HPLC unit with a built-in 2487 UV detector at 280 nm. Samples were manually injected at room temperature using a 100 μl sample loop (Thomson, Springfield, VA). Fab fragments, $\text{F(ab}')_2$ fragments (Calbiochem, Darmstadt, Germany), Fc fragments and intact HIgG (Bethyl Laboratories, Inc. Montgomery, TX) at a concentration of 1 mg/ml were loaded separately in PBS onto each chromatography column followed by elution with 2% acetic acid. Protein A Sepharose CL-4B and MAbsorbent A2P columns were packed and loaded with the same target proteins, followed by the elution method recommended by each manufacturer. The flow rate used was 50 $\mu\text{l}/\text{min}$ in the first 5 minutes and then changed to 100 $\mu\text{l}/\text{min}$ for elution. The fraction of bound target protein was determined by measuring the area of the chromatogram peak.

3.2.7 Selective binding of HIgG from cMEM on HWRGWV

A chromatographic approach was employed to examine the HIgG binding ability to one of the best ligands identified in secondary screening. A 5 cm long × 0.39 cm (ID) Omega column (0.6 ml) packed with dry HWRGWV resin was equilibrated with PBS binding buffer at pH 7.4 before sample loading. A sample of 100 µl of 5 mg/ml HIgG in either PBS or cMEM was injected at a flow rate of 50 µl/min for 10 minutes. The flow rate was increased for the remainder of the run to 0.4 ml/min. The column was washed sequentially with 6.5 ml binding buffer, 8 ml 0.5 M NaCl in binding buffer, 10 ml phosphate buffer (PB) at pH 4, and then 8 ml 2% acetic acid in water. For Protein A and A2P, smaller columns (0.1 ml) and less protein (0.1 mg HIgG) were employed. The chromatographic conditions followed the instructions from each manufacturer. The effluent was monitored by absorbance at 280 nm. All experiments were conducted at room temperature. Fractions were collected (3 ml for HWRGWV, 1 ml for controls) and concentrated 5.3 times by centrifugation at 4°C, 11,247×g for 90 minutes using a MicroCon YM-3 filter (regenerated cellulose, 3,000 MWCO, Millipore, Billerica, MA).

3.2.8 Cross-reactivity of HWRGWV with IgG subclasses and IgGs from different species

HIgG subclasses (HIgG1, HIgG2, HIgG3 and HIgG4), human serum IgM, IgD, and IgE, human secretory IgA and IgGs from different species (bovine, goat, mouse, rabbit, and chicken) were applied to an HWRGWV column (0.1 ml) to determine its cross-reactivity. All the Igs except IgA were isolated from blood. Human IgA was a colostrum secretory Ig. All proteins were purchased from Sigma (St. Louis, MO), except for HIgG1, which was purchased from Fitzgerald (Concord, MA). Samples (100 µl) at a concentration of 1 mg/ml were loaded in PBS (pH 7.4) at a flow rate of 50 µl/min for 5 minutes, followed with another 3 ml PBS flushing. The column was then washed in succession with 4 ml of each PBS + 0.5M NaCl, pH 4 PB and 2% acetic acid at a flow rate of 0.2 ml/min. Chromatograms were recorded at room temperature with the UV detector at 280 nm.

3.2.9 Gel electrophoresis

Concentrated fractions of chromatography peaks were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) on an XCell SuperLock™ Mini-Cell system from Invitrogen. The gels were stained by SimpleBlue SafeStain (Invitrogen, Carlsbad, CA) and their density was analyzed using the software ImageJ 1.32j (Wayne Rasband, National Institutes of Health, Bethesda, MD). The purity of the product was calculated as the fraction of the total area equivalent to the HIgG bands at 25 and 50 kDa, and the yields were estimated by normalizing the 25 kDa band to the HIgG standard.

3.3 Results and Discussion

3.3.1 Primary screening

The method chosen for detection of positive beads from the peptide library was to radiolabel the target proteins with ^{14}C -formaldehyde. It is based on the technique developed by Jentoff & Dearborn (20), optimized for on-bead detection by Mondorf *et al* (22) and further validated (12, 13, 15) by our group.

The method requires the acetylation of the amino groups present in the proteins, and has the potential to change the native conformation of a protein (20). In order to decrease the effect of labeling, only about 5% of the amino groups were targeted for acetylation. The influence of radiolabeling on the binding of the Fc fragment to commercial resins was assessed (Table 3-2). Radiolabeling seemed to decrease the binding of Fc fragment to both Protein A and A2P resins by almost 50% with a label extent of 2%. Previous experiments have determined that labeling less than 1% of the available amino groups typically generates difficulty in identifying positive beads because it produces weak signals and requires longer exposure times, thus increasing the background signal, as well as the risk of isolating false positives. It was decided to continue using the radiolabeled protein for evaluating positive beads because the advantages of the technique were more important than the reduced binding demonstrated when the target was radiolabeled. In this study, intact HIgG with less than 4% of the amino groups methylated and Fc fragment labeled at 2% of the available amino groups were used.

Four different libraries were screened during primary screening. The first library used was a large bead hexameric library built on Toyopearl AF-Amino-650 EC resin. As mentioned earlier, this resin has a particle size ranging in diameter from 100 to 300 μm . The use of large beads helps not only on the generation of positive signals during the exposure to the radiographic films, but also on the determination of the positive peptide sequence present in the selected bead. The other three libraries used in this study were small bead monomeric, trimeric and hexameric libraries. The base resin for these libraries was Toyopearl AF-Amino-650 M resin, which was around 65 μm in diameter. Small bead libraries were used mainly for confirmation purposes, while the large bead library was used to identify the hexamers showing binding to the target proteins.

Several factors need to be considered during primary screening: blocking solution, washing solution, target conformation and source, and the composition of the competing compounds. To increase the selection stringency, increasing numbers of blocking and competition steps were used in this study. After blocking with casein, cMEM was used as an additional blocker as well as a competing solute during the incubation of the library with the target protein. These steps were taken in order to reduce the chance of identifying weak binders or binders that could not stand the competition from contaminating proteins. Washing conditions can define the binding strength and the possible types of protein-target interactions. A pH 5 acetate buffer containing 0.05% (w/v) Tween 20 and 0.5-2 M NaCl was selected as the washing solution because the surfactant Tween 20 tends to eliminate weak binders due to hydrophobic interactions, while the presence of salt reduces the number of weak binders relying on charge-charge interactions. The change in pH during washing has the potential to decrease the number of positive beads by increasing the pressure for weak binders to release the bound protein. In order to direct the selection of IgG binding ligands towards the conserved Fc region of HIgG, radiolabeled Fc fragments were employed as the targets in addition to ^{14}C -HIgG.

Table 3-3 shows the results obtained during the primary screening using different strategies for ligand identification. A total of 4% (0.7 gram) of the large bead hexameric library was screened, yielding 437 positive beads. From this total, 252 beads were isolated from the experiments using Fc as target protein for both initial screen and re-screen (Fc/Fc

group), and the remaining 185 beads were isolated when using HIgG for initial screen, followed by Fc in the re-screen (HIgG/Fc group).

The selection of beads with stronger signal from the Fc/Fc group generated an 11-bead sub-group for peptide identification, and 8 more beads were sequenced from the group obtained from HIgG/Fc screen (Table 3-3) with a total of 19 peptides being identified. All ligands identified at this stage contained histidine (H, His) in the first position (N-terminus) of the peptide. Because this is an unusual result with very consistent homology, three random negative beads were sent for sequencing and no histidine was found in the first position (data not shown). This provided confidence that the finding of histidine in the first location of all the identified peptide was not an artifact from the sequencing or library synthesis.

In order to determine if there were suitable ligands without histidine in the first position, a biased small bead hexameric library was screened. This library was produced using the “split-couple-recombine” approach, but after the final amino acid residue was attached, the 18 aliquots were kept segregated, yielding 18 sub-libraries, each with a different final amino acid on the N-terminus. To produce the biased library, 17 of the sub-libraries were mixed in equal amounts, and a total of 663 mg of library without His at the N-terminus were screened against Fc fragment, yielding 7 positive beads. The results show that when histidine is not present, lysine (K, Lys) becomes the most prevalent amino acid in the first position. The presence of several undetermined positions (marked as X in Table 3-3) is due to the use of libraries on small base matrix resin, which yields a low signal during peptide sequencing. This is a problem often encountered with solid phase peptide libraries on small beads, especially those with longer peptide chains, that bear lower amounts of peptide, resulting in weaker signals during sequencing.

To test the binding of Fc fragment to beads containing a single amino acid ligand, a small bead monomeric library was screened. Six milligrams of library were used, and 4 positive beads were submitted for sequencing. It was found that not only histidine, but also tyrosine (Y, Tyr) and isoleucine (I, Ile) were able to bind the target protein when there is only one amino acid residue present. This is an indication that the ligands identified with the hexamer library may bind to the target proteins through the whole sequence instead of

through histidine alone, although histidine seems to play a very important role in the interaction.

To determine if it would be possible to find a three amino acid peptide that recognizes the Fc fragment, 75.8 mg of a trimer library were tested against Fc fragment. The results shown in Table 3-3 exhibit a strong presence of histidine (3 positions), with tyrosine and lysine also being identified in the first position. It is interesting to note that the same sequence (HIW) was identified twice during the screening. However, the excellent homology observed in the screening results from the large bead hexamer library was not observed when using trimers, suggesting that trimers may lack in binding power when compared to hexamers.

It is remarkable that the first amino acid (N-terminus) in all the positive beads identified from the large bead library is histidine regardless of the various screening conditions used in the experiments. This level of homology with the same amino acid showing at the same location on a hexamer is unusual and has rarely been seen when using non-biased OBOP solid phase combinatorial peptide libraries. Lam *et al.* (18) screened a linear pentameric peptide library against a monoclonal anti- β -endorphin antibody using a fluorescence assay technique for detection, with 6 reactive beads identified out of 2 million, all having YG on the N-terminus. Smith *et al.* (23) reported 7 sequences with proline in the second position of all peptides and 5 out of 7 being arginine on the third position during screening of a peptide nonameric library against human class I major histocompatibility complex by an enzyme-linked colorimetric approach. Cwirla *et al.* (24) screened a phage-display hexapeptide library by biopanning and found that all 51 beads identified contained tyrosine as the N-terminal residue.

When compared to other studies using radiolabeling as the detection method for the screening of non-biased combinatorial peptide libraries, the homology found is even more unusual. Wang *et al.* (15) identified 11 hexapeptide sequences that bind staphylococcal enterotoxin B (SEB), and only 4 (with one doubtful call) started with tyrosine (Y), while Gurgel *et al.* (13) did not identify a consensus when screening for ligands for α -lactalbumin. Higher consensus was observed by Lou *et al.* (25) who screened approximately 1 million peptide beads from a biased linear library XIYXXXX (X is any amino acid except Cys) and

identified 5 ligands with three having EE on the third and fourth position by using ^{35}P -labeled phosphorylation of peptide on beads. This study is the only one identified where the screened peptides present a 100% homology with histidine on the N-terminus when using the radiolabeling screening approach.

Histidine has been previously identified as a ligand for IgG (26-34). Histidine was coupled on Sepharose or silica particles through its carboxyl group (27-29) or immobilized via its amino group on poly(ethylene vinyl alcohol) hollow-fiber membrane (30-32) or on poly(2-hydroxyethylmethacrylate) beads (34). Immobilized histidine ligands were found to be very sensitive to the binding conditions, such as buffer system composition (30, 31), pH value of the buffer (31) and salt concentration (29). Regardless of the coupling method used to produce the histidine-containing matrix, HIgG was adsorbed in 25 mM Tris-HCl at pH 7.5 – 7.6 (27, 29-31) and desorbed with the binding buffer containing NaCl from 0.05 M to 1 M. However, other proteins such as trypsin and lysozyme could also be bound and eluted under these conditions (35). The mechanism of HIgG adsorption was reported to be mostly due to ionic interactions due to the imidazole ring which are assisted by its free terminal group (29). Nevertheless, the interactions were weak because a low salt concentration (less than 1 M NaCl) was able to achieve elution.

Apart from the first position, histidine appears only twice in all sequences determined (HDVFHT and HVHYYW), suggesting that its placement in the ligand is also very significant. The second amino acid from the N terminus among the ligands identified from the large bead library experiments also showed a high degree of homology. Seven out of 11 were hydrophobic (5 aromatic: 1 tyrosine, 2 phenylalanine (F, Phe), 2 tryptophan (W, Trp)) when screened and re-screened against Fc fragment and all eight were hydrophobic (5 aromatic and 5 aliphatic) when screened against intact HIgG and then re-screened against the Fc fragment. The probability of randomly isolating 19 leads with histidine at the N-terminus from a linear hexameric library built with 18 amino acids is $(1/18)^{19}$ or 1.4×10^{-24} , while the probability of discovering 10 leads with an HX motif (X being any aromatic amino acid residue) on one terminus is $[(1/18)^{10} \times (3/18)^{10}]$, or 4.6×10^{-21} . The homologous sequences indicate that the combination of an aromatic amino acid and histidine may play an important role in peptide binding to HIgG and Fc fragment of HIgG.

3.3.2 Secondary screening

Secondary screening experiments were meant to confirm the ability of the peptide ligands identified during the primary screening to efficiently and preferentially bind HIgG in non-competitive (when in PBS buffer) and competitive (when diluted in cMEM) formats. The experiments are also used to eliminate any peptide ligands that present weak binding, decreasing the number of potential candidates for further studies. Intact HIgG was chosen as the target in order to assure that the identified ligands were able to bind the target protein in its native form. Selected hexamers were chosen from the different families identified during primary screening (HF-, HI-, HV-, HW-, HY-, and K-).

In addition to the hexamers from screening experiments, other hexamers were also synthesized and tested. HAAAAA was synthesized in order to evaluate if His alone would display the same binding behavior as in a more complex hexapeptide. The poly-Ala added is intended to act as a spacer arm. Peptide HFRRHL is a section derived from the sequence GFRKYLHFRRHLL, a subunit of a multiple antigenic peptide [(GFRKYLHFRRHLL)₄(KRG)₂KG] used to purify polyclonal antibodies raised against the same peptide (36). The sequence YYWLHH was originally identified by Wang *et al.* (15) to isolate SEB from complex mixtures and was included to test the strength of HIgG binding to ligands that have a high content of histidine, but not in the N terminus. The ligand WHWRKR was identified by Gurgel *et al.* (13) to isolate alpha-lactalbumin from whey protein isolate (WPI) and it is included here to test its ability to bind HIgG since it also contains tryptophan and a histidine near the N terminus. An acetylated version of HYFKFD was included in order to evaluate the importance of the terminal charge carried by all the peptides through their terminal amino group. It is important to notice that in this peptide only the terminal amino group was acetylated, while the amino group in the Lys residue remained unchanged. DFKFYH is an inversion of the sequence HYFKFD. It was tested to evaluate the importance of orientation on binding. The same applies to KLQWVH, an inversion of HVWQLK. KRGFYY is a hexamer designed based on the results obtained from the small bead library, with the undetermined positions being filled by aromatic amino acids.

Two trimers, HIW and KGE, were also evaluated at this stage. Toyopearl Amino 650 M resins, the base matrix of the peptide library, as well as its fully acetylated form, were also

included in the secondary screening experiments as negative controls. As a positive control, a Protein A resin was included in the experimental design.

The results of secondary screening of all the resins are listed in Table 3-4. Among the ligands tested under both non-competitive and competitive conditions, only the two trimers had better performance under competition than without. However, the overall performance of these two ligands was poor, showing binding of only 10.7% of the applied protein. Competition decreased the amount of HIgG bound to all hexamer peptide resins tested. The bound fraction was reduced under competitive conditions to about 60% to the total bound without competition, except for WHWRKR, which bound only 16.4% (10.9% of total).

Peptides HFRRHL, HVHYYW, HWRGWV, HYFKFD and YYWLHH, one from each consensus family, exhibited the best binding of the set, since they were able to adsorb more than 30% of the applied HIgG in the presence of cMEM. The peptide HWRGWV exhibited the best performance among the screened peptides (59.7% binding), with others binding between 30-40% of the target protein. Even though these results are not as high as the 89% binding obtained with the Protein A resin, they represent a significant amount of binding of HIgG in a single step. The peptide YYWLHH, originally identified as a binder for SEB, also bound a significant amount of HIgG, but subsequently it was determined that this was primarily by non-specific hydrophobic interactions that exhibited very little specificity. Other peptides containing hydrophobic patches bound much less HIgG indicating that hydrophobicity alone is not a sufficient determinant for HIgG binding.

Other ligands with the same amino acids at the first two positions (HF-, HI-, HV-, HW-, and HY-) exhibited remarkable differences in their ability to bind HIgG. The poor performance of HAAAAA indicates that the presence of histidine only is not sufficient to ensure binding of HIgG. These observations indicate that the better hexamer ligands may bind HIgG through the whole sequence and that the side chain of histidine plays an important role in capturing the target protein.

With the exception of YYWLHH, all ligands without histidine at the N-terminus (K- and W-) bound less than 20% of the applied HIgG in the competitive mode. Acetylated HYFKFD (Ac-HYFKFD) captured as much HIgG as the un-acetylated ligand, indicating that charge interactions through the terminal amino group are not important in the binding

mechanism of this ligand. The low binding obtained by the base resin, Toyopearl Amino 650M also contributes to this conclusion.

A comparison of the binding of the reverse sequence pair HVWQLK and KLQWVH reveals that neither of them bound HIgG very efficiently (15 and 12%, respectively). However, the pair formed by the positive lead HYFKFD and its reverse form, DFKFYH, bound 39% and 8%, respectively under competitive conditions. This indicates that the peptide orientation can be important in the capture of HIgG by small peptide ligands immobilized on these solid supports.

3.3.3 Effect of sequence truncations

Pentameric, tetrameric and hexameric modifications of positive ligands identified in primary screening were further analyzed for binding ability of HIgG in the presence of cMEM (competitive conditions). Pentameric and tetrameric sequences were obtained by removing the C-terminus amino acid(s), while the hexameric modifications were achieved by replacing the original amino acid residue with another amino acid or by just changing the order of the residues. Table 3-5 shows the amount of protein present in each of the fractions generated during the experiments as indicated in the methods section describing secondary screening. It can be seen that all tetramers bound less HIgG than their corresponding pentamers which in turn bound less than the hexamers. The most influenced peptide was HVHYYW: deletion of the last two amino acids (YW) led the binding percentage to decrease 10 times (36.1% vs. 3.8%), while the deletion of HH from YYWLHH only reduced the binding percentage of protein by a factor of 2 (65.2% vs. 36.2%). Regardless of whether the deleted amino acids were charged or uncharged, the percentage of protein eluted by 1M NaCl remained almost the same, at less than 7%. All the modified ligands showed similar elution patterns when compared to their original forms; a small amount of HIgG was eluted with 1M NaCl and comparably much more eluted with 2% AcOH. This indicates that the interactions between ligands and HIgG remained relatively unchanged even after the ligands were modified. Nevertheless, the binding was greatly weakened with the deletion of C-terminal amino acids. Clearly, deletion of the carboxyl terminal amino acid(s) compromises the selectivity and the affinity of ligands for HIgG.

Two very similar sequences, HHLWYY and HHLYYW, both of which are mutations of YYWLHH and only different in the order of the last three amino acids, performed differently in adsorption of HIgG, with HHLWYY binding noticeably more protein than HHLYYW. This suggests that a tryptophan residue closer to the N- terminus is important in capturing HIgG, which is in agreement with the stronger binding of HYWKFD, a modification of HYFKFD, as shown in Table 3-4. In addition, the weaker HIgG adsorption of mutation HWRRWV than its original form HWRGWV confirmed that the binding of the ligands by the protein is through the whole amino acid sequence, with the knowledge that histidine and an aromatic amino acid on the N- terminus can play an important role in these interactions.

By amino acid alignment, the hexamers that bound more than 30% HIgG under competitive conditions could be grouped into two families. Family I includes HYFKFD, HFRRHL and HWRGWV, which has a combination of Histidine + aromatic amino acid + positively charged amino acid. Family II has ligands HVHYYW, HHLYYW and HHLWYY, which encompasses Histidine + non-aromatic amino acid + hydrophobic amino acid. Peptide YYWLHH, which bound 65.2% of HIgG, has no His on the N- terminus and was not identified from our original library screening. As will be described later, this peptide was not as specific as the peptides in Families I and II identified by the combinatorial library screening approach.

3.3.4 Tertiary screening - binding of Fc fragment to selected ligands

HIgG consists of a large number of different antigen specific antibodies or isotypes. A relatively conserved region (Fc) on the IgG molecule, however, is present in all these isotypes. In order to identify a ligand that is able to broadly recognize all the isotypes, the primary screening protocol was designed to identify Fc-specific ligands by using radiolabeled Fc as the target. The ability of the best HIgG-binding ligands to bind specifically to the Fc fragment was tested by exposing HIgG and its three fragments, Fc, Fab and F(ab')₂, to columns packed with different peptide ligand resins.

Six hexamer resins belonging to the two ligand families, as well as ligand YYWLHH, were investigated using a chromatographic column format. The percentages of bound HIgG

and its fragments on all tested ligands as well as the negative and positive controls, calculated according to the area of the chromatograms obtained are listed in Table 3-6. Two ligands were selected as negative controls (HAAAAA and KRGFYY). HAAAAA has H in the N-terminus, just like ligands in Families I and II, and KRGFYY has a hydrophobic patch on the C-terminus, as in Family II ligands. Neither intact HIgG nor its fragments bound to HAAAAA, while only 18.7% of HIgG and small amounts of each of the fragments bound to KRGFYY. Two commercially available resins (Protein A and A2P) were used as positive controls, and these chromatograms are shown in Figure 3-1. A2P bound all of the applied fragments of HIgG, as claimed by the manufacturer, while Protein A bound the majority of Fc and HIgG, but also small amounts of Fab and F(ab')₂, in agreement with the performance described in previous work (37-40). Another commonly used affinity ligand for HIgG, Protein G, not included in this study, is known to bind both the Fc and Fab fragments, with even higher affinity for Fab than Protein A (41, 42).

Figure 3-2 shows the chromatograms of exposure of Family I ligands to HIgG and its fragments. A comparison of Figures 1 and 2 indicates that Family I ligands, just like Protein A, bound HIgG and Fc efficiently, but bound only small amounts of Fab and F(ab')₂ fragments. Family II ligands bound more than 70% intact HIgG, but did not bind well to any of the three fragments (at most 51%). It must be noticed, however, that Fc was the fragment form that displayed the highest degree of binding. The peptide YYWLHH behaved like A2P, binding HIgG, Fc and F(ab')₂ fragments equally well, and adsorbing less, but still significant amounts, of Fab (Table 3-6). Overall, Family I ligands were able to recognize Fc best and the selectivity to Fc is comparable to Protein A.

We have not been able to identify in the literature a small ligand for IgG purification that targets specifically the Fc part of an antibody. The binding sites of two commonly used IgG binders, Protein A and Protein G, are located in the Fc region of HIgG, but both ligands are large proteins, as opposed to our low molecular weight ligands. Reported variants of the Fc binding domain of Protein A displayed specific binding to Fc fragment with association constants in the range of 10^6 - 10^8 M⁻¹ (37, 43-46), but these ligands still consisted of at least 33 amino acids. Gulich *et al.* (9) engineered one of the Fc binding domains of Protein G, and a variant with 63 amino acid residues was identified that had improved alkaline stability.

The sequences of the ligands found in this work were compared to the sequences of the Fc binding domains in Protein A and Protein G, but no clear homology was observed with any of these regions.

Fasina *et al.* (47) did not show any data on the ability of tetrameric tripeptide TG19318 to recognize the Fc portion of HIgG, although it was a peptide selected from a tripeptide tetramer library by its ability to inhibit the interactions between Protein A and biotinylated immunoglobulins. The 12-mer linear peptide EPIHRSTLTALL, sharing 42% homology with the Fc-binding domain of Protein A, was identified by phage display technique using the Fc fragment of anti-Tac MAb as the target (36). However, the authors did not present any information on the binding of Fc fragment by the peptide after immobilization.

It has been reported that histidine immobilized on a poly(ethylene vinyl alcohol) membrane bound only Fab and F(ab')₂ but not Fc fragment of plasma HIgG through its α -amino group (30). However, a contradictory result was described that only the Fc domain but not the Fab domain of placental HIgG was found to bind to histidine immobilized on AH-Sepharose 4B through its carbonyl group (27). The authors of this work also observed a discrepancy in elution volumes when the IgG variants differing only in their primary structures at the complementarity determining regions (CDR) were applied on the Histidyl-AH-Sepharose 4B column, indicating the involvement of the Fab region in the binding of HIgG to the resin. In our experiments, HAAAAA immobilized on Toyopearl AF-Amino beads through its carboxyl group revealed no affinity to either intact plasma HIgG or any of its fragments. These results imply that the supports and the orientation of the leads can play an important role on the interaction of HIgG with the ligand.

Commercial resin MAbsorbant A2P (ProMetic Biosciences), containing a small triazine ligand, binds all tested IgG fragments, as well as the intact molecule. Todorova-Balvay *et al.* (48) found that transition metal ions showed lower affinity to F(ab')₂ than to F'c (the Fc fragment without the hinge region) and intact HIgG. However, the binding of proteins to metal ions depends on the accessibility of His residues that may vary in the Fab region for different antibodies. In comparison to the ligands mentioned above, the small

peptides in Family I (HWRGWV, HFRRHL and HYFKFD), showed very good specificity to the Fc fragment of HIgG, with retention comparable to Protein A.

3.3.5 Selective binding of HIgG from cMEM by HWRGWV

As demonstrated above, Family I ligands were able to bind HIgG predominantly through its Fc fragment. Among Family I ligands, HWRGWV was found to be most effective in binding HIgG under competitive conditions. This peptide was chosen for additional, more detailed, characterization. This section describes the ability of HWRGWV immobilized on a resin to purify HIgG from a complex mixture.

The competitive medium used in this study, cMEM, is a complex mixture of proteins, peptides, vitamins and other nutrients, with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB). FCS consists of serum proteins, with a large amount of serum albumin, a small amount of immunoglobulins (including bovine IgG), transferrin, and lipoproteins (49). Tryptose is an enzymatic protein digest, consisting of a large number of peptide fragments. The purification of HIgG in one step using affinity chromatography from such a mixture is a difficult challenge due to competition from nonspecific binding of other species.

Figure 3-3 shows chromatograms resulting from the injection of pure HIgG (marked I in the figure) and HIgG spiked into cMEM (marked II). It can be seen from the injection of pure HIgG that the column was very efficient in capturing HIgG, as a flow-through fraction was barely noticed in line I. Most of the protein was eluted by phosphate buffer at pH 4.0, generating fraction P2. The composition of each of the fractions collected during the run can be seen in the gel electrophoresis patterns shown in Figure 3-4. Peak P3 (lane 11) probably contains a large number of proteins at low concentration, which translates into a single lane in the SDS-PAGE gel. Lane 10 contains peak P2 of the injection of pure HIgG. The four chain fragments of reduced HIgG are evident in Figure 3-4 and labeled as i, ii, iii, and iv respectively. By comparing the signals of band iv from lanes 10 and 2 (control HIgG) we can observe the strong concentration effect of the ligand column. Lanes 8 and 9, corresponding to peaks P1 and P1' of the 0.5 M NaCl elution, contained a small amount of HIgG. This kind of partial elution at different steps is a common phenomenon for affinity

chromatography of HIgG using small ligands (50), Protein A, Protein G or the variants of their HIgG binding domains (51-53) as adsorbents. The slight peak broadening in the eluted fractions is possibly due to the presence of isoforms of HIgG.

When HIgG spiked into cMEM was introduced into the column (Figure 3-3, line II), a large flow-through peak (FT) appeared. This was followed by two peaks during the salt wash step (P1 and P1'), one peak with a shoulder from pH 4 PB elution (P2 and P2'), and a small peak obtained during elution with 2% acetic acid (P3). Lanes 12 to 17 in Figure 3-4 show the composition of the different fractions collected during the elution of HIgG + cMEM from the column. Fraction P2 contained most of the HIgG, in its different isoforms (bands marked i to iv). The main contaminant in P2 was BSA in its monomer form, as well as the BSA dimer. Most of the BSA present was, however, eluted during the salt wash step (fractions P1 and P1', lanes 13 and 14, Figure 3-4).

The results obtained when using immobilized HWRGWV resin were compared with separations carried out using two commercial ligands (Protein A and A2P) whose chromatograms and SDS-PAGE analysis are shown in Figures 5 and 6. Instead of the step elution used for the peptide resin, the manufacturers' individual recommended elution conditions for each resin (citric acid at 10mM for protein A and 50 mM for A2P) were used. Both columns displayed a large flow-through peak (Figure 3-5). The elution peak from A2P (lane 4, Figure 3-6) contained some BSA, while Protein A displayed an HIgG eluate free of contaminants (lane 6, Figure 3-6). In order to make comparison easier, Figure 3-6 also has a reproduction of lane 15 from Figure 3-4. The HIgG-rich elution fraction (P2) from HWRGWV resin showed HIgG bands and some BSA.

The purities and yields of isolated HIgG were estimated from SDS-PAGE images by densitometry, and are listed in Table 3-7. The calculated purity and yield for HWRGWV resin were based on the composition of fraction P2 only. It is shown clearly that the purity and yield of HIgG isolated from HWRGWV resin are comparable to the values obtained by using the A2P resin. As expected, the Protein A resin gave a higher purity, but a slightly lower yield. One point to notice is that the elution condition of this hexamer ligand, using buffer at pH 4, was milder than the elution solutions recommended for both Protein A resin and A2P resin, at lower than pH 3. Furthermore, we believe the purity and the yield using

the HWRGWV resin could be enhanced significantly by additional optimization of the elution buffer and methods. This initial chromatographic evaluation of the HWRGWV ligand demonstrates that this hexamer peptide has the potential to isolate HIgG from cMEM proteins containing at least 10% FCS.

3.3.6 Cross-reactivity of HWRGWV

In order to evaluate the ability of HWRGWV to recognize other Igs, human serum Igs including IgG1-IgG4, IgE, IgD and IgM, human secretory IgA, purified immunoglobulins from bovine, goat, mouse, rabbit and chicken were applied to an HWRGWV column. The chromatograms obtained for each protein are shown in Figures 7-9.

The injection of human IgG subclasses 1, 2, 3, and 4 are shown in Figure 3-8. Unlike Protein A, which has no affinity to human IgG3, all subclasses of human IgG were recognized by HWRGWV resin. All HIgG subclasses exhibited sharp pH 4 PB elution peaks. IgG4 presented a sharp peak, but with lower signal than the others. The fact that all HIgG subclasses are able to bind to HWRGWV resin suggests that the hinge region, which differs in length and primary sequence among the four forms of IgG, is not significantly involved in the binding. This is also indicated by the results in Figure 3-1 in which HWRGWV was found to bind much more weakly to F(ab')₂ than to the Fc fragment, both fragments of IgG containing the same hinge region.

The HWRGWV peptide resin retention of immunoglobulins produced by humans, secretory IgA, serum IgG, IgM, IgD, and IgE are shown in Figure 3-7. Compared to hIgG, IgD has four extra glycosylation sites at the end of the Fc portion, and IgE has a longer tail in addition to the extra glycans. From the similarity of the chromatograms of IgD and IgE to IgG, it could be deduced that the structural difference among the three immunoglobulins has no significant influence on their binding to the HWRGWV resin. The flow through peak of IgD run was actually the impurities of the starting material as studied by size exclusion chromatography (data not shown). It should be noticed, however, that the binding capacity of IgE might be smaller than that of the IgG due to the observation of larger flow through peak but smaller Ig elution peak. Unlike IgD and IgE, secretory IgA and IgM are structurally different from IgG since they exist as dimers and pentamers respectively. HWRGWV resin

did not bind IgA as efficiently as to bind IgG, a behavior that is similar to the performance of Protein A and Protein G, both of which preferentially adsorb IgG (54). The weak binding of IgA may be attributed to the steric hindrance to Fc binding caused by the secretory component winding around the Fc part of the IgA dimer. More than 93% of IgM was captured by HWRGWV and the bound protein was eluted in a broad peak by pH 4.0 PB. The binding between IgM and the peptide surface might be the result of non-specific interactions as a result of the larger size of this protein (5 times larger than IgG). These non-specific interactions between IgM and HWRGWV might explain the observed broad elution peak. However, IgM might also be bound specifically through the individual Fc fragments in each monomer, and the peak broadening could be caused by a distribution in the number and strength of interaction of individual IgM monomers adsorbed to the surface.

HWRGWV resin was effective in retaining bovine, goat, mouse rabbit and chicken IgG and displayed similar elution patterns when compared to human IgG (Figure 3-9). The degree of homology between the human Fc fragment and the other mammalian Fc fragments tested varies from 53% to 63%. In terms of mammalian IgG binding, HWRGWV is more like Protein G which binds all these IgGs with similar strength (55). Protein A binds human IgG very strongly but exhibits weak binding to bovine and goat IgGs (55). The degree of homology between the human and chicken IgG Fc fragments is only about 30%, less than the homology to mammalian IgGs. The amino acid alignment of Fc portions on human and chicken IgGs was performed using the protein blast software “blastp” (<http://www.ncbi.nlm.nih.gov>) (Figure 3-10). There are no more than four consecutive amino acids that are homologous between these two species. This implies that HWRGWV interacts with amino acids on human and chicken IgG that are in approximately the same spatial orientation in their tertiary structures. It is still possible though that the peptide ligand binds to a protein segment other than the Fc portion of chicken IgG. In either case, the ability of the peptide ligand to bind chicken IgG is remarkably different from those of Protein A and Protein G which are known to have no affinity to chicken IgG. This could be used advantageously, for example, for the large-scale purification of chicken IgG.

3.4 Conclusions

Protein A exhibits limited binding to several species and subclasses of IgG, which has hampered its use in commercial applications. This had led to a search for other Fc-binding ligands with broader specificity. The combinatorial peptide library approach described was able to identify a family of peptides with a specific composition from the N-terminus: His + aromatic amino acid + positively charged amino acid that is able to bind selectively to the Fc fragment of HIgG. The binding strength of HIgG to the various peptide resins depends on the entire sequence of the peptide and the histidine side chain plays a very important role in the protein-ligand interaction as evidenced by the specific binding of the peptides to the Fc fragment instead of Fab or F(ab')₂ fragments from HIgG. As a model ligand, HWRGWV was selected as the candidate for purification of HIgG from cMEM complex. HWRGWV is a ligand that, unlike Protein G that also has affinity for Fab fragments, preferentially binds HIgG through its Fc fragment. It adsorbs all subclasses of HIgG, as opposed to Protein A, which does not interact with the Fc portion of HIgG3. HWRGWV retains all mammalian IgG tested, chicken IgG, human serum IgM, IgD, IgE and to a less extent human secretory IgA, which demonstrates its broad specificity. The efficient adsorption of human IgM may allow HWRGWV to be used in the purification of IgM from a monoclonal source, for which there is no specific ligand. The IgM selectivity of this ligand over other proteins, however, needs further investigation. Nevertheless, HWRGWV has the potential to be a good reagent for IgG detection and purification of either monoclonal or polyclonal antibodies due to its broad specificity and selective recognition of Fc fragments. The efficient recovery of IgG from cMEM suggests that the described ligand could be of interest in IgG purification applications.

3.5 References

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Table 3-1 Small ligands for HIgG purification

Ref.	Ligand	Support	IgG Source
(56)	Sulfamethazine	Poly(glycidyl methacrylate)	Human blood serum
(36)	EPIHRSTLTALL	Amino-NuGel	Culture
(57)	Mannosylerythritol lipid	Poly(2-hydroxyethyl methacrylate)	Purified HIgG
(58)	Multiple antigenic peptide: AV13: GFRKYLHFRRHLL (AV13) ₄ K ₂ KG AV15: VRLGWLLAPADLDAR (AV15) ₄ (KRG) ₂ KG	Epoxy-activated Eupergit C30N	Anti-multiple antigenic peptides sera
(47)	Protein A mimetic TG19318: (RTY) ₄ K ₂ KG	Epoxy-activated Eupergit C30N	Human blood serum
(10)	D-Protein A mimetic TG19320: D-(RTY) ₄ K ₂ KG	Poly-acrylamide/azlactone-activated gel (Emphaze matrix)	Serum
(27)	Histidine	Aminoethyl Sepharose 4B	Placental serum
(59)	Thiophilic MEP HyperCel	Hydrophobic matrix	Cell culture (5%FCS)
(60)	MAbsorbent A1P/A2P	6% Cross-link agarose	Human blood serum
(61)	2-(3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-sym-triazine, 22/8	Sepharose 6B	Human blood serum
(62)	Metal ions: Ni ²⁺ , Zn ²⁺ , Co ²⁺ , Cu ²⁺	Poly(hydroxyethyl methacrylate)	Human blood serum
(63)	Metal ions: Cu ²⁺	Poly(methylmethacrylate-2-methacryloylamidohistidine)	Platelet-poor plasma

Table 3-2. Fc binding to Protein A and A2P resins before and after radiolabeling with ^{14}C .
The extent of labeling of the Fc fragment was 2%.

Bound protein	Unlabeled Fc, mg/ml resin	^{14}C -Fc, mg/ml resin
Protein A	9.9	4.9
A2P	16.1	9.1

Table 3-3. Sequences obtained after primary screening.

Sequences read from left to right beginning with the N-terminus. X is an amino acid that could not be identified by Edman degradation.

Library	Large bead ^a hexameric	Large bead ^a hexameric	Small bead ^b monomeric	Small bead ^b trimeric	Small bead ^b hexameric (No H on N-terminus)
1st-screening target	^{14}C -Fc	^{14}C -HIgG	^{14}C -Fc	^{14}C -Fc	^{14}C -Fc
Re-screening target	^{14}C -Fc	^{14}C -Fc	/	/	/
Sequence	HYGLGW	HWGPTK	Y	HIW	APHHLL
	HEILYW	HIQLDG	H	KGE	KGXQXX
	HFDKGF	HYIDAK	I	YHQ	PTHLFP
	HWRGWV	HVHYW	Y	HIW	KLQMVX
	HANGFL	HPWYVT			KXXGX
	HRPKIF	HWIDPL			KRXXX
	HFDKGF	HYFKFD			KGGXX
	HETRFS	HLKWYA			
	HWGTIA				
	HDFVHT				
	HVWQLK				

a. Bead diameters: 100-300 μm

b. Bead diameters: 40 - 90 μm

Table 3-4. Results of secondary screening experiments.

Percentage of binding of target protein to selected ligands tested in secondary screening against ^{14}C -HIgG. The competitive mode was done with ^{14}C -HIgG in cMEM and non-competitive mode in PBS buffer. Sequences in bold face were selected for tertiary screening.

Consensus	Peptide	Non-competitive,%	Competitive, %
H-	HAAAAA	/	1.6
HF-	HFDKGF	10.7	6.0
	HFRRHL	/	31.7
HI-	HIQLDG	/	10.0
	HIW	7.9	12.9
HV-	HVHYYW	55.5	36.1
	HVWQLK	/	15.1
HW-	HWIDPL	/	19.9
	HWRGWV	/	59.7
	WHWRKR	66.3	10.9
HY-	Ac-HYFKFD	/	35.7
	HYFKFD	69.3	39.4
	HYIDAK	/	8.6
	DFKFYH	/	8.0
K-	KGE	6.1	7.3
	KLQWVH	/	12.2
	KRGFYY	/	17.3
YYW	YYWLHH	/	65.2
Positive control	Protein A	/	89.0
Negative controls ^a	Acetylated amino resin	14.7	4.15

Amino resin

11.5

0.69

a. Acetylated and non-acetylated Toyopearl AF Amino 650 M resin

Table 3-5. Binding of HIgG in cMEM to sequence modifications of best binders identified in Table 3-4.

Original sequence	Modification	Bound, %	Unbound, %	1M NaCl wash, %	2% AcOH wash, %	Remain on resin, %
	HFRR	12.6	87.4	2.2	8.9	1.5
HFRRHL	HFRRH	17.5	82.5	2.2	12.9	2.4
	HFRRHL	31.7	68.3	2.4	20.9	8.4
	HVHY	3.8	96.2	0.8	1.5	1.5
HVHYYW	HVHYY	18.0	82.0	1.1	12.8	4.1
	HVHYYW	36.1	63.9	1.2	29.8	5.1
	HWRG	18.5	81.5	3.9	11.9	2.7
HWRGWV	HWRGW	45.0	55.0	4.4	35.7	4.9
	HWRRWV	43.0	57.0	0.9	37.8	4.3
	HWRGWV	59.7	40.3	1.2	51.7	6.8
	HYFK	10.9	89.1	2.6	6.1	2.2
HYFKFD	HYFKF	31.3	68.7	3.6	23.8	3.9
	HYWKFD	54.3	45.7	6.5	41.9	5.9
	HYFKFD	39.4	60.6	4.1	30.5	4.8
	YYWL	36.2	63.8	1.4	30.6	4.2
YYWLHH	HHLWYY	58.5	41.5	0.9	50.3	7.3
	HHLYYW	48.9	51.1	1.1	42.1	5.7
	YYWLHH	65.2	34.8	2.6	55.8	6.8

Table 3-6. Percentage of HIgG and its fragments bound to ligands.

The percentages were calculated according to the areas of chromatographic peaks (Figures 1 and 2) eluted by 2% AcOH for peptide ligands, 10 mM citric buffer at pH 3.0 for Protein A resin, and 50 mM citric buffer for A2P resin.

	Ligand	Fab	F(ab')₂	Fc	HIgG
Negative controls	HAAAAA	0	0	0.1	0
	KRGFYI	0.1	0.7	7.0	18.7
Family I	HWRGWV	3.5	24.9	96.1	100
	HFRRHL	0.4	14.1	98.1	100
	HYFKFD	1.8	14.1	98.0	100
Family II	HHLWYY	1.8	16.5	51.1	73.3
	HHLYYW	1.5	7.4	32.7	85.9
	HVHYYW	0.9	5.7	20.5	87.8
	YYWLHH	71.0	98.8	97.2	93.1
Positive controls	Protein A	10.4	15.9	95.8	89.8
	A2P	93.1	97.5	93.5	98.5

Table 3-7. Purity and yield of HIgG separated from cMEM by chromatography.

Data obtained by densitometry analysis of SDS-PAGE gels using ImageJ software.

Ligand	Purity, %	Yield, %
HWRGWV	68	65
Protein A	96	61
A2P	70	74

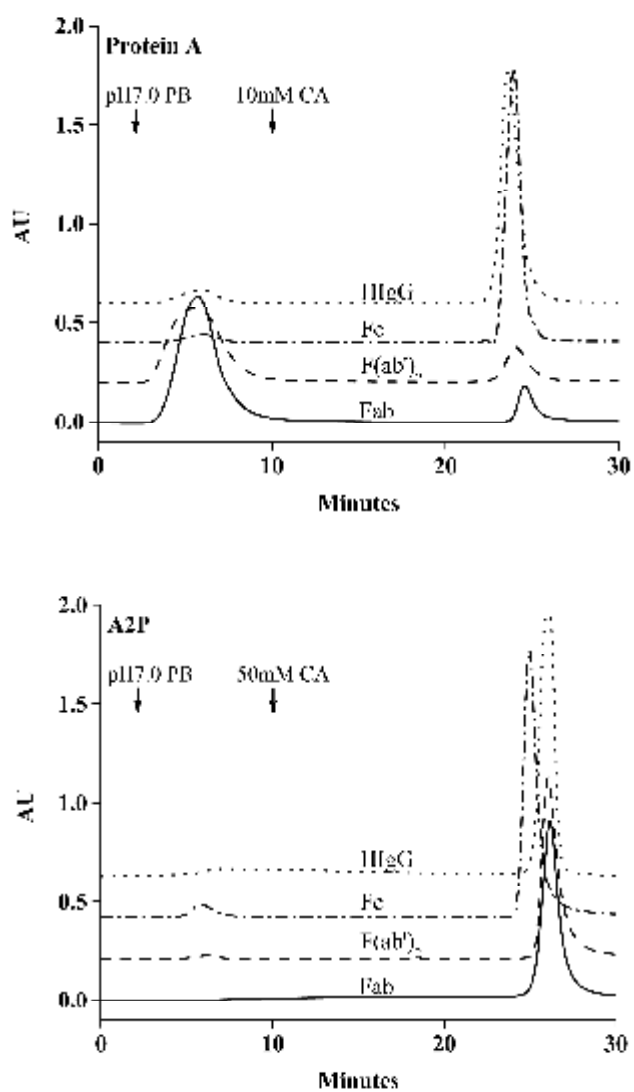


Figure 3-1 Binding of HIgG, Fab, F(ab')₂, and Fc on Protein A and A2P.

Protein injected: 0.1 mg. Samples were loaded in 20 mM PB at pH 7.0 and eluted with 10 mM citric acid (CA) at pH 3.0 for Protein A. For A2P, samples were loaded in 25 mM PB at pH 7.0 and eluted with 50 mM CA.

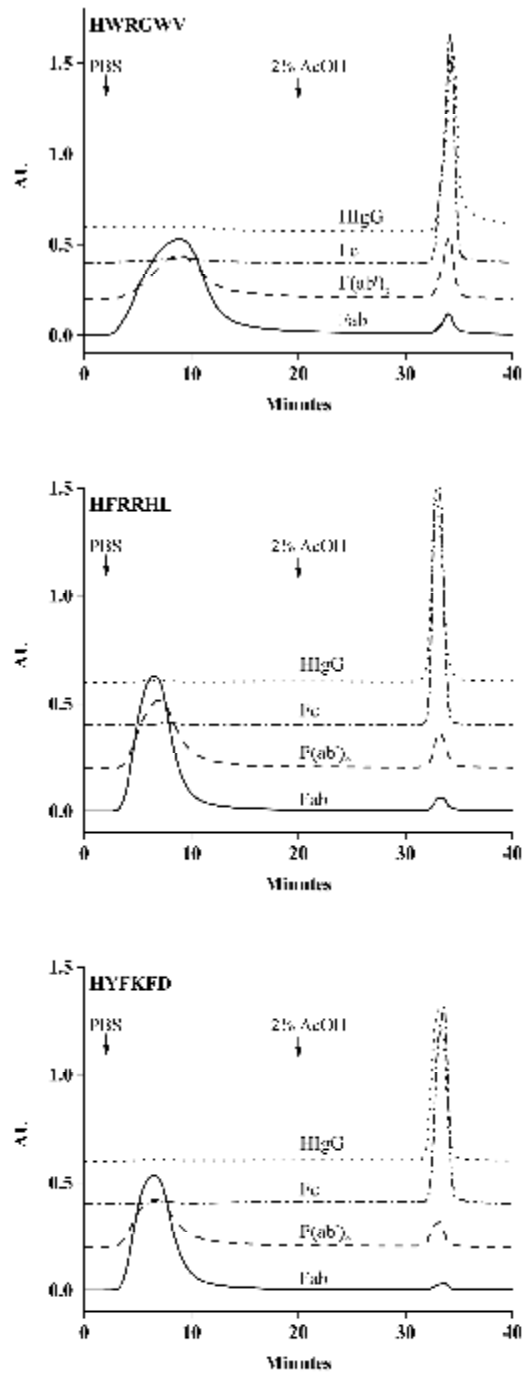


Figure 3-2 Binding of HIgG, Fab, F(ab')₂, and Fc on HWRGWV, HFRRHL and HYFKFD columns.

Protein injected: 0.1 mg. Samples were loaded in PBS and eluted with 2% AcOH.

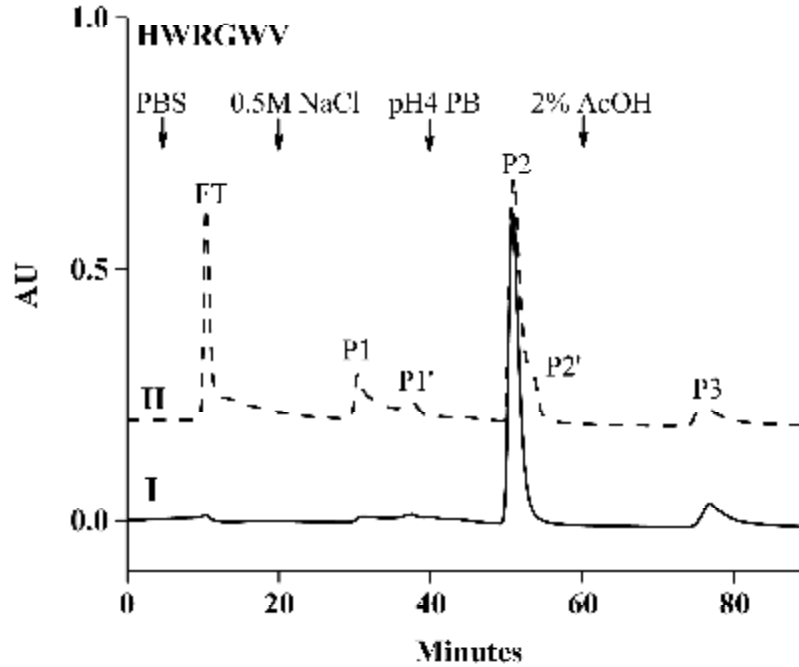


Figure 3-3 Separation of HIgG from cMEM eluting with pH 4 PB on a 0.6 ml HWRGWV column.

Samples were loaded in PBS at pH 7.4, and sequentially washed for 20 minutes each with 0.5 M NaCl, pH 4 PB and 2% acetic acid (AcOH). The flow rate was 0.4 ml/min, and the concentration of HIgG was 5 mg/ml and the injection volume was 100 μ l. HIgG was dissolved in PBS (Line I) or in cMEM (Line II). The detected peaks were flow through (FT), salt wash peaks (P1 and P1'), PB wash peaks (P2 and P2') and 2% AcOH wash peak P3. The volumes of the collected peaks were 3 ml.

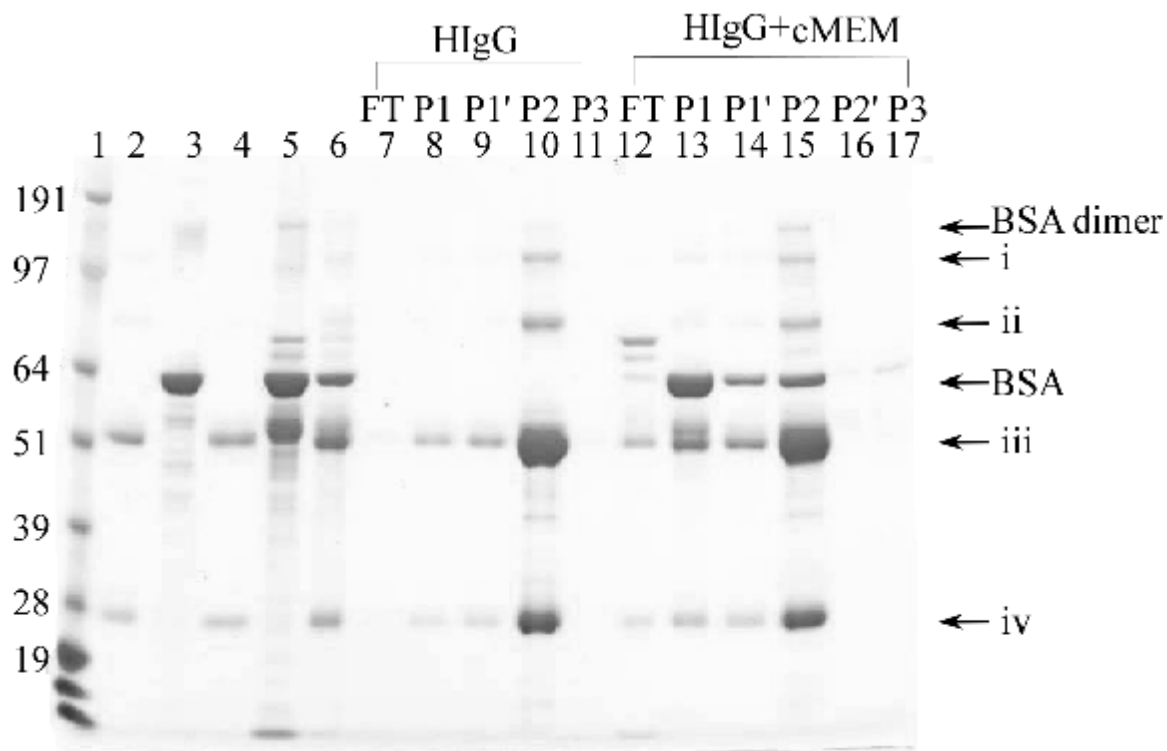


Figure 3-4 SDS-PAGE of the separation peaks denoted in Figure 3.

Lane 1: molecular marker; lane 2: HIgG standard; lane 3: BSA standard; lane 4: Bovine IgG (BIgG) standard; lane 5: 20% cMEM; lane 6: 1:50 dilution of loading material (HIgG+cMEM); lanes 7 – 11 correspond to the flow through (FT), peaks P1, P1', P2 and P3 of pure HIgG injection, respectively. Lanes 12-17 correspond to the flow through (FT), peaks P1, P1', P2, P2', and P3 of HIgG in cMEM, respectively. Lables i-iv indicate the different fragments of HIgG. The gel was run under reducing conditions.

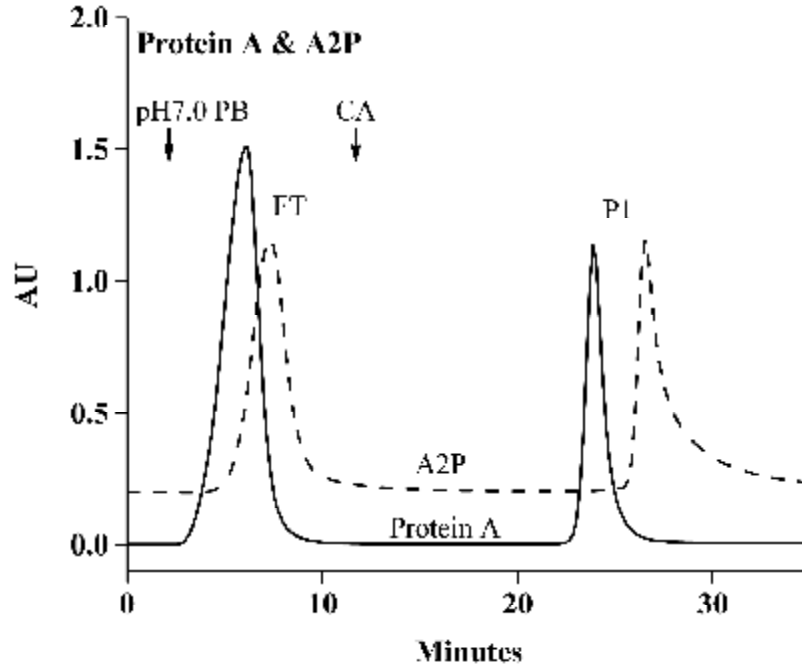


Figure 3-5 Separation of HIgG from cMEM on two commercial resins, Protein A and A2P, under conditions recommended by each manufacturer.

Samples were loaded in pH7 PB and eluted with citric acid (CA) at 10 mM for Protein A and 50 mM for A2P. Column volume: 0.1 ml; concentration of HIgG: 1mg/ml; sample loop: 100 μ l. FT and PI denote the flow through and elution peaks respectively. The volumes of the collected peaks were 1ml.

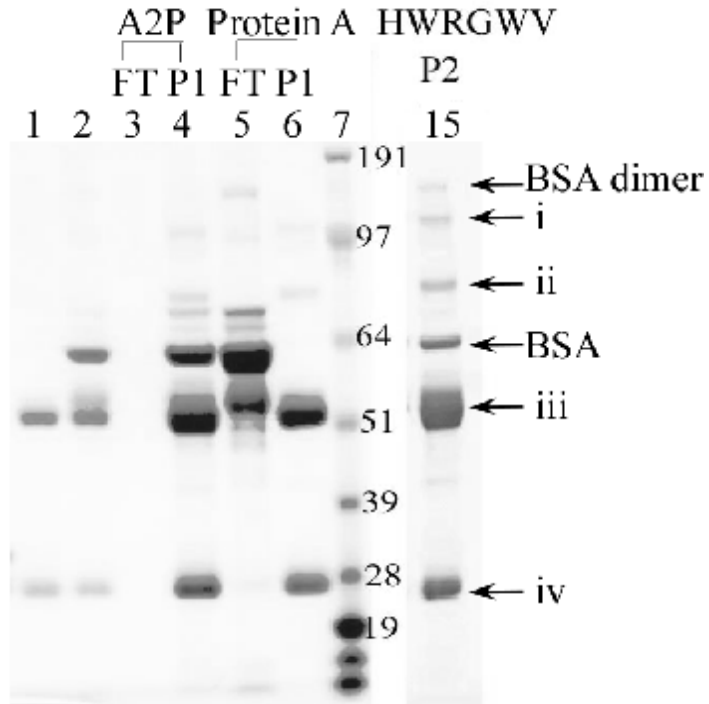


Figure 3-6 SDS-PAGE of the separation peaks shown in Figure 5.

Lane 1: HIgG standard; lane 2: 1:10 dilution of starting material (HIgG+cMEM); lanes 3 and 4 correspond to the flow through (FT) and P1 peaks from the A2P column, respectively. Lanes 5 and 7 correspond to the flow through (FT) and P1 peaks from Protein A column, respectively; lane 7: molecular marker; lane 15 is peak P2 from HWRGWV column indicated in Figure 3. Labels i-iv indicate the different fragments of HIgG. The gel was run under reducing conditions.

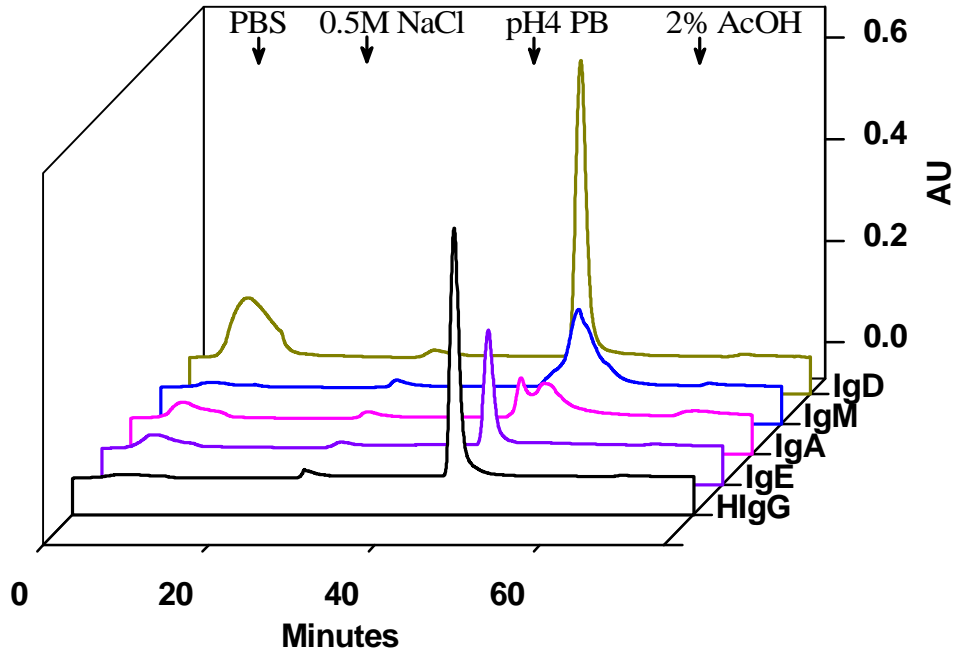


Figure 3-7 Human IgE, IgD, IgM from blood plasma and human secretory IgA binding to HWRGWV.

Igs were loaded in PBS at pH 7.4 and then successively washed with PBS+0.5M NaCl, pH 4 PB buffer and 2% AcOH at the time indicated by arrows. Protein injected: 0.1 mg.

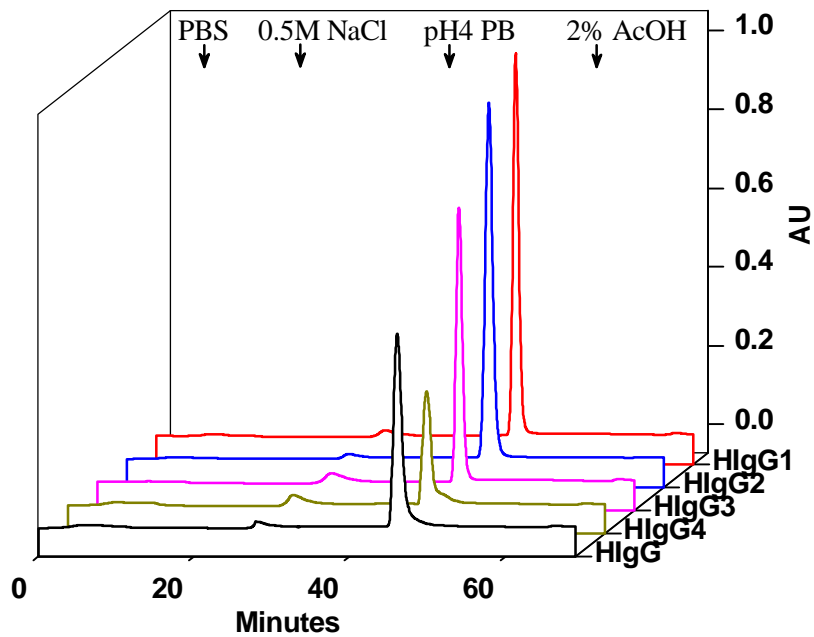


Figure 3-8 HWRGWV binding of HIgG subclasses and human IgM and IgA.

From front to back are IgA, IgM, IgG1, IgG2, IgG3, IgG4 and HIgG from blood serum. Igs were loaded in PBS at pH 7.4 and then successively washed with PBS+0.5M NaCl, pH 4 PB and 2% AcOH at the time indicated by arrows. Protein injected: 0.1 mg.

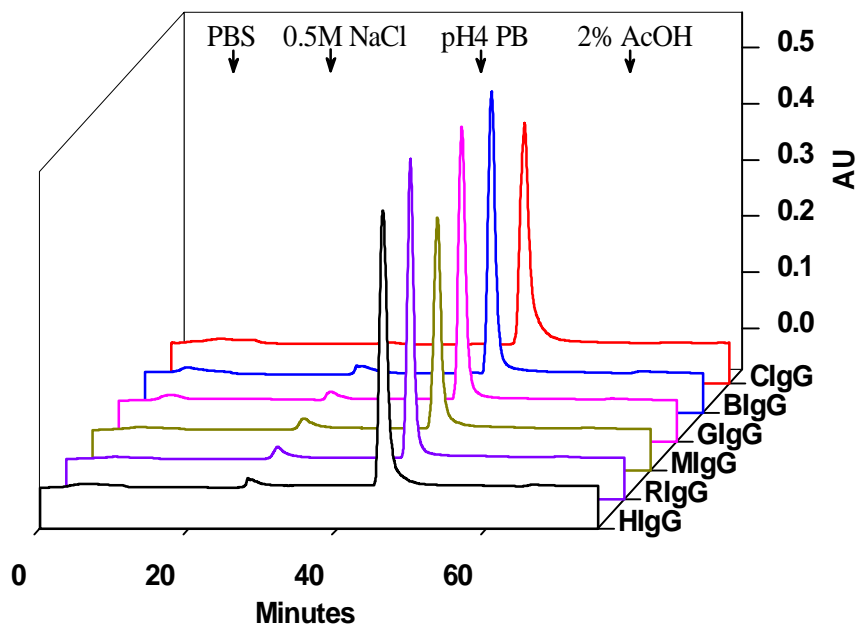


Figure 3-9 Binding of different mammalian IgGs on an HWRGWV column.

From front to back are IgGs from human (HIgG), bovine (BIgG), goat (GIgG), mouse (MIgG) and rabbit (RIgG). IgGs were loaded in PBS at pH 7.4 and then successively washed with PBS+0.5M NaCl, pH 4 PB and 2% AcOH at the time indicated by arrows. The amounts of injected protein were 0.1 mg in each case.

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Score = 131 bits (297), Expect = 7e-29
Identities = 68/220 (30%), Positives = 119/220 (54%), Gaps = 17/220 (7%)

Query 58  LYAIPSPGGE-LYISLDAKLRCLVNVLP-SDSSLSVTWTRE--KSGNLRPDPMLQEHFN 113
      ++ PP P + L IS  ++ C+VV++ D + W + + N + P ++ +N
Sbjct 19  VFLFPPKPKDTLMI SRTPEVTCVVVDVSHEDPQVKFNWYVDGVQVHNAKTKPR--EQQYN 76

Query 114 GTYSASSAVPVSTQDWLSGERFTCTVQHEELPLPLSKSVYRNTGPTTPPLIYPFAPHPEE 173
      TY S + V Q+WL G+ + C V ++ LP P+ K++ + G P +Y P EE
Sbjct 77  STYRVVSVLTVLHQNWLDGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE 136

Query 174  LLSRVTL SCLVRGFRPRDIEIRWLRDHRVAPATEFVTAVLPEERTANGAGGDGDTFFV 233
      ++ ++V+L+CLV+GF P DI + W + P + TT P ++G +FF+
Sbjct 137  MTKNQVSLTCLVKGFYPSDIAVEW--ESNGQPENNYKTT---PPVLDSGD-----SFFL 185

Query 234  YSKMSVETAKWNGGTVFACMAVHEALPMRFSQRTLQKQAG 273
      YSK++V+ ++W G VF+C +HEAL ++Q++L G
Sbjct 186  YSKLTVDKSRWQQGNVFSVMSVHEALHNHYTQKSLSLSPG 225

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Figure 3-10 Amino acid alignment between Fc fragments of human (subject) and chicken

IgG.

**Chapter 4. Characterization of hexamer peptides for
immunoglobulin G isolation as affinity media**

4.1 Introduction

The increasing importance of antibodies as therapeutics (1) has led to a need for more efficient, robust, and lower cost processes to purify antibodies from serum, cell culture, and ascitic fluid. Chromatography plays a major role in industrial scale antibody purification. Ion-exchange chromatography (IEC) (2), hydrophobic interaction chromatography (HIC) (3), size exclusion chromatography (SEC) (4), and affinity chromatography (AC) (5, 6), have all been used in immunoglobulin G (IgG) capture and purification. Affinity chromatography allows the possibility of obtaining several fold purification with high recovery in fewer steps (7). Half of all therapeutic antibodies currently in the market have been purified by affinity chromatography using Protein A or Protein G as ligands (8). These ligands are costly because they have to be highly purified from bacterial sources; they have the potential for causing immunogenic response in patients upon possible leakage into the product, and they tend to lose activity as a result of harsh elution (pH 3), washing and sterilization conditions. As a result, there is a great deal of interest in identifying alternative affinity ligands for antibody purification.

Special attention has been paid to the use of small ligands for affinity purification of antibodies due to their advantages of being more stable (no three-dimensional structure), less immunogenic, and less expensive than large protein ligands. The use of high throughput screening techniques, sometimes combined with molecular modeling, has generated new and powerful small ligands for potential Protein A replacement. Roque et al. (9) have summarized several synthetic ligands, including peptidic and non-peptidic ligands, that have been discovered for antibody purification. Some of them have been extensively studied and are already commercialized, such as the hydrophobic charge induced ligand MEP (4-mercapto ethyl pyridine) marketed as BioSeptra® MEP HyperCel® (10-14), the Protein A mimetic peptide Kaptiv-GY based on the sequence (RTY)₄K₂KG (TG19318) (15-17), the mixed-mode chromatographic ligand FastMabs A (18, 19), and MAbSorbant A2P derived from a triazine derivative ligand 22/8 (20, 21). Meanwhile, the search for new ligands to be used in antibody purification is still quite intensive in both industry and academia (22, 23).

Until recently (24), none of the small ligands developed to bind to antibodies behaved like Protein A in their binding specificity to the Fc fragment. The ability to bind through the Fc fragment is an important function since it allows the ligand to be used as a universal binder for whole antibodies and Fc-fusion proteins, potentially diminishing the time required to develop separation processes for new antibodies.

In a previous study (24), our group was able to show that the linear hexamer peptide ligands HYFKFD, HFRRHL, and HWRGWV, identified by a three-step screening process using a solid-phase hexamer peptide library, exhibited the ability to bind human IgG (hIgG) through its Fc portion. This was the first report of short ligands that were able to mimic the binding specificity of Protein A for the Fc fragment of hIgG. This important property enables the use of these small ligands for binding Fc-fusion proteins, as well as orienting peptides on surfaces for immunoassays, therapeutics and diagnostics. In addition, HWRGWV was shown to be able to bind all subclasses of hIgG, hIgE, hIgD, hIgM, polyclonal IgGs from several other mammal and chicken species, and, to a less extent, secretory hIgA.

This chapter characterizes the ability of these linear Fc-binding peptide ligands, as well as a non-Fc specific peptide, HVHYYW, to bind hIgG. Binding isotherms are presented with measured dissociation constants and equilibrium binding capacities. Their chromatographic performance in the purification of hIgG from complete mammalian cell culture medium (cMEM) containing 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB) were also determined. Among them, HWRGWV was studied in greater detail to determine the influence of temperature and ligand density on hIgG adsorption and separation.

4.2 Experimental

4.2.1 Materials

All peptide resins, HYFKFD, HWRGWV, HFRRHL, and N-terminal acetylated HWRGWV (Ac-HWRGWV) were synthesized directly on Toyopearl AF-Amino-650 M (particle size 65 μm) (Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY). All three peptide ligand resins were synthesized at a peptide density of 0.1 meq/g, while HWRGWV resins were also synthesized at different peptide densities ranging from 0.02 to

0.55 meq/g. Protein A Sepharose CL-4B resin (Protein A) was purchased from Amersham Biosciences (Piscataway, NJ). MAbsorbent A2P immobilized on PuraBead 6% cross-linked agarose gel (A2P) was a gift from ProMetic Biosciences (Burtonsville, MD). Phosphate buffered saline of pH 7.4, human IgG, sodium chloride, sodium acetate, monobasic sodium phosphate, dibasic sodium phosphate, glacial acetic acid were purchased from Sigma (St. Louis, MO). Fab and F(ab')₂ fragments were from Calbiochem (Darmstadt, Germany). Fc fragments were from Bethyl Laboratories, Inc. (Montgomery, TX). NuPAGE Novex 4-12% Bis-Tris gels, NuPAGE MOPS Running Buffer, NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent, Seebblue plus2 pre-stained molecular weight marker, and SimpleBlue SafeStain were all from Invitrogen (Carlsbad, CA). Human IgG ELISA kit and Micro-BCA assay kit were from Alpha Diagnostic International (San Antonio, TX) and Pierce (Rockford, IL), respectively. Cell culture media EMEM was from Quality Biological (Gaithersburg, MD). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, Utah) and Becton Dickinson (Sparks, MD), respectively. MicroCon YM-3 filters (regenerated cellulose, 3,000 MWCO) and Durapore 0.45 µm filters were purchased from Millipore (Billerica, MA). Microbore columns of 30 mm long × 2.1 mm (ID) were from Alltech (Deerfield, IL) and a Waters 626 LC system including a 2487 dual wave-length UV detector (Milford, MA) was used for the chromatography separations. MGW Lauda RM6 circulating bath from Brinkmann (Westbury, New York) was employed for temperature control.

4.2.2 Adsorption isotherm measurements

Adsorption isotherms of resins with different peptide ligands and different peptide densities were measured in a set of batch experiments at room temperature. All the experiments were performed at least in duplicate. Resins were weighed as dry powder, washed thoroughly and equilibrated with phosphate buffered saline (PBS) at pH 7.4 containing 10 mM phosphate buffer, 2.0 mM KCl and 138 mM NaCl. Centrifugal filters (0.5 mL) with 0.45 µm Durapore membranes were used as adsorption vessels. Four hundred microliters (400 µL) of hIgG solutions with concentrations ranging from 0.05 to 10 mg/mL in PBS were added separately to the adsorption vessels containing 10 mg resin and incubated

on an orbital shaker for 2 hours. The unbound hIgG fractions were collected by centrifugation and the protein concentrations were determined using a Micro-BCA assay or by UV-absorbance at 280 nm. The amount of bound hIgG was calculated by mass balance. The data were fit to a Langmuir isotherm model

$$q = \frac{q_m C}{K_d + C} \quad (\text{Eq. 4-1})$$

where q , C , K_d , and q_m are the concentration of the bound protein (mg-protein/g-resin), the concentration of the free protein (mg-protein/mL-solution), the dissociation constant (mg/mL), and the maximum capacity (mg protein/g resin) respectively.

4.2.3 Fragments of hIgG binding on HWRGWV

Fab, F(ab')₂, Fc fragments and intact hIgG binding on the HWRGWV resin were examined the way same as described in Chapter 3 (section 3.2.6).

4.2.4 Chromatographic isolation of hIgG from cMEM

Resins were dry packed in 30 mm × 2.1 mm (ID) Microbore columns (0.1 mL) and washed with PBS. Before sample loading, columns were equilibrated with pH 7.4 loading buffer consisting of either PBS or PBS + 1M NaCl. Human IgG was spiked into cMEM to form a complex starting material with the desired hIgG concentration, ranging from 0.5 to 10 mg/mL. The cMEM was formulated by combining EMEM with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB). The salt concentration and pH of the starting material at this state were the same as PBS (7.4). Sodium chloride was added to the starting material when PBS + 1M NaCl was the loading buffer. Human IgG spiked in cMEM was otherwise directly applied to the columns without any adjustment. Samples were injected at a flow rate of 50 μL/min for enough time to pass the same volume of the loading buffer as that of sample loop. The flow rate was then increased for the remainder of the run to 0.2 mL/min. When PBS was used as the binding buffer, the column was washed sequentially with 2 mL binding buffer, 4 mL washing solution, 4 mL elution buffer, and then 4 mL 2% acetic acid in

water to clean the columns. When PBS + 1M NaCl was used as the binding buffer, the column was washed sequentially with 1 mL binding buffer, 4 mL elution buffer, and then 4 mL 2% acetic acid. The washing solution was either PBS + 1M NaCl or PBS + 1M NaAc (sodium acetate) and elution buffer was 0.2M phosphate buffer (PB) at pH 4 or 6 or 0.2M acetate buffer (AB) at pH 4. For Protein A and A2P, both immobilized on agarose gel, the chromatographic conditions followed the instructions from each manufacturer. The effluent was monitored by absorbance at 280 nm. The temperature was controlled by an MGW Lauda RM6. Collected fractions were directly subject to ELISA assays or concentrated by centrifugation at 4°C, 11,247×g for 90 minutes using a MicroCon YM-3 filter or electrophoresis analysis.

4.2.5 Sample analysis for yields and purities

The protein profiles of chromatography samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under conditions previously described in Chapter 3 (section 3.2.9) and the purity of hIgG peaks was calculated by densitometry measurement using software ImageJ 1.32j (National Institutes of Health, Bethesda, USA). The yield of hIgG purification was calculated according to the hIgG concentrations determined by enzyme-linked immunosorbent assay (ELISA) using a human IgG ELISA kit from Alpha Diagnostic International.

4.3 Results and discussion

4.3.1 Isotherms and isolation of hIgG by hexamer peptide ligands

4.3.1.1 Adsorption isotherms

Adsorption isotherms of hIgG using resins with ligands HYFKFD, HFRRHL, HVHYYW, and HWRGWV at a ligand density of 0.1 meq/g on Toyopearl AF Amino 650M, as well as to two commercially available IgG purification resins used as positive controls (Protein A agarose CL-4B and MAbsorbent A2P agarose gel) were determined (Figure 4-1) with hIgG concentrations in the range 0.05 - 10 mg/mL in PBS at room temperature. A2P is a “Protein A mimic” synthetic triazine ligand developed by ProMetic Biosciences. The

isotherm adsorption data were directly fitted to a Langmuir model and the obtained dissociation constant (K_d) and maximum capacity (q_m) of each resin are listed in Table 4-1. Since the A2P resin was supplied in 20% ethanol the binding capacity for this material was reported in units of mg/mL of gel. The capacity of all other resins was measured by dry weight. In order to allow for a direct comparison, the capacity data were converted into mg/mL-drained-gel using estimated swelling ratios for the Toyopearl 650 M material (4.7 mL/g) and for the Protein A resin (4 mL/g). Table 4-1 also lists the surface coverage of hIgG on the resins calculated based on q_m and known surface area of the beads. The surface area of dry Toyopearl Amino 650M resin was 30 m²/g, while the equivalent areas of Protein A and A2P resins are unknown.

The maximum equilibrium binding capacities of the hexamer peptide resins at the ligand density of 0.1 meq/g were in the range of 27.0 - 33.6 mg/mL-drained resin which was comparable to A2P resin, 35.1 mg/mL, and slightly higher than that of Protein A, 22.0 mg/mL. The capacities of the two positive controls derived from isotherms were in the range claimed by the manufacturers, 20-40 mg/mL for A2P and 20-30 mg/mL for Protein A. Based on the known hydrodynamic dimensions of IgG (235×44×44 Å) (25), closely packed “end-on” and “side-on” monolayers of unperturbed IgG molecules would give surface concentrations of 13 and 2.4 mg/m², respectively. Since the surface area of the base Toyopearl AF Amino 650M resin is approximately 30 m²/g, a maximum binding capacity of 120-160 mg/g yields a surface protein density of 4 - 5.3 mg/m². This is of the same order of magnitude as the monolayer predictions, indicating that the hIgG molecules are forming a monolayer on the surface of the ligand resins when in equilibrium at saturation concentrations.

The dissociation constant (K_d) values for the peptide ligands were in the order of 10⁻⁵ M which are within the normal range (10⁻⁴ - 10⁻⁸M) for affinity chromatography resins (26). This value also fell in the range of 10⁻⁵ - 10⁻⁷ M usually found with short peptides as affinity ligand for proteins (27-29). The K_d for the peptide resins were about two orders of magnitude larger than for the positive controls used in this study, as well as the reported values for ligands MEP Hypercel (12) and A2P (17). However, as will be shown later, the weaker affinities can offer an advantage, since they allow for milder elution conditions of captured

IgG. The milder elution condition may reduce aggregation and denaturation of the product. It is also possible for these ligands with dissociation constants of about 10^{-5} to be used in quick and dynamic separation and to be operated in high-performance mode (30).

4.3.1.2 Peptide selection based on hIgG isolation from cMEM

In order to select a peptide resin for more detailed characterization, a preliminary purification experiment to test the ability of the peptide resins to isolate hIgG from cMEM was carried out. The yields and purities were not expected to be as high as possible in this experiment since buffer, peptide density, and other conditions had not been optimized. To simulate the purification of IgG from cell culture media, hIgG was spiked into cMEM, and the mixture was directly applied to 0.1-mL chromatographic columns packed with each of the four identified ligands (HVHYYW, HWRGWV, HYFKFD, and HFRRHL). The peptide resins had a ligand density of 0.1 meq/g-dry-resin, except for HWRGWV, which had a slightly lower density of 0.08 meq/g-dry-resin. The columns were loaded in PBS, washed with PBS+1M NaCl to remove non-specifically bound proteins, and bound IgG was then eluted by 0.2M phosphate buffer at pH 4. The purity and recovery of collected hIgG fractions (P2) were analyzed by SDS-PAGE and ELISA, and the results are listed in Table 4-2.

HWRGWV, HFRRHL, and HYFKFD resins demonstrated comparable results in terms of the ability to isolate hIgG from cMEM (Table 4-2). The preliminary chromatographic isolation of hIgG in one purification step achieved purities and recoveries of around 90% and 50%, respectively. They all were therefore good candidates for further investigation as antibody purification affinity ligands, with the possible less stringent elution conditions and proper capacities for hIgG. Ligand HVHYYW was the only one among these four ligands that did not have an aromatic residue in position 2, and it showed a lower yield (25.4%) when compared to the others, even though it had K_d and q_m values similar to the other three ligands when pure IgG was utilized. This indicates that His followed by an aromatic amino acid in the ligand is necessary for efficient IgG adsorption from complex protein mixtures. Since HWRGWV gave the highest purity and recovery it was chosen for further study. The influence of peptide density, temperature, binding buffer, and elution conditions on hIgG

isolation from the complex protein mixture are discussed in the following sections. It will be seen that both higher purity and recovery ($\geq 95\%$) were obtained with HWRGWV, indicating its strong potential for antibody isolation from cell culture media.

4.3.2 Characterization of the ligand HWRGWV

4.3.2.1 Influence of peptide density on binding of hIgG fragments

The influence of peptide density on the binding of hIgG fragments was investigated, with the knowledge that HWRGWV at a ligand density of 0.08 meq/g bound hIgG specifically through its Fc region (Chapter 3). One hundred micrograms of pure hIgG and its fragments, Fc, Fab, and F(ab')₂, in PBS at pH 7.4 were separately injected into HWRGWV columns with different ligand substitution levels (0.022 – 0.55 meq/g). The retained target protein or fragments were eluted with 2% AcOH and the bound percentages of the loading amount calculated using the elution peak area are shown in Table 4-3. Ac-HWRGWV at a peptide density of 0.08 meq/g was also examined and included in Table 4-3 alongside with non-modified ligands to better understand the influence of the N-terminal charge of the normal peptide on the binding of the IgG fragments.

The peptide density on the HWRGWV resins had a significant influence on their binding of intact hIgG and hIgG fragments (Table 4-3). At a low density of 0.022 meq/g, the resin retained about 60% hIgG but had no efficient capture of any fragments. When the peptide density increased to 0.04 meq/g, more than 96% of Fc and hIgG were retained while less than 21% F(ab')₂ and even less Fab were bound. HWRGWV at 0.08 meq/g specifically bound the Fc fragment of hIgG, in similar amounts to the resin with peptide density of 0.04 meq/g. This indicates that a peptide density threshold is necessary for HWRGWV to efficiently capture Fc and hIgG. With further increase of the peptide density to 0.22 and 0.55 meq/g, the resins retained the ability to bind Fc and intact hIgG but both bound higher percentages of Fab and F(ab')₂. The high peptide density apparently contributed to an increase in non-specific interactions with Fab and F(ab')₂ fragments. The increased bound amount of F(ab')₂ was more than two times higher than that of Fab, which suggests that the hinge region may facilitate the binding of F(ab')₂ to the peptide resin.

With the knowledge that the isoelectric points of the majority of hIgG subclasses are in the range of 7 – 9, the majority of the IgG molecules are positively charged at pH 7.4, the same as the net charge on HWRGWV ligand at this pH. The similar charges on the ligand and the protein might suggest that electrostatic interactions are not the dominant driving force for binding of hIgG. Therefore, it could be expected that N-terminal acetylation should not influence the binding of hIgG or its fragments. This is supported by our experimental data obtained with Ac-HWRGWV ligand. As shown in Table 4-3, Ac-HWRGWV exhibited no noticeable difference from HWRGWV on the binding of either intact hIgG or fragments of hIgG at the experimental pH value of 7.4, when amino groups are positively charged on the normal ligand.

4.3.2.2 Influence of peptide density on the adsorption isotherms of hIgG to HWRGWV

Resins with peptide densities of 0.022, 0.04, 0.08, 0.22, and 0.55 meq/g were prepared and their adsorption isotherms of hIgG in PBS were measured at room temperature (Figure 4-2A). The adsorption data were fit directly to a Langmuir model with the K_d and q_m values listed in Table 4-4. The average stoichiometry calculated as the ratio of ligand density to maximum capacity is also included in this table to indicate the number of peptides that might be in contact with one hIgG molecule at different ligand densities. The isotherm for Ac-HWRGWV at a peptide density of 0.08 meq/g is also reported in Figure 4-2B and Table 4-4 for comparison. The correlation coefficients for the Langmuir fits to the isotherm data were very good ($R^2 > 0.96$) when the peptide densities were equal to or less than 0.22 meq/g but the fit was not as good ($R^2 = 0.91$) at the highest peptide substitution level (0.55 meq/g). This might be due to the non-specific adsorption of antibody through the Fab fragments at higher peptide density, in addition to the specific binding of Fc. This two-mode adsorption is not consistent with the uniform adsorption assumption of the Langmuir model.

The binding capacity decreased slightly at low peptide density with increases in the peptide substitution and then remained fairly constant after the peptide density reached values of 0.04 meq/g or higher, which was the threshold for efficient IgG capture. This observation is in contrast to the increased binding capacity with increasing ligand density observed with sorbents with less specific protein-ligand interactions, like hydrophobic

chromatographic sorbents (31). However, these results are similar to those reported by Wang and Carbonell (32) when the hexamer ligand YYWLHH was used to purify *Staphylococcal* enterotoxin B (SEB) spiked into *E. coli* lysate. The nearly constant capacity beyond the threshold density suggests that the surface might be totally covered by a monolayer of hIgG molecules regardless of the peptide density.

The dissociation constants of hIgG adsorption decreased when the HWRGWV peptide density on the resin increased (Table 4-4). Decreased K_d values indicate a more intense, stronger, contact between the protein and the sorbent at higher ligand densities.

The stoichiometry of hIgG-ligand interaction progressively increased with the peptide density as indicated in Table 4-4. At low peptide densities, the ratio of ligand to protein at the maximum capacity was approximately 21:1, while at the highest peptide density the ratio was approximately 600:1. In view of the increased non-specific binding of Fab and $F(ab')_2$ at the higher peptide densities, the increased binding strength (decreased K_d) might be due to increased non-specific binding of hIgG Fab fragments.

The hIgG adsorption isotherm of Ac-HWRGWV resin was similar to that of HWRGWV at the same ligand substitution level, with the same q_m and K_d values within the experimental variance of $\pm 0.2 \times 10^{-5}$ M. This observation again suggests that changes in ionic strength should not affect binding of hIgG since hIgG is not retained on the resin through electrostatic interactions.

As discussed above, HWRGWV with peptide densities of 0.04 and 0.08 meq/g specifically retained Fc fragments of hIgG, while higher affinity (lower K_d) was associated with higher density (0.08 meq/g). Therefore, a ligand density of 0.08 meq/g was used in most of our later studies to keep both the specificity and the affinity to the Fc portion of hIgG.

4.3.2.3 Effect of peptide density and temperature on hIgG isolation from cMEM

To study the effect of temperature and ligand density on the dynamic isolation of hIgG from cMEM solutions, chromatographic experiments were carried out using HWRGWV affinity resins at different peptide densities and at different temperatures. The preliminary chromatographic conditions as described in section 4.3.1.2 were employed. Representative chromatograms and corresponding SDS-PAGE gels are shown in Figure 4-3. The average

purities and recoveries of eluted hIgG using pH 4 PB are listed in Table 4-5. When hIgG-spiked cMEM was applied to the HWRGWV column at 0.04 meq/g (Figure 4-3A, dash-dotted line), three peaks appeared in the chromatogram: flow-through peak (FT), 1M salt wash peak (P1), and pH 4 PB elution peak (P2). The first 3 lanes in Figure 4-3B show the protein profiles of the three peaks, where P2 contained most of the hIgG, in its different isoforms (bands i – iv), as can be seen by comparing the respective bands in the standard hIgG lane (Figure 4-3B, lane 8). The main contaminant in P2 was BSA in its monomer form at about 65 kDa. Most of the BSA present in the starting material seemed to have been held by electrostatic interactions in the HWRGWV column as it was eluted during the salt wash step (fraction P1, lane 2 in Figure 4-3B). This made it possible to optimize the chromatographic conditions for purity and/or recovery improvement.

Figure 4-3B also shows the SDS-PAGE gel of the three chromatographic peaks from HWRGWV resins with peptide densities of 0.04 (lanes 1-3) and 0.22 (lanes 4-6) meq/g and the hIgG peak P2 from densities of 0.08 (lane 10) and 0.55 meq/g (lane 11). P2 from an Ac-HWRGWV column under the same conditions is also included in the Figure 4-3B (lane 12). Only P2 is shown for the last two densities because the chromatograms of ligand density 0.08 and 0.55 meq/g had similar profiles to those of 0.04 and 0.22 meq/g, respectively. The band profiles obtained with 0.04 and 0.22 meq/g peptide densities were similar except for the different proportions of proteins in each fraction. More BSA was present in P2, while fewer proteins flowed through for the column with 0.22 meq/g HWRGWV resins. Both reduced FT and P1 fractions at higher ligand density could be due to increased non-specific interactions.

It is also noticeable by comparing the purities and yields achieved with different ligand densities at 21°C (Table 4-5) that the four peptide densities could be sorted into two groups based on chromatographic performance: a low density (0.04 and 0.08 meq/g) group and a high density (0.22 and 0.55 meq/g) group. The purities and yields of the low density group were around 90% and 50%, respectively, while those of the high density group were approximately 80% for both the yield and the purity. Improved recovery at high density was compensated for the loss in purity of isolated hIgG, indicating that raising ligand density elevated not only the binding specific to hIgG but also the non-specific binding of

competitive proteins. However, the disparity arising with ligand density was trivial unless the gap between the densities was significantly large.

Purities and yields of the runs at different temperatures are also tabulated in Table 4-5. The data show that increasing temperature did not affect the purity of the resulting hIgG but caused a slight decrease in the overall yield, similar to the findings for Protein A that the differences of purities and yields between different temperatures were insignificant (33). This suggests that the binding between hIgG and the ligand is not dominated by hydrophobic interactions. This also indicates that HWRGWV may be able to purify labile antibodies at low temperature without loss of its chromatographic performance.

4.3.3 Optimization of chromatographic conditions

This section describes the optimization of chromatographic conditions based on the observation that the isolated hIgG when loading in PBS was contaminated mainly by BSA. It was also evident that BSA was retained on the HWRGWV column predominantly by electrostatic interactions since the majority of BSA was eluted with 1M salt. Two strategies were employed to improve the purification efficiency: (1) increasing the salt concentration by adding 1M NaCl to the loading buffer, to eliminate BSA binding; and (2) lessening the elution stringency by using pH 6 PB to release only hIgG. Another buffer system, acetate buffer (AB), for washing and elution was also examined to determine if buffer ions affected the purification. All experiments were carried out at room temperature on a 0.1 mL column packed with 0.08 meq/g HWRGWV, and also on a 0.1 mL Ac-HWRGWV column (0.08 meq/g) in an attempt to better understand the nature of the ligand-protein interaction in the system. The yields and purities, analyzed respectively by ELISA and SDS-PAGE, of isolated hIgG are listed in Table 4-6 and compared to the results obtained with Protein A and A2P ligands. IgG was loaded in PB to the two commercial ligands as recommended by the manufacturers. Representative chromatograms and the corresponding SDS-PAGE gels are displayed in Figure 4-4.

For the first strategy, high salt concentration in the binding buffer was employed to suppress BSA binding to HWRGWV, hence improving the product purity and yield (Table 4-6). To do this, the salt concentration in hIgG spiked cMEM was increased by adding NaCl

to 1M and the solution was then loaded to the column in PBS + 1M NaCl at pH 7.4. Bound hIgG was released with 0.2M PB at pH 4. Sodium chloride was chosen as the BSA binding-depressing salt because it has limited ability to promote hydrophobic interactions (34). Increasing salt concentration of loading via addition of 1M NaCl effectively increased the chromatographic separation of hIgG on the HWRGWV column. The yield of isolated hIgG (Table 4-6) was boosted from 52% to 98% when the loading concentration of hIgG was 10 mg/mL. The results were reproducible with the HWRGWV resin from the same synthetic batch while lower recovery was obtained when the resins of different batches were used (Appendix).

Since both the hydrophobic and electrostatic interactions were excluded as the dominant forces for hIgG adsorption, the high salt concentration in the loading buffer seems to decrease the retention of BSA on the affinity medium instead of improving the hIgG binding. This is evident in Figure 4-4A where a much larger flow-through peak was observed (Figure 4-4A, HWRGWV) when compared to the FT fraction in PBS loading (Figure 4-3); this flow-through peak contained most of the BSA (Figure 4-4B, lane 1). The purity of isolated hIgG by HWRGWV ligand was also slightly increased at high salt loading (Table 4-6), even though a small amount of BSA monomers (62 kDa band) and dimers at ~130 kDa (Figure 4-4B, lane 2) were present. The presence of BSA monomers was similar to that of A2P (Figure 4-4B, lane 16) and the binding of BSA dimers was probably due to non-specific hydrophobic interactions. BSA dimers, as well as the salt consumption, might be able to be reduced by using sodium caprylate in the loading buffer, since caprylate is known to interact with albumin and hence prevent BSA from binding to columns (21, 35). When compared to the runs loaded in PBS, loading in high salt concentration progressively decreased the buffer volume for the chromatography process from 12 mL (Figure 4A) to 4 mL (Figure 5A) and decreased the process time by a factor of 3 at the same flow rate. Reduced processing time was another plus, along with the milder elution condition at pH 4, to keep the activity of antibodies. The high salt loading in PBS with 1M NaCl was also applied on another two Fc-binding peptides HYFKFD and HFRRHL (Table 4-7). The high salt loading conditions resulted in higher yield and moderate improvement in purity. However, the increase in yield on HWRGWV was considerably larger.

The second strategy attempted to increase yields and purity of hIgG was to elute the protein with PB at pH 6 instead of pH 4. The hope was that this would elute hIgG but not BSA from the HWRGWV column since BSA is predominantly retained by electrostatic forces. The isoelectric point (pI) of BSA is in the range from 4.6 (36) to pH 5.5 (37, 38), depending on its isoform. It is also known that histidine becomes positively charged when pH decreases to below 6.0 while arginine and the N-terminal amine are always positively charged at acidic pH. When the elution pH was 4, positively charged BSA was extracted from positively charged resins together with hIgG. At pH 6.0 BSA is negatively charged and hence is kept retained by electrostatic interactions on positively charged HWRGWV resins. It is apparent from the data in Table 4-6 that, when using pH 6 PB to elute hIgG, the purity was increased from 91% to 93%. The purity increased from 95% to 98% by carrying out the adsorption step at high salt conditions. The purity of 98% achieved with pH 6 elution at high salt loading condition was comparable to those of Protein A and A2P. Bound hIgG being able to be eluted at pH 6, thanks to the weak interactions ($K_d = 10^{-5}$ M) between hIgG and HWRGWV, would be an advantage in isolation of pH sensitive antibodies. However, higher purity was compensated by lower yield noting that only 75% of bound hIgG was eluted at pH 6.

Changing the salt type in the washing step from sodium chloride to sodium acetate and the elution buffer from phosphate buffer (PB) to acetate buffer (AB) had insignificant influence in separation (Table 4-6).

Experiments carried out by overloading the column with hIgG in PBS were performed on the HWRGWV column with injections of 20 and 30 mg/mL of hIgG instead of 10 mg/mL-gel, hoping that the components of stronger interactions (hIgG) could displace the components of weaker interactions (BSA), resulting in higher purity of product. The purities of isolated hIgG from the overloading runs are included in Table 4-6. The results in the table show that the displacement effect did not occur with the HWRGWV ligand, implying that an equilibrium state may be reached between the competitive binding of BSA and hIgG to the ligand under the previous loading condition of 10 mg/mL-gel.

Similar trends, qualitatively and quantitatively were observed when the above strategies were employed to purify hIgG from cMEM on an Ac-HWRGWV column (Table 4-6 and

Figure 4-4). Like HWRGWV, purity was improved from 81.4% to 96% when the salt concentration in the loading buffer was increased to 1M and was further enhanced to 99% when pH 6 PB elution was coupled to the high salt loading condition. Meanwhile, the yield increased from 69.3% to 95.5% with the application of the high salt loading approach but dropped back to 69.7% when the two strategies were combined because of the partial elution. It can also be seen in Figure 4-4 that when loading in high salt concentration, the protein profile in hIgG peak from Ac-HWRGWV resin (Figure 4-4B, lane 7) was very similar to that of HWRGWV (Figure 4-4B, lane 2). The similarity between the two ligands was also observed under PBS loading conditions (Figure 4-3B, lanes 10 and 12), indicating that the N-terminal charges had negligible influence on capturing the main contaminant BSA retained by electrostatic interactions. Therefore, the charges on the side chain of arginine probably had a key role in BSA retention. It might be possible to block the charges on arginine to eliminate the BSA binding, but the influence of arginine on the binding of hIgG is not known. Overall, it was demonstrated again that Ac-HWRGWV performed analogously to HWRGWV even in dynamic hIgG isolation, suggesting that the N-terminal charges played an insignificant role in both adsorption and isolation of hIgG from cMEM.

The initial concentration (C_0) of hIgG was found to influence the yield and purity of the isolated hIgG when the total loading amount was kept constant at 10 mg/mL-gel ($C_0V_0 = 1$ mg) (Figure 4-5). Generally both purity and yield decreased with decreases in C_0 from 10 to 0.5 mg/mL. The purity, for example, diminished slightly from 95% to 93% when C_0 was reduced from 10 to 5 mg/mL, it fell from 93% to 78% when C_0 was reduced further from 5 to 2 mg/mL and kept dwindling with decreased hIgG concentration. The yield decreased slightly but remained higher than 88% when the hIgG initial concentration was decreased from 10 to 1 mg/mL and afterward the yield plunged from 88% to 45% when C_0 decreased from 1 to 0.5 mg/mL. When C_0 was reduced to 0.5 mg/mL, the purity and yield dropped respectively to 64% and 45%, values that are much smaller than those obtained with Protein A but still comparable to the results obtained on the A2P column (Table 4-6) under the similar conditions.

The resulting purity and recovery on HWRGWV ligand resins obtained in this study were comparable to those from literature data of commercial small ligands: MAbSorbent

A2P (Prometic BioSciences Inc., Wayne, NJ), MEP Hypercel (Life Technologies, Rockville, MD), and Kaptiv-GY (Interchim, Montlucon, France). Resin A2P was optimized to purify polyclonal IgG from hyper immunized ovine serum with both the yield and purity greater than 95% (21). HCIC has been studied for IgG purification, yielding purity and recovery of 44% and 75%, respectively, when the feedstock solution was from protein-free cell culture supernatant with IgG concentration at 0.0474 mg/mL (14). When dealing with cell culture supernatant containing 5% FCS, the purity and recovery of hIgG were found to be 69% and 76% respectively (13). Kaptiv-GY isolated hIgG from human serum with both purity and yield of about 90% (22). No literature data has been identified for this ligand in the purification of IgG from cell culture media.

Hexamer peptide HWRGWV exhibited the ability to purify hIgG from a medium containing 10% FCS and 5% TPB with purity and recovery, depending on the initial concentration of the antibody, comparable to the reported numbers listed above and the parallel experiments done on Protein A and A2P columns. However, HWRGWV was the only one among all of these small ligands known to mimic Protein A binding hIgG through its Fc portion. In addition, mild (up to pH 6) elution conditions could be applied to HWRGWV ligand to minimize the activity loss of the product, but with some loss in overall yields during the elution step.

4.4 Conclusions

Three Fc-binding linear hexamer peptide ligands (HWRGWV, HYFKFD, and HFRRHL) immobilized individually on Toyopearl AF Amino 650M resins showed dissociation constants in the range of 10^{-5} - 10^{-6} M and the potential of isolating hIgG from cMEM. IgG was eluted at a less acidic condition (pH 4) than with Protein A and A2P ligands immobilized on cross-linked agarose gels. Among these hexamers, HWRGWV demonstrated the ability to purify hIgG from cMEM in one step with both the purity and the yield as high as 95% when hIgG concentration in cMEM was 10 mg/mL. These results are comparable to those obtained using Protein A and A2P resins. Lowering the initial hIgG concentration decreased the product purity yield, whereas it was still comparable to the results from the A2P resin when C_0 was 0.5 mg/mL. The larger than 88% recovery at hIgG concentrations higher than 1

mg/mL is promising for antibody capture from protein solutions like cMEM, namely serum containing cell culture supernatant.

The peptide density of HWRGWV had a significant influence on the binding affinity of hIgG and the specificity to the Fc fragment, but barely affected the binding capacity of hIgG. The peptide density also influenced recovery and to a lower degree the purity of isolated hIgG. Resins with peptide densities of about 0.08 meq/g exhibited both the appropriate binding strength (7.3×10^{-6} M) to hIgG and the binding specificity to Fc fragments. Temperature had little effect on the chromatographic isolation of hIgG from cMEM when using HWRGWV as the affinity ligand.

N-terminal acetylation of HWRGWV had no influence on adsorption and isolation of hIgG from cMEM, indicating that the charge of the N-terminal histidine played no major role on the retention of hIgG to the HWRGWV ligand. Acetylation of N-termini also had negligible effects on capturing the main contaminant BSA, signifying that the charges on arginine probably were responsible for BSA retention. The positive results shown above indicate that small linear peptide ligands have great potential for use as Fc specific ligands for antibody capture and purification.

4.5 References

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Table 4-1 Apparent dissociation constant K_d and maximum binding capacity q_m obtained using a direct fit of the Langmuir equation to the raw isotherm data as shown in Figure 4-1. The ligand densities were 1.0 meq/g for all hexamer peptide resins.

Ligand	$K_d, \times 10^{-5}M$	q_m		$C_s, mg/m^{2**}$
		mg/g	mg/mL*	
A2P	0.039	N/A	35.1	N/A
Protein A	0.043	88.1	22.0	N/A
HWRGWV	1.0	134	28.4	4.5
HYFKFD	1.1	127	27.0	4.2
HFRRHL	2.6	158	33.6	5.3
HVHYYW	2.0	129	27.4	4.3

* The swell ratios of Protein A and hexamer peptide resins are assumed to be 4 and 4.7 mL/g-dry-resin, respectively.

** C_s denotes surface concentration of hIgG which is calculated by dividing q_m by the surface area of the hexamer peptide resin beads ($30 m^2/g$).

Table 4-2 Recovery and purity of hIgG purified from cMEM (duplicate runs) on different experimental ligands in chromatographic formats.

The averages of the data from duplicate runs are indicated as Avg. All data were obtained by using 0.1 mL columns with 1mg hIgG loaded.

Ligand	Purity, %			Recovery, %		
	Run1	Run2	Avg	Run1	Run2	Avg
HWRGWV	89.7	92.0	90.9±1.2	53.2	51.2	52.2±1.0
HYFKFD	89.9	83.5	86.7±3.2	43.2	40.7	42.0±1.3
HFRRHL	88.9	88.2	88.6±0.4	48.6	49.4	49.0±0.4
HVHYYW	93.5	83.3	88.4±5.1	25.4	25.3	25.4±0.1

Table 4-3 Bound percentage, calculated by the peak area, of hIgG and its fragments to HWRGWV at different peptide densities and to acetylated HWRGWV (Ac-) at 0.08 meq/g. Fab, F(ab')₂ and Fc are fragments of hIgG.

Density, meq/g	Fab, %	F(ab') ₂ , %	Fc, %	hIgG, %
0.02	0.6	2	22	58.5
0.04	2.6	21.2	95.9	96.8
0.08	4.3	24.3	94.8	100
0.08, Ac-	4	28.8	97.1	100
0.22	14.5	97.7	98.1	97.2
0.55	74.1	96.6	91.4	100

Table 4-4 Apparent dissociation constant (K_d), maximum binding capacity (q_m), and superficial stoichiometry (S) at different ligand densities acquired by direct fit of the Langmuir isotherm to the raw data as shown in Figure 4-2.

Density, meq/g	q _m , mg/g	K _d , ×10 ⁻⁵ M	R ²	S, Peptide#/IgG
0.022	158.1	3.0	0.980	21
0.04	147.8	2.0	0.960	41
0.08	131.8	0.73	0.985	91
0.08, Ac-	130.8	0.38	0.972	92
0.10	133.6	1.0	0.995	112
0.22	136.8	0.26	0.963	241
0.55	137.6	0.095	0.911	600

Table 4-5 Purity (P) and recovery (R) of isolated hIgG from cMEM on the HWRGWV columns (0.1 mL CV) at different peptide densities and temperatures. Densitometry and ELISA were employed respectively for P and R assessment.

Density	0.04 meq/g		0.08 meq/g		0.22 meq/g		0.55 meq/g	
T, °C	P, %	R, %	P, %	R, %	P, %	R, %	P, %	R, %
4	/	/	91.1±0.3	63.8±3.8	/	/	85.7±0.3	107.3±0.9
11	/	/	89.6±0.2	62.2±2.3	/	/	84.6±1.5	100.7±1.3
21	91.7±1.3	61.0±0.2	90.9±1.2	52.2±1.0	84.2±0.8	82.8±1.9	83.9±0.7	81.9±1.6

38	/	/	88.5±0.2	44.4±0.1	/	/	81.8±1.4	81.4±7.4
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Table 4-6 Purity and recovery, together with the average (avg) of the duplicate runs, of hIgG product separated from cMEM at different chromatographic conditions.

Injection volume (V_0), initial hIgG concentration (C_0), loading buffer, elution buffer, and affinity media were varied in the investigation. The purity and yield were calculated by densitometry and ELISA respectively.

V_0 , μl	C_0 , mg/mL	Elution buffer	Purity, %			Recovery, %		
			Run1	Run2	Avg	Run1	Run2	Avg
HWRGWV								
1mg hIgG loaded in PBS								
100	10	pH4 PB	89.7	92.0	90.9±1.2	53.2	51.2	52.2±1.0
100	10	pH4 AB	89.9	92.0	91.0±1.1	45.9	42.7	44.3±1.6
100	10	pH6 PB	93.6	92.3	93.0±0.7	39.3	39.9	39.6±0.3
1 mg hIgG loaded in PBS + 1M NaCl								
100	10	pH4 PB	95.5	94.1	94.8±0.7	98.7	96.7	97.7±1.0
100	10	pH6 PB	98.1	97.2	97.7±0.4	67.0	68.6	67.8±0.8
Over-loading in PBS								
200	10	pH4 PB	90.2	/	90.2	/	/	/
300	10	pH4 PB	84.5	/	84.5	/	/	/
Ac-HWRGWV								
1mg hIgG loaded in PBS								
100	10	pH4 PB	82.7	80.0	81.4±1.4	69.3	/	69.3
1 mg hIgG loaded in PBS + 1M NaCl								
100	10	pH4 PB	96.1	95.0	96±0.6	95.7	95.2	95.5±0.2
100	10	pH6 PB	98.3	99.0	99±0.3	70.3	69.1	69.7±0.6
Over-loading in PBS								
300	10	pH4 PB	81.7	/	81.7	/	/	/
300	10	pH4 AB	80.3	/	80.3	/	/	/
Positive control, 1mg hIgG loaded in corresponding concentration of PB								
Protein A								
100	10	pH3 CA	100	/	100	99.5	94.3	96.9±2.6
2mL	0.5	pH3 CA	98.5	98.9	98.7±0.2	79.2	89.1	84.2±4.9
A2P								
100	10	pH3 CA	97.3	/	97.3	95.3	93.3	94.3±1.0
2mL	0.5	pH3 CA	76.6	67.6	72.1±4.5	58.4	46.9	52.7±5.8

Table 4-7 Initial values for purity and yield for loading conditions on resins HWRGWV, HYFKFD, and HFRRHL: PBS with 1M NaCl vs. PBS loading.

Ligand	Yield, %		Purity, %	
	PBS	PBS + 1M NaCl	PBS	PBS + 1M NaCl
HYFKFD	42	65.8	86.7	92
HFRRHL	49	62	88.6	90.6
HWRGWV	52.2	94.8	90.9	97.7

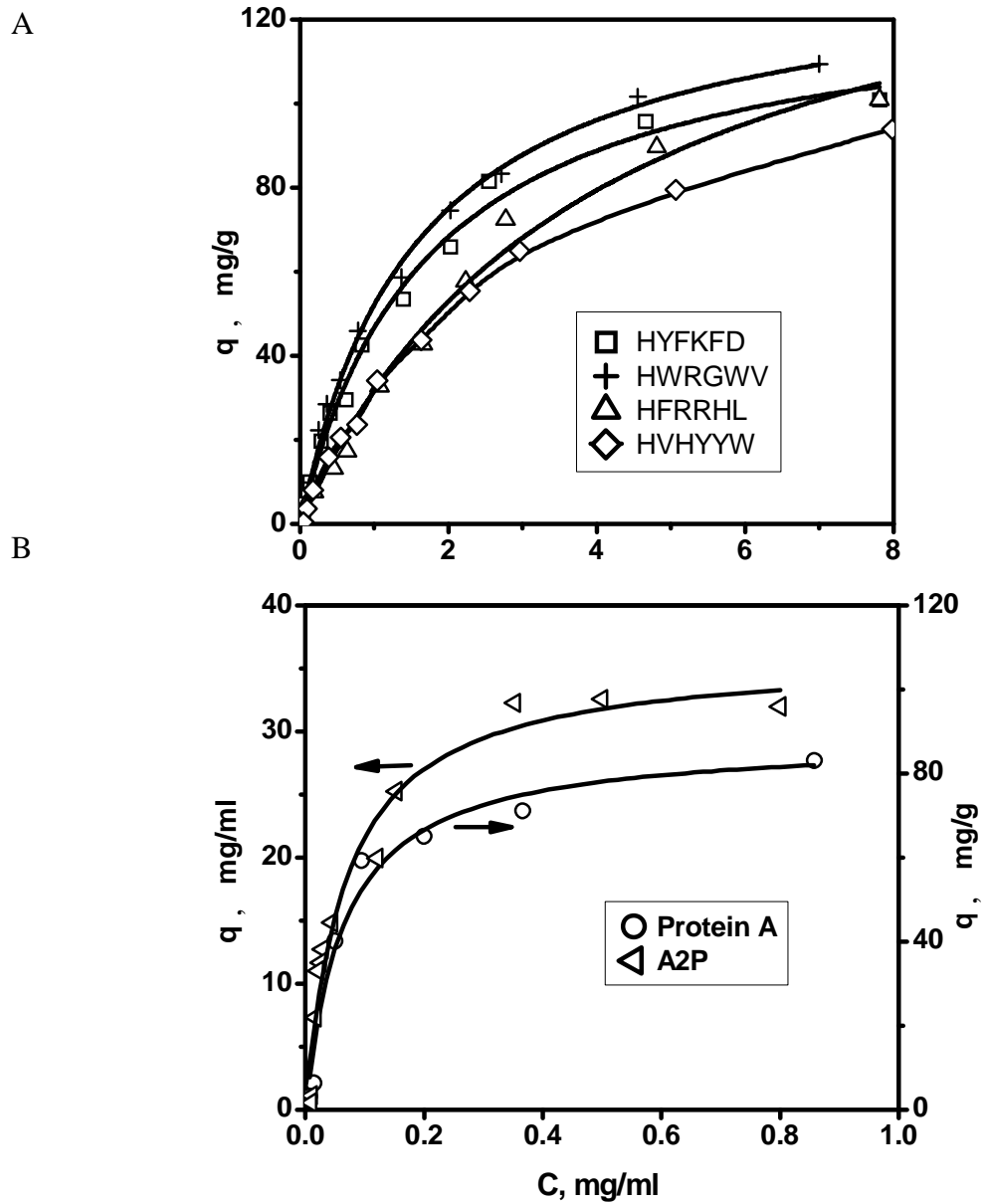
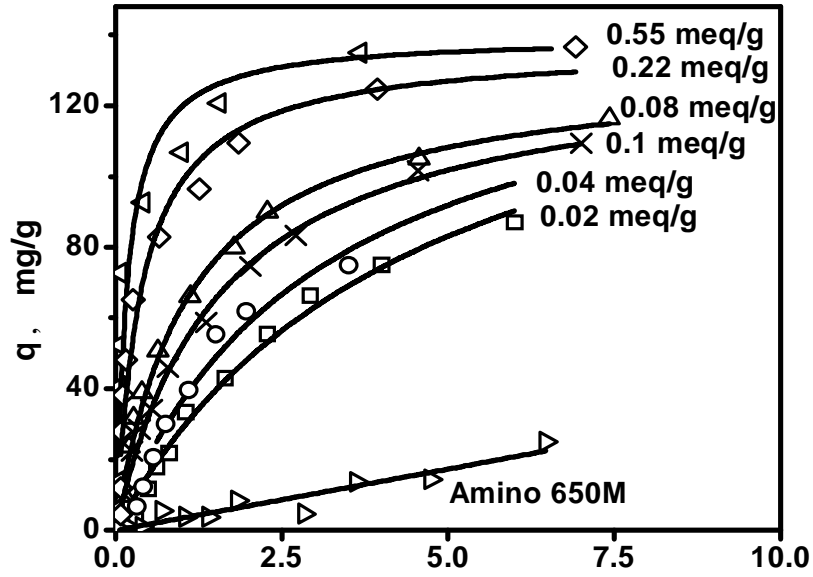


Figure 4-1 Langmuir fits (lines) of isotherms for hIgG adsorption to (A) hexamer ligands and (B) positive controls resins. The symbols are the original experimental data.

A



B

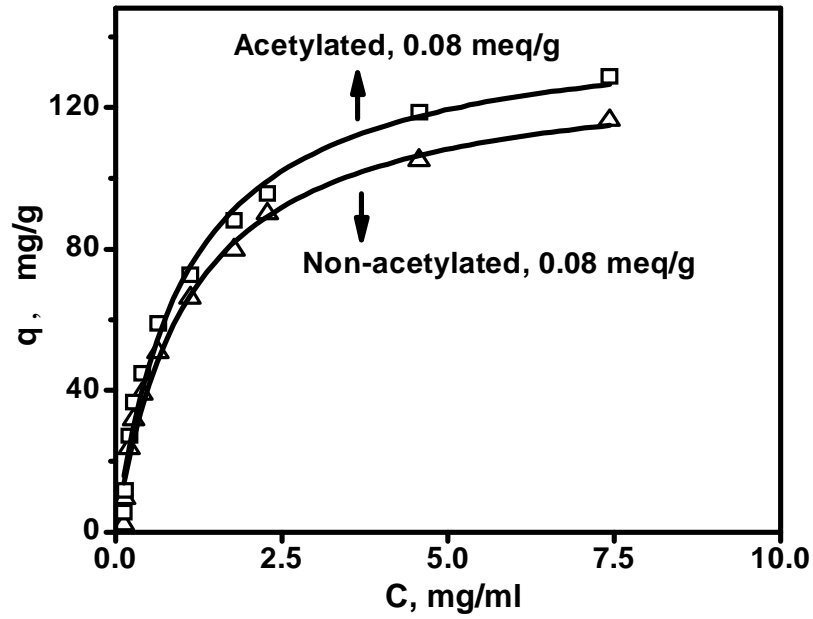


Figure 4-2 Langmuir fits (lines) of isotherms for hIgG adsorption to (A) HWRGWV resins at different ligand densities and (B) to acetylated HWRGWV resin. Ligand density are indicated in the graphies. Amino 650M is the base resin included as a negative control. Symbols are the experimental data.

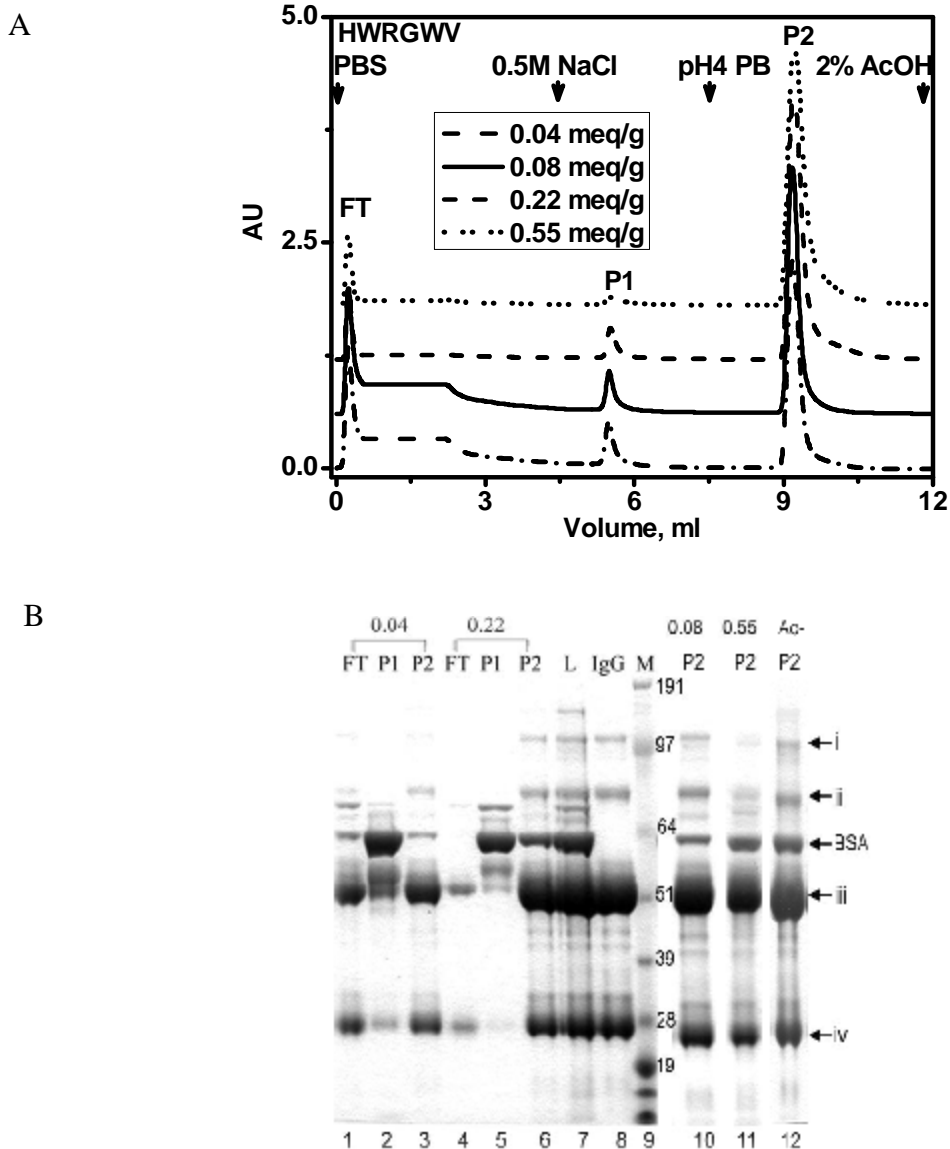


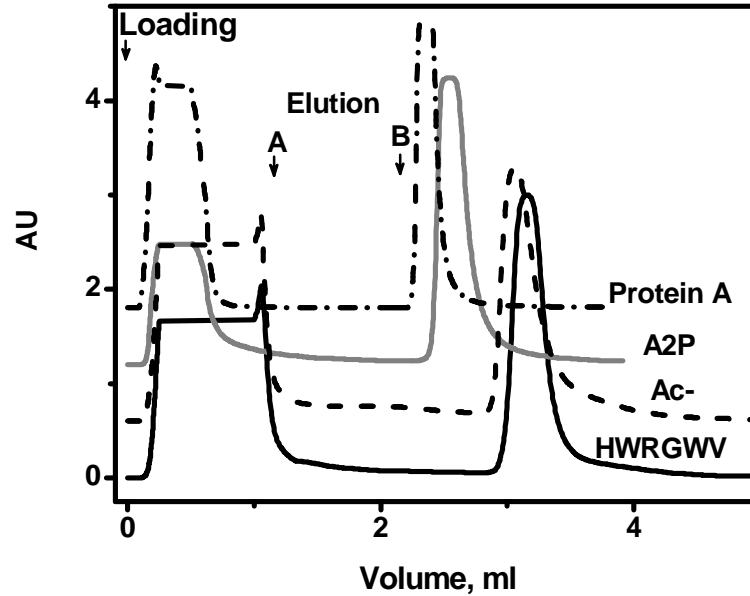
Figure 4-3 (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM eluting on HWRGWV resins at different densities.

A: Samples were loaded in PBS at pH 7.4, and sequentially pass through 20 minutes each of 1 M NaCl for washing, pH 4 PB for elution and 2% acetic acid (AcOH) for cleaning. The flow rate was 0.2 mL/min, the concentration of hIgG 10 mg/mL and the injection volume 100 μ L. The detected peaks were flow through (FT), salt wash peaks (P1), and PB wash peaks (P2). The volumes of the collected peaks were 2 mL.

(B) SDS-PAGE gel of the peaks denoted in panel A, as well as loading material (L) with a 1:5 dilution, standard hIgG (IgG), and molecular weigh marker (M). The first two text lines mark the ligand density in meq/g and peaks of the chromatograms. The three slices of 0.08, 0.55 meq/g and Ac- were from another different gels not fully shown in this paper. Ac-

denotes another run on Ac-HWRGWV under the same chromatography conditions. Lables i-iv indicate the different fragments of hIgG. The gel was run under reducing conditions.

A



B

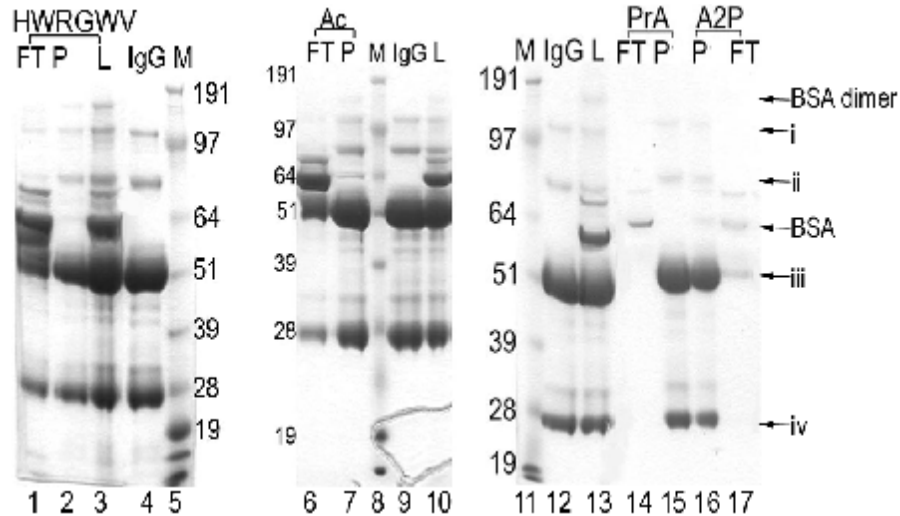


Figure 4-4: (A) Representative chromatograms and (B) SDS-PAGE for hIgG separations from cMEM using different affinity resins.

A: For HWRGWV and Ac-HWRGWV columns, protein complex was loaded in PBS + 1M NaCl and bound hIgG was eluted with pH 4 PB. Samples were loaded in 0.02M PB (pH 7) to Protein A and eluted with 10 mM pH 3 citric acid (CA). A2P column was loaded with 0.025M PB at pH 7 and bound proteins were eluted with 50 mM pH 3 CA. Column volume: 0.1 mL; concentration of hIgG: 10mg/mL; sample loop: 100 μ L.

B: Reduced SDS-PAGE gels of the chromatographic peaks on HWRGWV, Ac-HWRGWV (Ac-), Protein A (PrA) and A2P columns denoted in panel A, where flow through (FT) and hIgG elution peak (P) for each column were analyzed, together with loading material (L) with a 1:5 dilution, standard hIgG (IgG), and molecular weigh marker (M). The first two lines of text mark the affinity ligands and the corresponding chromatographic peaks in hIgG

separation from cMEM.

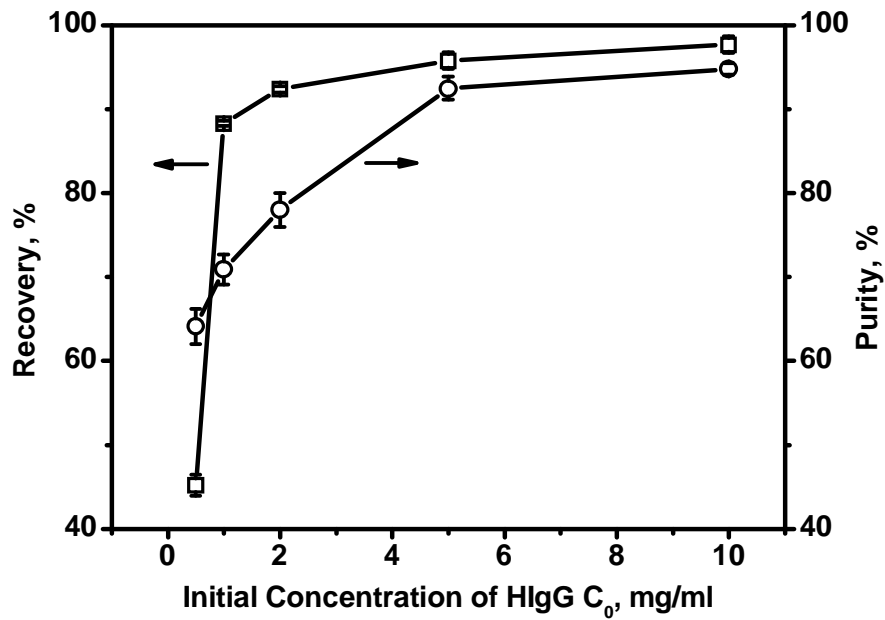


Figure 4-5 Influence of initial hIgG concentration in cMEM on the purity and recovery of the product isolated by the HWRGWV column.
 Loading buffer: PBS + 1 M NaCl; elution buffer: pH 4 PB; column volume: 0.1 mL; amount of hIgG loaded: 1 mg.

**Chapter 5. Use of HWRGWV for hIgG purification from human
blood plasma II+III paste**

5.1 Introduction

Antibody products can be produced as polyclonal antibodies from (anti)-serum or monoclonal antibodies (mAbs) from cell culture. Each mAb product is specific to a single epitope of an antigen, and the antibody molecules all exhibit the same specificity, affinity, and isotype. In contrast to mAbs, polyclonal antibodies are the product of many responding clones of cells and are, in consequence, heterogeneous at many levels: in the classes and subclasses of the antibody produced, their specificity, titer, and affinity. The heterogeneity of polyclonal antibodies, together with the abundance of impurity proteins in serum, renders the isolation of antibodies from serum difficult. Intravenous immunoglobulin G (IVIG), a product from human serum, has been found effective in the treatment of immune deficiency, Kawasaki syndrome, and immune thrombocytopenic purpura disease (1). With an increasingly large number of therapeutical applications being discovered for IVIG, it has now become the leading plasma product, ahead of albumin and factor VII. The brand names of the IVIG products comprise Carimune® (CSL Behring, Bern, Switzerland), Gamunex® (Talecris Biotherapeutics, Research Triangle Park, NC), Gammagard® (Baxter Healthcare Corporation, Deerfield, IL), Gammar® (Aventis Behring L.L.C., King of Prussia, PA), among others. Seventy years after the development of the Cohn methods (2, 3), the cold ethanol fractionation technique is still the common method to process human plasma in industry today for IVIG production. The persistence of this method is mainly due to the scarceness of the plasma source that requires maximum outcome of plasma proteins, the large volume of processed plasma, the huge amount of proteins to be treated per lot, and the strict regulation associated with IVIG production (4).

To pursue better product quality and higher yields, alternative precipitation agents and modern chromatographic purification techniques have been integrated into the Cohn method (5-8). The chromatography steps are usually of ion exchange mode and the precipitation agents commonly used are caprylic acid and polyethylene glycol (PEG). After cold ethanol precipitation to form Cohn paste II+III, at least another three main steps, including column chromatography and/or precipitation, are usually employed to achieve a yield in the range

from 39.1% to 64% and the purity required by FDA regulatory standards. Tanaka et al. (9) directly isolated hIgG from cryoprecipitated human plasma by using four ion-exchange and two size-exclusion chromatography steps, with a final yield of 31.8% which was lower than those when Cohn paste II+III were used as the starting material.

Currently, the use of affinity chromatography is very limited for IVIG preparation, either as a mainstream purification tool or as a polishing step. Affinity chromatography has the potential to improve the yield and purity with fewer processing steps, potentially decreasing the cost of IVIG production. However, Protein A or Protein G are not used in human plasma fractionation because of the high cost, the risks of leaching, the harsh elution conditions associated with the process, and the large quantity of gel required to produce tons of IgG. In one study, MAbsorbent A2P was determined not suitable for large scale purification of IVIG because of its low dynamic capacity (16 mg IgG/mL resin), low recovery (below 80%), and strong retention of some residual IgG, as stated by Buchacher and Iberer (1). In contrast, another study (10) showed when MAbsorbent A2P was coupled with Fractogel DEAE chromatography, IgG product was obtained with a purity (by electrophoresis and HPLC) equivalent to commercial standards, but the resulting yield was not reported. An affinity cascade for plasma protein isolation was announced by ProMetic Biosciences (11), claiming a 50% purity and 87% yield in IgG fraction after an affinity column to IgG. A mixed-mode hydrophobic charge induction (HCIC) ligand explored for antibody purification from cell culture media was also probed for IVIG production (1). HCIC was nevertheless considered incapable to be dedicated to the purification of polyclonal IgG from plasma due to the low binding capacity and the low selectivity in complex solutions like plasma. It would be highly desirable to have an inexpensive affinity ligand able to result in high purity and/or yield for a less costly IVIG manufacturing process.

The structure of the matrix, its chemical properties, and mechanical stability influence the ligand orientation and configuration, ligand binding capabilities, protein accessibility to ligands, maximum operational pressure limit, and consequently the purification efficiency (12, 13). Furthermore, differences in material costs affect the process economics. Hence, an economical downstream process can be achieved by selection of less expensive but robust matrixes, ligands, and chromatographic procedures.

As described in Chapter 4, hIgG can be purified from mammalian cell culture media (cMEM) formulated with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB) by using the short peptide ligand HWRGWV synthesized on Toyopearl AF-Amino resin (HWRGWV-Toyopearl). Both the purity and yield of isolated hIgG were higher than 95%. In this chapter, the immobilization of HWRGWV on Sepharose CL-4B is described and the ability of the produced resin (HWRGWV-Sepharose) to purify hIgG from cMEM was compared with the results obtained on HWRGWV-Toyopearl. In addition, experiments using HWRGWV-Toyopearl for hIgG isolation from Cohn paste II+III are described.

5.2 Experimental

5.2.1 Materials

II+III paste of human blood plasma was purchased from Monobind Inc. (Lake Forest, CA). Peptide resins HWRGWV and N-terminal acetylated HWRGWV (Ac-HWRGWV) at ligand densities of 0.08 meq/g were synthesized directly on Toyopearl AF-Amino-650 M (particle size 65 μm) (Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY). Soluble peptide Fmoc-HWRGWV was also synthesized by Peptides International. 0.22 μm Ultrafree-MC low-binding Durapore membrane filters and MicroCon YM-3 regenerated cellulose filters with MWCO of 3,000 were obtained from Millipore (Billerica, MA). NuPAGE Novex Bis-Tris gels, NuPAGE MOPS Running Buffer, NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent, Seeblue plus2 pre-stained molecular weight marker, and SimpleBlue SafeStain were all from Invitrogen (Carlsbad, CA). Sepharose CL-4B was obtained from GE Healthcare (Pittsburg, PA). The following products were all purchased from Sigma (St. Louis, MO): phosphate buffered saline (PBS) of pH 7.4, human IgG (hIgG), bovine serum albumin (BSA), sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate, glacial acetic acid, 3,3'-diaminodipropylamine (DADPA), 2,4,6-trinitrobenzenesulfonate (TNBS), 1,1'-carbonyl diimidazole (CDI), N,N'-diisopropylcarbodiimide (DCC), hydroxylbenzotroazole hydrate (HOBT), dimethylformamide (DMF), dichloromethane (DCM), Fmoc-alanine. Cell culture medium

EMEM was from Quality Biological (Gaithersburg, MD). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT) and Becton Dickinson (Sparks, MD), respectively. Human IgG ELISA kit was from Alpha Diagnostic International (San Antonio, TX). Micro BCA assay kit was purchased from Pierce (Rockland, IL). A Waters 626 LC system integrated with 2487 UV detector (Waters, Milford, MA) was used for all chromatography runs unless otherwise mentioned. Empty PEEK-lined Omega columns with a volume 0.1 mL were from Upchurch (Oak Harbor, WA). A UV-160 UV-Vis recording spectrophotometer was from Shimadzu (Kyoto, Japan).

5.2.2 Coupling of DADPA spacer arm to Sepharose CL-4B activated via CDI

Sepharose gel (1 mL) was washed in a glass container with 5×2 mL DMF to remove the residual water. The gel was then suspended as a 50% slurry in DMF. 1, 1'-carbonyldiimidazole (CDI, 38 mg, MW 162) was added to the slurry to activate the gel by tumbling for 1 h at room temperature. After reaction, the gel was washed with DMF 5×2 mL to remove the imidazole produced during the reaction. The activated gel was then immediately mixed with DADPA (0.48 mL, 3.40 mmol, MW 131.2) in 1 mL DMF to react for 4 h at room temperature. After the reaction, the gel was sequentially washed with 2×2 mL of DMF, PBS, and then DMF to completely remove un-reacted DADPA. The stable amide linkages were then coupled to the matrix with primary amines as the terminal groups. The TNBS test was employed for both the solution and the resin to determine the completeness of washing and success of coupling respectively.

5.2.3 Attachment of Fmoc-HWRGWV to DADPA coupled Sepharose CL-4B

0.5 mL of DADPA coupled Sepharose CL-4B was aliquoted to immobilize Fmoc-HWRGWV. The pre-activating solution was prepared by dissolving Fmoc-HWRGWV (MW 1061, 0.30 g) and hydroxybenzotrozoale hydrate (HOBt, MW 135, 0.038 g), yielding 1:1 in molar ratio, in 0.5 mL DMF. The pre-activation solution was then added to the DADPA-activated resins (0.5 mL) and incubated for 5 min. After that, 0.75 mL of N, N'-diisopropylcarbodiimide (DCC) was added to the mixture tumbling at room temperature for 24 hours. The Fmoc-HWRGWV-modified resins were subsequently washed with 2×0.5 mL

DMF for 1 hour, 2 × 1 mL methanol, 4 × 1 mL 1:1 DMF:DCM, and then 3 × 1 mL DMF for 5 minutes with each wash. A parallel immobilization using Fmoc-ala was performed as a control.

5.2.4 Fmoc deprotection and ligand density measurement

Fmoc groups were deprotected from the peptides immobilized on the resins by incubating the resins modified as described above with 1 mL 20% piperidine in DMF for 5 minutes and another 1 mL of the same solution for 15 minutes. The resins were then washed with 8 × 1 mL DMF. All the piperidine and DMF wash supernatants were collected together and diluted with methanol before the optical density (A) was measured at 310 nm on a Shimadzu UV-160 UV-Vis spectrophotometer. The content of the Fmoc-piperidine adduct, namely, the peptide density on the resins (S_n) was then calculated in $\mu\text{mol/mL}$ resin according to equation

$$S_n = \frac{V_s \times n \times A}{7604 \times V_{\text{resin}}} \times 1000 \quad (\text{Eq. 5-1})$$

Here n is the dilution factor of wash solution; V_s and V_{resin} are respectively the volume of wash solution and resin in mL.

5.2.5 TNBS test for amines

Compounds containing primary amines were qualitatively visualized by reaction with 2,4,6-trinitrobenzenesulfonate (TNBS) to form a brown complex. The TNBS test procedure was described by Hermanson et al. (14).

5.2.6 Evaluation of IgG retention by HWRGWV immobilized on Sepharose CL-4B

Moist Sepharose CL-4B gel immobilized with HWRGWV was packed in a 30 mm long × 2.1 mm (ID) Omega column (volume 0.1 mL) and washed with 20% methanol before being equilibrated in loading buffer. The loading buffer was PBS or PBS + 1M NaCl. PBS is an aqueous solution containing 10 mM phosphate, 2.7 mM KCl, and 138 mM NaCl at a pH of 7.4. The starting material was either hIgG in PBS or hIgG spiked in cMEM with

concentrations of hIgG being 1 and 10 mg/mL, respectively. The salt concentration in the starting material was adjusted to the same as that of the loading buffer. cMEM was formed by combining EMEM with 10% FCS and 5% TPB, to mimic the mammalian cell culture supernatant. The chromatography procedures were the same as those described in Chapter 4 with the wash and elution solutions being PBS + 1M NaCl and 0.2M phosphate buffer (PB) at pH 4, respectively. Another column packed with alanine-bound Sepharose CL-4B (A-Sepharose) was subjected to the same procedure as a negative control. Triplicate chromatographic runs were examined for each column at room temperature.

5.2.7 HIgG separation from II+III paste by HWRGWV linked on Toyopearl resins

The effect of residual ethanol in the starting material on hIgG retention on an HWRGWV column was assessed by an ethanol spiking experiment. HIgG (1 mg/mL) in PBS was spiked with 3% (v/v) ethanol and the ethanol spiked IgG solution (100 μ L) was injected in a 30 mm long \times 2.1 mm (ID) Omega column (0.1 mL) packed with HWRGWV resins. PBS was used as the loading buffer and the chromatographic procedures were the same as those indicated in Chapter 4 where washing and elution buffer were PBS + 1 M NaCl and pH 4 phosphate buffer (PB), respectively. The retention time was compared with the one when pure hIgG in PBS was employed.

Cohn paste II+III was re-suspended at different concentrations in PBS or PBS with NaCl. The amount of NaCl added to PBS was from 0 to 1 M. The paste solution was passed through a 0.22 μ m Durapore membrane filter before being loaded to the columns. The ethanol content in re-suspended solution was checked by HPLC coupled with a refractive index detector (Shimazu, Tokyo, Japan) on a Phenomenex Rezex ROA(H+) (7.8 \times 300 mm) column (Phenomenex, Torrance, CA) running in 0.01 M sulfuric acid at 0.6 mL/min. Antibody separation was carried on columns packed with HWRGWV or N-terminal acetylated HWRGWV (Ac-HWRGWV) immobilized on Toyopearl AF-Amino 650M under the following conditions: the loading buffers were PBS or PBS with different concentrations (0 - 1 M) of NaCl; the bound protein was stripped with 0.2 M phosphate buffer (PB) at pH 4 and the column was cleaned with 2% (v/v) acetic acid after each run; the typical flow rate

was 0.05 mL/min during loading and maintained 0.2 mL/min for the rest of the run. Peaks were collected and assayed by ELISA and SDS-PAGE.

5.2.8 ELISA and SDS-PAGE analysis for purity and yield

The same procedures described in Chapter 4 were used to determine the purity and yield of isolated hIgG (ELISA and SDS-PAGE).

5.3 Results and discussion

5.3.1 HlgG separation from cMEM by HWRGWV immobilized on Sepharose CL-4B

It has been shown that HWRGWV directly synthesized on Toyopearl AF-Amino 650M resin (HWRGWV-Toyopearl) was able to isolate hIgG from cMEM with more than 95% purity and yield (see Chapter 4). To determine the performance of the ligand when immobilized on a different solid support, agarose-based Sepharose CL-4B was used, with immobilization and ligand binding capability being evaluated.

Sepharose CL-4B is one of the most commonly employed supports because of its macroporosity, low non-specific adsorption, good chemical stability, relatively low cost, and availability of functional groups for easy modification and ligand coupling (15, 16). Due to the undesirable hydrophobic character of the spacers present on the commercialized Sepharose derivatives which are suitable for peptide coupling via carboxyl groups, diaminodipropylamine (DADPA) was used as a spacer, which is more hydrophilic and contains no net charge. The resulting structure is depicted in Figure 5-1A.

HWRGWV was attached to DADPA-activated Sepharose CL-4B through its carboxyl terminal using the HOBt/DCC method and the resulting structure is shown in Figure 5-1B. The density of HWRGWV immobilized on Sepharose CL-4B was 36.7 $\mu\text{mol/mL}$ (Table 5-1), measured by the concentration of de-protected Fmoc groups using equation 5-1. The control immobilization of alanine generated a density of 33.0 $\mu\text{mol/mL}$ on Sepharose CL-4B. Alanine was used as a negative control since it had no ability to retain hIgG (17). The resulting ligand density of 36.7 $\mu\text{mol/mL}$ is typical for small ligands immobilized on Sepharose CL-4B (16, 18) and higher than the ligand density of 17 $\mu\text{mol/mL}$ on the Toyopearl resin used in this work (Table 5-1).

The HWRGWV-modified Sepharose CL-4B (HWRGWV-Sepharose) was packed into a 0.1 mL column for IgG binding and isolation assessment. Pure IgG in PBS at a concentration of 1 mg/mL was loaded to the column. The column was then sequentially washed with 2 mL PBS, 4 mL PBS with 1M NaCl to remove the non-specifically retained protein, 4 mL PB at pH 4 to elute the bound hIgG, and 4 mL 2% AcOH to clean the column. Binding of pure hIgG in PBS was compared between the two HWRGWV modified solid supports, Sepharose CL-4B and Toyopearl AF-Amino resins (Figure 5-2). The overall chromatograms were similar, where negligible amounts of IgG were present in the flow through (FT), a small amount was washed off with 1 M NaCl solution (P1), and most was stripped with pH 4 PB (P2). However, the amount of hIgG eluted with pH 4 on Toyopearl and Sepharose CL-4B were respectively 94% and 75% of the initial amount injected, evaluating by the peak areas. The differences are also appreciable by visual comparison of the chromatograms (Figure 5-2). IgG elution peak (P2) from the Sepharose CL-4B was less sharp and symmetric than those on Toyopearl resin. The peak tailing on Sepharose might be due to the size exclusion characteristic of the Sepharose beads whose pore size distribution are not as narrow as that of Toyopearl (Table 5-1). A smaller ratio of the peak areas P2 to P1 for Sepharose was also noticed, suggesting that more IgG was retained by the Sepharose via ion-ion interactions. The electrostatic interactions could arise from the contact between IgG and the base Sepharose resin, since the free amines remaining on DADPA-activated Sepharose after peptide immobilization are not capped while those on the Toyopearl base resin are capped by acetylation. This is also evident in Figure 5-2, where Sepharose modified with alanine (A-Sepharose) in the same way as with HWRGWV displayed a salt wash peak (P1). Modification of the matrix, such as acetylation, might be able to block the electrostatic interactions. Nevertheless, the majority of the hIgG was retained by HWRGWV-Sepharose and the bound IgG could be eluted with pH 4 PB, suggesting the ability to isolate IgG from protein mixtures

Two chromatographic procedures were examined to compare the performance of HWRGWV-Sepharose and HWRGWV-Toyopearl to separate hIgG from cMEM (Figure 5-3 and Figure 5-4). Human IgG-spiked cMEM was loaded into the column in PBS or in PBS + 1 M NaCl solution after the salt concentration of the starting material was adjusted to 1M,

Table 5-2 lists the yields assayed by ELISA and the purities by SDS-PAGE of isolated hIgG on the two matrices at both loading conditions.

The chromatographic profile and protein gel profiles resulting from each peak of PBS loading on the two columns packed with HWRGWV on different matrices are shown in Figure 5-3. The chromatograms were similar with three peaks showing: flow-through (FT), salt wash peak (P1) and bound protein elution peak (P2) (Figure 5-3A). The corresponding gels (Figure 5-3B) show that the protein bands in P1 and P2 were similar with a small difference shown between loaded material (L) and the flow-through (FT). P1 contained mostly BSA at about 64 kDa and P2 consisted of mainly IgG, with BSA being the main contaminant protein. The seemingly larger amount of BSA in the loaded material (lane L, Figure 5-3B) on the gel of HWRGWV-Toyopearl was actually due to the smaller amount of protein loaded on this gel (2.6 μg of hIgG) than on the gel to its left (10.3 μg of hIgG, lane 13) which corresponds to the HWRGWV-Sepharose column at the same loading conditions. Less BSA was displayed in the FT fraction of HWRGWV-Sepharose which might be ascribed to the stronger ionic interactions exhibited by this resin. When the elution peaks (P2) were subjected to ELISA and densitometry analyses, similar yields (about 50-60%) and purity (about 90%) were obtained on both supports (Table 5-2). When high salt loading conditions were employed (Figure 5-4A), two peaks resulted in the chromatograms where one was flow-through (FT) and the other the IgG elution peak (P). More protein flowed through (larger FT) on HWRGWV-Sepharose and less was recovered in the elution peak (smaller P) when compared with HWRGWV-Toyopearl. The protein profile (Figure 5-4B) in each peak was similar for both solid supports except that a lot more hIgG seems to be displayed in the FT on HWRGWV-Sepharose. The purities calculated by densitometry on the two matrices were similar at around 90% but the yields by ELISA assay were different: 98% on Toyopearl and 64% on Sepharose (Table 5-2). The volumetric ligand density on the Toyopearl material was 17 $\mu\text{mol/mL}$, only half the density on Sepharose. However, in the loading conditions examined, HWRGWV-Toyopearl resin generated yields of either higher or equal to those on Sepharose, at the same time without any loss in purity. This might be indicative of the difference in the ligand availability caused by the structure of the base matrix. Toyopearl AF Amino is a porous polyacrylate resin where the ligands are oriented in a “brush form” on the

surface, which perhaps allows for a higher degree of ligand cooperativity in binding target proteins.

Comparing the yields and purities that resulted from the two loading conditions on each matrix (Table 5-2), it is evident that both supports showed similar slight increases in purity by adding salt, but they had a much different changes in yield when switching the salt concentration in the loading buffer to 1M. The yield on HWRGWV-Toyopearl experienced a dramatic increase from 51% to 98% when changing the loading buffer from PBS to PBS + 1M NaCl. This type of increase was not observed on HWRGWV-Sepharose, where the yields were identical for both loading conditions. That is to say that, unlike the case for Toyopearl, addition of salt in the loading step had no effects on binding hIgG from the protein mixtures. It is not clear why there was such a difference observed between the two matrices.

Two small ligands based on polysaccharide supports have been commercialized for IgG separations. One is MAbSorbent A2P from Prometic BioSciences Inc. (Wayne, NJ) and the other HCIC ligand MEP Hypercel from Life Technologies (Rockville, MD). The former is immobilized on 6% cross-linked agarose and the latter linked on cross-linked cellulose. Resin A2P was optimized to purify polyclonal IgG from hyper immunized ovine serum with both the yield and purity greater than 95% (19). HCIC has been studied for IgG purification, yielding purity and recovery of 44% and 75%, respectively, when the feedstock solution was from protein-free cell culture supernatant with IgG concentrations at 0.0474 mg/mL (20). When dealing with cell culture supernatant containing 5% FCS, the purity and recovery of hIgG were found to be 69% and 76% respectively (21). Compared to these ligands, HWRGWV-Sepharose achieved a yield of 64% which is similar to HCIC but lower than MAbSorbent A2P. The observed 89% purity is higher than that of HCIC and close to that obtained with A2P.

The similar IgG binding and elution pattern on the two matrices suggests that the predominant binding behavior of the ligand HWRGWV was not significantly altered by changing the solid support. The lower yield and the slightly lower purity were demonstrated on the HWRGWV-Sepharose resin in this preliminary study. However, it should be possible

to improve the purity and yield obtained on HWRGWV-Sepharose with optimized chromatographic conditions, ligand density, and matrix modification strategies.

5.3.2 hIgG isolation from II+III paste by HWRGWV immobilized on Toyopearl AF-Amino

Cohn II+III paste was employed as the crude hIgG source to explore the possibility of using the HWRGWV-Toyopearl resin in IVIG production. HWRGWV-Toyopearl was used since it produced better yields in hIgG isolation from cMEM. The influence of the ethanol content in the feed stream on hIgG retention on the column was determined by spiking ethanol into the material. No influence on the results of the chromatography was observed when pure hIgG solution containing up to 3% (v/v) ethanol was analyzed. The residual ethanol concentration in the paste suspension of 50 mg/mL was about 1% (v/v), analyzed on a Phenomenex Rezex ROA(H+) column, so the re-suspended paste was directly applied to the column after filtration through a 0.22 μ m filter.

In this initial study aiming at the separation of hIgG from the Cohn II+III paste, different loading conditions were examined, including the paste concentration in the column feed (C_{paste}), and the salt concentration in the paste re-suspension solvent (C_{SS}) and in the loading buffer (C_{SL}) (Table 5-3). The basic loading and re-suspension buffer was PBS, and NaCl was used to adjust the salt concentration when necessary. The concentration of hIgG in the suspension was about 6.6 ± 0.4 mg/mL as analyzed by ELISA when the paste concentration was 50 mg/mL of re-suspension solution. The yields reported in Table 5-3 were calculated according to the hIgG concentration of the re-suspended paste.

Three starting material concentrations were evaluated on the HWRGWV-Toyopearl column. Cohn paste at 100, 50 and 25 mg/mL were re-suspended in PBS or PBS with NaCl at different salt contents, yielding hIgG concentrations of 13.2, 6.6, and 3.3 mg/mL in suspensions (Table 5-3 and Figure 5-5). The chromatograms of the II+III paste runs at different re-suspension concentrations consist of three peaks: a broad flow-through (FT), a sharp pH 4 elution peak (P) and a small 2% AcOH peak (P3) (Figure 5-5). The loading of materials with higher concentrations show larger areas of FT and P while P3 is not affected as much. IgG elution peaks (P) were subject to ELISA and SDS-PAGE analyses for yield and

purity calculations. When the paste concentration decreased from 100 to 50 mg/mL, the yield increased 30% while no change in purity was observed (Table 5-3, runs 1 and 2). The low yield associated with the highest starting material concentration (100 mg/mL) could be due to overloading of the column, which is confirmed by the presence of a much larger flow-through peak (FT) (Figure 5-5A, left) and a higher hIgG concentration in this peak (Figure 5-5A, right). It should be realized that the FT of 100 mg/mL loading is out of scale at the set sensitive of 2 AUFS. When the paste concentration decreased from 50 to 25 mg/mL (runs 3 vs. 4 and 7 vs. 8), the yield decreased about 18 - 27% while the purity increased about 8%. The concentration of 50 mg/mL achieved the highest yield gain at little expense of purity, regardless of the salt concentration in the dissolving and loading solvents (0, 0.5, or 1 M) (Table 5-3). A starting material concentration of 50 mg/mL was therefore selected for the most runs.

Increasing the residence time of the starting material in the HWRGWV-Toyopearl column from 2 min to 10 min by decreasing the loading flow rate from 0.05 to 0.01 mL/min (Table 5-3, runs 7 and 8) actually decreased the hIgG yield from 69.8% to 47.1% without changing the purity (~ 79%). The loading flow rate was hence kept at 0.05 mL/min for all other runs

Salt concentrations in the re-suspension and loading solutions were changed for the chromatographic isolation of hIgG from the II+III paste at a paste concentration of 50 mg/mL to examine their influence on yield and purity (Table 5-3). Representative chromatograms are shown in Figure 5-6, where the run of hIgG spiked in cMEM loaded in PBS + 1M NaCl on HWRGWV-Toyopearl (black line) is included for comparison. There are some differences in the chromatographic profiles between the paste runs and the cMEM run. IgG elution peak (P) consisted of a long tail, which might be from the impurities eluted by pH 4 PB since such a tail was not observed for the cMEM loading. Upon washing the column with 2% AcOH, an extra washing peak (P3) was present, indicating that some proteins in plasma were bound on HWRGWV resin so tightly that had to be stripped by solvent with low pH. The chromatograms of the paste runs at different conditions are similar. However, the yields and purities calculated according to the elution peak are different.

When the same re-suspension and loading buffers were employed, the same yield (59%) and purity (76%) were obtained at two salt concentration levels of 0.5 M (run 3) and 1M (run 7). When the salt concentration in the loading buffer was maintained at 1 M while changing the salt concentration in the suspension, the yields were 63.1% in PBS (run 2), 69.8% in PBS + 0.5 M NaCl (run 5), and 58.6% in PBS + 1M NaCl (run 7) and the purities were 67.3%, 78.3%, and 79.1%, respectively. On the other hand, suspending the paste in PBS + 0.5 M NaCl produced both the highest yield and purity. Increasing the salt concentration in loading solution from 0.5 to 1M, while holding the salt concentration in suspension at 0.5 M NaCl, improved the yield from 59.7% to 69.8%, with both the purities being close to 76%. Overall, re-suspending the II+III paste in PBS + 0.5 M NaCl and loading in PBS + 1 M NaCl rendered both the highest yield (69.8%) and purity (78.3%) on the HWRGWV-Toyopearl column. These results suggest that different combinations of the salt concentrations in loading and re-suspension buffers might lead to different purity and yield values, where lower salt concentration in suspension solvent than in loading buffer favored yield, but not necessarily purity.

For the Ac-HWRGWV-Toyopearl column, different combinations of salt concentration in loading and re-suspension solvents were also investigated but with lower levels of salt, 0 - 0.5 M, to reduce the salt consumption (Table 5-3). The chromatogram profile on the acetylated resin is similar to that on the HWRGWV column (Figure 5-6, green line) with three peaks (FT, P, P3) present. Like in the case of the HWRGWV-Toyopearl resin, the salt concentrations influence the yield and purity of the hIgG product. Both the yield and purity were found to increase with increases in the salt concentration when the same buffer was used for the paste re-suspended and sample loading (runs 9 and 12). The yield increased from 30.2% to 57.0% while the purity increased to a much lower degree from 75.2% to 80.0%. Increasing the salt concentration from 0 to 0.25 M in the suspension solution, while keeping the salt concentration in loading buffer at 0.25 M (runs 10 and 12), decreased the yield slightly from 64.1% to 57.0% and, on the other hand, increased the purity from 69.9% to 80.0%. If the salt concentration in suspension was kept at 0 M, gradually increasing the salt concentration in the loading buffer from 0 to 0.25 and then to 0.5 improved the yield steadily from 30.2% to 64.1% and then to 82.1% (runs 9-11). Different trends were displayed in

purity where the lowest purity was found for the loading in 0.25 M NaCl (69.9%), while the higher purity of close to 76% was obtained for the loadings in 0 and 0.5 M. Therefore, it was true as well for the acetylated ligand that keeping the salt concentration in suspension lower than that in loading buffer increased yield, but again at the expense of higher purity.

Among the combinations investigated, the paste re-suspended in PBS while loaded in PBS + 0.5 M NaCl (Table 5-3, run 11) generated the highest yield (82.1%) and close to highest purity (77.3%). Figure 5-6 displays the chromatogram of run 11 (left, green line) and its corresponding electrophoresis gel (right). The SDS-PAGE gel shows that the major impurity BSA was present in the FT, and P3 contained some impurities and some IgG. The purity of the product peak (P) was 77.3%, including a little BSA and some other proteins. The best yield obtained in this initial study (82.1%) was higher than the one step yield (74%) reported in the literature on ion exchange columns (22) and on MAbsorbent A2P (1). It was lower than the one step yield of 92% on an IEC column listed by Parkkinen et al. (7), noting that the 92% yield was achieved by loading the paste II+III pre-treated with caprylic acid and PEG while the pre-treatment recovered about 76% IgG in the paste.

It is difficult to compare the results obtained in the present study with the purity and yield results found in some of the previous literature (5, 6, 8, 9) since the values were reported as the final values for the process, rather than a single chromatographic step. However, with a yield of 82.1%, with good possibilities for further optimization, this ligand might be worth exploring further for its use in the purification of IgG from plasma or Cohn II+III paste. It is worth mentioning that a buffer containing caprylate would be good to prevent BSA binding on the ligand and hence may increase the yield and/or purity of the product IgG (6, 19). More experiments should also be performed aiming to determine the contents of subclasses, IgG aggregates, IgA, IgM, DNA, RNA, and lipid impurities, as well as the effectiveness of virus removal in order to put this ligand in industrial use for IVIG preparation (1, 5, 6).

5.4 Conclusions

HWRGWV was successfully coupled onto Sepharose CL-4B with a volumetric ligand density twice as high as that on Toyopearl. The changing of the matrix from Toyopearl AF-

Amino 650M to Sepharose CL-4B had no marked influence on the purity of hIgG isolated from cMEM by HWRGWV columns. Comparing between HWRGWV linked on Sepharose and Toyopearl matrices, a lower yield was observed on Sepharose CL-4B when starting material was fed in PBS + 1 M NaCl, but not when fed in PBS. The better performance of HWRGWV-modified Toyopearl matrix with a lower ligand density shows an advantage of a porous medium where a higher degree of cooperativity facilitates the protein binding and isolation. When HWRGWV-Toyopearl was used to separate hIgG from Cohn II+III paste, a yield of 82.1% and a purity of 77.3% were obtained in a single chromatography step. Based on the promising results obtained in this preliminary study, further optimization of chromatographic conditions would be likely to yield enhanced separation performance.

5.5 Acknowledgements

We would like to thank those who helped with the experimental work, Dmitri Moundous for his work on hIgG separation from Cohn II+III paste and Jason Kelly for his effort in analyzing the ethanol content in the paste suspension.

5.6 References

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Table 5-1 Characteristics of the support matrixes Sepharose CL-4B and Toyopearl AF-Amino 650M given by the manufacturers.

Matrix	Sepharose CL-4B	Toyopearl AF-Amino 650M
Exclusion limit, kDa	20,000	5,000
Optimal separation range, kDa	70 – 20,000	N/A
Particle size, μm	45 - 165	40 - 90
Surface area, m^2/mL	/	6.2
Packing pressure, psi	3.6	30
pH stability	2 - 14	2 - 13
Density of HWRGWV, $\mu\text{mol}/\text{mL}$	36.7 ^a	17 ^b

a: experimental data from this work

b: calculated from 0.08 meq/g (given by Peptides International) by assuming that 1 g of dry Toyopearl resin is equivalent to 4.7 mL when fully swollen

Table 5-2 Comparison of purities and yields obtained using peptide ligand HWRGWV immobilized on Sepharose CL-4B and Toyopearl AF-Amino resins under different buffer conditions.

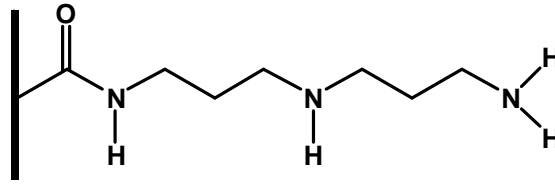
Loading buffer	PBS		PBS+1M NaCl	
	yield	purity	yield	purity
Sepharose CL-4B	64.8	86	63.7	89.4
Toyopearl Amino	51.2	90.9	97.7	94.8

Table 5-3 IgG separation from reconstituted Cohn II+III paste on HWRGWV or acetylated HWRGWV (Ac-HWRGWV) coupled on Toyopearl-AF-Amino resins. Concentrations of the reconstituted paste (C_{paste}), NaCl salt added to PBS in suspension (C_{SS}) and in loading buffer (C_{SL}), as well as the purity and yield of recovered IgG after each run are displayed.

Ligand	Run #	C_{paste} , mg/ml	C_{SS} , M	C_{SL} , M	Yield, %	Purity, %
HWRGWV	1	100	0	1	32.1 ± 1.9	68.5
	2	50	0	1	63.1 ± 2.1	67.3
	3	50	0.5	0.5	59.7 ± 4.9	74.1
	4	25	0.5	0.5	41.2 ± 7.6	85.5
	5	50	0.5	1	69.8 ± 7.2	78.3
	6 ^a	50	0.5	1	47.1 ± 0.8	79.5
	7	50	1	1	58.6 ± 7.8	79.1
	8	25	1	1	31.8 ± 6.5	86.6
Ac-HWRGWV	9	50	0	0	30.2 ± 1.4	75.2 ± 2.7
	10	50	0	0.25	64.1 ± 4.7	69.9 ± 7.0
	11	50	0	0.5	82.1 ± 0.5	77.3 ± 0.5
	12	50	0.25	0.25	57.0 ± 5.2	80.0 ± 0.2

a. Loading was finished at 0.01 mL/min rather than 0.05 mL/min as for all other runs

A



B

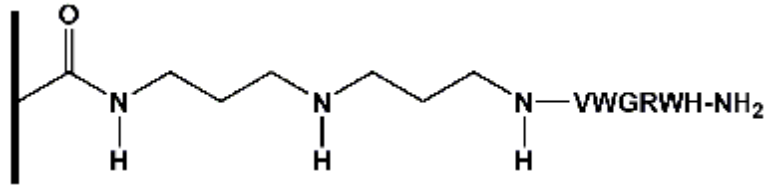


Figure 5-1 Resin structure on Sepharose CL-4B after (A) DADPA coupling and (B) peptide ligand immobilization.

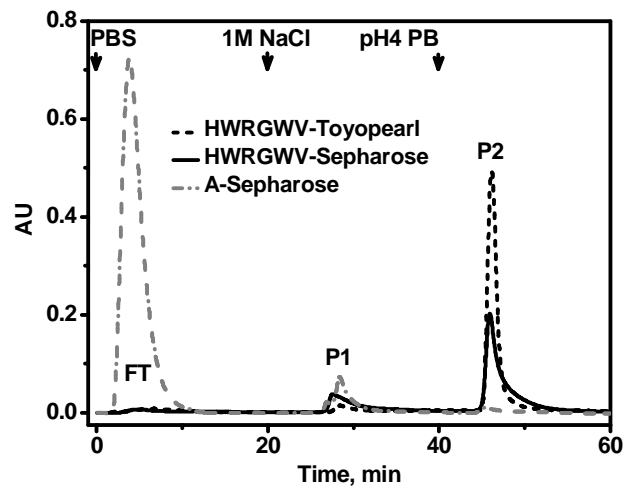


Figure 5-2 Chromatograms of pure hIgG loading on HWRGWV-Sepharose CL-4B (solid line), HWRGWV-Toyopearl (dashed line), and Ala modified Sepharose CL-4B (A-Sepharose, dash-dotted line).

HIgG was loaded in PBS, washed with PBS + 1 M NaCl and eluted by pH 4 PB. FT, P1, and P2 correspond to the flow through, salt wash, and IgG elution peaks, respectively. Column volume: 0.1 mL; sample loop: 0.1 mL; hIgG concentration: 1 mg/mL.

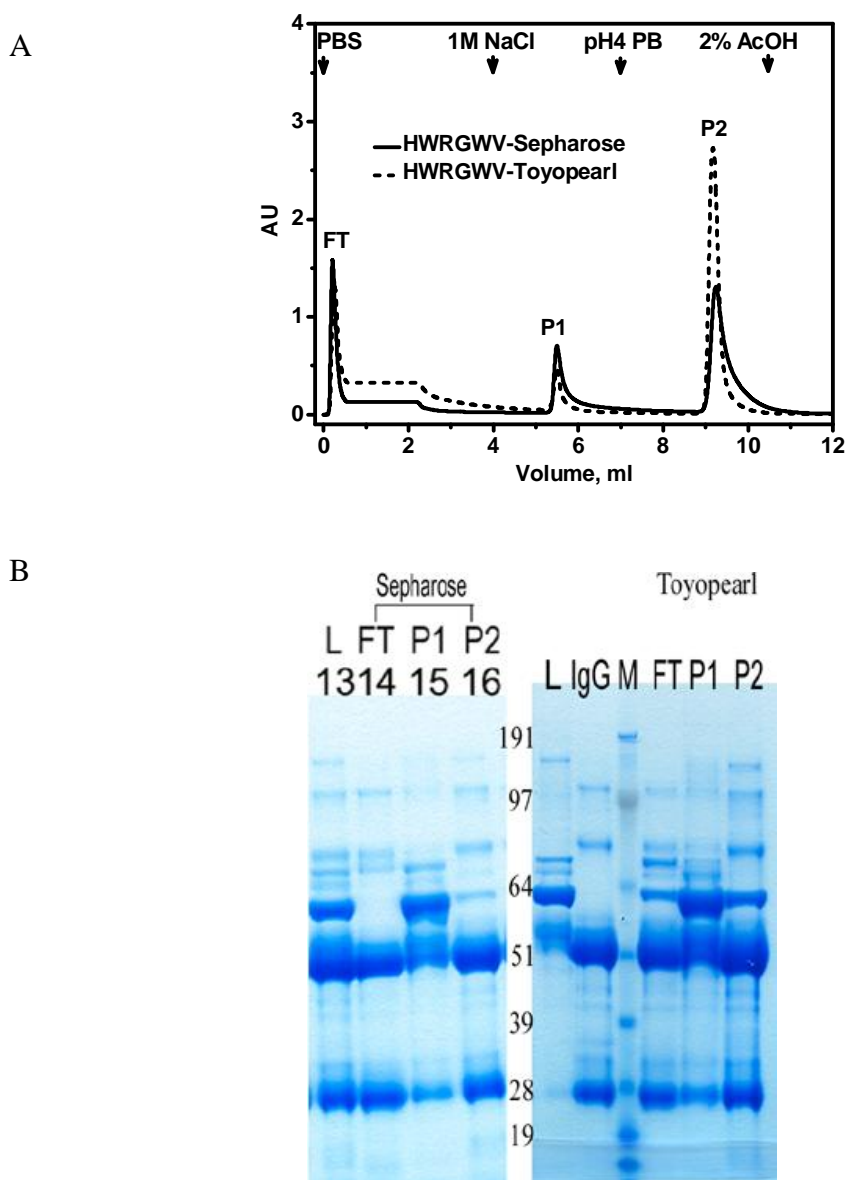
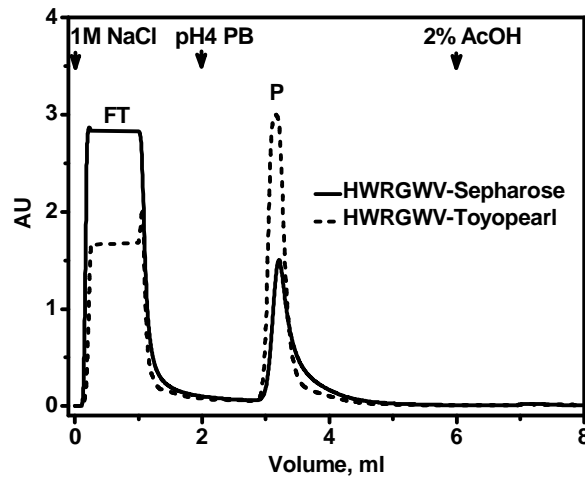


Figure 5-3 Comparison of (A) Chromatograms and (B) SDS-PAGE analyses for IgG isolation from cMEM by HWRGWV immobilized on Sepharose CL-4B (solid line) and Toyopearl AF-Amino 650M (dashed line) resins at PBS loading condition.

A: FT, P1, and P2 refer to the flow through, salt wash, and hIgG elution peaks. hIgG spiked cMEM at the concentration of 10 mg/mL was the starting material (L).

B: The gels were run under reducing conditions and M, IgG, and L stand for molecular marker, hIgG standard, and starting material, respectively. The protein loading amounts in lanes L for Toyopearl and Sepharose were respectively 2.6 μ g and 10.3 μ g, both calculated by hIgG contents.

A



B

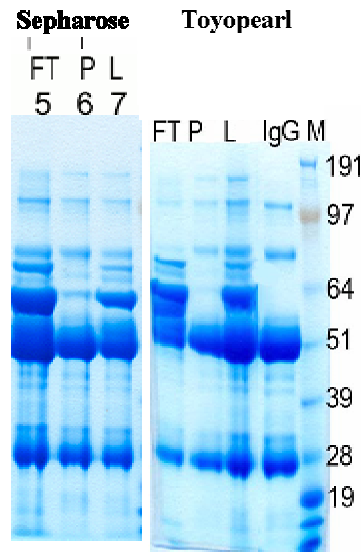
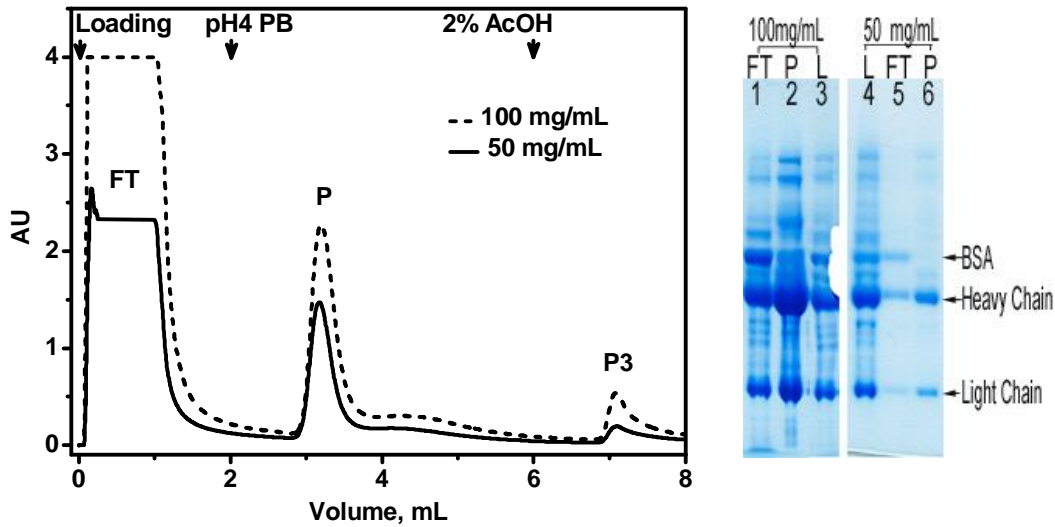


Figure 5-4 Comparison of (A) Chromatograms and (B) SDS-PAGE analyses for IgG isolation from cMEM by HWRGWV immobilized on Sepharose CL-4B (solid line) and Toyopearl AF-Amino 650M (dashed line) resins at PBS + 1 M NaCl loading condition.

A: FT and P refer to the flow through and hIgG elution peaks. hIgG spiked cMEM + 1M NaCl at the concentration of 10 mg/mL were the starting material (L).

B: The gels were run under reducing conditions and M, IgG, and L stand for molecular marker, hIgG standard, and starting material, respectively. The protein loading amount in lanes L was 10.3 μ g, calculated by hIgG content.

A



B

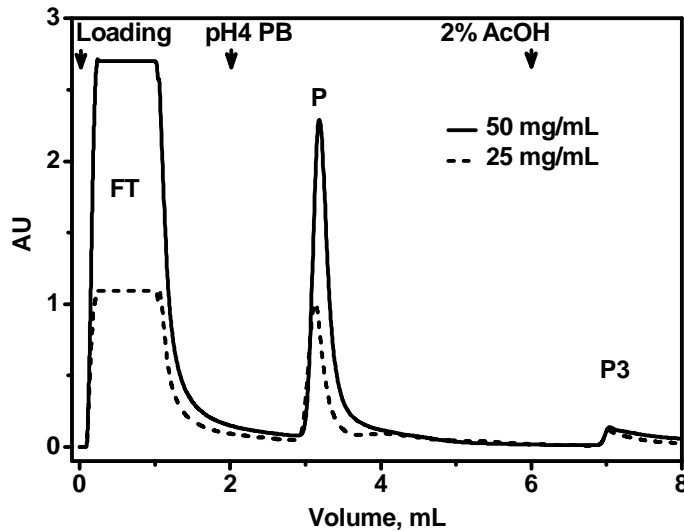
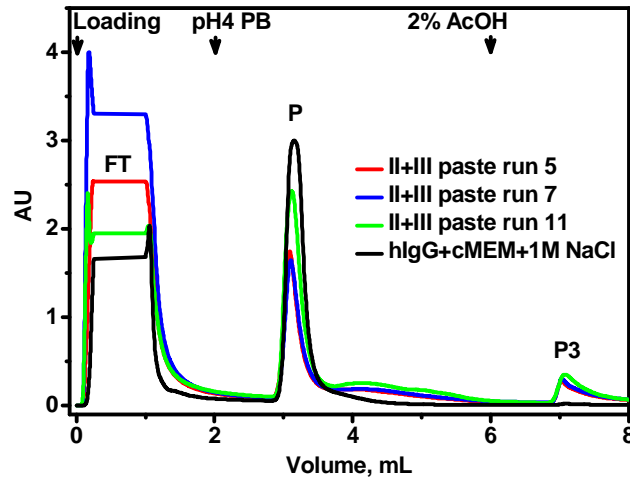


Figure 5-5 II+III paste runs on HWRGWV-Toyopearl at different paste suspension concentrations.

A: The II+III paste suspension at the indicated concentration in PBS was loaded to the HWRGWV-Toyopearl column in PBS + 1M NaCl. The corresponding chromatography runs are 1 and 2 (Table 5-3) for the paste suspension concentrations 100 and 50 mg/mL, respectively. The flow-through (FT) and elution peaks (P) were analyzed by SDS-PAGE (right) where L is the starting material for each run.

B: The II+III paste suspension at the indicated concentration in PBS + 0.5M NaCl was loaded to the HWRGWV-Toyopearl column in PBS + 0.5M NaCl. The corresponding chromatography runs are 3 and 4 (Table 5-3) for the paste suspension concentrations of 50 and 25 mg/mL, respectively. Column volume: 0.1 mL; sample loop: 0.1 mL.

A



B

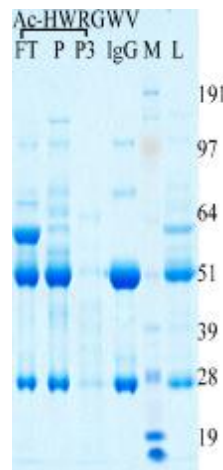


Figure 5-6 Separation of hIgG from II+III paste or cMEM on acetylated or non-acetylated HWRGWV columns at different conditions.

A: Chromatograms of hIgG isolation from cMEM (black) and II+III paste (red, blue, and green lines). The starting materials were injected to the columns in corresponding loading buffer followed by a pH 4 PB elution and a 2% AcOH cleaning. hIgG spiked in cMEM at a concentration of 10 mg/mL (black) was loaded on the HWRGWV-Toyopearl column after its salt concentration was adjusted with NaCl to 1M. For the II+III paste runs, the paste re-suspension and loading buffers are listed in Table 5-3. The II+III paste runs 5 and 7 were carried out on the HWRGWV-Toyopearl column while the run 11 was done on the Ac-HWRGWV-Toyopearl column. FT, P, and P3 are the peaks from the chromatography run corresponding to flow-through, pH 4 PB elution, and 2% AcOH cleaning peaks, respectively. Column volume: 0.1 mL; sample loop: 0.1 mL.

B: The SDS-PAGE gel corresponding to the II+III paste run 11 (Panel A, green). FT, P, and P3 are the chromatography peaks. M is the Seebule pre-stained protein marker and L the starting material. IgG denotes the pure hIgG standard.

Chapter 6. Stability study of Fc-binding peptide resins

6.1 Introduction

In production scale chromatography the column is usually run in over-load conditions and the resin needs to be reused extensively to make it cost-effective.. As a result, column lifetime is one important feature in process design and process cost. During multiple uses of a chromatographic medium, protein, nucleic acid or lipid build-up, ligand loss, and degradation of the matrix support can diminish the capacity of a resin and lead to a decline in performance. It has been suggested (1), through experiments on strong ion exchange media, that the column lifetime depends more on the degree of column fouling and the ease to clean the medium than the chemical stability of the matrix. Fouling occurs when remnants in a column accumulate and may partially block bead pores, prevent target proteins from binding to ligands by occupying the active sites, or reduce the pore size or void volume by depositing in and/or between the particles. These fouling effects can reduce the capacity, and sometimes the selectivity, of the ligand, and increase the pressure drop of the column. The effects of column fouling on ion exchange resins (2-4), column cleaning and ligand leakage of Protein A and Protein G (5-7), as well as resin storage for peptide affinity media (8) have been studied to evaluate the resin lifetime in preparative chromatography mode. From those studies, it was concluded that fouling could be eliminated by filtrating the loading source [PRETREATING WITH WHAT?], using guard columns, applying different loading strategies and employing appropriate column cleaning steps. Among them, a proper column cleaning and regeneration procedure is critical to prevent the fouling problem and maintain column performance. Column cleaning methods depend on the chemical compatibility of the ligands and can enhance the resin storage stability.

Short peptides as affinity ligands have gained great interest (9-13) due to their unique advantages of being more stable, less immunogenic, less expensive, and having less stringent elution conditions than protein ligands. However, only a few studies (8, 11, 12, 14, 15) have been identified in the literature examining the stability of peptide ligands under process conditions. Most of the studies were focused on the number of injection cycles and no more

than 15 cycles were carried out on the tested columns, except for Kaufman et al. (15), who found that over 180 injections of Cohn fraction I paste suspension could be employed on the column packed with FLLVPL-resin for fibrinogen purification without any loss of column performance.

This chapter describes a run-storage cycle experiment to investigate the performance stability of three columns packed with the Fc-binding hexamer peptide resins HWRGWV, HYFKFD, and HFRRHL (13). The number of theoretical plates and asymmetry factors were measured periodically to monitor the column integrity. The yields and purities of hIgG isolated from cMEM at a small scale preparative mode were calculated for each cycle to determine the separation efficiency of the columns.

6.2 Materials and Methods

6.2.1 Materials

Peptide resins HWRGWV, HFRRHL, and HYFKFD at ligand densities of around 0.1 meq/g were synthesized directly onto Toyopearl AF-Amino-650 M (particle size 65 μ m) (Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY). Empty PEEK-lined Omega columns with a volume of 30 mm \times 2.1 mm (ID) (0.1 mL) were from Upchurch (Oak Harbor, WA). MicroCon YM-3 regenerated cellulose filters with MWCO of 3,000 were obtained from Millipore (Billerica, MA). NuPAGE Novex Bis-Tris gels, NuPAGE MOPS Running Buffer, NuPAGE LDS Sample Buffer, NuPAGE Reducing Agent, Seebue plus2 pre-stained molecular weight marker, and SimpleBlue SafeStain were all from Invitrogen (Carlsbad, CA). All the following products were purchased from Sigma (St. Louis, MO): phosphate buffered saline (PBS) of pH 7.4, human IgG (hIgG), sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate, glacial acetic acid, and chromatography-grade methanol. Cell culture media EMEM was from Quality Biologicals (Gaithersburg, MD). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT) and Becton Dickinson (Sparks, MD), respectively. Human IgG ELISA kit was from Alpha Diagnostic International (San Antonio, TX). Waters 626 LC system and 2487 UV detector were all from Waters (Milford, MA).

6.2.2 Run and storage cycle for resin stability

A run-storage cycle experiment was designed where the resin storage time for each cycle was increased while keeping the chromatographic runs the same in each cycle. On day 1 of the experiment, one specific peptide was packed and conditioned in the way described in section 6.2.3. Four successive IgG separation runs were performed under the conditions described in section 6.2.4 followed by three acetone injections to check the column efficiency (section 6.2.5). The column was then rinsed with 20% methanol and stored in the same solution for 5 days at 4°C. A 20% aqueous solution of methanol was used for resin storage to keep the media from microbial growth. On day 7, four hIgG separation runs and three acetone injections were carried out again before the column was stored for 9 days under the same conditions described above. The procedure was continued in this manner, with the same injections as those on day 7 being conducted on days 17, 36, 63, 90, 155 and the resins being stored in 20% methanol between the days of injection experiments. Each peptide column was tested at different dates but with the same cycle plan until a significant drop in yield was observed. Typically, flow-through and IgG elution peaks of the second and fourth hIgG purification runs were collected on each day for ELISA and SDS-PAGE analyses.

6.2.3 Column preparation

The tested ligands HWRGWV, HYFKFD, and HFRRHL were based on Toyopearl AF-Amino-650M with the peptide density of around 0.1 meq/g. The peptides were synthesized from their C-termini to N-termini on the matrix and the free amines remaining on the base matrix after peptide synthesis were capped with acetyl groups. All three resins were dry-packed individually in 30 mm × 2.1 mm (ID) Omega columns and swelled with 20% methanol. Columns were equilibrated with loading buffer (PBS + 1 M NaCl) before sample injections.

6.2.4 Chromatographic conditions

A high-salt loading condition, i.e. the starting material loaded in PBS with 1M NaCl, was employed in the hIgG separation experiments. The starting material was hIgG-spiked cMEM, where cMEM was formed by combining Minimum Essential Medium (EMEM) with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB). The concentration of

hIgG in cMEM was 10 mg/mL and the salt concentration was adjusted to 1 M by NaCl. The starting material of 0.1 mL was injected into the column at a 0.05 mL/min flow rate for 5 minutes. The flow rate was then increased to 0.2 mL/min for the remaining steps. After sample loading, the column was sequentially washed with 2 mL loading buffer, 4 mL of 0.2M phosphate buffer (PB) at pH 4.0 to elute hIgG, 4 mL of 2% acetic acid in water for column cleaning, and then was re-equilibrate with loading buffer for the next injection. All runs were performed on a Waters 626 LC system with a 2487 UV detector and the effluent was monitored by absorbance at 280 nm.

6.2.5 Column efficiency

The packed column performance was checked using residence time distribution (RTD) analysis. This entailed inspecting the columns for possible deterioration in bed quality using injections of 0.02 mL 5% (v/v) acetone in water at a flow rate of 0.2 mL/min where the effluent concentration was monitored by absorbance at 280 nm. The two factors of bed quality monitored in this experiment were the number of theoretical plates (N), and the asymmetry factor (A_s), calculated with the following equations (16),

$$N = 5.54(V_R/\omega_{1/2})^2 \quad (\text{Eq. 6-1})$$

$$A_s = b/a \quad (\text{Eq. 6-2})$$

Here V_R is the retention time in minutes, $\omega_{1/2}$ is the recorded peak width at half of the peak height in minutes, and a and b are the first and second half-peak widths at 10% peak height, respectively.

6.2.6 Sample analysis for yields and purities

The protein profiles of both flow-through and eluates from hIgG purification runs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA) for purity and yield calculations in the same way as described in Chapter 4.

6.2.7 Unpacked resin examination for ligand change and bead morphology change

The peptide columns were unpacked after the termination of the stability experiment. Used resins were either sent to Peptides International for amino acid analysis by HCl hydrolysis or examined under an Olympus CKX41 microscope for bead morphology changes.

6.3 Results and Discussion

6.3.1 Column efficiency

Residence time distribution (RTD) analysis is often used to characterize and monitor the packing efficiency and column performance (17-20). The number of theoretical plates (N) and asymmetry factor (A_s) can be analyzed under analytical chromatographic conditions using small molecule inert tracers. A large N and an A_s of 1 are desired for efficient column performance. Acetone (5% by volume) was utilized in this work for the RTD analysis by assuming a Gaussian peak profile and typical results are shown in Figure 6-1. Since the same column geometry was utilized for all ligands which had the same solid support, these RTD factors were directly compared without any normalization. All three peptide resins showed a slight decreasing trend of N over the course of the experiment (Figure 6-1, A), where the HYFKFD column was terminated after 66 days experiment because of the significant drop in yield while the other two ligand columns were kept being examined for 210 days. The drop in N was insignificant for all the three columns, suggesting that no notable integrity breaches in columns were detected after 210 days (66 days for HYFKFD) of run and storage cycles.

Figure 6-1B shows the asymmetry factor calculated for each day of runs throughout the experiment. The asymmetry factor (A_s) determined for the HWRGWV column in this experiment was about 1.8, larger than these of HFRRHL and HYFKFD which maintained constant at 1. The large A_s may indicate the presence of a large headspace, void spaces, or channels resulting in the tailing of the pulsed-input peak. However, because of the more significant extra-column effects for short columns (21, 22), A_s values between 0.8 and 1.8 are still acceptable. The HWRGWV column was therefore utilized without repacking. It is shown in Figure 6-1B that the change in A_s was insignificant over the duration of the experiment except for the increase in A_s observed on day 210 for the HWRGWV column.

The cause of this increase is not known. Nevertheless, the overall low variation in A_s and N indicates the packing produced a stable bed configuration, at least, in the first 100 days of the experiment.

6.3.2 Chromatograms and SDS-PAGE analysis gels

Relatively similar chromatogram and corresponding SDS-PAGE profiles, as well as their trends of change over the course of experiments, were noticed for the three ligand columns when used for hIgG isolation from cMEM. Taking the HWRGWV column as an example, the chromatograms are composed of a flow-through peak (FT) and a hIgG elution peak (P) on both day 1 and day 170 (Figure 6-2) where the FT peak is a result of flow-through protein not bound by the column during the loading and washing steps and the P peak represents the elution step with pH 4 phosphate buffer. As evident in Figure 6-2 that larger FT and smaller P were observed on the day corresponding to the end of the experiment than on day 1. BSA is the major impurity in the starting material and it can be seen in the SDS-PAGE gel at 65 kDa as monomers and at about 130 kDa as dimers (Figure 6-3A, lane 7). On day 1, the majority of protein in the FT was BSA (at about 64 kDa) (Figure 6-3A, lanes 1 and 3) and hIgG was the dominant component in the P samples with a little impurity BSA (lanes 2 and 4). On day 170 noticeably more hIgG, but similar amount of other proteins, were evident in the FT bands (Figure 6-3B, lanes 4 and 6),. The P samples (Figure 6-3B, lanes 5 and 7) seemed to have higher purity of IgG when compared to those on day 1, which might be due to the lower recovery on day 170 and therefore a lower total amount of protein loaded on the gel. The IgG elution peaks of the separation runs from the three columns were subjected to ELISA and SDS-PAGE analyses for yield and purity assessment as to be discussed in detail in the following sections.

6.3.3 Yield change over time

The yields calculated by ELISA analyses of the elution peaks are shown in Figure 6-4 as a function of time beginning from the first-day runs on the three columns. Each data point represents the average of two runs generated on the same day. HWRGWV had the highest yield among the three ligands, about 25% higher than those on HFRRHL, and HYFKFD had the lowest over the duration of the experiment. The yield observed on day 1

with HWRGWV (82%) was lower than those from previous experiments (95%, Chapter 4), which might be due to the packing of the column used in this experiment, whose asymmetry factor was 1.8 while a maximum A_s value of 1.3 was determined for the columns packed in previous separation experiments.

The analysis of the data presented in Figure 6-4 shows an overall decreasing trend of the yield for hIgG separation from cMEM using all three peptide ligands. The yield of the HYFKFD resin dropped more radically than the other two ligand columns, lowered by almost 50% over a 66-day period with 20 hIgG-spiked cMEM injections, while only 15% of yield decrease was observed in a period of 90 days with 24 separation runs for both HFRRHL and HWRGWV resins. The rapidly decreasing yield on HYFKFD eliminated this ligand from the stability studies. The resin was dismissed after 66 days of testing and subjected to amino acid analysis by peptide hydrolysis with HCl. The ratio among the amino acids in HYFKFD were still H:Y:F:K:D = 1:1:2:1:1, the same as the original residue composition of the ligand. Even though peptide hydrolysis cannot distinguish the alteration of amino acids, this result indicates that there had been no detectable peptide bond breakage between amino acids of the sequence. However, this shed little light on ligand loss/alteration during the separation and storage practice. Hence, the other two resins encompassed in this work were not subject to the same ligand hydrolysis analysis after the experiment was terminated.

For both HFRRHL and HWRGWV ligands, the yield decreased by 15% in the first 90 days with 24 runs and the longest single storage period of 26 days (Figure 6-4). The back-pressure on the columns was around 40 psi in the first 90 days of the experiment. A much more dramatic yield drop was observed on day 155 for both ligands, decreasing by 40% from day 90 to day 155 during which the resins were kept at 4°C in 20% methanol. Higher pressure drop (approximately 80-90 psi) during operation was also noticed on day 155 than on preceding days. When these two columns were connected back onto the HPLC on day 170 after 14 days of storage, it was found that the pressure drop of the columns was as high as 80-90 psi, while the yields on HFRRHL and HWRGWV were further dropped to 20% and 15%, respectively. After another 39-day storage, no further change was observed in either back-pressure or yield with these two resins. These observations indicate that the resins

were damaged. There are several factors that can cause damage to resins: bead crushing, resin fouling, ligand leakage, ligand alteration/degradation or any combination of them. The first two factors could cause back pressure build-up. Our problem could be the result of the first two or the combination of the first two with other effects. Visual observation of the beads under a microscope after unpacking did not indicate any gross morphological change in bead size or shape, nor particle breakage (Figure 6-5). Therefore, fouling is more likely the main reason, especially when increased back-pressure of the column is accompanied by a yield drop of more than 70%.

The severe fouling found on day 150 for the HWRGWV column was not likely just due to the separation runs carried out on the column since only 24 hIgG separation injections have been conducted. HWRGWV was found to be able to retain both the yield and purity for 40 separation runs with a shorter storage period (less than 15 days) at 4°C in 20% methanol. It is highly possible that the fouling in the two columns is due to the longer storage interval (69 days) between the cycles of day 90 and day 155. Even though 20% solution of a short-chain alcohol is mild to proteins, the prolonged incubation could turn proteins into fully denatured configuration (23, 24). The fully denatured proteins could be then retained on the resins so tightly that their removal with 2% AcOH became impossible, which in turn increased the fouling problem by interacting with other components in the feed at next running cycle. This also explains the similar degree of decrease in yield on day 170 after a 14-day storage. Additional cleaning steps prior to storage seem to be necessary for these resins to prevent the fouling problem.

6.3.4 Purity change over time

The purities of isolated hIgG from the three peptide ligands were very similar, ranging between about 85 to 95%, and displayed little change over the test time of 210 days (66 days for HYFKFD) (Figure 6-6). The consistently high purity means that the selectivity to hIgG of the resins was retained over the run and storage period. The decreased capacity, but not the selectivity, along with the increased back pressure might be very likely attributed to the fouling of the columns, even though the ligand cleavage from the solid supports cannot be completely excluded.

In this study, HFRRHL and HWRGWV displayed similar trends over the duration of the test. A much more rapid drop in yield but the similar changes in purity and RTD analysis were found on the HYFKFD column. The quick deterioration exhibited by HYFKFD might be related to the peptide characteristics since all the three ligands had the same solid support and were synthesized directly onto the matrix from the C-termini using the same chemistry. Ligand HYFKFD is more hydrophobic at the N-terminal end with two aromatic amino acids in presence while the other two ligands have only one. The more hydrophobic N-terminal may have stronger retention for some impurities which cannot be eluted by 2% AcOH and finally causes severe fouling problems.

Column fouling should be greatly reduced by employing good column cleaning approaches as usually done for chromatography in industry since 2% AcOH is a very weak solvent for column cleaning. Chaotropic agents such as guanidine hydrochloride, guanidinium thiocyanate, and urea of different concentration in solutions of various pH values (5, 15), pepsin (1), 0.1 – 1 M sodium hydroxide (6, 7, 25), among others, have been utilized for column cleaning to remove tightly bound species after chromatographic operations. Instead of using 2% AcOH as the final cleaning agent, more stringent solvents as listed above should be applied to the column every several runs and before the storage. This should improve the storage stability of the media and maintain the column performance. Alternative solvents to 20% methanol for storage, such as sodium azide at different pH values, might help to eliminate the protein denaturation and in turn reduce the fouling of the column.

There were three prior contributions identified in the literature that investigated the stability of IgG-binding small peptide ligands under preparative chromatography conditions. Peptide ligand D-PAM, (Arg-Thr-Tyr)₄-Lys₂-Lys-Gly with all amino acids in D-configuration, could be repeatedly used for at least 15 cycles (8) and N-benzyloxycarbonyl-L-tyrosine did not show any yield reduction after 10 injections (11), where low pH (below 2.3) was employed as the final wash of the column in both cases. More stringent cleaning cycles using 0.5 M NaOH were applied by Bellofiore et al. (12) on linear peptides of 22-mer M[46-2] (AEGEFINVPMMVDGITMGDPAK) and 10-mer m[9-18] (PMMVDGITMG) immobilized on Sepharose Fast Flow via the N-terminal amine groups. They found that the

capacity of the resins was maintained for at least 9 cycles after 5-10% ligand leakage with the first NaOH treatment. The resistance of m[9-18] to the enzymatic hydrolysis of trypsin and α -chymotrypsin up to 2 hours was confirmed in batch formats at room temperature. With shorter storage periods, HWRGWV has been found to be able to handle 40 separation cycles without remarkable loss in yield and purity under the conditions of the current work. We did not separately perform the proteolytic resistance studies on the Fc-binding peptides. However, we had an HWRGWV column running with trypsin, pepsin, as well as endoproteinases Lys-C and Glu-C, containing feed streams under the conditions to be described in Chapter 7. Thirty cycles with 2 minutes residence time for each cycle were conducted without noticeable change in chromatograms and hIgG binding ability. Therefore, compared to the peptide ligands in the literature, HWRGWV exhibited comparable stability for preparative chromatographic isolation of IgG.

6.4 Conclusions

Three Fc-binding peptide columns were examined for their stability by reporting the purity, yield and RTD analysis. HWRGWV and HFRRHL maintained constancy of these parameters in the first 90 days with the longest single storage period of 26 days. A 64-day storage of resin in 20% methanol after 24 hIgG separation runs resulted in a dramatic decrease in yield and sudden increase in column back-pressure while other parameters were maintained. HYFKFD experienced a quick yield drop in 66 days of experiment, while purity and RTD analysis showed little change. The yield drop on the three columns could most likely be ascribed to the fouling of the media by adsorbed proteins that are not able to be eluted by 2% AcOH, the cleaning agent used in this work. Prolonged storage of the media in 20% methanol after their usage for IgG purification without special column regeneration made the problem more profound. A good column cleaning and sanitization procedure using more stringent than 2% AcOH agents should be able to enhance the column lifetime. Other than the fouling, HFRRHL and HWRGWV or at least the latter are stable under the process conditions applied in this study.

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6.6 References

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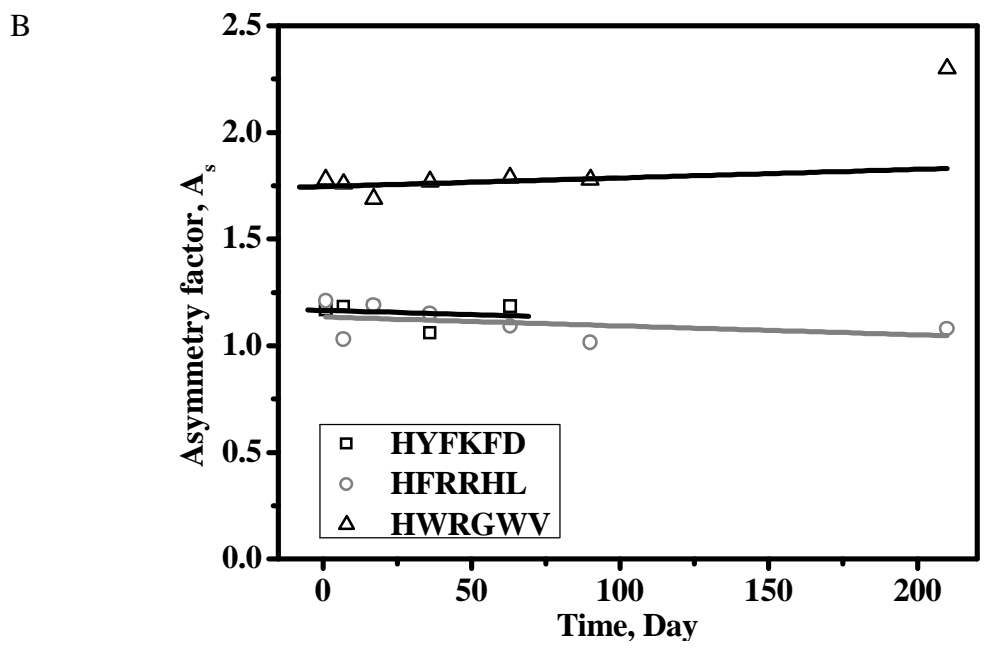
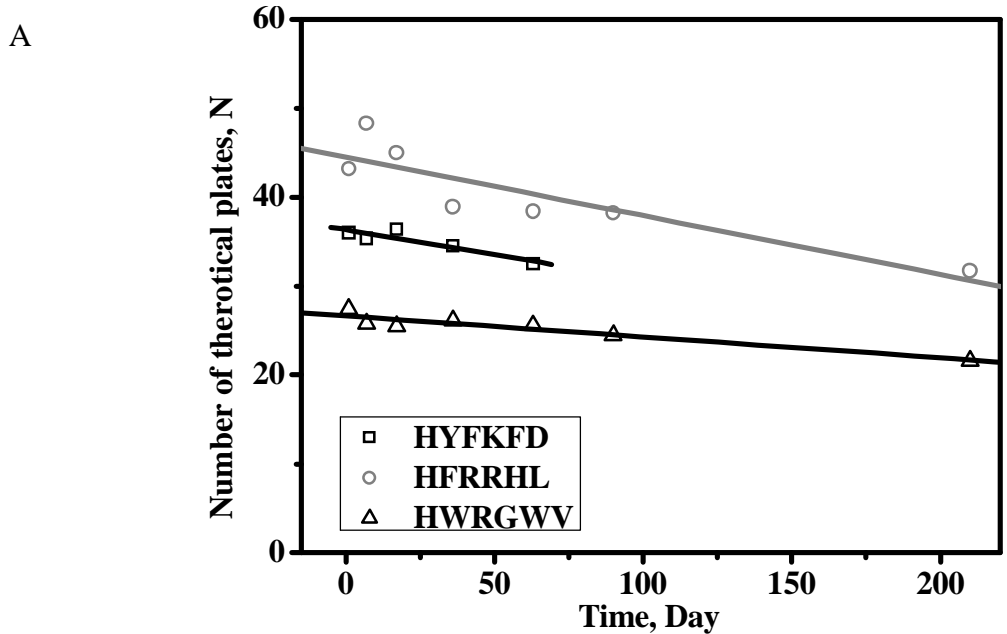


Figure 6-1 (A) Number of theoretical plates and (B) asymmetry factor trends for all resins over the time of the experiment.

The experimental points (symbols) were linearly fitted (lines) to better see the trends.

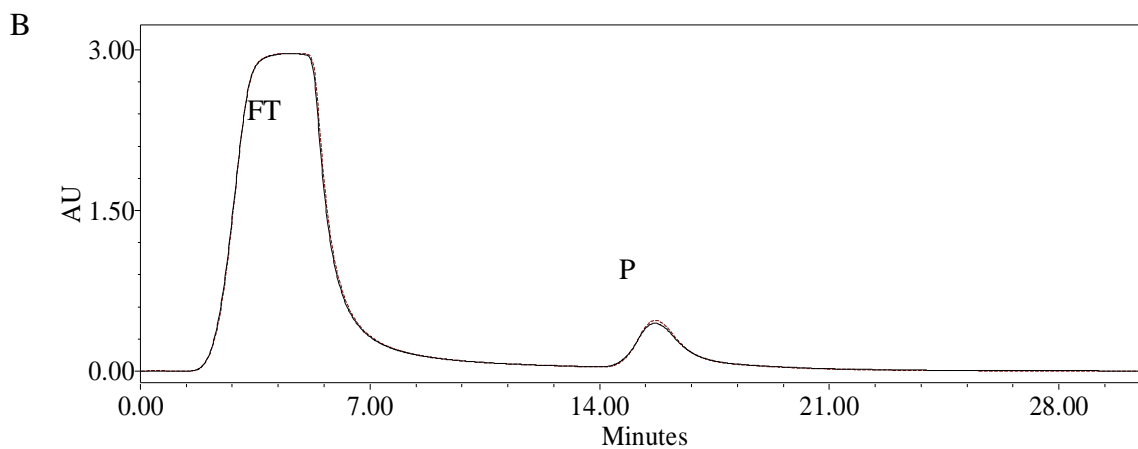
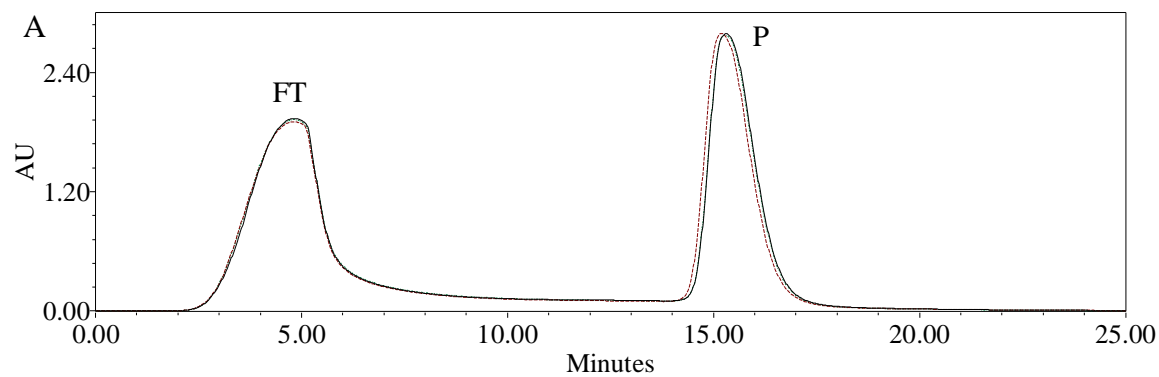
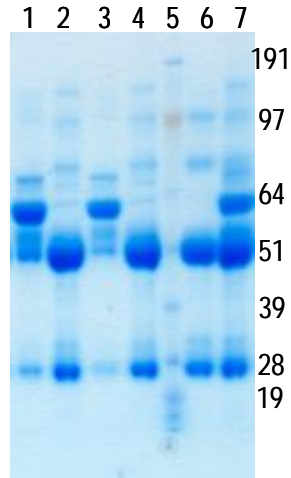


Figure 6-2 Duplicate chromatograms from HWRGVW on (A) day 1 and (B) day 170. The first peak is the flow-through (FT) and the second (P) the IgG elution peak by 0.2 M phosphate buffer at pH 4. Sample injection volume was 100 μ L and the initial hIgG concentration in cMEM was 10 mg/mL.

A



B

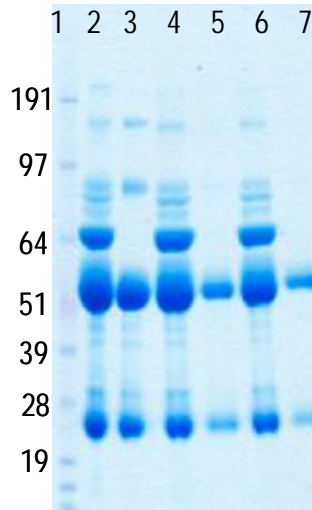


Figure 6-3 SDS-PAGE gels of the peaks from hIgG separation runs from HWRGWV on (A) day 1 and (B) day 170.

A: The gel is corresponding to the chromatograms in Figure 6-2A and was run under reduced condition. Lanes 1 and 3: FT; lanes 2 and 4: P; lane 5: MW marker; lane 6: pure hIgG; lane 7: starting material hIgG-spiked cMEM.

B: The gel is corresponding to the chromatograms in Figure 6-2B and was run under reduced condition. Lane 1: MW marker; lane 2: starting material; lane 3: pure hIgG; lanes 4 and 6: FT; lanes 5 and 7: P.

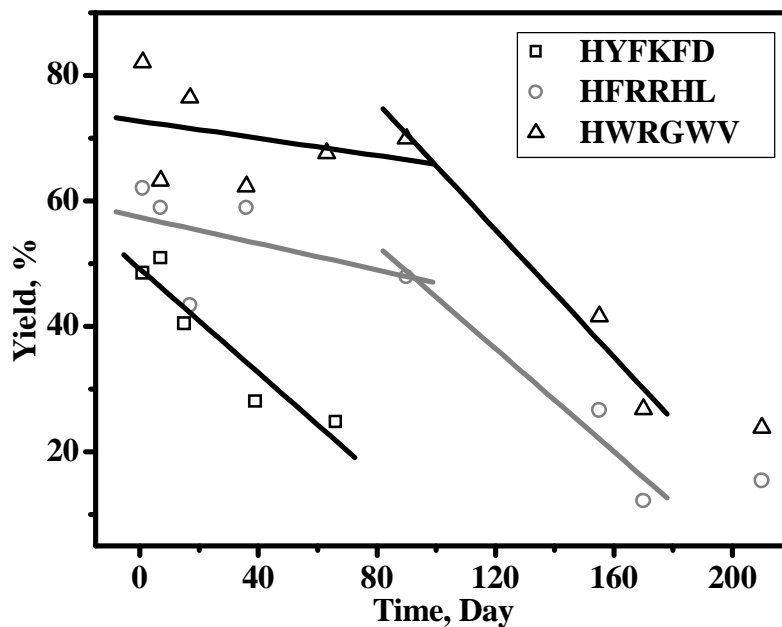


Figure 6-4 Yield trends over the course of the experiment for ligands HWRGWV, HYFKFD, and HFRRHL calculated by ELISA. The experimental points (symbols) in segment were linearly fitted (lines) to better see the trends.

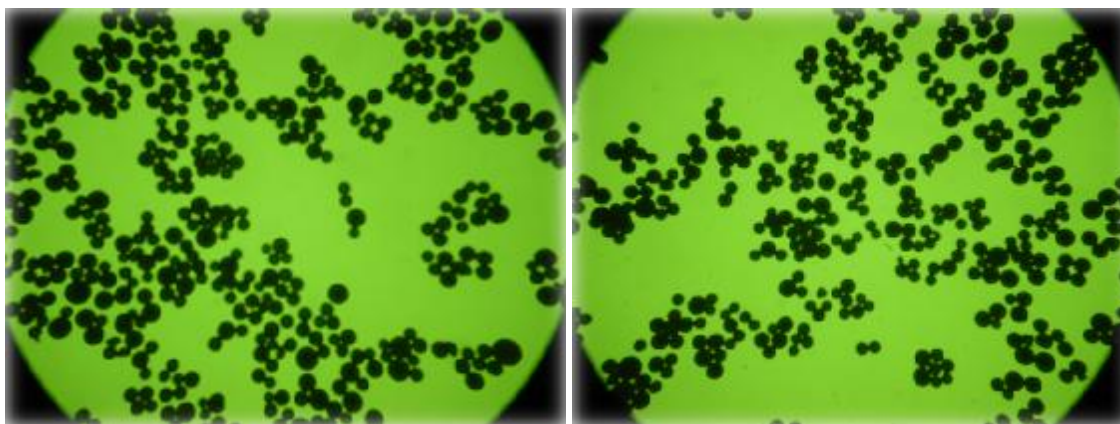


Figure 6-5 HWRGWV beads before (left) and after (right) the stability study under 200× magnification.

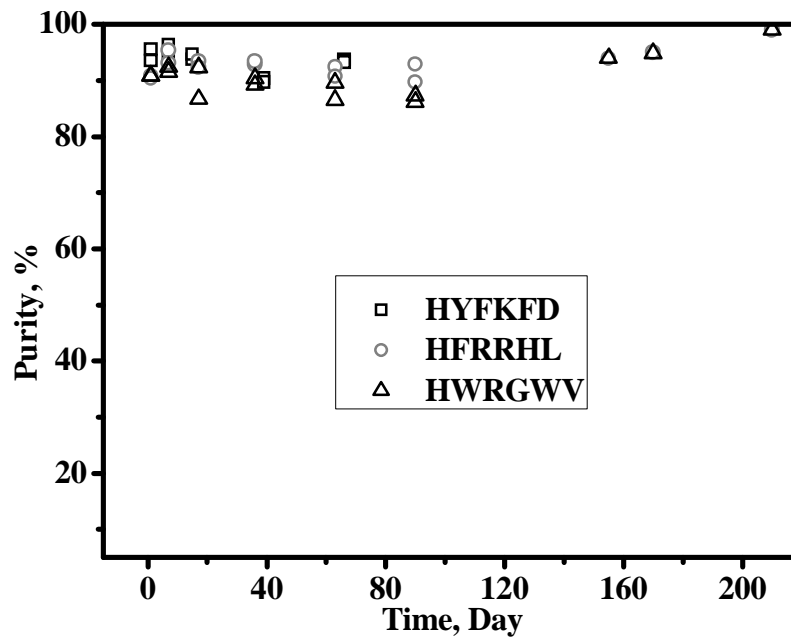


Figure 6-6 Isolated hIgG purity change over time for ligands HWRGWV, HYFKFD, and HFRRHL estimated by densitometry.

Chapter 7. Interactions between human immunoglobulin G and the affinity ligand HWRGWV

7.1 Introduction

Human immunoglobulin G (hIgG, IgG) (Figure 7-1) is comprised of two identical heavy (H) chains with a molecular weight of 50 kDa and two 25 kDa identical light (L) chains which are connected together by disulfide bonds (1). Each polypeptide chain is constituted of several domains that are formed by antiparallel strands (2) and have about 100 amino acid residues. Starting from the amino termini, the L chains include the V_L and C_L domains while the H chains encompass the V_H , C_{H1} , hinge, C_{H2} , and C_{H3} regions. The “V” label indicates a variable region that exhibits striking differences in amino acid composition within the same class of immunoglobulins, while the “C” label denotes a constant region where the amino acid composition is relatively invariant within the same class of immunoglobulins. The C_{H1} and V domains of both the heavy and light chains together comprise the antigen-binding Fab portion which, together with the hinge region, constitutes the $F(ab')_2$ fragment as shown in Figure 7-1. The hinge, C_{H2} , and C_{H3} domains constitute the Fc fragment region, and without the hinge region the fragment is identified as Fc' . The Fc' fragment can be further reduced by pepsin digestion to a pFc fragment (Lys334 – Lys447) consisting of only one C_{H3} domain (2). HIgG is a glycoprotein with hydrocarbons attached at Arg297 in the C_{H2} domain (Figure 7-1) The degree of glycosylation affects the binding of certain proteins to the hinge and C_{H2} domains, such as the binding of Fc receptors ($Fc\gamma R$ s) and complement protein C1q (3). However, it has little influence on antigen binding and the binding in the C_{H3} domain (3). The amino acid sequence of hIgG1 H chain (PDB code: 2IG2) is shown in Figure 7-2 with the domains indicated by arrowed lines.

Human IgG consists of four subclasses: IgG1, 2, 3, and 4 (4). Their Fc portions share a 95% amino acid sequence homology. The main differences between these Fc portions are in the lengths, the numbers of disulfide bonds, and the amino acid compositions of their hinge regions. This hinge region variance results in differences in binding to $Fc\gamma R$ s between the subclasses (5). IgG1, 2, and 4 can bind Protein A while IgG3 cannot because its residue 435 is not a histidine which is necessary for Protein A binding (6, 7).

Two consensus binding sites exist on the Fc portion of IgG, the hinge proximal region of the C_{H2} domain for Fc γ R_s binding and the junction of the C_{H2} and C_{H3} domains where Protein A, Protein G, neonatal Fc receptor (FcRn), and rheumatoid factor are retained (8). The degree of antibody glycosylation influences the binding at the hinge proximal site but has little influence on interactions at the inter-C_{H2}-C_{H3} binding site. The existence of both the C_{H2} and C_{H3} domains is necessary for the binding at the inter-C_{H2}-C_{H3} binding site (9). The binding of the receptor at the hinge proximal site is usually monovalent while binding at the junction of the C_{H2} and C_{H3} domains allows for bivalency (3). For both consensus binding sites, most of the amino acids that are in contact with the ligand molecules upon binding are located in the loops of these regions. The flexibility of the loop structures is considered an important factor for the consensus binding sites to interact with a variety of different molecules (10, 11).

Small peptides designed for drug applications or for the capture and purification of IgG have been found to have the ability to mimic the binding specificity of large proteins. Some of them have been found to interact with IgG at these two binding sites. The cyclic Fc-III peptide (DCAWHLGELVWCT) (10), identified by screening a phage display library for binding to Fc, was found to compete effectively with Protein A. Crystallography studies showed that the peptide targeted the C_{H2}-C_{H3} interface of Fc. The cyclic tripeptide (CFHH)₂KG, selected against mouse IgG and named FcRM, resembled the loop structure of Fc γ R_{III} in binding the lower hinge region (Pro230 – Ser239) of the Fc fragment as determined by nuclear magnetic resonance (NMR) (12), despite the fact that, according to the chromatographic data, this peptide also displayed a second binding site localized in the Fab portion of mouse IgG. Linear peptides with sequences taken from the loop residues of the Fc receptor for bovine IgG2 were tested and modified, with the minimum sequence Phe82 – Val85 (FIGV) finally identified for its ability to inhibit the binding of the receptor to bovine IgG2 (13). A synthesized peptide whose sequence represented the segment Thr256 – Pro 271 in the C_{H2} domain of human IgG1 was able to compete with hIgG in binding to the Fc γ R_{IIa} receptor (14) and displayed some of the functions associated with whole hIgG molecules (15). Although the exact binding sites on the proteins for some of these peptides were not

identified the concept that a small peptide can play the same role as large proteins in specific protein-protein interactions is clear.

Mutagenesis (16-18) and crystallography (7, 19-22) are two powerful but arduous techniques employed in revealing the interactions involving IgG. Other methods such as NMR (23), digestion coupled with amino acid sequencing (24), and binding inhibition (13, 14), among others, have also been applied to locate the sites of interactions between proteins and ligands.

As described in previous chapters of this thesis, the hexamer ligand HWRGWV has the ability to specifically retain the Fc fragment of hIgG, but it does not bind to the Fab or F(ab')₂ fragments. It also is able to selectively bind to hIgG from a complex protein mixture, it binds to all four human IgG subclasses, as well as IgGs from a wide range of animal sources. This chapter describes efforts to determine the possible interaction site of the Fc-binding peptide HWRGWV on hIgG. Several approaches were taken in making this determination. First, hIgG was deglycosylated to see if the binding of IgG to the peptide was affected because it binds near the hinge region. Second, the binding of hIgG to the peptide column was investigated in the presence of Protein A or Protein G to determine if the peptide competes with these proteins for binding to the IgG molecule. If so, this would indicate that the peptide binds to the Protein A and Protein G binding sites described previously. Finally, enzyme digests of hIgG were adsorbed to HWRGWV columns and the resulting peptides were sequenced by mass spectrometry (MS). These results suggest that the HWRGWV peptide binds to a small loop (Ser383 – Asn389) localized in the pFc fragment of hIgG. The loop is composed of a sequence of seven amino acids (SNGQPEN) that have great possibility to interact with the amino acids of the peptide ligand.

7.2 Experimental

7.2.1 Materials

Peptide resin HWRGWV at a ligand density of 0.08 meq/g was synthesized directly on Toyopearl AF-Amino-650 M (average particle size 65 μ m) (Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY). Soluble Protein A from *Staphylococcus aureus* and

recombinant Protein G were purchased from Rockland (Gilbertsville, PA) and GE Healthcare (Piscataway, NJ), respectively. ECL Plus Western blotting detection reagents were also from GE Healthcare. Endoglycosidase peptide:N-glycosidase F (PNGase F) was obtained from New England Biolabs, Inc. (Ipswich, MA). Phosphate buffered saline of pH 7.4, human IgG, pepsin, endoproteinase Lys, endoproteinase Gly, Tris-buffered saline with 0.1% Tween 20 (TBST), sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate, glacial acetic acid, trifluoroacetic acid (TFA), and formic acid were purchased from Sigma (St. Louis, MO). Fc fragment of hIgG was obtained from Calbiochem (San Diego, CA). NuPAGE Novex Bis-Tris gels, 4 - 20% Tris-Glycin gels, NuPAGE MOPS running buffer, NuPAGE MES running buffer, Tris-Glycin SDS running buffer, NuPAGE LDS Sample Buffer, Tris-Glycin SDS sample buffer, NuPAGE Reducing Agent, Seebue plus2 pre-stained molecular weight marker, Mark 12 non-staining protein marker, pre-stained multi-colored standard, Invitrolon PVDF membrane, Silverstain Express silver staining kit, SimpleBlue SafeStain, and WesternBreeze Chromogenic Western blot immunodetection kit were all from Invitrogen (Carlsbad, CA). Biotinylated Galanthus Nivalis lectin (GNL) and peroxidase-labeled streptavidin were from Vector Laboratories (Burlingame, CA). Kodak Biomax MR autoradiography film was purchased from Fisher (Atlanta, GA). MicroCon YM-3 filter (regenerated cellulose, 3,000 MWCO) and Durapore 0.22 µm filter were purchased from Millipore (Billerica, MA). (Savannah, GA). A Protein Pak 300 SW (7.5 × 300 mm) column and a Waters 626 LC system including a UV detector were used for the chromatography separations (Waters, Milford, MA). An MGW Lauda RM6 circulating bath from Brinkmann (Westbury, NY) was employed for temperature control. Empty PEEK-lined Omega columns with a volume of 0.1 mL were from Upchurch (Oak Harbor, WA). An Alltech Adsorbosphere UHS C18 column (150mm x 4.6mm, 5µm particle size, Alltech, Nicholasville, KY) was used for reverse-phase chromatography. Human IgG ELISA kit and Micro-BCA assay kit were from Alpha Diagnostic International (San Antonio, TX) and Pierce (Rockford, IL), respectively. Cell culture media EMEM was from Quality Biological (Gaithersburg, MD). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT) and Becton Dickinson (Sparks, MD), respectively. The instrument and accessories for MS analysis are as follows: The MS unit was a hybrid LTQ-

FT (Thermo Finnigan, San Jose, CA). Chromatography was done on a 75 μm i.d. PicoFrit capillary column (New Objective, Woburn, MA), with a 5 μm C18 silica stationary phase (Agilent, Palo Alto, CA). A PAL Autosampler (LEAP Technologies, Carrboro, NC), a custom built C8 OPTI-PAK trap cartridge (Optimize Technologies, Oregon City, OR), a 10-port switching valve (VICI, Houston, TX), and a Chorus 220 nano-flow pump (CS Analytics, Zwingen, Switzerland) were used to transfer samples. Acetonitrile (ACN) used in MS analysis was purchased from Burdick and Jackson (Muskegon, MI) while ACN for any other experiments was from Sigma. A Savant SpeedVac concentrator was provided by Thermo Fisher Scientific (Waltham, MA).

7.2.2 hIgG deglycosylation using PNGase F

hIgG was incubated with 7500 units of PNGase F at a protein to enzyme ratio of 6 μg to 75 U in a total of 600 μl volume adjusted with PBS (10 mM phosphate buffer, 2.0 mM KCl and 138 mM NaCl, pH 7.4) for 8 hours at 37 $^{\circ}\text{C}$, with the completeness of the reaction being checked by lectin blot. A parallel control experiment was carried out at the same conditions without PNGase F. The hIgG-PNGase F mixture was either directly loaded to the HWRGWV column or first separated on a size exclusion column (SEC). A Protein Pak 300 SW column (7.5 \times 300 mm) was utilized to isolate deglycosylated hIgG and enzyme PNGase F from a mixture of 200 μL running by PBS at 0.5 mL/min. The collected deglycosylated hIgG fraction was either directly used for isothermal adsorption measurements or concentrated down to 100 μL to be loaded to the HWRGWV column.

7.2.3 Deglycosylated hIgG binding to HWRGWV column

A volume of 100 μL digestion solution or purified deglycosylated hIgG solution at a concentration of 1 mg/mL was loaded and eluted at the same conditions determined previously for the peptide HWRGWV column (25). Briefly, samples were loaded at a flow rate of 50 $\mu\text{L}/\text{min}$ for 5 minutes, followed by flushing with another 3 mL PBS; the column was then washed in succession with 4 mL each of PBS + 0.5M NaCl, pH 4 PB and 2% acetic acid at a flow rate of 0.2 mL/min. The bound and unbound fractions were collected and detected by lectin blot analysis.

7.2.4 SDS-PAGE and lectin blot to detect the glycosylation

Samples of PNGase F digestion and fractions from the HWRGWV column were loaded in 4-12% Tris-Bis SDS-PAGE gel and the gels were run in MOPS running buffer under reducing conditions. Pre-stained Seeblue plus2 protein marker was included to indicate the molecular weight of the proteins. Invitrolon PVDF membranes were employed to blot proteins separated by electrophoresis at a voltage of 100 V for 80 minutes. The blocking and washing solutions for the lectin blot detection were WesternBreeze blocking solution and WesternBreeze washing solution + 0.01% SDS, respectively. All the steps executed later were at room temperature unless otherwise mentioned. Protein blotted membranes were blocked in 10 mL blocking solution for 1 hour and then incubated with 10 mL of 2 µg/mL biotinylated GNL diluted in blocking solution overnight at 4 °C. After washing in washing solution three times for 10 minutes each, membranes were incubated with 0.05 µg/mL peroxidase-labeled streptavidin for 1 hour. After the same washing step, membranes were developed using ECL plus detection solution and exposed to a Kodak Biomax MR autoradiography film.

7.2.5 Isothermal adsorption measurement of deglycosylated hIgG to HWRGWV resin

The isotherms of deglycosylated and normal hIgG adsorption to HWRGWV resins were carried out in centrifugal filters (0.5 mL) with 0.22 µm Durapore membranes in duplicate. Four hundred microliters of protein solutions with concentrations ranging from 0.010 to 10 mg/mL in PBS were prepared by serial dilution. The most diluted protein solution was first added to the adsorption vessel containing 10 mg (in dry weight) resin washed and equilibrated in PBS after being pre-swelled in 20% methanol. The protein solution was incubated with resins in an orbital shaker for 2 h. After spin removal of the solution, the solution with the second to the lowest concentration (400 µL) was added and incubated with resins in the same way as mentioned. The procedure continued with all protein solutions from low to high IgG concentration. The unbound hIgG concentration in each supernatant was determined using a Micro-BCA assay. The accumulated amount of bound hIgG was calculated by mass balance. The data were fit to a Langmuir isotherm model

$$q = \frac{q_m C}{K_d + C} \quad (\text{Eq. 7-1})$$

where q , C , K_d , and q_m are the concentration of the bound protein (mg-protein/g-resin), the concentration of the free protein (mg-protein/mL-solution), the dissociation constant (mg/mL), and the maximum capacity (mg protein/g resin) respectively.

7.2.6 Competitive binding of Protein A and Protein G in hIgG adsorption on HWRGWV resin

Protein A and Protein G competition experiments were carried out using two approaches. One method was to inject the mixture of Protein A and hIgG or its fragments in PBS to the HWRGWV column under the chromatographic conditions described in section 7.2.3. The molar ratio of Protein A to IgG or its fragments was 2:1. The other approach was to load pure hIgG at a concentration of 1 mg/mL in PBS + 0.5M NaCl to a HWRGWV column and then to perform a stepwise elution of the bound antibody with Protein A reconstituted in PBS + 0.5M NaCl at 1, 2, 4, and 6 times the molar amount of injected hIgG. The column was finally cleaned with 2% AcOH. Similar experiments were also conducted by using Protein G at a molar ratio to IgG of 5:1. The fractions collected every 10 minutes were subjected to SDS-PAGE analysis on 4 – 20% Tris-Glycin gels under reduced conditions running in Tris-Glycin SDS buffer at 125V for 2 hours. Pre-stained multicolor protein standards were included in each gel as markers. The gels were stained with Coomassie blue when concentrated samples were applied and silver-stained when samples were directly loaded. Sample concentration was accomplished by centrifugation at 4 °C, 11,247×g for 2 hours using MicroCon YM-3 filters.

7.2.7 Enzymatic fragmentation of whole hIgG and its Fc fragment

For limited proteolysis with pepsin (Sigma, St. Louis, MO), hIgG lyophilized powder was solubilized in 0.1 M citrate buffer at pH 4 and incubated with pepsin at a protein-to-enzyme ratio of 25:1 (w:w) at 37 °C. At set times ranging from 30 minutes to 22 hours, 24- μ L samples were removed and added to a mixture of SDS-PAGE sample buffer (15 μ L) and

reducing agent (3 μ L). The samples were immediately boiled for 10 minutes and then frozen for later use. Another set of 150 μ L samples were removed and pepsin was inactivated by adding 220 μ L 0.1 M Tris-HCl buffer at pH 8.4 for binding studies on an HWRGWV column. The same procedure was applied to the Fc fragment of hIgG.

For endoproteinase Lys-C and Glu-C cleavage, the Fc fragment was reconstituted in 0.1 M Tris-HCl buffer at pH 8.4 and incubated with the enzymes at 37 °C for 24 hours. The protein:enzyme ratios were 100:1 (w:w) for Lys-C and 50:1 for Glu-C, respectively. CNBr digestion of Fc was carried out in 70% formic acid in water at Fc:CNBr of 1:100 in the dark at room temperature for 24 hours. The digestion solution was dried by N₂ before being reconstituted in PBS for HWRGWV binding examination and SDS-PAGE analysis. The SDS-PAGE samples were prepared the same way as described above.

7.2.8 Binding of the enzymatic digests to HWRGWV column

One hundred microliters of inactivated pepsin digestion solution or the direct digestion solutions of all other enzymes were loaded to a 0.1-mL HWRGWV column at 0.05 mL/min, the flow rate then being increased to 0.1 mL/min after 6 minutes. The column was washed with loading buffer PBS for 20 min and bound proteins or fragments were eluted with 2% acetic acid (AcOH). Both flow-through and elution peaks were collected and analyzed by SDS-PAGE.

7.2.9 Reverse-phase HPLC (RP-HPLC) to separate the enzymatic digests

An Alltech Adsorbosphere UHS C18 column was used to separate the digests and fractions eluted from the HWRGWV column at ambient temperature. The solvents were as follows: solvent A: 0.1% (v:v) trifluoroacetic acid (TFA) in water; solvent B: 0.1% (v:v) TFA in acetonitrile (ACN). The column was equilibrated with 100% A. Samples were loaded at 0.1 mL/min, and after five minutes the flow rate was increased to 0.5 mL/min and kept at this level for the rest of the run. The gradient was linearly increased to 27% B over 100 min, then to 100% B over 60 min, and subsequently held at 100% B for 10 min before being changed back to the initial condition. Chromatograms were monitored at 280 nm and 214 nm. The manually collected peaks were either directly resolved by SDS-PAGE or dried in a Savant SpeedVac and subsequently used for MS analysis.

7.2.10 SDS-PAGE analysis of HWRGWV-bound fragments

To analyze the intact hIgG or its Fc fragment digestion by enzymes, 12% Tris-Bis SDS-PAGE gels loaded with target samples were run in MES running buffer under reducing or non-reducing conditions. Mark 12 non-staining protein marker was included to indicate the molecular weight of proteins and the gels were visualized by silver-staining. The target samples could be the digestions, the bound and unbound fractions from the HWRGWV column, or collected RP-HPLC peaks.

7.2.11 MS analysis of HWRGWV-bound fragments

All mass spectra were acquired on a hybrid LTQ-FT mass spectrometer in the positive-ion mode. Automatic gain control (AGC) was set to 1×10^6 and the capillary temperature was set at 250 °C. Both direct infusion and LC-MS/MS were utilized in this investigation.

Reversed phase liquid chromatography was performed at room temperature using a 75 μm i.d. \times 100 mm PicoFrit capillary column with a 15 μm emitter tip packed in-house with 5 μm C18 silica stationary phase. The mobile solvents A and B were respectively 2% ACN and 98% ACN in water with 0.2% formic acid as the ion pairing reagent in both. Samples were injected using a PAL Autosampler and over the course of 10 minutes trapped and washed on a custom built C8 OPTI-PAK trap cartridge with 100% A at 1 $\mu\text{L}/\text{min}$ until a 10 port switching valve was triggered to move the sample in-line with the gradient. Elution was carried out by a Chorus 220 nano-flow pump at 500 nL/min with the gradient as follows: 2% B for 6 minutes followed by a linear gradient from 2 – 55% in 30 minutes and then 55 – 59% B in 5 minutes.

The sequences acquired by MS were determined by matching with the known hIgG amino acid sequence from protein databases (PDB: 2IG2). ExPASy.org (<http://ca.expasy.org/tools/findpept.html>) was used to search the sequences that could match the fragments with molecular weight larger than 10 KDa within the error of 5 ppm. For small species, Bioworks software v2.1 was used to search the databases for any match, and each positive result was confirmed by manual interpretation of the MS data. Thank to who kindly provided the MS data.

Both the MS analysis and data interpretation were conducted by D. Keith Williams, Jr. and Dr. David Muddiman in the Department of Chemistry at NCSU.

7.3 Results and discussion

7.3.1 Deglycosylated hIgG binding to HWRGWV

To examine the effect of glycosylation on hIgG binding to the HWRGWV peptide resin, hIgG was treated with endoglycosidases peptide:N-glycosidase F (PNGase F) and the deglycosylated hIgG was then applied to HWRGWV resins for binding assessment. A lectin blot was employed for glycosylated hIgG detection, where the glycosylated hIgG binds the biotinylated GNL, which can be visualized by chemiluminescence detection after labeling with peroxidase-conjugated streptavidin. In contrast, the deglycosylated IgG can not be detected due to the lack of the carbohydrates that interact with the GNL.

The completion of the deglycosylation reaction after 8 hours of incubation in PNGase F was checked by SDS-PAGE (Figure 7-3A) and lectin blot (Figure 7-3B) which were also employed for HWRGWV-bound and -unbound fraction analyses (lanes 6-8). Complete removal of glycans on the γ -chain by PNGase F led to a size shift of about 4 kDa in the IgG heavy chain (Figure 7-3A, lane 3), compared to the molecular weight of the heavy chain before the reaction (Figure 7-3A, lane 2). It also can be seen that the size of the heavy chain was not reduced for normal hIgG incubated at the same conditions without PNGase F (Figure 7-3A, lanes 4 and 5), indicating that the decrease of the heavy chain molecular weight in lane 3 was due to the action of PNGase F. Lectin blots (Figure 7-3B) verified the success of the deglycosylation where the heavy chain band in lane 3 disappeared due to the lack of glycans, while the heavy chains in all other lanes (2, 4, and 5) were still visible. Therefore, the deglycosylation reaction of hIgG was completed within 8 hours of incubation with PNGase F.

Binding of deglycosylated hIgG to the HWRGWV column at a peptide density of 0.08 meq/g was investigated with either the single hIgG protein purified by SEC or the protein mixture with PNGase F. The chromatograms are shown in Figure 7-4 and the corresponding SDS-PAGE and lectin blot analyses are shown in Figure 7-3 (lanes 6 – 8). As shown in Figure 7-4, HWRGWV showed no difference in retention and release between the normal and deglycosylated hIgG, with the majority of IgG being eluted by pH 4 PB in a sharp peak

(P2) and small amounts remaining in the flow-through (FT) and 0.5 M NaCl wash peaks. These are the same chromatographic patterns observed for normal hIgG and hIgG incubated in PBS without PNGase F for 8 hours at 37 °C. The presence of the eluted deglycosylated hIgG with pH 4 PB was confirmed by the lectin blot analysis (Figure 7-3B, lane 6). It is also evident that PNGase F was not retained by the ligand column since it was present only in the flow through FT (Figure 7-3A, lane 7) but not in the peak P2 (lane 8) when the IgG-PNGase F mixture was loaded directly. The ability of the ligand HWRGWV to bind both normal and deglycosylated hIgG is similar to that of Protein A on which normal and carbohydrate-deficient IgG exhibited identical binding abilities (26). From this experiment it can be concluded that the hexamer ligand HWRGWV does not bind to IgG through a lectin-like interaction, and that is likely not binding to the hinge proximal site.

The isotherm of deglycosylated hIgG adsorption on HWRGWV was found to overlap with that of normal hIgG (Figure 7-5), suggesting the same dissociation constant for both IgG forms on HWRGWV. This was also confirmed by the chromatographic elution experiments, where it was impossible to separate the two forms of hIgG on HWRGWV using either a salt gradient or pH gradient (data not shown). In summary, the similar elution profiles for the normal and deglycosylated hIgG indicate that the ligand binding sites on IgG were not affected by the degree of glycosylation. It is possible to conclude from this result that the ligand does not bind to the IgG through the hinge proximal region of the C_H2 domain. Otherwise, deglycosylation would have influenced these interactions (3, 21).

7.3.2 Competition of the peptide with Protein A or Protein G for binding hIgG

The possibility that HWRGWV binds to the consensus inter-C_H2-C_H3 interaction site on Fc was explored through competitive binding experiments with Protein A and Protein G. Competition between Protein A and the hexamer ligand HWRGWV for IgG binding was examined with two different approaches in a column format, where Protein A was in solution while the HWRGWV ligand was immobilized on Toyopearl AF-Amino 650M. The first approach was aimed to investigate the ability of HWRGWV to retain a Protein A-hIgG complex. To do this, pure Protein A, as well as its mixtures with hIgG or hIgG fragments in PBS, were injected into the HWRGWV column (Figure 7-6). The ratio of Protein A to

antibodies in the mixtures was 2:1. At this ratio Protein A is in great excess since the Protein A-hIgG binding stoichiometry in solution is approximately 1:3.3 as reported by Jungbauer and Hahn (27). The stoichiometry of Protein A binding to Fab is unknown, although Protein A – Fab interactions have been confirmed in several different studies (28-31). It is shown in Figure 7-7 that Protein A, when injected alone into the column, was non-specifically retained by the ligand column and could be washed off with 0.5 M NaCl in PBS. The salt wash peak was significantly smaller when Protein A was applied to the HWRGWV column in mixtures with antibodies, suggesting the association of Protein A with hIgG and its fragments prevents the Protein A from binding to the column. When the mixtures were injected, the majority of Fc and hIgG molecules bound to Protein A were retained and eluted in pH 4 phosphate buffer; this is the same behavior as that of pure IgG on the HWRGWV column. As seen in the SDS-PAGE gel (Figure 7-7A), the flow-through (fractions F1 and F2) and salt wash (fractions F3 and F4) peaks contained mainly Protein A (42 kDa) that was in excess for antibody binding. The pH 4 PB elution peak (fraction F5) comprised both Protein A and antibodies (Figure 7-7A). Fab fragments in the Protein A mixture remained unbound (Figure 7-6), the same as when only the pure fragment was loaded to the column (25). Most of the Fab fragment was present in the flow-through (F2) and a small amount remained associated with Protein A in the salt wash (F4) and pH 4 elution (F5) peaks (Figure 7-7B). The phenomenon that Protein A exited the peptide column always as the complex with antibodies is understandable because Protein A has a higher affinity for all antibody fragments than HWRGWV, about 10^8 M^{-1} for hIgG and its Fc fragment and about $10^5 - 10^6$ M^{-1} for its Fab portion (32). The affinity of HWRGWV to hIgG is about 10^5 M^{-1} (Chapter 4). The similarity of the chromatography patterns between pure IgG or its fragments and their mixtures with Protein A lead to the conclusion that the adsorption of hIgG and its fragments on HWRGWV column were not influenced by the presence of Protein A in the feed streams; namely, there was no competition between HWRGWV and Protein A in binding hIgG and Fc.

The second approach was to employ Protein A in PBS with 0.5M NaCl as an elution agent for hIgG bound on the HWRGWV column (Figure 7-8). The solvent PBS + 0.5 M NaCl was used to prevent Protein A from binding to the HWRGWV resin. Both the chromatogram (Figure 7-8A) and the gel (Figure 7-8B) show that the major amount of hIgG

was eluted by the 2% AcOH elution step (lanes 8 and 9) but not by Protein A which was applied in a large molar excess (6 times the amount of hIgG) (lanes 4-6). When Protein A was applied in a stepwise fashion (Figure 7-9), most of the IgG was also stripped only with the 2% acetic acid elution. Based on these results, the conclusion can be drawn that Protein A is unable to break the interaction between hIgG and HWRGWV, and vice-versa, HWRGWV does not affect the association between hIgG and Protein A. In other words, the interaction sites on hIgG for HWRGWV are different from those for Protein A.

Similar studies were also performed using recombinant Protein G (Figure 7-10 - Figure 7-12). The Protein G used in this study was a recombinant protein produced in *Escherichia coli* and contains two IgG-binding regions (33). Also, it is well known that hIgG has two identical binding sites for Protein G at the junction of the C_H2 and C_H3 domains. Therefore, the stoichiometry for Protein G and hIgG binding is 1:1, which is confirmed by our ITC experiment (Appendix 2). In this competitive binding study between Protein G and HWRGWV, Protein G was applied in great excess where the molar ratios of Protein G to hIgG or its fragments were 5 to 1. Pure Protein G was examined first by loading it to the HWRGWV column (Figure 7-10). It shows that the majority of Protein A remained in the flow-through fraction and 0.5 M NaCl wash, while a small amount was distributed in both the pH 4 PB and 2% AcOH elution fractions. When the mixture of Protein G-Fab was injected (Figure 7-10 and Figure 7-11A), both proteins were washed off before pH 4 elution. In the case where the mixture of Protein G and hIgG were loaded on to the HWRGWV column (Figure 7-10), large flow-through and salt wash peaks were present. This is due to the excess of Protein G present in the injected mixture, as seen in the corresponding electrophoresis pattern (Figure 7-11B), where fractions 2 – 4 are shown to be composed mainly of Protein G. As was the case for Protein A, Protein G was eluted together with hIgG by low pH solvents (Figure 7-11B, F5 – F7). The protein profile for the run with Protein G-Fc mixture (Figure 7-10 and Figure 7-11B) was similar to that of Protein G-IgG. When Protein G was used as a stripping agent (Figure 7-12) it was not able to remove HWRGWV-bound hIgG from the column as evidenced by the fact that fractions 4 – 7 (Figure 7-12B) were composed of only Protein G. Similar to the case with Protein A, hIgG was eluted only during the 2% AcOH step, as shown in fraction 8 (Figure 7-12B). Hence, Protein G was

unable to disrupt the interaction between hIgG and HWRGWV on the resin, again suggesting the existence of distinct binding sites on hIgG for the two ligands. The similar results obtained with protein A and Protein G might have been expected since both Protein A and Protein G are known to bind to IgG in a region localized around the junction of the C_H2 and C_H3 domains even though they may interact with different amino acids on Fc at this portion (7, 34).

7.3.3 Binding site study by MS analysis of hIgG fragments

As shown in the previous sections, the carbohydrate moiety in the Fc portion had no influence on the interactions between the Fc-binding ligand HWRGWV and hIgG, and Protein A and Protein G do not compete with HWRGWV for IgG binding. It is evident that HWRGWV binds to the Fc fragment at a site different from the conventional hinge proximal or the inter-C_H2-C_H3 binding sites. To determine the potential site of binding to HWRGWV on hIgG, MS techniques were used to sequence peptide fragments of hIgG and Fc that were found to bind to the peptide resin.

Enzymatic fragmentation of hIgG and Fc was conducted before the hydrolyzation products were passed through an HWRGWV column. The composition of the retained fragments was then analyzed by SDS-PAGE and MS. Three enzymes (pepsin, Glu-C, and Lys-C) were used for hIgG or Fc fragmentation to produce small fragments consisting of different amino acid residues. The main results are summarized in Table 7-1 and are discussed in detail in the sections that follow.

7.3.3.1 hIgG and Fc fragmentation and fragment analysis

7.3.3.1.1 Pepsin digestion of hIgG

Pepsin was selected as a fragmentation enzyme to generate pFc fragments for HWRGWV binding study. Pepsin at pH 4 is known (2) to hydrolyze hIgG at the lower hinge region into a F(ab')₂ fragment and a Fc' fragment which is further digested into a pFc fragment (C_H3 domain, 12 kDa) and numerous small pieces from the C_H2 domain (Figure 7-13). The optimal peptic incubation time was examined. The peptic digests of hIgG at different times were neutralized and loaded to the HWRGWV column, followed by 2%

AcOH elution (Figure 7-14A). It can be seen that the flow-through peak (FT) increased while the elution peak (P1) decreased with increasing incubation time, suggesting that the digestion of hIgG was getting more complete with time. The loaded material (L), as well as the bound (P1) and unbound (FT) fractions from the HWRGWV column were analyzed by SDS-PAGE (Figure 7-14B). Two bands at about 36.5 and one band at 21.5 kDa were from pepsin (MW 34.5 kDa) as indicated in lane 3. The hydrolyzation of hIgG by pepsin was evidenced by the appearance of fragments at 25 kDa, 12 kDa, possibly representing Fc' and pFc fragments respectively, and other smaller pieces at 3.5 kDa on the SDS-PAGE gel (Figure 7-14B) in the lanes labeled as L. The weakening of the 25 kDa band with time shows the digestion progression and the disappearance of both 25 kDa and 12 kDa implied the completion of the digestion. Analyzing the lanes containing HWRGWV-bound (P1) and unbound (FT) fractions, it can be noted that the peptide ligand was not able to retain fragments smaller than 3.5 kDa that were present only in the FT whereas P1 contained mainly the fragments of 25 and 12 kDa. By visual comparison, the highest ratio of 12 to 25 kDa fragments, speculated to correspond to the ratio of pFc to Fc', appeared in L and P1 (lanes 7 and 9) after a two hour incubation of hIgG with pepsin. As a result, the samples obtained with 2 hours digestion including the digest, the HWRGWV-unbound and -bound fragments, corresponding to the materials of the lanes 7 – 8 in Figure 7-14B, were chosen for RP-HPLC separation.

The RP-HPLC separation was completed on an Alltech Adsorbosphere UHS C18 column with a gradient of ACN (Figure 7-15A). The peptic digest (blue line) was separated into a large array of small peaks, a sharp peak at 116 min, a broad peak at 127 min, and a few peaks after 150 min. Most peaks eluted before 116 min were also contained in the FT (red line) but not in the P1 (black line) from the HWRGWV resin. In contrast, the peak at 116 min was present only in P1. The peaks at 127 min and after 150 min appeared in all three samples. Since the peaks after 150 min for all three runs were identical, the ones at 116 and 127 min from the P1 sample were collected and analyzed again by SDS-PAGE under reduced conditions (Figure 7-15B and Table 7-1). It is evident that Peak 116 contained mainly the 12 kDa fragment, presumably pFc (Figure 7-15B, lane 5) whereas peak 127 was comprised of not only the 12 kDa fragment, but also the F(ab')₂ fragment by comparison with the standard lanes (Figure 7-15B, lanes 2-4). The emergence of the F(ab')₂ fragment might be the result of

the concentration effect of the RP-HPLC since they are not as distinct in the gel before the RP-HPLC run (Figure 7-14B). The binding of some F(ab')₂ on HWRGWV was not a surprise since HWRGWV showed a weak ability to bind F(ab')₂ (25). The 12 kDa fragment in the peak at 116 min, which might be one of the H chain domains, was collected from the RP-HPLC run for mass spectrometry analysis.

Two species were found in the peak of 116 min (Figure 7-16) with molecular weights of 11.698 and 12.698 kDa, respectively corresponding to the sequences Leu79 – Leu194 and Glu333 – His435 of hIgG (Figure 7-2). The first species matches part of the Fab portion, and the second matches the pFc moiety of hIgG. The results confirmed the existence of pFc at 12 kDa in the SDS-PAGE analyses. The detection of Leu79 – Leu194 fragments was probably due to the ability of the HWRGWV ligand to bind a small amount of F(ab')₂ fragment (25). The existence of the Glu333 – His435 fragment suggests that HWRGWV may bind Fc through the interaction with the residues within the pFc region.

7.3.3.1.2 Pepsin digestion of Fc

Pepsin treatment of Fc was also investigated the same way as for hIgG and a similar trend was noticed (Figure 7-17). The HWRGWV-bound (P1) peak became smaller while the unbound peak (FT) increased (Figure 7-17A), implying a smaller content of bound fragments with increasing incubation time in pepsin. This was also confirmed by the depletion of the 50 and 25 kDa bands, believed to be Fc and Fc' fragments, with the increase of the incubation time in lanes labeled as L (Figure 7-17B). The band at 12 kDa from pFc also began to decrease after 5.5 hours of digestion, indicating the completion of the hydrolysis process. For this reason, the digestion time was selected as 2 hours.

After the Fc fragment was hydrolyzed by pepsin for 2 hours and then injected to the HWRGWV column, the digest and the FT and P1 fractions were further analyzed (Figure 7-18 and Table 7-1). These three samples correspond to the lanes 9 – 11 of the gel shown in Figure 7-17B. The RP-HPLC chromatogram of the FT (red line) had a similar peak profile as the digest (blue line) but was different from P1 (black line) which had only the peak at 120 minutes. The peaks at 120 min of both the FT and P1 runs were collected and analyzed by reduced SDS-PAGE gel (Figure 7-18B). It can be seen that the two peaks had a similar

protein profile (lanes 4 and 6), mainly containing the Fc' and pFc fragments. The appearance of the pFc fragment in the FT might be due to the overloading of the HWRGWV column based on the observation that a much larger 120 min peak was present in the RP-HPLC of the original digest.

The RP-HPLC peak at 120 min of the peak P1 was collected and analyzed by MS (Figure 7-19 and Table 7-1). Two overlapping fragments were resolved, one being Lys334 – Gly446 and the other Lys334 – His435 with molecular weights of 12.730 and 11.732, respectively. The first fragment is the whole pFc fragment. The second one belongs to the first one and is only one amino acid different from the peptide detected in the peptic digest of hIgG. The finding of these two fragments confirmed the binding of HWRGWV to the pFc fragment of hIgG. MS failed to detect the larger fragment Fc' (Figure 7-18B, lane 6), possibly due to the ionization competition between the large and small fragments. It is easier to ionize smaller fragments that finally enter the electrospray droplet (35). This problem was also encountered, to a different extent, when analyzing all other samples, where smaller fragments were detected but not the larger ones that were visible in the gel.

7.3.3.1.3 Digestion of Fc with Endoproteinases Lys-C and Glu-C

The Fc fragment of hIgG was also cleaved by endoproteinases Lys-C and Glu-C to produce fragments different than those of peptic digests. Lys-C selectively cleaves the peptide bonds at the C-terminal of lysine and Glu-C breaks the bonds at the C-terminal of glutamic acid and aspartic acid residues (36). After incubation of Fc individually with the endoproteinases, the digests were applied to the HWRGWV column. The HWRGWV-bound and unbound fractions were then separated by RP-HPLC before MS analysis. All significant peaks from both the peptide column and RP-HPLC were collected and analyzed by non-reducing SDS-PAGE (Figure 7-20), where Fc was partially reduced to form a band at around 25 kDa (lanes 6 and 14). After the treatment with Lys-C and Glu-C, Fc was incompletely hydrolyzed (lane L), as deduced by the appearance of the fragments smaller than 21.5 kDa and the remnant of the Fc bands at 25 and 50 kDa (lanes 4 and 7). By comparing the corresponding FT (lanes 2 and 8) and P1 (lanes 3 and 9) fractions from the HWRGWV runs, the elution peak P1 contained a band that was not present in the flow-through fraction (FT) of

each of the runs, the 12 kDa band for Lys-C and the 15 kDa band for Glu-C digestion. These two fragments might be one of the domains existing in Fc. The P1 fraction of the Lys-C digestion was loaded on the C18 column and a large peak at 120 min was detected. A tailed peak at 120 min appeared in both FT and P1 fractions of Glu-C digestion in the RP-HPLC separation. All these peaks were included in Figure 7-20 (lanes 1 and 10 – 13). In the detectable molecular weight range of SDS-PAGE, not much difference was observed in the protein profiles between the peaks before and after the RP-HPLC. To more precisely determine the mass of the fragments, the peaks from the RP-HPLC were analyzed by MS. The RP-HPLC, SDS-PAGE, and MS results are also summarized in Table 7-1.

An 11.228 kDa peptide was identified by MS in the P1 of the Lys-C digestion (Figure 7-21). The peptide was composed of the amino acid residues Lys334 – His435 of hIgG, the same sequence found in the peptic digestion of Fc. This finding further confirmed the interactions between the pFc and the HWRGWV ligand. Some smaller fragments were also found, the sequence Phe275 – Lys288 from the C_H2 domain, and sequences Gln362 – Lys370 and Gly371 – Lys392 located inside the larger fragments Lys334 – His435. The finding of these small fragments opens up the possibility of narrowing down the binding site of HWRGWV on pFc.

In the case of the Glu-C digestion of Fc, both the FT and P1 were analyzed by MS. The FT fraction was included to identify the fragments that may partially overlap with, and thereof shorten, the bound fragments. The MS was not able to detect any fragments larger than 3 kDa visible in SDS-PAGE gel. Some small fragments were found: Leu234 – Asp249, Leu234 – Glu258, Val259 – Asp268, and Gly281 – Gln294 in P1; Val273 – Gln283 and Lys334 – Glu357 only in FT but not P1.

Digestion of Fc with cyanogen bromide (CNBr) was performed to try to cut the Fc fragment into fractions that differed from those obtained by the other fragmentation methods (37, 38). The cleavage produced several fragments visible in SDS-PAGE gel but further MS analysis was unsuccessful in resolving any peptides in this sample. The pFc fragment were also further cleaved with CNBr, NH₂OH, and Lys-C to generate smaller pieces that might be capable of being retained on the peptide ligand. The subsequent MS analysis, however, did not reveal any fractions that were smaller than pFc.

7.3.3.2 HWRGWV binding sites on Fc

Figure 7-22 aligns all the segments sequenced by MS that are within the Fc portion of hIgG. It is clearly shown that pFc (Lys334 – Gly447) and its sub-fragments have been found in several digestions to be retained by the HWRGWV resins, indicating the interactions between the pFc and the HWRGWV ligand. Since the fragment Lys334 – Glu357 (yellow line) cannot be retained by HWRGWV, the interaction site on pFc might be in Met358 – His435 portion. Inside this portion, fragments Gln362 – Lys370 and Gly371 – Lys392 were individually found to interact with peptide ligand HWRGWV. As shown in Figure 7-23, the amino acids in the segment Gln362 – Lys370 form a β -strand and all seven residues in the middle of this strand are fully buried inside the Fc 3-D structure (in dark blue). Therefore, the HWRGWV binding site on Fc is unlikely localized in this segment. In contrast, the Gly371 – Lys392 portion of Fc contains a loop structure (Ser383 – Asn389) that is exposed to the bulk solvent (Figure 7-23), and hence is able to be in contact with the ligand HWRGWV. The amino acid sequence of the loop is SNGQPEN, rich in glutamine (Q) and asparagine (N). These two amino acids can form hydrogen bonds with histidine (H), tryptophan (W), and arginine (R), the N-terminal residues in the ligand. Glycine (G) and proline (P) in this loop have the ability to interact with the ligand residues glycine (G) and tryptophan (W) by hydrophobic forces. The solvent accessibility of glutamic acid (E) is less than that of the other residues in the loop, but in a dynamic binding environment it is possible for the electrostatic interaction at neutral pH between positively charged glutamic acid (E) and negatively charged arginine (R) of the ligand. Therefore, it is likely that the HWRGWV interaction site on Fc is localized to the Gly371 – Lys392 portion.

Other fragments localized in the hinge region and C_H2 domain are basically belong to two large segments Leu234 – Asp265 and Val284 – Gln294, considering both the bound and unbound peptide fragments (Figure 7-22). The former segment is unlikely the HWRGWV original binding site since the degree of glycosylation is influential to the interactions that involve the residues in this hinge proximal portion (39-41). The portion Val284 – Gln294 is localized on the opposite side of the complement C1q binding motif of hIgG (Asp270, Pro329, Pro331 and Lys322) (42). Since deglycosylated hIgG is not able to bind C1q (43), the interactions between HWRGWV and Gly284 – Gln294 might be also affected by

removal of the carbohydrates on hIgG. Therefore, the binding of the ligand HWRGWV to the Val284 – Gln294 portion on Fc is less likely. Otherwise, the deglycosylation would be expected to affect the interactions between HWRGWV and hIgG. The appearance of the fragments Leu234 – Asp265 and Val284 – Gln294 in the HWRGWV-bound fraction might be due to non-specific interactions.

The combined data described in this section indicate the possibility that HWRGWV interacts with the pFc portion of hIgG (Figure 7-24). It is very likely that the active interaction region is the loop Ser383 – Asn389 (light green line) contained within the segment Gly371 – Lys392 (dark green line). Protein A and Protein G make contact with amino acids mainly localized in the two loops formed by Leu251 – Ser254 and Met428 – Gln438 (7). Also, the contacting surface area on IgG for Protein A and Protein G does not cover the loop Ser383 – Asn389 which is away from the C_H2-C_H3 interface and more close to the C-termini of the H chains (Figure 7-25). This might be the explanation for the non-competition in binding hIgG between HWRGWV and Protein G or Protein A. The amino acids in the loop Ser383 – Asn389 can interact with the HWRGWV ligand by several forces: hydrogen bonding, hydrophobic, and possibly electrostatic forces, resulting in a specific interaction between hIgG and the ligand. This is in agreement with our previous finding from chromatographic experiments (Chapter 4) that hIgG is retained in an HWRGWV column via some specific interactions with the Fc portion.

7.4 Conclusions

It was found that the deglycosylated hIgG could be retained on the HWRGWV resin as well as the normal hIgG with the similar affinities. It is hence concluded that the peptide ligand does not bind hIgG through a lectin-like interaction. It is also unlikely for HWRGWV to interact with hIgG through the sites that are affected by the degree of glycosylation of IgG, for example the hinge proximal region. Meanwhile, there is no competition observed between HWRGWV and Protein A or Protein G in binding hIgG, indicating that the HWRGWV does not bind to hIgG at the inter-C_H2-C_H3 site. HWRGWV has the ability to retain the pFc fragment of hIgG and may interact with the amino acid residues localized in the loop Ser383 – Asn389. This loop is away from the C_H2-C_H3 interface and more close to

the carboxyl termini of the hIgG heavy chains. The binding of the loop to HWRGWV is specific, involving several forces of hydrogen bonding, hydrophobic, and possibly electrostatic interactions.

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Table 7-1 Analysis of HWRGWV-bound (P1) fractions in different digestion samples by RP-HPLC, SDS-PAGE, and MS

Enzyme	Pepsin	Pepsin	Pepsin	Lys-C	Glu-C
Digested protein	HIgG	HIgG	Fc	Fc	Fc
Peak retention time in RP-HPLC, min	116	127	120	120	120
MW by SDS-PAGE, kDa	12	12, >21.5	12, 25	12, >25	12, 15, >25
Possible fragment of hIgG by SDS-PAGE	pFc	pFc, F(ab') ₂	pFc, Fc'	pFc, Fc	Fc domains, Fc
MS detected fragment	pFc, Leu79-Leu194	/	pFc	pFc, Gln362 – Lys370, Gly371 – Lys392	Fragments <3 kDa

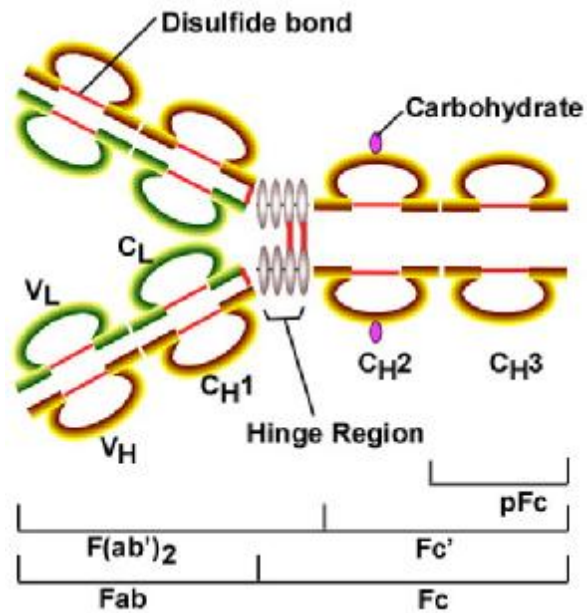


Figure 7-1 Structural diagram of human IgG1

Green line: light chain; brown line: heavy chain. Each “C” like structure denotes one of the IgG domains. The enzymatic fragments Fab, Fc, F(ab')₂, Fc' and pFc are indicated below the diagram.

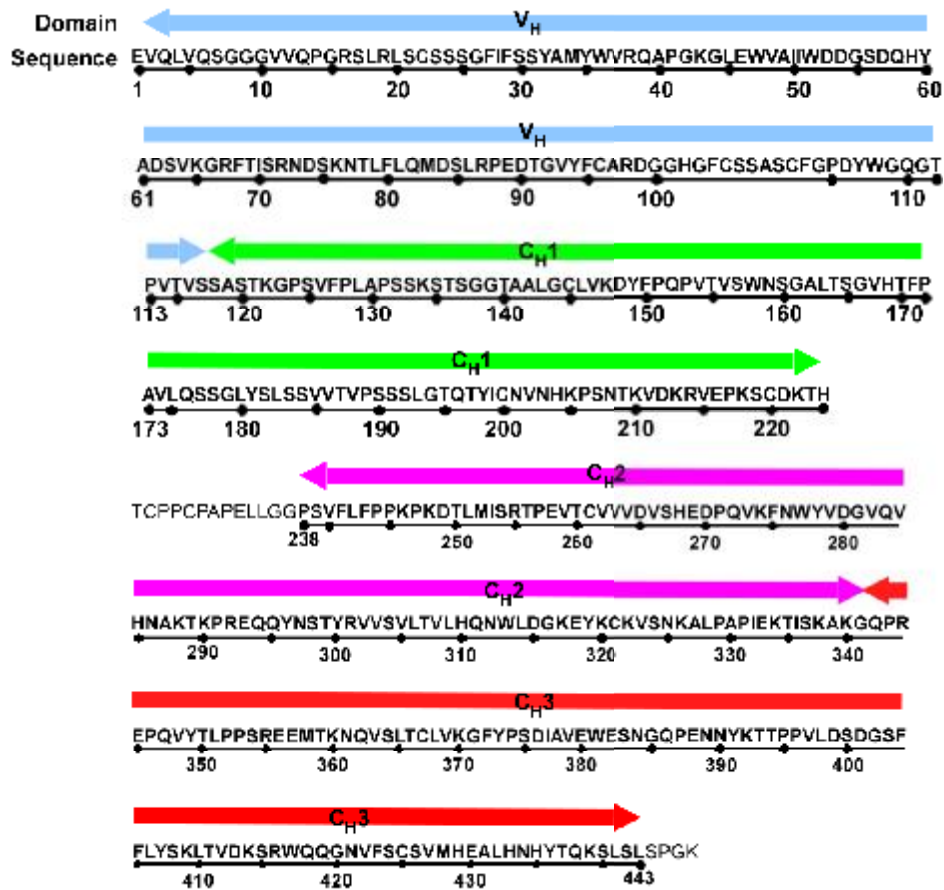


Figure 7-2 Amino acid sequence of hIgG1 heavy chain with individual domains being indicated by colored arrows (PDB codes: 2IG2 from www.rcsb.org).

A

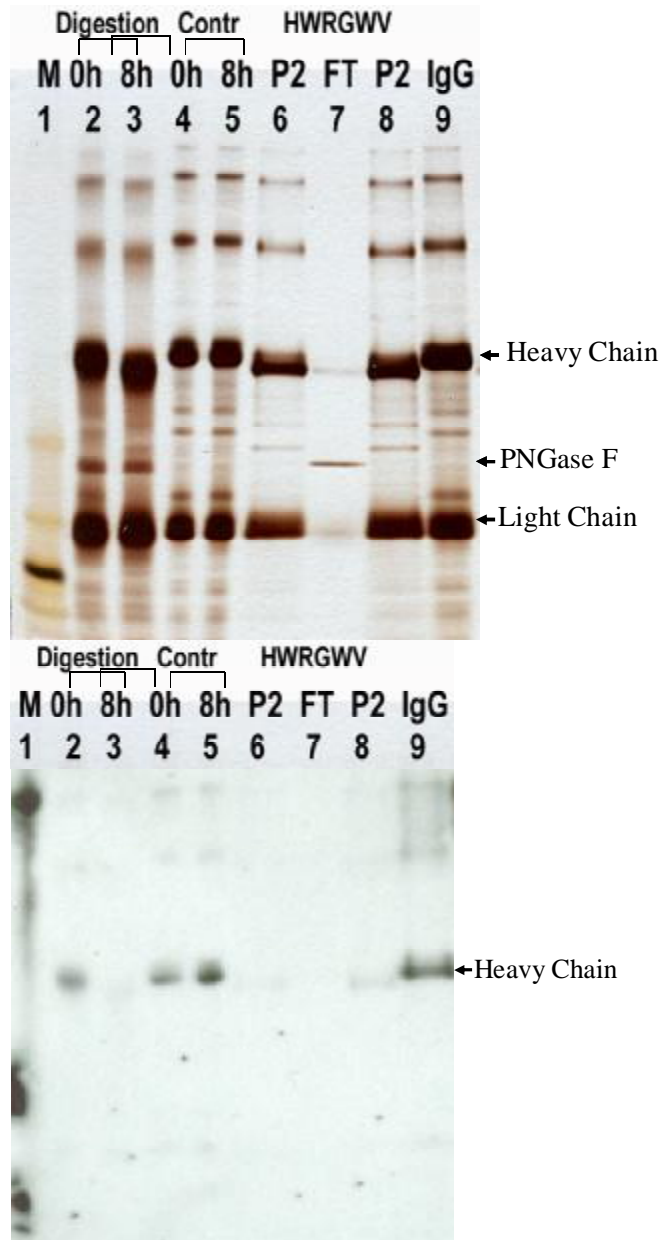


Figure 7-3 Deglycosylated hIgG detection by (A) silver stained SDS-PAGE gel and (B) the corresponding lectin blot.

The gel was run under reduced conditions. 0h (lanes 2 and 4) and 8h (lanes 3 and 5) indicate the hIgG incubation time in hours with (digestion) and without (contr) PNGase F. Lanes 1 (M) and 9 (IgG) are molecular marker and hIgG standard, respectively. Lanes 6-8 correspond to the peaks indicated in Figure 7-4. Specifically, lane 6 was the P2 from the HWRGWV column loading of SEC isolated deglycosylated hIgG while lanes 7 and 8 were from the

direct loading of PNGase F digestion solution to the column.

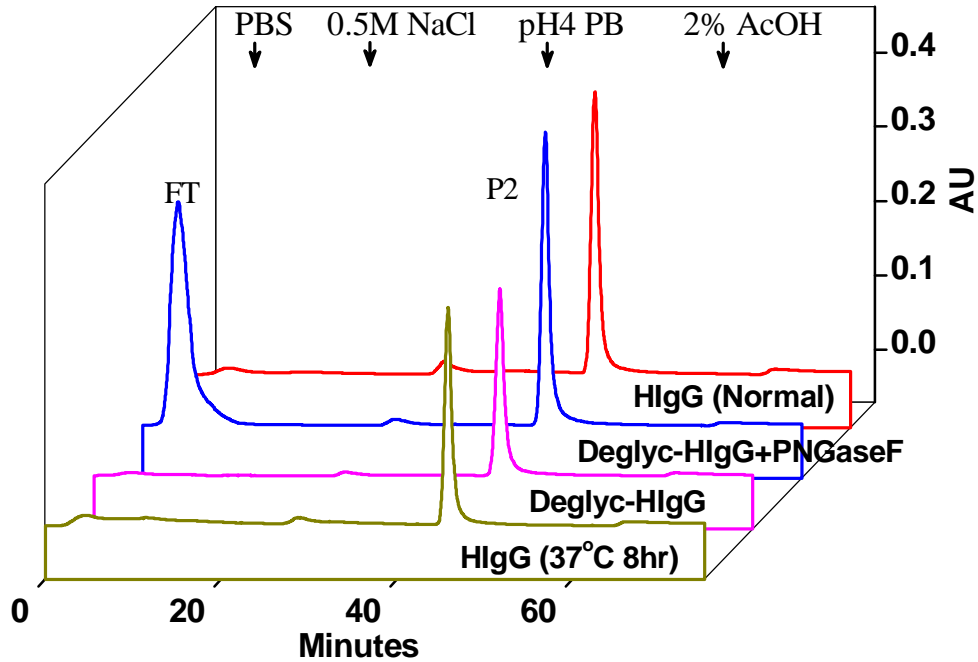


Figure 7-4 Deglycosylated hIgG binding on HWRGWV column.

Samples were loaded in PBS at pH 7.4, and sequentially washed for 20 minutes each with 0.5 M NaCl, pH 4 PB and 2% acetic acid (AcOH). The flow rate was 0.2 ml/min. Both the column volume and the injection volume were 100 μ L. The solutions of hIgG (normal),

hIgG heated in PBS at 37 $^{\circ}$ C for 8 hours (37 $^{\circ}$ C 8hr), the mixture of deglycosylated hIgG with

PNGase F (Deglyc-hIgG+PNGaseF), and deglycosylated hIgG purified by SEC (Deglyc-hIgG) were separately loaded to the column. FT and P2 refer to the flow through and hIgG elution peaks from the HWRGWV column.

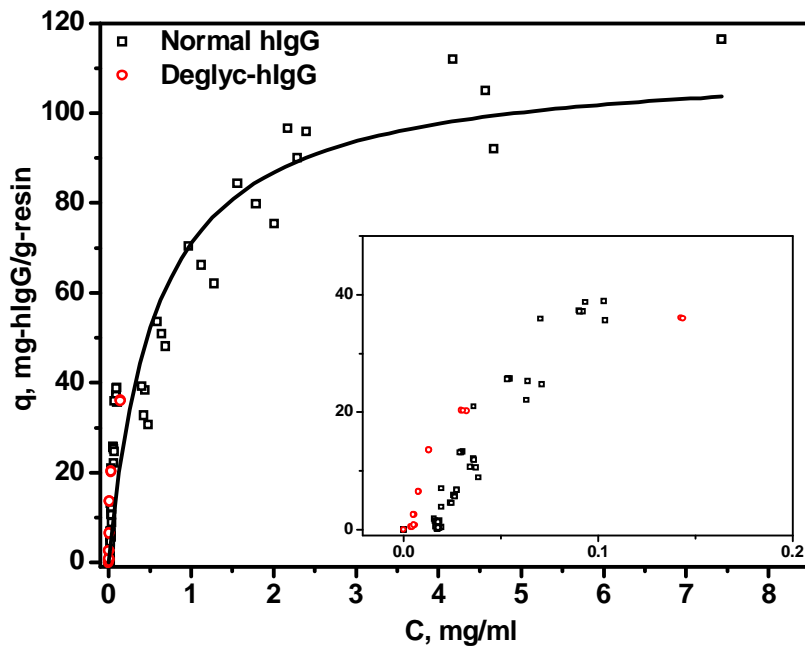


Figure 7-5 Isotherms of normal and deglycosylated hIgG (Deglyc-hIgG) binding to HWRGWV resins with a ligand density of 0.08 meq/g. Symbols are the experimental data and the line is from the Langmuir fitting of normal hIgG adsorption data. The data at the origin area are enlarged in the insert.

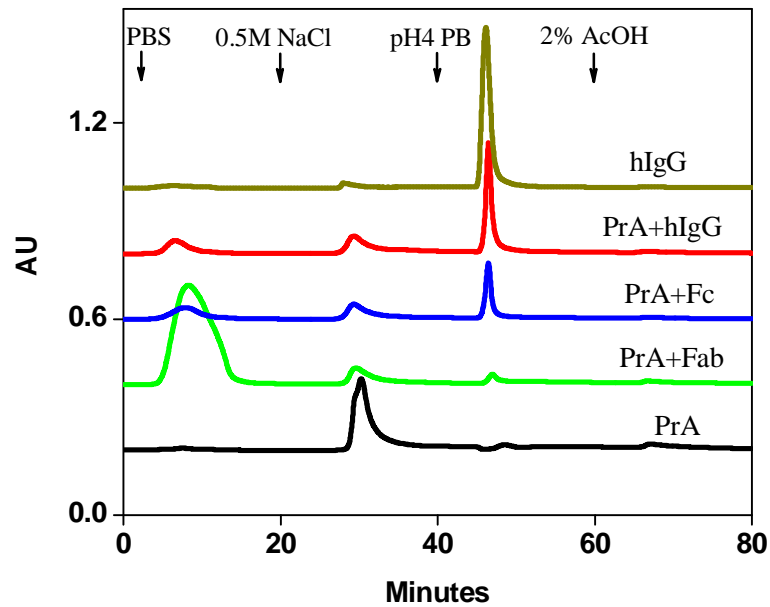
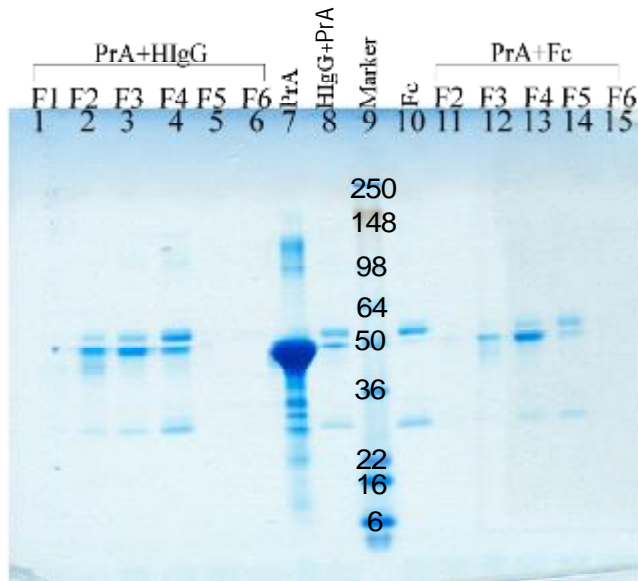


Figure 7-6 Chromatograms of Protein A (PrA) mixtures with hIgG and its fragments binding on the HWRGWV column.

The loading concentrations of pure Protein A and hIgG were 1 mg/mL. The molar ratio of Protein A to hIgG and its fragments was 2:1 and the concentrations of hIgG, Fc, and Fab in the mixtures were 1, 0.311, and 0.58 mg/mL, respectively. Fractions were collected in every 10 minutes and analyzed by SDS-PAGE.

B



C

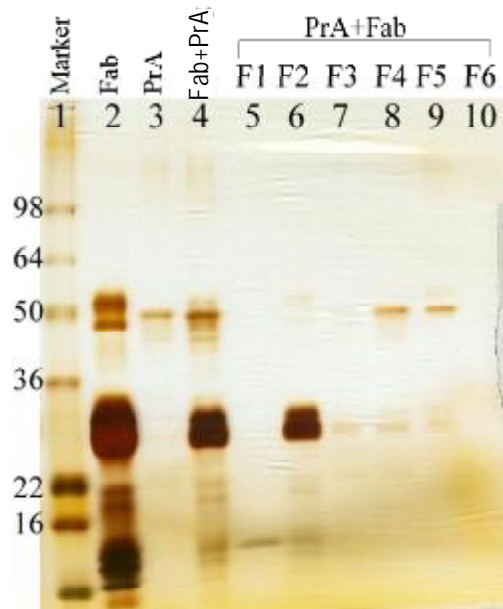


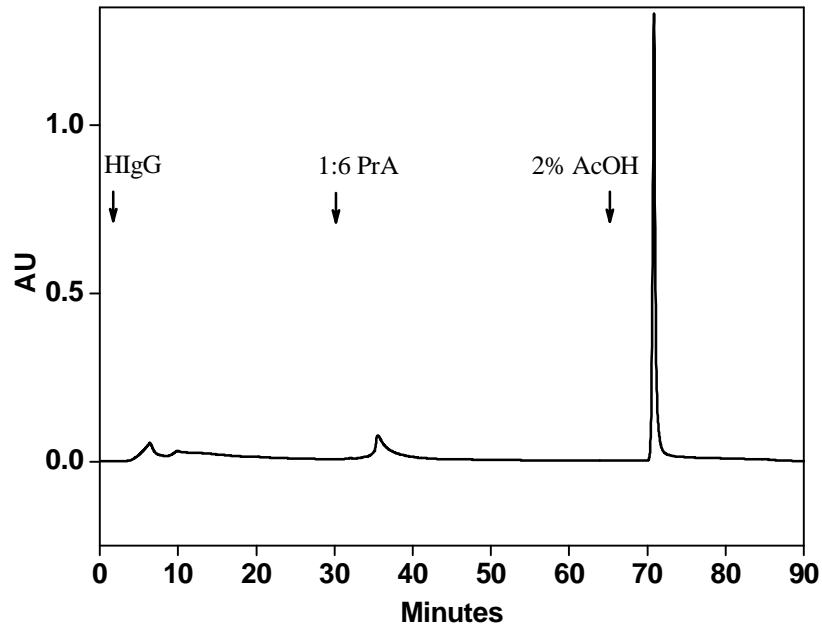
Figure 7-7 SDS-PAGE analyses of the fractions (F) collected in antibody-Protein A (PrA) mixtures run on the HWRGWV column as shown in Figure 7-6.

F1 and F2 were from flow-through, F3 and F4 from 0.5 M NaCl wash, F5 and F6 from pH 4 PB elution, and F7 from 2% AcOH cleaning. The marker in the gels was pre-stained multi-colored standard for SDS-PAGE.

A: Reduced fractions from the PrA+hIgG run and non-reduced samples from PrA+Fc run. Staining: Coomassie Blue; reducing agent: dithiothreitol (DTT).

B: non-reduced samples from the PrA+Fab run. Staining: silver-staining.

A



B

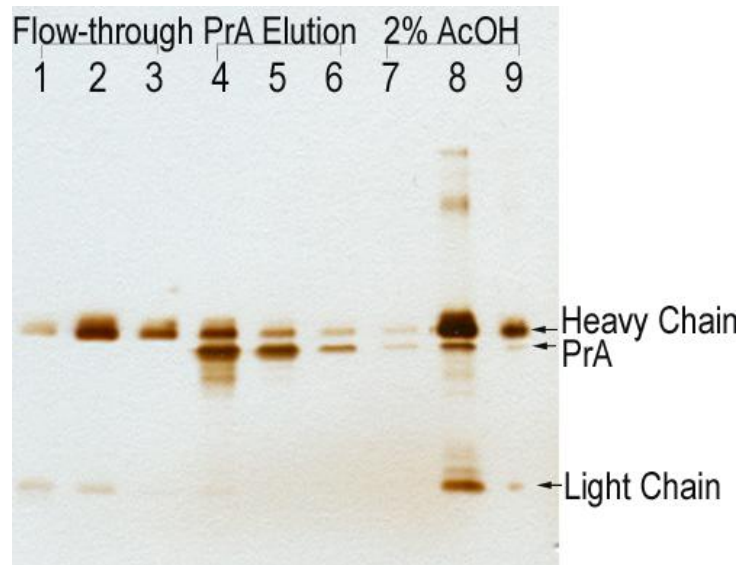
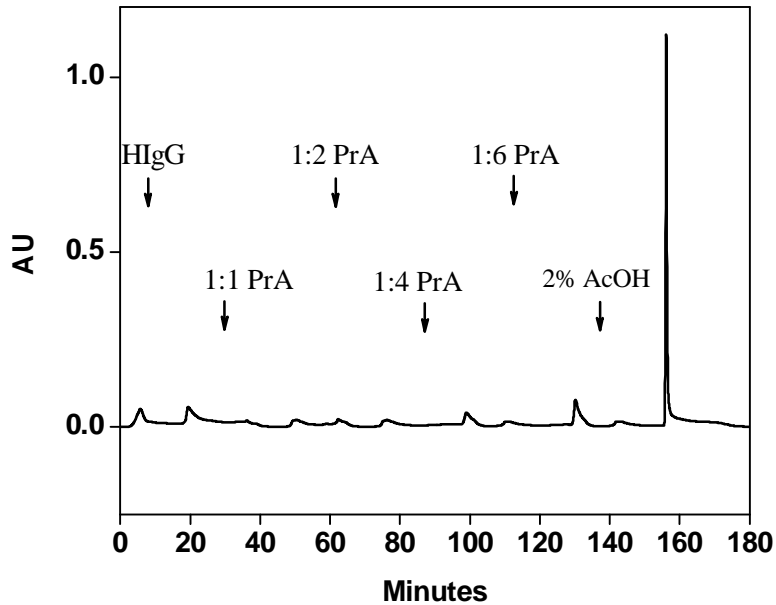


Figure 7-8 (A) Chromatogram and (B) SDS-PAGE gel of HWRGWV-bound hIgG elution by Protein A (PrA) and 2% AcOH.

A: hIgG was injected to the column in PBS + 0.5 M NaCl, washed with PrA in the same buffer, and eluted by 2% AcOH in water. The concentrations of hIgG and Protein A were 1 and 1.68 mg/mL to form a hIgG:PrA molar ratio of 1:6. Fractions were collected every 10 minutes and analyzed in the gel (B).

B: Lanes 1 – 9 are corresponding to the fractions 1 – 9. The gel was silver stained for visualization.

A



B

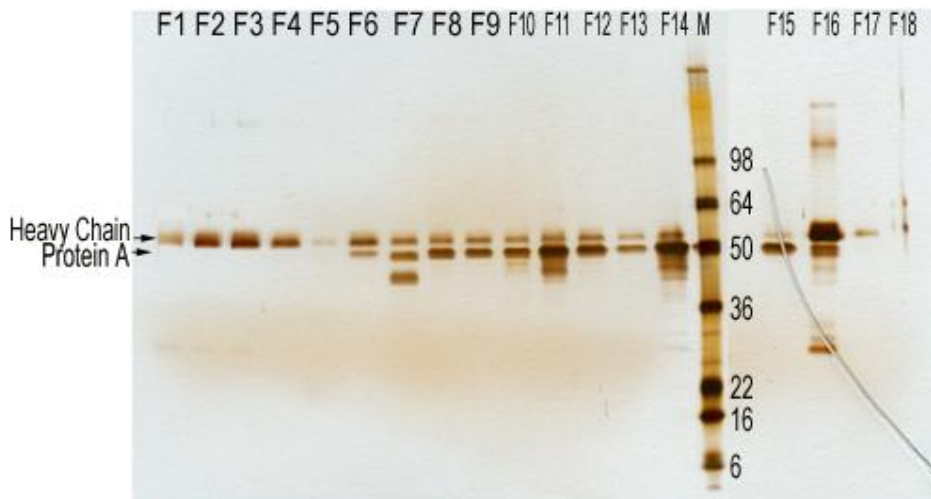


Figure 7-9 Elution of HWRGWV-bound hIgG by Protein A (PrA) at different hIgG to PrA molar ratio in stepwise and 2% AcOH.

A: Chromatogram; The hIgG concentration was 1 mg/mL in PBS + 0.5 M NaCl, and Protein A in the same buffer was applied in 1, 2, 4, and 6 times moles of the amount of hIgG.

B: Corresponding SDS-PAGE gel. F1 – F18 represent the fractions collected every 10

minutes, where F1 – F3, F4 – F6, F7 – F9, F10 – F12, F13 – F15, and F16 – F18 correspond to flow-through, 1:1PrA, 1:2 PrA, 1:4 PrA, 1:6 PrA, and 2% AcOH elutions, respectively.

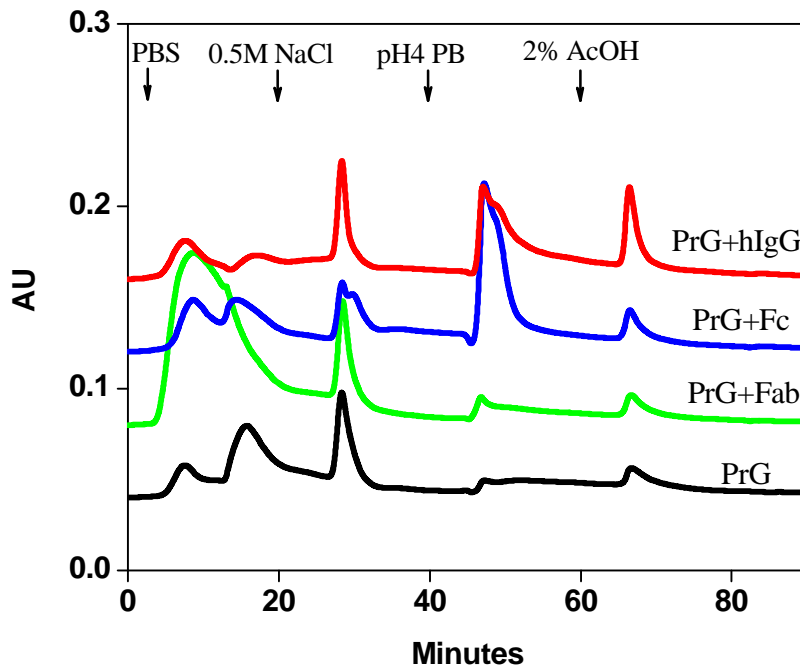


Figure 7-10 Chromatograms of Protein G (PrG) mixtures with hIgG and its fragments on the HWRGWV column.

The loading concentration of pure Protein G was 1 mg/mL. The molar ratio of Protein G to hIgG and its fragments was 5:1 and the concentrations of hIgG, Fc, and Fab in the mixtures were 1, 0.5, and 0.58 mg/mL, respectively. Fractions were collected in every 10 minutes and analyzed by SDS-PAGE.

A



B

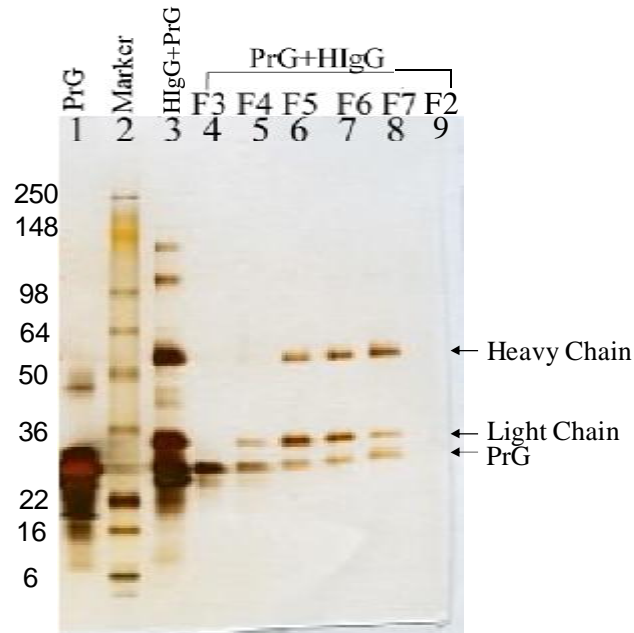
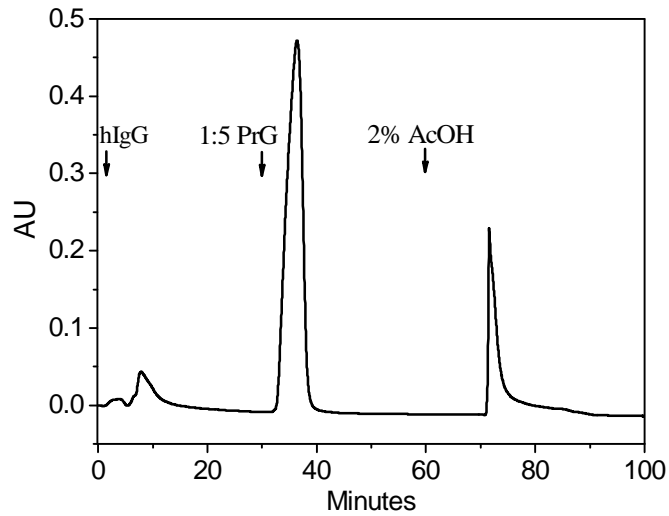


Figure 7-11 SDS-PAGE analyses of the fractions (F) collected in (A) PrG+Fc and PrG+Fab runs and (B) PrG+hIgG run on the HWRGWV column as shown in Figure 7-10. F1 and F2 were from flow-through, F3 and F4 from 0.5 M NaCl wash, F5 and F6 from pH 4 PB elution, and F7 from 2% AcOH cleaning. The marker in the gels was pre-stained multi-colored standard for SDS-PAGE. Fc' is the single chain of Fc. Staining: silver-staining;

reducing agent: dithiothreitol (DTT).

A



B

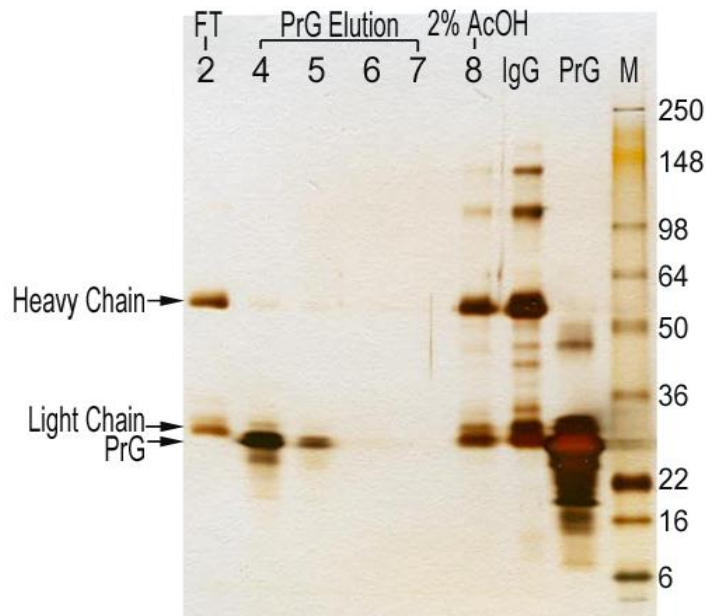


Figure 7-12 (A) chromatogram and (B) SDS-PAGE gel of HWRGWV-bound hIgG elution by Protein G (PrG) and 2% AcOH.

A: hIgG was injected to the column in PBS + 0.5 M NaCl, washed with PrG in the same buffer, and eluted by 2% AcOH in water. The concentrations of hIgG and Protein G were 1 and 1.13 mg/mL, generating a hIgG:PrG of 1:5 by molar. Fractions were collected every 10 minutes and analyzed by SDS-PAGE (B)

B: Lanes 2 – 8 corresponding to the fractions 2 – 8. The gel was silver stained for

visualization. FT and M refer to flow-through and marker, respectively.

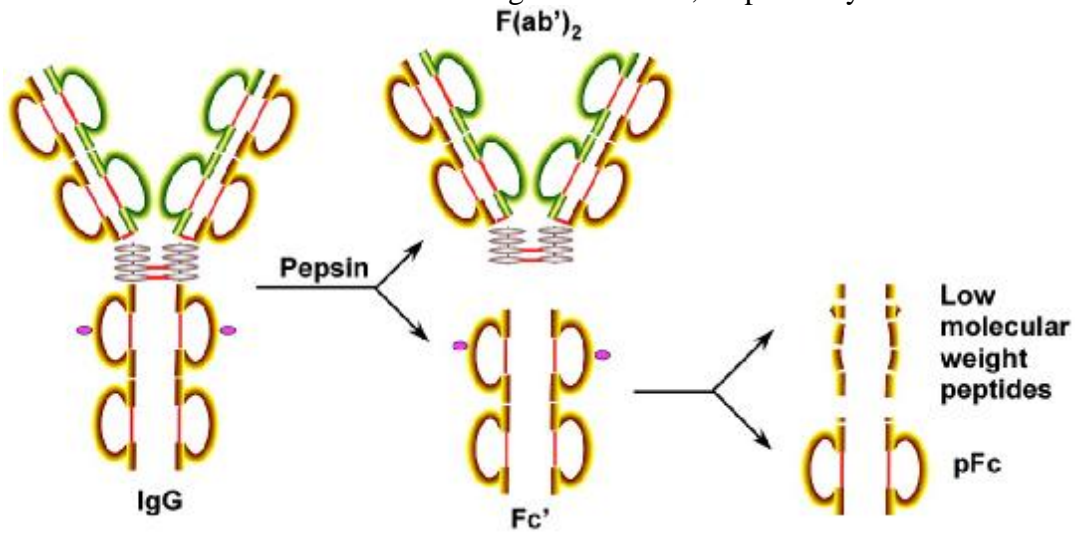


Figure 7-13 Pepsin cleavage sites of human IgG1.

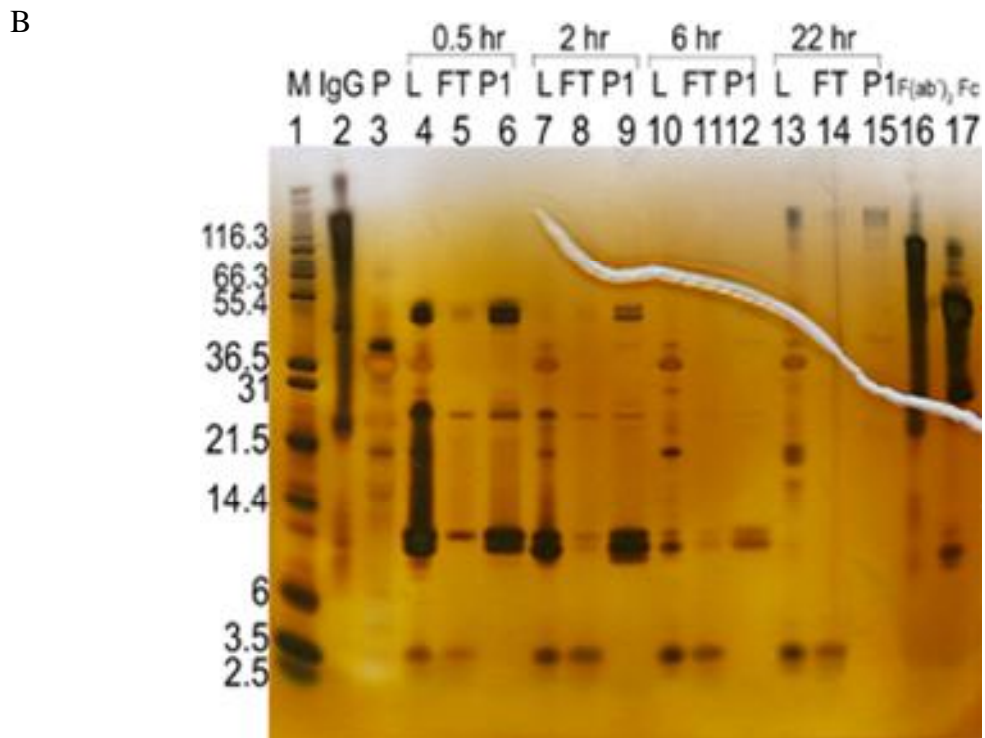
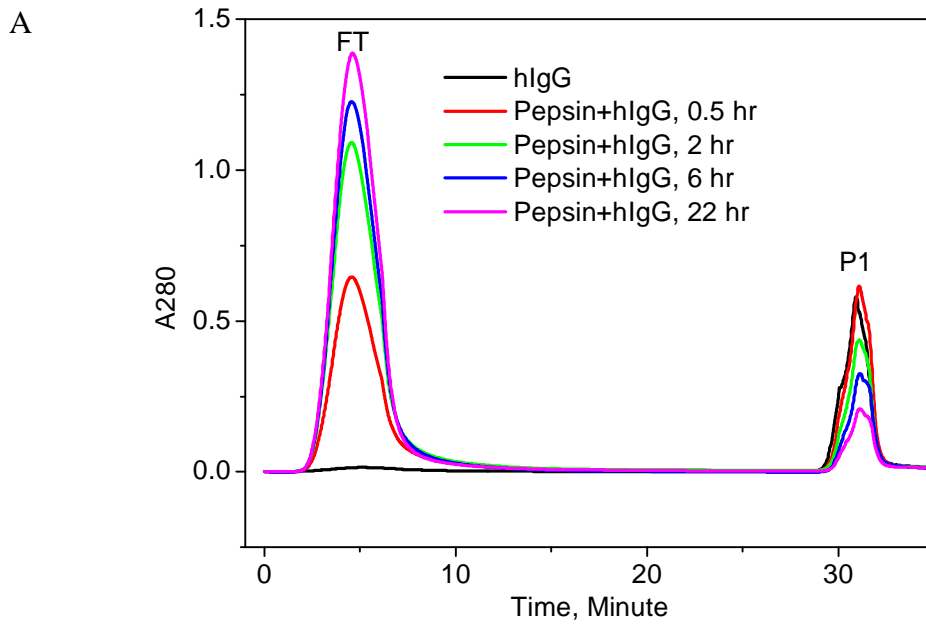
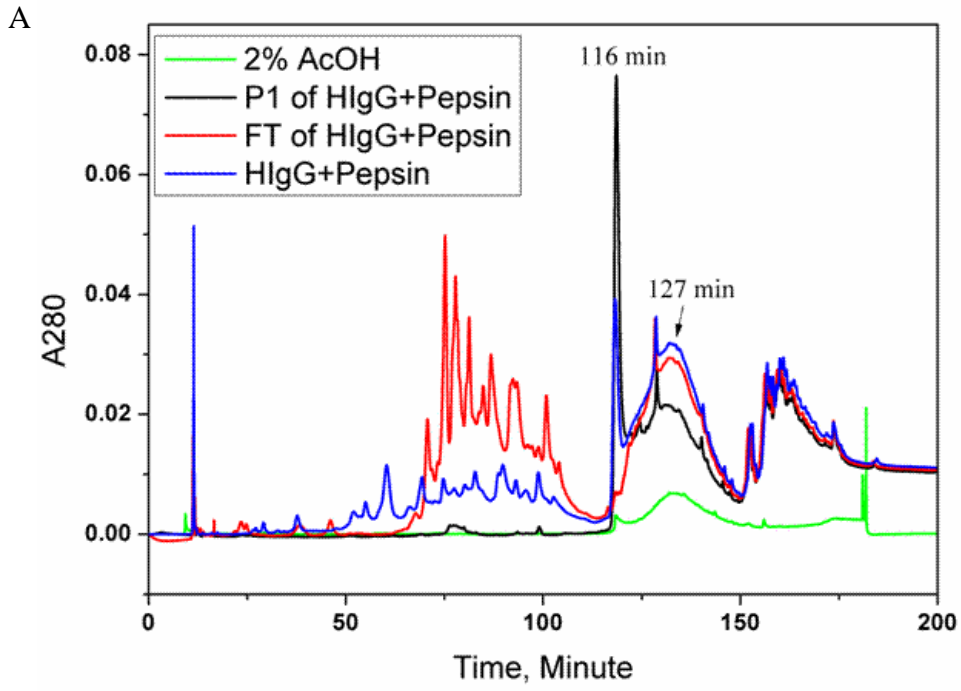


Figure 7-14 (A) Loading of peptic digests of hIgG at variable incubation time on an HWRGWV column and (B) the corresponding non-reducing SDS-PAGE gel.

A: Column volume: 0.1mL; loading buffer: PBS; elution buffer: 2% AcOH; FT: flow-through peak; P1: elution peak.

B: M: Mark12 protein standard; P: pepsin; L: loading of digests at different incubation time

points indicated.



B

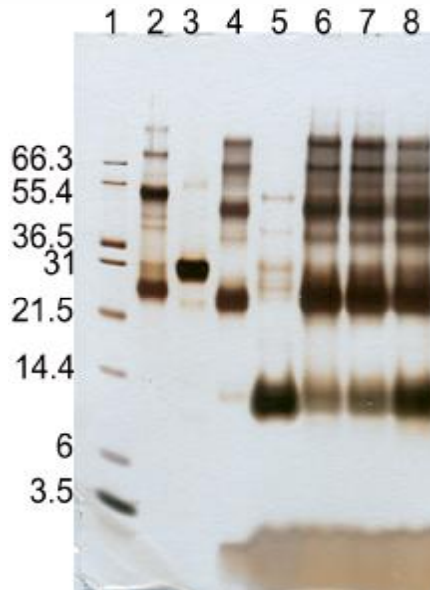
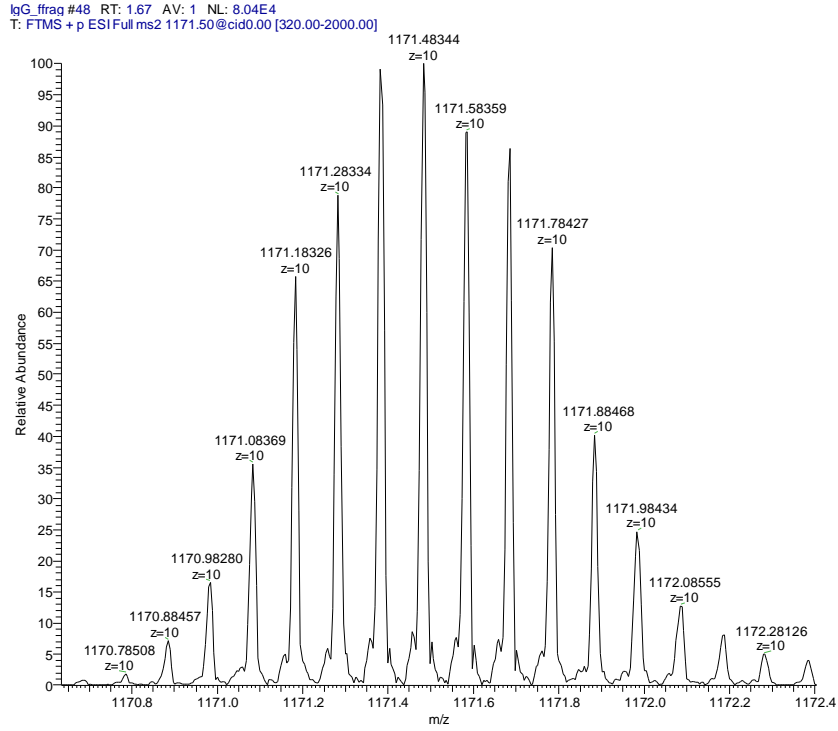


Figure 7-15 (A) RP-HPLC separation of the fractions from pepsin digestion of hIgG and (B) reduced SDS-PAGE gel of RP-HPLC peaks at 116 and 127 min when the HWRGWV-bound fraction (P1) was loaded.

A: 2% AcOH (Black line) is the baseline for the P1 analysis (Brown line) since the bound fragments were eluted from HWRGWV by 2% AcOH.

B: Lane 1: Marker; lane 2: hIgG; lane3: Fc; lane 4: F(ab')₂ ; lane 5: peak 116; lanes 6-8: peak 127 collected in three fractions.

A



B

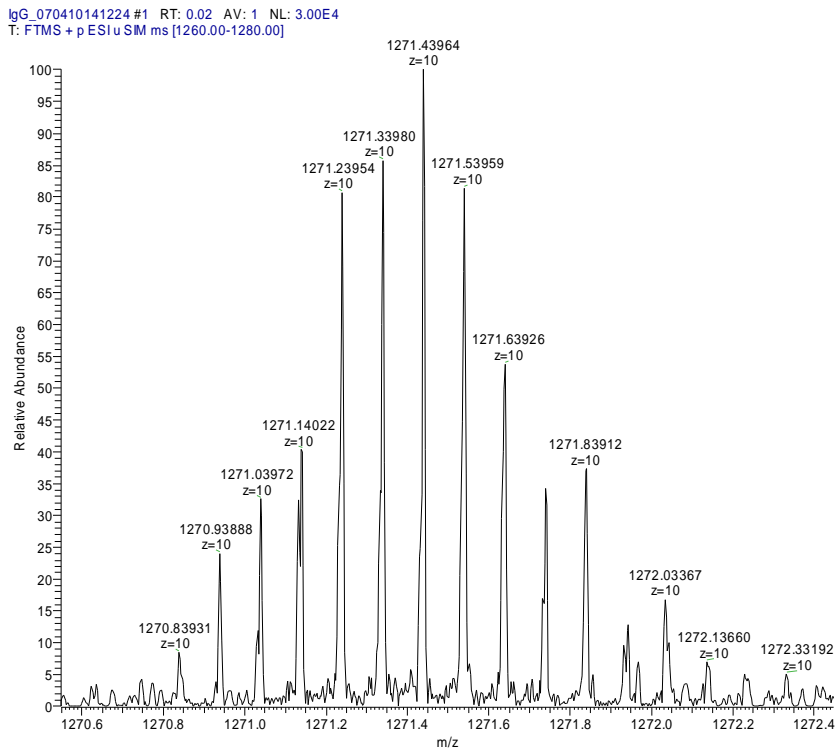


Figure 7-16 Intact MS of HWRGWV-bound species from peptic digest of hIgG. Species with amino acid residues from (A) Glu333 – His435 and (B) Leu79 – Leu194 were identified.

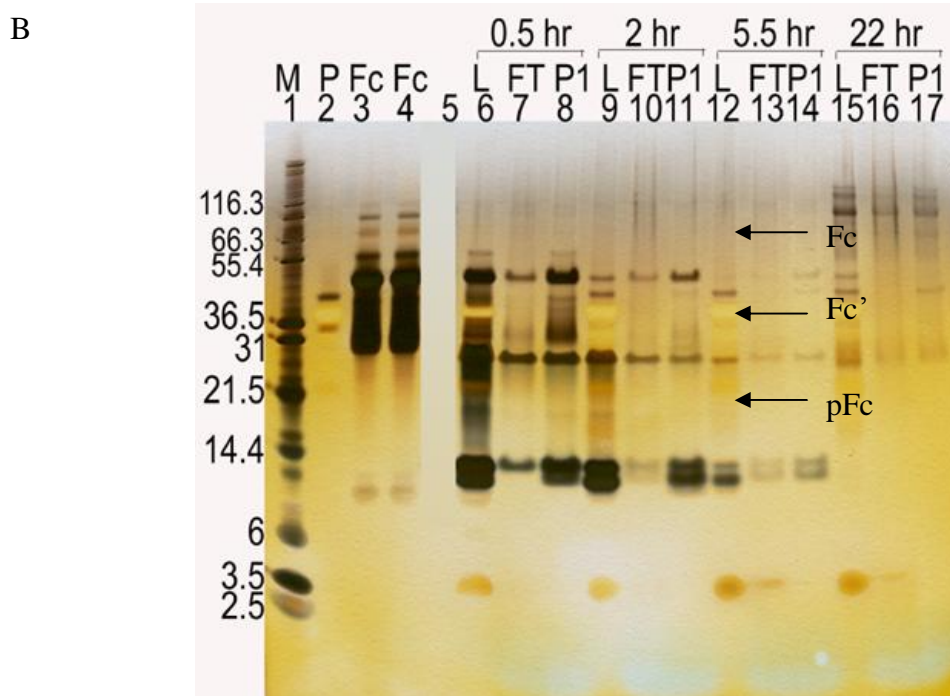
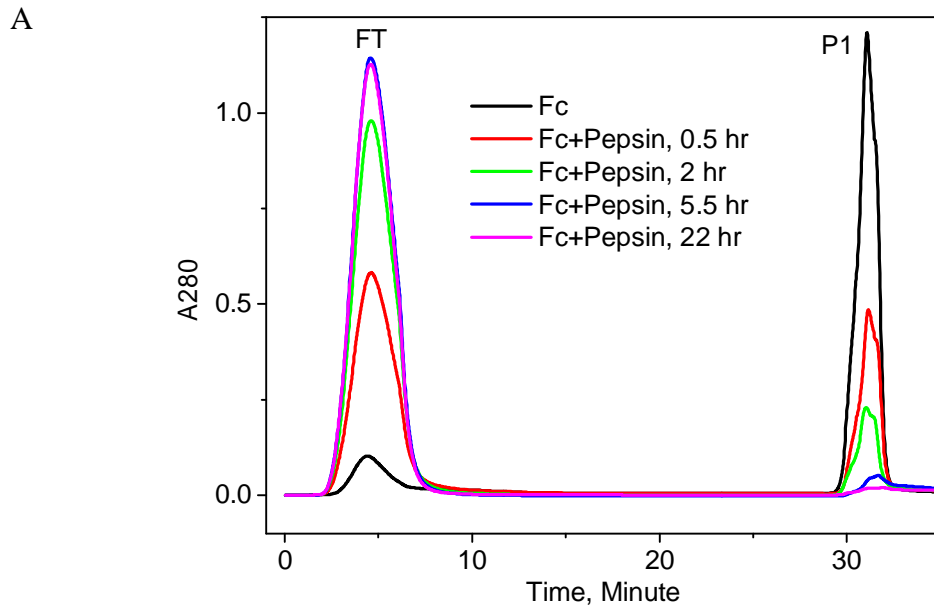


Figure 7-17 (A) Loading of peptic digests of Fc at variable incubation times on HWRGWV and (B) corresponding SDS-PAGE gel under non-reducing conditions.

A: Column volume: 1mL; loading buffer: PBS; elution buffer: 2% AcOH; FT: flow through peak; and P1: elution peak.

B: M: Mark12 protein standard; P: pepsin; L: loading of digests at different times indicated.

Lane 5 was blank.

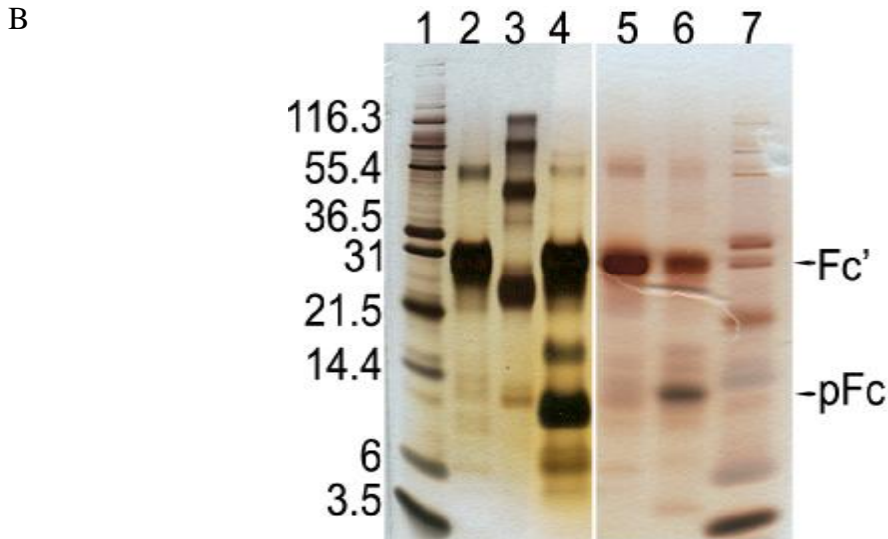
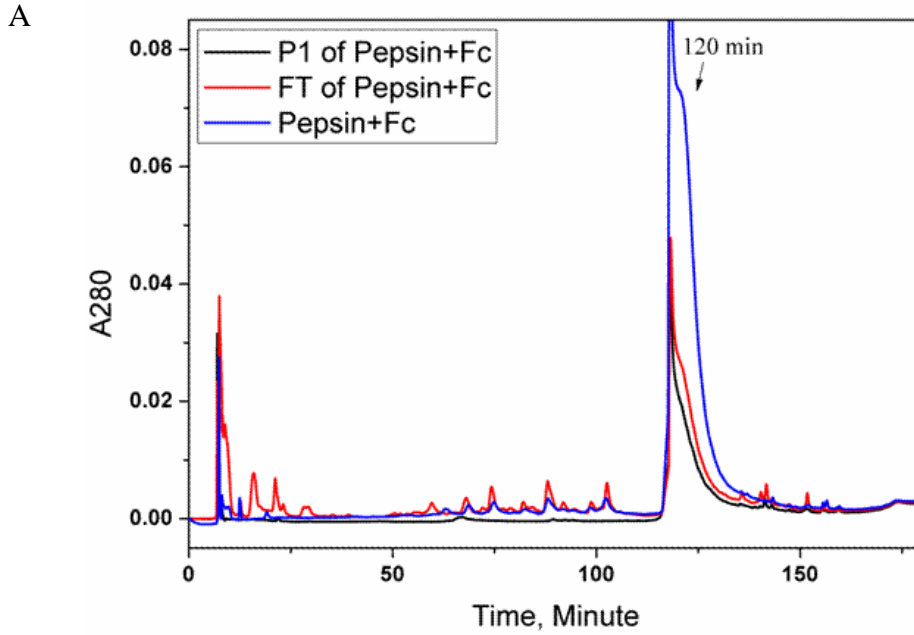
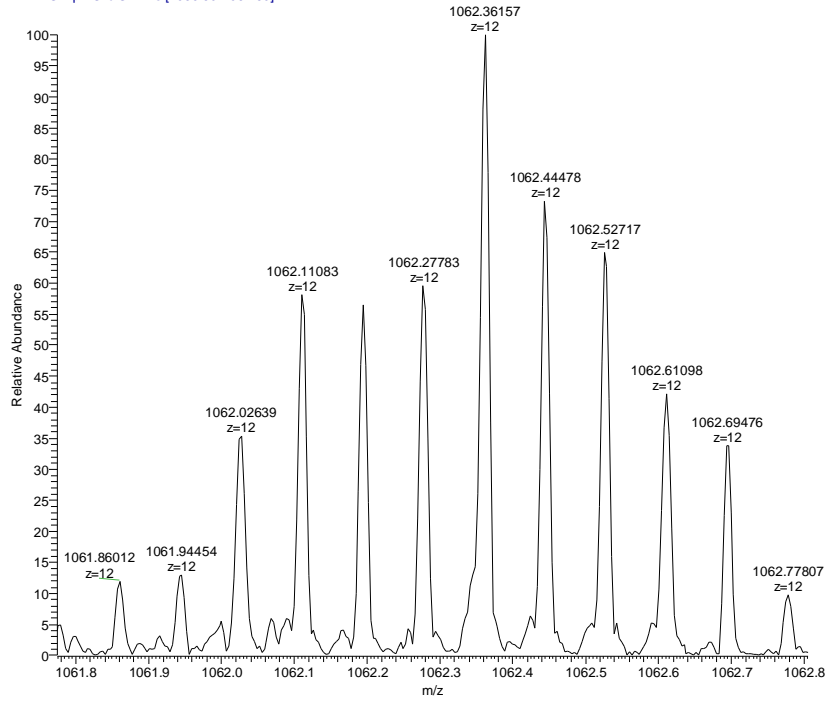


Figure 7-18 (A) RP-HPLC of pepsin digestion of Fc, HWRGWV- bound (P1) and unbound (FT) fragments of the digestion and (B) reduced SDS-PAGE gel of peaks at 120 min from RP-HPLC runs of P1 and FT fragments.

For the gel: Lanes 1&7: molecular weight marker; lane 2&5: Fc fragment of hIgG; lane 3: F(ab')₂ fragments of hIgG; lane 4: Peak at 120 min of the FT fraction run on RP-HPLC; lane 6: peak at 120 min of P1 fraction run on RP-HPLC.

A

IgG_FC_P1_SIM1062#1 RT: 0.00 AV: 1 NL: 9.12E4
T: FTMS + p ESIu SIM ms [1059.50-1064.50]



B

IgG_FC_#107 RT: 2.06 AV: 1 NL: 3.07E4
T: FTMS + p ESI w SIM ms [1000.00-1200.00]

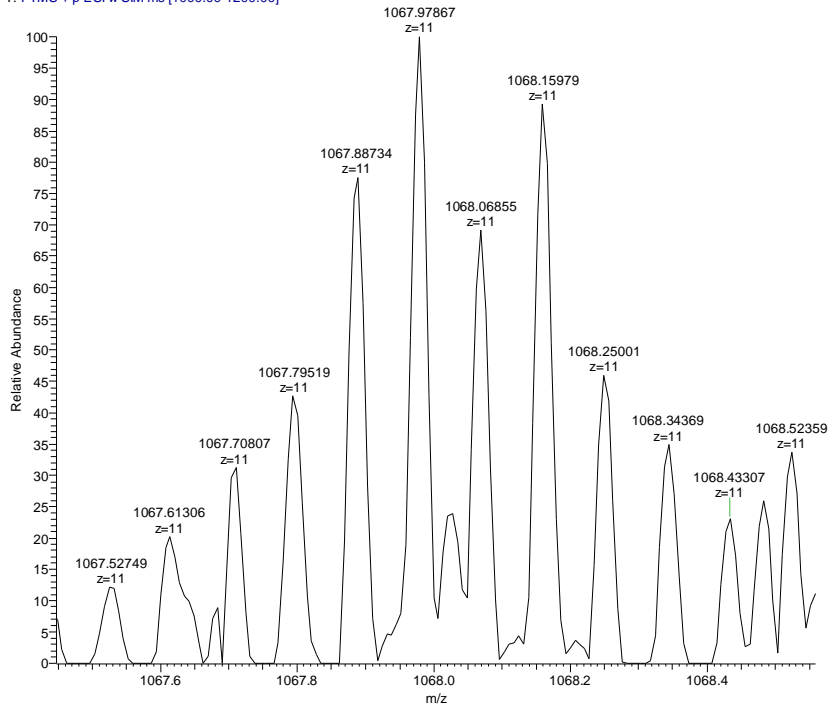


Figure 7-19 Intact MS of HWRGWV-bound species from peptic digest of Fc. Species with amino acid residues from (A) His334 – Gly446 and (B) Lys334 – His435 were

identified.

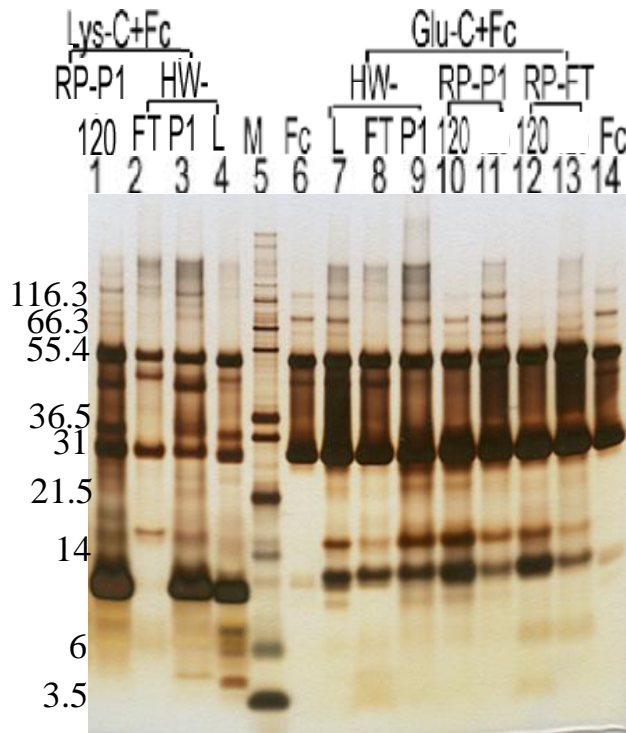


Figure 7-20 Non-reducing SDS-PAGE gel of the Fc digested separately by endoproteinases Lys-C and Glu-C.

HW- means the digestions (L) separated on an HWRGWV column and the unbound flow through (FT) and bound peak (P1) fractions that were collected. RP-P1 and RP-FT stand for the RP-HPLC analyses of the P1 and FT peaks collected from the HWRGWV column. The retention times of the RP-HPLC peaks in lanes 1, 10, and 12 are 120 min. Lanes 11 and 13 are the tails of the peaks at 120 min in lanes 10 and 12 respectively.

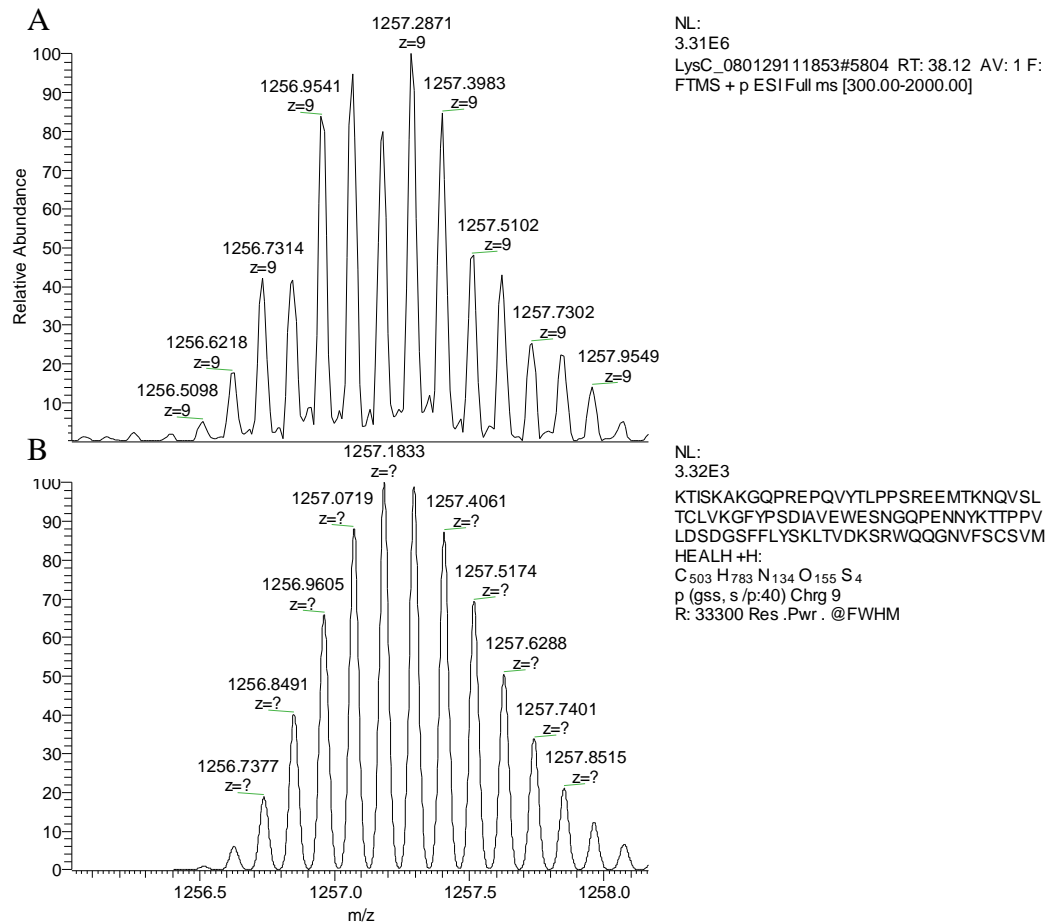


Figure 7-21 MS of the HWRGWV-bound piece from Lys-C digestion of Fc fragment.
A: From a Lys C sample; B: Simulation of the sequence found utilizing FindPept on
ExPASy.org with a difference of about 4 ppm.

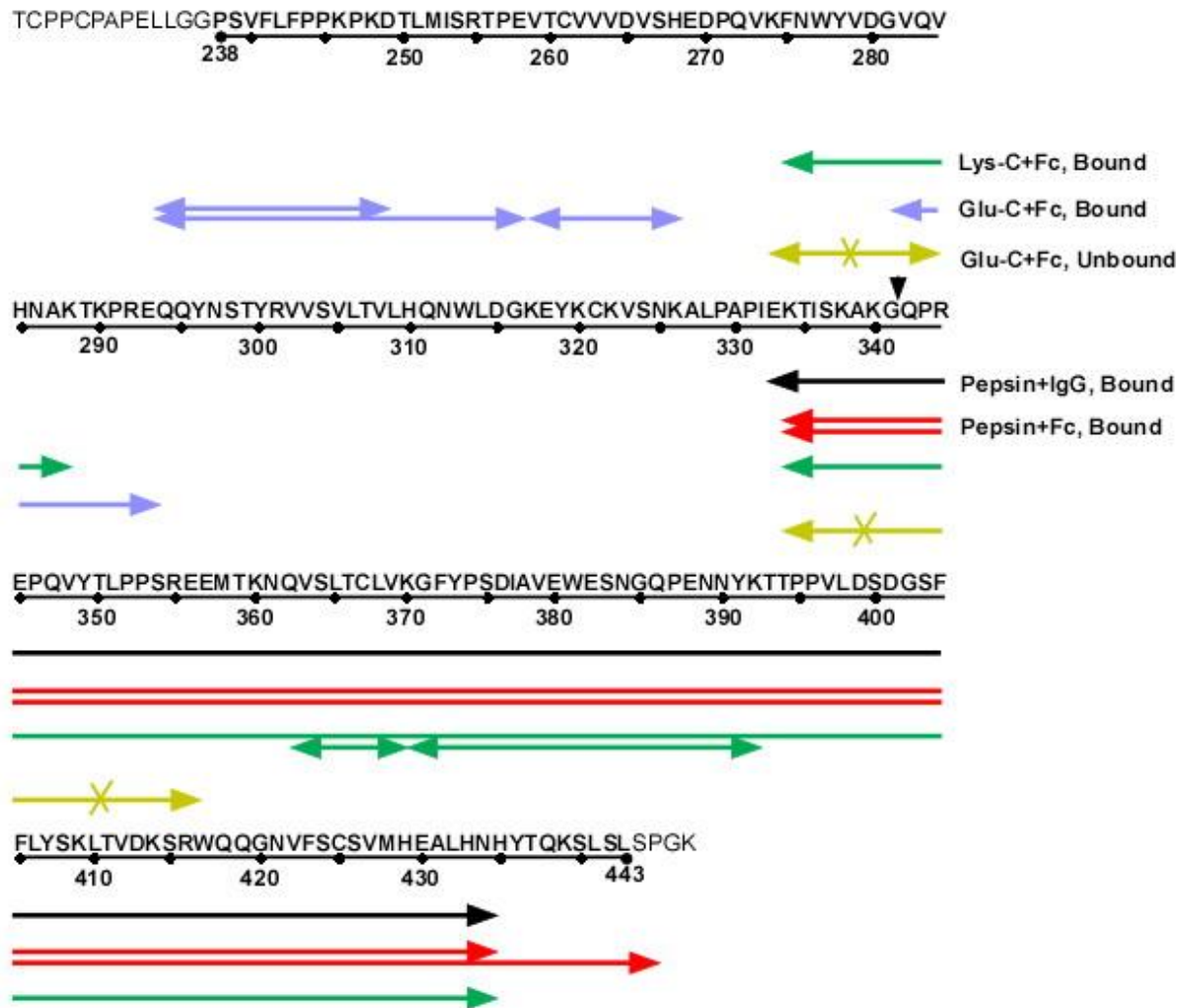
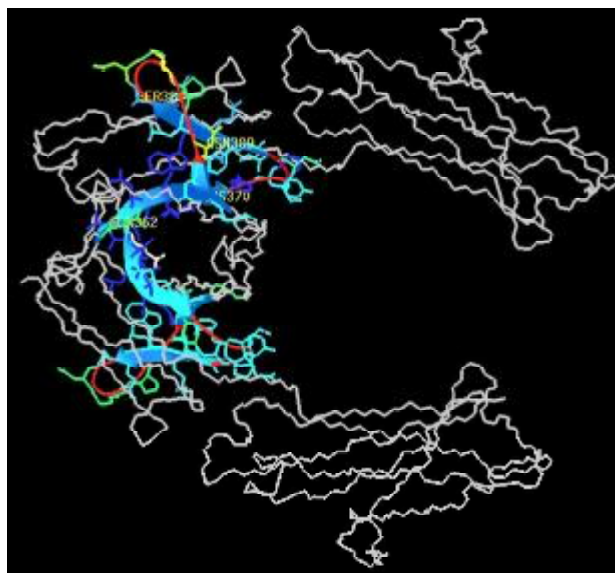


Figure 7-22 Alignment of peptide segments obtained from proteinase cleavage of hIgG or its Fc fragment.

The Fc sequence and numbering are from PDB 1FCC. HWRGWV- bound and unbound species of pepsin, Lys-C, and Glu-C digestions are indicated with arrowed lines of different colors. The black arrow between 341 and 342 residues indicates the separation point of C_H2 and C_H3 domains.

A



B

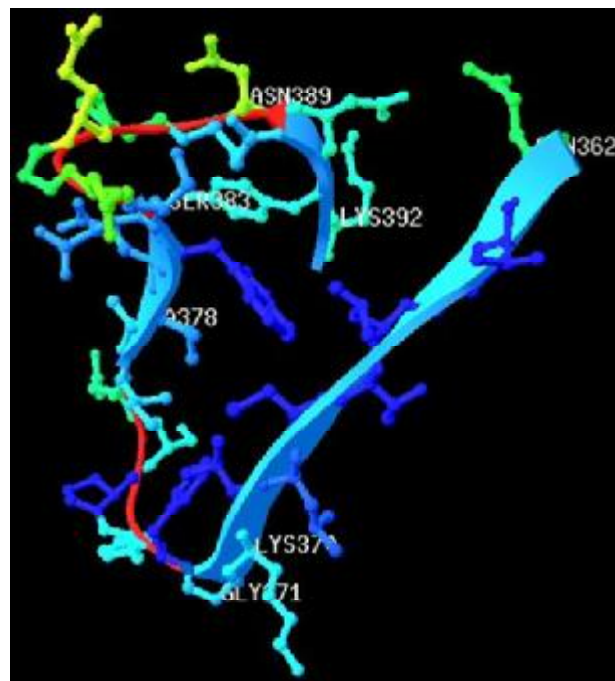


Figure 7-23 (A) the position of segments Gln362 – Lys370 and Gly371 – Lys392 in the Fc portion of hIgG (PDB: 1FCC) and (B) solvent accessibility of the amino acid residues in these two segments.

Stands and coils are depicted in blue and red, respectively. The backbones of the polypeptide chains other than in Gln362 – Lys392 region are in gray; the amino acid residues in this region are colored according to the solvent accessibility where the color from dark blue to

light blue to green attributes to the residues of fully berried to highly exposed. Images were produced using Swiss-PdbViewer V4.0.

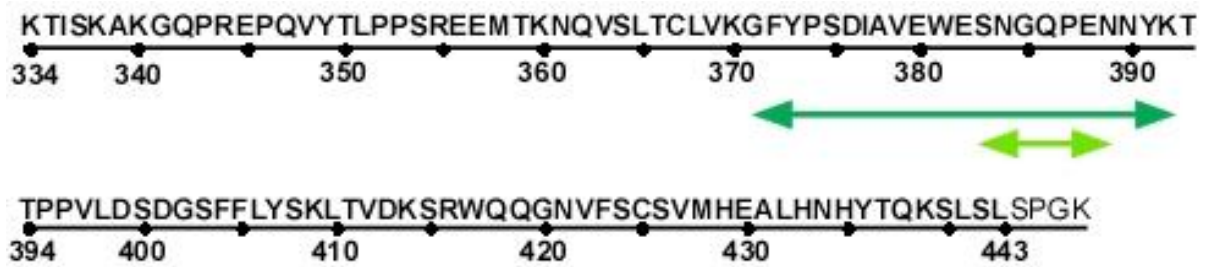


Figure 7-24 amino acid sequence of pFc, HWRGWV binding segment (dark green line) and the loop (light green line) possibly in contact with the ligand.

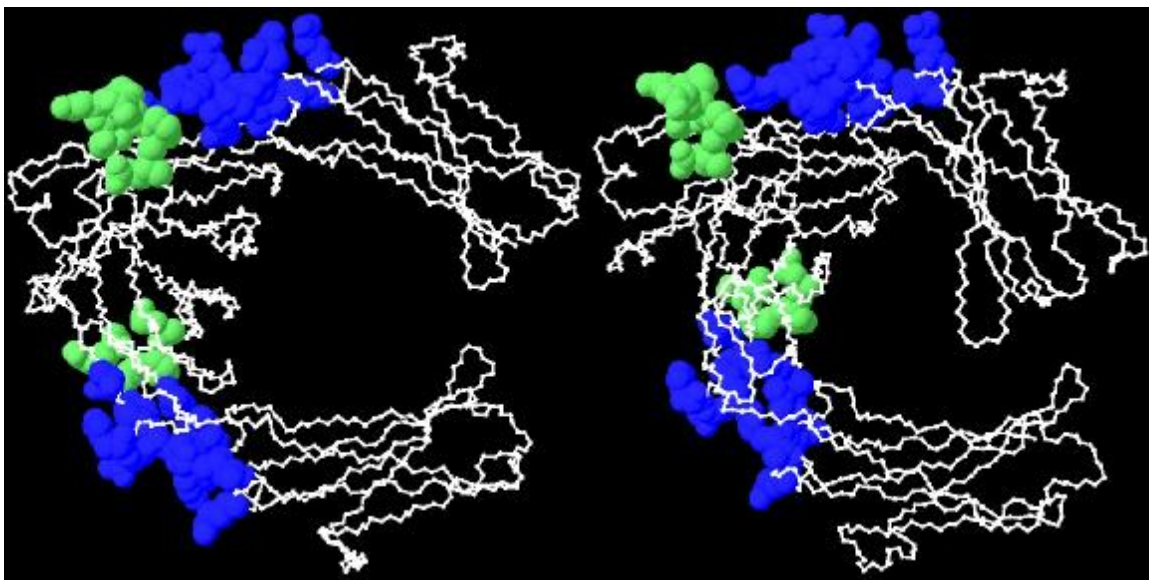


Figure 7-25 View from different angles of the HWRGWV binding loop (green) and Protein A and Protein G binding site (blue) in 3-D structure of Fc.

Images were produced using Swiss-PdbViewer V4.0.

Chapter 8. Conclusions and recommendations

8.1 Conclusions

8.1.1 Fc-binding peptides identified by screening a solid-phase library using Fc as the target

Radiolabeled Fc was employed as the target during the screening of a solid phase combinatorial hexamer peptide library in order to identify an Fc-specific ligand able to broadly recognize all antibody isotypes and Fc-fusion proteins. Homogenous peptides were identified with the histidine as the N-terminal amino acid in all sequences. The histidine side chain is believed to play an important role in the protein-ligand interactions. However, the selectivity of the ligands to hIgG depends on the entire peptide sequence rather than a single amino acid residue since HAAAAA had no ability to retain hIgG. Also truncations and modifications of the positive peptide leads bound less hIgG than their original hexamers. Among the positive ligands, a family with a specific composition from the N-terminus: His + aromatic amino acid(s) + positively charged amino acid(s) exhibited the ability to selectively bind Fc over Fab fragments of hIgG, indicating the success of the screening strategy where the desired binding-portion of a protein were used as the target. The family consists of three peptides, HWRGWV, HFRRHL, and HYFKFD. Their specificity to the Fc fragment of hIgG makes them potential replacements for Protein A.

8.1.2 HWRGWV selected as the best ligand for study

HWRGWV, HFRRHL, and HYFKFD demonstrated similar protein binding properties. They retained most of the Fc and hIgG available, but little Fab and F(ab')₂ at a ligand density of about 0.1 meq/g. The dissociation constants and maximum binding capacities for hIgG fell in the range of 10⁻⁵-10⁻⁶ M and 27.0 - 33.6 mg/mL-drained resin, respectively. When hIgG spiked in cell culture media containing 10% fetal calf serum (cMEM) was applied to the columns packed with the peptide ligands, similar yields and purities were obtained. However, HWRGWV exhibited slightly higher purity and yield at this condition and stood out in the secondary screening and the chromatographic separation of hIgG when the feed was loaded in a high-salt solvent.

8.1.3 Antibody binding and isolation characteristics of HWRGWV

The Fc-binding peptide HWRGWV has a broad binding spectrum, being able to adsorb all subclasses of hIgG, all mammalian IgG tested (including the species of rabbit, bovine, goat and mouse), chicken IgG, human serum IgM, IgD, IgE and to a less extent human secretory IgA. The adsorption and desorption profiles of these antibodies are very similar except for IgA and IgM which displayed broader elution peaks. Human IgG adsorption to HWRGWV is independent of its glycosylation. Also, Protein A and Protein G showed no competition with HWRGWV in binding hIgG. It is possible that HWRGWV binding site on the Fc portion is localized in Gly371 – Lys392 of the C_H3 domain and interact with the amino acids SNGQPEN in the loop structure of Ser383 – Asn389.

Unlike the ligands selected through screening of phage-displayed libraries which usually lost the affinity to the targets upon immobilization, HWRGWV identified from a solid-phase technique demonstrated great ability to purify hIgG from cMEM as an affinity ligand. Using HWRGWV-modified Toyopearl resins as the affinity media, both the purity and the yield obtained could be as high as 95% in one step when hIgG concentration in cMEM was 10 mg/mL. Operation temperature and N-terminal acetylation of HWRGWV have insignificant influence on both adsorption and isolation of hIgG from cMEM. Increasing the peptide density of HWRGWV improves its binding affinity to hIgG but decreases the specificity to Fc fragment, while barely affecting the binding capacity for hIgG and isolation of IgG from cMEM. Using the ligand with a density of around 0.1 meq/g can simultaneously retain its selectivity to Fc and appropriate affinity for binding. The initial hIgG concentration in the feed influences both the purity and recovery of the product. The feed applied to the column should have an antibody concentration larger than 1 mg/mL to achieve an efficient separation. The ligand maintained the binding ability for hIgG when changing the solid support from Toyopearl AF-Amino 650 to Sepharose CL-4B. HWRGWV-bound Toyopearl also displays promising results in our initial study to separate hIgG from Cohn paste II+III where a yield of 82.1% and a purity of 77.3% were obtained in one step.

8.2 Recommendations for future work

8.2.1 HWRGWV ligand

Its ability to isolate antibodies from cell culture supernatants should be examined. Meanwhile, the adsorption of DNA, lipid, host cell proteins to the ligand should be determined, as well as its ability to bind virus. It should be also established the column cleaning and sanitization procedures. Generally, 4 M guanidine hydrochloride, 4 M urea followed by 70% ethanol with 2% AcOH (1), 0.1 M sodium hydroxide (2), and Triton X-100 (3) could be the candidates for a peptide column regeneration.

It might be possible to use HWRGWV to remove the abundant proteins in plasma. More than 90% the mass of plasma proteins is constituted by albumin, Igs, haptoglobin, antitrypsin, and transferrin (4). Special attention has been paid to simultaneously remove these abundant proteins in plasma in proteomic research for new biomarker discovery (5). HWRGWV might be able to serve the purpose since it demonstrated the ability to bind BSA and IgG, the most abundant proteins in plasma, as well as some other proteins in plasma.

The ligand might be used to separate Fab and Fc from a feed since the selectivity of the ligand to Fc decreases with increasing peptide density. The separation might be done by passing the feed stream sequentially through two HWRGWV columns packed with low density and high density resins, respectively.

The binding site study done in this work provides some evidence that the HWRGWV binding site may be localized in the C_H3 domain of hIgG. Other investigations can be conducted to complete the study. The ability of HWRGWV to not retain a single C_H2 domain of hIgG should be examined in order to exclude this portion for HWRGWV binding. Overlapped peptide segments from pFc should be produced and investigated for their ability to interact with HWRGWV to confirm the binding site location. In addition, competitive binding between Fc and HWRGWV retained segments of pFc may provide some information about the of 3-dimensional structure of a peptide fragment on interactions between HWRGWV and hIgG.

8.2.2 Peptide ligand stability

Peptide ligands have been identified in numerous researches for separation of biological molecules (6, 7). However, most of them are prone to degradation with the presence of enzymes and strong basic chemicals, which limit their application in industry. There are several ways to improve the stability of a peptide ligand. Using D- amino acids to construct the peptide can improve the proteolysis resistance of the ligand (3). Cyclic peptides will be another choice. Cyclic peptides are more resistant to protease degradation due to the lack of conformational freedom and display high affinity (8). Using the technique to produce cyclic peptides, it might be possible to cross-link a peptide ligand to increase the stability. Instead of using amino acids, their chemical analogs could be employed to improve the ligand compatibility with NaOH, a common agent for column cleaning and sanitization (9, 10).

8.3 References

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- (10) www.gehealthcare.com/protein-purification. (2006) Use of sodium hydroxide for cleaning and sanitizing chromatography media and systems.

Appendix 1. Comparison of HWRGWV resins from different synthesis batches

A1.1 Introduction

Four batches of HWRGWV have been obtained from Peptides International (Louisville, KY) at a ligand density of about 0.1 meq/g. The ligand was synthesized directly on Toyopearl AF-Amino-650 M (average particle size 65 μm , Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry. Despite the fact that all resin batches were produced following the same methodology, different performances were observed during binding and isolation of human IgG from mammalian cell culture media (cMEM). This appendix is aimed to directly compare the performance of HWRGWV resins from different batches.

A1.2 Experimental

A1.2.1 Adsorption isotherm measurement

The methods for adsorption isotherm measurement and purity and yield calculations were the same as those described in Chapter 4 (section 4.2.2).

A1.2.2 Breakthrough curve construction

The breakthrough curves were obtained on a Waters 626 LC system with a UV detector (Waters, Milford, MA). Columns of 0.1 ml (30 mm \times 2.1 mm (ID), Alltech, Deerfield, IL) were dry packed, swelled with 20% methanol, washed and equilibrated with PBS. Human IgG in PBS at 1 mg/mL was applied to the columns at different flow rates of 0.05 – 0.5 mL/min. The dynamic binding capacity (DBC) was calculated at 5% of breakthrough.

A1.2.3 Chromatographic separation of hIgG

Two chromatographic conditions have been used: loading in PBS or high salt (PBS + 1M NaCl). The high salt loading condition was the same as that described in Chapter 4 (section 4.2.4) for all batches tested. The PBS loading condition was similar to that described in Chapter 3 (section 3.2.7) with a few variations for batch #4. The salt wash started at 50 min for the batch #4 instead of at 20 min. CMEM without tryptose phosphate broth (TPB)

was used for the batch #4 at this loading condition while cMEM with 5% TPB was used for the batch #1.

A1.3 Results and discussion

The experiments that have been carried out on the HWRGWV resins are summarized in Table A1-1. Three batches of Toyopearl base resins were used in peptide synthesis so far and peptide batches #3 and #4 used the same batch of Toyopearl resins. The HWRGWV resin of batch #2 had a slightly lower ligand density than all others. Since the density variance within a narrow range has insignificant influence on hIgG binding and isolation (Chapter 4), the four batches were compared without considering the density difference. Since batch #3 had very poor chromatography performance, it was not characterized for any other properties.

A1.3.1 Adsorption isotherms

Figure A1-1 shows the adsorption isotherms of batches #1, #2, and #4 measured at room temperature. It can be seen that the batches #1 and #2 are similar in shape, while the batch #4 clearly displayed two phases in hIgG binding. In the case of the first two batches, the data were fitted to a Langmuir model and the fitted static binding capacity (q_m) and dissociation constant (K_d) were tabulated in Table A1-2. For batch #4, the data in the first binding phase were fitted to a Langmuir model and obtained parameters are included in Table A1-2. Also included in this table is the highest amount of hIgG bound to the resin in the second phase, graphically read as 140 mg/g. The K_d values are same for these three batches, within the experimental variance of $\pm 0.2 \times 10^{-5}$ M. The binding capacities for batch #1 and #2 are same but slightly lower than batch #4, considering the experimental variance of about 3 mg/g for q_m . Since all isotherm measurements were done under the same experimental conditions, the different binding behavior of batch #4 might be ascribed to the peptide resin material.

A1.3.2 Breakthrough curves

The breakthrough curves were constructed on the batches #2 and #4 at different flow rates (Table A1-3). The dynamic binding capacity of the batch #4 was only the half of batch #2 at all flow rate examined, less utilization of the total capacity for batch #4 when calculated

according to the static capacity of its first binding phase. This suggests that there are some differences present in the materials of the two HWRGWV resins.

A1.3.3 hIgG isolation from cMEM

Comparing batches # 2, 3, and 4 (Table A1-4) in hIgG isolation from cMEM at exactly the same chromatographic conditions, the purities were higher than 90% in all three batches with batch #4 being the highest at 97%. The variation in yield is significant, ranging from 67% to 97%, with batch #2 the highest and batch #3 the lowest. The lower yield observed for batch #4 than for batch #2 is in agreement with the dynamic binding capacity results. Comparing the chromatograms of batches 2 and 4, the IgG elution peaks (P) are similar while the flow-through peak (FT) on batch #4 is broader (Figure A1-2). It is not clear why such a difference was observed other than the two HWRGWV resins were produced at different time on different batches of Toyopearl base resins.

The chromatographic performance of batches #1 and #4 were tested by loading hIgG spiked in cMEM to the columns in PBS (Table A1-4). Due to the differences in the experimental conditions as described in the method section, the chromatograms are not same (Figure A1-3). However, the same yield was observed with batch #4 when it is compared to batch #1 and the purity on batch #4 was higher (Table A1-4).

A1.4 Conclusions

The variation among the different batches of HWRGWV resins is obvious. There are two reasons that might be accounted for. One is the difference in the individual batch of base matrix and the other the technique to directly synthesize the peptide ligand on the solid support. The peptides on the resins built in this way may vary in both the length and the amino acid composition, which in turn influence the performance of the peptide resins.

A1.5 Acknowledgements

We would like to thank Amith Naik for his test done with the batch #4 resin.

Table A1-1 Four batches of the HWRGWV resins with their synthesys lot numbers, ligand densities, and testing methods.

Batch #	1	2	3	4
Lot #	219424	220043	222201	224281
Ligand density, meq/g	0.1	0.08	0.1	0.11
Batch isotherm	Yes	Yes	No	Yes
Breakthrough curve	No	Yes	No	Yes
Chromatography loading condition for hIgG isolation	PBS	PBS + 1M NaCl	PBS + 1M NaCl	PBS, PBS + 1M NaCl

Table A1-2 Static binding capacity (q_m) and affinity (K_d) calculated by the batch isothermal measurement.

Batch	q_m, mg/g	K_d, ($\times 10^{-5}$ M)	R^2
#1	133.6	1.0	0.99
#2	131.8	0.73	0.98
#4	94.8 ^a /140 ^b	0.36	0.99

- a. Q_m obtained by fitting the data of first binding phase to a Langmuir model
- b. Graphically read the highest amount of bound hIgG in the second binding phase

Table A1-3 Comparison of the dynamic binding capacity (DBC) of the batches #2 and #3 at different flow rate.

Flow rate, mL/min	Velocity, cm/min	DBC at 5%, mg/mL	
		Batch #2	Batch #4
0.5	14.4	3.29	/
0.3	8.67	5.08	2.73
0.2	5.78	7.98	3.7
0.1	2.89	11.48	5.87
0.06	1.73	11.27	/

0.05	1.45	/	6.1
------	------	---	-----

Table A1-4 Purity and yield comparison among different batches of HWRGWV resins where $C_{0,hIgG}$ and CV are the initial concentration of hIgG in cMEM and the column volume, respectively.

Batch	Loading buffer	CV, mL	$C_{0,hIgG}$, mg/mL	Purity, %	Yield, %
#1	PBS	0.6	5	68	65 ^a
#4	PBS	0.6	5	93	63
#2	PBS + 1M NaCl	0.1	10	94.8	97.7±1.0
#3	PBS + 1M NaCl	0.1	10	92.7	67.0±0.4
#4	PBS + 1M NaCl	0.1	10	97.3	80±0.7

a. Calculated by densitometry (see Chapter 3), all others were measured by ELISA

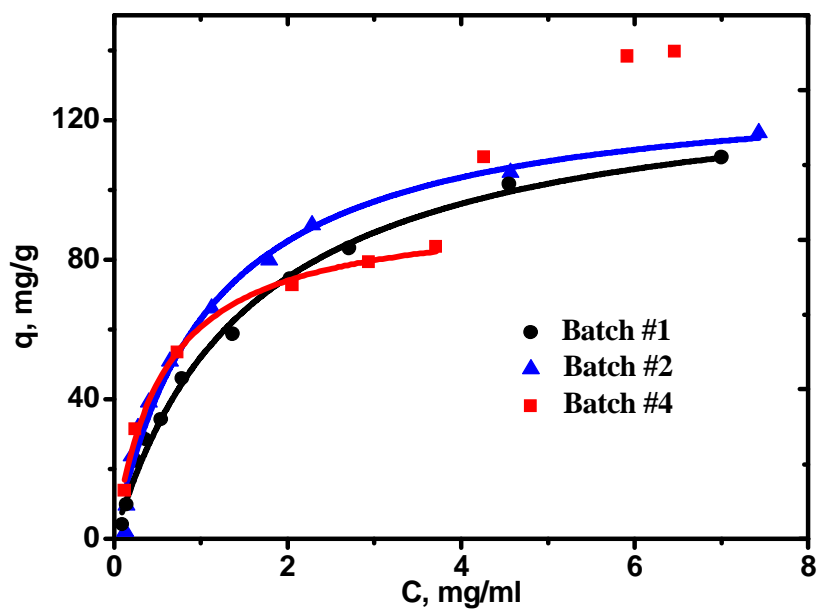


Figure A1-1 hIgG adsorption isotherms on the HWRGWV resins of batches #1, 2 and 4

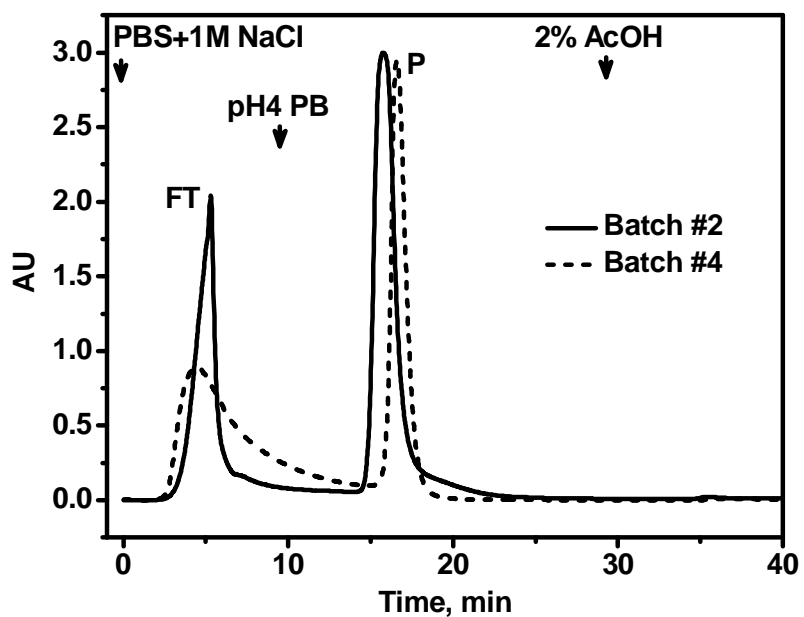
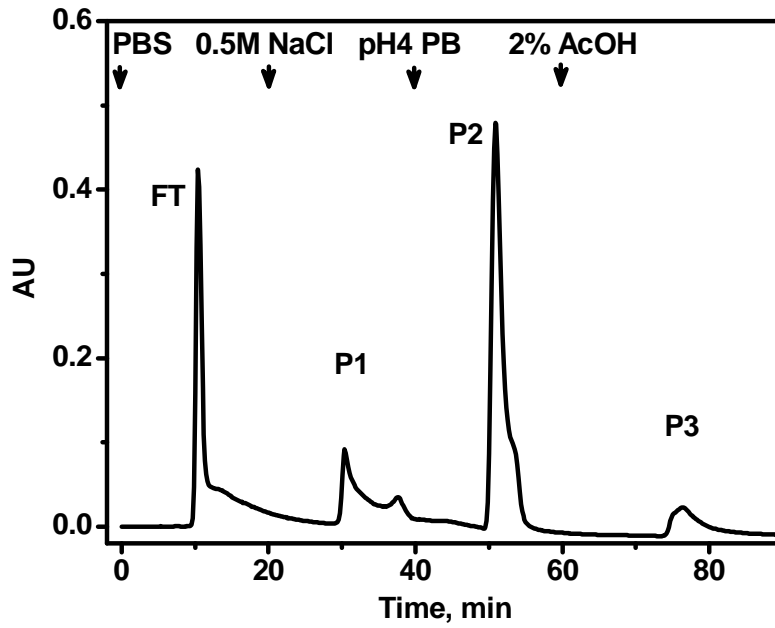


Figure A1-2 hIgG isolation from cMEM on the batches #2 and #4 at the high salt loading condition.

HlgG was loaded in PBS+1M NaCl, washed with the same buffer, and eluted by pH 4 PB. The column was then cleaned with 2% AcOH. FT: flow-through; P: hIgG elution peak.

A



B

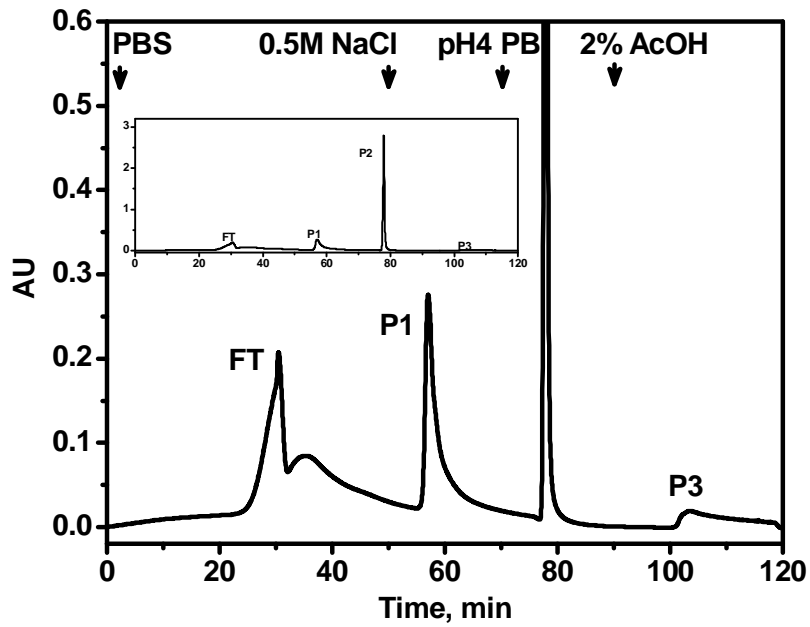


Figure A1-3 hIgG isolation from cMEM with (A) batch #1 and (B) batch #4 under the PBS loading condition.

FT: flow-through; P1: salt wash; P2: hIgG elution peak; P3: 2% AcOH wash. The insert in figure B is a full-scale view of the chromatogram.

Appendix 2. A little about thermodynamics of hIgG-HWRGWV interactions

A2.1 Introduction

Thermodynamic information can provide some insight on protein-ligand interactions since thermodynamics of interactions depend on the number and type of bonds formed in the system. If the binding take place on a solid surface, adsorption isotherms can be measured in either batch format or column format on chromatographic resins (1). If the interactions occur in solution, isothermal titration calorimetry (ITC) is the technique broadly employed in study the interactions between proteins and ligands (2). ITC allows the determination of enthalpy change (ΔH), free energy change (ΔG), and entropy change (ΔS) in a single experiment by stepwise measuring the heat generated during the titration.

This appendix describes the thermodynamics of the interaction between HWRGWV ligand and hIgG, both on the resin and in solution. Isotherms of hIgG adsorption on the HWRGWV resins at different temperatures were measured in a batch format. The binding between hIgG and HWRGWV in solution was examined by ITC and compared with the isothermal adsorption experiment.

A2.2 Experimental

A2.2.1 Materials

Peptide resin HWRGWV at a ligand density of 0.08 meq/g was synthesized directly on Toyopearl AF-Amino-650 M (average particle size 65 μm) (Tosoh Bioscience, Inc., Montgomeryville, PA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International (Louisville, KY). Soluble peptide HWRGWV and its amide HWRGWV-HN₂ were also synthesized by Peptides International. Soluble recombinant Protein G was purchased from GE Healthcare (Piscataway, NJ). Phosphate buffered saline (PBS) of pH 7.4 and human IgG (hIgG) were all purchased from Sigma (St. Louis, MO). An MGW Lauda RM6 circulating bath from Brinkmann (Westbury, NY) was employed for temperature control. Durapore 0.22 μm filter were purchased from Millipore (Billerica, MA). SpectraporCE MWCO 500 Da (width 16 mm) dialysis tubing was provided by Spectrum Laboratories Inc. (Savannah, GA). Micro-BCA assay kit was from Pierce (Rockford, IL).

Microcal VP-ITC microcalorimeter and Origin 5.0 software were provided by MicroCal (MicroCal, Northampton, MA).

A2.2.2 hIgG adsorption isotherm measurements on HWRGWV resin at different temperatures

The isothermal adsorption measurements of normal hIgG at different temperatures ranging from 4 to 20 °C were carried out on HWRGWV resin with a ligand density of 0.08 meq/g using the procedure indicated in chapter 4. The enthalpy change (ΔH), free energy change (ΔG), and entropy change (ΔS) of adsorption were calculated from the measured temperature dependence of K_d using a van't Hoff plot

$$\ln K_d = \frac{\Delta H^0}{RT} - \frac{\Delta S^0}{R} \quad (\text{Eq. A2-1})$$

The enthalpy can be obtained from the slope ($\Delta H/R$) of a plot of $\ln K_d$ vs. $1/T$. The entropy (ΔS) was then calculated by the expression

$$\Delta G^0 = RT \ln K_d = \Delta H^0 - T\Delta S^0 \quad (\text{Eq. A2-2})$$

assuming that ΔH^0 and ΔS^0 are independent of T . This assumption is equivalent to neglecting the temperature dependence of the heat capacity change (ΔC_p) upon adsorption. In these equations, R is the gas constant and T the absolute temperature in Kelvin.

A2.2.3 Isothermal titration calorimetry

Lyophilized HWRGWV (MW 894Da) and its amide HWRGWV-NH₂ (MW 910Da), as well as hIgG, were reconstituted in PBS to the desired concentrations. Solutions were dialyzed (MWCO 500Da) at 4 °C against the same buffer after adjusting the pH to 7.4. Soluble Protein G diluted in PBS was employed as a positive control. All ITC experiments were conducted at 26 °C in a Microcal VP-ITC microcalorimeter with a stirring speed of 300 rpm. The samples were degassed under vacuum prior to the titration. The volumes of the syringe and the cell were ~270 and 1453.5 μL , respectively. The ligands in the syringe were titrated into the cell containing hIgG solutions. The heat of ligand dilution was measured by injecting the ligand into the buffer solution without protein. The data were analyzed with the Origin 5.0 software provided by MicroCal (MicroCal, Northampton, MA), where the

calorimetric thermograms (heat change as a function of time) were analyzed by integrating the area under each peak to determine the heat (Q) of injection. Different models were tried during fitting of the data and the best fitting with the smallest errors were chosen to fit the K_d values. Two models were finally used: a one binding site model and two sequential binding sites model (3). The dissociation constant K_d in the former model can be expressed by

$$K_d = \frac{(1-\Theta)[X]}{\Theta} \quad (\text{Eq. A2-3})$$

where $[X]$ is the free concentration of the ligand Protein G and Θ is the fraction of the binding sites on hIgG that is occupied by Protein G.

The equilibrium constants in the two sequential binding sites model are defined as

$$K_{d1} = \frac{[M][X]}{[MX]} \quad K_{d2} = \frac{[MX][X]}{[MX_2]} \quad (\text{Eq. A2-4})$$

where $[M]$ is the free concentration of protein hIgG; $[MX]$ and $[MX_2]$ are the free concentration of protein-ligand complexes.

A2.3 Results and discussion

A2.3.1 Batch adsorption isotherms

In order to understand the driving force for hIgG binding to HWRGWV resin, the effect of temperature on hIgG adsorption to HWRGWV resins at 0.08 meq/g was examined by batch isothermal adsorption experiments at different temperatures (4 °C, 11 °C, and 20 °C) (Figure A2-1). The data were fitted to a Langmuir model and the resulting K_d and q_m values are tabulated in Table A2-1. The van't Hoff plot of $\ln K_d$ versus $1/T$ is shown in Figure A2-2 and it can be seen that the data can be linearly fitted to a line, indicating that the heat capacity of adsorption (ΔC) is independent of temperature. Enthalpy change (ΔH) was then calculated according to the intercept of the van't Hoff plot (Table A2-1). Free energy change (ΔG) and entropy change (ΔS) were finally calculated using equations A2-1 and A2-2 and also listed in Table A2-1. The K_d values for hIgG adsorption to HWRGWV slightly increased from 2.3×10^{-6} to 7.3×10^{-6} M when the temperature was elevated from 4 to 20 °C. The maximum

capacity q_m seems not to be influenced by the interaction temperature. The calculated enthalpy and entropy changes were -48.2 kJ/mol and -0.0662 kJ/mol/K, respectively. The adsorption of hIgG to HWRGWV resin is shown to be an enthalpy-driven process (negative ΔH) with unfavored (negative) ΔS . Governing hydrophobic interactions could be excluded with the observation of the negligible ΔC_p since a large negative ΔC_p (4) should be expected for a dominant contribution to binding from the hydrophobic effect. This is in agreement with the chromatographic data where temperature has insignificant influence on hIgG retention on the HWRGWV resins. The binding does not seem to be dominated by charge-charge interactions either since enhanced salt concentration was not effective in eluting hIgG as demonstrated in our chromatographic experiments. Therefore, binding between hIgG and HWRGWV might be a combination of several interaction modes of the enthalpy-favored driving forces, such as van de Waals forces, charge-charge interactions, and hydrogen bonding (5), resulting in a specific recognition.

A2.3.2 In-solution ITC

To determine the numbers of hIgG binding sites on the HWRGWV ligand, and the binding strength in solution, ITC experiments were carried out using HWRGWV and its amide (HWRGWV-NH₂). The amide peptide was formed by treating the HWRGWV peptide with ammonium hydroxide. Protein G binding to hIgG was used as a positive control. Meanwhile, the ligand titrations into the solvent were conducted as negative controls, which did not give appreciable peaks of heat change caused by ligand dilution.

The titration profiles of Protein G into hIgG are shown in Figure A2-3. The raw ITC data of Protein G titration were very well fitted to a one binding site model (Equation 7.4). This model does not distinguish the identical binding sites. Human IgG contains two identical Protein G binding sites with each being located on one heavy chain. The Protein G used in this study was a recombinant protein produced in *Escherichia coli* and contains two IgG-binding regions (6). It is known as well that hIgG has two Protein G-binding sites localized in the junction of C_{H2} and C_{H3} domains. Therefore, the stoichiometry of this reaction is 1:1, consistent with the ITC data. The K_d value given by fitting was 3.16×10^{-8} M

which is similar to the chromatographically measured value of 3.3×10^{-8} M (7) for protein G from *E. coli* interacting with immobilized hIgG.

Titration profiles of HWRGWV into hIgG are shown in Figure A2-4. Raw ITC data were fitted using the one binding site model, resulting in a weak binding interaction with a K_d value of 1.6×10^{-4} M, much smaller than the K_d value obtained in the batch isothermal adsorption experiment on chromatographic resin. The difference between the two experiments might be due to the free carboxyl groups exist on soluble but not on immobilized ligands since the peptides are immobilized through the C-termini.

The titration profile of the amidated ligand into hIgG is shown in Figure A2-5. The two sequential binding sites model (Equation 7.5) was found to give the best fit to the data. Two sets of thermodynamic parameters, one for each binding site, were generated by the data fit (Table A2-2). In this model, the binding of the ligand to the second site is dependent on the occupation of the first site by the ligand. However, a good fitting to this model does not inform about the intrinsic relationship of the two sites which could be identical or non-identical, independent or interacting (3). Nevertheless, one can be confident of the number of ligand bound on a receptor molecule when this model is used in fitting. In the case of our system, two amide HWRGWV ligands can be bound on each hIgG molecule while the relationship between the two interaction sites is not known.

The overall dissociation constant ($K_{d1} \times K_{d2}$), enthalpy ($\Delta H_1 + \Delta H_2$) and entropy ($\Delta S_1 + \Delta S_2$) changes of HWRGWV-NH₂ binding to hIgG were respectively 7.98×10^{-6} M, -63.0 kJ/mol and -0.112 kJ/mol/K, the same order of magnitude as these obtained by isothermal adsorption experiments on HWRGWV resins at different temperatures. The agreement between the ITC results in solution and the batch isothermal data on resins implies that the binding between hIgG and HWRGWV-NH₂ resemble the binding of hIgG to HWRGWV immobilized on the resins via the carboxyl terminal. The amidated form of HWRGWV seems to resemble the immobilized ligand better than the normal form, possibly due to the blockage of the charge on the terminal COOH group of the free peptide. The thermodynamic data indicates that the binding of HWRGWV-NH₂ to hIgG is an enthalpy-driven reaction compensated by a negative entropy contribution, as was the case for the immobilized peptide ligand.

A2.4 Conclusions

Thermodynamics of hIgG binding to HWRGWV were examined by two different techniques. Isotherm measurements were conducted in a batch format on HWRGWV chromatographic resins and ITC experiments were carried out in solution using soluble HWRGWV and its amide. Both experiments show that hIgG binding to HWRGWV is an enthalpy-driven process with a negative entropy distribution. The immobilized ligand is better resembled by the amide peptide HWRGWV-NH₂ whose C-terminal charges are blocked. This is reasonable since the peptide is immobilized through its C-terminal. It also shows that each hIgG molecule can bind two HWRGWV ligands but the relationship between the two binding sites needs to be further determined.

A2.5 References

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Table A2-1 Effect of temperature on the adsorption of hIgG to HWRGWV ligand at a density of 0.08 meq/g.

T, °C	q_m, mg/g	K_d, (×10⁻⁶M)	ΔG, kJ/mole	ΔH, kJ/mol	ΔS, kJ/mol/K
4	129.8	2.3	-29.9		-0.0652
11	127.9	5.2	-28.7		-0.0678
20	131.8	7.3	-28.8		-0.0654
				-48.2	Average = -0.0662

Table A2-2 Thermodynamic parameters determined for the binding of HWRGWV-NH₂ to hIgG by fitting the ITC raw data at 26 °C with the two sequential binding sites model.

Parameter	Value
[ligand] ₀ : [hIgG] ₀	36
Solvent	2×PBS
K _{d1} , M	7.46×10 ⁻⁴
ΔH ₁ , kJ/mol	-6.35
ΔS ₁ , kJ/mol/K	0.0386
K _{d2} , M	1.07×10 ⁻²
ΔH ₂ , kJ/mol	-56.6
ΔS ₂ , kJ/mol/K	-0.151
K _{d1} ×K _{d2}	7.98×10 ⁻⁶
ΔH ₁ +ΔH ₂	-62.95
ΔS ₁ + ΔS ₂	-0.1124

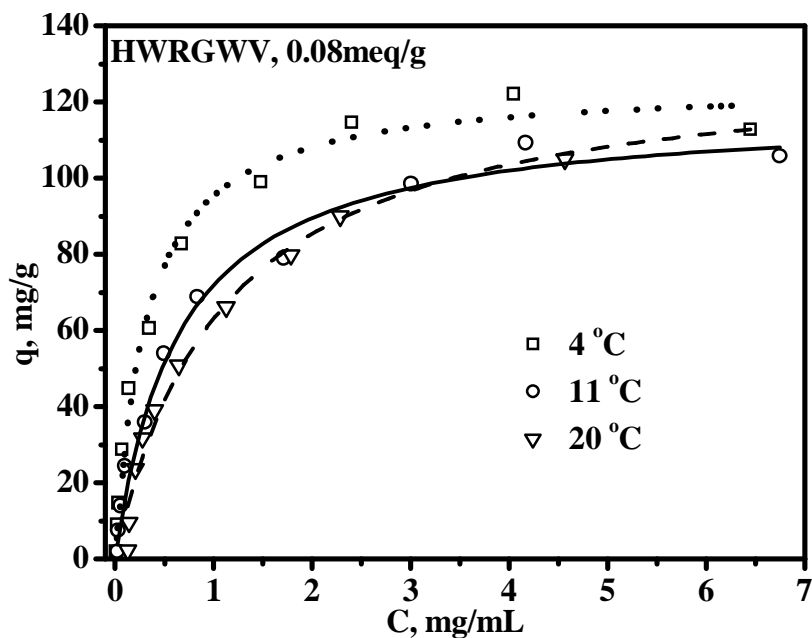


Figure A2-1 Influence of temperature on hIgG adsorption isotherm of HWRGWV at the ligand density of 0.08 meq/g.

Symbols represent the average experimental data and the lines are generated from the Langmuir fitting. Dotted line: 4 °C; solid line: 11 °C; and dashed line: 20 °C.

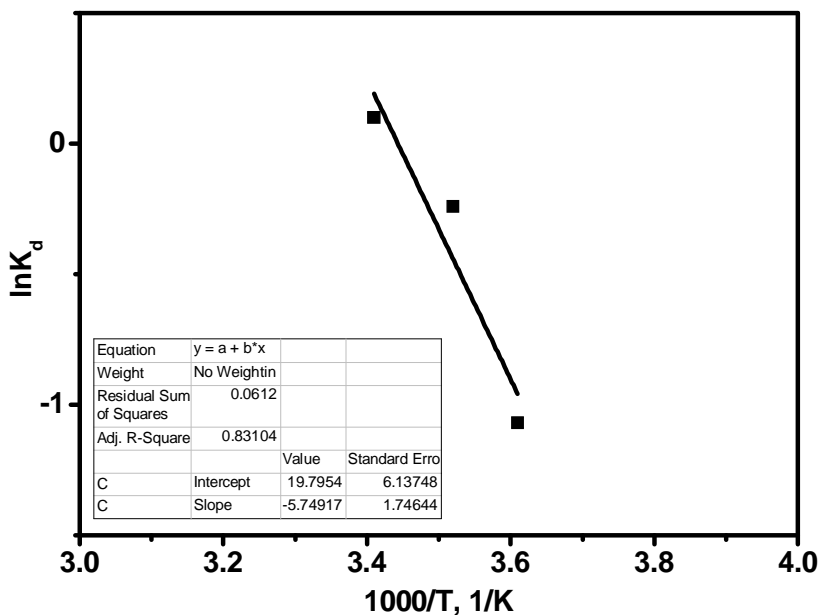
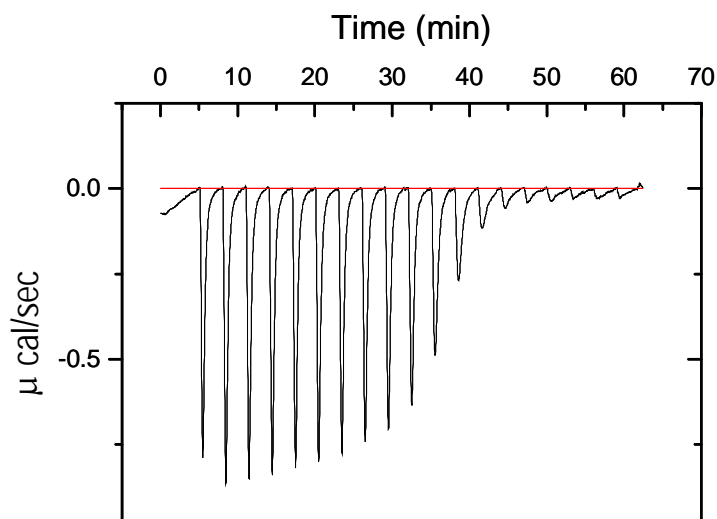


Figure A2-2 Van't Hoff plot of hIgG binding to the HWRGWV resins

A



B

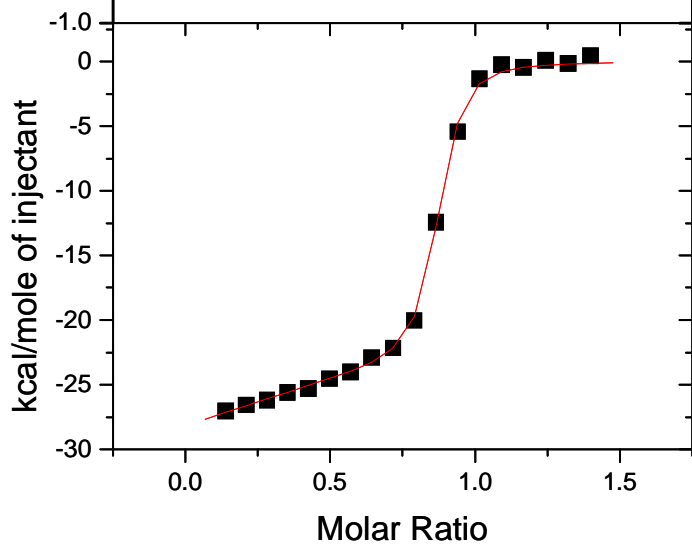
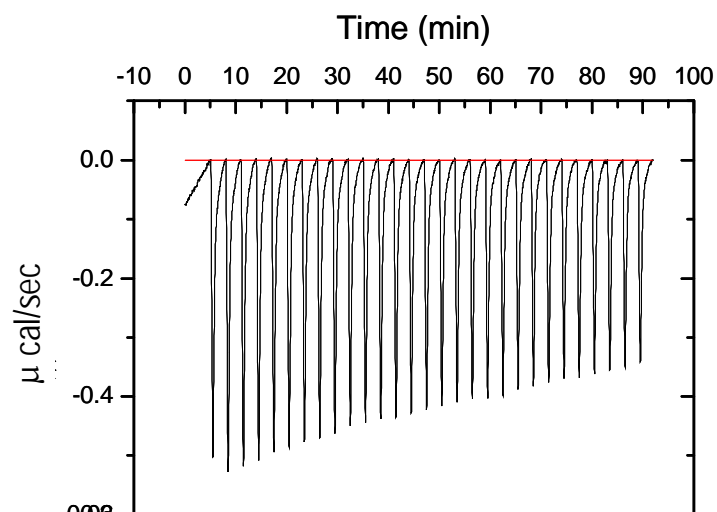


Figure A2-3 Analysis of Protein G binding to hIgG using ITC at 26 °C.

A: Raw titration data showing the heat response resulting from each 9 μ l injection of 0.111765 mM Protein G into the ITC cell containing 0.0099552 mM hIgG.

B: Peak area normalized to the moles of ligand added and nonlinear least squares fit (line) to a one binding site model. The molar ratio denotes the ratio of ligand to protein.

A



B

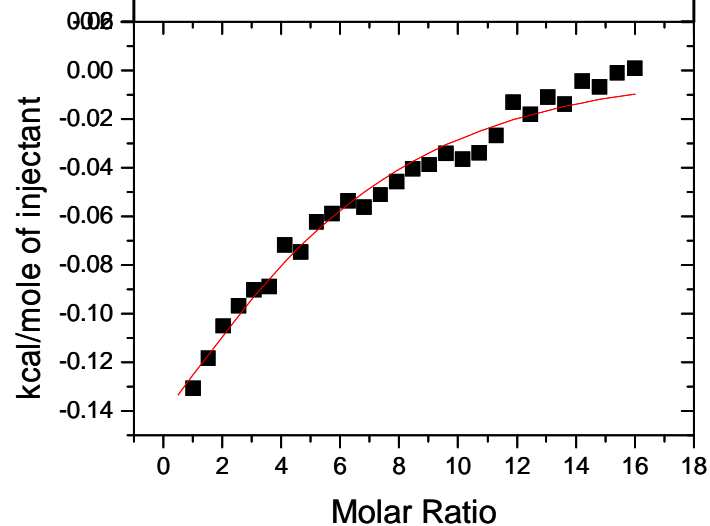
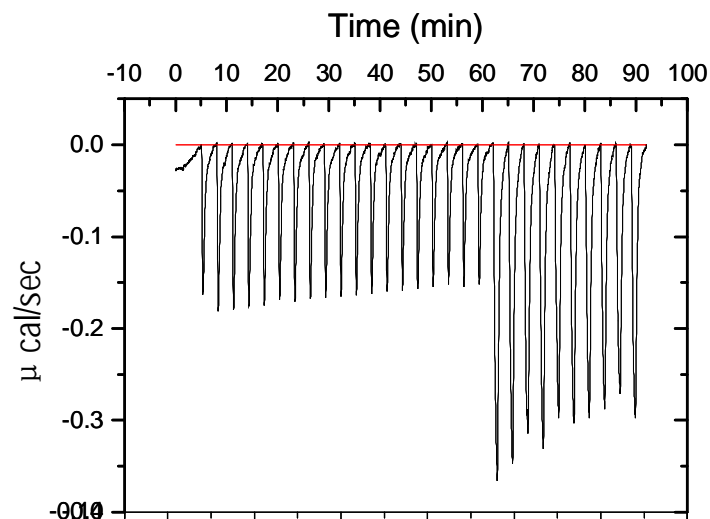


Figure A2-4 Analysis of soluble HWRGWV binding to HIgG using ITC at 26 °C.

A: Raw titration data showing the heat response resulting from each 10 μ l injection of 6.17393 mM HWRGWV into 0.169367 mM HIgG.

B: Peak area normalized to the moles of ligand added and nonlinear least squares fit (line) to a one binding site model. The molar ratio denotes the ratio of ligand to protein.

A



B

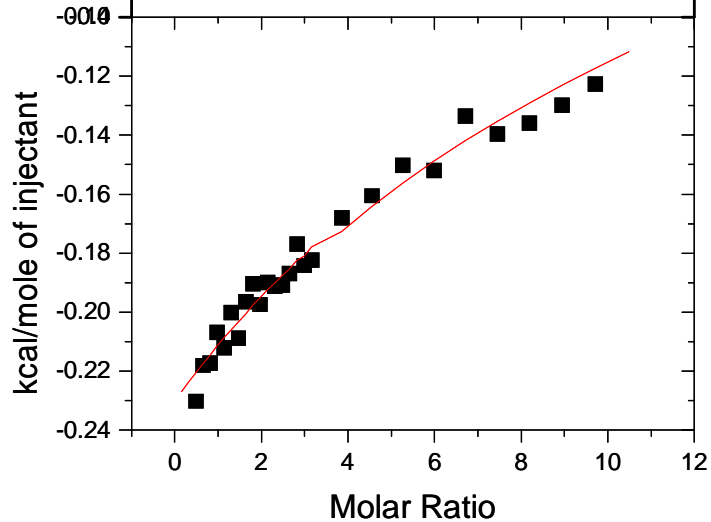


Figure A2-5 Analysis of soluble HWRGWV- NH₂ binding to hIgG using ITC at 26 °C.
 A: Raw titration data showing the heat response resulting from each injection of 6.2227 mM HWRGWV- NH₂ into 0.1721 mM hIgG; Titration volume: 5 μ L \times 19 + 20 μ L \times 6.

B: Peak area normalized to the moles of ligand added, and nonlinear least squares fit (line) to a two sequential binding sites model. The molar ratio denotes the ratio of ligand to protein.