

ABSTRACT

MENEGATTI, STEFANO. Design, Selection, and Development of Novel Peptide Ligands for Bioseparations and Diagnostics. (Under the direction of Dr. Ruben G. Carbonell.)

The relevance of protein-based biopharmaceuticals has increased dramatically in the past decades and a variety of products are now available for human therapy. Antibodies in particular are currently the most heavily consumed protein therapeutics, with a current market volume expected to reach 1 trillion US\$ in 2015 and a compound annual growth rate (CAGR) of 3-6%. Meeting the increasing demand for these therapeutics at lower prices while complying with increasingly stringent regulatory environments, calls for the development of new technologies and platform approaches for efficient downstream protein purification. Extended use of affinity chromatography holds great promise in meeting the urgent demand for affordable high-quality biological products. This technology, however, is still dependent on the use of biological ligands, such as Protein A, Protein G, and Protein L, that have significant issues associated with their high cost, harsh elution conditions, narrow specificity, low chemical stability, and immunogenicity in patients if they leach into the product stream. Small, robust, synthetic ligands may offer an effective alternative to protein ligands. Peptides in particular combine levels of affinity and specificity similar to those of biological ligands with high chemical and biochemical stability, broader specificity, low immunogenicity and ease of synthesis that can reduce costs.

The work in this thesis aims to discover and characterize novel peptide ligands to produce efficient, robust, and affordable affinity adsorbents for improved downstream purification of biologics. Two main areas have been investigated: (a) the development of linear hexapeptide – based adsorbents for the purification of human antibodies and (b) the design and screening of novel libraries of cyclic peptides for the discovery of novel ligands.

The research conducted on the characterization and development of competitive peptide-based affinity adsorbents comprises: (a.1) testing existing peptide ligands for the purification of antibodies from a variety of sources; (a.2) optimizing the protocol of ligand coupling on chromatographic resins to increase adsorbent binding capacity; (a.3) a method of modification of the resin's surface chemistry to increase the adsorbent's chemical stability in harsh alkaline conditions; and (a.4.) a combined computational and chemical strategy for the design of protease-stable peptide ligands. The resulting peptide affinity adsorbents compete well with advanced Protein A – based adsorbents in terms of product yield and purity, dynamic binding capacity (~ 50 – 60 g/L), resistance to alkaline cleaning and sanitization, and biochemical stability in the presence of proteolytic enzymes.

In the second part of this work, two methods are presented for the design, synthesis, and screening of libraries of cyclic peptides for the identification of novel affinity ligands. The first method involves the generation and screening of (b.1) a biological mRNA-display library of cyclic peptides, and the second method (b.2) uses a synthetic solid-phase library of “reversible cyclic peptides”. Both libraries have been screened for the identification of ligands for human antibodies. The results of these studies indicate that these libraries are very promising tools for the discovery of robust, selective and affordable peptide ligands.

The methods presented herein offer a new set of tools, not only for affinity ligand discovery, but also for finding new drugs and diagnostic methods. Besides their technological value, these studies also offer insights into the mechanisms of non-covalent interaction that underlie the phenomena of biorecognition and protein activity.

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Design, Selection, and Development of Novel Peptide Ligands
for Bioseparations and Diagnostics

by
Stefano Menegatti

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Chemical Engineering

Raleigh, North Carolina

2013

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DEDICATION

A Lilly ♥

“Sicut lilium inter spinas”

As a lily among thorns

Song of Solomon 2,2

BIOGRAPHY

Stefano Menegatti was born on October 29th, 1984, in Ferrara, a town on the northern edge of what used to be the Byzantine Exarchate of Ravenna and is now the vestige of a once vibrant center of the Italian Renaissance. Stefano attended the local high school, where, besides mathematics and natural sciences, he discovered a passion for Latin literature and ancient and medieval philosophy. Stefano moved to Bologna in 2003, where he became a Fellow of the School of Advanced Studies *Alma Mater Studiorum* and began the five-year program in the Department of Chemical Engineering. There, he started his research on affinity purification of proteins under the supervision of Dr. Giulio Sarti, who was his advisor for both his B.Sc. and M.Sc. theses. While a student at Bologna, Stefano was hired as an editor at the Center of Scotistic Studies and collaborated in the production of the electronic edition of the *Opera Omnia* of John Duns Scotus (1266 – 1308). In Bologna, Stefano also met Dr. Ruben Carbonell, at the time visiting the department and delivering seminars on his research. In July 2008, Stefano joined Dr. Carbonell's Bioseparations Group as a visiting scholar at NC State University in the Department of Chemical and Biomolecular Engineering, to conduct research for the completion of his Master thesis. After defending his dissertation in December 2008, Stefano returned to NC State University to rejoin Dr. Carbonell's group as a Ph.D. candidate. After four years of intense work, Stefano is defending his doctoral dissertation, after which he will join the Department of Chemical Engineering at University of California Santa Barbara, to begin his post-doctoral appointment under the supervision of Dr. Samir Mitragotri.

ACKNOWLEDGMENTS

The list of people to whom I want to express my gratitude is fairly long. They all, in different ways, have made my life rich and exciting and they all deserve the most wholehearted “thanks” I can express in writing. The order in which their names appear here does not reflect in any way a priority in my heart and thoughts.

First, I want to thank my advisor, Dr. Ruben Carbonell. Ruben is more than an advisor. He has been like a second father to me. As a good parent, he has rewarded me when I deserved, and scolded me when it was right to do so. Our professional relationship has been extremely fruitful. Ruben has always given me the opportunity, the intellectual freedom, and the resources to be a successful student and scholar. Without any doubt, he is the best advisor I could have possibly had. I look forward to working with him in the years to come.

My gratitude and affection go to Dr. Harold “Hal” Hopfenberg and his sweetest wife Patsy. I never met my grandfathers. The war, the hardness of their lives took them away before I could meet them. Harold, the “Professore” as we used to say, is the grandfather I never had. Generous, very bright and curious, in love with Italy, its traditions and cuisine, Hal always has the best advice for any situation. He and his wife Patsy, a fine artist and avid reader, have always welcomed me in their house, where I felt at home while being 4000 miles away from my hometown.

Ruben and Hal are dedicated and industrious servants of this institution. Both have served as department head and head of the Kenan Institute. Excellent teachers, sons, husbands, fathers, and grandfathers, they represent a model of human and professional integrity.

Wholehearted thanks to professors Giulio Cesare Sarti e Carlo Gostoli. Attending their courses in thermodynamics and transport phenomena has turned the direction of my career goals from industry to academia. Their advice and help always came together with friendship. My thoughts also go to the other professors in Bologna, in particular to Serena Bandini, Ferruccio Doghieri, and Carlo Magelli.

The academic environment at NC State University has been my home, and for a few nights literally, for more than four years. Here, I was granted access to excellent facilities and, most importantly, I have become part of a unique group of students and scholars, of which many have become good friends. In the department of Chemical Engineering, my gratitude goes to both the senior faculties and the junior members. Thanks to Dr. Orlin (the accent goes on the “i”, not on the “O”) Velev and Dr. Jan “Giovannino” Genzer, for their advice, their company, the merry time we have spent together. Thanks to Dr. Chase Beisel, Dr. Michael Dickey, Dr. Kirill Efimenko, Dr. Wesley Henderson, Dr. Balaji Rao, Dr. Gregory Reeves, Dr. Fangxin Li, and Dr. Erik Santiso. They all, I am sure, will succeed in their academic careers and will make this department a center of excellence for chemical engineering in the years to come.

Special thanks go to the members of the faculty who served in my doctoral committee, Dr. Flickinger, Dr. Haugh, Dr. Khan, and Dr. Gurgel. To them goes my gratitude for the help, direction, and kind support.

I would also thank the NC State scholars at the department of Biochemistry, in particular Dr. Kevin Blackburn, Dr. Michael Goshe, and Dr. Benjamin Bobay. To them goes

my gratitude for the time they generously spent with me discussing and conducting experiments, and for having always granted me free access to their instrumentations.

I also would like to thank the administrative staff, Diane, Sandra, Shirley, and Angela. Special thanks to Sandra and Rajani for their advice and the help provided with the endless paperwork. To them all goes my gratitude along with that of my fellow students, as we all recognize that only thanks to their work we can manage our research and studies.

Finally, I would like to thank the professors that have kindly agreed to write recommendation letters for my applications, in particular Dr. Giorgio Carta, at the University of Virginia, and Dr. Steven Cramer, at the Rensselaer Polytechnic Institute.

It's now the turn of friends and family. Among the past and present members of the Bioseparations group, I would like to thank Amith, Kevin, Omon, Billy, and Mike. Without Amith, my doctoral work would not have been so productive. The synergy we created by working together has doubled our scientific production in terms of both width and depth. I owe to him part of my success as a doctoral student. Omon and I overlapped for less than two years in the group. A genuinely kind man, behind the cool posture he inherited from his Norwegian descent, Omon has helped me find my way around when I first arrived in town. I am sure he will become a "big shot" in a biotech company one day and I will have to ask an appointment to see him. Outside of our group, I would like to thank Heath Johnson. Heath is like a brother to me, he is the person here in Raleigh with whom I can share my philosophical up to the highest speculative peaks of metaphysics. He is also a great cook and I hope one day we will be in the food business together. Thanks to Kevin Ward for his excellent

collaboration in the lab and all the peptides he has synthesised for me, as well as to Chase and Jessica for the great time we had together.

The friends I met during the five years spent at the School of Advanced Studies in Bologna are among the dearest people to me. Here I want to thank Claudio “Claudiolino” Vandi, Lorenzo “Zonzolino” Battarra, Marco Castellani, aka “il Castellani”, Francesco “Fragolino” Fermani,, and Pierluigi “Gigi” Lecce. The years spent together in Bologna have been a wonderful moment of life. Claudio and I have been sharing a room for five years. I miss our late night movies and ice cream (although I am the only one who got fat for it) or running around in downtown Bologna. With Claudio, Lorenzo, Alberto, Ilaria and the others, we have created a vibrant philosophical circle, where much of my following logic and metaphysical speculations found their seminal reasons. Special thanks go to Marco, who gave me the first directions on how to apply to the graduate program at NC State. Without him, that very special evening, this document would not even exist.

Finally, the most special dedication is for loved ones. To my parents goes my gratitude for having dedicated their lives, all their time, and their energies, to me from the moment I was born. In different ways, which correspond to their radically different personalities, I feel they made their best to keep a united family with all the resources in order for me to become a successful and happy adult. My father was born during WWII, under the bombs, which tells something about his character. He grew up as a boy and a young man during a time when Italy had to be turn from a pile of ruins into a working economy. He, and his brother had no real childhood or youth, all that was given to them was an underpaid job. Yet, they both managed to build a house for themselves, for their kids and

save money. My mother, the last daughter of two farmers barely able to make their signature, has managed to gain a degree in medicine and a specialization in hematology. Where we live, she is a respected doctor. Having seen the number of gifts she receives from her patients for Christmas, Easter, and other celebrations, they must consider some sort of Nobel laureate. I consider myself as the combination of the two. My father made up with creativity and unsystematic reading what he lacked in formal education. From him, I got my creative, nonconformist, and irascible personality. My mother's recipe for success is hard work and her sweetest character. From her, I got my working discipline and some sweetness to balance my paternal inheritance. It is thanks to them, that I can spring up high in the sky of life.

Besides my parents, the most important place in my thoughts is for my little Lilly. She truly is to me “sicut lilium inter spinas”, the lily among thorns, of the Song of Solomon (2, 2). We met at the dawn of an extremely eventful period of my doctoral career, when papers, patents, experiments, meetings, applications, tough decisions crammed into my life. She has been my help, my inspiration, and my comfort, and managed to do all this while on the other side of the ocean. To her I dedicate my thoughts, my achievements in science, my writings, my life, and my love. She is studying medicine and, as hard working and compassionate as she is, she will make a great doctor. With her my life is complete, and I feel as happy and protected as I could be.

To her goes the dedication of this work, of my future works, of my whole self.

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**Chapter 1. The hidden potential of small synthetic molecules and
peptides as affinity ligands for bioseparations**

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A version of this chapter has been prepared as

Review paper for invited submission to Pharmaceutical Bioprocessing

Abstract

While extensively used as drugs, small synthetic molecules have not yet been widely applied in the industry as affinity ligands for the purification of biopharmaceuticals. Yet, a substantial amount of published research indicates that synthetic ligands, like triazine scaffolds, amino acids, and peptides show a great deal of promise for becoming the next generation affinity ligands for bioseparations. In this review, we present a comprehensive account on small synthetic ligands, from triazine dyes to the most recent polycyclic peptide ligands, selected for targeting high-value biopharmaceuticals, such as immunoglobulins, blood factors and therapeutic enzymes. These ligands could play a significant role in improving downstream processing and helping the bioprocessing industry overcome the urgent issues of costs and availability posed by growing economies on the global pharmaceutical market.

Keywords: bioseparations, affinity chromatography, synthetic ligands, triazine scaffolds, peptides, biopharmaceuticals, antibody purification.

1.1. Introduction

The impact of biopharmaceuticals on human therapy has increased exponentially in the last three decades and a variety of products are now available for the treatment of severe diseases [1-5]. While the demand for biotherapeutics is growing rapidly, owing to higher life expectancy in advanced countries and the access of developing countries to the global drug market [6-11], their high costs still preclude the availability of these drugs for specific therapies to large segments of the world's population [12-14]. On the other hand, with the expiration of many patents in the near future and the introduction of biosimilars to the market, biomanufacturers will be exposed to a more globally competitive climate, that is perhaps more receptive to technological innovation [15-18]. Meeting the large demand for products at lower prices while complying with increasingly stringent regulatory environments [19], requires a significant rethinking of manufacturing strategies, in particular introduction of new technologies and the design of sustainable platform approaches to purification [20-22].

The most critical area in the biomanufacturing scenario is downstream processing. In order to guarantee the high purity (> 99.9%) standards required for human therapeutics [23,24], current processes comprise multiple purification steps, which can result in low yields and often account for a significant fraction of the overall manufacturing costs. To overcome the limitations of existing processes, it is essential to develop a cost-effective, robust, and compact platform technology that can easily respond to variations in product titre, impurity profile, and feed volume [22,25,26]. Among the available technologies, affinity

chromatography is the most specific and effective technique for protein purification [27-29]. Affinity chromatography exploits the principle of biomolecular recognition, that is, the ability of biologically active molecules to form specific and reversible complexes with affinity ligands, and has been proposed as a capture step for high value therapeutics, such as blood factors, enzymes and antibodies [30-34]. Polyclonal and monoclonal antibodies in particular, with their high potential for immunotherapy and drug delivery, are currently the most heavily consumed protein therapeutics [35-38].

The affinity purification of antibodies, however, is still based on the use of biological ligands, such as Protein A, Protein G, and Protein L [39-42]. These ligands suffer from several drawbacks, such as 1) low chemical stability, 2) immunogenicity, with the risk associated with the leaching of ligand fragments in the product mainstream, and 3) harsh elution conditions made necessary by the high binding affinity, which can cause product aggregation and compromise the biological activity of the eluted protein [43-45].

To address these issues, over the last two decades engineered versions of Protein A have been presented, which offer higher stability and binding capacity (40 – 60 mg/mL) [39,46]. These newer protein-based adsorbents, however, are very expensive (15,000 – 20,000 \$/liter) and the immunogenicity of leachates is still a concern. To overcome these drawbacks, academia and industry have proposed synthetic ligands as chemically robust, efficient, non-immunogenic alternatives to protein ligands [27,47]. Among the various classes of compounds introduced in the last three decades, the most promising are triazine

scaffolds and peptides [28,48-50]. Owing to their synthetic nature and small size, these molecules have higher stability and can be produced at a lower price.

1.2. Triazine ligands

This class of ligands comprises triazinyl scaffolds substituted with polyaromatic ring systems and electron exchanging groups that mimic the binding of natural substrates. Lowe and coworkers pioneered the discovery and application of these compounds for the affinity purification of proteins, and they discovered a number of specific ligands, particularly for immunoglobulins and enzymes [51-58]. The bifunctional ligand 22/8, for example, functionalized with anilino and tyramino substitutions (Figure 1.1A), mimics the IgG-binding dipeptide motif Phe132-Tyr133 of Protein A [57,59]. The ligand shows good affinity ($K_D = 9.6 \cdot 10^{-6}$ M) and broad specificity, as it can capture antibodies of different classes, such as IgA and IgM, and animal species, such as chicken, cow, rabbit, pig, horse, rat, goat, sheep and mouse. The agarose-based adsorbent shows binding capacity above 50 g/L and withstands the harsh conditions required for resin cleaning and sanitization. The ligand can be used to recover IgG from plasma with high purity (> 90%), but relatively low yield (67-69%), and requires drastic elution conditions (0.1M glycine-HCl at pH 2.9) that can cause product aggregation and denaturation.

Based on ligand 22/8, two triazinic compounds have been designed and commercialized by ProMetic Biosciences Inc. under the name of MabSorbent A1P and A2P. The latter has an antibody binding capacity ranging from 30 to 40 g/L, but is affected by

antifoam agents and its use is limited to polyclonal serum or cell cultures without Pluronic F68 [28]. A second triazinic compound, called ligand 8/7, functionalized with 4-aminobenzamide and aminobutanoic substitutions (Figure 1.1B), was selected for the purification of Fab, F(ab')₂ and scFv fragments [60,61]. Ligand 8/7 binds both the κ and λ light chain of IgG with good affinity ($K_D = 2.6 \cdot 10^{-6}$ M). The agarose-based adsorbent, however, shows low binding capacity (~ 2 g/L). Recently, the same group has developed a new synthetic, although non-triazinic, ligand that mimics Protein G for the purification of mammalian immunoglobulins, including camelid IgGs that only contain a heavy chain. The ligand, called as A2C 11/1, binds IgG with a K_D of $4.78 \cdot 10^{-6}$ M, and, as indicated by *in silico* docking, targets the residues Asn35 and Trp43 of Protein G [62]. The adsorbent A2C-Sepharose binds antibodies from sera of different species, such as human, cow, goat, mouse, sheep, pig, rabbit, and rat, with a static capacity of about 25 g/L and returns a product purity of approximately 65%.

Another triazine-based ligand, Ligand 8-6, was identified for the affinity purification of IgY. The ligand, immobilized on Sepharose, was used to recover IgY from chicken, duck, and pigeon yolk, with resulting capacity of 74.8 g/L, and recovery and purity up to 75% and 90% respectively [63]. Other similar ligands have been developed for the purification of antibody therapeutics from plant extracts, such as Phe-Trz-Asp LAK-mimetic and 4E10lig ligands, identified for the purification of anti-HIV mAB 2F5 and 4.E10 from corn and tobacco extract respectively [64,65].

1.3. Amino acids and peptides

The second major class of synthetic ligands comprises amino acids and peptides. Single amino acids, like tryptophan, phenylalanine, and histidine have been promoted as “mixed mode” affinity ligands [28,34,66]. These ligands bind the target protein through a combination of hydrophobic, π -stacking, hydrogen bonding and electrostatic interactions. Naik *et al.* have proposed the use of tryptophan immobilised on polymethacrylate matrix Sepabeads for the purification of polyclonal IgG from serum and monoclonal IgG from a cell culture supernatant, with purity and recovery of 90% and 85% respectively [67]. Vijayalakshmi *et al.* have proposed the use of immobilised histidine for the purification of catalytic antibodies from the serum of lupus patients [68]. The histidyl-aminohexyl-Sepharose gel allowed a 60% recovery of the antibodies under gentle elution conditions and at high purity.

Peptides comprise a class of molecules of great interest in biotechnology and medicine. These compounds, which can be considered as segments of proteins, are natural candidates for mimicking the fine mechanisms of biomolecular recognition that result in specific binding to target biomolecules. Owing to their high chemical and structural versatility, as well as good chemical stability, peptides are attractive leads for the discovery of drugs, molecular probes, and affinity ligands for the purification of high-value biomolecules [24,28,33,69-74]. Rational and combinatorial approaches have been developed for the identification of peptide affinity ligands. The former aim to tailor a peptide sequence that fits into the preselected binding pocket of a protein target using data of the crystal

structures available on data bases and computational tools that simulate protein-peptide interactions *in-silico* [32,49]. The combinatorial approach is based on the design of peptide libraries and screening processes to identify ligands that bind to a given target protein. Several library formats exist, either biological, such as phage-, yeast-, ribosome-, and mRNA-display libraries, or synthetic, in both solid and liquid phase [75-80]. Lam and co-workers introduced the use of solid-phase combinatorial peptide libraries [81-85]. Our laboratory has extensively used these tools to discover peptide ligands for the affinity purification of a variety of target proteins from complex mixtures. Table 1.1 presents a list of peptide ligands identified in our laboratory using solid phase libraries built on chromatographic resins for affinity separations.

The search for peptide ligands has focused primarily on three main targets, i.e. immunoglobulins, blood factors, and therapeutic enzymes. Monoclonal antibodies (mAbs) are regarded as the most effective protein therapeutics for treating cancer and autoimmune diseases. More than twenty Mab-based drugs have received FDA approval and are now on the market [98-100]. These products generated an annual revenue of about \$ 50 billion in 2012, and throughout 2015 the estimated compound annualised growth rate (CAGR) is about 9.2% [101]. Currently, industry favours Protein A chromatography for the capture step in the antibody purification process [42]. This protein ligand, however, suffers from problems of high cost and low chemical stability, in particular towards the harsh alkaline conditions used for periodical cleaning and sanitization, and the associated risk of release of immunogenic fragments in the product mainstream [102]. Other technical concerns are related to the high binding strength and narrow binding specificity of Protein A. Due to the low dissociation of

the Protein A – antibody complex ($K_D = 10^{-8} - 10^{-9}$ M), elution needs to be performed at very low pH (~ 2.5), with the risk of product denaturation and irreversible aggregation [103,104]. Furthermore, Protein A does not bind the human IgG₃ subclass, shows weak binding to mouse IgG₁ and bovine IgG₁, and does not bind goat and chicken antibodies [105]. Much research has been carried out to discover Protein A mimetic peptides that bind the constant portion (Fc) of antibodies with milder affinity and broader specificity to enable the development of industrial processes of affinity purification of polyclonal antibodies from plasma fractions and more cost-effective purification of monoclonal antibodies from recombinant sources. Several peptide sequences have been identified for affinity purification of antibodies from complex media and are reported in Table 1.2.

Among these antibody-binding peptides, three Protein A – mimetic sequences show particularly interesting characteristics. The first is the tripeptide tetramer (Arg-Thr-Tyr)₄-Lys₂-Lys-Gly (Figure 1.2A), identified by Fassina and co-workers and known as TG19318 or PAM (Protein A mimetic), that binds the Fc portion of IgG [112,113,118]. PAM has been reported to show a broad range of specificity towards antibodies from different sources (human, cow, horse, pig, mouse, rat, rabbit, goat, sheep, and chicken) and classes (IgG, IgY, IgM, IgA, and IgE), yielding approximately 95% pure protein under optimized conditions [114-117]. The ligand is also highly stable to harsh sanitization conditions, such as long incubation times with 1M NaOH. By replacing all amino acids with D stereoisomers a protease-resistant version of the peptide, called D-PAM, has been developed [119,120].

Recently, Lund *et al.* have identified a peptide ligand, D₂AAG (Figure 1.2.B), which specifically binds the Fc fragment of IgG with $K_D = 10^{-5}$ M and is stable to contact with 0.1M NaOH [122]. The adsorbent prepared by coupling this peptide on agarose-based WorkBeads resin (Bio-Works) shows a dynamic binding capacity of up to 48 g/L and purifies IgG from harvest cell culture fluid with purity and recovery above 90%. The adsorbent was also shown to separate aggregated IgG from non-aggregated IgG, indicating that the ligand could be used both as a primary capture step for IgG purification as well as a subsequent polishing step.

Finally, our group has developed three hexapeptide sequences, HWRGWV (Figure 1.2.C), HYFKFD, and HFRRHL that bind the Fc region of human IgG, with a K_D in the range 10^{-5} to 10^{-6} M [123,130]. This relatively low binding strength allows the elution of the antibody at milder conditions (pH 4.0 – 5.0) than those required by Protein A (pH 2.5), with reduced risk of aggregation and loss of product bioactivity [131]. Characterization on HWRGWV by mass spectrometry and docking simulations indicate that the ligand-binding site can be found in the loop Ser383-Asn389 (SNGQPEN), located in the CH3 domain of the pFc portion of hIgG [132]. This HWRGWV sequence was extensively characterized for its ability to isolate IgG from a variety of complex sources, including cell culture media, commercial CHO cell culture supernatants, Cohn II+III fraction of human plasma, transgenic milk and whey, and plant extract, with product yields and purities up to 90% and 95% respectively [126,128,129]. Further studies have been performed to improve the chemical stability and binding capacity of peptide-based resins. A method of surface resin modification was developed to enable the production of an alkaline-stable peptide-based

adsorbent. The adsorbent HWRGWV-Toyopearl produced by peptide synthesis on the modified chromatographic resin was used over 200 cycles of IgG purification, each followed by alkaline cleaning and sanitization with aqueous 0.1M NaOH, without significant loss of capacity and selectivity [127]. Further, a highly efficient protocol of ligand coupling was developed in order to optimize ligand distribution and accessibility on the resin pore surface. The adsorbents HWRGWVC-Toyopearl (peptide density of 60 μ moles/mL) and HWRGWVC-WorkBeads (peptide density of 50 μ moles/mL) gave dynamic binding capacities of 40 g/L and 60 mg/mL (residence time 5 min) respectively [136]. The ligand was also found able to bind IgG from different species, i.e. mouse, rat, cow, goat, rabbit, and chicken, and purify them from crude sera [unpublished results]. This indicates that the peptide could be used for purifying MAbs from hybridoma cell culture and ascites fluids. To prevent the proteolytic degradation of the peptide ligand by the enzymes present in these fluids, in particular trypsin and chymotrypsin, variants of the peptide ligands have been developed by replacing natural amino acids with non-natural residues [137]. The variant $HW_{Met}CitGW_{Met}V$ (W_{Met} : N_{in} -methyl-tryptophan, Cit: citrulline) shows resistance to both proteases over a high number of injections.

The ligand HWRGWV was also used to bind human immunoglobulins of different classes, such as IgA and IgM [124,125]. Chromatographic tests with HWRGWV-Toyopearl resin at varying concentration of peptide ligand were performed to separate IgG, IgA and IgM from a cell culture supernatant and Cohn II+III fraction. After pretreatment with caprylic acid precipitation or combination of caprylic acid and polyethylene glycol precipitation, three elution fractions were obtained. The first fraction contained pure IgG (>

95%), while the two subsequent fractions were respectively enriched in IgA (42% hIgA and 56% hIgG) and IgM (46% hIgM, 28% hIgA and 24% hIgG).

Considerable research has also been directed towards the discovery of ligands for the purification of blood coagulation factors, the glycoproteins Factor VII, VIII, and IX, and Von Willebrand factor. The deficiency of these factors causes haemophilia (Factors VII, VIII, and IX) and bleeding diathesis (von Willebrand factor) of the skin and mucous membranes. To restore correct haemostasis, patients are treated with injection of purified factors obtained either from human blood plasma or recombinant sources. Due to the requirements of high purity (> 99.9%) as well as immunological and virus safety, the cost of coagulation factor concentrates is very high and limits the implementation of prophylactic therapy as well as extended therapeutic and surgical use in patients suffering with bleeding disorders. Currently, these biomolecules are purified through a sequence of chromatographic steps, including immunoaffinity chromatography, that have a significant impact on the product costs. In order to reduce the number of purification steps and hence reduce the costs, several research teams have sought small peptide ligands for the affinity purification of blood coagulation factors. A list of these sequences is reported in Table 1.3. On average, these ligands offer good product recovery and purity from plasma or recombinant sources. Several studies have also been performed to increase the resistance to proteolysis and the binding capacity of the peptide-based affinity adsorbents.

Finally, several peptide ligands have also been identified for the purification of therapeutic enzymes. The application of enzymes in pharmaceutical and food industry,

scientific research, and human therapy has increased considerably in the last decade and a growing number of products are waiting for FDA approval or are entering Phase III of clinical evaluation [146-150]. Enzymes are used in clinical settings for either analytical or therapeutic purposes. Therapeutic enzymes are currently used to treat a broad spectrum of diseases, from food intolerance and allergies to leukaemia, ulcers, fibrosis, and inflammations [151,152]. These products generate substantial revenues worldwide and their market in the US alone was estimated at \$ 1.15 – 1.17 billion in 2011 [153]. Currently, after extraction and concentration, enzymes are purified via chromatographic processes comprising up to five steps [154]. Combinatorial libraries of peptides have been used for the identification of sequences with affinity for enzymes. Several examples are listed in Table 1.4.

Several other peptide ligands have been identified for protein affinity purification. The sequence IQHPQ was identified by Lam *et al.* to bind Streptavidin [165]. Two other sequences, LNIVSVNGRHX and DNRIRLQAKXX (where X represents an undetermined residue), were identified by Pennington for alpha-6-b-1-integrin [166]; the heptamer HWWWPAS was found by Dong *et al.* for insulin [167]; the peptide EFDWNH was discovered by Lehman for anti-insulin antibodies [168]; GLERPE for prion protein by Lathrop [169]; four hexapeptides, APRQPP, DQDQDT, EGKQRR, and HQHRQR, were found by Noppe for Lactoferrin [170]; several peptides for β -actin were discovered by Miyamoto [171]; and finally, a peptide for α -n-b-3 integrin, RGD, was found by Xiao *et al.* [172].

1.4. Cyclic peptide ligands

Small linear peptides show a great deal of promise, owing to their abilities for specific biorecognition, chemical stability, low immunogenicity and toxicity, and ease of synthesis. Yet, a new generation of ligands, cyclic peptides, is gaining interest in the wider scenario of biotechnological applications. Due to their conformational rigidity, cyclic peptides possess higher affinity, specificity and enzymatic stability compared to their linear counterparts, and they are therefore promising candidates in the areas of drug discovery and identification of ligands for bioseparations [173-176]. The first libraries of cyclic peptides used for ligand discovery were prepared by a phage-display technique, where a disulfide bond between two cysteines framed the peptide sequence to attain cyclization. A list of disulfide-bridged cyclic peptides that bind specifically to target proteins is presented in Table 1.5. In all these studies, the affinity of the selected cyclic peptides was found to be higher than those of the corresponding linear sequences.

Since the pioneering work of McLafferty on phage-display libraries [182], many disulfide-bridged cyclic peptides with high affinity for protein targets have been identified. However, the lability of the disulfide bond limits the application of these cyclic peptides to research and medicinal environments. Under reducing conditions, such as high pH, the -S-S- bond hydrolyses and the cyclic peptide loses its structure. Hence, disulfide-bridged cyclic peptides cannot find application as affinity ligands for purification of protein therapeutics in the industry, where alkaline conditions are regularly employed for resin cleaning and regeneration [183-185]. Biological peptide libraries, like phage-display, bacterial display,

yeast display, etc. remain very powerful tools for identifying high-affinity ligands, owing to the mechanism of sequence selection by directed evolution. This process consists in iterating cycles of library generation and selection, which gradually defines homology patterns and enables the identification of a final candidate ligand with very high affinity and selectivity. Methods for attaining peptide cyclization on biological libraries other than the formation of disulfide bonds have been developed to discover cyclic peptide ligands. Roberts and co-workers, for example, have published a method for peptide cyclization on mRNA-display libraries, which comprises a liquid-phase crosslinking reaction between two primary amino groups using the homobifunctional linker disuccinimidyl glutarate (DSG) [186]. Using this technology, a library was prepared and screened against the Gα1 protein to identify a cyclic peptide that binds the target with “antibody-like affinity” ($K_D = 2.1$ nM). The cyclic peptide was found to bind the target protein 15-fold more tightly than its linear counterpart and to possess enhanced proteolytic stability [187]. The cyclization method as described by these researchers, however, suffers from moderate yields, confined in the range of 30% to 50%, and is open to formation of undesired by-products resulting from poly-modification and intermolecular crosslinking. To avoid these side reactions, we have recently proposed a variant of this peptide cyclization on a “reversible solid-phase format” [188]. After translation, the liquid-phase library of mRNA-peptide hybrids is adsorbed on solid phase, reacted with DSG, washed and eluted. The spatial segregation of the peptides on a solid phase and the optimized crosslinking conditions avoid the formation of unwanted byproducts and afford high peptide cyclization yield and library purity (> 90%). Library screening against IgG-Fc was performed under very stringent conditions to ensure the identification of

candidate binders in a single step. The selected sequences showed a marked similarity in amino acid composition, with a predominance of histidine, aromatic (W, F, and Y) and basic (R and K) residues, similarly to the above mentioned linear hexapeptides selected from a solid phase synthetic library screened against the same target. The selected sequence cyclo[Link-MWFRHY-K] was shown to bind IgG from different mammalian species, namely human, mouse, rabbit, chicken, goat, and cow, and was used to purify therapeutic monoclonal antibodies of IgG₁ and IgG₄ subclasses from two commercial cell culture supernatants. The product yield and purity (both at 95%) as well as the binding strength ($K_D = 7.6 \times 10^{-6}$ M) were slightly higher than those given by the linear hexapeptides.

While having a track record of success, biological libraries suffer from some limitations. They hardly allow the use of non-natural amino acids and other synthetic groups, and their screening process is fairly laborious. Solid-phase synthetic libraries, on the other hand, enable a wide range of chemical and structural variations. Several strategies of peptide cyclization have been proposed, which employ both natural and non-natural amino acids. The former include end-to-tail, end- or tail-to-side chain, and side chain-to-side chain cyclization by reaction between glutamic or aspartic acid and lysine residues [189,190]. Among the latter, Ring Closing Metathesis (RCM) and Click chemistry are notable techniques. RCM refers to the intramolecular olefin metathesis catalyzed by a Ruthenium-based Grubbs' reagent between two allylglycines located at the ends of the peptide [191-196]. Click chemistry consists of a Huisgen cycloaddition between the alkyne and the azide residues of non-natural amino acids, such as propargylglycine and an azido amino acid, leading to a triazole link [197-201]. The major issue related to these methods is the difficulty of

sequencing the cyclic peptides carried by the beads selected from library screening. The techniques routinely used for sequencing linear peptides, Edman degradation and single stage MS/MS, cannot be employed for cyclic peptides. Some MS-based methods have been reported for sequencing cyclic peptides, although they entail considerable effort and high level of uncertainty. In a mass spectrometer, in fact, the cyclic peptide undergoes ring opening at multiple positions to produce a complex mixture of fragments, making spectral interpretation difficult and highly uncertain [202-207].

A few strategies have been proposed to circumvent the difficulties involved in the post-screening hit identification. Pei and coworkers have developed a method for the synthesis of a one-bead-two-peptide (OBTP) library. This procedure comprises the segregation of each library bead into an outer layer, which displays cyclic peptides and is accessible to the target protein, and an inner core, which contains the peptide linear precursors and is impervious to proteins. After selecting the beads that bind the target protein through the cyclic ligands located in the outer layer, the linear peptides in the inner core are sequenced by partial Edman degradation (PED/MS) [203]. This method has been successfully applied to the identification of cyclic peptides ligand against a variety of targets, reported in Table 1.6. Recently, the same method has been extended to the identification of peptoids and peptide/peptoid hybrids [208].

Our group also has proposed a strategy for easy and high throughput sequencing of cyclic peptides leads, based on the use of a solid-phase library of reversible cyclic peptides [212]. The cyclic structure comprises two ester bonds that frame the functional sequence, *i.e.*

the portion of the peptide that directly interacts with the target protein, as shown in Figure 1.3. Ester bonds are sufficiently stable at neutral pH to allow screening the library with the peptides retaining their cyclic structure, whereas they are rapidly hydrolyzed in alkaline conditions. Beads selected from library screening are treated with an alkaline aqueous-organic solution, which cleaves the functional sequence from the resin surface and releases it in liquid phase. The linearized sequences are then analyzed by single step ESI-MS/MS. This method has been demonstrated through the selection of an IgG-binding cyclic peptide from a small model library. The sequence cyclo[(N_α-Ac)-S(G)-RWHYFK-Lact-E] was selected, synthesized on a chromatographic resin, and tested for antibody binding. To avoid ligand loss due to hydrolysis during the elution step (acid buffer) and resin sanitization (alkaline buffer) and to maintain the ligand cyclic structure, the ester bonds were replaced with amide bonds. This modification has no consequence on the structure of the ligand, which retains its binding capacity as demonstrated by chromatographic binding tests.

1.5. Polycyclic peptides

Recent advances in chemical and biochemical synthesis have opened the way to a new generation of highly complex peptide architectures. Multi-domain peptides, that is, ligands capable of binding the target molecule via multisite interactions, are very promising candidates for attaining the high levels of affinity required in difficult bioseparations [213]. Bicyclic and tricyclic peptides are regarded as promising structures for creating ligands that mimic the hand-in-glove binding mechanism characteristic of natural protein-protein

biorecognition. Recently, Heinis *et al.* have presented a method for producing phage-display libraries of bicyclic peptides. The formation of bicyclic peptides is achieved by reacting three cysteine residues located on the peptide with tris-(bromomethyl)benzene linker. The library was screened against human kallikrein and a ligand was identified that binds the protein target with $K_D = 1.5 \times 10^{-9}$ M [213-216]. A study was also presented on the effect of peptide length on the efficiency of the bis-cyclization reaction and the affinity of the resulting ligands [217]. In a recent work, the same team has presented a method for the synthesis of tricyclic peptides that comprises the use of the above mentioned linker to form the first two cycles, followed by the enzyme transglutaminase that bridges between lysine and asparagine thereby forming the third loop [218]. Our group has developed a method for the synthesis of bicyclic and tricyclic peptides on solid phases. These structures comprise independent peptide cycles connected by a spacer arm (Figure 1.4). This conformation is expected to allow better access of the cyclic binding units into neighbor binding sites, thus promoting an “avidity effect” within the polycyclic ligand for the target biomolecule.

1.6. Conclusions

This literature survey indicates that small, easy to synthesize, non-immunogenic, and cost-effective synthetic molecules offer promising alternatives to protein ligands for bioseparations. Of special interest is the class of peptide ligands, which, in the past three decades, has shown a tremendous improvement in terms of both numbers and performance for a wide variety of high-value biological targets. Peptides combine the advantages of small

synthetic molecules, such as biochemical stability and ease of synthesis, with an affinity and specificity similar to those of biological protein ligands. The potential of peptides is further increased by the constant development of novel synthetic and computational tools. The introduction of non-natural amino acids and of new reaction mechanisms open the way to higher levels of molecular complexity, and thus affinity, specificity, and stability, at lower synthesis costs. New refined computational tools for molecular design and simulation of protein-peptide interactions offer an invaluable help to chemists and engineers for better understanding the phenomena of molecular biorecognition and for the design of more effective ligands and drugs. With all these resources, peptides offer a great deal of promise for the identification of novel ligands for developing new diagnostics, therapeutics and bioseparation technologies. Yet, while peptide drugs have found numerous applications, the potential of peptides as affinity ligands for downstream bioprocessing has been but minimally utilized. Under pressure for radical changes in downstream processing technologies precipitated by the demand for affordable biopharmaceutics from growing economies and an aging world population we believe that this class of compounds will play a strong role in future, low cost, sustainable bioprocessing technologies.

1.7. References

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Table 1.1. Peptide ligands for the affinity purification of proteins.

Sequence	Target	Reference
WQEHYN, WQETYQ, and YENYGY	Fibrinogen	[86]
VIWLVR	α -1-proteinase inhibitor	[87]
WHWRKR	α -lactalbumin	[88,89]
FLLVPL	Fibrinogen	[90]
YYWLHH	Staphylococcal enterotoxin B (SEB)	[91-93]
WRW	Porcine ParvoVirus (PPV)	[94-96]
GVNFEVVG / GVNFTVVG, and GVFFELVG / GVFFEIVG	S-protein	[97]

Table 1.2. Immunoglobulin-binding peptide ligands.

Sequence	Target	Reference
APAR	Granulocyte macrophage-colony stimulating factor (GM-CSF) monoclonal antibody	[106]
PDTRPAP	Carcinoma-associated anti-MUC1 mucin antibody C595, diabody fragment C595	[52,107-109]
EPIHRSTLTALL	anti-Tac IgG1 antibody (HAT)	[110,111]
(Arg-Thr-Tyr) ₄ -Lys ₂ -Lys-Gly (TG19318 or PAM)	IgG-Fc of different animal species, and IgM, IgA, IgE, and IgY	[112-118]
(DArg-DThr-DTyr) ₄ -DLys ₂ -DLys-DGly (D-PAM)		[119,120]
AEGEFINVPMMVDGITMGD PAK (M[46-2])	Murine anti-human tumor-associated tenascin-C (Tn-C) mAb	[121]
PMMVDGITMG	Murine anti-human tumor-associated tenascin-C (Tn-C) Fab	[121]
D ₂ AAG	IgG-Fc	[39,122]
HWRGWV, HYFKFD, and HFRRHL	IgG-Fc of different animal species, and IgM, IgA, and IgY	[123-132]

Table 1.2. Continued

DPQYRALMGENQDLRKRE GQYQDKIEELE	IgA	[133]
50-mer	IgA	[134]
CCHQRLSQRK	IgM	[135]

Table 1.3. Peptide affinity ligands for blood coagulation factors.

Sequence	Target	Reference
Ac-RVRSFY	Von Willebrand Factor	[138]
RDRKWNCTDHVC	Factor VIII (light chain)	[139]
EYKSWEYC EYHSWEYC EYISWEYC	Factor VIII	[140,141]
WEYC and WDYC (3-IAA)EYC (3-IAA)DYC (3-IAA) Ψ (CH ₂ NH)EYC (3-IAA)-EY Ψ (CH ₂ NH)C	Factor VIII	[142-144]
YANKGY	Factor IX	[145]

* Note: IAA: indolacetic acid. Ψ (CH₂NH) indicates that the peptide bond (CO-NH) has been replaced by CH₂NH.

Table 1.4. Peptide ligands for the affinity purification of enzymes.

Sequence	Target	Reference
EIFPGNSKTYAAE (fragment of anti-lysozyme antibody) AVLERAARSVLLNAP, STLLPPELSETPNAT, and ELAPEDPEDSALLED (analogous to the amino acid sequence of herpes simplex glycoprotein D)	Lysozyme	[155,156]
YDLRYDRERA, GQGWDYDLRYDRERA, KLASNYDTNGDGWI(Nle), LASNYDTNGDGWI(Nle), KLASNYDTNGDGWI(Nle)GLWELGKGPQR VWNVGYGTLS	Lysozyme	[157]
YNFEVL	Ribonuclease S	[145,158]
YIYGSKF	p60(c-src) protein tyrosine kinase	[159]
NWMMF	Glycosomal phosphoglycerate kinase (gPGK)	[160]
VAR, TAR, VPR, and TPR	Trypsin	[161]
D-His-Pro-Phe-His-Leu-Ψ(CH ₂ NH)-Leu-Val-Tyr	Renin	[162]
TRWLVIYFSRPYLVAT	α-amylase	[163]

Table 1.4. Continued

FHENWS	α -amylase	[164]
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Table 1.5. Cyclic peptides (by disulfide bond) selected from phage-display libraries.

Sequence	Target	Reference
Cyclo[CHPQFC], cyclo[CHPQFPC], and cyclo[CHPQGPPC]	Streptavidin	[177]
Cyclo[CD(R/L)A(S/T)P(Y/W)C]	Avidin and neutravidin	[178]
CCFSWRCRC	Chymotrypsin	[179]
AEGTGDHL-cyclo[CGAWFRPC]- DAEPGEGGGGK	Factor VIII	[180]
H ₂ N-cyclo[CFHHC] ₂ -KG	IgG (Fc and Fab fragments)	[181]

Note: C indicate cysteine residues employed for peptide cyclization

Table 1.6. Cyclic peptide ligands identified from screening OBTP libraries.

Sequence	Target	Reference
Cyclo[ARYQSRVE] and cyclo[AIYQSRVE]	Human prolactin receptor	[175]
Cyclo[AFCpYCNVLNE] and cyclo[AIKpYQNYLNE]	SH2 domains of Grb2	[209]
Cyclo[ANIpYDNVLNE]	Tensin	[209]
Cyclo[YHIGIGPVIVITAAE] and others	Calcineurin/NFAT interaction complex	[210]
Cyclo[aWYQ-Fpa-Nle-aaaEK]-K and cyclo[aI-Fpa-RYWaaE]K	HIV-1 Capsid / Human Lysyl-tRNA Synthetase complex	[211]

Note: uppercase indicate L-amino acids, lowercase indicate D-amino acids.

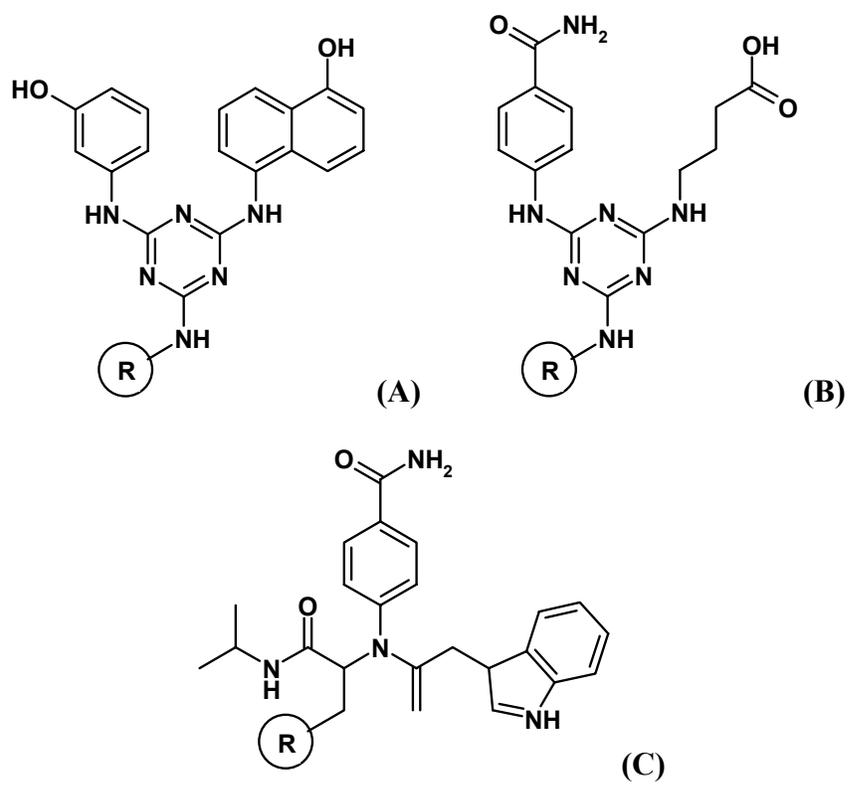


Figure 1.1. (A) ligand 22/8; (B) ligand 8/7; (C) ligand A2C 11/1; R: Sepharose resin.

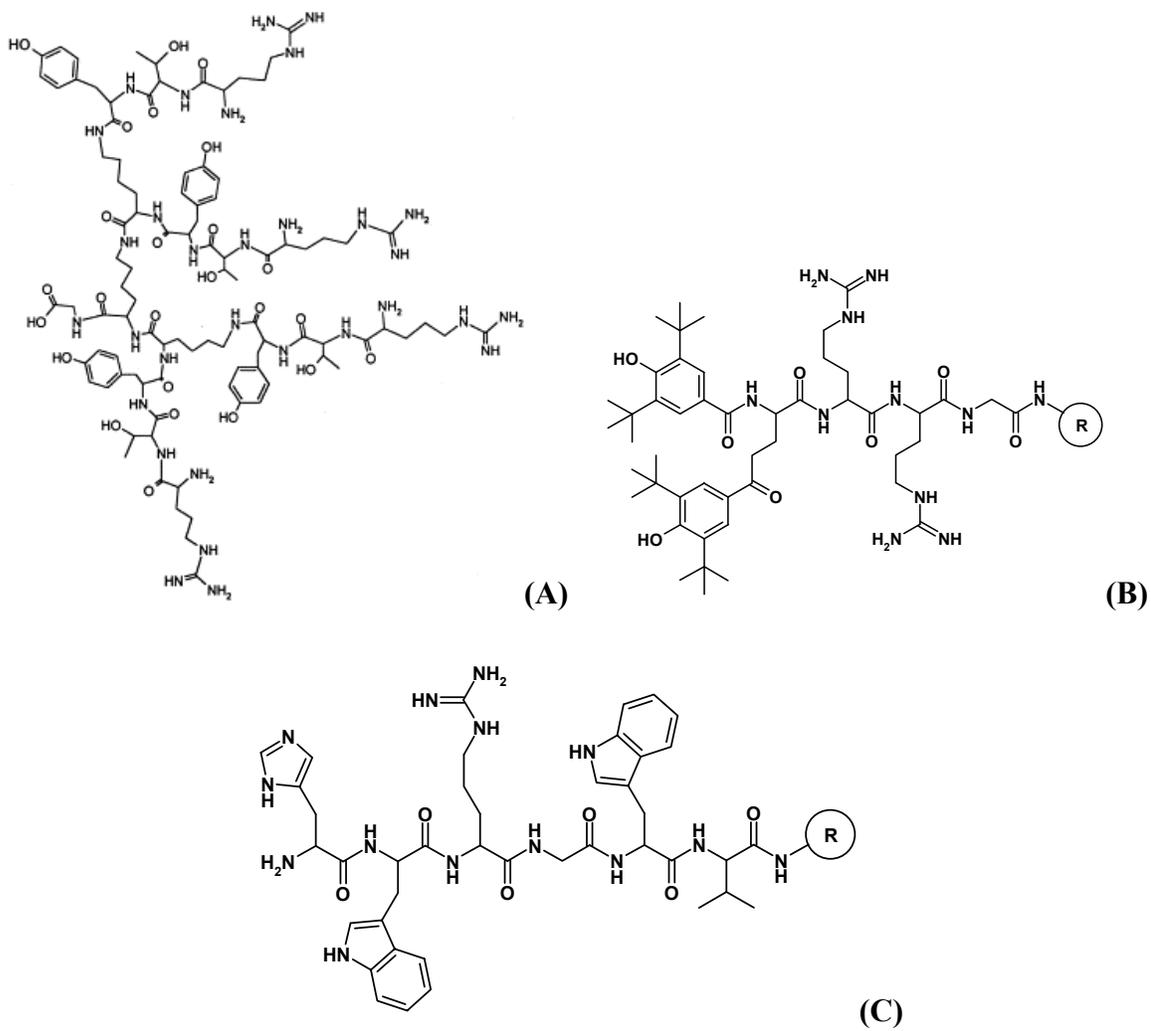


Figure 1.2. (A) TG19318; (B) ligand D₂AAG; (C) HWRGWV; R: polymer resin.

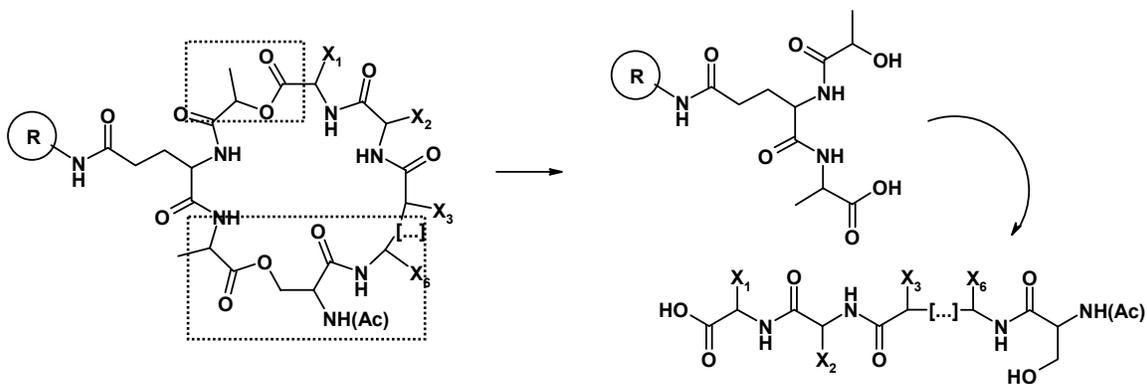


Figure 1.3. Model structures of the cyclic dilactone cyclo[(N_α-Ac)S(A)-X₁X₂X₃X₄X₅X₆-Lact-E] and linearized structure Ac-S-X₁X₂X₃X₄X₅X₆.

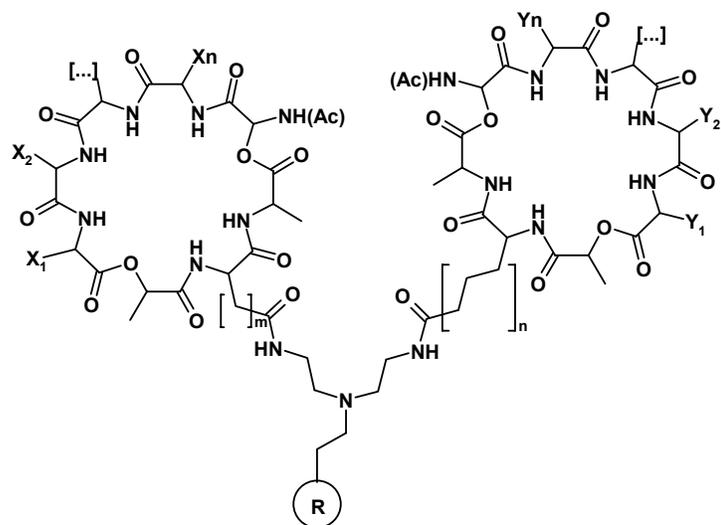


Figure 1.4. Structure of bicyclic peptide.

**Chapter 2. Performance of hexamer peptide ligands for affinity purification of
Immunoglobulin G from commercial cell culture media**

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A version of this chapter has been published in

Journal of Chromatography A 2011, 1218 (13), 1691-1700.

Abstract

Previous work has reported on the identification and characterization of the hexapeptide ligands HWRGWV, HYFKFD, and HFRRHL for the affinity capture of IgG through specific binding to its Fc fragment. This paper addresses issues related to the successful application of these ligands, on a commercial methacrylate chromatographic resin, for the purification of IgG from mammalian cell culture fluids. The concentrations of sodium chloride and sodium caprylate in the binding buffer were optimized to maximize the purity and yield of IgG upon elution. Screening of several regeneration conditions found that either 2M guanidine-HCl or a combination of 0.85% phosphoric acid followed by 2M urea resulted in complete recovery of the IgG adsorption capacity and that the column could be reused over many cycles. The hexapeptide ligands were used for the purification of humanized and chimeric monoclonal antibodies from two commercial CHO cell culture fluids. The chimeric MAb of IgG1 subclass was purified using the HWRGWV resin whereas the humanized MAb of IgG4 subclass was purified using the HWRGWV, HYFKFD and HFRRHL resins. The purities and yields obtained for both the MAbs were found to be higher than 94% and 85% respectively. These results compare well with the yields and purities obtained using Protein G columns. The residual DNA and host cell protein reduction obtained by the HWRGWV resin was in the range of 4 Log reduction value (LRV) and 2 LRV respectively, comparable to those reported for Protein A resins. The dynamic binding capacity of all three peptide resins for the humanized monoclonal antibody was in the range of 20 mg/mL.

Keywords. Hexapeptide ligand, IgG purification, Monoclonal antibody, Affinity chromatography, Regeneration.

2.1. Introduction

Monoclonal antibodies (MAbs) and Fc-fusion proteins had annual sales of \$34 billion in 2008 [1]. At that time, Mabs and Fc-fusion proteins accounted for 275 of the biotherapeutic products in the FDA pipeline in various stages of clinical trials. These important biologics have transformed the treatment of many cancers, autoimmune and infectious diseases and they offer tremendous potential to treat a wide range of other acute conditions. However, MAb-based therapies are very expensive, with costs of several thousand dollars per dose, making them unaffordable to many patients. With growing competition from follow-on biologics and increased pressure from the high cost of healthcare, the success of next generation MAbs may be largely governed by the economic factors [2, 3].

Progress in mammalian cell culture and fermentation technologies have enhanced antibody productivity from milligrams to grams per litre with titres $> 5\text{g/L}$ becoming increasingly common [4, 5]. However, downstream processing technology, which accounts for anywhere between 50-80% of total production costs [6], has not kept up with advances in upstream processes. A major contributor to downstream processing costs of antibodies is the use of Protein A or Protein G affinity product capture steps following cell removal. Since Protein A and G bind very strongly to antibodies, product elution is normally done at pH 3, which can result in antibody aggregation, while also serving as a viral inactivation step. Repeated cycles of binding and elution, together with periodic cleaning and sanitization of the resin using 0.1-0.5N NaOH, can result in loss of activity of the Protein A and Protein G chromatographic supports as a result of denaturation of the three-dimensional tertiary

structures of these ligands. Even though engineered versions of Protein A and Protein G for greater stability are available [7, 8], they are also more expensive. To address these issues, there has been great interest over the years, on the part of both industry and academia, on the discovery and validation of more efficient and less costly affinity ligands for antibodies. Among the various types of alternative ligands developed, small synthetic ligands have received a lot of attention due to their higher stability and lower cost relative to large protein ligands. Several such synthetic ligands have been developed for antibody purification [9-23]. Nevertheless, these small ligands have not made huge inroads into the market for Protein A, mostly due to their lack of specificity. Resins with larger ligands, such as camelid antibodies exhibit excellent specificity but can be as expensive as Protein A and G.

Our research group has identified peptide ligands from combinatorial solid-phase hexapeptide libraries for the purification of various biomolecules such as α -1-proteinase inhibitor [24], α -lactalbumin [25], staphylococcal enterotoxin B [26-28] and fibrinogen [29]. Three hexapeptide ligands that bind to the Fc fragment of hIgG, HWRGWV, HYFKFD, and HFRRHL, have been identified and preliminary comparisons of their binding properties have been made to both Protein A and the small triazine-based ligand MAbSorbent A2P [30,31]. Mass spectrometry analysis and molecular docking calculations have been used to determine the putative binding site of HWRGWV on the Fc portion of hIgG [32]. These small peptide ligands exhibit higher specificity than small organic ligands, but lower binding avidity than Protein A and Protein G. They have the advantage of being able to be synthesized chemically under cGMP conditions, offering the potential for lower production costs. Most of the work done on these peptide ligands has involved chromatographic resins on which the peptide

ligand is synthesized directly on the chromatographic support using standard solid phase peptide synthesis techniques. This direct synthesis method may offer significant cost advantages over the more common practice of synthesizing pure peptide ligand, followed by attachment to the chromatographic resin from a suitable solvent at a prescribed surface density.

During experiments on the capture of IgG by the HWRGWV resin from complete minimal essential medium (cMEM) containing 10% fetal calf serum it was found that there was some contamination of the product by bovine serum albumin (BSA) [31]. This nonspecific interaction of BSA to the peptide resin is most likely electrostatic in nature and is believed to be due to un-reacted groups and/or truncated peptides which are formed during the solid phase peptide synthesis of the peptide on polymethacrylate-based Toyopearl Amino 650M resin. In order to prevent nonspecific BSA binding, the effects of NaCl and sodium caprylate in the equilibration and binding buffers were studied. The resulting yield and purity of IgG under these conditions are presented in this work. In addition, results are presented on the identification of suitable regeneration agents for retaining the performance of the peptide ligand column over many cycles. Regeneration conditions for affinity resins are highly dependent on the nature of the ligand and the support, as well as on the type of impurities that are to bind strongly to the column [33]. The ability to regenerate and clean affinity supports is an important consideration for industrial applications.

The present work also reports on the efficacy of the peptide resins in purifying humanized and chimeric monoclonal antibodies from Chinese Hamster Ovary (CHO) cell

culture fluids. Both of these MAbs are commercial products in their clarified cell culture fluids. The chimeric MAb of IgG1 subclass was purified using the HWRGWV resin whereas the humanized MAb of IgG4 subclass was purified using HWRGWV, HYFKFD and HFRRHL resins. Results are presented on the dynamic binding capacity, yield and purity of MAbs obtained during the peptide affinity capture step, as well as on the ability of the HWRGWV peptide resin to effectively remove residual DNA and host cell proteins (HCPs). The results are compared to those found using the industry's standard Protein A and G columns.

2.2. Experimental

2.2.1. Materials

HWRGWV, HYFKFD, and HFRRHL resins with a peptide density of 0.15 meq/g were obtained from CreoSalus (KY, USA). The peptide was synthesized directly on Toyopearl AF-Amino-650M (particle size 65 μ m, Tosoh Bioscience, Montgomeryville, USA) using the fluorenylmethyloxycarbonyl (Fmoc) coupling chemistry. Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (TX, USA). Sodium chloride, sodium acetate, sodium thiocyanate, ethylene glycol, glycine, guanidine HCl, urea, hydrochloric acid, glacial acetic acid, phosphoric acid were obtained from Fischer Scientific (PA, USA). Phosphate buffer saline at pH 7.4, ethanol, methanol and isopropanol were from Sigma (MO, USA). All the solvents were of analytical grade. Cell culture medium (Eagle Minimum Essential medium, EMEM) was from Quality Biological (MD, USA). Fetal calf

serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (UT, USA) and Becton Dickinson (MD, USA), respectively.

The complete mammalian cell culture medium (cMEM) used for NaCl, sodium caprylate and regeneration studies was prepared by combining EMEM with 10% FCS and 5% TBP. For the purification experiments, two CHO cell culture supernatants containing monoclonal antibody (MAb) were obtained from two different biopharmaceutical manufacturers that asked for confidentiality. The monoclonal antibodies, MAb1 and MAb2 belong to different IgG classes. MAb1 is a humanized IgG4 whereas MAb2 is a chimeric IgG1. The concentration of MAb1 in the supernatant was 1.5 mg/mL and the concentration of MAb2 in its supernatant was 2.3 mg/mL. In addition to the MAbs, these supernatants contained the usual set of impurities found in mammalian cell culture fluids: pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components.

Micro BCA assay kits were purchased from Pierce (IL, USA). NuPAGE® Novex gels (4–12% Bis–Tris), NuPAGE® MOPS and MES running buffers, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus2® pre-stained molecular weight marker, SimpleBlue™ SafeStain were all from Invitrogen (CA, USA). A HiTrap™ Protein G column was purchased from GE Healthcare (NJ, USA). A Waters 626 LC system integrated with 2487 UV detector (Waters, MA, USA) was used for all chromatography runs unless otherwise mentioned. Microbore stainless steel columns with dimensions 30 mm long × 2.1 mm I.D. were from Altech-Applied Science (PA, USA). All experiments were carried out at room temperature.

2.2.2. Influence of NaCl concentration on IgG yield and purity

Thirty five milligrams of HWRGWV resin were dry-packed in 30 mm x 2.1 mm I.D. microbore columns (0.1 mL). The resin was swollen with 20% methanol and then washed with phosphate buffered saline (PBS), pH 7.4. A sample solution of IgG was prepared by spiking 10 mg of IgG into 1 mL of cMEM. The effect of salt concentration in the equilibration and binding buffers was studied at 0.25 M, 0.5 M, 0.75 M and 1 M NaCl. NaCl concentrations listed are in addition to the salts already present in PBS (0.14 M NaCl). The NaCl concentration in the binding buffer was adjusted to the desired concentration listed above. After equilibrating the column with the binding buffer, 100 μ L of feed was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/hr). The column was washed with 4 mL of binding buffer at flow rate of 0.4 mL/min (692 cm/hr). Elution was then performed with 4 mL of 0.2 M acetate buffer, pH 4 at a flow rate of 0.4 mL/min. The effluent was monitored by absorbance at 280 nm. Fractions were collected and concentrated five times by centrifugation at 4°C, 20817 x g for 30 min using an Amicon Ultra centrifugal filter (3000 MWCO, Ultracel[®], Millipore, MA, USA). These fractions were then used for analysis of IgG purity and yield.

2.2.3. Influence of sodium caprylate on IgG yield and purity

The HWRGWV column was packed and swollen as described in Section 2.2.2. A sample solution of IgG was prepared by spiking 10 mg of IgG into 1 mL of cMEM. The effect of sodium caprylate in the binding buffer was studied at different concentrations of

sodium caprylate *viz.* 0 mM, 25 mM, 50 mM and 75 mM. The sodium caprylate concentrations in the binding buffer and in the feed mixture were adjusted to the above listed concentrations in a base buffer of PBS containing 0.25 M NaCl. The chromatographic steps, fraction collection and preparation were same as described in Section 2.2.2.

2.2.4. Sample analysis for yields and purities

The amounts of IgG in the collected fractions were quantified by HPLC using a 1-mL HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions, as described by Yang *et al.* [30], using NuPAGE® Novex 4-12% Bis-Tris gels in a Xcell SuperLock™ Mini-Cell system (Invitrogen). Sample preparation was done by adding 5 µL of NuPAGE® LDS buffer and 2 µL of NuPAGE® reducing agent to 13 µL of sample and boiling the resulting mixture for 10 minutes. Gels were Coomassie-stained by using SimpleBlue™ SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 kDa.

2.2.5. Batch screening of various regeneration solutions

The following regeneration agents at different concentrations were selected for the screening study: sodium thiocyanate (1 M, 2 M, 3 M), urea (2 M, 5 M, 8 M), guanidine-HCl (1 M, 2 M, 6 M), 0.1 M glycine pH 2.5, 0.85% (v/v) phosphoric acid, 0.85% phosphoric acid followed by 2M Urea in sodium acetate pH 4 buffer, 10% (v/v) ethanol, 10% (v/v) isopropanol, 50% (w/w) ethylene glycol. Fourteen aliquots of 10 mg of HWRGWV resin were swollen in 20% (v/v) methanol for 30 min. After equilibration with PBS pH 7.4, each aliquot was incubated in 1 mL of cMEM mixture containing 10 mg/mL IgG for 2 hours. Resins were thoroughly washed in PBS to remove the unbound proteins and then incubated for 1 hour in 1.5 mL of regeneration solution. The supernatants were collected and analyzed for the total protein content by bicinchoninic acid (BCA) method. The regeneration efficiency was calculated as ratio of protein desorbed by the regeneration agent to the total protein loaded on the resin. To further test the regeneration, 20 μ L of regenerated resins were washed with PBS, boiled with 20 μ L SDS buffer (mixture of 25% NuPAGE® LDS buffer and 10% NuPAGE® reducing agent) for 10 minutes and the resulting supernatants were analyzed by SDS-PAGE.

2.2.6. Secondary screening of the selected regeneration agent

The most efficient regeneration agents were further tested in sequential cycles of chromatographic runs. HWRGWV resin was dry-packed in 0.1 mL column, swollen and washed with PBS as described in Section 2.2.2. The resin was equilibrated with PBS

containing 1 M NaCl. A sample of 100 μ L of cMEM containing 10 mg/mL IgG was loaded onto the column in the equilibration buffer at a flow rate of 0.05 mL/min (87 cm/hr). The column was washed with 4 mL of binding buffer at a flow rate of 0.4 mL/min (692 cm/hr). Elution and regeneration were then performed simultaneously with 4 mL of selected regeneration agent.

2.2.7. Purification of monoclonal antibodies from CHO cell culture supernatants

Thirty-five milligrams of the peptide resins, HWRGWV, HFRRHL or HYFKFD, were dry packed in 0.1 mL columns, swollen and washed as described in Section 2.2.2. After washing, the resins were equilibrated with PBS + 1 M NaCl. Samples of 100 μ L of CHO cell culture supernatants were loaded onto the columns at flow rate of 0.05 mL/min (87 cm/hr). The unbound proteins were washed from the column by using 4 mL of equilibration buffer. Product elution was carried out by using 4 mL of 0.2 M sodium acetate buffer, pH 4, or 0.2 M glycine-HCl, pH 3. Cleaning was performed by 4 mL of 0.85% (v/v) phosphoric acid and regeneration was done by 4 mL of 2 M urea in sodium acetate pH 4 buffer. All the chromatographic steps after loading were performed at a flow rate of 0.4 mL/min (693 cm/hr). Chromatographic fractions were collected and analyzed for purity and yield using the methods described in Section 2.2.4. In the purification of MAb1 all three resins were tested (HWRGWV, HFRRHL and HYFKFD), while in the purification of MAb2 only the HWRGWV resin was used.

2.2.8. Determination of host cell protein (HCP) content

The host cell protein contents of the chromatographic fractions were determined using HCP ELISA kits from Cygnus Technologies (NC, USA). The high sensitivity protocol described by the manufacturer was used in the analysis. Briefly, the anti-CHO coated microtiter wells were filled with 100 μ L of standards and samples and incubated on a rotator at 200 rpm for 1 hour at room temperature. The wells were then washed three times with 300 μ L washing buffer and 100 μ L of anti-CHO:HRP was added to each of the wells. These wells were incubated for 2 hours under the same conditions as described previously. After incubation, the wells were washed four times and filled with 100 μ L of 3,3',5,5' tetramethyl benzidine (TMB) substrate solution. The color was allowed to develop for 30 minutes at room temperature. The reaction was terminated by adding 100 μ L of 0.5 M sulphuric acid to each well. The amount of hydrolyzed substrate was measured by μ Quant Microplate reader (BioTek Inc, VT, USA) at 450 nm. A 4-parameter logistic fit was used to calculate the HCP (ng/mL) content in the chromatographic fractions. The HCP values determined in terms of ng/mL were converted into ng/mg of MAb to take into account the dilution of fractions. The log reduction value (LRV) obtained was determined by taking a \log_{10} ratio of HCP in the load to the HCP in the elution fractions.

2.2.9. Determination of DNA content

The DNA content of the fractions was determined by quantitative polymerase chain reaction (qPCR) using a TaqMan® Residual CHO DNA Detection Kit (Applied Biosystems,

CA, USA) and a 7500 Fast system RT-PCR (Applied Biosystems, CA, USA). All the chromatographic fractions were subjected to buffer exchange with 10 mM Tris-HCl, pH 7.5 using an Amicon® Ultra centrifugal filter (3000 MWCO, Ultracel®, Millipore, MA, USA). CHO DNA standards were prepared from the stock DNA solution (30 ng/μL). Ten microliters of standards, sample of different dilutions and controls, all in triplicates were added to the 96-well plate. Then, 20 μL of PCR reaction mixture consisting of the primers, probes and internal positive control were added to the wells. The plate was set up on the RT-PCR system and reaction carried out according to the manufacturer's protocol.

2.2.10. Determination of dynamic binding capacities

The three peptide resins HWRGWV, HFRRHL and HYFKFD were packed in chromatographic columns, washed and equilibrated with PBS + 1 M NaCl as described in Section 2.2.7. An acetone pulse (5%, v/v) was applied to the columns to determine the total column void volume. Two milliliters of CHO cell culture supernatant containing humanized MAb1 was then loaded directly onto each of the peptide columns. Chromatographic fractions of 0.25 mL were collected and analysed using a HiTrap Protein G column. Breakthrough volume was determined at the point where MAb1 concentration in the flowthrough fraction reached 10% of its feed concentration. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume the dynamic binding capacity of the peptide resins was determined.

2.3. Results

2.3.1. Influence of NaCl concentration on IgG yield and purity

Three cycles of purification of IgG from a cMEM mixture spiked with IgG (10 mg/mL) were carried out for each NaCl concentration (0 M, 0.25 M, 0.5 M, 0.75 M, 1 M) in the equilibration and binding buffers. The purifications were carried with the HWRGWV-Toyopearl resin. Figure 2.1 shows the chromatograms and SDS-PAGE results of the runs with the different NaCl concentrations in the different equilibration and binding buffers. The purity of eluted IgG determined by densitometric analysis and the yield estimated by Protein G HPLC is shown in Figure 2.2. It can be observed from the SDS-PAGE results (Figure 2.1B) that, with increases in the NaCl concentration, the amount of the major impurity, albumin, in the flowthrough fraction increases while that in the elution fraction decreases. Figure 2.2 shows that both the yield and purity of IgG increased with increased NaCl concentration in the equilibration and binding buffers. The purity increased from 80% to 95% with increases in NaCl concentration from 0 M to 1 M. The yield of IgG increased from 77% to 92%. These results confirm that the non-specific binding of albumin is electrostatic, and is mostly due to presence of free amino groups of the base matrix or/and some truncated peptides that are formed during the solid phase synthesis of peptide on the polymethacrylate-based Toyopearl Amino resin. The presence of NaCl prevents the non-specific electrostatic binding of albumin to the amino groups in the resin, making the peptide groups freely available to IgG binding and thereby increasing the binding capacity.

2.3.2. Influence of sodium caprylate on IgG yield and purity

Albumin binds to several fatty acids such as caprylate to form a stable complex. BSA has at least ten binding sites for caprylate [34]. The high affinity interaction between caprylate and albumin could be exploited to reduce the nonspecific binding of BSA to the peptide resin. During purification of IgG using commercial affinity resins such as MabSorbent A2P [35] and MEP Hypercel [36] it is recommended to have post-load wash steps with buffers containing low concentrations of sodium caprylate to remove the bound albumin. It was therefore decided to investigate the performance of HWRGWV resin under different concentrations of sodium caprylate (0 mM, 25 mM, 50 mM and 75 mM). PBS buffer containing 0.25 M NaCl was used as a base buffer since it was found that, in the complete absence of NaCl, there was no influence of sodium caprylate in improving the purity of IgG (data not shown). The chromatograms and SDS-PAGE analysis of the IgG purification from cMEM using different concentrations of sodium caprylate are shown in Figure 2.3. From the SDS-PAGE analysis it can be seen that the addition of sodium caprylate increases the amount of BSA in the flowthrough, thereby increasing the purity of eluted IgG. Increasing the sodium caprylate concentration from 0 mM to 50 mM increased the IgG purity from 78% to 94%. Further increases in sodium caprylate concentration to 75 mM increased the purity of IgG to 97% but there was a significant loss of IgG in the flowthrough fraction, as can be observed in Figure 2.3B, lane 75 mM FT. In this case the higher amount of sodium caprylate might be masking hydrophobic interactions of the HWRGWV resin with IgG, and since hydrophobic interactions form an important part of peptide-IgG binding [31], this leads

to a decrease in the IgG binding capacity of the peptide resin. As a result, the optimum concentration of sodium caprylate to obtain high purity and yield of IgG is about 50 mM.

2.3.3. Batch screening of regeneration solutions

In earlier studies [30, 31] column regeneration was performed with a 2% acetic acid wash. However, repeated use of the regenerated column (15 cycles) under these conditions showed a loss of binding capacity of HWRGWV resin (results not shown). As a result, batch studies were carried out to identify an efficient regeneration agent for HWRGWV-Toyopearl. Several commonly used regeneration agents were screened at different concentrations: sodium thiocyanate (1 M, 2 M, 3 M), urea (2 M, 5 M, 8 M), guanidine-HCl (1 M, 2 M, 6 M), 0.1 M glycine pH 2.5, 0.85% (v/v) phosphoric acid followed by 2 M urea in sodium acetate pH 4 buffer, 10% (v/v) ethanol, 10% (v/v) isopropanol and 50% (w/w) ethylene glycol. The regeneration efficiency results were calculated as a percentage (ratio) of the total protein desorbed relative to the total protein loaded onto the resins and are shown in Figure 2.4. As can be observed, guanidine-HCl (2 M, 6 M) and 0.85% phosphoric acid followed by 2 M urea were the most effective regeneration agents in desorbing the proteins from the resins. In order to further confirm these results, the regenerated resins were washed with PBS, boiled with the SDS buffer (mixture of NuPAGE® LDS buffer and NuPAGE® reducing agent) for 10 minutes and the supernatants were analyzed to determine the amount of protein still remaining bound to the resins. From Figure 2.5 it can be seen that the resins regenerated with guanidine-HCl (2 M, 6 M) or 0.85% phosphoric acid followed by 2 M urea had the least

amounts of bound protein, thus confirming their high regeneration efficiency. Since use of 6 M guanidine-HCl would lead to very high system pressure due to its high viscosity, only 2 M guanidine-HCl and 0.85% phosphoric acid followed by 2 M urea were selected for further chromatographic studies.

2.3.4. Secondary screening of the selected regeneration agents

Different cycles of IgG purification from a cMEM mixture using HWRGWV resin were carried out to more fully characterize the regeneration properties of the two most promising buffer systems: 0.85% phosphoric acid followed by 2 M urea; and 2 M guanidine-HCl. The effects of reducing the concentration of guanidine-HCl from 2 M to 1 M were also studied.

2.3.4.1 Regeneration with 0.85% phosphoric acid followed by 2 M urea

Twenty cycles of purification of IgG from cMEM using HWRGWV resin were performed with a regeneration regime consisting of 0.85% phosphoric acid wash followed by 2 M urea. As can be seen from the chromatograms and SDS-PAGE results (Figure 2.6) the performance of the peptide resin was consistent over this range of cycles. The IgG yield and purity over the purification cycles was 85% ($\pm 3\%$) and 94% ($\pm 2\%$), respectively.

2.3.4.2. Regeneration with 2M guanidine-HCl

Twenty cycles of purification of IgG from cMEM using HWRGWV resin were performed with 2 M guanidine-HCl regeneration after each cycle. From the chromatograms

(Figure 2.7), it can be seen that the performance of the resin was maintained throughout the 20 purification cycles. The IgG yield for the purification cycles was 84% ($\pm 3\%$). For further purification cycles it was decided to test the use of 1 M guanidine-HCl instead of the 2 M concentration. As can be seen from Figure 2.7, the performance of the peptide resin was consistent from the 20th cycle to the 30th cycle, indicating that even 1 M guanidine-HCl can efficiently regenerate the resin. Based on the above studies it can be concluded that both guanidine-HCl (2 M and 1 M), and 0.85% phosphoric acid followed by 2 M urea, were able to successfully regenerate the peptide resin.

2.3.5. Purification of monoclonal antibodies from CHO cell culture supernatants

2.3.5.1. Purification of MAb1

HWRGWV resin was used for purification of a monoclonal antibody (MAb1) from a clarified cell culture fluid. MAb1 is a humanized MAb of IgG4 subclass and is a widely used therapeutic product. The concentration of MAb1 in the cell culture supernatant was 1.5 mg/mL. In addition to MAbs, the cell culture supernatant contained pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components. The peptide resin was packed into the column and equilibrated with PBS + 1 M NaCl. The supernatant was then directly loaded onto the column without any pre-treatment. Washing, elution and regeneration steps were carried out as per the conditions optimized in the studies discussed in Sections 2.3.1 and 2.3.3. Washing was performed with 1 M NaCl and elution was performed with 0.2 M acetate buffer, pH 4. The chromatogram of purification of MAb1

is shown in Figure 2.8A. It can be seen that the elution has a split peak. For comparison, a binding study of pure human polyclonal IgG at same concentration as that of MAb (1.5 mg/mL) was carried out under similar chromatographic conditions. In this case, a single peak was obtained during elution (Figure 2.8B). The split peak in the case of MAb1 may be due to the presence of MAb aggregates in the cell culture supernatant that seem to bind more strongly to the ligand. Therefore, the purification of MAb1 was carried out at a lower elution pH of 3 using 0.2 M glycine-HCl buffer, resulting in a single elution peak (Figure 2.9A). The SDS-PAGE of the chromatographic fractions (Figure 2.9B) shows that the HWRGWV had selectively captured the MAb. The purity calculated by the densitometric analysis was 95% and the yield estimated by HPLC analysis was found to be 85%. As seen below, these results for yield and purity of the MAb1 compared very favourably with those obtained using a Protein G column.

The HCP and DNA content of the chromatographic fractions are shown in Table 2.1. The HWRGWV resin was able to reduce the HCP from 671 ng/mg MAb to 28 ng/mg MAb, achieving a log reduction value (LRV) of 1.4, which is comparable to the LRVs (1.4 - 2.3) reported for Protein A [37] and is better than those reported for mimetic ligands such as MEP Hypercel and Mabsorbent A2P [38]. The HWRGWV resin was also found to be very efficient in clearing the DNA, as no residual DNA was detected in the elution fractions.

To test the reusability of the HWRGWV resin, 10 cycles of MAb1 purification were carried out. Figure 2.9A shows that the chromatograms of all the purification cycles were

identical. The average purities and yields were 94% and 85% respectively, thus confirming the complete regeneration and reusability of HWRGWV resin within the range tested.

The two additional peptide resins, HYFKFD and HFRRHL, were also used for the purification of MAb1 from the cell culture supernatant. The conditions were the same as those used with HWRGWV resin. Five purification cycles were carried out with each of the two resins. The SDS-PAGE results of the MAb1 purification (5th cycle) using HYFKFD and HFRRHL are shown in Figure 2.10. From the SDS-PAGE it can be seen that both HYFKFD and HFRRHL selectively capture and purify MAb1. The average purity of all the runs was 93% and 95% for HYFKFD and HFRRHL respectively. The corresponding MAb1 yields were 86% and 84% respectively.

The performance of the peptide resins was then compared to results obtained using a Protein G column by carrying out purification of MAb1 using a HiTrap Protein G column. In order to keep the operating conditions similar to those used for the peptide resins, 1 mL of cell culture supernatant was loaded onto a 1-mL Protein G column (as 0.1 mL of supernatant was loaded on 0.1 mL peptide resin column). Equilibration was performed with 25 mM phosphate pH 7 buffer and elution was carried out with 0.1 M glycine HCl pH 2.5 buffer (as per the manufacturer's instructions). The SDS-PAGE gels comparing the Protein G purification fractions and the HWRGWV elution fraction are shown in Figure 2.11. It can be observed that the elution fraction of HWRGWV shows a purity similar as that of the Protein G elution fraction. Also, the MAb1 yield using the Protein G column was estimated to be 78%, which was lower than that obtained by HWRGWV (85%) and other two peptide resins.

The reason for the low yields might be the presence of misfolded IgG, which might have the proper molecular weight but does not bind to either Protein G or to the peptide ligands due to blocked binding sites.

The performance of the peptide resins also compare well with other commercially available small ligands such as MAbSorbent A2P (ProMetic BioSciences, NJ, USA), MEP HyperCel (Pall Corp, NY, USA), and Kaptiv-GY (Interchim, Montlucon, France). Mabsorbent A2P, an aromatic triazine derivatized ligand was used to purify MAb from cell culture supernatant with both yields and purity above 95% [39]. However, its binding to IgG is weakened in the presence of pluronic acid, phenol red, surfactants and other hydrophobic moieties. Therefore, cation exchange chromatography was used prior to application of the antibody on Mabsorbent. Hydrophobic charge induction chromatography (HCIC) based MEP HyperCel resin has been used to isolate chimeric MAb from CHO cell culture supernatants resulting in yield and purity of 75% and 44% respectively [40]. In another application, MEP HyperCel was used for the purification of monoclonal IgG1 from cell culture supernatant containing 5% fetal bovine serum [41]. The MAb yield and purity in this case was 76% and 69% respectively. Kaptiv-GY, a tetrameric tripeptide was used to purify different classes of MAbs with yields and purity above 90% [42]. It is not known, with any of these ligands, whether the binding occurs specifically through the Fc fragment.

2.3.5.1.1. Determination of dynamic binding capacity

The dynamic binding capacities (DBC) of three peptide resins for MAb1 were determined by breakthrough experiments. A 2 mL aliquot of CHO cell culture supernatant

was loaded onto 0.1 mL of each of the three peptide resins at a flow rate of 0.05 mL/min (87 cm/hr). The breakthrough curves are shown in Figure 2.12. As shown in the Table 2.2, the dynamic binding capacities of HWRGWV, HYFKFD and HFRRHL for MAb1 at the 10% breakthrough point were estimated to be 18.4 mg/mL of resin, 17.6 mg/mL of resin and 19.3 mg/mL of resin, respectively. Considering the fact that the breakthrough experiments were carried out in the presence of all cell culture components and impurities, the DBC obtained by the peptide resins is comparable to those generally reported for Protein A resins [43].

2.3.5.2. Purification of MAb2

The HWRGWV peptide resin was also used for purification of another monoclonal antibody (MAb2) from CHO cell culture supernatant. MAb2 is a chimeric MAb of IgG1 subclass containing murine light and heavy chain variable region sequences and human constant region sequences. The concentration of MAb2 in the supernatant was 2.3 mg/mL. The peptide resin was packed into the column and equilibrated with PBS + 1 M NaCl. The cell culture supernatant was loaded directly onto the column without any pre-treatment. Elution was carried out using 0.2 M acetate buffer pH 4. The remaining chromatographic conditions were the same as used for purification of MAb1. Chromatograms and SDS PAGE (non-reducing) analysis of four cycles of MAb2 purification using HWRGWV resin are shown in Figure 2.13. The average yield and purity of the four purification runs was 84.5% and 95% respectively, which are similar to the values obtained for MAb1.

The host cell protein (HCP) and DNA content of the chromatographic fractions are shown in Table 2.3. The HCP levels were reduced from 389,773 ng/mg MAb to 9,790 ng/mg

MAB resulting in a log reduction value (LRV) of 1.6, which is comparable to those reported for Protein A [37]. The peptide resin also showed a very high DNA clearance capability. The DNA concentration was reduced from 17,580 ng/mg MAb to 1.06 ng/mg MAb resulting in a LRV of 4.22, which is in the higher range of DNA LRV values (2.9 – 3.0) reported for Protein A matrices [44].

For comparison, a Protein G column from GE Healthcare (HiTrap Protein G) was used for the purification of MAb2 from CHO cell culture supernatant. A fraction of 1 mL of supernatant was loaded on a 1-mL Protein G column. All the operating conditions were same as used for the HWRGWV resin. The yield and purity (Figure 2.13B) of MAb2 obtained were 82% and 96% respectively, similar to the values obtained with the HWRGWV resin.

2.4. Conclusions

The salt concentration in the binding buffer was found to be an important parameter during the purification of IgG by the hexamer peptide resin HWRGWV. Increases in the NaCl concentration in the binding buffer increased both the yield and purity of IgG. Using a 1 M NaCl concentration, purity and yields above 90% were obtained for the purification of IgG from cMEM. MS analysis (not shown) of the cleaved peptide from the base matrix reveals truncated peptide sequences formed during the solid phase synthesis of the peptide on the polymethacrylate based Toyopearl resin. These truncated sequences may be responsible for non-specific adsorption of albumin and other proteins. Since these truncated sequences have a terminal amino group, the interaction is primarily electrostatic at a pH 7.4, the pH

used for the protein capture experiments. The addition of NaCl prevents the binding of these impurities and makes the peptide groups more freely available for IgG binding. In cases where the use of high NaCl concentrations is not convenient, it has been found that low concentrations of sodium caprylate (50 mM) can be used to achieve high IgG purity and yield. In this case, the sodium caprylate binds to specific sites on the albumin molecule to reduce nonspecific adsorption to the HWRGWV resin. If the sodium caprylate concentration is too high (75 mM or higher), it begins to interfere with the specific adsorption of IgG to the HWRGWV resin.

Efficient cleaning and regeneration agents are needed to ensure the consistent performance and long life of the resins. Various regeneration and cleaning agents have been reported in the literature and used commercially. For the HWRGWV resin, guanidine-HCl (1 M or 2 M) and 0.85% phosphoric acid followed by 2 M urea were found to be efficient washing and regeneration agents. Batch and column studies with cMEM confirmed the effectiveness of the selected regeneration protocols.

The three peptides HWRGWV, HYFKFD and HFRRHL were used to successfully purify a humanized MAb1 of IgG4 subclass. This MAb, which is a widely used therapeutic product, was purified from CHO cell culture supernatant with a purity and yield above 90% and 85% respectively. The yields and purity results obtained with the peptide resins were equal or better than results obtained with a Protein G column. The HCP and DNA reduction levels obtained with HWRGWV resin were comparable to values reported for Protein A resins. The dynamic binding capacities (DBC) of the three peptide resins for the MAb (IgG4)

determined directly by loading the cell culture supernatant were in the range of 17-19 mg/mL of resin. The HWRGWV peptide resin was also used to successfully purify a chimeric MAb of IgG1 subclass from CHO cell culture supernatant. The purity obtained was 95% and the yield was 84.5%. The yield and purity obtained with the peptide resin were similar to those that obtained with a Protein G column. The HWRGWV resin was also very efficient in reducing the DNA and host cell proteins with log reduction values of 4.22 and 1.6 respectively.

Studies are in progress to characterize, validate and improve the solid phase synthesis of peptides on the resin to minimize truncated peptide sequences and obtain a highly homogeneous population of hexamer peptide on the resins. This should significantly enhance the binding capacity and specificity of the peptide resins thus resulting in a highly specific, small peptide affinity adsorbent for large-scale purification of antibodies and Fc fusion proteins.

2.5. Acknowledgements

The authors would like to thank Nathaniel Hentz and Rebecca Semcer from BTEC/NCSU for their assistance with DNA quantitation. The authors also gratefully acknowledge the support of the North Carolina Biotechnology Center and the Kenan Institute for Engineering, Technology & Science at NC State for financial support of this project.

2.6. References

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Table 2.1. Host cell protein and DNA content of the chromatographic fractions of purification of MAb1 (IgG4) using HWRGWV resin.

Fractions	HCP (ng/mg MAb)	DNA (pg/mg MAb)
Load	671	1898.35
Flowthrough	90052	68337
Elution	28	ND

Table 2.2. Dynamic binding capacities of peptide resins for MAb1 (IgG4).

Peptide Resin	DBC for MAb1 at 10% Breakthrough (mg /mL of resin)
HWRGWV	18.4
HYFKFD	17.6
HFRRHL	19.3

Table 2.3. Host cell protein and DNA content of the chromatographic fractions of purification of MAb2 (IgG1) using HWRGWV resin.

Fractions	HCP (ng /mg MAb)	DNA (ng/mg MAb)
Load	389773	17580
Flowthrough	15128536	278993
Elution	9790	1.06

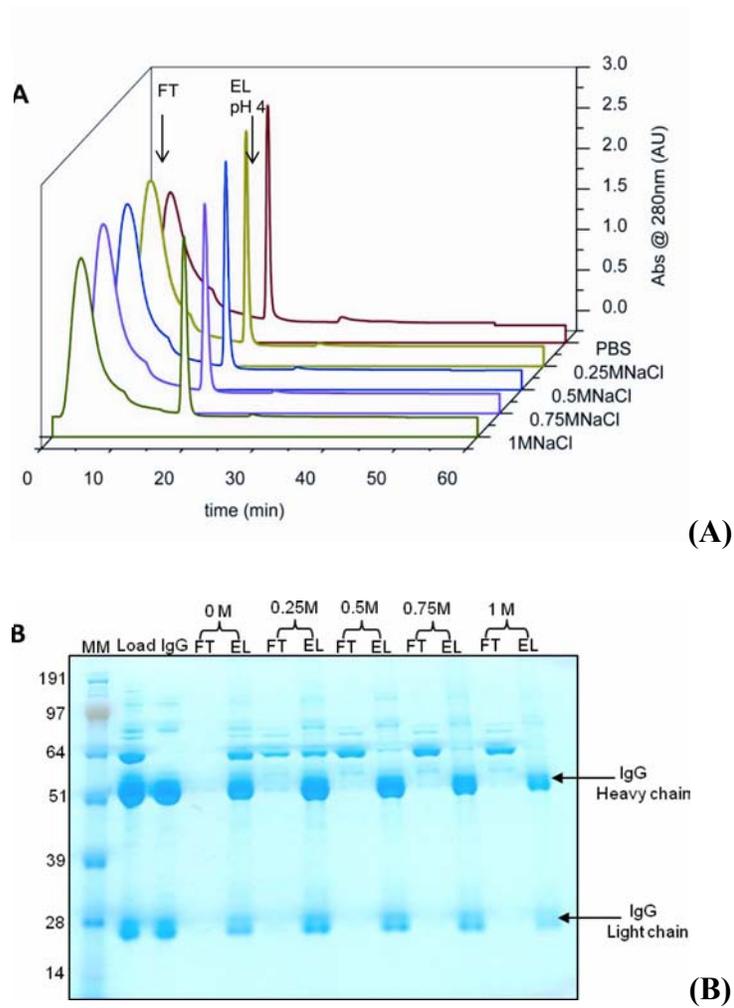


Figure 2.1. (A) Chromatograms of purification of IgG from cMEM-IgG (10mg/mL) mixture with HWRGWV resin under different NaCl conditions used during equilibration and binding. (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM-molecular weight marker, FT - flowthrough fraction, EL - elution fraction.

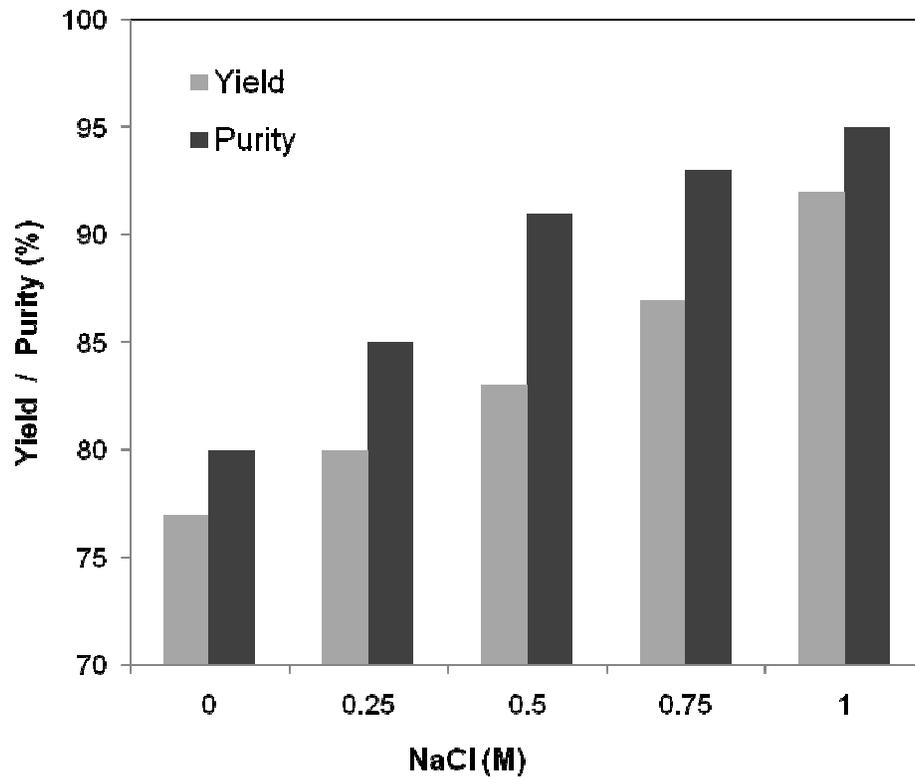
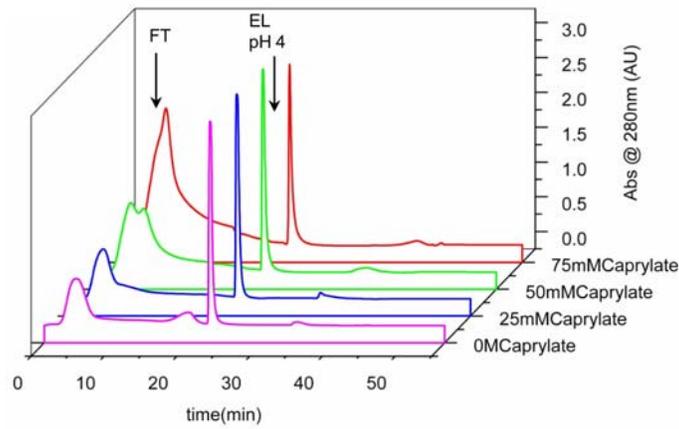
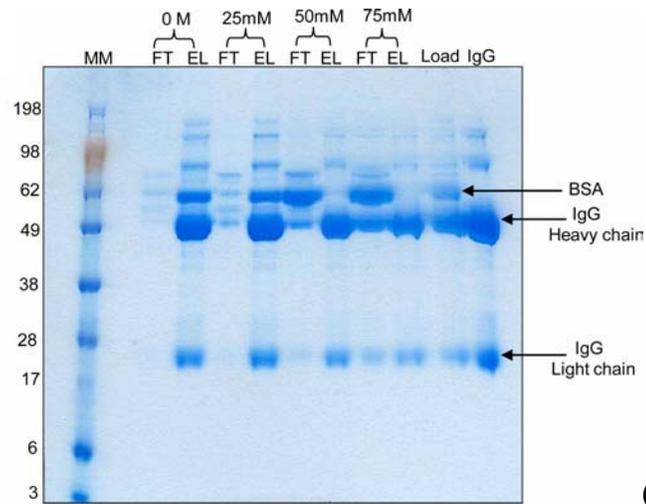


Figure 2.2. Influence of NaCl concentration in the equilibration and binding buffer on yield and purity of IgG purified from cMEM-IgG mixture using HWRGWV resin. Data presented are averages of triplicate runs.



(A)



(B)

Figure 2.3. (A) Chromatograms of purification of IgG from cMEM-IgG (10mg/mL) mixture under different concentrations of sodium caprylate in the equilibration and binding buffer using HWRGWV resin. (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM-molecular weight marker, FT- flowthrough fraction, EL-elution fraction.

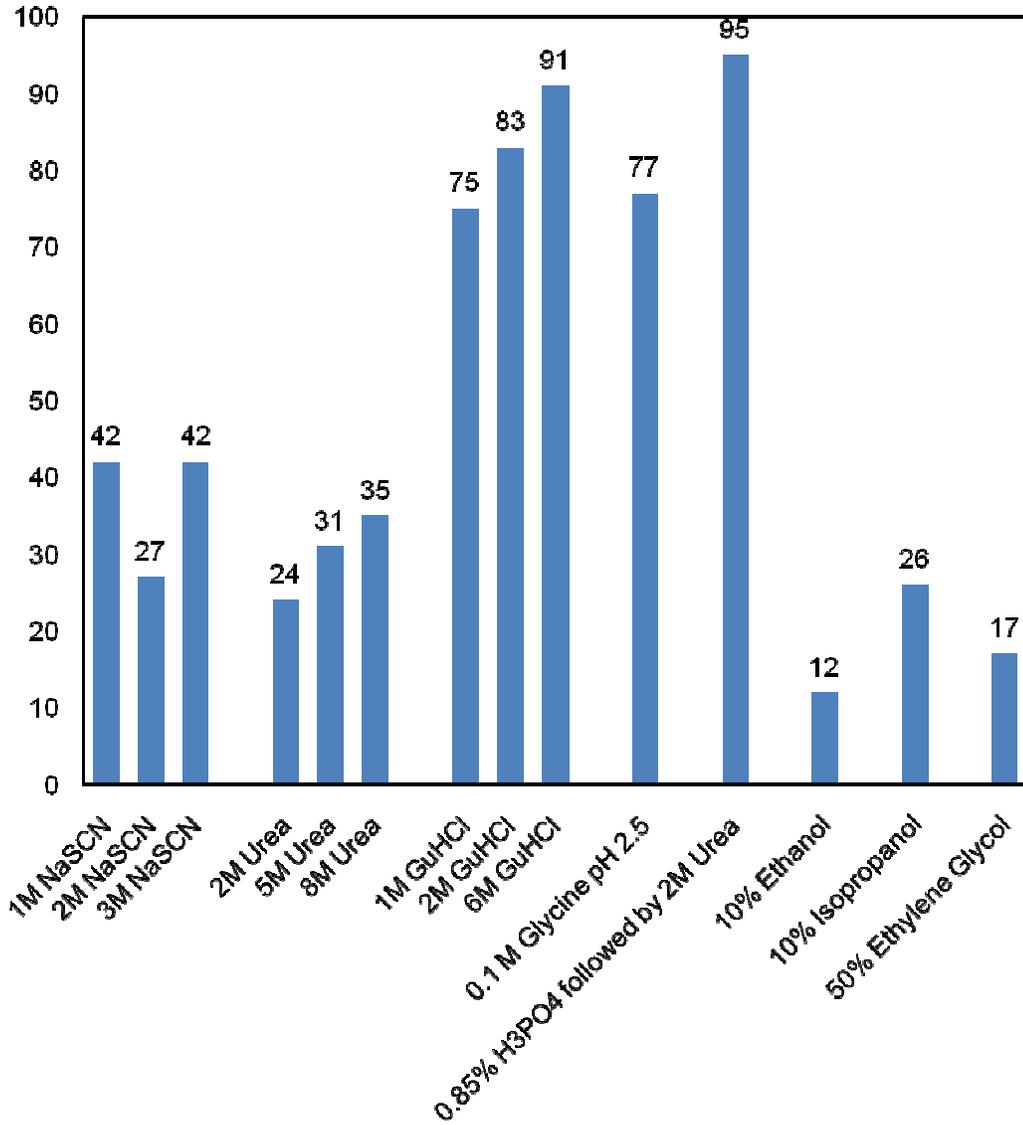


Figure 2.4. Regeneration efficiency of HWRGWV resin (percentage of protein desorbed from resin to total protein loaded) using different regeneration agents.

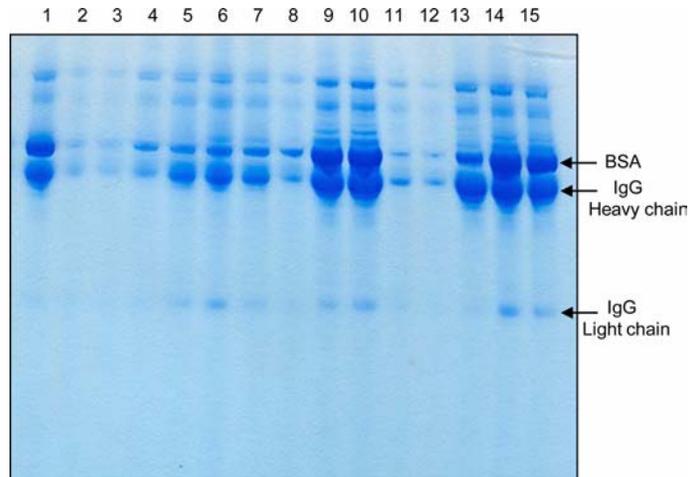


Figure 2.5. SDS-PAGE (reducing conditions) of supernatants obtained by boiling the regenerated HWRGWV resin with SDS buffer. Lanes: 1- No regeneration agent, 2- 6M GuHCL, 3 – 2M GuHCl, 4 – 1M GuHCl, 5- 5M NaSCN, 6- 2M NaSCN, 7-1M NaSCN, 8- 0.1M Glycine HCl 2.5, 9- 2M Urea, 10- 5M Urea, 11- 0.85% phosphoric acid, 12- 0.85% phosphoric acid followed by 2M Urea, 13- 50% (w/w) ethylene glycol, 14- 10% (v/v) ethanol, 15- 10% (v/v) Isopropanol.

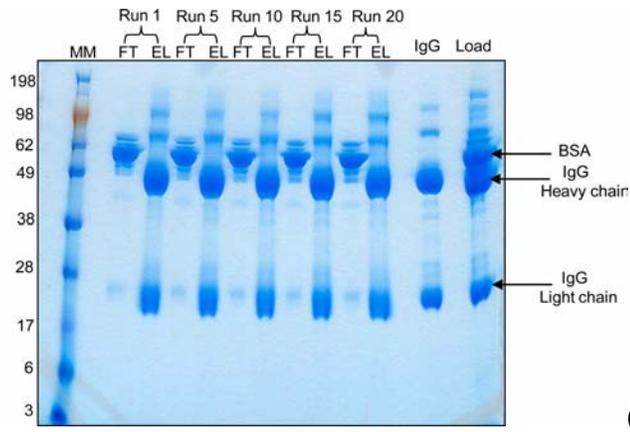
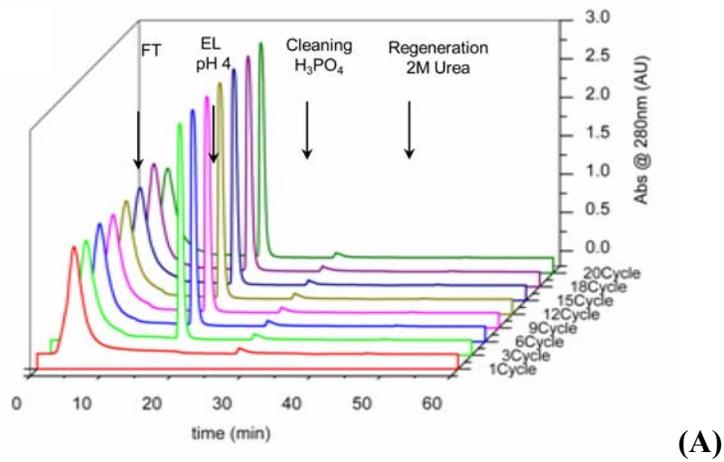


Figure 2.6. (A) Chromatographic HWRGWV regeneration studies with 0.85% phosphoric acid followed by 2 M urea. (B) SDS-PAGE (reducing conditions) of chromatographic fractions. Labels: MM- Molecular weight marker, FT- flowthrough fraction, EL-elution fraction.

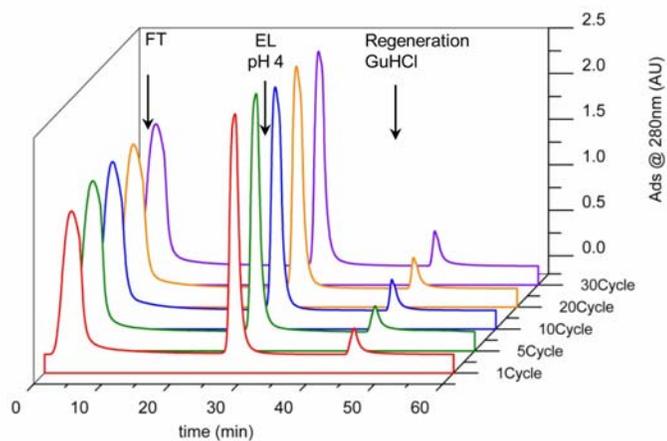


Figure 2.7. Chromatographic regeneration studies of HWRGWV resin using 1M and 2M Guanidine HCl.

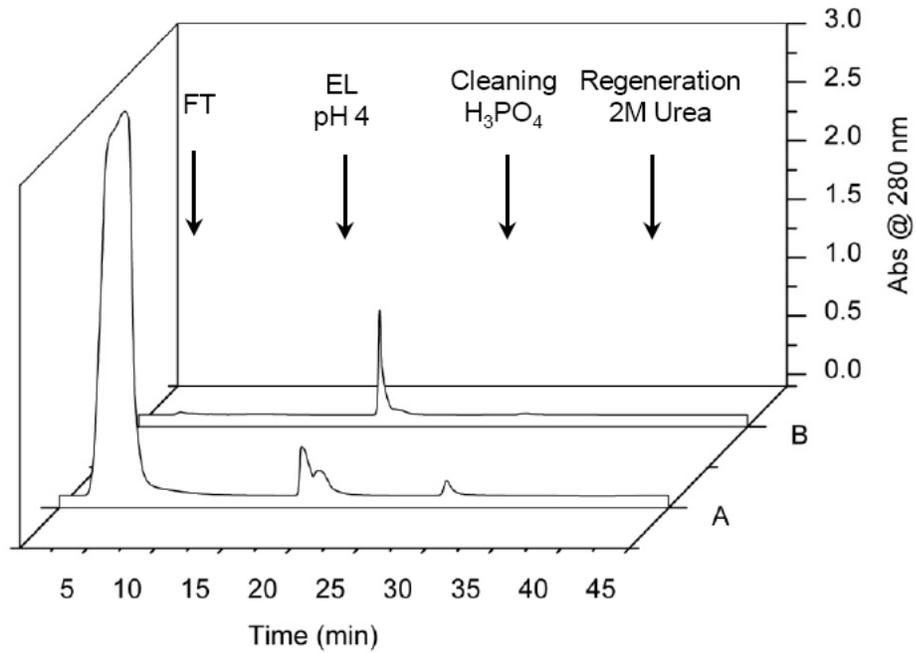
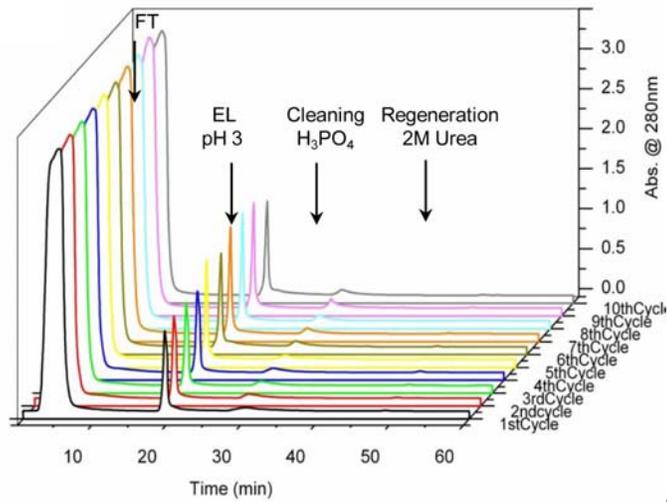
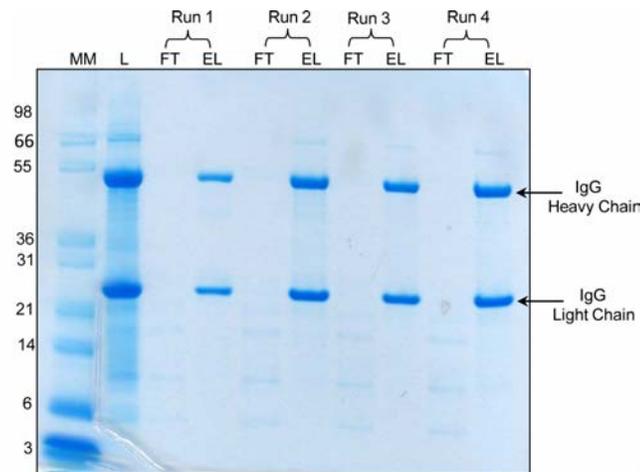


Figure 2.8. (A) Chromatogram of purification of MAb1 (IgG4) from cell culture supernatant with elution at pH 4 using HWRGWV resin. (B) Chromatogram of binding of pure human polyclonal IgG under similar conditions as that used for MAb purification.



(A)



(B)

Figure 2.9. (A) Comparison of ten cycles of purification of MAb1 (IgG 4) from cell culture supernatant using HWRGWV resin. (B) SDS-PAGE (reducing conditions) of the chromatographic fractions. Labels: MM-molecular weight marker, FT- flowthrough fraction, EL-elution fraction.

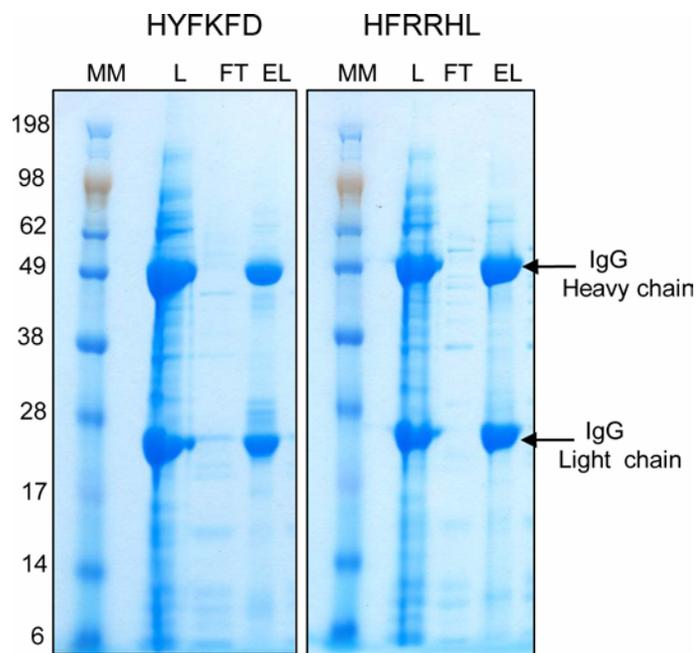


Figure 2.10. SDS PAGE analysis (reducing conditions) of chromatographic purification of MAb1 (IgG4) using HYFKFD and HFRRHL resins. Labels: MM- Molecular weight marker, FT- flowthrough fraction, EL-elution fraction.

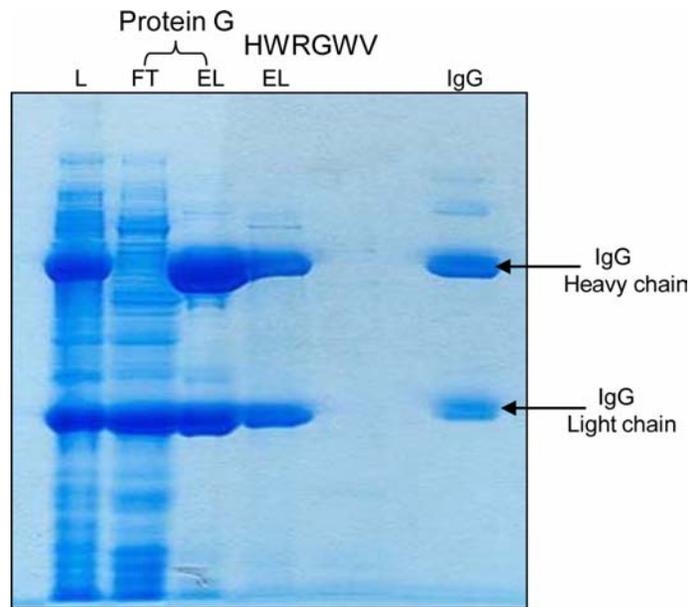


Figure 2.11. SDS PAGE analysis (reducing conditions) of chromatographic purification of MAb1 (IgG4) using Protein G and HWRGWV resin.

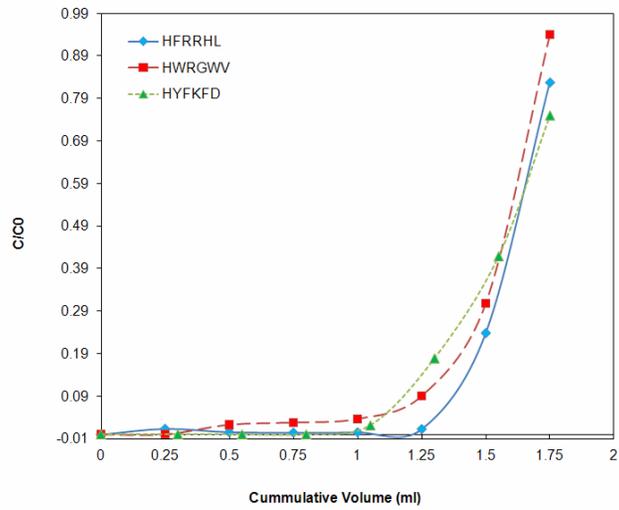
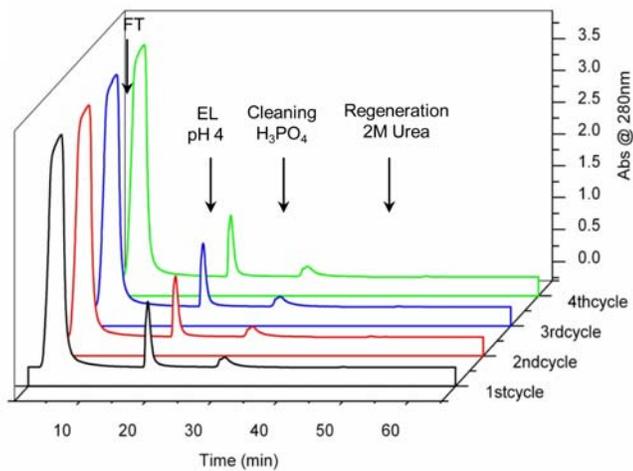
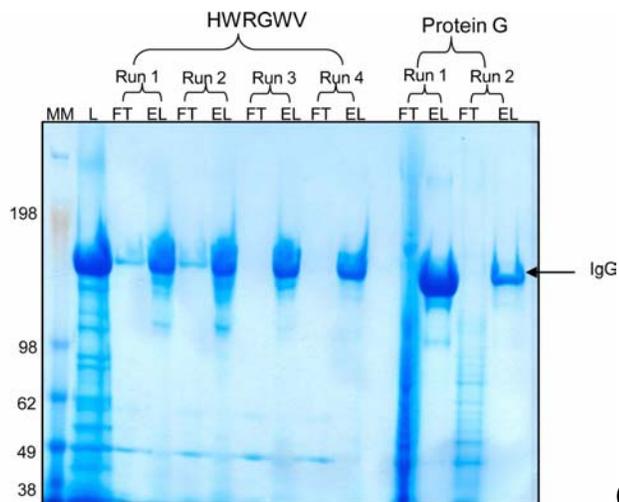


Figure 2.12. Breakthrough curves of HWRGWV, HYFKFD, and HFRRHL resins for MAb1 (IgG4).



(A)



(B)

Figure 2.13. (A) Chromatogram of four cycles of MA2 (IgG1) purification from cell culture supernatant using HWRGWV resin. (B) SDS-PAGE analysis (non-reducing conditions) of the chromatographic fractions of MA2 purification runs using HWRGWV and Protein G resins.

**Chapter 3. Purification of polyclonal antibodies from Cohn Fraction II+III, skim milk
and whey by affinity chromatography using hexamer peptide ligand**

Stefano Menegatti, Amith D. Naik, Patrick V. Gurgel, Ruben G. Carbonell

A version of this chapter has been published in

Journal of Separation Science 2012, 35(22), 3139-3148.

Abstract

HWRGWV, a peptide that binds specifically to the Fc fragment of hIgG, was used for the purification of IgG from Cohn Fraction II+III of human plasma and from bovine skim milk and whey. The concentration of sodium chloride and sodium caprylate in the binding buffer as well as the pH of the elution buffer were optimized to achieve high IgG yield and purity. Under optimized conditions, IgG was recovered from plasma fractions with yield and purity up to 84% and 95% respectively. IgG was also purified from skim milk with 74% yield and 92% purity and from whey with 85% yield and 93% purity. Purification experiments were also performed with Protein A resin and the results were found to be similar to those obtained with the peptide adsorbent.

Keywords: Affinity chromatography; Immunoglobulin G; Milk; Peptide ligands; Plasma

3.1. Introduction

Therapeutic antibodies, both monoclonal and polyclonal, are a leading class of biopharmaceuticals for the treatment of a vast variety of diseases, from infections to cancer and autoimmune diseases [1, 2]. Antibody-based drugs access the human body in different forms, such as immunostimulators or conjugated to cytotoxic drugs, cytokines or radionuclides [3-7]. Engineered monoclonal antibodies (mAbs) are regarded as the paradigm of this class [8, 9]. Their use is expected to increase in the coming decades and novel methods for producing and tailoring binding specificities are becoming available [10]. Nevertheless, the widespread application of monoclonal antibodies is still hampered by their cost and availability. Furthermore, the monovalent nature of monoclonal therapeutics can be limiting when the target properties are not well known [11]. Polyclonal antibodies (pAbs), on the other hand, can be produced in higher amounts at lower costs. Moreover, pAb-based therapeutics exhibit polyvalent interactions against multiple epitopes and targets and are therefore best suited for the prevention or treatment of some diseases [12]. Plasma-derived polyclonal intravenous immunoglobulin (IVIG) preparations have been successfully applied to the prophylactic prevention of infectious diseases in immunodeficient patients and find increasing use against autoimmune and inflammatory problems [13-15]. To date, IVIG is the major plasma product on the global blood product market, with a steadily increasing annual consumption [16]. Polyclonal antibodies have been produced from transgenic plants and transgenic animals [17-19] as well as from human plasma. These sources have a potential to lower the upstream processing costs thereby further reducing the costs of polyclonal antibodies.

The cornerstone of large-scale production of polyclonal antibodies from human plasma has been ethanol fractionation, pioneered by Cohn, which separates plasma proteins by tuning pH, ionic strength, ethanol content and temperature [20-22]. The numbered Cohn fractions contain the following main constituents: I, fibrinogen; II, γ -globulins; III, β -globulins; IV, α -globulins; V, albumin; VI, glycoproteins. Cohn Fraction II+III paste has become the starting material of further techniques for the purification of IVIG. However, these processes suffer from problems of low yields and low purity, thus necessitating the need for additional chromatographic steps, thereby reducing the overall process yield [23, 24]. Protein A/G affinity chromatography, which is the industrial standard for the purification of monoclonal antibodies, might not be commercially feasible for the purification of polyclonal antibodies on large scale. This is due to problems of high viscosity of serum and high concentration of impurities that bind non-specifically to the adsorbents. Moreover, Protein A matrices do not bind to the human IgG₃ subclass and also suffer from limitations of high cost, difficult elution and chemical lability towards the cleaning and sanitization conditions [25-28]. Purification of IgG from lacteal secretions of transgenic animals is also difficult due to the variety of impurities and low concentration. Typical IgG concentrations are quite low, being in the order of 0.7 mg/mL [29]. Similarly to human plasma, Protein A/G affinity chromatography is not feasible for milk, as the general problems of high cost as well as toxicity and immunogenicity of leachates are still a concern.

To overcome these problems, synthetic ligands based on peptides, amino acids, triazine scaffolds and thiophilic compounds have been suggested for purification of antibodies [30]. Among them, peptide ligands have been shown to mimic the binding of large

protein ligands and offer robustness and low costs. Our group has identified and developed the hexameric peptide HWRGWV for purification of IgG [31, 32]. This peptide binds specifically the Fc fragment of hIgG. Yield and purity of monoclonal antibodies recovered from cell culture supernatant media were around 85% and 95% respectively, comparable with those obtained using Protein A / G adsorbents [33]. Furthermore, unlike Protein A, the peptide ligand binds human IgG₃ as well as bovine and goat IgGs. It also allows elution in milder conditions than those of Protein A [ref.], it is chemically stable and the method for the on-resin synthesis allows up to 100 reuses of the adsorbent with alkaline sanitization (0.5M NaOH) after every cycle (reference our paper on alkaline stability). This work presents the use of the affinity adsorbent HWRGWV Toyopearl resin for the purification of IgG from Cohn fraction II+III of human plasma and from bovine skim milk and whey. The chromatographic protocols have been optimized to maximize the product yield and purity for the three polyclonal sources. The salt concentration and composition of the binding buffer have been studied by exploring the use of sodium chloride and sodium caprylate. Also the pH of the eluting agent has been varied in order to achieve an optimum level of IgG yield and purity.

3.2. Experimental

3.2.1. Materials

HWRGWV resin having a peptide density of 0.15 meq/g was purchased from CreoSalus (Louisville, KY, USA). The peptide was synthesized on Toyopearl AF-Amino-

650M resin (particle size 65 μm) via conventional Fmoc coupling chemistry. Human polyclonal Immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Sodium chloride, sodium acetate, sodium caprylate, glycine, urea, hydrochloric acid, glacial acetic acid, phosphoric acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Phosphate buffer saline at pH 7.4 and Cohn's Fraction II+III were from Sigma Aldrich (Saint Louis, MO, USA). Cohn's paste II+III was dissolved in PBS pH 7.4 to achieve a concentration of approximately 5 mg/mL and filtered sequentially through a 0.44 μm and a 0.22 μm filter from Pall Corporation (Port Washington, NY, USA). Skim milk was purchased from the local grocery store. Whey was prepared from skim milk by precipitating the casein with 0.1 N HCl. The casein was separated from the whey by centrifugation and then the pH of the whey was adjusted to pH 7 with 1 M Tris HCl, pH 8. NuPAGE® Novex gels (4-12% Bis-Tris), NuPAGE® MOPS running buffer, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlueplus2® pre-stained molecular weight marker, SimpleBlue SafeStain were all from Invitrogen (Carlsbad, CA, USA). Recombinant Protein A immobilized on Toyopearl HW-65F (Toyopearl AF-rProtein A-650F) was a kind gift from Tosoh Bioscience (King of Prussia, PA, USA). HiTrap™ Protein G column was purchased from GE Healthcare (Piscataway, NJ, USA). A Waters 626 LC system integrated with 2487 UV detectors (Milford, MA, USA) was used for all chromatography runs. Microbore stainless steel columns (30 mm long x 2.1 mm I.D.) were purchased from Altech-Applied Science (State College, PA, USA). All experiments were carried out at room temperature.

3.2.2. Purification of IgG from Cohn's Fraction II+III

3.2.2.1. Effect of elution pH

Thirty five milligrams of HWRGWV Toyopearl resin were packed in a 30 mm x 2.1 mm I.D. Microbore column (0.1 mL) and swollen with 20% v/v methanol. Cohn Fraction II+III feed sample was prepared as described in Section 3.2.1. Four elution buffers were prepared in a base of 0.2 M sodium acetate buffer by adjusting the pH at 4, 4.5, 5 and 5.5. The resin was equilibrated with PBS pH 7.4 buffer containing 0.25M NaCl. One hundred microliters of feed sample was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of equilibration buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of the selected elution buffer at the flow rate of 0.4 mL/min (696 cm/h). Cleaning and regeneration was performed by 4 mL of 0.85% phosphoric acid followed by wash of 4 mL of 2 M urea in sodium acetate pH 4 buffer. The effluent was monitored by absorbance at 280 nm. Fractions were collected and concentrated five times by centrifugation at 4°C, 20817 x g for 30 min using an Amicon® Ultracentrifuge filter (3000 MWCO, Ultracel®, Millipore, Billerica, MA, USA). These fractions were then used for analysis of IgG purity and yield as described in Section 3.2.2.5.

3.2.2.2. Effect of conductivity

The HWRGWV column was packed and swollen as described in Section 3.2.2.1. Cohn fraction II+III for the injection was prepared as described in Section 3.2.2.1. The effect of conductivity in terms of salt concentration in the binding buffer was studied at 0 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M and 0.5 M NaCl. The concentrations listed are in addition to the salts

already present in PBS (0.14 M). After equilibrating the column with the binding buffer, 100 μ l of feed was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of binding buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer pH 5 at the flow rate of 0.4 mL/min (696 cm/h). Column cleaning, regeneration, system monitoring as well as fraction collection and analysis were performed as described in Section 3.2.2.1.

3.2.2.3. Effect of sodium caprylate

The HWRGWV column was packed and swollen as described in Section 3.2.2.1. The effect of sodium caprylate in the binding buffer was studied at different concentrations of sodium caprylate *viz.* 0 mM, 20 mM, 40 mM and 60 mM. The sodium caprylate concentrations in the binding buffer were adjusted to the above listed concentrations in a base buffer of PBS containing 0.2 M NaCl. The chromatographic steps, fraction collection and analysis were same as described in Section 3.2.2.2.

3.2.2.4. Purification with Protein A resin

Toyopearl AF-rProtein A-650F resin was packed in a 30 mm x 2.1 mm I.D. microbore column (0.1 mL) and equilibrated with PBS pH 7.4. Cohn Fraction II+III feed sample was prepared as described in Section 3.2.2.1. One hundred microliters of feed sample was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of equilibration buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.1 M Glycine buffer pH 2.5 at the flow rate of 0.4 mL/min

(696 cm/h) (as per manufacturer's instructions). System monitoring as well as fraction collection and analysis were performed as described in Section 3.2.2.1.

3.2.2.5. *Sample analysis for yields and purities*

The amount of IgG in the collected fractions was quantitated by HPLC using a 1 mL HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (for purification of IgG from Cohn II+III) or non-reducing (for purification of IgG from skim milk and whey) conditions, as described by Yang *et al.* [31, 32], using NuPAGE[®] Novex 4-12% Bis-Tris gels in a Xcell SuperLock[™] Mini-Cell system (Invitrogen, Carlsbad, CA, USA). Sample preparation was done by adding 5 μ L of NuPAGE[®] LDS buffer and 2 μ L of NuPAGE[®] reducing agent to 13 μ L of sample and boiling the resulting mixture for 10 minutes. Gels were Coomassie-stained by using SimpleBlue[™] SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, Bethesda, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 KDa.

3.2.3. Purification of IgG from whey and skim milk

3.2.3.1. Effect of conductivity on IgG purification from whey

The HWRGWV column was packed and swollen as described in Section 3.2.2.1. Whey was spiked with IgG to obtain an IgG concentration of 10 mg/mL. The effect of salt concentration in the binding buffer was studied at 0 M, 0.25 M, 0.5 M, 0.75 M and 1 M NaCl. The concentrations listed are in addition to the salts already present in PBS (0.14 M). After equilibrating the column with the binding buffer, 100 μ L of whey was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of binding buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer pH 4 at the flow rate of 0.4 mL/min (696 cm/h). Column cleaning and regeneration, as well as system monitoring as well as fraction collection and analysis were performed as described in Section 3.2.2.1.

3.2.3.2. Effect of sodium caprylate on the purification of IgG from whey

The HWRGWV column was packed and swollen as described in Section 3.2.2.1. The effect of sodium caprylate in the binding buffer was studied at different concentrations of sodium caprylate *viz.* 0 mM, 20 mM, 40 mM and 60 mM. The sodium caprylate concentrations in the binding buffer were adjusted to the above listed concentrations in a base buffer of PBS containing 0.25 M NaCl. The chromatographic steps, fraction collection and analysis were same as described in Section 3.2.3.1.

3.2.3.3. Purification with Protein A resin

The experiment was performed as described in Section 3.2.2.4 with 100 μ L of whey spiked with IgG (10 mg/mL) as feed sample.

3.2.3.4. Purification of IgG from skim milk

The HWRGWV column was packed and swollen as described in Section 3.2.2.1. Skim milk was spiked with IgG to obtain an IgG concentration of 10 mg/mL. PBS pH 7.4 buffer containing 0.5 M NaCl was used as binding buffer. The chromatographic steps, fraction collection and preparation were same as described in Section 3.2.3.1.

3.3. Results

3.3.1. Purification of IgG from Cohn's Fractions II+III

3.3.1.1. Effect of elution pH

Cohn Fraction II+III from human plasma was employed as crude hIgG source to explore the possibility of using the HWRGWV Toyopearl resin for IVIG production. The feed sample was prepared by dissolving the lyophilized Cohn fraction in PBS buffer pH 7.4 to obtain an IgG concentration of 5 mg/ml. A preliminary purification study was carried out by loading 100 μ L of feed sample on HWRGWV Toyopearl resin. Elution was performed at pH 4 using 0.2 M sodium acetate buffer. From the SDS PAGE (Figure 3.1) it can be observed that purity of eluted IgG was very low (about 45%). The IgG seems to be contaminated by IgA, IgM and albumin. The presence of other globulins was anticipated, as HWRGWV is known to

bind all Igs, in particular IgG, IgA and IgM, although with different strength [31]. At low pH, however, all these proteins are eluted together from the resin. This result suggested that the optimization of elution pH was the first step to be undertaken.

The effect of the elution pH was studied by using 0.2 M sodium acetate buffer at pH 4, 4.5, 5 and 5.5. The chromatograms and the SD-PAGE are presented in Figure 3.2. As the elution pH increased from pH 4 to pH 5.5, the purity of IgG increased from 76 % to 96 % (Table 3.1). Among the Igs, IgG is the most loosely bound to HWRGWV ($K_{d,IgG} > K_{d,IgA} > K_{d,IgM}$) and is thus eluted at higher pH values [34]. Due to their polymeric structure, IgA and IgM bind more tightly to the HWRGWV ligand via multipoint interaction, hence requiring relatively stringent elution conditions, such as low pH buffers. The IgA and IgM that remained bound to column were desorbed during regeneration (Figure 3.2B, Lanes 4, 7, 10 and 13) by 0.85% phosphoric acid buffer (~ pH 2). Although increasing the elution pH improves the purity of IgG, it also leads to a decrease of yield. Increasing the elution pH from 5 to 5.5 enhanced the purity from 91% to 95%, but also decreased the yield considerably from 85% to 76% (Table 3.1). A considerable amount of IgG remained bound to the column and was removed during regeneration (Figure 3.2B, Lanes 4). Therefore, 0.2 M acetate buffer pH 5 was then chosen as the best eluting agent, as it offers a good balance between yield and purity.

3.3.1.2 Effect of conductivity

To further improve the recovery of IgG from plasma fraction a detailed study of the effect of ionic strength of the equilibration buffer on IgG yield and purity was conducted.

Ionic strength of the equilibration buffer was studied in terms of NaCl concentration. Sodium chloride is known to improve product purity by preventing the non-specific electrostatic binding of some proteins, albumin in particular, to the free amino groups and/or truncated peptide sequences of the HWRGWV resin [33]. In this study, three cycles of purification from Cohn's Fraction II+III were carried out for each NaCl concentration (0 M, 0.1 M, 0.2M, 0.3 M, 0.4 M and 0.5 M) in the equilibration and binding buffers. Figure 3.3 shows the typical chromatograms and SDS-PAGE results of the runs with the different NaCl concentrations, while Table 3.2 reports the values of IgG yield and purity.

It can be observed from the SDS-PAGE (Figure 3.3B) that, with increase in NaCl concentration, the purity of IgG in the eluted fraction increased, from 81% with PBS up to 96% with 0.5 M NaCl in PBS. Higher NaCl concentrations increased the amount of albumin (67 KDa) in the flowthrough fraction, thus leading to an increase in the purity of eluted IgG. Sodium chloride also seemed to prevent the electrostatic binding of some other impurities to the resin. However, as reported in Table 3.2, with an increasing NaCl concentration a loss of IgG was observed in the flowthrough fraction and to a lower extent in the regeneration fraction, resulting in lower yields. It appears that the addition of NaCl enhances the binding strength of some IgG subclasses and weakens the interaction of some others. Increasing the NaCl concentration from 0 M to 0.5 M lowered the IgG yield from 89% to 53% (Table 3.2). Results indicate that 0.2M NaCl is the salt concentration that gives a good compromise between yield and purity (respectively 84% and 89%).

3.3.1.3 Effect of sodium caprylate

Short fatty acids, such as caprylic acid and sodium caprylate, are commonly employed for protein precipitation and inactivation of enveloped viruses in plasma and cell culture [16, 35, 36]. As caprylate shows high affinity for albumin [37], washes of sodium caprylate are adopted to remove the albumin bound to affinity adsorbents for IgG purification. Technical specifications for commercial affinity resins MabSorbent A2P [38] and MEP Hypercel [39] recommend performing post-load wash steps with buffers containing low amounts of caprylate to remove the bound albumin. Similarly, previous studies by our group suggest that small amounts of sodium caprylate added to the binding buffer exert a favorable effect on IgG recovery from complex mixtures [33]. It was therefore decided to test whether sodium caprylate can further improve the performance of HWRGWV resin. Purification studies were carried out by addition of sodium caprylate to the equilibration and binding buffer optimized in Section 3.1.2 (PBS + 0.2 M NaCl). Two different concentrations of sodium caprylate (20 mM and 40 mM) were studied, comparing with buffer containing no caprylate. The elution was carried out at the conditions optimized in the Section 3.3.1.1 (pH 5). The chromatograms and the SDS-PAGE analysis of the IgG purification from Cohn Fraction II+III at the various caprylate concentrations are reported in Figure 3.4.

It is evident from the SDS-PAGE that the combination of small amounts of sodium chloride and sodium caprylate significantly enhances the yield and purity of IgG recovered by HWRGWV resin. Increasing the sodium caprylate concentration from 0 mM to 40 mM

increased the IgG purity from 87% to 92% (Table 3.3). Although the addition of caprylate prevents the binding of some high molecular weight contaminants, which thus flow through, the study of the SDS-PAGE (Figure 3.4B) indicates another mechanism favoring the IgG purity. It is observed that the addition of caprylate increases the adsorption strength of the main impurities, which are thus not eluted at pH 5 but are released only later during the regeneration (~ pH 2). With increasing the caprylate concentration, the number and amount of impurities decrease in elution lanes 3, 6 and 9 and correspondingly increase in regeneration lanes 4, 7 and 10. On the other hand, the IgG yield remained unchanged at 84% (Table 3.3) with increasing caprylate concentration. As a result, the optimum concentration of sodium caprylate to obtain high yield and purity of IgG is about 40mM.

In summary, chromatographic purification employing (a) PBS containing 0.2 M NaCl and 40 mM sodium caprylate as equilibration buffer and (b) 0.2 M sodium acetate pH 5 as elution buffer resulted in a IgG purity and yield of 93% and 84% respectively. The yield obtained in this study was higher than the one step yield (74%) on ion exchange columns [40] and on Mabsorbent A2P (< 80%) [16] used for purification of IVIG from human plasma and Cohn fraction II+III respectively. Parkkinen and coworkers had obtained a yield of 92% using an ion exchange column [41]. However, this yield was achieved by loading the Cohn Fraction II+III pre-treated with caprylic acid and PEG, and that the yield of the pre-treatment step was about 75% IgG.

3.3.1.4 Purification with Protein A resin

Purification of IgG from Cohn II+III fraction was also performed using Toyopearl AF-rProtein A-650F. The chromatographic conditions employed followed the instructions of the manufacturer. Figure 3.5 shows the SDS-PAGE of the collected flowthrough and elution fractions. The chromatographic run afforded a very high product yield (96%), but low purity (74%). IgM and IgA were found to be the main contaminants in the eluted fractions. IgM and IgA are known to bind Protein A, although less strongly than IgG [42]. Similarly to the results presented for the peptide ligand in Section 3.3.1.1, the very acidic conditions employed for elution (pH 2.5) caused the desorption of all the bound proteins, therefore lowering the IgG purity.

3.3.2 Purification of IgG from whey and skim milk

3.3.2.1 Effect of ionic strength on IgG purification from whey

HWRGWV resin was used for the purification of IgG from whey. Since transgenic whey was not available, the feed was simulated producing whey in our laboratory and spiking polyclonal IgG into it to reach an IgG concentration of 5 mg/mL. In order to optimize the purification protocol, a study was performed to determine the effect of salt concentration of the equilibration or binding buffer on IgG yield and purity. Accordingly, a set of chromatographic purifications was run using PBS pH 7.4 containing different concentrations of NaCl (0 M, 0.25 M, 0.5 M, 0.75 M and 1 M) as equilibration and binding buffers. The chromatograms and SDS-PAGE results are presented in Figure 3.6. With plain PBS buffer (0

M NaCl) almost all major impurities, i.e. β -lactoglobulin, α -lactalbumin and albumin bound to the resin and contaminated the eluted IgG. Addition of 0.25 M NaCl to PBS in the equilibration buffer prevented the binding of albumin and α -lactalbumin, but β -lactoglobulin was still found in the elution, thus giving an IgG purity of 65% (Table 3.4). By increasing the salt concentration to 0.5 M NaCl in PBS, the binding of β -lactoglobulin to the resin was eliminated and a highly pure IgG (91%) was obtained. Further increases in NaCl concentration up to 0.75 M and 1 M did not induce any appreciable improvement in IgG purity. This study indicates that non-IgG proteins present in whey, such as albumin, α -lactalbumin and β -lactoglobulin bind electrostatically to the HWRGWV resin. Previous studies (not shown) have revealed the presence of truncated peptide sequences formed during the synthesis of the peptide ligand on polymethacrylate based Toyopearl resin. These truncated sequences do not bind the IgG but are likely to cause the non-specific electrostatic adsorption of albumin and other proteins to the affinity adsorbent. The salt concentration did not have any significant effect on IgG yield, which remained constant at a value of approximately 83% through the whole range of NaCl concentration (Table 3.4). Based on these results, PBS buffer containing 0.5 M NaCl was selected as optimal equilibration and binding buffer.

3.3.2.2 Effect of sodium caprylate on IgG purification from whey

Purification studies of IgG from Cohn II + III fraction (Section 3.1.3) have shown that the addition of sodium caprylate in the equilibration buffer increases the purity of eluted IgG. It was therefore decided to study the effect of sodium caprylate on the purification of IgG from

whey. Accordingly, a set of chromatographic purifications were run using sodium caprylate added to PBS pH 7.4 containing 0.25 M NaCl as equilibration and binding buffer. Four different concentrations of sodium caprylate (0 mM, 20 mM, 40 mM and 60mM) were studied. The chromatograms and SDS-PAGE are presented in Figure 3.7. From the SDS-PAGE it can be seen that the addition of sodium caprylate prevents the binding of albumin and α -lactalbumin. At the concentration of 60 mM of caprylate both whey albumins are completely removed. However, sodium caprylate does not have any effect on β -lactoglobulin, which results as the major contaminant of IgG in the eluted fraction. Moreover the addition of sodium caprylate lowers the IgG yield from 85% (0 mM) to 60% (60 mM).

3.3.2.3 Purification with Protein A resin

The purification of IgG from whey was also performed using Toyopearl AF-rProtein A-650F. Figure 3.8 shows the SDS-PAGE of the collected flowthrough and elution fractions. Protein A allowed the recovery of IgG with high yield and purity, respectively 94% and 96%.

3.3.3.4 Purification of IgG from skim milk

The HWRGWV resin was then challenged with skim milk spiked with IgG at a concentration of 5 mg/mL. The chromatographic conditions were same as those optimized for the purification of IgG from whey (Section 3.2.1). Equilibration, binding and washing were carried out with PBS buffer pH 7.4 containing 0.5 M NaCl, while elution was performed with 0.2 M sodium acetate buffer, pH 4. Three cycles of IgG purification from skim milk were run. The chromatograms and SDS-PAGE are shown in Figure 3.8. All the major impurities, i.e. β -lactoglobulin, α -lactalbumin and albumin were in the flowthrough

fraction (lane 5 and 6, Figure 3.8B), demonstrating the specificity of the ligand towards IgG, even when challenged with proteins known to have high non-specific binding properties. The purity of IgG determined by densitometric analysis of SDS-PAGE was found to be 92%, while the average IgG yield was found to be 74%. The lower IgG yield as compared to that obtained in the purification from whey is mainly due to the presence of casein in skim milk. Casein is known to foul the chromatographic resins and the pretreatment of milk is hence a step universally accepted in the purification of recombinant proteins from transgenic milk [43]. As the skim milk was loaded directly onto the peptide resin, the casein bound non-specifically to the resin and/or blocked the pores of the resin leading to a decrease in IgG binding capacity. The large peaks observed during the cleaning step might correspond to the bound casein. In summary, the performance of HWRGWV resin in purifying IgG from skim milk and whey was comparable and in some cases better than the other affinity ligands reported in literature. MEP HyperCel hydrophobic charge induction chromatographic (HCIC) resin (Pall Corp, NY, USA) was used to purify monoclonal antibody from transgenic goat milk [44]. Although the purity reported was higher than 95%, the transgenic milk was subjected to two filtration steps before loading the milk onto the MEP Hypercel. Thiophillic chromatography and Protein G – Sepharose 4FF were used to purify IgG from bovine whey [45]. The purity of eluted IgG obtained was 74% and 81% respectively.

3.4. Conclusions

This study has demonstrated the applicability of the peptide-based adsorbent HWRGWV Toyopearl resin to the purification of polyclonal IgG from complex mammalian sources. As the ligand HWRGWV binds to the Fc portion of the immunoglobulins, the separation of IgG from IgA and IgM required the optimization of elution conditions. Because of their polymeric nature, IgA and IgM bind more tightly to the peptide ligands compared to IgG. This allows the separation of IgG from the other Igs by using milder elution conditions. Increasing the pH from 4 to 5.5 increased the IgG purity from 76% to 96%. However, the yield decreased from 95% to 76%. As a result, the optimized elution pH was found to be 5.5, which gave 85% yield and 91% purity. In order to further increase the purity of IgG, further work was performed to study the effect of conductivity and concentration of sodium caprylate in the binding buffer. While higher NaCl concentration enhanced the product purity, it also negatively affected the yield, by shielding the electrostatic component of IgG (subclasses) binding to the ligand. Therefore, 0.2 M NaCl in PBS was considered the best choice, allowing 84 % yield and 89 % purity. Furthermore, it was found that the addition of small amounts (20mM to 40mM) of sodium caprylate to PBS containing 0.2 M NaCl enhanced the IgG purity in the eluted fraction. In particular, the use of 40 mM sodium caprylate allowed 84% yield and 92% purity. As expected, sodium caprylate does not affect the IgG binding, but rather the behavior of the other contaminants, seemingly by preventing their binding to the resin. The performance of peptide resin was found to be comparable to that of Protein A resin, which recovered IgG from Cohn Fraction II+III with 96% yield and 74% purity.

The HWRGWV resin was also successfully used for the purification of IgG from whey. In this case the optimization was aimed towards the separation of IgG from albumin, α -lactalbumin and β -lactoglobulin. The use of PBS pH 7.4 containing 0.25 M NaCl as binding buffer prevented the binding of albumin and α -lactalbumin to the resin. Further increase in the NaCl concentration to 0.5 M resulted in pure IgG (91%) in the elution fraction. The addition of small amounts of sodium caprylate in the binding buffer prevented the adsorption of albumin and α -lactalbumin, but did not affect the binding of β -lactoglobulin. In summary, the use of PBS pH 7.4 buffer containing 0.5 M NaCl and 0.2M sodium acetate buffer pH 4 for binding and elution respectively were found to be the best conditions for the purification of IgG from whey. The purity and the yield obtained were 91% and 83% respectively, which were comparable to those obtained with Protein A resin. The peptide ligand was also successfully used for the purification of IgG from skim milk. While the purity obtained was 92%, the yield was found to be 74%. The slightly lower yield is mainly imputed to the presence of casein in the feed.

In conclusion, this work demonstrates the potential of peptide-based affinity adsorbent HWRGWV Toyopearl resin for the large-scale purification of polyclonal IgG from complex sources.

3.5. References

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Table 3.1. Influence of elution pH on yield and purity of IgG purified from Cohn Fraction II+III using HWRGWV Toyopearl resin.

Elution pH	IgG Yield	IgG Purity
pH 5.5	76%	96%
pH 5	85%	91%
pH 4.5	91%	84%
pH 4	95 %	76%

Table 3.2. Influence of salt concentration in equilibration and binding buffer on yield and purity of IgG purified from Cohn Fraction II+III using HWRGWV Toyopearl resin.

NaCl concentration in PBS	IgG Yield	IgG Purity
0 M	89%	81%
0.1 M	86%	85%
0.2 M	84%	89%
0.3 M	76%	92%
0.4 M	65%	94%
0.5 M	53%	96%

Table 3.3. Influence of sodium caprylate concentration in the equilibration and binding buffer on yield and purity of IgG purified from Cohn Fraction II+III using HWRGWV Toyopearl resin. Data presented are averages of triplicate runs.

Caprylate concentration	IgG Yield	IgG Purity
0 mM	85% \pm 2.5%	87%
20 mM	84% \pm 2.0%	90%
40 mM	84% \pm 2.7%	93%

Table 3.4. Influence of salt concentration in equilibration and binding buffer on yield and purity of IgG purified from whey using HWRGWV Toyopearl resin. Data presented are averages of triplicate runs.

NaCl concentration in PBS	IgG Yield	IgG Purity
0 M	86% ± 1.9%	50%
0.25 M	82% ± 1.5%	65%
0.5 M	83% ± 2.1%	91%
0.75 M	83% ± 2.0%	91%
1 M	84% ± 2.5%	92%

Table 3.5. Influence of sodium caprylate concentration in equilibration and binding buffer on yield and purity of IgG purified from whey using HWRGWV Toyopearl resin. Data presented are averages of triplicate runs.

NaCl concentration in PBS	IgG Yield	IgG Purity
0 M	85%	69%
20 mM	74%	81%
40 mM	62%	83%
60 mM	60%	84%

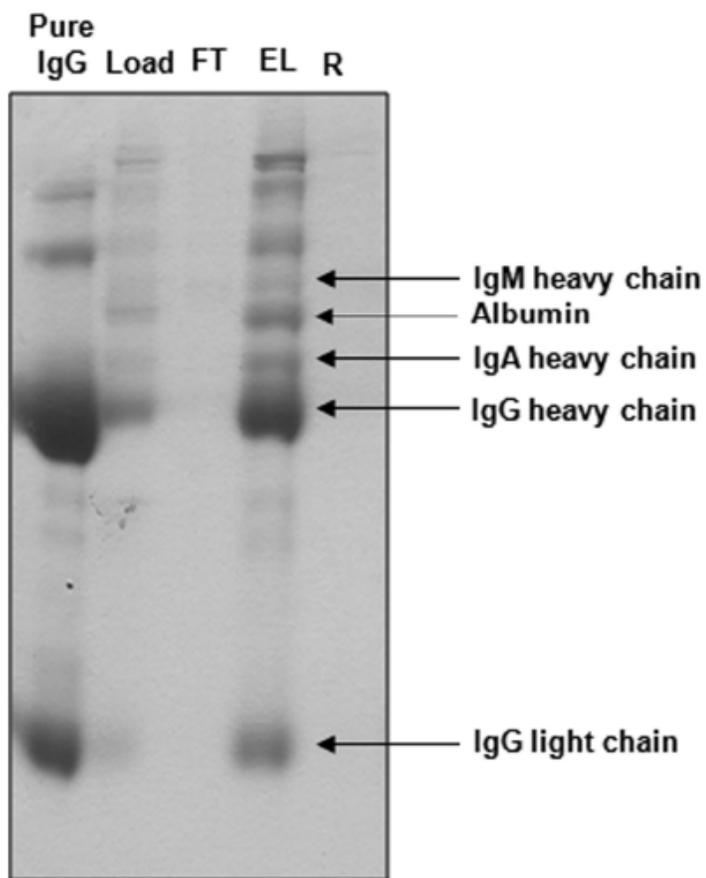


Figure 3.1. SDS PAGE analysis (reducing conditions) of chromatographic purification of IgG from Cohn Fraction II+III using 0.2 M sodium acetate buffer pH 4. Labels: FT – flowthrough fraction, EL – elution fraction, R – regeneration fraction.

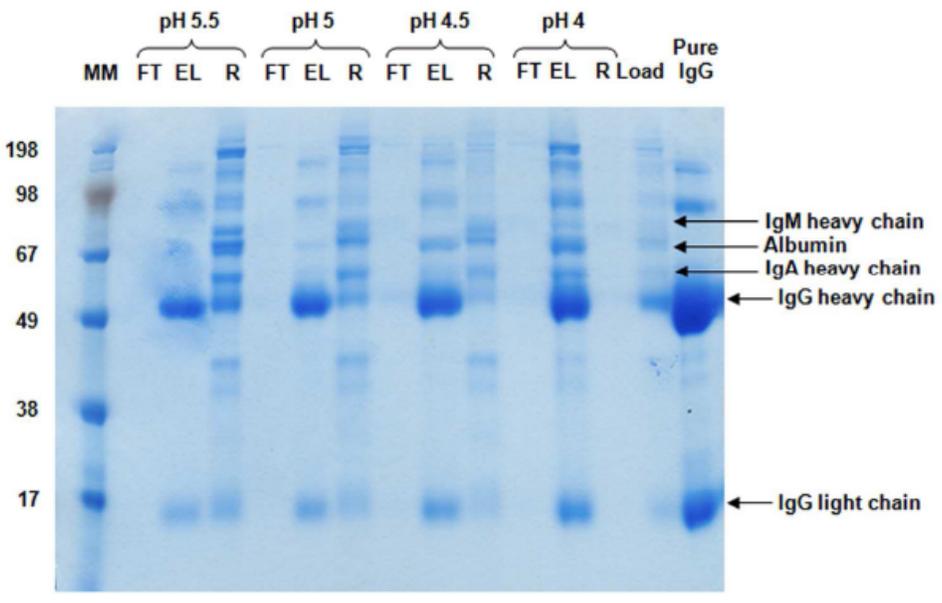
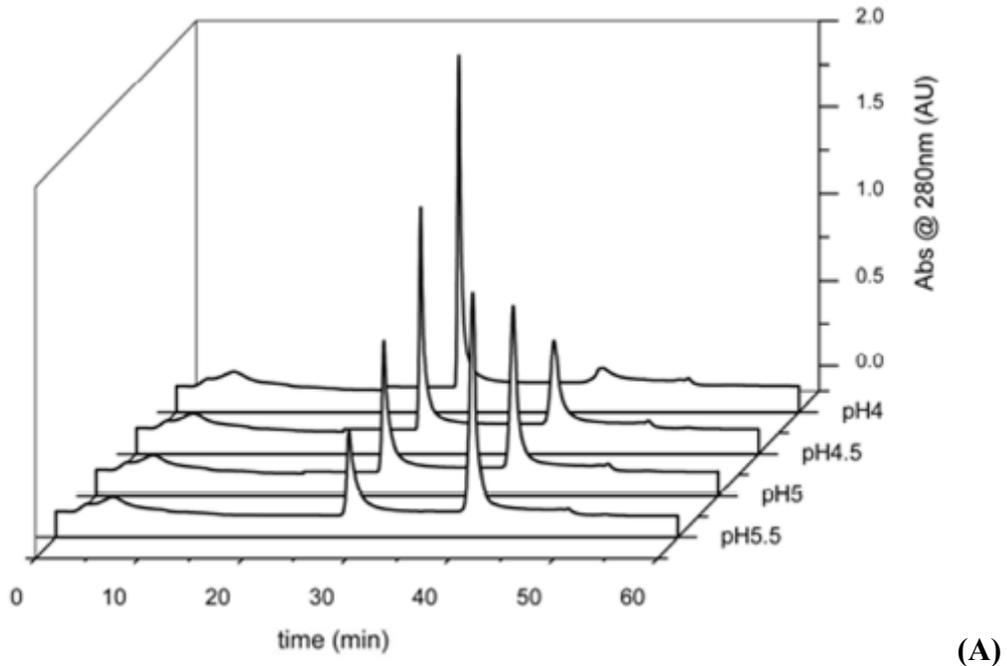


Figure 3.2. (A) Chromatograms of purification of IgG from Cohn Fraction II+III under different elution pH conditions. (B) SDS-PAGE (reducing conditions) of flowthrough, elution and regeneration fractions. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction; R – regeneration fraction.

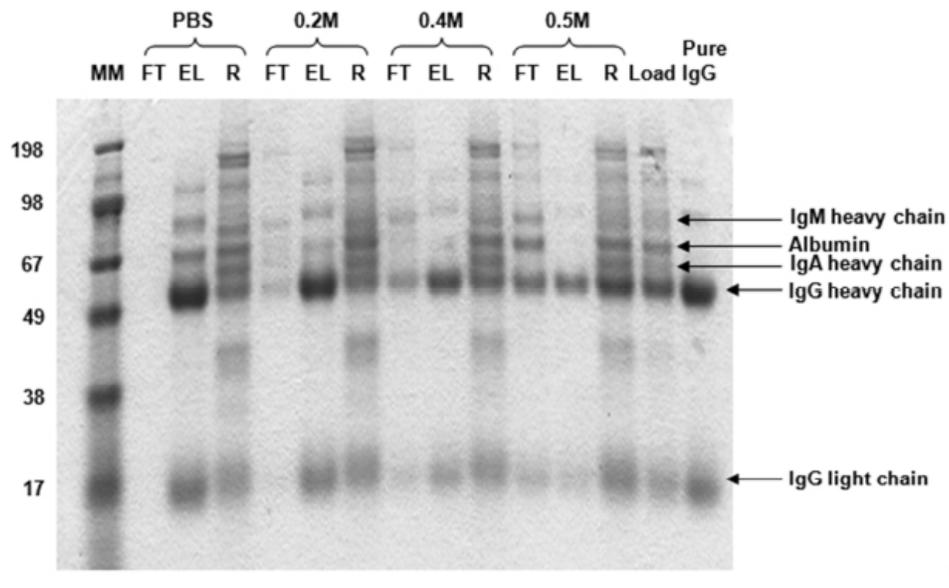
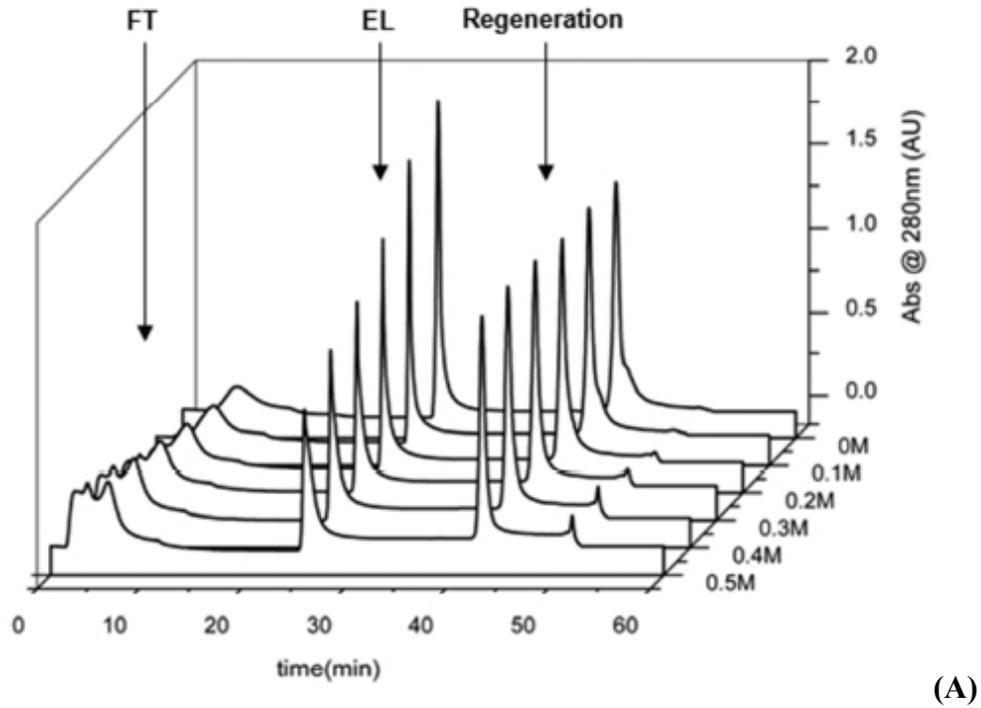


Figure 3.3. (A) Chromatograms of purification of IgG from Cohn’s Fraction II+III under different NaCl conditions used during equilibration and binding buffer. (B) SDS-PAGE (reducing conditions) of flowthrough, elution and regeneration fractions. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction; R – regeneration fraction.

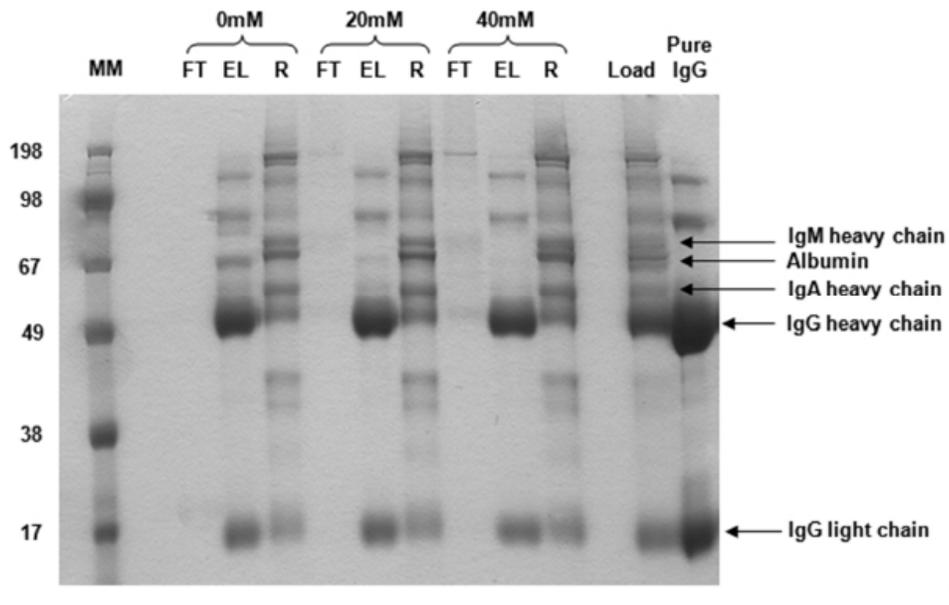
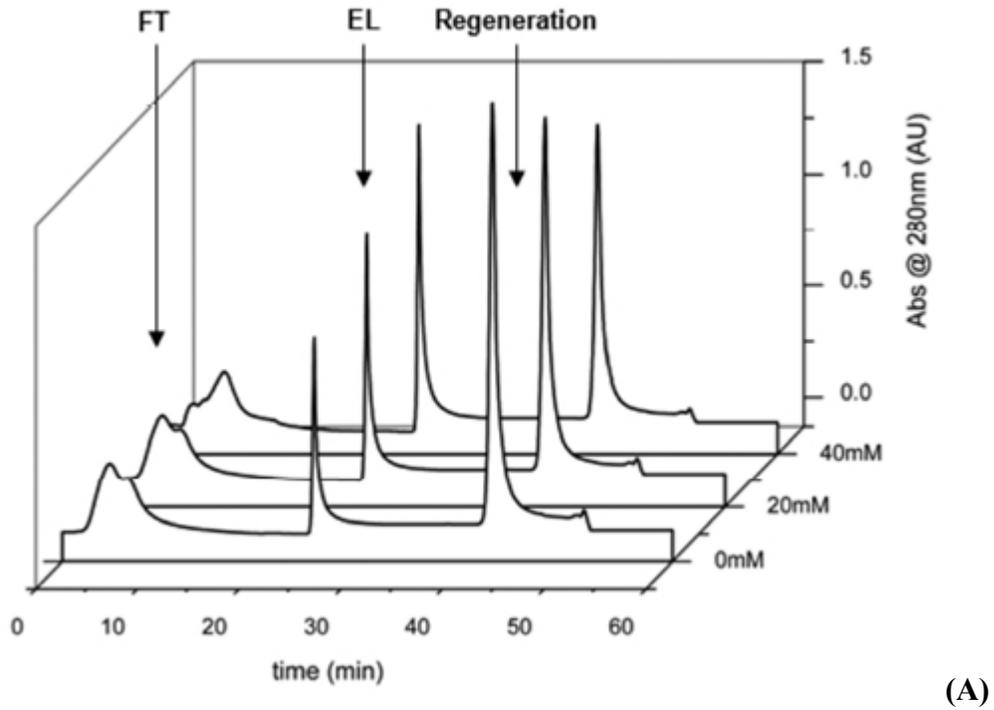


Figure 3.4. (A) Chromatograms of IgG purification from Cohn Fraction II+III under different concentrations of sodium caprylate in the equilibration and binding buffer. (B) SDS-PAGE (reducing conditions) of flowthrough, elution and regeneration fractions. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction; R – regeneration fraction.

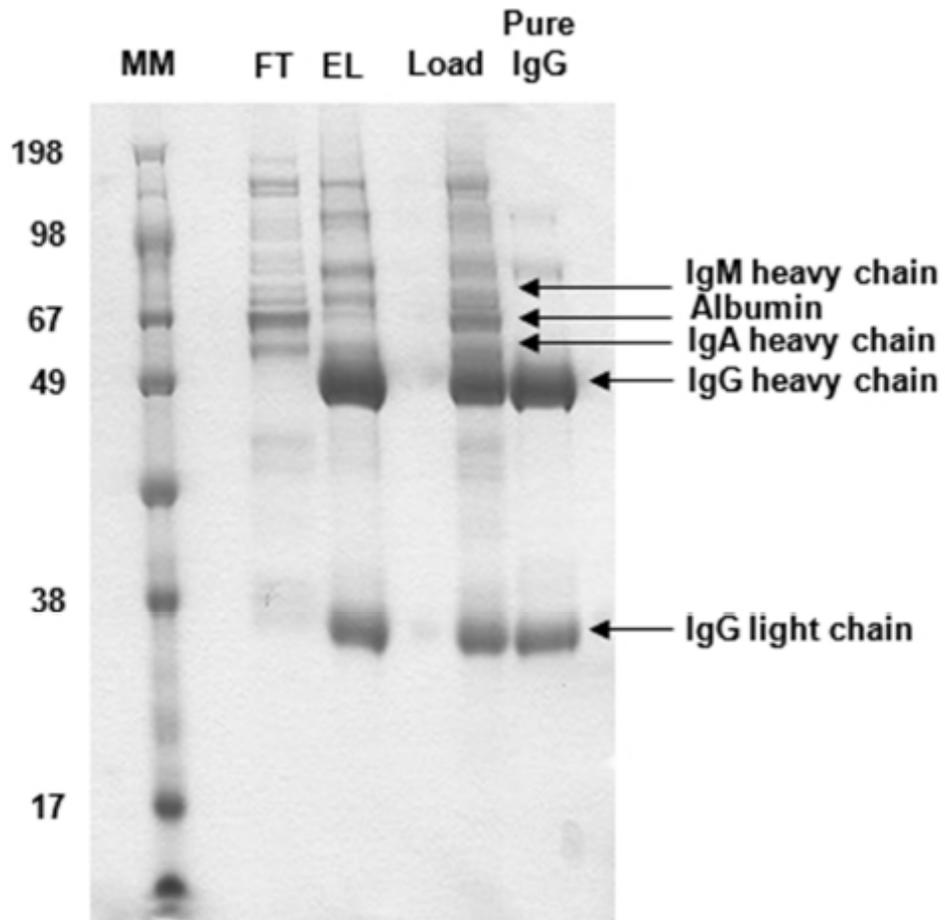
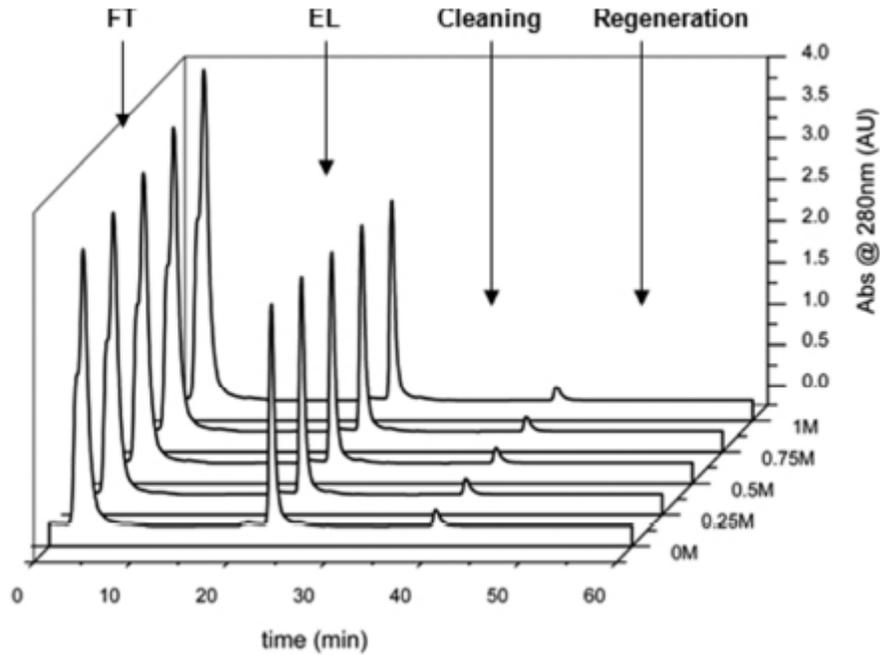
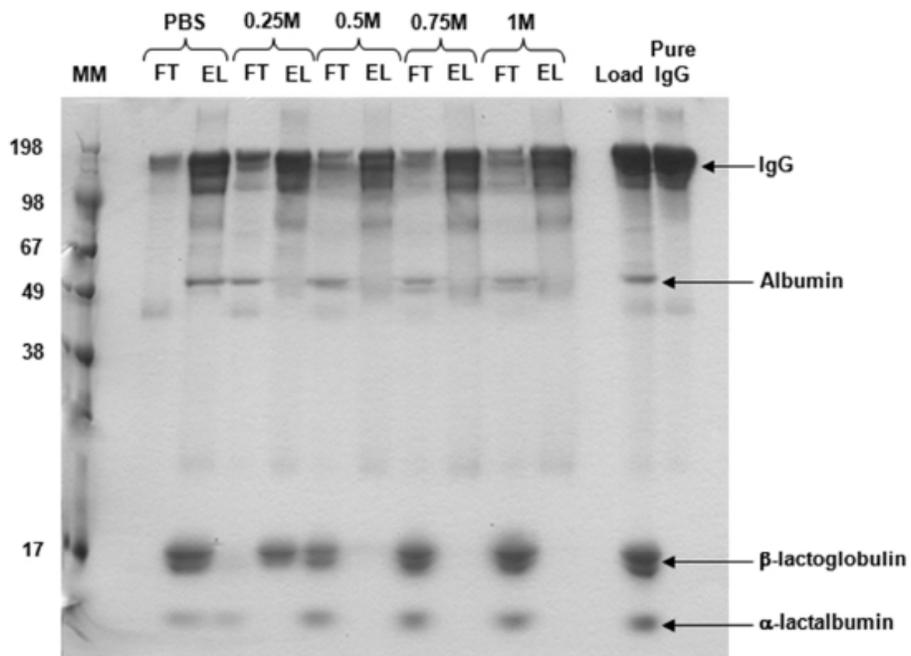


Figure 3.5. SDS PAGE analysis (reducing conditions) of chromatographic purification of IgG from Cohn Fraction II+III using r-Protein A resin. Labels: MM – Molecular weight marker, FT – flowthrough fraction, EL – elution fraction.

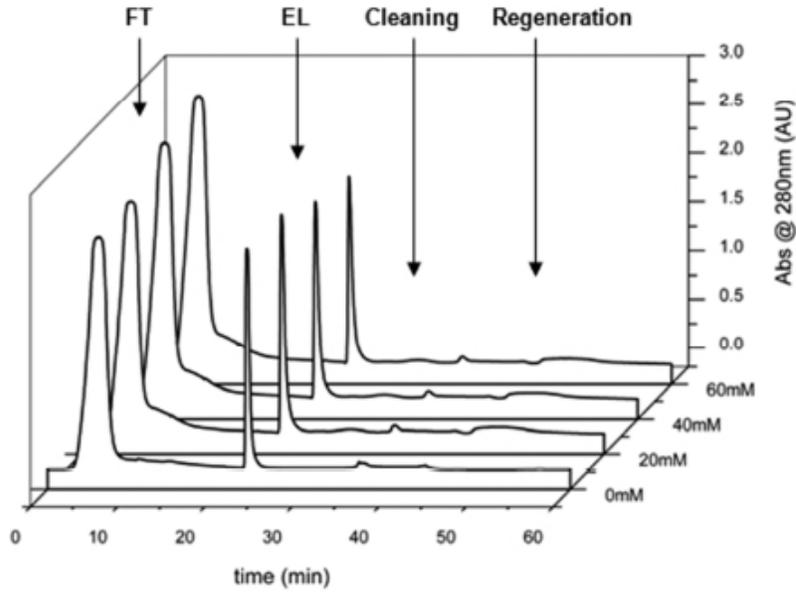


(A)

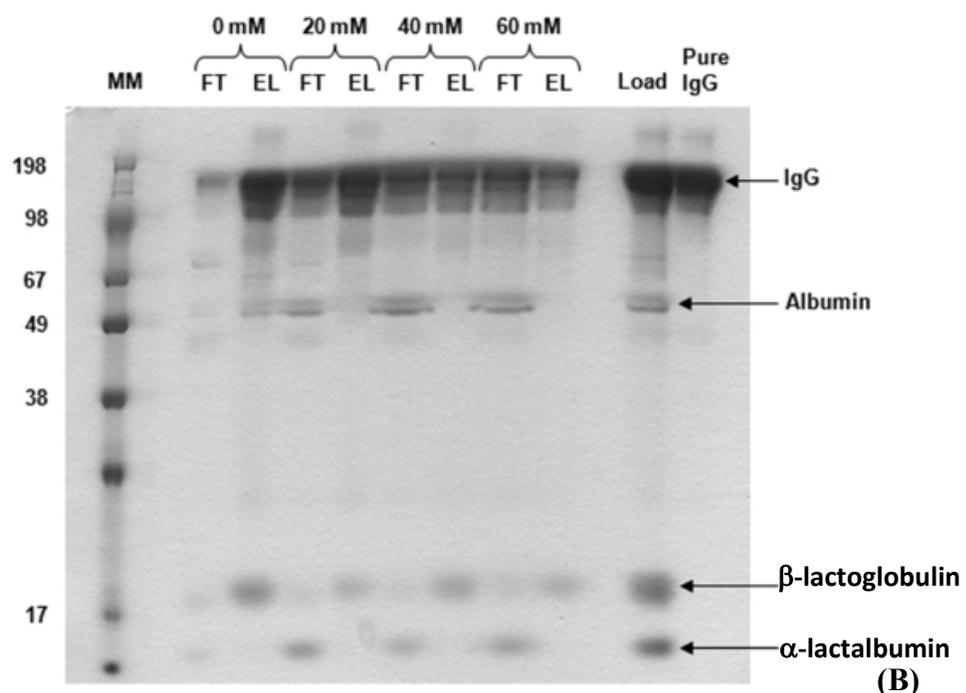


(B)

Figure 3.6. (A) Chromatograms of IgG purification from whey with HWRGWV resin under different NaCl concentrations in the equilibration and binding buffer. (B) SDS-PAGE (non-reducing conditions) of flowthrough and elution fractions. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.



(A)



(B)

Figure 3.7. (A) Chromatograms of IgG purification from whey under different concentrations of sodium caprylate in the equilibration and binding buffer. (B) SDS-PAGE (non-reducing conditions) of flowthrough, elution and regeneration fractions. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction; R – regeneration fraction.

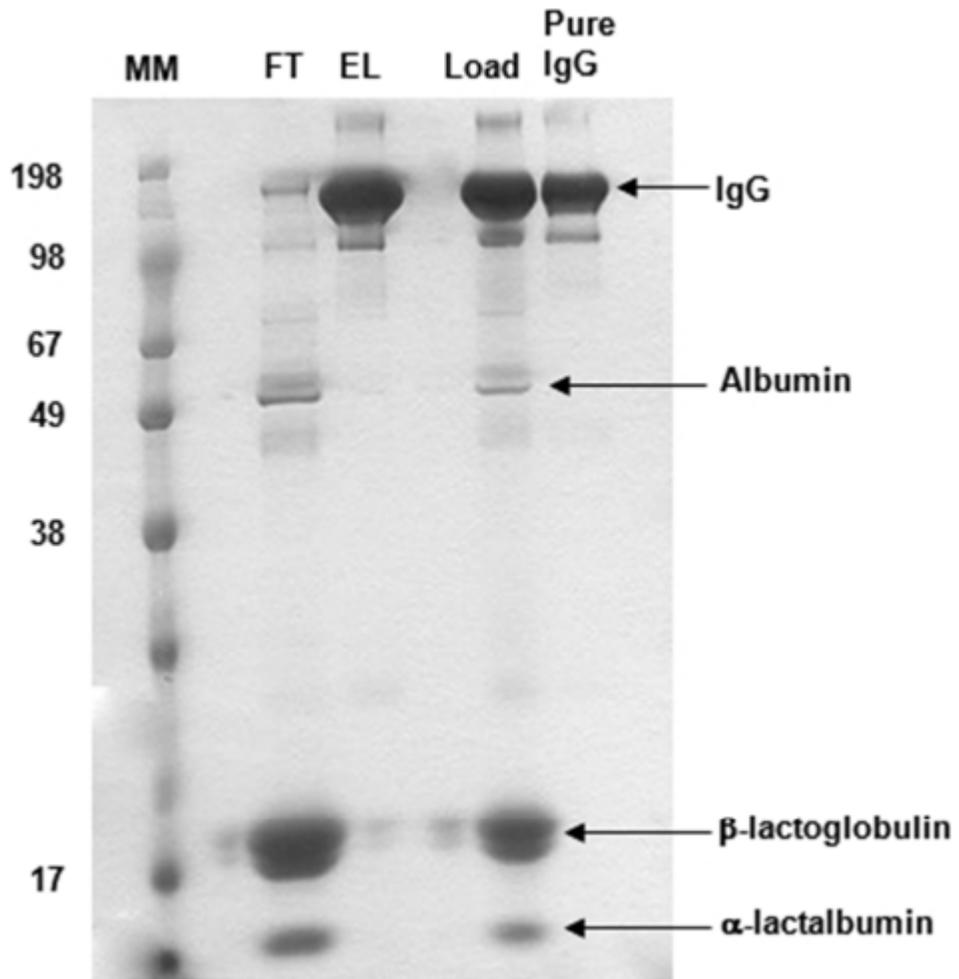
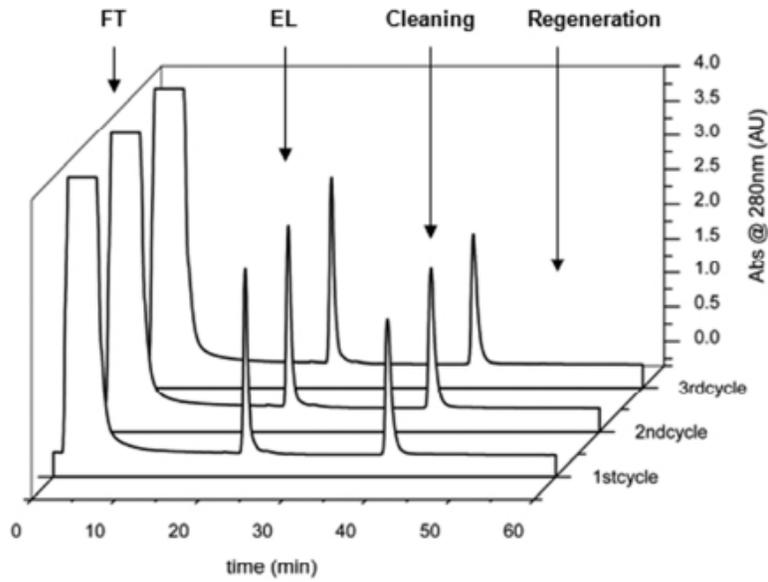
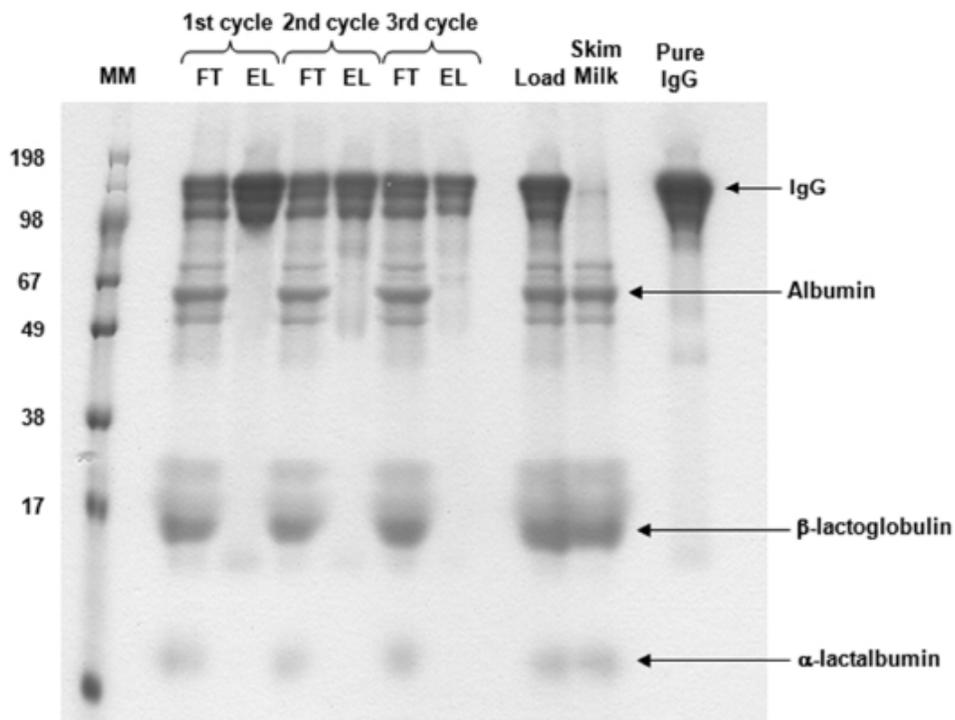


Figure 3.8. SDS PAGE analysis (non-reducing conditions) of chromatographic purification of IgG from whey using recombinant Protein A matrix. Labels: MM – Molecular weight marker, FT – flowthrough fraction, EL – elution fraction.



(A)



(B)

Figure 3.9. (A) Chromatograms of IgG purification from skim milk using HWRGWV resin. (B) SDS-PAGE (non-reducing conditions) of chromatographic fractions of purification of IgG from skim milk using HWRGWV resin. Labels: M – molecular weight marker; FT – flowthrough fraction; EL – elution fraction.

**Chapter 4. Process for Purification of Monoclonal Antibody Expressed in Transgenic
Lemna Plant Extract Using Dextran-Coated Charcoal
and Hexamer Peptide Affinity Resin**

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*A version of this chapter has been published in
Journal of Chromatography A 2012, 1260, 61-66.*

Abstract

The production of therapeutic proteins using transgenic plants offers several advantages, including low production cost, absence of human pathogens, presence of glycosylation mechanisms, and the ability to fold complex therapeutic proteins into their proper conformation. However, impurities such as phenolic compounds and pigments encountered during purification are quite different from those faced during purification from mammalian cell culture supernatants. This paper deals with the development of a pretreatment and affinity separation process for the purification of a monoclonal antibody from transgenic *Lemna* plant extract. A pretreatment step is described using dextran-coated charcoal for the removal of pigments and phenolic compounds without reducing the antibody concentration. Then, the peptide affinity ligand HWRGWV coupled to a commercial polymethacrylate resin is used for the capture and purification of MAb from the pretreated plant extract. The final yield and purity of the MAb obtained were 90% and 96% respectively. The performance of the hexamer peptide resin after the pretreatment step was found to be similar to that obtained with a commercial Protein A resin.

Keywords: Dextran-coated charcoal; Transgenic plants; Hexapeptide ligand; IgG purification; Monoclonal antibody; Phenolics; *Lemna minor*.

4.1. Introduction

The impact of antibody-based biopharmaceuticals on human therapy has increased exponentially in the last three decades and a variety of products is now available for the treatment of severe diseases [1]. Monoclonal antibodies (MAbs) are regarded as the new frontier of therapeutics [2,3] and already more than twenty MAb-based drugs have received FDA approval and are currently employed to fight cancer and infectious, cardiovascular and autoimmune diseases [4,5]. The demand of these products is increasing, with dosages in some cases reaching amounts of 1 g per patient per year [6]. However, the high production cost of these products is still a barrier to their wide application and access to specific therapies is still quite limited [7,8]. There is ongoing research in both upstream and downstream areas to reduce production costs. The IgG product titers in mammalian host cells have reached values of up to 5g/L [9]. Also, there is increasing interest in the use of transgenic plants for the production of antibodies. Transgenic plants offer potential lower production costs, ease of scalability, lack of human or animal pathogens and the ability to produce target proteins with desired structures and functionalities. Several plant-made pharmaceuticals, including plasma proteins, enzymes, collagen, fibrinogen, insulin, and vaccines are already available on the market [10]. Currently, several MAbs produced by transgenic plants are in different stages of clinical trials [11]. Among the many different varieties of plants that can be used as transgenic hosts, tobacco, maize and duckweed (*Lemna*) have been reported to have a high potential for commercialization [11]. In particular, *Lemna*, a small aquatic plant has high potential due to its ease of production, high protein content,

and fast growth rate (36 h doubling time, rapid clonal expansion). This plant has been used to produce numerous therapeutic proteins including MAbs [12,13].

The purification of proteins from plant sources, however, presents several challenges related to the presence of water soluble non-protein impurities, such as amino acids, organic acids, carbohydrates, pectin, nucleic acids, and phenolic compounds [14-16]. The latter in particular consist of a very wide range of molecules, from low molecular weight phenolic acids and aldehydes, benzoquinones, and tyrosine derivatives (100–200 Da), to medium size hydroxycinnamic acids, coumarins, and chromones (200 – 500 Da), to high molecular weight xanthoids, flavonoids, stilbenoids, anthraquinones, and polyphenols (0.5 – 1 kDa). Furthermore, the pigments, such as chlorophylls (0.6 – 0.9 kDa), carotenoids (0.35 – 0.65 kDa), anthocyanins and betalains (0.3 – 0.8 kDa) form an additional class of highly complex organic molecules. These two classes of compounds, phenolics and pigments, are a major concern, as they can interact with the MAbs via hydrogen bonding, oxidative coupling, ionic and/or hydrophobic interactions [17,18], therefore decreasing their bioactivity. Moreover, phenolic compounds have a tendency to foul the expensive purification columns [18], lowering resin lifetime and thereby increasing the overall process cost. Thus, for the efficient purification of MAbs from plant extracts, there is a need for a pretreatment step that removes phenolic compounds, pigments, and other impurities without affecting the MAb concentration. The few reports in the literature which deal with this topic include the use of ion exchange chromatography [19], aqueous two-phase partitioning [20,21] and membrane filtration [22,23], yet there is still a lot of interest in efficient, cost effective alternatives for removing pigments and phenolics from plant cell supernatants. Among these alternatives,

activated charcoal based adsorption has been widely employed for the removal of phenolics [24]. However, charcoal is also known to adsorb proteins non-specifically [25]. A variant of charcoal, which has been reported to exhibit lower protein binding, is dextran-coated charcoal. This material has been used in immunoassays of human hormones in biologic fluids [26,27]. In this paper we present for the first time the use of dextran-coated charcoal for the removal of pigment and phenolic compounds without affecting the concentration of MAb. The effects of the volume ratio of dextran-coated charcoal to plant extract and exposure time have been studied in detail and compared with activated charcoal in terms of ratio of phenolic removal over IgG loss.

After the removal of phenolic impurities and pigments, the plant extract was subjected to an affinity chromatography step to capture and purify the monoclonal antibody. The most commonly used affinity ligand for antibody capture is Protein A. However, Protein A based adsorbents present several drawbacks, such as high cost (\$ 10,000 – 15,000 per liter), high immunogenicity and low chemical and biological stability towards alkaline CIP conditions. To address these concerns, our group has identified and developed linear hexamer peptides (HWRGWV, HYFKFD and HFRRHL) that bind to the Fc region of IgG [28]. Recent work in our laboratory indicates that the ligand HWRGWV is able to capture whole antibody products of different subtypes from industrial mammalian cell cultures that include antifoam, host cell proteins, DNA, RNA, vitamins and many other contaminants [29].

In this work we describe the use of the peptide affinity ligand for the capture and purification of monoclonal antibody from *Lemna minor* extract which has been pretreated

with dextran-coated charcoal. For comparison we have also performed analogue purification studies with Protein A.

4.2. Experimental

4.2.1. Materials

Transgenic *Lemna minor* plant extract producing human IgG₁ was kindly provided by Biolex Therapeutics (Pittsboro, NC, USA). Dextran-coated charcoal and activated charcoal were obtained from Sigma (Saint Louis, MO, USA). Dextran-coated charcoal was prepared from acid washed charcoal powder and dextran (MW 64 – 70 kDa) combined in a 10:1 w/w ratio. HWRGWV-Toyopearl resin with a peptide density of 0.15 meq/g was obtained from CreoSalus (Louisville, KY, USA). The peptide was synthesized directly on Toyopearl AF-Amino-650M (particle size 65µm, Tosoh Bioscience, Montgomeryville, PA, USA) using the fluorenylmethyloxycarbonyl (Fmoc) coupling chemistry. Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Sodium acetate, sodium chloride, sodium carbonate, glycine, hydrochloric acid, and phosphoric acid were obtained from Fischer Scientific (Pittsburgh, PA, USA). Phosphate buffer saline at pH 7.4, gallic acid, Folin-Ciocalteu reagent was from Sigma (Saint Louis, MO, USA). NuPAGE® Novex gels (10% Bis-Tris), NuPAGE® MES running buffers, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus2® pre-stained molecular weight marker, SimplyBlue™ SafeStain were all from Life Technologies (Grand Island, NY, USA). A HiTrap™ Protein G column was purchased from GE Healthcare

(Piscataway, NJ, USA). A Waters 626 LC system integrated with 2487 UV detector (Milford, MA, USA) was used for all chromatography runs unless otherwise mentioned. 0.1 mL stainless steel columns with dimensions 30 mm long \times 2.1 mm I.D. were from Restek (Bellefonte, PA, USA). All experiments were carried out at room temperature.

4.2.2. Pretreatment of plant extract with dextran-coated charcoal and activated charcoal

The effect of dextran-coated charcoal or activated charcoal on the removal of phenolic compounds and MAb from the plant extract was studied using different ratios of charcoal to plant extract at different time intervals. An amount of 1 mg or 5 mg or 10 mg or 20 mg of dextran-coated charcoal or activated charcoal was taken in microcentrifuge tubes and contacted with 1 mL of plant extract by end-to-end rotation for different time intervals (1 min, 2 min, 4 min, 10 min, 20 min, 30 min, 45 min, 1 h, and 2 h). After incubation the mixture was centrifuged and the supernatant collected for analysis of phenolic compounds and MAb.

4.2.3. Quantitation of phenolic compounds and MAb

The quantitation of phenolic compounds in pretreated samples was done by Folin-Ciocalteu method adapted for a 96-well microtiter plate as described in [30]. Gallic acid was used to generate a standard curve ranging from 1 to 40 mg/L. A volume of 200 μ L of each sample was added to the microtiter plate and mixed with 13 μ L of Folin-Ciocalteu reagent.

After 10 min, 37 μ L of freshly prepared 20% w/v sodium carbonate was added to the mixture. The mixture was incubated for 30 min at 37°C. The absorbance was read at 760 nm on a microplate reader (Biotek Synergy H4, Winooski, VT USA). The quantification of MAb was done by IgG using a 1-mL HiTrap™ Protein G column. Human polyclonal IgG was used to generate the standard curve.

4.2.4. Purification of IgG from pretreated stock with HWRGWV and Protein A resins

Thirty five milligrams of HWRGWV-Toyopearl resin were packed in a 30 mm x 2.1 mm I.D. column and swollen with 20% v/v methanol. The resin was equilibrated with PBS buffer, pH 7.4. One milliliter of pretreated plant extract sample was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of equilibration buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.2M Glycine HCl, pH 3 at the flow rate of 0.4 mL/min (696 cm/h). Cleaning and regeneration were performed by adding 4 mL of 0.85% phosphoric acid. The effluent was monitored by absorbance at 280 nm. Fractions were collected and stored at 4°C for analysis of phenolic compounds and MAb. For determination of MAb purity, the samples were concentrated ten times by centrifugation at 4°C, 13000 x g for 15 min using an Amicon® Ultra centrifugal filter (3000 MWCO, Ultracel®, Millipore, Billerica, MA, USA).

Toyopearl AF-rProtein A-650F resin was packed in a 30 mm x 2.1 mm I.D. column and equilibrated with PBS pH 7.4. Loading and washing conditions were the same as used for the peptide resin. Elution was then performed with 4 mL of 0.1 M Glycine buffer pH 2.5 at the

flow rate of 0.4 mL/min (696 cm/h) (as per manufacturer's instructions). System monitoring as well as fraction collection and analysis were performed as described above.

4.2.5. Purity Analysis by SDS-PAGE

The purity of IgG in the collected fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described by Yang *et al.* [28], using NuPAGE® Novex 10% Bis-Tris gels in a Xcell SuperLock™ Mini-Cell system. Sample preparation was done by adding 5 µL of NuPAGE® LDS buffer and 2 µL of NuPAGE® reducing agent to 13 µL of sample and boiling the resulting mixture for 10 min. Gels were Coomassie-stained by using SimplyBlue™ SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 kDa.

4.2.6. Determination of dynamic binding capacity

An important parameter of an affinity resin is its dynamic binding capacity (DBC) for the target protein. DBC is the total amount of target protein that the resin can bind under actual flow conditions. It is determined from breakthrough curve, which is obtained by continuously loading the protein solution onto the resin column and measuring the outlet protein concentration. The point at which the outlet protein concentration reaches 10% of feed concentration is called 10% breakthrough point and is commonly used for estimating DBC.

The experimental setup for determining the DBC of the HWRGWV peptide resin was as follows. The resin was packed and equilibrated as described in Section 2.4. An acetone pulse (5%, v/v) was applied to the column to determine the total column void volume. Twenty milliliters of pretreated plant extract was then loaded directly onto the peptide column at flow rate of 0.02 mL/min (35 cm/h). Chromatographic fractions of 0.5 mL were collected and analyzed for MAb content. The calculated MAb concentrations of outlet fractions were used to generate a breakthrough curve and thereby determine the 10% breakthrough point and the corresponding breakthrough volume. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume the dynamic binding capacity of the peptide resins was determined.

4.3. Results

4.3.1. Pretreatment of plant extract with dextran-coated charcoal and activated charcoal

To determine the optimum time and amount of dextran-coated charcoal required for removal of phenolic compounds from a plant extract, batch binding studies were carried out with different loadings of dextran-coated charcoal (0.1, 0.5, 1, and 2% w/v), at varying time intervals ranging from 1 min to 2 h. Comparative studies were also carried out with activated charcoal under similar conditions. The results are shown in Figures 4.1A-4.1D. As can be seen from Figure 4.1a, at 0.1% loading both dextran-coated charcoal and activated charcoal removed about 35% of the phenolic compounds from the plant extract after 2 h of incubation.

It should be noted that in a contact time of only 1 min the phenol content was reduced to approximately half (15%) of the maximum removal (35%) indicating the fast adsorption kinetics of the charcoal. From Figures 4.1B-1D it can be observed that increasing the amount of charcoal increased the removal of phenolic compounds. For 0.5% and 1% loadings of dextran-coated charcoal the removal of phenolic compounds was 64% and 83% respectively in 2h, which was similar to that obtained with activated charcoal. A further increase in the amount of charcoal to 2% loading did not significantly increase the removal of phenolic compounds. Though the results described here are for an exposure time of 2 h, it can be seen from Figure 4.1C and Figure 4.1D that after a contact time of 10 min the adsorption equilibrium was reached and even a contact time of 4 min was sufficient to remove around 80% of the phenolic compounds from the plant extract.

The next step was to determine the effect of pretreatment with charcoal on the MAb concentration of plant extract. The results are shown in Figures 4.2A-4.2D. As can be seen from Figure 4.2A, at 0.1% charcoal loading there was no loss of MAb from plant extract for both dextran-coated charcoal and activated charcoal. Even after 2 h of exposure the concentration of MAb in the plant extract remained the same as the initial concentration (0.12 mg/mL). However, as shown earlier, 0.1% charcoal loading removed only 35% of the phenolic compounds, so that higher loading of charcoal is required to have a more effective pretreatment of the plant extract. When the charcoal loading was increased to 0.5% there was a 10% MAb loss in case of activated charcoal whereas no MAb loss was observed for dextran-coated charcoal treatment (Figure 4.2B). A further increase to 1% loading resulted in about 35% MAb loss for activated charcoal whereas with dextran-coated charcoal only a

slight MAb decrease of 5% was observed (Figure 4.2C). The advantage of using dextran-coated charcoal becomes more evident when the amount of charcoal is further increased to 2%. At 10 min contact time, which was found sufficient to remove most of phenolic compounds, activated charcoal caused a 56% reduction of MAb concentration of plant extract, whereas dextran-coated charcoal led to only a 15% MAb loss. After 2h of contact time the activated charcoal adsorbed almost all the MAb from the plant extract (Figure 4.2D).

These results thus prove the effectiveness of dextran-coated charcoal in removing the phenolic compounds without causing a significant loss in MAb concentration. Based on the above studies the optimum amount of charcoal loading is 1% (w/v) for a contact time of 4 mins. Under these conditions the dextran-coated charcoal removes 75% of the phenolic compounds without causing any loss in the MAb concentration of plant extract. Figure 4.3 demonstrates the visual change in colour of the plant extract before and after treatment with dextran-coated charcoal under the optimized conditions and illustrates the complete removal of pigments.

The selective removal of phenolic compounds and pigments from the plant extract by the dextran-coated charcoal has been explained in terms of the sieving property of the dextran layer [31]. Dextran allows small organic molecules, either aliphatic or aromatic, carrying polar functional groups, like phenols and pigments, to penetrate through the coating and become adsorbed on the substrate surface mainly by hydrophobic interactions. On the contrary, larger molecules such as proteins do not penetrate through the hydrophilic layer and are not retained by the adsorbent [32,33].

4.3.2. Purification of SDS-PAGE from pretreated stock with SDS-PAGE and Protein A resins

The next step was to develop a chromatographic step for the capture and purification of MAb from the plant extract. The SDS-PAGE binding peptide ligand SDS-PAGE identified by our group [28] was selected for the affinity chromatographic purification of MAb from plant extract. The plant extract was pretreated with dextran-coated charcoal using the optimized conditions from batch studies (Section 3.1). Two milliliters of plant extract was mixed with 20 mg of dextran-coated charcoal for 10 min. The mixture was then centrifuged and the treated plant extract was loaded onto a 0.1 mL SDS-PAGE-Toyopearl resin column at flow rate of 0.05 mL/min (87 cm/h). The chromatogram and SDS-PAGE are shown in Figure 4.4. From the SDS-PAGE (Figure 4.4B, Lanes 3 and 4) it can be seen that the peptide resin was able to capture all the MAb, while the impurities passed through the column unbound. The majority (89%) of the phenolic compounds which were still present in the plant extract even after pretreatment, passed through the column unbound and were collected in the FT (Table 4.1). Only a trace amount of phenolic compounds was found in the eluted fraction (7%). The MAb purity, as determined by densitometric analysis of the elution lanes on the SDS-PAGE, was 96%. The yield of MAb as determined by Protein G SDS-PAGE was found to be 90 %.

In order to compare the performance of the peptide resin with widely used affinity adsorbents, the pretreated plant extract was loaded onto Protein A–Toyopearl resin. The chromatographic conditions were same as used for the peptide resin. Chromatograms and

SDS-PAGE results are shown in Figure 4.4. The performance of the Protein A column was similar to that of the peptide resin in terms of both MAb purity, as can be seen from the SDS-PAGE (Figure 4B, lanes 6 and 7), and phenolic removal (Table 4.1).

4.3.3. Determination of dynamic binding capacity

The dynamic binding capacity (DBC) of the peptide resin for the MAb was determined by breakthrough experiment. A 20 mL of plant extract was loaded onto 0.1 mL of peptide resin at a flow rate of 0.02 mL/min (35 cm/h). Fractions were collected at different intervals and the MAb concentration in the fractions was determined by HPLC using HiTrap Protein G column. The calculated MAb concentrations were plotted vs. cumulative volume to obtain a breakthrough curve, shown in Figure 4.5. The fraction at which the MAb concentration reached 0.012 mg/mL (10 % of feed MAb concentration) was taken as the breakthrough point. The volume corresponding to the 10% breakthrough point was corrected by subtracting the void volume of the system. This corrected breakthrough volume was used to determine the total MAb bound to the HWRGWV resin and thereby the dynamic binding capacity, which was estimated to be 12 mg/mL. This value of DBC is notable, considering that the MAb concentration of the plant extract was low (0.12 mg/mL).

4.4. Conclusions

Transgenic plants, such as *Lemna*, offer a promising alternative to lower the cost of manufacturing antibodies. To makes sure that the economic benefit gained by the use of this

low-cost expression system is retained, an efficient downstream processing needs to be developed. The impurities present in plant extracts, *viz.* phenolic compounds and pigments foul the expensive chromatographic resin and can also interact with the MAb, affecting its bioactivity. In this work we have shown for the first time the use of dextran-coated charcoal to remove the phenolic compounds and pigments without significantly affecting the MAb concentration of the plant extract. Comparative studies carried out with activated charcoal under similar conditions clearly showed the advantage of using the dextran-coated charcoal towards MAb recovery. Detailed studies were carried out to optimize the amount of the charcoal and contact time required for the pretreatment step. The uptake of the phenolics was fast and equilibrium was reached in approximately 20 min in all the studies. Treatment with 1% (w/v) of dextran charcoal for 4 min was sufficient to remove around 75% of the phenolic compounds without causing any MAb loss.

The low concentration of MAb in the plant extract demands a highly specific affinity adsorbent to capture and purify the MAb with high yields and purity. We had earlier identified and developed a hexameric peptide ligand HWRGWV showing high specificity to the Fc region of IgG. This peptide adsorbent was successfully used for the capture and purification of MAb from the pretreated plant extract. The MAb yield obtained was 90% and the purity was 96%. This performance of the peptide adsorbent was similar to that obtained with Protein A resin under identical conditions. The peptide adsorbent was characterized in terms of its dynamic binding capacity (DBC) for MAb. Although the MAb concentration in the feed was very low (0.12mg/mL), the DBC obtained was remarkable (12 mg/mL).

Overall, the purification system comprising of pretreatment with dextran-coated charcoal and affinity purification by HWRGWV resin has the potential to become a cost-effective process for high recovery of MAb from transgenic plant extracts.

4.5. Acknowledgements

The authors would like to thank Lynn Dickey of Biolex Therapeutics for providing us the transgenic *Lemna* plant extract. The authors also gratefully acknowledge the support of the Biomanufacturing Training and Education Center (BTEC/NCSU) and the Kenan Institute for Engineering, Technology & Science at NC State for financial support of this project.

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Table 4.1. Amount of phenolic compounds (%) present in the chromatographic fractions.

Fractions	% Phenolic compounds HWRGWV-Toyopearl	% Phenolic compounds Protein A-Toyopearl
Flowthrough	89.1 %	88 %
Elution	7.3 %	8.8 %
Regeneration	2.6 %	1.7 %

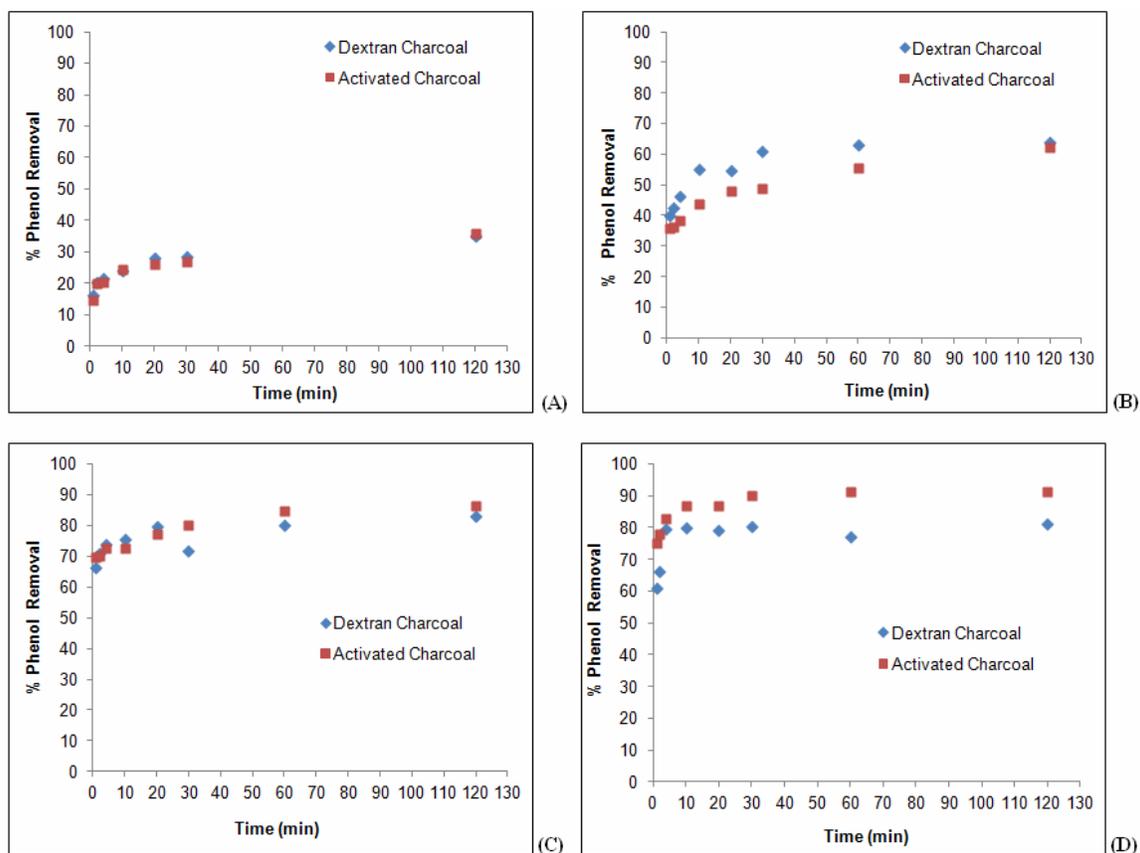


Figure 4.1. (A) Removal of phenolic compounds from 1mL plant extract treated with 1mg of dextran-coated charcoal or activated charcoal at different time intervals. (B) Removal of phenolic compounds from 1mL plant extract treated with 5mg of dextran-coated charcoal or activated charcoal at different time intervals. (C) Removal of phenolic compounds from 1mL plant extract treated with 10mg of dextran-coated charcoal or activated charcoal at different time intervals. (D) Removal of phenolic compounds from 1mL plant extract treated with 20mg of dextran-coated charcoal or activated charcoal at different time intervals.

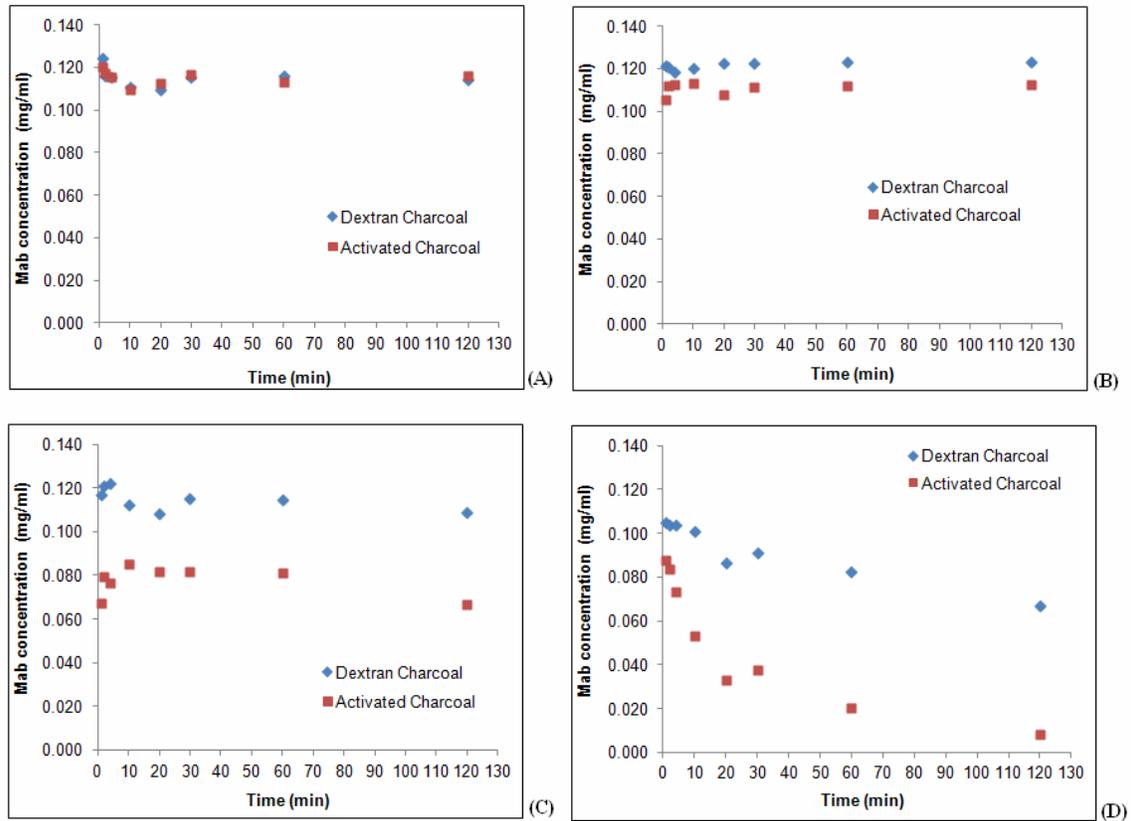


Figure 4.2. (A) Concentration of MAb of plant extract (1mL) treated with 1mg of dextran-coated charcoal or activated charcoal at different time intervals. (B) Concentration of MAb of plant extract (1mL) treated with 5mg of dextran-coated charcoal or activated charcoal at different time intervals. (C) Concentration of MAb of plant extract (1mL) treated with 10mg of dextran-coated charcoal or activated charcoal at different time intervals. (D) Concentration of MAb of plant extract (1mL) treated with 20mg of dextran charcoal or activated charcoal at different time intervals.



Figure 4.3. (A) Lemna minor plant extract treated with 1% (w/v) dextran-coated charcoal for 4 mins. (B) Lemna minor plant extract.

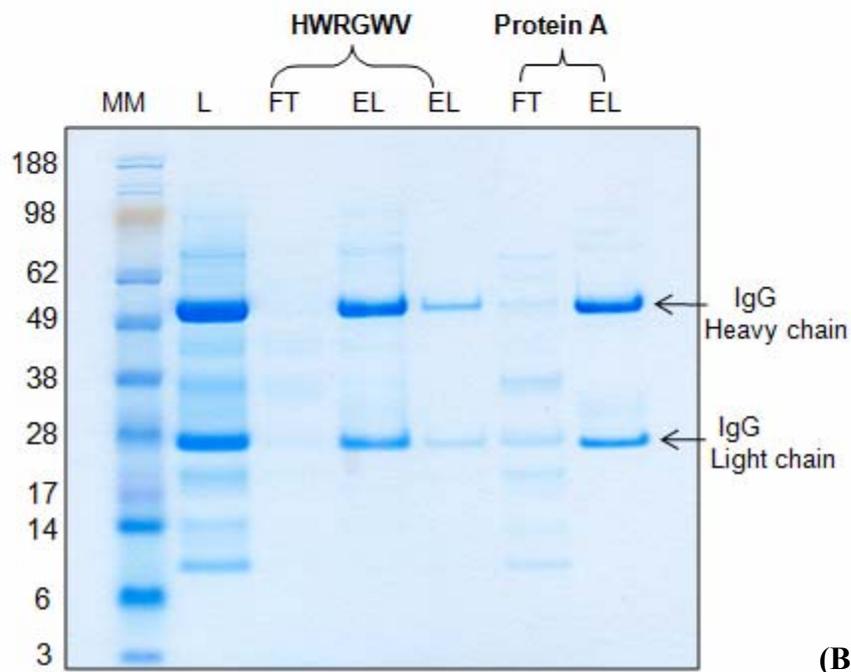
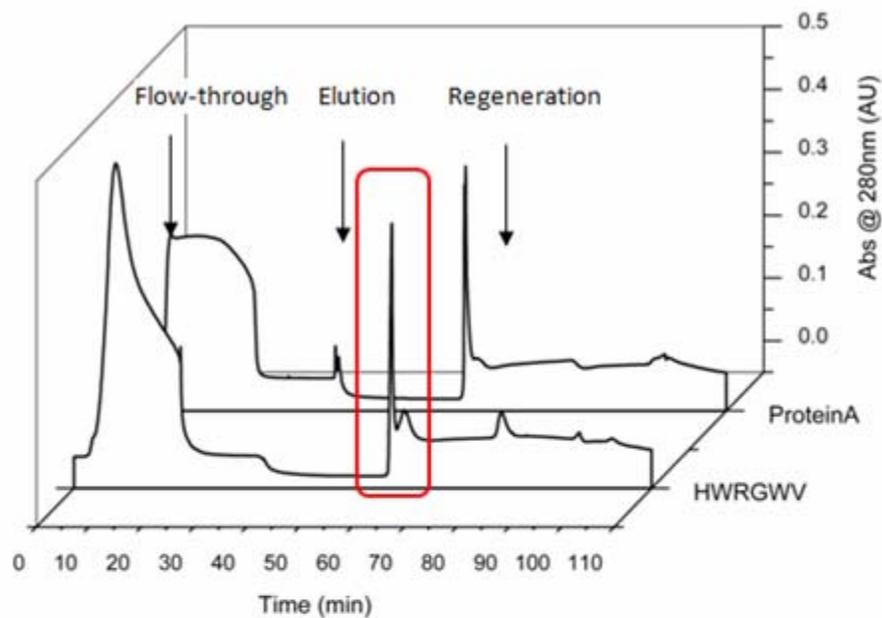


Figure 4.4. (A) Chromatogram of purification of MAbs from plant extract using HWRGWV-Toyopearl resin or Protein A-Toyopearl resin. (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM-molecular weight marker, FT- flowthrough fraction, EL- elution fraction.

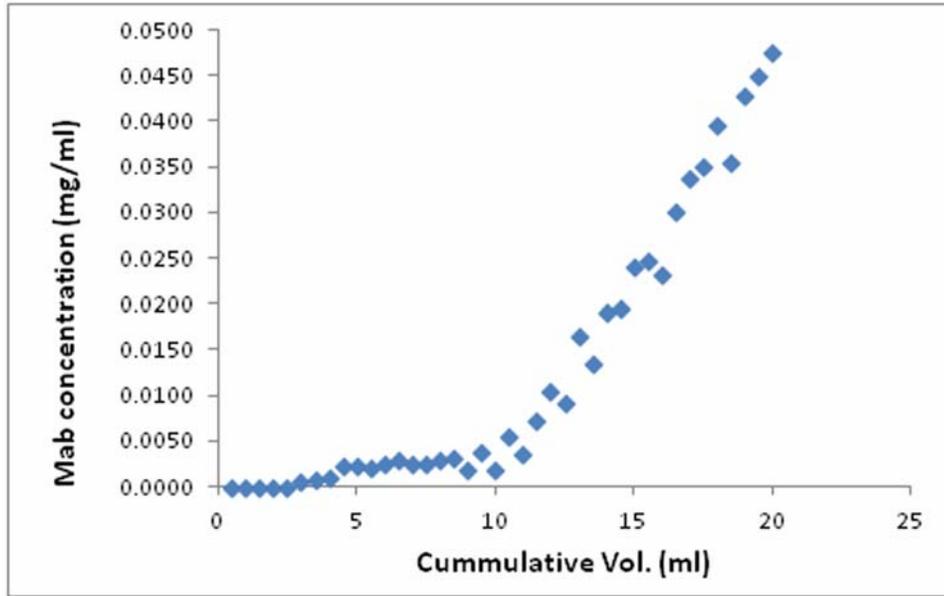


Figure 4.5. Breakthrough curve of HWRGWV-Toyopearl resin for MAb from plant extract

**Chapter 5. Peptide-based affinity adsorbents with high binding capacity
for the purification of monoclonal antibodies**

A version of this paper has been published in

Industrial Engineering Chemistry Research 2012, doi: [10.1021/ie302345w](https://doi.org/10.1021/ie302345w).

Abstract

High binding capacity and selectivity are key features for the successful application of affinity adsorbents for antibody purification. This study presents the development of affinity resins based on hexapeptide ligand HWRGWV for recovering monoclonal antibodies from cell culture fluids. Methods are presented for the immobilization of the peptide ligand and its variants on polymethacrylate and agarose based chromatographic supports using two main coupling strategies. The first one involves the formation of a peptide bond between the amino groups on the substrate and the peptide C-terminus activated with the uronium coupling agent HATU. The second approach involves resin activation with iodoacetic acid, followed by coupling of a cysteine-terminated variant of the ligand to form a thioether bond. The reaction conditions of peptide coupling were optimized to maximize the binding capacity of the resulting adsorbents. The peptide resins were characterized by measuring their static IgG binding capacities. The measured static binding capacity ranged from 35 to 48 mg/mL. The dynamic binding capacities (DBC) of four selected adsorbents were also determined, and they ranged from 35 to 42 mg/mL with a 5-minute residence time. All the resins exhibited high selectivity towards the Fc fragment of IgG. The affinity resins were used to purify two MAbs, a chimeric IgG₁ and a humanized IgG₄, from commercial CHO cell culture fluids. The resulting yields and purities for both MAbs were found to be in the range of 87 – 93% and > 94 % respectively, which compare well with the purity and yield values obtained using commercially available Protein A media. Finally, the peptide resin with the highest IgG binding capacity, HWRGWVC-WorkBeads, was tested for 20 DBC cycles which included

cleaning in place with 0.1 M NaOH after every cycle. The resin showed a high degree of reusability and alkaline stability, as it maintained 90% of its initial capacity.

5.1. Introduction

Over the last four decades, antibody-based biopharmaceuticals have vastly impacted human therapy and a variety of products are now available for the treatment of severe diseases [1]. Monoclonal antibodies (MAbs) are in the vanguard of novel therapeutics and the FDA has approved more than twenty MAb-based drugs [2-5]. In 2015, this class of products is expected to generate revenues of \$67 billion, with a majority targeting cancer and autoimmune diseases [6]. However, the high price of these products is still adverse to their wide application and access to specific therapies is still quite limited [7, 8]. Product isolation, purification and polishing account for a large portion of the overall manufacturing costs and make up a considerable market barrier [9]. Highly engineered biological ligands employed in affinity chromatography, such as the recombinant Protein A and G, while being excellent binders, are very expensive (\$8,000-\$15,000 per liter of resin) [10-15]. Moreover, these ligands require harsh elution conditions (pH 2.5-3) that can lead to antibody aggregation in resulting product yields, as well as chemical degradation of the resin with prolonged reuse. The leaching of Protein A fragments into the process stream raises regulatory issues and are of concern to the manufacturers.

Researchers from both academia and industry are searching for new specific, chemically robust and economical synthetic ligands as alternatives [16-18]. Among the proposed ligand compounds, peptides represent an important and growing class [19-21]. Our research group has identified three hexapeptide ligands, HWRGWV, HYFKFD and HFRRHL, which bind IgG through the Fc portion, thus mimicking the binding mechanism of

Protein A [22]. Additionally, the ligands have shown the ability to purify human immunoglobulins A (hIgA) and M (hIgM) from complex media [23]. The peptide HWRGWV was further characterized for its ability to isolate IgG from a variety of complex sources, including cell culture media, CHO cell culture supernatants, Cohn II+III fraction of human plasma, transgenic milk and whey, and plant extract [24, 25]. The product yields and purities that resulted from these experiments were always comparable to those obtained with Protein A media. A method of resin surface modification has been designed to produce modified HWRGWV-Toyopearl resins that can withstand alkaline cleaning and sanitization in place with aqueous 0.1M NaOH over 200 cycles without significant loss of capacity and selectivity [26].

Up to now, these adsorbents exhibited a dynamic binding capacity for IgG of about 20 mg/mL, which is considerably lower than the more industrially accepted levels (~ 40 mg/mL) given by Protein A media. The cause of this low binding capacity likely resides in the method adopted by our group for the preparation of the peptide-based affinity adsorbents, which consists in building the peptide directly on polymethacrylate-based Toyopearl amino resin by solid phase peptide synthesis using conventional Fmoc/tBu coupling chemistry [27]. As this resin is not particularly well suited for peptide synthesis, this process results in truncated peptide sequences, which are far less selective and avid than the hexamer ligand. Additionally, solid phase peptide synthesis involves the use of very harsh reagents such as trifluoroacetic acid and piperidine. Resins based on agarose, controlled pore glass and dextran are not stable in these solvents and hence, this method cannot be applied to these matrices.

In order to overcome these issues, this work presents a set of studies on the coupling of the whole purified peptide ligand HWRGWV and its variants on two different base matrices, Toyopearl Amino-High Capacity (HC), a polymethacrylate based resin, and WorkBeads 40 ACT, an agarose based resin. Two coupling chemistries were chosen for ligand immobilization. One employs the coupling agent, HATU (2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate Methanaminium) to form an amide bond between the peptide C-terminus and the amino groups on the resin surface (Figure 1A). The second one consists in coupling the peptide modified with a cysteine on the C-terminus onto the iodoacetic acid activated substrate by formation of a thioether bond (Figure 5.1B). For the HATU-based chemistry, four peptide sequences were coupled to the resin: HWRGWV, Ac-HWRGWV, Ac-HWRGWVA, and Ac-HWRGWVG. It has been previously shown that acetylation of the amino group does not affect IgG binding but it does provide additional chemical stability to the peptide [28]. The effects of peptide molar concentration and activation protocols on the efficiency of peptide coupling were analysed. Based on the values of static binding capacity (SBC) of the resulting adsorbents, the best performing resin for each base matrix, namely Ac-HWRGWVG-Toyopearl HC and Ac-HWRGWVG-WorkBeads resins, were selected for further characterization and purification studies. For the iodoacetic acid activation-based chemistry, HWRGWVC was used and two analogous adsorbents were prepared, HWRGWVC-Toyopearl HC and HWRGWVC-WorkBeads resins. These resins were characterized in terms of dynamic binding capacity (DBC) at different linear velocities, and selectivity towards the Fc and Fab fragments of IgG. The adsorbents were employed for the purification of two monoclonal antibodies, a chimeric IgG₁ and a

humanized IgG₄, from commercial CHO cell culture fluids. Finally, the resin with the highest capacity, HWRGWVC-WorkBeads, was studied for 20 DBC cycles which included alkaline cleaning in place after every cycle.

This paper is written in honor of our friend and colleague of many years, Prof. Giulio Sarti of the University of Bologna, in recognition of his 65th birthday. Prof. Sarti has done seminal research in many areas of membrane transport, including the field of bioseparations. He and his collaborators have produced some excellent papers on the use of affinity ligands on porous membranes for antibody product capture from cell culture [29-35].

A few years ago, our research group had sent to Bologna the affinity peptide ligand HWRGWV discussed in this work for his group to attach to membranes and test its performance in IgG capture. Because at that time we had not yet developed an optimized protocol for attaching this peptide to solid supports with high efficiency, the results were not encouraging. The results presented in this paper provide unequivocal proof that the peptide ligand attached to surfaces can result in adsorption matrices with very high binding capacity, perhaps even in membranes some day.

5.2. Experimental

5.2.1. Materials

Toyopearl Amino-High Capacity (HC) and Toyopearl AF-rProtein A-650 F were gifts from Tosoh Bioscience (King of Prussia, PA, USA), WorkBeads 40 ACT resin was obtained from Bio-Works Sweden AB (Bromma, Sweden). MabSelect SuRe LX was

received as a gift from GE Healthcare (Piscataway, NJ, USA). HWRGWV-Toyopearl resin with a peptide density of 0.15 meq/g (34 μ moles/mL) was obtained from CreoSalus (Louisville, KY, USA). The purified peptides (purity > 95%) HWRGWV, Ac-HWRGWV, Ac-HWRGWVA, Ac-HWRGWVG, and HWRGWVC were purchased from Genscript (Piscataway, NJ, USA). The coupling agent (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) was purchased from ChemPep Inc. (Wellington, FL, USA). Ammonium hydroxide, iodoacetic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), triethylamine, glycine, sodium acetate, sodium carbonate, sodium chloride, hydrochloric acid, glacial acetic acid, phosphoric acid, dimethylformamide (DMF) (extra dry), dichloromethane (DCM), methanol, and ethanol were from Fisher Scientific (Pittsburgh, PA, USA). Diisopropylethylamine (DIPEA), phosphate buffer saline (PBS), pH 7.4, and Kaiser test kit were from Sigma Aldrich (Saint Louis, MO, USA). Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Fc and Fab fragments of human polyclonal IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Two CHO cell culture supernatants containing monoclonal antibodies, a chimeric IgG₁ and a humanized IgG₄, were obtained from two different biopharmaceutical manufacturers that asked for confidentiality. The concentration of IgG₁ and IgG₄ in the supernatant were 2.3 and 1.5 mg/mL respectively. In addition to the MAbs, these supernatants contained pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components. NuPAGE[®] Novex gels (4–12% Bis–Tris), NuPAGE[®] MOPS and MES running buffers, NuPAGE[®] LDS sample buffer, NuPAGE[®] reducing agent, SeeBlue plus2[®] pre-

stained molecular weight marker, SimpleBlue™ SafeStain were all from Life Technologies (Carlsbad, CA, USA). A Waters 626 LC system integrated with 2487 UV detectors (Milford, MA, USA) or Agilent 1100 LC system equipped with an autosampler (Santa Clara, CA, USA) were used for all chromatography runs. Microbore stainless steel columns 30 mm long x 2.1 mm I.D. were from Alltech-Applied Science (Somerset, PA, USA). All chromatographic runs were carried out at room temperature.

5.2.2. Amination of WorkBeads 40 ACT

WorkBeads 40 ACT resin, originally in 20% ethanol, was rinsed with DI water and 50 mM carbonate buffer, pH 9.8. A solution of 25% v/v NH₄OH in 100 mM carbonate buffer, pH 12.0 was added (in 1:1 volume ratio) to WorkBeads 40 ACT resin and incubated overnight at room temperature. The resin was rinsed with 50 mM carbonate buffer, pH 9.8, ethanol, and DMF.

5.2.3. Coupling of peptide ligands on Toyopearl Amino-HC and aminated WorkBeads 40 ACT resins via HATU chemistry

Toyopearl Amino-HC resin was swollen in 20% v/v methanol for 2 h and then rinsed with anhydrous DMF. The following peptide sequences, i.e. HWRGWV, Ac-HWRGWV, Ac-HWRGWVA, and Ac-HWRGWVG were coupled to the amino resin by HATU chemistry at varying molar excess and peptide concentration. The molar ratio peptide: HATU: DIPEA of 1 : 2 : 4 was maintained for all couplings. Six values of molar excess,

namely the ratio between the number of equivalents of peptide and the number of equivalents of amino groups on the resin surface, were employed, i.e. 0.1, 0.25, 0.5, 0.75, 1, and 2. For each molar excess, three peptide concentrations in DMF, namely 50 mM, 100 mM, 150 mM, were explored. For each peptide concentration, an anhydrous DMF solution of peptide and HATU was made and then mixed with DIPEA. Immediately after mixing, the solution was added to the resin and incubated overnight at room temperature under vigorous shaking. The resin was then sequentially rinsed with DMF, ethanol and PBS, pH 7.4. Elemental analysis to determine peptide density on the two highest binding adsorbents prepared by HATU chemistry was performed on 25 mg of resin at the Environmental and Agricultural testing service at the Department of Soil science at North Carolina State University.

5.2.4. Coupling of HWRGWV-C on Iodoacetic acid-activated Toyopearl Amino-HC and aminated WorkBeads 40 ACT resins

For iodoacetic acid based coupling, studies were carried out to determine the effect of peptide density on the IgG binding capacity of resins. Toyopearl Amino-HC and aminated WorkBeads (500 μ L each) were taken in 3 mL fritted tubes and washed extensively with ethanol, followed by water. Each resin was then reacted with 120 mg of iodoacetic acid (IAA) and 100 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 500 μ L of 0.1 M MES buffer, pH 4.7 for 2 h at room temperature. The activated resins were thoroughly washed with water, ethanol, and DMF. The washed Toyopearl and WorkBeads resins were then distributed into ten aliquots of 50 μ L. Varying amounts of HWRGWVC peptide (0.5 mg

- 17 mg) were dissolved in 100 μ L of DMF containing 25 μ L triethylamine and then added to the resins respectively. The reaction was carried out overnight at room temperature. The resins were finally filtered and washed with DMF. The collected supernatants and wash fractions were spectrophotometrically analyzed at 280 nm to determine the amount of peptide coupled to the resins by mass balance.

5.2.5. Determination of static binding capacity

An IgG solution at a concentration of 7.5 mg/mL was prepared in PBS, pH 7.4 and filtered using a 0.22 μ m low protein binding PVDF filter (Millipore, MA, USA). The peptide resin was equilibrated in PBS for 30 min and filtered to obtain a wet paste. A volume of 1 mL of IgG solution was added to 50 μ L of wet resin. The slurry was incubated in mild shaking for 30 min at room temperature. The resin was washed with 9 mL of PBS, pH 7.4. Protein elution was performed by adding 4 mL of 100 mM glycine-HCl, pH 2.5 to the resin and incubating the slurry in mild shaking for 15 min at room temperature. All fractions, i.e. flow-through, washing and elution, were collected and analyzed by spectrophotometry at $\lambda = 280$ nm to determine the IgG content.

5.2.6. Determination of dynamic binding capacity

One hundred microliters of peptide resin, Protein A-Toyopearl or MabSelect SuRe LX resin was packed into a microbore 30 mm x 2.1 mm I.D. column. An acetone pulse (0.01M) was applied to the column to determine the total column void volume. After

equilibration with PBS pH 7.4, 1 mL of 10 mg/mL IgG in PBS, pH 7.4 was loaded to the column at a linear flow velocity of 35 cm/h (5 min residence time) or 87 cm/h (2 min residence time). Breakthrough volume was determined at the point where the IgG concentration in the flow-through reached 10% of its feed concentration. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume, the dynamic binding capacity of the peptide resins was determined.

5.2.7. Determination of resin selectivity (Fc vs. Fab)

The peptide resin was packed as described in Section 5.2.6. Sample solutions of Fc and Fab, both at a concentration of 1 mg/mL, were prepared in PBS, pH 7.4. After equilibrating the column with PBS, 50 μ L of Fc or Fab solution was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of PBS at flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer, pH 4 at a flow rate of 0.2 mL/min (348 cm/h). The effluent was monitored by UV absorbance at 280 nm. The peak areas of the flow through and elution fractions of IgG were used to determine the % binding.

5.2.8. Purification of monoclonal antibodies from CHO cell culture supernatants

One hundred microliters of peptide resin were packed in a microbore column and washed as described in Section 5.2.6. After washing, the resins were equilibrated with PBS. Samples of 100 μ L of CHO cell culture supernatants were loaded onto the columns at a flow

rate of 0.05 mL/min (87 cm/hr). The unbound proteins were washed from the column by using 2 mL of equilibration buffer. Product elution was carried out by using 4 mL of 0.2 M sodium acetate buffer, pH 4. For Protein A-Toyopearl resin elution was carried out with 4mL of 0.1 M glycine-HCl buffer, pH 2.5. Cleaning and regeneration were performed with 4 mL of 0.85% (v/v) phosphoric acid. All the chromatographic steps after loading were performed at a flow rate of 0.2 mL/min (348 cm/h). Fractions were collected and concentrated five times by centrifugation at 4°C, 20817 x g for 30 min using Amicon Ultra centrifugal filter (3,000 MWCO) (Millipore, MA, USA). These fractions were then used for analysis of IgG purity and yield.

5.2.9. Sample analysis for yields and purities

The amounts of IgG in the collected fractions were quantified by HPLC using a 1 mL HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, using NuPAGE® Novex 4-12% Bis-Tris gels in a Xcell SuperLock™ Mini-Cell system (Life Technologies). Sample preparation was performed by adding 5 µL of NuPAGE® LDS buffer and 2 µL of NuPAGE® reducing agent to 13 µL of sample and boiling the resulting mixture for 10 minutes. Gels were coomassie-stained by using SimpleBlue™ SafeStain. The IgG purity was determined by densitometric analysis of coomassie-stained gels by means of

ImageJ 1.32j software (National Institutes of Health, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 kDa.

5.2.10. Reusability studies of HWRGWVC-WorkBeads

One hundred microliters of HWRGWVC-WorkBeads resin was packed into a microbore column and equilibrated with PBS, pH 7.4. Human IgG (10 mg/mL in PBS, pH 7.4) was loaded onto the column at a linear flow velocity of 35 cm/h (5 min residence time). Breakthrough volume was determined at the point where the IgG concentration in the flow-through reached 1 % of its feed concentration. This breakthrough volume was corrected by subtracting the void volume and based on this corrected volume, the dynamic binding capacity of the resin was determined. IgG was eluted from the column with glycine-HCl, pH 2.5 and the peptide column was regenerated by washing with 0.1 M NaOH for 30 minutes. The experiment was repeated for 20 cycles.

5.3. Results

5.3.1. Amination of WorkBeads 40 ACT

Amination of WorkBeads 40 ACT was performed to convert the bromoalkyl groups on the resin surface into primary amino groups. After reaction with ammonium hydroxide, a Kaiser test performed on a resin aliquot confirmed that the conversion to primary amines was successful.

5.3.2. Determination of static binding capacity of resins prepared by HATU-based coupling chemistry and iodoacetic acid-based activation

Four peptide sequences, namely HWRGWV, Ac-HWRGWV, Ac-HWRGWVA, and Ac-HWRGWVG were coupled onto Toyopearl Amino-HC and aminated WorkBeads resins using HATU-based coupling chemistry. The three variants of the base sequence HWRGWV were chosen based on the mechanism of peptide coupling using HATU. First, peptides with a free N-terminus can form oligomers either in solution or on the resin surface. The former are likely to fold into random coil hence becoming less reactive, while the latter might have impaired binding ability. Therefore, two versions of the peptide ligand HWRGWV, one with a free N-terminus and one with acetylated N-terminus, were compared. Our previous studies have shown that acetylating the N-terminus of HWRGWV does not affect its binding to IgG [28]. Therefore, in this work the N-terminus acetylated peptides were used.

Second, the steric hindrance of the side group of the C-terminal amino acid is hypothesized to have an impact on the efficiency of ligand coupling. Therefore, amino acids with a smaller side group such as alanine and glycine were added as terminal amino acids next to valine. To the best of our knowledge, there has been no published report that involves the HATU-mediated coupling of a whole peptide to a resin surface in which C-terminal steric effects were explored. Therefore, to study these effects on the C-terminus, while keeping the N-terminus protected, the sequences Ac-HWRGWVA and Ac-HWRGWVG were chosen. Toyopearl Amino-HC, a polymethacrylate based resin, was chosen for its chemical stability towards the organic solvents and reagents employed for

coupling the peptide ligands, as well as for its mechanical resistance to the high flow rates and pressures characteristic of affinity chromatography. Furthermore, these resins show low non-specific protein binding and high functional density (approx. 200 $\mu\text{mol-NH}_2/\text{mL}$), which enables high ligand concentration on the resin. WorkBeads 40 ACT resin is a highly cross-linked agarose matrix functionalized with bromoalkyl groups at a density of 80 to 100 $\mu\text{mol}/\text{mL}$ mounted on a 4-16 atoms spacer arm. As mentioned in Section 5.3.1., the resin can be easily aminated by $\text{S}_{\text{N}}2$ reaction with ammonia in alkaline conditions. These amino groups are needed for coupling the peptide via HATU chemistry (Figure 5.1A). Although WorkBeads have a functional density considerably lower than that of Toyopearl Amino-HC, they have been proven to be a good support for the preparation of affinity adsorbents for antibody purification. Lund *et al.* recently reported an affinity adsorbent comprising a small synthetic ligand coupled onto WorkBeads, which gave a MAb dynamic binding capacity of 48 mg/mL [21].

The peptide molar excess and the peptide concentration were varied to optimize the ligand coupling. The former is defined as the ratio between the number of equivalents of peptide in the reaction solution and the number of equivalents of amino groups on the resin surface. Six values of coupling molar excess were tried for all resins, i.e. 0.1, 0.25, 0.5, 0.75, 1, and 2. For each molar excess, three values of peptide concentration were tried, namely 50mM, 100mM, and 150mM. The resulting peptide-based adsorbents were tested to determine the IgG static binding capacity according to the protocol presented in Section 5.2.5.

Figure 5.2 shows the correlation between IgG static binding capacity and the peptide molar excess for each peptide sequence coupled onto Toyopearl Amino-HC. In each plot three curves are presented, each corresponding to a peptide concentration in the coupling solution. These results suggest that a peptide concentration of at least 100 mM is required to achieve a satisfactory coupling yield, which affords higher capacity resin.

As anticipated, the acetylation on the peptide N-terminus impacted favourably on ligand coupling. In fact, while HWRGWV-Toyopearl-HC gave a maximum binding capacity of 24 mg/mL, Ac-HWRGWV-Toyopearl-HC offered a considerably higher capacity of 33 mg/mL. On the other hand, a clear dependence of coupling efficiency and hence binding capacity upon the steric effect of the C-terminal amino acid was not observed. As Figure 5.2 shows, the addition of alanine and glycine did not substantially improve the binding capacity of the resulting adsorbents, which were found to be 33.5 and 35.0 mg/mL, respectively. It was further observed that increasing the peptide molar excess only up to 0.5 – 0.75 effectively resulted in higher binding capacity, while for higher values of molar excess in the coupling solution no further increase was observed. This might indicate that at higher density the peptide ligands begin to hinder IgG binding. A similar phenomenon has been observed with MAb-binding ion exchange chromatography resins [36].

The same evaluation of the effects of acetylation and addition of an amino acid on the peptide C-terminus upon the adsorbent binding capacity were repeated on aminated WorkBeads. The same ranges of molar excess (0.1 – 2) and peptide concentration in the coupling solution (50 mM, 100 mM, and 150 mM) were employed. Figure 5.3 shows the

correlations of the IgG static binding capacity versus peptide molar excess and concentration for each ligand coupled on WorkBeads.

WorkBeads showed a markedly different behaviour compared to Toyopearl Amino-HC. First, as shown in Figure 5.3, the acetylation of the peptide N-terminus did not afford any improvement in binding capacity of the resulting adsorbent HWRGWV-WorkBeads over Ac-HWRGWV-WorkBeads. Conversely, the addition of alanine or glycine to the peptide C-terminus resulted in a considerable increase in binding capacity, from a maximum value of 22 mg/mL for Ac-HWRGWV, up to values of 24.8 mg/mL for Ac-HWRGWVA and 37.5 mg/mL for Ac-HWRGWVG. However, similar to what was observed for Toyopearl Amino-HC, a minimum peptide concentration of 100 mM was needed in the coupling solution to yield adsorbents with satisfactory capacity. As another point of similarity with Toyopearl Amino-HC, the capacity of WorkBeads resins reached a plateau at a peptide molar excess of 0.75. As a comparison between Figures 5.2 and 5.3 indicates, a molar excess (moles of ligand per mole of amino groups on resin surface) of approximately 0.75 is needed to maximize static binding capacity on both resins. Also, for both Toyopearl-HC and WorkBeads, the highest IgG binding capacity was obtained by the Ac-HWRGWVG variant and furthermore, the peptide density of both resins were similar, 20 μ moles/mL and 16 μ moles/mL respectively.

To prove the versatility of the peptide ligands with respect to different coupling strategies, the variant HWRGWVC was immobilized on iodoacetic acid activated resins. The reaction between the thiol group on C-terminal cysteine and the iodide group on the resin

surface leading to the formation of a thioether bond is known to be highly efficient [37]. Therefore, this approach was adopted to prepare two adsorbents, HWRGWVC-Toyopearl Amino-HC and HWRGWVC-WorkBeads. Studies were carried out to determine the effect of peptide density on the IgG binding capacity of the resins which are summarized in Figure 5.4.

As can be seen in Figure 5.4, for both Toyopearl Amino-HC and Workbeads, increases in peptide density resulted in increased IgG binding capacity of the resins. However, a saturation limit was observed for both resins beyond which there was no further increase in IgG binding capacity with increase in peptide density. In the case of Toyopearl Amino-HC, the optimum peptide density was 98 $\mu\text{moles/ml}$, which gave a binding capacity of 38 mg/ml. For WorkBeads the optimum density was 26 $\mu\text{moles/ml}$ that resulted in a binding capacity of 48 mg/mL. This static binding capacity was very similar to that obtained with rProtein A-Toyopearl resin (55 mg/mL), but lower than the value obtained with MabSelect SuRe LX (75 mg/mL). The higher peptide utilization of the WorkBeads resin as compared to Toyopearl Amino-HC resin might be due to a better spatial orientation of the immobilized peptide on the agarose crosslinked resins compared to the polymeric microporous particles of Toyopearl. Even though the highest static binding capacity was obtained with HWRGWVC-WorkBeads, we also selected Toyopearl-HC based resin and the best resins from the HATU-based chemistry for further characterization studies.

Finally, to prove the reproducibility of the two ligand coupling strategies, the top binding resin from each synthetic route was synthesized ten additional times and its static

binding capacity was measured, as per Section 5.2.5. Both chemistries showed high degrees of reproducibility, as the studied resins yielded average static capacities within 2 mg/mL of those values given in Figures 5.2-5.4 with coefficients of variation of less than 8%.

5.3.3. Determination of dynamic binding capacity

Based on the highest values of static binding capacity reported in Section 5.3.2., four adsorbents were selected for determining dynamic binding capacity (DBC), Ac-HWRGWVG-Toyopearl, Ac-HWRGWVG-WorkBeads, HWRGWVC-Toyopearl, and HWRGWVC-WorkBeads resin. For comparison, DBC experiments were carried out with the Protein A based resins, Toyopearl AF-rProtein A-650F and MabSelect SuRe LX. The DBC measurements were performed by injecting 1 mL of IgG solution in PBS at 10 mg/mL, at two different linear velocities, i.e. 35 cm/h and 87 cm/h. The resulting chromatograms are given in Figure 5.5, which show expected flow-through and elution peaks, denoted FT and EL, respectively in Figure 5.5A. All peptide based resins gave smooth flow-through (breakthrough) curves, as the HWRGWV ligand binds all subclasses of the polyclonal human IgG used in experimental proceedings [22]. However, Protein A does not bind IgG₃, therefore a step-like breakthrough curve was observed in Figure 5.5E for the rProtein A-Toyopearl.

The dynamic binding capacity (DBC) was measured when the IgG concentration in the flowthrough reached 10% of the feed concentration. As shown in Figure 5.6, the DBC of the resins at a linear velocity of 35 cm/h (5 min residence time) was in the range of 36- 42

mg/mL. These values are considerably higher than the DBC obtained with HWRGWV adsorbents prepared by direct on-resin peptide synthesis (20 mg/mL) [27]. It should also be noted that the peptide resins prepared in this study had a lower peptide density (16 – 26 μ moles/mL) than the peptide resins prepared by solid phase peptide synthesis (SPPS) and reported in our earlier studies (0.15 meq/g, 34 μ moles/mL). The better utilization of peptide ligands of adsorbents prepared in this work can be attributed to the absence of truncated peptide sequences which are formed during SPPS. The truncated sequences bind impurities non-specifically reducing both the purity and the binding capacity of the IgG eluted from the HWRGWV resins.

The DBC of HWRGWVC-WorkBeads (42 mg/mL) was comparable to that obtained with rProtein A-Toyopearl resin (49 mg/mL) but lower than MabSelect SuRe LX (56 mg/mL). Work is ongoing in our laboratory to further increase the binding capacity of peptide adsorbents by using different linkers and preliminary studies have resulted in peptide adsorbents with binding capacity of 67 mg/mL. As can be seen in Figure 5.6, a strong dependence of DBC on linear velocity was observed. All adsorbents including rProtein A-Toyopearl and MabSelect SuRe LX show a decrease in capacity upon increasing linear velocity from 35 cm/h to 87 cm/h.

5.3.4. Determination of resin selectivity (Fc vs. Fab)

The ligand selectivity towards the Fab and Fc fragments of IgG was determined via chromatographic binding studies. Each antibody fragment at a concentration of 1 mg/mL was

loaded onto the column. Elution was performed with 0.2 M acetate buffer, pH 4. As can be seen from Figure 5.7, all the peptide adsorbents showed very high binding for Fc fragment (94 -99 %) compared to Fab fragments (3-14 %) thus confirming the high specificity of peptide adsorbents towards the Fc region of IgG. This selectivity is highly desirable for the purification of Fc fusion proteins.

5.3.5. Purification of monoclonal antibodies from CHO cell culture supernatants

HWRGWV-based resins were used for purification of monoclonal antibodies from clarified cell culture fluids. For comparison, Protein A-Toyopearl resin was used for purification of MAbs. The two MAbs, a chimeric antibody of IgG₁ subclass and a humanized antibody of IgG₄ subclass at the concentrations of 2.3 mg/mL and 1.5 mg/mL respectively, were recovered from cell culture supernatants also containing pluronic acid, yeast hydrolysate, cholesterol, tropolone, amino acids, glucose, vitamins and cellular components. After equilibrating the peptide resin with PBS, the supernatant was loaded onto the column without any pre-treatment. Washing was performed with 1M NaCl in PBS and elution was then performed with 0.2 M acetate buffer, pH 4 for peptide resins and with 0.1 M glycine HCl, pH 2.5 for Protein A resin.

The resulting chromatograms for the four adsorbents employed are shown in Figure 5.8. All chromatograms contain four peaks corresponding to the separation events of impurity flow-through, non-specifically bound impurity elution, product elution and regeneration, which are denoted FT, NaCl Wash, EL and RE, respectively in Figure 5.8A. In

general, larger peaks were observed during purification of Mab from IgG₁ supernatants than IgG₄ supernatants, which coincides with the fact that the former is higher in concentration than the latter. SDS-PAGE of fractions collected from purifications using Ac-HWRGWVG-WorkBeads is given in Figure 5.9. As can be seen from the SDS PAGE (Lanes 2 and 6) the peptide resin was able to capture all the MAb from both supernatants and purify them with a very high purity (Lanes 4 and 8). In fact, in case of IgG1 purification, the peptide adsorbent was able to purify the Mab from the over-expressed IgG light chain present in the supernatant, thus confirming the high specificity of the HWRGWV ligand to the Fc region. Similar SDS PAGE profiles were observed for all the HWRGWV-based resins. The MAb yields and purities were in the range of 87 - 93% and 94 - 98 % respectively, similar to results obtained with rProtein A –Toyopearl resin (Table 5.1).

5.3.6. Reusability of HWRGWVC-WorkBeads

The peptide resin with the highest IgG binding capacity, HWRGWVC-Workbeads was tested for reusability and stability to alkaline cleaning conditions. Twenty cycles of IgG dynamic binding experiment were performed with the peptide resin. After every cycle the resin was washed with 0.1M NaOH solution for 30 mins. Figure 5.10 reveals that the resin showed a high degree of reusability during these runs, as it retained 90% of its initial dynamic binding capacity. Additionally, the resin showed no peptide leaching during the NaOH cleaning step, which is in good agreement with past results obtained by our group [26] thereby confirming the high stability of the HWRGWVC peptide.

5.4. Conclusions

Ligand coupling strategies play a vital role in the development of cost effective affinity adsorbents. Though several coupling chemistries have been reported in the literature, a judicious selection, development and optimization is essential for immobilizing a particular ligand to a support. Factors such as ligand solubility, ligand stability and base resin stability influence the selection of coupling strategy. In this work two different chemistries were examined for coupling the Fc binding peptide ligand-HWRGWV and its variants to polymethacrylate and cross-linked agarose-based resins. The HATU chemistry involved activating the peptide and then coupling it to the resin. On the other hand, the iodoacetic acid based chemistry involved activating the resin and then coupling the peptide to the resin. For HATU-based chemistry, in addition to HWRGWV, three variants were used. Variation included acetylation of the N-terminus of the peptide and/or addition of alanine or glycine at the C-terminus. Studies were carried out to optimize the peptide coupling conditions. The optimum values of peptide molar excess and concentration for all the variants were found to be in the range of 0.5-0.75 and 100mM respectively. For both Toyopearl HC and WorkBeads, the Ac-HWRGWVG ligand gave the highest IgG binding capacity. Ac-HWRGWVG-Toyopearl HC with a peptide density of 20 $\mu\text{moles/mL}$ resulted in IgG static binding capacity of 35 mg/mL and Ac-HWRGWVG-WorkBeads with a peptide density of 16 $\mu\text{moles/mL}$ gave 37.5 mg/mL.

The coupling conditions were also optimized for the iodoacetic acid based activation chemistry. HWRGWVC-Toyopearl with a peptide density of 98 $\mu\text{moles/mL}$ gave a binding

capacity 38 mg/mL and HWRGWVC-WorkBeads, with a peptide density of 26 μ moles/mL, gave a binding capacity 48 mg/mL. The dynamic binding capacities of the four resins at linear flow velocities of 35 cm/hr were in the range of 36-42 mg/mL. This value of DBC was similar to that obtained with rProtein A –Toyopearl resins. However, the DBC is still lower than the recent versions of Protein A resins such as MabSelect Sure LX (~ 60 mg/mL) and ProSep-vA Ultra Plus (~ 50 mg/mL). Work is ongoing in our lab to increase the binding capacity through introduction of various linkers with preliminary results giving a binding capacity of 67 mg/mL.

The peptide resins were further evaluated for Fc/Fab selectivity and for purification of two different MAbs from CHO cell culture supernatants. All resins showed very high Fc binding specificity (94 -99 %) as compared to Fab binding (3 – 10 %). The peptide resins were also able to capture and purify the MAbs with high yields (87- 93 %) and purities (94- 98 %), which are comparable to the results obtained with Protein A- resin. The HWRGWVC-WorkBeads resin showed high reusability and alkaline stability as it maintained 90% of its initial capacity over the course of 20 binding cycles which included cleaning with 0.1 M NaOH solution.

A comparison of the two chemistries used in this study highlights certain advantages of using the iodoacetic acid (IAA) based chemistry over HATU based chemistry for coupling the HWRGWV-based peptides to the chromatographic solid supports. First, the coupling efficiency obtained with IAA is higher than that resulting from HATU chemistry. Second, as only the resin is activated during the IAA chemistry, the unreacted peptide can be reused.

This reusability of peptide is not possible in the case of HATU chemistry. Finally, the amount of peptide bound to the resin can be readily determined by spectrophotometric analysis of the unreacted peptide in the reaction solvent. In the case of HATU chemistry, elemental analysis of the resin has to be performed for estimation of peptide density. The only advantage that the HATU-based chemistry offers is that it can be completed in a single step as compared to the two step reaction required for IAA-based chemistry.

In addition to coupling peptides on resins, these methods can be extended to couple the HWRGWV ligand to nonwoven membranes, chips and other surfaces for applications in purification, biosensors and diagnostics.

5.5. Acknowledgements

The authors would like to thank Takaaki Terasaka of the Fuji Silysia Chemical Co. and Tracy Hendrick of the NCSU Department of Chemical and Biomolecular Engineering for help in experimental proceedings. Matthew Groff and Ryan Hutchinson of GE Healthcare are thanked for the gift of MabSelect SuRe LX. Lisa Lentz of the NCSU Environmental and Agricultural Testing Service is acknowledged for conducting elemental analysis. The NCSU Biomanufacturing Training and Education Center (BTEC) is gratefully thanked for funding.

5.6. References

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Table 5.1. Yield and purity of IgG₁ and IgG₄ MAbs purified from cell culture supernatants using HWRGWV-based resins.

Adsorbent	IgG ₁		IgG ₄	
	Yield	Purity	Yield	Purity
Ac-HWRGWVG-Toyopearl HC	88 %	94 %	87 %	96 %
Ac-HWRGWVG-WorkBeads	90 %	96 %	90 %	98 %
HWRGWVC-Toyopearl HC	90 %	95 %	88 %	95 %
HWRGWVC-WorkBeads	93 %	96 %	90 %	98 %
rProtein A – Toyopearl	95 %	97 %	90 %	98 %

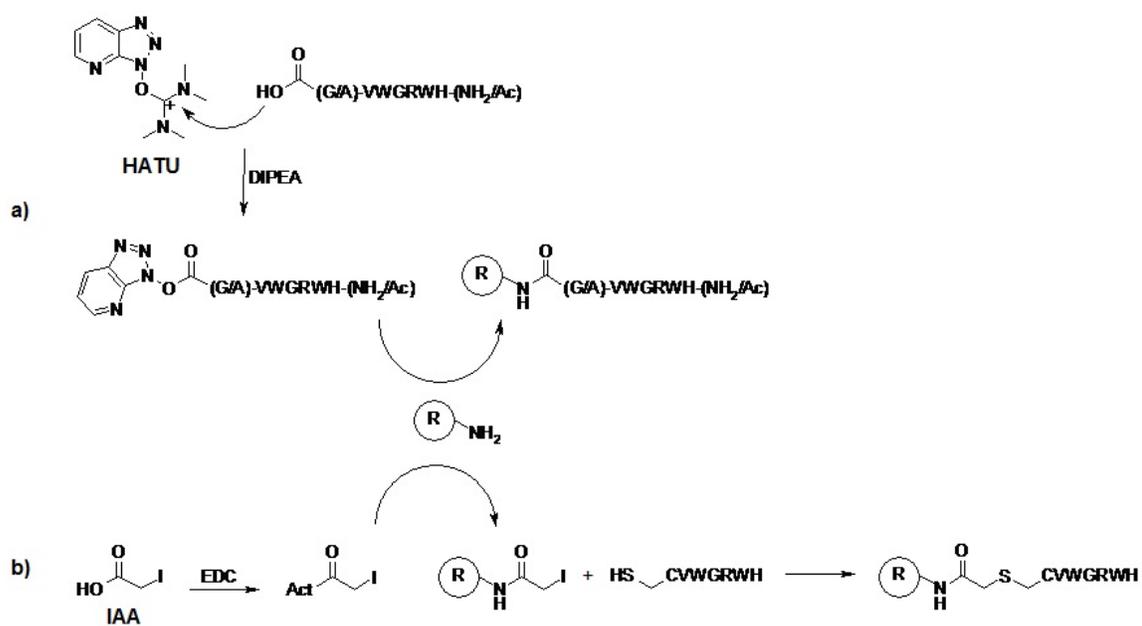


Figure 5.1. Peptide coupling chemistries. a) HATU coupling chemistry b) Iodoacetic acid activation coupling chemistry; DIPEA – Diisopropylethylamine, IAA – Iodoacetic acid, EDC- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide , NH₂/Ac- Free amino peptide/ N-terminus acetylated peptide, Act- EDC activated IAA, R- Resin.

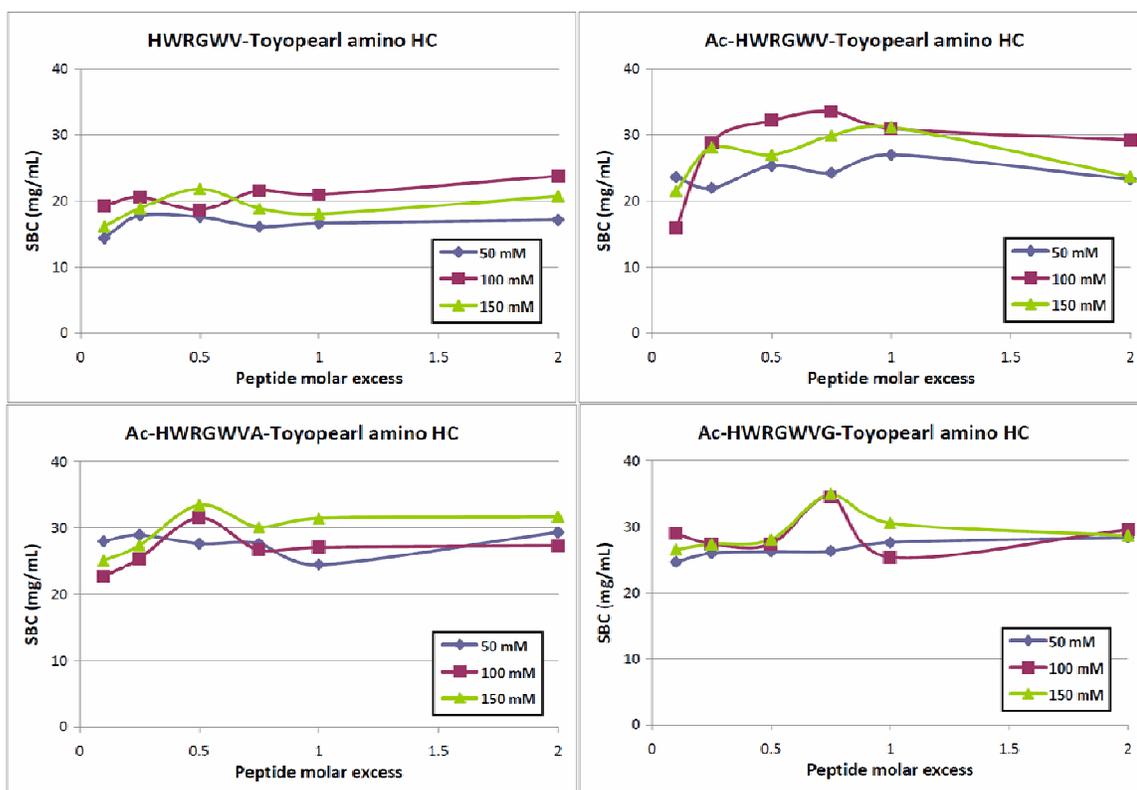


Figure 5.2. Static binding capacities of various Toyopearl Amino-HC resins prepared by HATU-based coupling chemistry.

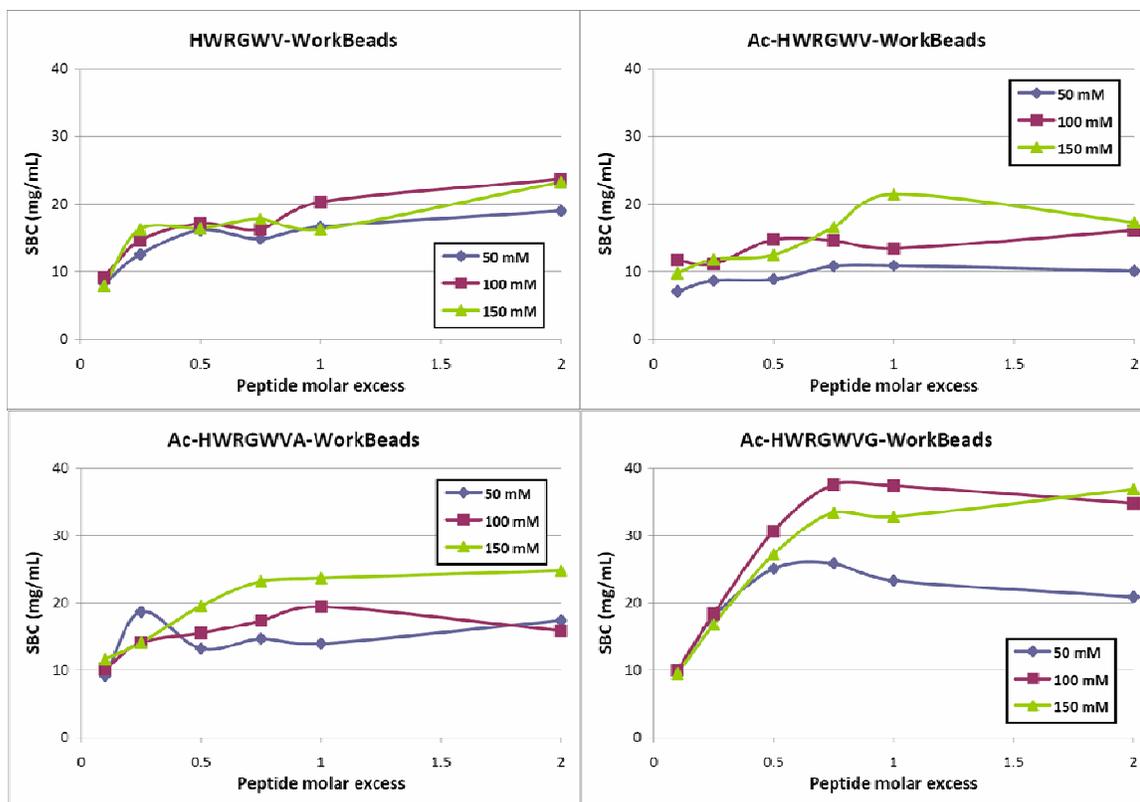


Figure 5.3. Static binding capacities of various WorkBeads resins prepared by HATU-based coupling chemistry.

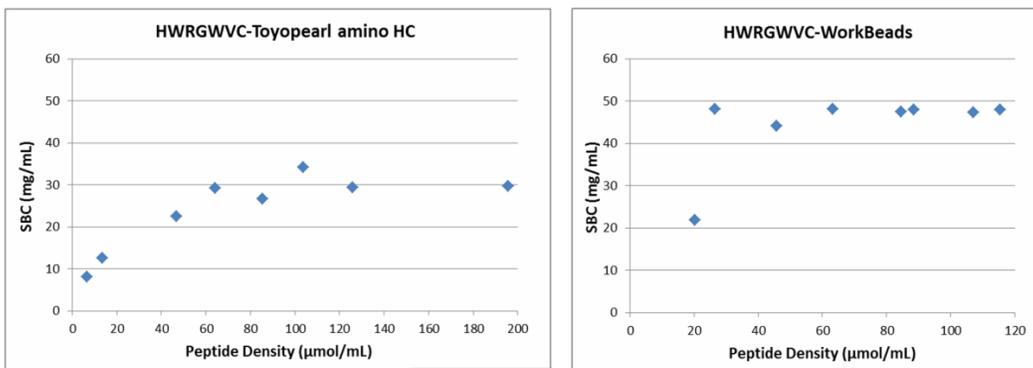


Figure 5.4. Static binding capacities of various resins prepared by iodoacetic acid activation-based coupling chemistry.

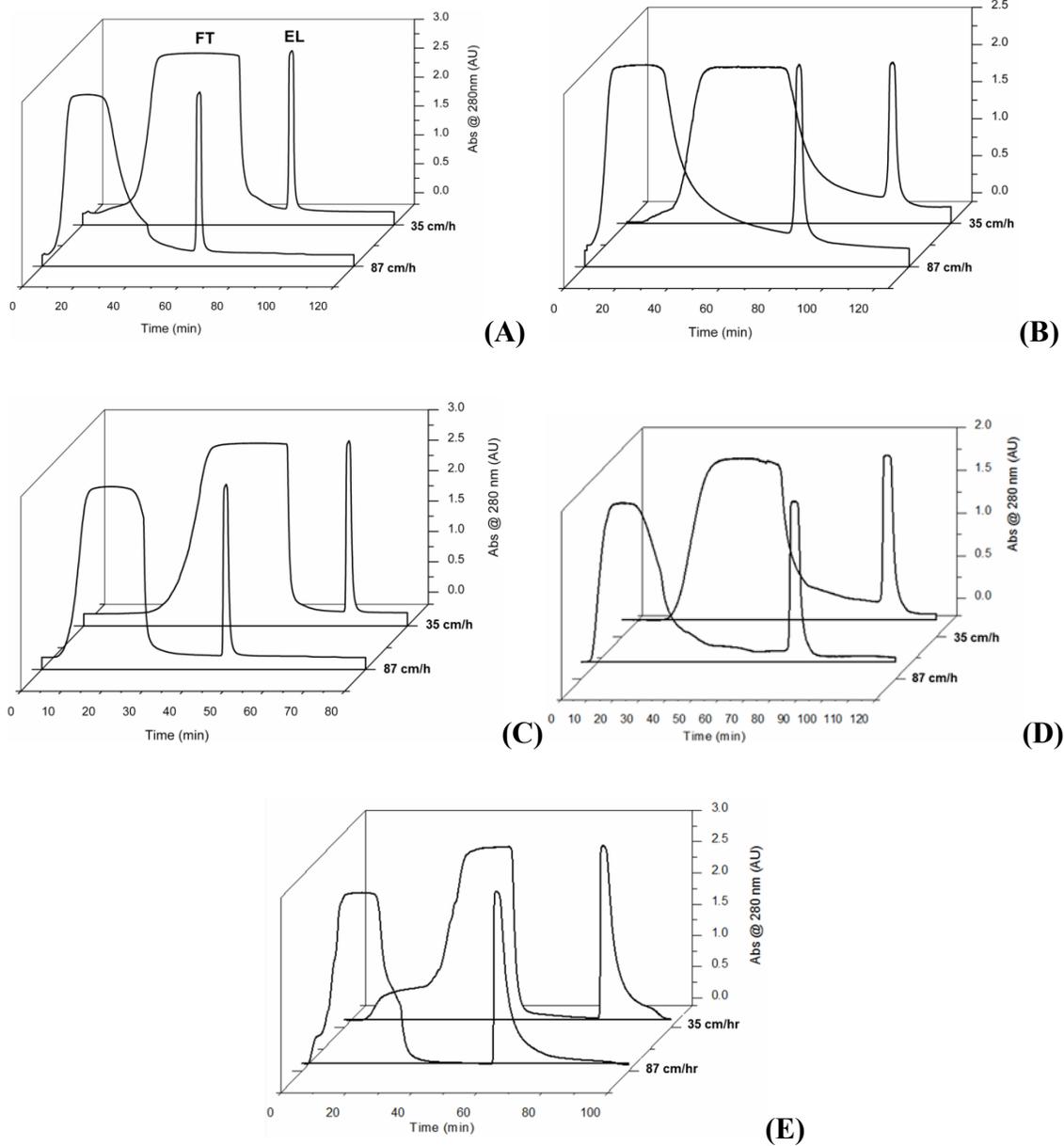


Figure 5.5. Breakthrough curves of peptide and Protein A adsorbents. IgG was loaded at a concentration of 10 mg/mL. The two linear velocities used were 35 cm/h and 87 cm/h, corresponding to residence times of 5 min and 2 min; (A) Ac-HWRGWVG-Toyopearl-HC; (B) Ac-HWRGWVG-WorkBeads; (C) HWRGWVC-Toyopearl-HC; (D) HWRGWVC-WorkBeads; (E) rProtein A-Toyopearl.

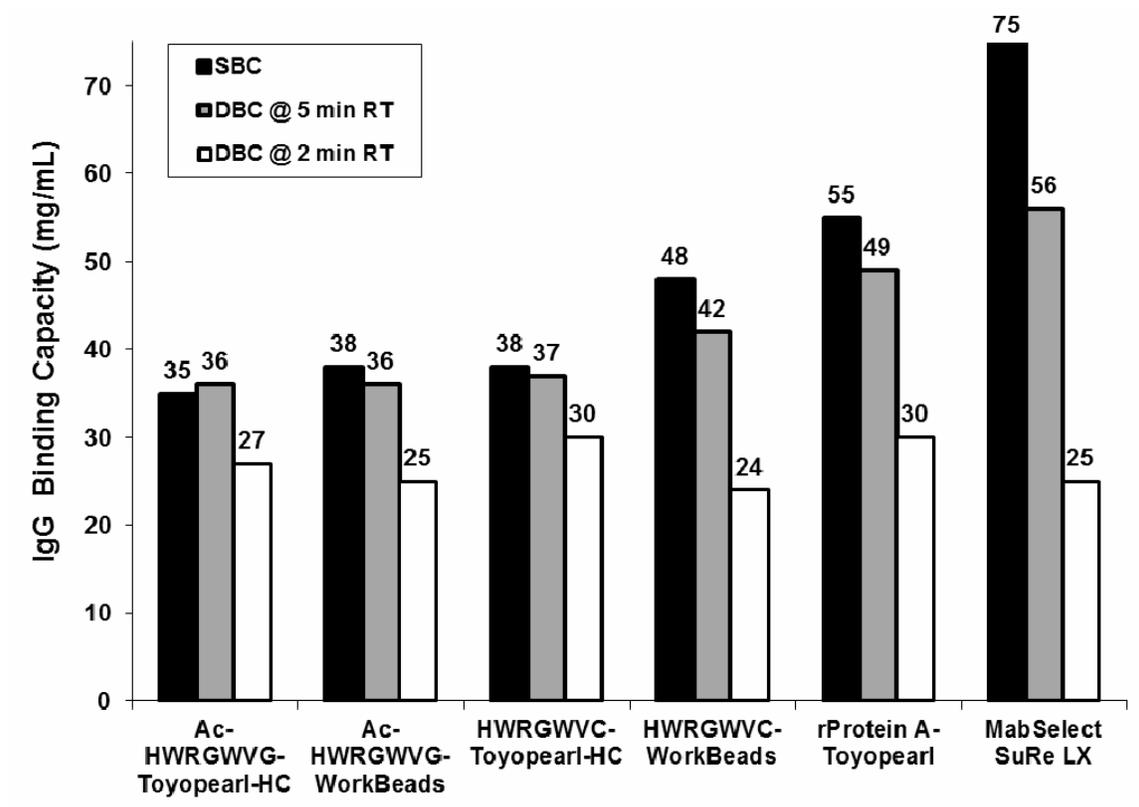


Figure 5.6. Summary of the values of various binding capacities of peptide-based and Protein A-based affinity adsorbents.

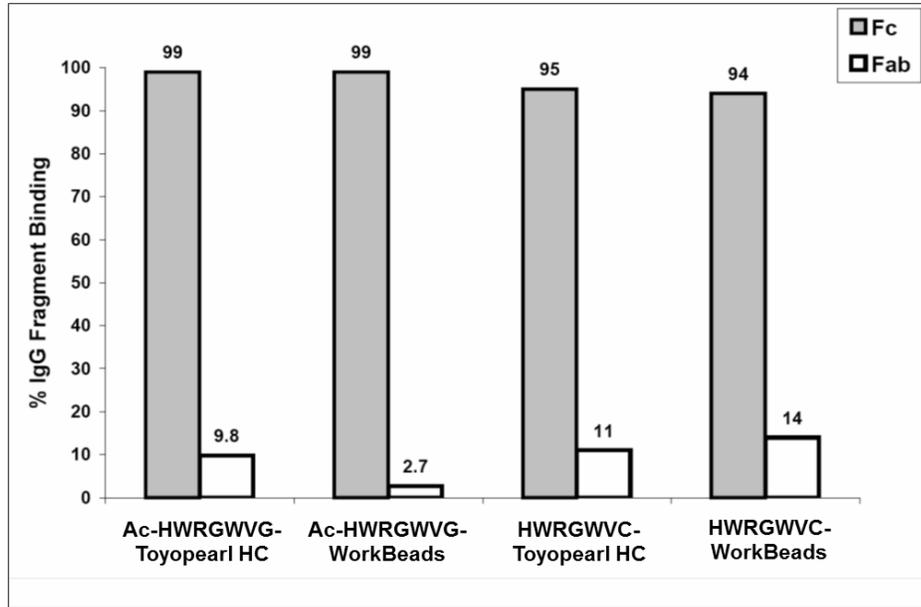


Figure 5.7. Binding studies of Fc and Fab fragments of human IgG on different resins.

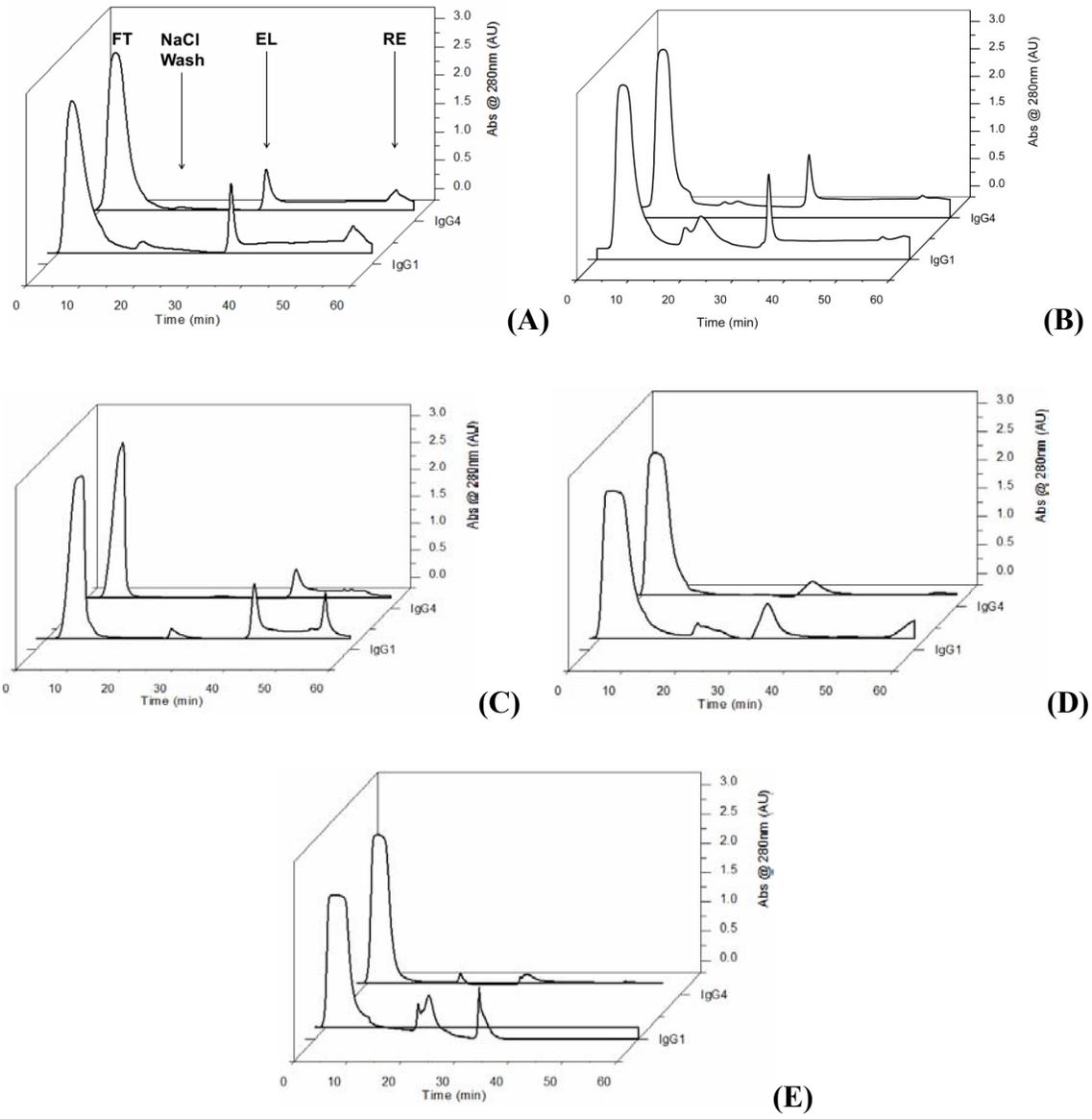


Figure 5.8. Chromatograms of purification of IgG₁ and IgG₄ from cell culture supernatants using HWRGWV-based resins. (A) Ac-HWRGWVG-Toyopearl-HC; (B) Ac-HWRGWVG-WorkBeads; (C) HWRGWVC-Toyopearl-HC; (D) HWRGWVC-WorkBeads; (E) rProtein A-Toyopearl.

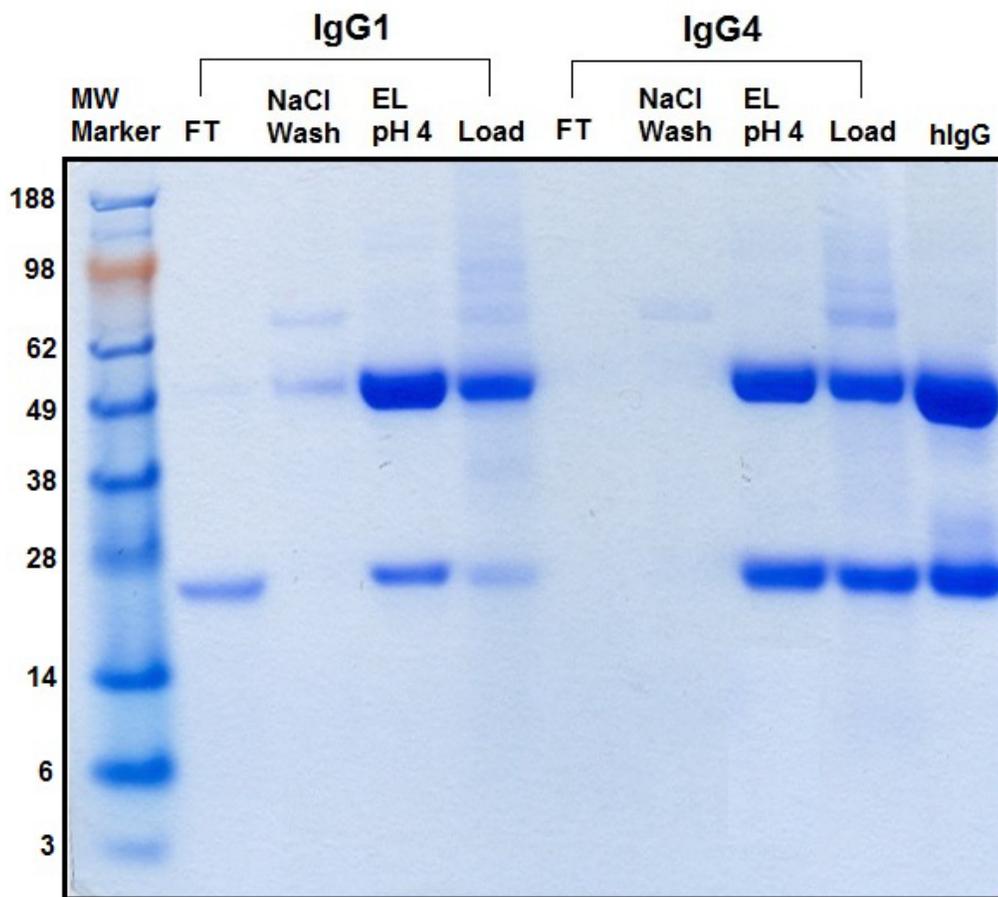


Figure 5.9. SDS-PAGE (reducing conditions) of flow-through and eluted fractions of IgG purifications from CHO cell culture supernatants containing IgG1 and IgG4 using Ac-HWRGWVG-WorkBeads resin. Labels: FT – flow-through fraction; NaCl Wash- wash fraction performed with 1 M NaCl in PBS, pH 7.4; EL – elution fraction, IgG- human Immunoglobulin G standard.

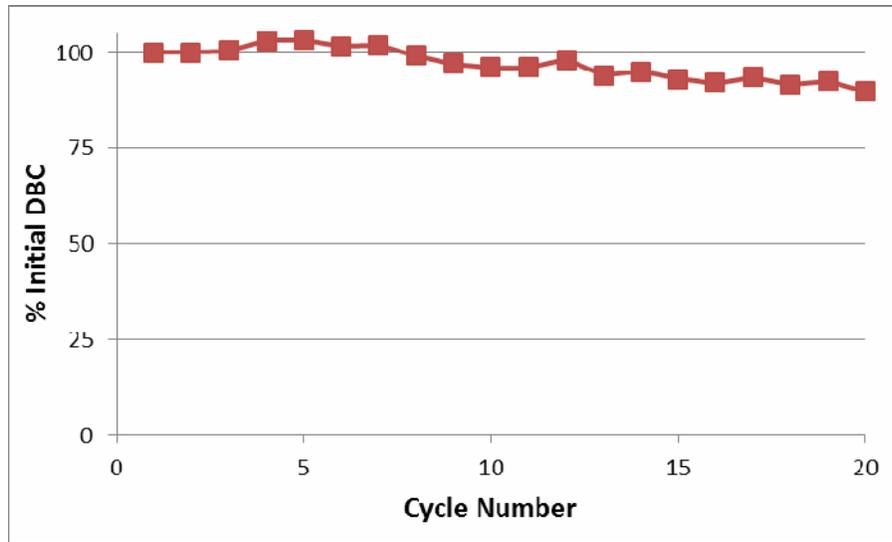


Figure 5.10. Percentage of initial dynamic binding capacity of HWRGWVC-WorkBeads over 20 cycles.

**Chapter 6. Alkaline-stable peptide ligand affinity adsorbents
for the purification of biomolecules**

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*A version of this chapter has been published in
Journal of Chromatography A 2012, 1245, 55-64.*

Abstract

A strategy of modification of resin surface chemistry is presented to produce hydrophilic peptide-based alkaline-stable affinity adsorbents for the purification of biopharmaceuticals from complex media. In this work, the peptide-based affinity adsorbent HWRGWV-Toyopearl resin for the purification of IgG is presented as an example. When prepared by direct peptide synthesis on the chromatographic matrix, the peptide-based resin showed lability under alkaline conditions. In fact, the regeneration with aqueous 0.1M NaOH caused the leaching of 40% of the peptide ligand, resulting in a decrease of IgG yield from 85% to 23%. It was found that the ligand leaching was caused by the coupling of a significant amount of peptide by alkaline-labile ester bonds. A method was designed to prevent the formation of ester bonds and allow the synthesis of the ligand exclusively on alkaline-stable bonds. The method consists in activating the hydrophilic base resin, blocking the hydroxyl groups responsible for alkaline lability and performing the peptide synthesis exclusively via alkaline-stable amide bonds. Repeated cycles of IgG purification from a cell culture medium were performed, each followed by cleaning with aqueous NaOH (0.1M, 0.5M and 1M). The IgG yield decreased from 91% to 85% after 200 purification cycles with 0.1M NaOH. However, the IgG purity remained almost constant at around 95% based on SDS-PAGE analysis. The procedure presented is rapid, efficient and inexpensive and does not require any equipment other than the usual instrumentation for peptide synthesis. The method also has a broad application since it is valid for any peptide ligand identified for the purification of a biopharmaceutical target.

Keywords: Peptide ligand; Affinity adsorbent; NaOH stability; Modification of surface chemistry; Solid-phase peptide synthesis; Cleaning-in-place.

6.1. Introduction

Biopharmaceuticals have emerged as a very important class of therapeutics for the treatment of a vast array of diseases. However, these products are very expensive to consumers and have not been able to reach a large segment of the world's population. The high cost is mainly imputed to the purification processes (downstream processing), which account for over 50-80% of the overall manufacturing costs [1]. Chromatography is a central figure in downstream processing, being the only scalable technique that has the ability to achieve the high purity standards required by the regulatory authorities for commercial biologicals [2]. Among the different types of chromatography, affinity chromatography has the highest potential for the selective purification of therapeutic proteins. In order to be applied on the industrial scale, an affinity adsorbent should exhibit high binding capacity and specificity, low cost and good chemical resistance toward the treatments of cleaning-in-place (CIP) and sanitization-in-place (SIP) periodically performed to ensure its safe reusability [3]. The industrial standard for CIP and SIP of chromatographic media is aqueous NaOH (0.1 M – 0.5 M), as it is the most effective agent in eliminating bacteria, endotoxins, and viruses and does not present any serious environmental disposal problem [4]. The widely used protein ligands for IgG purification, Protein A and Protein G, are expensive (\$ 8,000 – 15,000 per liter of resin) and can release immunogenic fragments into the process stream [5-10]. To overcome these problems, attempts have been made over the last two decades to develop specific, chemically robust and cost-effective synthetic ligands [11-13]. In particular, peptides are of great interest due to their high specificity and stability and low cost compared to biological ligands. Our group has identified several peptide ligands for the purification of

biomolecules from complex media [14-18]. Recently, three linear hexapeptide ligands HWRGWV, HYFKFD and HFRRHL were discovered that bind human IgG through its Fc portion, thus mimicking the binding mechanism of Protein A [19, 20]. These hexapeptide ligands successfully capture monoclonal antibodies from CHO cell culture supernatants and exhibit yield, purity, host cell protein removal and DNA removal that are comparable to the results obtained with Protein A and Protein G [21].

The method adopted by our group for the preparation of peptide-based affinity adsorbents consists in building the peptide directly on polymethacrylate-based Toyopearl AF-Amino-650M resin via conventional Fluorenylmethyloxycarbonyl (Fmoc)-based coupling chemistry [22]. The resin was chosen as it is suited for both peptide synthesis and chromatographic applications, due to its chemical resistance to organic solvents and reagents, mechanical stability, high hydrophilicity and low non-specific binding [23]. However, experimental work has shown that these adsorbents do not withstand the alkaline conditions (0.1 M – 0.5 M NaOH) used in CIP and SIP treatments. In fact, upon exposure to alkaline wash the peptide ligand is leached from the adsorbent in one cycle, resulting in a significant loss of binding capacity. To the best of our knowledge, the alkaline lability of affinity adsorbents produced by direct peptide synthesis on chromatographic media has not yet been described in literature.

At first we tested the stability of the peptide itself in alkaline conditions. The peptide was found to be stable in aqueous 1M NaOH for up to 1 h. This indicated that the cause of ligand loss was likely due to the chemical linkage between the peptide and the base matrix.

Based on the evaluation of the properties of the functional groups present on the hydrophilic amino resins, it was determined that the peptide synthesis chemistry could yield two different populations of peptides, one coupled onto amino groups and the other onto hydroxyl groups, via amide and ester bonds respectively. While the former are highly chemically stable, the latter are labile in alkaline conditions and could be responsible for the ligand leakage during the NaOH wash. As described in this paper, the fraction of peptides coupled through ester bond was found to be around 40%. Since the amino groups are much stronger nucleophiles than hydroxyl groups and their ratio on the resin is not disclosed, such high percentage of ester linkages were not expected.

As a result, a new strategy of resin surface modification was developed for synthesizing alkaline stable peptide adsorbents. The strategy consists of following steps: (i) activating the base matrix, (ii) selectively blocking the hydroxyl groups and (iii) performing the peptide synthesis exclusively on amino groups through alkaline-stable amide bonds.

This work presents as a case study the on-resin synthesis of the affinity adsorbent HWRGWV-Toyopearl for the purification of IgG from cell culture medium. First, it is shown that a CIP step performed with 0.1 M NaOH on a HWRGWV adsorbent produced by direct on-resin synthesis caused a massive ligand leaching resulting in a loss of IgG binding capacity. A preliminary study is then presented to demonstrate the validity of the modification protocol. A chromophore amino acid (tryptophan) was coupled on a batch of modified resin with the new synthesis protocol, which showed a negligible amino acid leakage when washed in alkaline conditions. Finally, the ligand HWRGWV was synthesised

on the modified resin to produce an alkaline-stable affinity adsorbent. Consecutive cycles of IgG purification were performed, each followed by an alkaline CIP step. After 200 cycles using regeneration with aqueous 0.1M NaOH solution, the IgG yield decreased from 91% to 85%. When the regeneration was performed with 0.5M NaOH and 1M NaOH, the IgG yield decreased respectively to 80% over 100 cycles and to 76% over 50 cycles. However, under all these conditions, the IgG purity remained approximately constant in the range of 93 – 96%.

6.2. Experimental

6.2.1. Materials

HWRGWV resin having a peptide density of 0.15 meq/g was purchased from Creosalus (Louisville, KY, USA). Fmoc coupling chemistry was used to synthesize the peptide directly on Toyopearl AF-Amino-650M resin (particle size 65 μm). The resins Toyopearl AF-Amino-650M and Toyopearl HW-65F were purchased from Tosoh Bioscience (King of Prussia, PA, USA). Toyopearl HW-65F is a base resin having hydroxyl groups and is derivatized to produce Toyopearl AF-Amino 650M that has amine functional groups, as well as available hydroxyl groups. The reagents for peptide synthesis, Fmoc-protected amino acids (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-Trp(Boc)-OH, Fmoc-Trp-OH, Fmoc-Val-OH) and the coupling agents (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU) and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium

hexafluorophosphate (HCTU)) were purchased from ChemPep Inc. (Wellington, FL, USA). Boc-Gly-OH, Boc-diaminoethane and carbonyl diimidazole were from Fischer Scientific (Pittsburgh, PA, USA). Diisopropylethylamine, ethanedithiol, thioanisole, anisole, piperidine (99%), trifluoroacetic acid and acetic anhydride were from Sigma Aldrich (Saint Louis, MO, USA). The solvents employed for peptide synthesis and analysis, dimethylformamide (extra dry), dichloromethane (extra dry), acetonitrile (HPLC grade) and trifluoroacetic acid (HPLC grade) were from Fisher Scientific (PA, USA). Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Sodium chloride, sodium hydroxide, sodium acetate, hydrochloric acid and glacial acetic acid were obtained from Fischer Scientific (Pittsburgh, PA, USA). Phosphate buffer saline (PBS) of pH 7.4, ethanol, methanol and isopropanol were from Sigma Aldrich (Saint Louis, MO, USA). All the solvents were of analytical grade. Cell culture medium (Eagle Minimum Essential medium, EMEM) was from Quality Biological (Gaithersburg, MD, USA). Fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT, USA) and Becton Dickinson (Sparks, MD, USA), respectively. The complete mammalian cell culture medium (cMEM) was prepared by combining EMEM with 10% FCS and 5% TPB. NuPAGE® Novex gels (4–12% Bis–Tris), NuPAGE® MOPS and MES running buffers, NuPAGE® LDS sample buffer, NuPAGE® reducing agent, SeeBlue plus2® pre-stained molecular weight marker, SimpleBlue™ SafeStain were all from Invitrogen (Carlsbad, CA, USA). A HiTrap™ Protein G column was purchased from GE Healthcare (NJ, USA). A Waters 626 LC system integrated with 2487 UV detectors (Waters, MA, USA) was used for all chromatography runs. This Waters 626 inert quaternary solvent pump can deliver a flow

rate between 0.01 and 20.00 mL/minute. Microbore stainless steel columns (30 mm long x 2.1 mm I.D.) were from Altech-Applied Science (Somerset, PA, USA). All experiments were carried out at room temperature.

6.2.2. On-column alkaline CIP of HWRGWV-Toyopearl resin prepared via direct on-resin synthesis

Thirty five milligrams of HWRGWV-Toyopearl resin were dry-packed in 30 mm x 2.1 mm I.D. Microbore columns (0.1 mL). The resin was swollen with 20% v/v methanol and then washed with PBS, pH 7.4. The column was equilibrated using PBS buffer containing 1 M NaCl. A 100 μ L feed solution of IgG, prepared by spiking 10 mg of IgG into 1 mL of complete cell culture medium (cMEM), was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 4 mL of equilibration buffer at flow rate of 0.2 mL/min (346 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer pH 4 at a flow rate of 0.4 mL/min (692 cm/h). The column was then regenerated with 0.1 M sodium hydroxide at the flow rate of 0.1 mL/min (174 cm/h) for 10 min. The effluent was monitored by UV absorbance at 280 nm. Different cycles of IgG purification followed by alkaline regeneration were performed. Fractions were collected and concentrated five times by centrifugation at 4°C, 20,817g for 30 min using an Amicon Ultra centrifugal filter (3000 MWCO, Ultracel[®], Millipore, MA, USA). These fractions were then used for analysis of IgG purity and yield (see Section 6.2.8).

6.2.3. Determination of ligand leaching from HWRGWV-Toyopearl resin in alkaline conditions

Thirty five milligrams of HWRGWV-Toyopearl resin was packed in a column and washed as described in Section 6.2.2.1. Three alkaline washing steps with 0.1M NaOH at the flow rate of 0.1 mL/min (174 cm/h) for 10 min were performed, each followed by a washing step with PBS pH 7.4 at the flow rate of 0.4 mL/min (692 cm/h) for 10 min. The alkaline washes were collected and analysed by C18 HPLC as described in Section 6.2.4.

6.2.4. Stability of the ligand in basic solution

A 100 µg/mL solution of pure HWRGWV peptide was prepared in 20:80 water:acetonitrile. The solution was divided into three samples, in which the NaOH concentration was brought respectively to 0.1 M, 0.5 M and 1 M by adding aqueous 10 M NaOH solution. Aliquots were withdrawn from each sample after 10, 20, 30, 45, 60 and 120 min and neutralized with an appropriate volume of aqueous 12 N HCl. The resulting fifteen samples were concentrated and desalted using C18 ZipTips® (Millipore, Bedford, USA). The initial ligand solution and the samples were analysed by C18 HPLC (RAININ Microsorb-MV HPLC Column VARIAN 86-200-E3, 3 µm particle diameter), running a linear gradient of water:acetonitrile from 95:5 to 60:40 in 30 min. The absorbance was monitored at 220 nm and 280 nm. The samples were also analysed by MALDI-TOF MS (Shimadzu Biotech Axima Assurance MALDI). A 0.75 µL of each sample was mixed with 20 µL of Universal MALDI Matrix (Fluka Analytical) and loaded onto the MALDI plate.

6.2.5. Determination of the number of amide and ester bonds in base resin

The UV-visible amino acid Tryptophan was coupled to Toyopearl AF-Amino-650 M and Toyopearl HW-65 F resins to determine the number of amide bonds and ester bonds formed during solid phase peptide synthesis and determining the amino density on each resin. One hundred milligrams of each of the resins were swollen in 20% v/v methanol in water for 2 h and washed with anhydrous DMF. An anhydrous DMF solution (1 mL) of Fmoc-Trp-OH (51.2 mg, 3 eq. to resin), HCTU (49.6 mg, 3 eq.) and diisopropylethylamine (DIPEA) (41.8 μ L, 6 eq.) was added to the resins. The mixture was shaken for 3 h and the resin was washed with DMF. Further couplings were repeated to saturate all the available amino groups, as monitored by Kaiser test. Finally, the Fmoc protection was removed by incubating the resins with 2 mL of 20%v/v piperidine in DMF solution for 30 min. The Fmoc released in the supernatant was analyzed spectrophotometrically at 301 nm and used for estimation of the amino acid density of the resins. The difference in amino acid density of the two resins was regarded as a measure of the amount of amino acid coupled through amide bonds. Another method for estimating the amount of amide and ester bonds was to determine the amount of tryptophan leached from the resins on exposure to NaOH solution. This was performed as follows: 35 milligrams of each resin batch were dry-packed in a 30 mm x 2.1 mm I.D. Microbore column (0.1 mL). The resin was swollen with 20% v/v methanol and then washed with PBS, pH 7.4. Aqueous 0.1 M NaOH was passed at the flow rate of 0.05 mL/min (87cm/h) for 30 min. The effluent was monitored by absorbance at 280 nm. The NaOH wash fraction was collected and analyzed spectrophotometrically at 280 nm to determine the amount of tryptophan leached.

6.2.6. Base-stable peptide ligand synthesis on resins containing hydroxyl and amine groups

Two hundred milligrams of Toyopearl AF-Amino-650M was swollen in 20% v/v methanol for 2 h and then rinsed with anhydrous DMF. The resin was reacted with Boc-Alanine via HCTU chemistry. An anhydrous DMF solution (1 mL) of Boc-Ala-OH (45.4 mg, 3 eq.), HCTU (99.3 mg, 3 eq.) and DIPEA (83.6 μ L, 6 eq.) was added to the resin. The mixture was shaken for 3 h and then the resin was washed with DMF. Further couplings were repeated to saturate all the available amino groups, as monitored by Kaiser test. The resin was dry-packed in 30 mm x 2.1 mm I.D. Microbore column (0.1 mL) and swollen in 20% v/v methanol. After equilibration with PBS pH 7.4, 0.1 M NaOH was flown through the column to remove the Boc-alanine coupled through ester bonds. The resin was split into two batches, (a) and (b), and rinsed with DMF. Acetylation was carried out only on batch (a) with acetic anhydride – DIPEA (1:1, 2 mL, 3 x 30 min), while batch (b) was kept non-acetylated. Boc protection was then removed from both resins by TFA in DCM (1:1, 2 mL, 2 x 30 min) and the amino groups were free-based by triethylamine (TEA) in DMF (1:1, 2 mL, 2 x 15 min). A Kaiser test was performed to check the presence of free amino groups available for coupling. Fmoc-tryptophan was coupled on both resins via HCTU chemistry. An anhydrous DMF solution (1 mL) of Fmoc-Trp-OH (51.2 mg, 3 eq.), HCTU (49.6 mg, 3 eq.) and diisopropylethylamine (DIPEA) (41.8 μ L, 6 eq.) was added to the resins. The mixture was shaken for 3 h and the resin was washed with DMF. Further couplings were repeated to saturate all the available amino groups, as monitored by Kaiser test. The same coupling was also performed on unmodified Toyopearl AF-Amino-650M to produce the batch (c) used as

control. The amino acid density was estimated on the three resins by Fmoc quantification as described in Section 6.2.2.3. The difference in amino acid density between batch (b) and (a) was considered as a measure of the amount of amino acid coupled through ester bonds. To determine the NaOH stability of the resins, 35 mg of each resin batch were dry-packed in 30 mm x 2.1 mm I.D. Microbore columns (0.1 mL). The resin was swollen with 20% methanol and then rinsed with PBS pH 7.4. Aqueous 0.1 M NaOH at the flow rate of 0.05 mL/min (87 cm/hr) was passed for 30 min. The effluent was monitored by absorbance at 280 nm.

6.2.7. Base-stable peptide synthesis on resins containing only hydroxyl groups

Two hundred milligrams of Toyopearl HW-65F resin was swollen in 20% v/v methanol for 2 h and then rinsed with anhydrous DMF. A 2 mL of 50% v/v resin slurry in anhydrous DMF was mixed with 385 mg of carbonyl diimidazole (CDI). The reaction was carried out for 1 h at room temperature in mild shaking. The activated resin was thoroughly washed to remove the unreacted CDI. The resin was reacted with (mono)Boc-diaminoethane (Boc-DAE). A DMF solution (1.5 mL) of 150 mg of Boc-DAE was added to the resin. The mixture was shaken overnight at 45°C and then the resin was washed with DMF. The resin was then split into two batches, (a) and (b). Acetylation was carried out only on batch (a) with acetic anhydride – DIPEA (1:1, 2 mL, 3 x 30 min), while batch (b) was kept non acetylated. Boc deprotection, Fmoc-tryptophan coupling, estimation of amino acid densities of resins (a) and (b) and NaOH stability testing were performed according to same procedure as described in Section 6.2.5.

6.2.8. Synthesis of HWRGWV on modified Toyopearl AF-Amino-650M and CIP test

The ligand HWRGWV was synthesized at the density of 0.11 meq/g on modified Toyopearl AF-Amino-650M resin via conventional Fmoc coupling chemistry. The Toyopearl AF-Amino-650M resin was modified according to procedure described in Section 6.2.5. This peptide resin was packed in a column and utilized for repeated cycles of IgG purification as described in Section 6.2.2.1., each followed by an alkaline cleaning at the flow rate of 0.1 mL/min (174 cm/h) for 10 min. The column was regenerated for 200 cycles with aqueous 0.1M NaOH and for 100 and 50 cycles with aqueous 0.5M NaOH and 1M NaOH respectively.

6.2.9. Sample analysis for yields and purities

The amount of IgG in the collected fractions was quantified by HPLC using a 1-mL HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, as described by Yang *et al.* [8]., using NuPAGE® Novex 4-12% Bis-Tris gels in a Xcell SuperLock™ Mini-Cell system (Invitrogen, CA, USA). Sample preparation was done by adding 5 mL of NuPAGE® LDS buffer and 2 mL of NuPAGE® reducing agent to 13 mL of sample and boiling the resulting mixture for 10 min. Gels were Coomassie-stained by using SimpleBlue™ SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institute of Health,

MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 KDa.

6.3. Results

6.3.1. Stability of commercial HWRGWV-Toyopearl resin to 0.1M NaOH

The peptide resin HWRGWV-Toyopearl produced by direct on-resin peptide synthesis via conventional chemistry was used to perform five cycles of purification of IgG from cMEM. Regeneration between the first and second cycle was performed with 0.85%v/v phosphoric acid, as described in previous work [21], while 0.1M NaOH was used in the following four cycles. The flow-through and elution fractions of the five cycles were collected and analyzed by Protein G chromatography and SDS-PAGE to determine product yield and purity. Results are reported in Table 6.1. The yield and purity of IgG from the first and second cycle were almost equal, being 85% and 91% respectively. However, after the first NaOH wash the resin exhibited a sharp decrease in IgG binding capacity. The alkaline wash caused a large ligand loss, which produced the very high peak observed during the NaOH regeneration, as reported in Figure 6.1A (2nd cycle). The product yield showed a 4-fold reduction, from 85% to 23% (Table 6.1). This is also indicated by the SDS-PAGE reported in Figure 6.1C, which shows that a considerable amount of IgG was found in the flow-through after the first alkaline wash (lane 4), and by the evident increase of the flow-through peak in the chromatograms reported in figure 6.1B. Unlike yield, however, an almost negligible decrease in IgG purity was detected (from 91% to 90%). Furthermore, the alkaline

washes that followed did not further affect the resin performance, as both yield and purity remained constant over the following cycles, respectively at the values of 21% and 90% (Table 6.1). This indicates that upon alkaline wash, the adsorbent loses only binding capacity but not selectivity towards IgG.

In order to determine the extent of ligand loss, three consecutive washing steps with 0.1M NaOH at the flow rate of 0.1 mL/min for 10 min were performed on a column packed with fresh resin, each followed by a rinse with PBS pH 7.4. The alkaline washes were collected and analysed by C18 HPLC to determine the mass of ligand leached from the resin. Results are reported in Table 6.2. While the first alkaline wash resulted in a 43% loss of ligand, following washes were found to remove only small amounts of peptide. This result agrees with the previous findings, indicating that the loss of resin performance is mainly caused by a loss of ligand resulting from the first alkaline wash. To the best of our knowledge, such drastic ligand leaching in alkaline conditions has not been reported for affinity adsorbents produced by direct peptide synthesis on chromatographic resin.

6.3.2. Stability of the ligand in basic solution

The ligand loss in alkaline conditions reported above is either due to the chemical instability of the ligand itself or to the lability of the bond between the ligand and the resin. By exposing the peptide to different NaOH concentrations for different time intervals, it was possible to determine the stability of the ligand in alkaline conditions, as described in Section 6.2.4. Figure 6.2 reports the C18 HPLC analysis of HWRGWV exposed to different NaOH

concentrations *viz.* 0.1 M, 0.5 M and 1 M over 1 h (Figure 6.2A) and 2 h (Figure 6.2B). The results indicate that NaOH has no effect on the ligand even at concentrations up to 1 M and exposure time up to 2 h, thus suggesting that the peptide itself is chemically stable. Similar results were obtained by the MALDI-TOF MS analysis of the samples (data not shown). It is hence concluded that the loss of resin performance is not due to peptide degradation but rather to the cleavage of the whole ligand from the resin surface. This is also consistent with the previous finding that the alkaline wash does not lower the product purity, but only the recovery.

6.3.3. Determination of the number of amide and ester bonds

The results presented in Sections 6.3.1 and 6.3.2 indicate that a considerable number of peptides are coupled to the resin via alkaline-labile bonds. This is due to the nature of the functional groups present on the resin and to the mechanism of peptide synthesis. During on-resin peptide synthesis the carboxyl terminus of the N-protected incoming amino acid is activated in solution and reacts with the nucleophilic groups located on the resin surface (Step 1) [25]. Toyopearl AF-Amino-650M resin, as well as any other amino resin used for chromatography, bears two kinds of nucleophilic groups, *viz.* amino and hydroxyl [26]. The activated carboxyl terminus of the first amino acid binds partly to the amino and partly to the hydroxyl groups, respectively through amide and ester bonds (Step 2). The synthesis proceeds with the subsequent amino acids thus leading to two populations of peptides, one coupled via amide bonds and the other via ester bonds. The latter, however, are readily

hydrolysed in alkaline conditions and are responsible for the ligand leaching during the NaOH wash (Step 3). This mechanism is shown in Figure 6.3.

It was therefore decided to estimate the relative number of amide and ester bonds formed during solid phase peptide synthesis. Because hydroxyl groups are less reactive than amine groups in these coupling chemistries, it was suspected that the density of ester bonds per gram of resin would be lower than the density of amide bonds per gram of resins. To determine this, Fmoc-protected tryptophan was coupled to Toyopearl AF-Amino-650M and Toyopearl HW-65F resins. The first resin bears both amino and hydroxyl groups, while the second resin bears only hydroxyl groups. Therefore, tryptophan was coupled on Toyopearl AF-Amino-650M via both amide and ester bonds, and it was coupled on Toyopearl HW-65F only via ester bonds. The amino acid density on these resins, measured by Fmoc quantification, was found to be 0.44 meq/g and 0.17 meq/g respectively (Table 6.3). The difference (0.27 meq/g) was considered to represent the density of amino acid coupled through amide bonds. Consequently, it was estimated that ester bonds make up 38.6% of the total.

The two resin batches were then subjected to alkaline wash (with 0.1 M NaOH) and the amount of tryptophan leached was determined by spectrophotometric analysis of the alkaline flow-through fractions at 280 nm, as described in Section 6.2.5. Results are reported in Table 6.4. The fraction of the coupled tryptophan that was leached by 0.1M NaOH (38.2%) was hence regarded as the amount of amino acid coupled by ester bonds. Results reported in Table 6.3 and Table 6.4 indicate that approximately 40% of the amino acids (or

peptides) are coupled to the resin by ester bonds. This remarkably high amount of ester bonds is due to the strength of the coupling protocol, which involves repeated reactions with high excess of amino acid and coupling agent. These conditions are however necessary to provide the adsorbent with a ligand density sufficient to guarantee an appropriate binding capacity. Based on these results, it was concluded that the hydroxyl groups needed to be capped prior to peptide synthesis in order to have the ligand coupled exclusively by amide bonds. Furthermore, the capping is to be reversible, in order to restore the resin hydrophilicity after the synthesis is completed. The protocols developed for these peptide synthesis reactions are provided in the Sections that follow.

6.3.4. Base-stable peptide synthesis on resins containing hydroxyl and amine groups

The solution for synthesizing alkaline stable peptide adsorbents consists in modifying the resin surface chemistry by (i) activating the base matrix, (ii) selectively blocking the hydroxyl groups and (iii) performing the peptide synthesis exclusively on amino groups through alkaline-stable amide bonds. The method counts five steps (Figure 6.4):

1. Introduction of Boc-protected amino groups: Boc-alanine is coupled to Toyopearl AF-Amino-650M resin, partly by amide and partly by ester bonds. The latter are then hydrolysed by means of an alkaline wash (0.5 M NaOH), which leaves on the resin only the Boc-alanine coupled through amide bonds.

In alternative, base Toyopearl HW-65F resin, which bears only hydroxyl groups, is activated (e.g. with carbonyl diimidazole, tosyl chloride or epichlorohydrin) and reacted with Boc-diaminoethane.

2. Capping of hydroxyl groups. The hydroxyl groups are capped by acylation.
3. Deprotection of amino groups. The Boc protection is removed by the appropriate cleaving solution and the amino groups are free-based. The resin thus bears free amino groups and acylated hydroxyl groups.
4. Peptide synthesis. The peptide is synthesised exclusively on amino groups.
5. Decapping of hydroxyl groups. After the peptide synthesis is completed, a second alkaline wash (0.5 M NaOH) is performed to remove the acylation from the hydroxyl groups and clean the resin.

The presented strategy exhibits some flexibility. The protocol does not require Boc-Alanine specifically, as it can be carried out with any other similar molecule. Alanine was chosen because it is usually employed as a spacer amino acid on which the peptides are synthesized. Also, α -amino-protecting groups other than Boc can be used, such as Z (benzyloxycarbonyl), provided that they are base stable. Boc was chosen for this study because it is readily available and easily removable in acid conditions. Finally, the acylation of hydroxyl groups can be performed with a variety of agents, such as acetic anhydride, acetyl chloride or benzyl chloride.

By coupling a single amino acid (tryptophan) it was possible to test the new surface chemistry protocol. Three batches were prepared on Toyopearl AF-Amino-650M resin.

Batch (a) was prepared by following the whole synthetic procedure, while batches (b) and (c) were controls. Sample (b) was not acetylated before the Boc removal, while sample (c) was prepared by direct coupling on the unmodified amino resin. The amino acid density was measured by Fmoc quantitation. Batch (a) had a density of 0.25 meq/g, while resins (b) and (c) had densities of 0.42 meq/g and 0.44 meq/g, respectively. Alkaline washes with 0.1 M NaOH were performed on-column on the three batches of resin. As can be seen from the chromatograms (Figure 6.5), a negligible amount of tryptophan was leached from batch (a), while non-acetylated batch (b) and unmodified batch (c) showed an equally large amino acid leakage. These results indicate that the method presented is effective in achieving the resin stability toward alkaline CIP treatments and that the capping of hydroxyl groups before amino acid coupling is a critical step. This was also confirmed by the values of amino acid density of the three resins. Batch (a) had 40% lower amino acid density than Batch (b) and Batch (c). This correlated well with results presented in Section 6.3.3 where the amount of ester bonds formed was estimated to be approximately 40% of the total.

6.3.5. Base-stable peptide synthesis on resins containing only hydroxyl groups

An alternative protocol of surface chemistry modification was performed on a base Toyopearl HW-65F resin, which bears only hydroxyl groups. After activation by CDI chemistry, the resin was reacted with Boc-diaminoethane to introduce protected amino groups on the resin (Figure 6.4, step 1/b). As before, two batches of resin were prepared, (a) and (b), the latter being unacetylated before the Boc removal. The chromatographic results

(Figure 6.6) of the 0.1 M NaOH wash performed on the two resins are similar to those presented in Section 6.3.4, proving that, as far as alkaline stability is concerned, the protocols are equally effective.

6.3.6. Testing alkaline stability and performance of HWRGWV synthesized on modified Toyopearl AF-Amino-650M

HWRGWV was synthesized via Fmoc-coupling chemistry on Toyopearl AF-Amino 650M resin modified according to procedure described in Section 6.3.4. The peptide adsorbent was tested for its alkaline stability by performing repeated cycles of IgG purification from cMEM, each followed by an alkaline cleaning with aqueous NaOH. Two hundred cycles were performed employing 0.1 M NaOH, 100 cycles employing 0.5 M NaOH and 50 cycles employing 1 M NaOH. Chromatograms and SDS-PAGE of the collected fractions are reported in Figure 6.7 – 6.9, while the IgG yield and purity are reported in Tables 6.5 – 6.7.

These results indicate that the affinity adsorbent can be reused for a high number of cycles without resulting in a critical loss of binding capacity. In fact, even in the harsh regenerating conditions of 1M NaOH, the IgG yield decreased from 88% to 76%. This performance of the peptide-based affinity adsorbent is better than MabSelect Xtra and Protein A Sepharose 4 Fast Flow (GE Healthcare) [10], and comparable to that reported for alkaline stable version of Protein A, MabSelect SuRe (GE Healthcare) [24], under similar conditions of NaOH concentration (0.5M) and contact time (10 min). Because of the stability

of the peptide ligand under the cleaning conditions, it can be concluded that the decrease of IgG yield observed with the peptide resin is likely the result of a gradual deterioration of the polymethacrylate resin over a large number of cleaning cycles in alkaline conditions [27].

6.4. Conclusions

Providing adsorbents characterized by long lifetime reduces the manufacturing costs and ultimately the product price. A method of surface chemistry modification of chromatographic resins was presented for the synthesis of alkaline stable peptide-based affinity adsorbents. A case study based on an IgG affinity adsorbent-HWRGWV-Toyopearl resin demonstrated that the adsorbent can be cleaned with aqueous NaOH at different concentrations (0.1 M, 0.5 M and 1 M) and reused for a large number of purification cycles. The performance of the peptide-based affinity resin was found to be comparable to that reported for alkaline stable Protein A – based MabSelect SuRe resin. In particular, product purity remained almost unaltered throughout the purification cycles.

The method applies to the preparation of affinity adsorbents by on-resin peptide synthesis via conventional Fmoc/tBoc chemistry. This approach is efficient, economical and secures the successful development of the peptide-based adsorbent, as the ligand is identified within a library that is synthesised on the same matrix as used for the chromatographic purification.

Furthermore, the proposed technique consists in only one additional step before the peptide synthesis. It is a very efficient and easily controlled reaction, inexpensive and does

not require any additional equipment. It does not affect the properties of the resin for affinity chromatography and also does not influence the performance of the peptide ligand. Also this technique is universal, being valid for any peptide sequence identified for the purification of a desired biopharmaceutical target.

6.5. References

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Table 6.1. IgG yield and purity of the four cycles of purification using HWRGWV-Toyopearl resin with intermediate NaOH washes.

Cycle	IgG yield	IgG purity
1	85%	92%
2	85%	91%
3	23%	90%
4	21%	90%
5	21%	90%

Table 6.2. Extent of ligand leaching with increasing number of 0.1M NaOH washes.

NaOH wash	Mass of leached HWRGWV (mg)	Density of HWRGWV on the resin (meq/g)	% Overall Ligand loss
0	---	0.15	---
1	1.88	0.086	43%
2	0.088	0.083	45%
3	0.059	0.081	46%

Table 6.3. Determination of the amount of tryptophan coupled through ester bond via Fmoc quantification.

Resin	Density of Trp on the resin (meq/g)	% of Trp coupled by ester bonds
Toyopearl AF-Amino-650M	0.44	38.6%
Toyopearl HW-65F	0.17	100%

Table 6.4. Determination of the amount of tryptophan coupled through ester bond via quantification of amino acid leaching.

Resin	Density of Trp on the resin (meq/g)	Mass of Trp on the resin* (mg)	Mass of Trp leached (mg)	% of Trp coupled by ester bonds
Toyopearl AF-Amino-650M	0.44	3.145	1.201	38.2%
Toyopearl HW-65F	0.17	1.215	1.210	99.6%

* Referred to the amount of resin packed in a column (35mg).

Table 6.5. IgG yield and purity from different purification cycles using HWRGWV synthesized on modified Toyopearl resin. Cleaning was performed with aqueous 0.1 M NaOH.

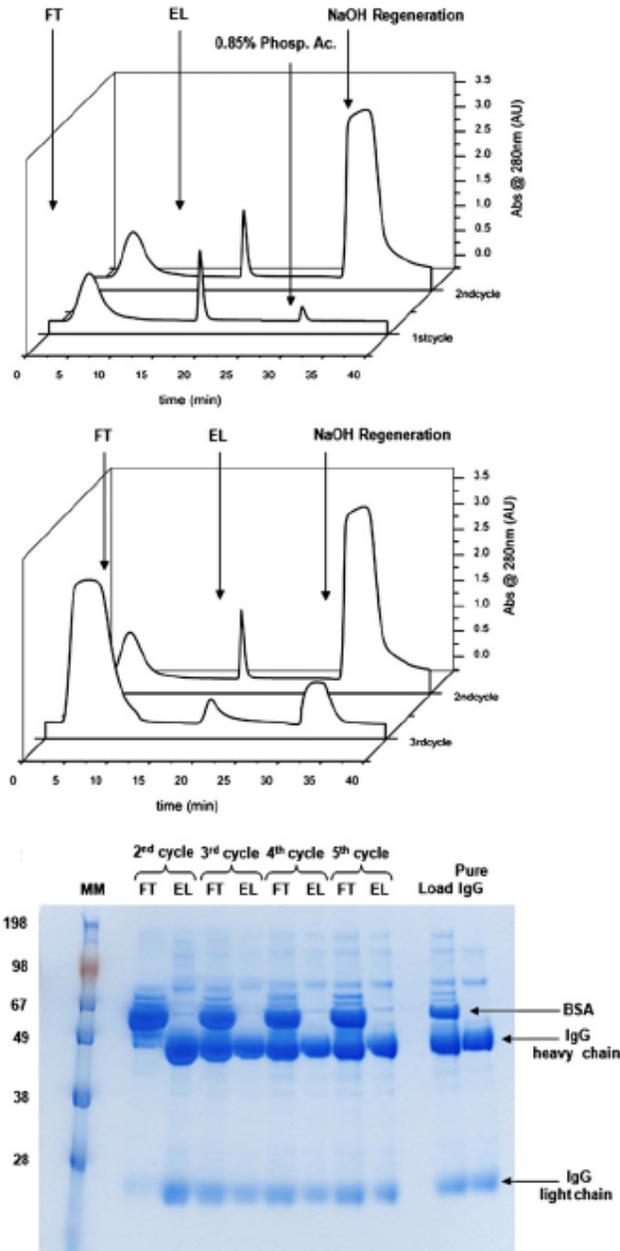
Cycle # (0.1 M NaOH wash #)	IgG yield	IgG purity
1 (0)	91 %	96 %
25 (24)	90 %	95 %
50 (49)	88 %	94 %
100 (99)	87 %	94 %
200 (199)	85 %	93 %

Table 6.6. IgG yield and purity from different purification cycles using HWRGWV synthesized on modified Toyopearl resin. Cleaning was performed with aqueous 0.5 M NaOH.

Cycle # (0.5 M NaOH wash #)	IgG yield	IgG purity
1 (0)	89 %	98 %
25 (24)	87 %	97 %
50 (49)	85 %	96 %
75 (74)	82 %	95 %
100 (99)	80 %	95 %

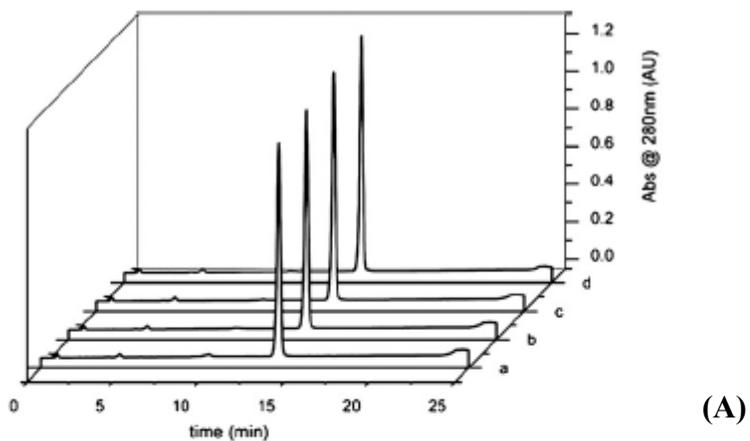
Table 6.7. IgG yield and purity from different purification cycles using HWRGWV synthesized on modified Toyopearl resin. Cleaning was performed with aqueous 1 M NaOH.

Cycle # (1 M NaOH wash #)	IgG yield	IgG purity
1 (0)	88 %	96 %
10 (9)	84 %	95 %
20 (19)	82 %	93 %
30 (29)	79 %	93 %
50 (49)	76 %	94 %

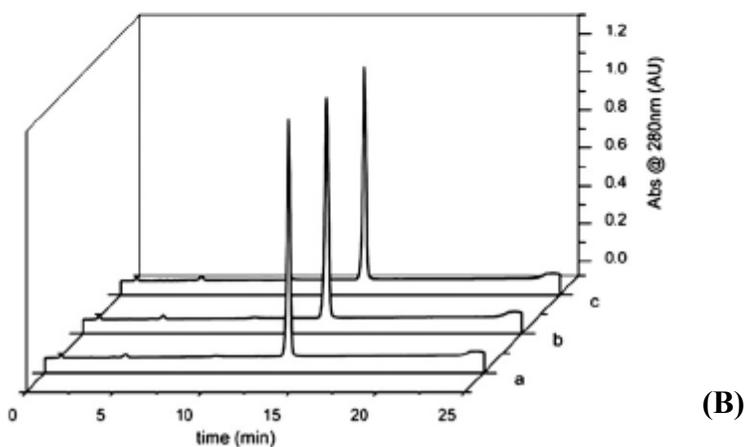


(C)

Figure 6.1. (A) Chromatograms of first two cycles of IgG purification from cMEM using HWRGWV-Toyopearl resin. The resin was regenerated with 0.85% phosphoric acid in the first cycle and with 0.1M NaOH in the second cycle. (B) Chromatograms of second and third cycle of IgG purification from cMEM using HWRGWV-Toyopearl resin with intermediate NaOH wash. (C) SDS-PAGE (reducing conditions) of flow-through and eluted fractions of IgG purification cycles using HWRGWV-Toyopearl resin with intermediate NaOH wash. Labels: MM-molecular weight marker, FT- flow-through fraction, EL-elution fraction.



(A)



(B)

Figure 6.2. (A) C18 HPLC analysis of stability of pure peptide exposed to different concentrations of NaOH for 1 hour: a) 0.1 M NaOH, b) 0.5 M NaOH, c) 1 M NaOH, d) 0 M NaOH (control). (B) C18 HPLC analysis of stability of pure peptide exposed to different concentrations of NaOH for 2 hour: a) 0.5 M NaOH, b) 1 M NaOH, c) 0 M NaOH (control).

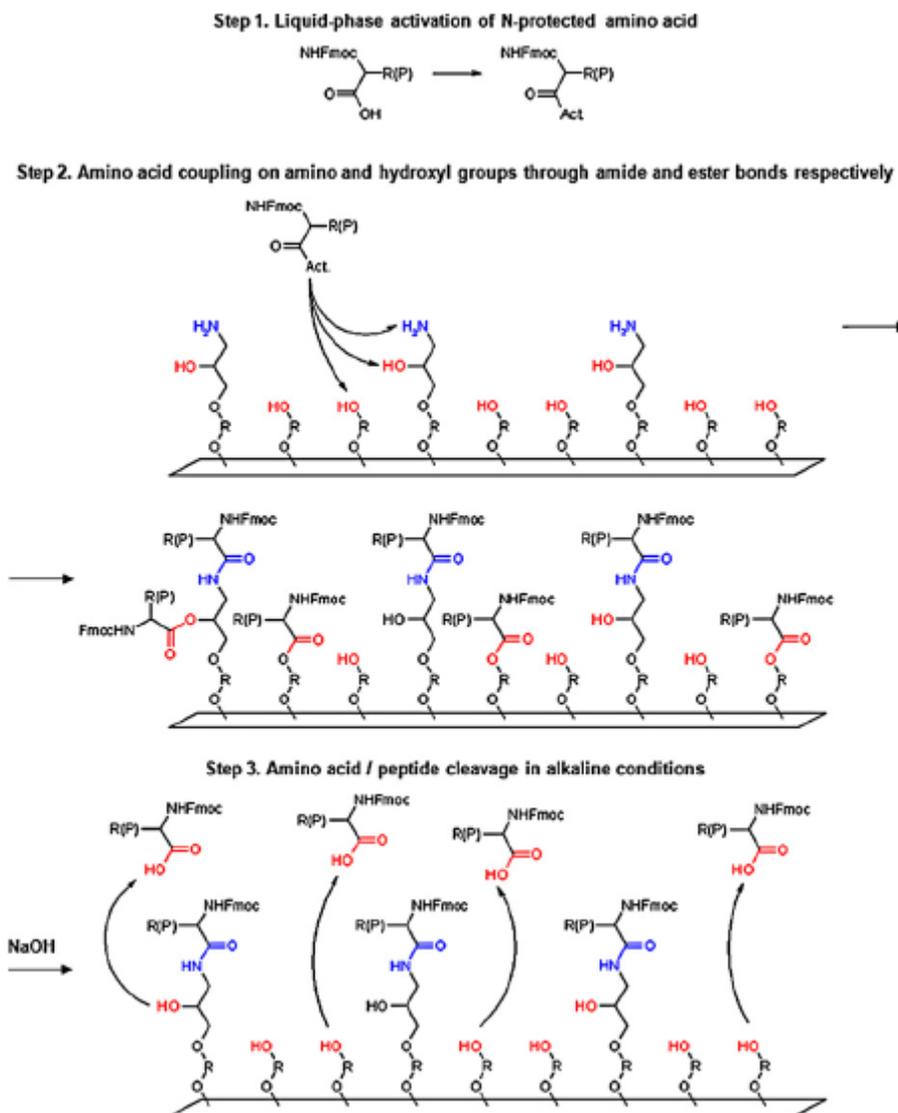


Figure 6.3. Mechanism of peptide synthesis on Toyopearl AF-Amino-650M resins and ligand leaching in alkaline conditions.

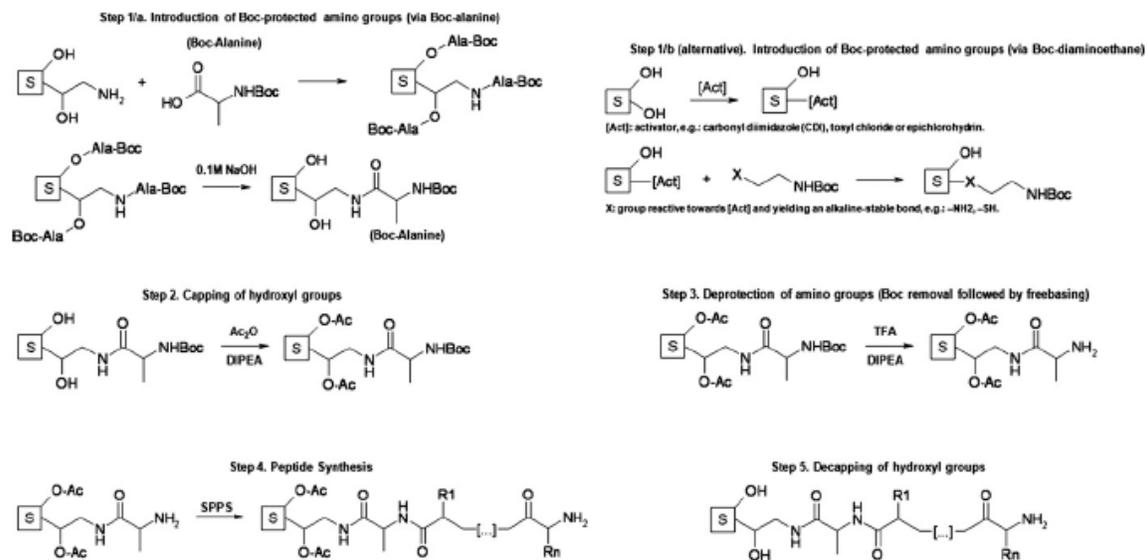


Figure 6.4. Protocol for blocking hydroxyl groups to prevent ester bond formation.

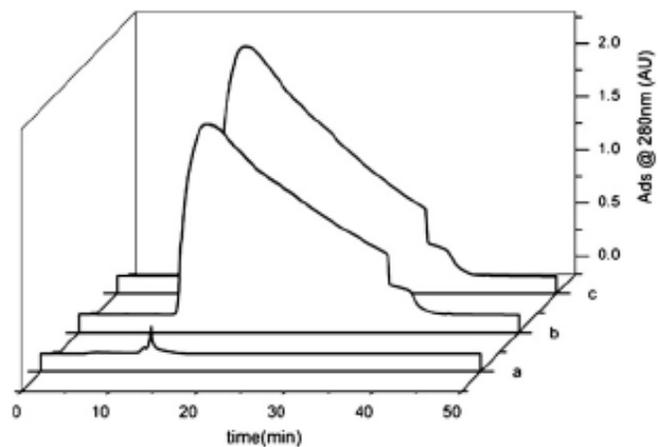


Figure 6.5. Chromatograms of 0.1 M NaOH wash of tryptophan-coupled resin batches: (a) modified Toyopearl AF-Amino-650M; (b) modified but non-acetylated; (c) unmodified Toyopearl amino resin.

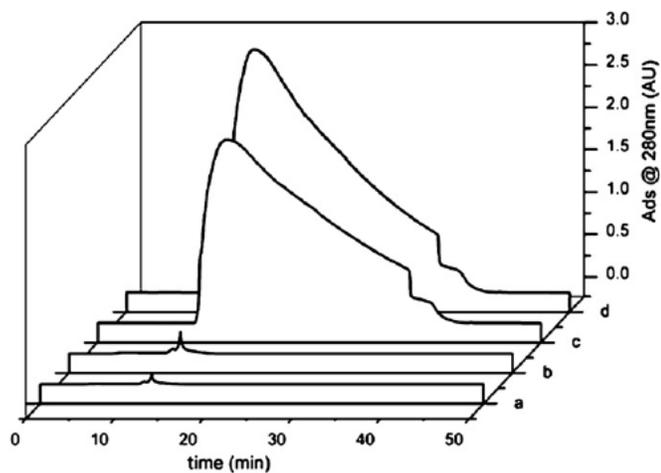


Figure 6.6. Chromatograms of 0.1 M NaOH wash of tryptophan coupled resins : (a) modified Toyopearl HW-65F (batch a as per Section 6.3.5); (b) modified Toyopearl AF-Amino-650M (as per Section 6.3.4); (c) unmodified Toyopearl AF-Amino-650M resin (as per Section 6.3.4); (d) modified Toyopearl HW-65F without acetylation (batch b as per Section 6.3.5).

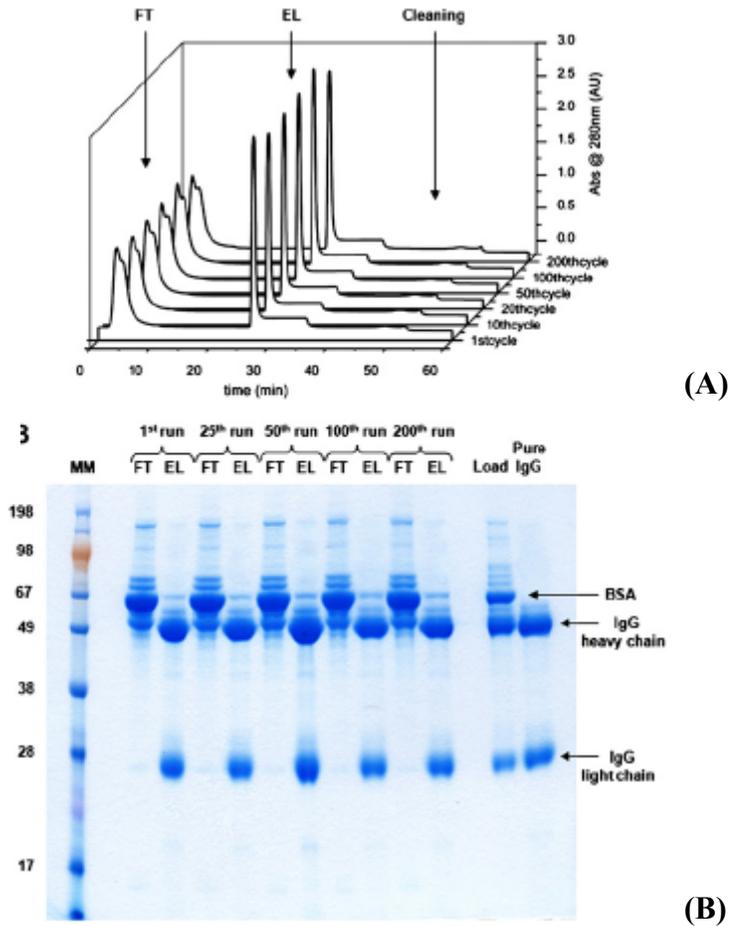


Figure 6.7. (A) Chromatogram of IgG purification runs using HWRGWV-Toyopearl resin with intermediate aqueous 0.1 M NaOH cleanings. (B) SDS-PAGE (reducing conditions) of flow-through and eluted fractions of IgG purification from cMEM at 1st, 25th, 50th, 100th and 200th cycles. Labels: MM-molecular weight marker, FT- flow-through fraction, EL-elution fraction.

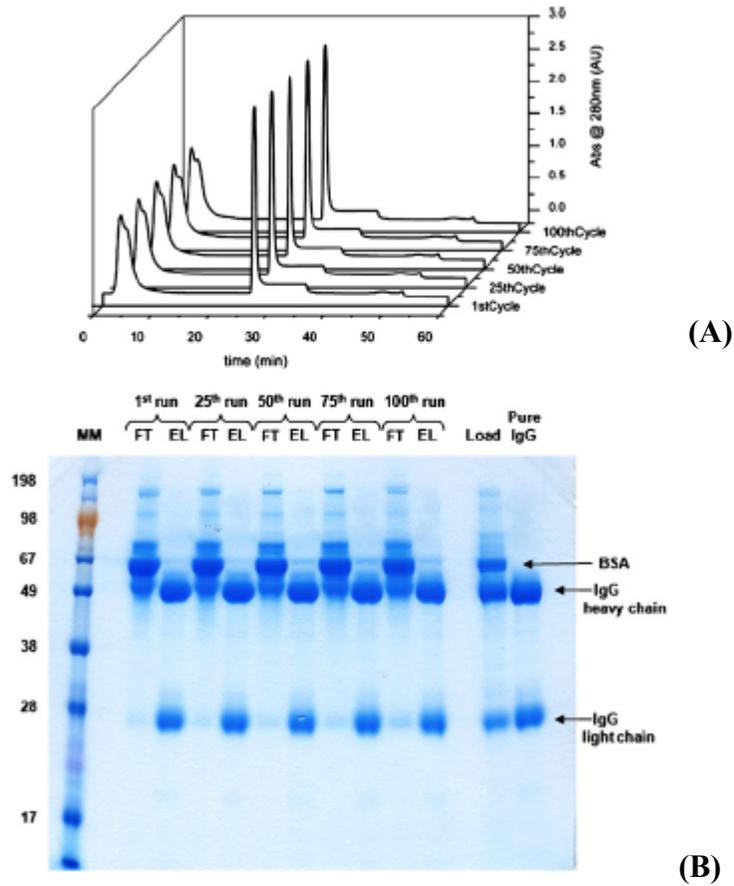
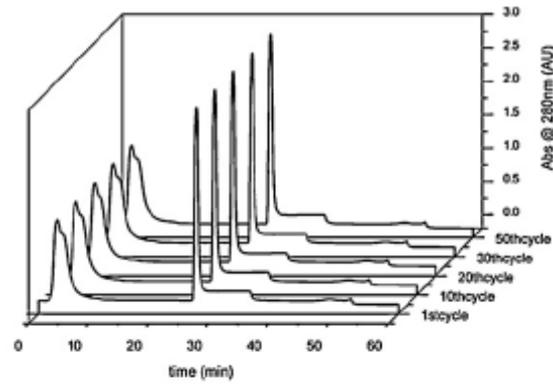
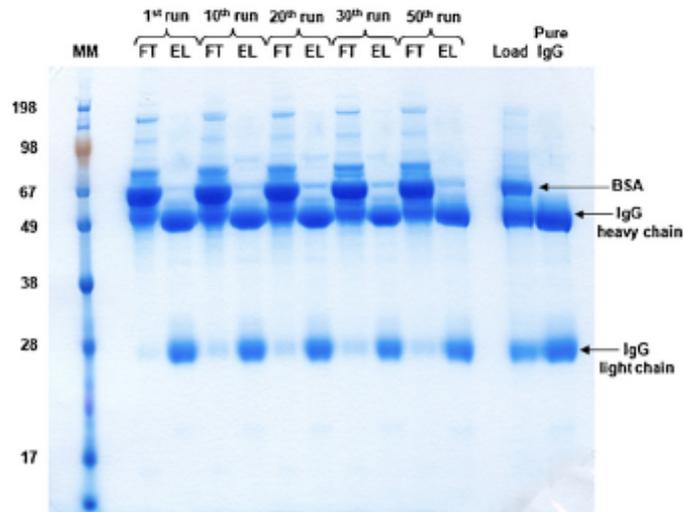


Figure 6.8. (A) Chromatogram of IgG purification runs using HWRGWV-Toyopearl resin with intermediate aqueous 0.5 M NaOH cleanings. (B) SDS-PAGE (reducing conditions) of flow-through and eluted fractions of IgG purification from cMEM at 1st, 25th, 50th, 75th and 100th cycles. Labels: MM-molecular weight marker, FT- flow-through fraction, EL-elution fraction.



(A)



(B)

Figure 6.9. (A) Chromatogram of IgG purification runs using HWRGWV-Toyopearl resin with intermediate aqueous 1 M NaOH cleanings. (B) SDS-PAGE (reducing conditions) of flow-through and eluted fractions of IgG purification from cMEM at 1st, 10th, 20th, 30th and 50th cycles. Labels: MM-molecular weight marker, FT- flow-through fraction, EL-elution fraction.

**Chapter 7. Protease-resistant hexapeptide ligands for the purification
of antibodies from animal plasma**

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*A version of this chapter has been prepared for submission to
Pharmaceutical Bioprocessing*

Abstract

A strategy is presented for developing variants of peptide ligands with enhanced biochemical stability for purifying antibodies from animal sera. Antibody binding peptides HWRGWV, HYFKFD, and HFRRHL, previously discovered by our group, were modified with non-natural amino acids to gain resistance to proteolysis, while maintaining target affinity and selectivity. As trypsin and α -chymotrypsin were chosen as models of plasma proteolytic enzymes, the basic (arginine and lysine) and aromatic (tryptophan, phenylalanine, and tyrosine) amino acids were replaced with non-natural analogs. Using the docking software HADDOCK, a virtual library of peptide variants was designed and screened *in-silico* against the known HWRGWV binding site on the pFc fragment of IgG (Ser 383 – Asn 389). A pool of selected sequences that exhibited the largest computed free energy of binding were synthesized on chromatographic resin and the resulting adsorbents were tested for IgG binding and resistance to proteases. The ligand variants exhibited binding capacities and specificities comparable to the original sequences, but had much higher proteolytic resistances. The sequences HW_{Met}CitGW_{Met}V and HF_{Met}CitCitHL were used for purifying polyclonal IgG from IgG-rich fractions of human plasma, with yields and purity above 90%. The method proposed herein represents a general strategy for increasing the biochemical stability as well as the affinity and selectivity of any natural or synthetic peptide ligand for bioseparations or biotechnological applications.

Keywords: Peptide affinity ligands; Polyclonal antibodies; Affinity chromatography; Proteolysis; protease; Molecular docking.

7.1. Introduction

The purification of immunoglobulins from mammalian sera for therapeutic and research purposes is an issue of considerable relevance in biotechnology and biomanufacturing [1,2]. Plasma-derived polyclonal intravenous immunoglobulin (IVIG) preparations have been successfully applied to the prophylactic prevention of infectious diseases in immunodeficient patients and find increasing use against autoimmune and inflammatory disorders [3,4]. To date, IVIG is the major plasma product on the global blood product market, with a steadily increasing annual consumption [5]. Further, polyclonal antibodies derived from the serum of immunized animals are also currently employed in medical research for developing immunoassays, therapeutic treatments and new strategies of drug delivery [6-9]. Serum can also be a source of monoclonal antibodies, as is the case in hybridoma technology, which, although quite dated, is still a powerful research tool for the development of monoclonal antibodies [10]. As hybridoma colonies are grown in culture media mainly with high concentrations of bovine serum, the purification of monoclonal antibodies from these fluids resembles in fact the recovery of polyclonal antibodies from animal sources [11]. Protein A and Protein G, the most commonly used affinity ligands for antibody purification, are not well suited for this type of antibody purification [12,13]. Besides the known issues of high cost, low chemical stability, immunogenicity, and harsh elution conditions caused by the low dissociation constant ($\sim 10^{-8}$ M), there are some additional concerns [14,15]. Protein A does not bind human IgG₃ subclass, shows weak binding of mouse IgG₁ and bovine IgG₁, and does not bind goat and mouse IgG or subclasses of chicken IgY [16]. Protein G, while binding all human IgG subclasses and the majority of

animal antibodies, also captures albumin, by far the major protein constituent in plasma and serum, and hence it is not normally used for antibody purification from plasma [17]. Engineered forms of Protein G without the albumin binding site have been developed [18], but they are very costly and the issues of stability and immunogenicity remain a concern. To overcome these issues, synthetic ligands have been developed for antibody purification, which are more affordable and chemically robust, less toxic and less immunogenic when compared to protein ligands [19,20]. Our research group has identified three peptide ligands, HWRGWV, HYFKFD and HFRRHL, which bind IgG through the Fc portion, thus mimicking the binding mechanism of Protein A [21-23]. These sequences bind all human antibody subclasses as well as many animal (bovine, mouse, rabbit goat, llama, and avian) antibodies, and have been used for the purification of monoclonal and polyclonal antibodies from a variety of sources, including Cohn fraction II+III of human plasma [24-26]. In all these studies, product yield and purity were always found to be comparable to those given by Protein A. Yet, owing to their milder binding strength ($K_D \sim 10^{-5} - 10^{-6}$ M), they allow antibody elution from affinity columns under gentler conditions (pH 4.0 – 5.0), thus preventing aggregation and maintaining activity. Much work has also been carried out to increase the chemical stability and dynamic binding capacity (DBC) of these peptide ligand adsorbents. As a result of these optimization studies, the HWRGWV-Toyopearl adsorbent showed high resistance to 0.5M NaOH over continuous cycles of use and DBC values in the range of 50 g/L [27,28]. These ligands could hence enable the development of industrial scale affinity purification of monoclonal and polyclonal antibodies from serum and plasma.

A problem that both protein and synthetic peptide ligands face when used for the

purification of polyclonal antibodies from animal plasma is the presence of proteolytic enzymes, such as trypsin and α -chymotrypsin [29,30]. These proteases cleave peptide chains at the carboxyl end of basic (arginine and lysine) and aromatic (tryptophan, phenylalanine, and tyrosine) amino acids respectively [31,32]. Upon prolonged exposure of the affinity adsorbent to serum, trypsin and α -chymotrypsin cause substantial degradation of protein or peptide ligands with consequent loss of binding capacity. For protein ligands, like Protein A / G, this problem is aggravated by the release of immunogenic fragments in the product mainstream. As a preventive measure, protease enzyme inhibitors are often added to the feed mixture before injection [33]. These inhibitors, however, are costly and need to be removed from the final product.

A radical solution to these issues is to produce variants of peptide ligands comprising non-natural amino acids. These variants are expected to combine good target affinity and selectivity with high resistance against proteases. Verdoliva *et al.* have proposed the synthesis of a peptide ligand using D-stereoisomers of amino acids, which, unlike the naturally occurring L-forms, are not recognized and attacked by proteases [34,35]. D-amino acids, however, are very costly and are prone to other kinds of chemical degradation, such as those caused on the amino acid functional groups by acid and alkaline solutions used for protein elution and resin sanitization respectively [36-39]. To overcome these obstacles, chemically modified forms of L-amino acids can be employed instead of D-amino acids to produce peptide variants that, while retaining the target affinity and selectivity of the original sequences, exhibit high enzymatic resistance and chemical stability. To this end, a method is herein presented for the design and identification of these ligands which comprises three

steps: 1) design of a virtual library of variants of known peptide ligands using non-natural amino acids, 2) library screening *in-silico* against the target biomolecule by molecular docking simulations, 3) synthesis of the selected variants on chromatographic resins and testing of the resulting adsorbents for target binding and resistance to proteases. Tryptophan, phenylalanine, tyrosine, and lysine in the antibody binding peptides HWRGWV, HYFKFD, and HFRRHL were replaced with alkylated analogs, while citrulline was used in place of arginine. Other variants were created using N_{in} -formyl-tryptophan and 4-carbamoyl-phenylalanine, as well as by replacing glycine with aspartic acid in HWRGWV. The library was then screened against the known HWRGWV binding site in the pFc region of IgG (Ser383 – Asn389) using the docking software HADDOCK (version 2.1) [40,41]. This program simulates protein-peptide interaction and through external software estimates the free energy of binding based on the evaluation of van der Waals interactions, hydrogen bonding, deformation penalty, hydrophobic effects, atomic contact energy, softened van der Waals interactions, partial electrostatic, additional estimation of the binding free energy, dipole-dipole interactions, and the presence of water [40-45]. The selected sequences were synthesized directly on the polymethacrylate-based chromatographic resin Toyopearl AF-Amino-650M and tested for IgG binding and resistance against trypsin and α -chymotrypsin. The sequences $HW_{Met}CitGW_{Met}V$, $HF_{Met}CitCitHL$, and $HY_{Met}F_{Met}K_{(Met)2}F_{Met}D$ were chosen for purifying polyclonal antibodies (IVIG) from Cohn fraction II + III of human plasma. Finally, a study on the effect of conductivity of the binding buffer on IgG yield and purity was performed to compare the binding mechanism of the parental peptide HWRGWV and its variants $HW_{Met}CitGW_{Met}V$ and $Ac-HW_{Met}CitGW_{Met}V$. The latter was shown to attain higher

IVIG yield and purity than HWRGWV at lower conductivity of the binding buffer, thereby offering significant cost reduction for large scale downstream process.

7.2. Experimental

7.2.1. Materials

Protected amino acids and coupling agents for peptide synthesis were purchased from ChemImpex Inc. (Wood Dale, IL, USA). Diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triisopropylsilane (TIPS), ethanedithiol (EDT), phosphate buffer saline (PBS) pH 7.4, Cohn fraction II + III and Kaiser test kit were from Sigma Aldrich (Saint Louis, MO, USA). N,N'-dimethylformamide (DMF), dichloromethane (DCM), sodium acetate, sodium chloride, acetic acid glacial, 85%v/v phosphoric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Toyopearl AF-Amino-650M resins were a kind gift from Tosoh Bioscience (King of Prussia, PA, USA). Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech Bio, Inc. (Kernville, TX, USA). NuPAGE[®] Novex gels (4–12% Bis-Tris), NuPAGE[®] MOPS running buffer, NuPAGE[®] LDS sample buffer, NuPAGE[®] reducing agent, SeeBlueplus2[®] pre-stained molecular weight marker, SimpleBlue[™] SafeStain were all from Life Sciences (Carlsbad, CA, USA). The HiTrap[™] Protein G column was purchased from GE Healthcare (Piscataway, NJ, USA). A Waters 626 LC system integrated with 2487 UV detectors (Waters, MA, USA) was used for all chromatographic runs. Microbore stainless steel columns 30 mm long x 2.1 mm I.D were purchased from Alltech-Applied Science (Somerset, PA, USA). All chromatographic runs

were carried out at room temperature.

7.2.2. Molecular modeling

The coordinate files for the peptide variants were generated using the open source graphic chemical structure visualization program PyMOL (The PyMOL Molecular Graphic System, Version 1.2r3pre, Schrodinger, LLC.). Parameter and topology files for the modified amino acids were determined by observing the closest matching natural residues and copying those qualities to design the non-natural amino acid entry. For example, N_{in}-methylated and formylated tryptophan structures were designed based on the parameter and topology files for the standard tryptophan residue. 4-Methyl-, 4-methoxy-, and carbamoyl- phenylalanine were designed based on the parameter and topology files for the standard phenylalanine residue; in particular the carbamoyl functionality was obtained from asparagine. Citrulline was modeled based upon arginine's delta carbon and asparagine. Files for methylated and dimethylated lysine, Lys(Me) and Lys(Me)₂, were already coded in HADDOCK [40]. The partial charge on a single atom was assigned so as to maintain electrical neutrality on the functional groups, identically to all other parameter and topology files for natural amino acids in HADDOCK. Parameter and topology file of the amino acid modifications were checked against submissions to the PRODRG server, prescribed as a standard verification process (davape1.bioch.dundee.ac.uk/prodgr). The coordinate file for hIgG was obtained from the RCSB Protein Data Bank (PDB, 1FCC) [46]. The residues Ser383-Asn389 (SNGQPEN) on hIgG were defined as “active” and used as target for ligand docking. Protein A was included

in the target PDB file as a global restraint to prevent interaction between the peptide ligands and the Protein A binding site (residues 341-443) on hIgG. Molecular modeling was performed using the program HADDOCK (version 2.1) [40,41]. For each peptide variant, residues 1-2 were targeted to residues 389-387 of hIgG, residues 3-4 were targeted to residues 386-383 of hIgG, while residues 5-6 were not targeted since these were considered to be too close to the resin surface to interact well with the binding site. Default HADDOCK parameters (*e.g.*, temperatures for heating/cooling steps, number of molecular dynamics sets per stage, etc.) were used in the docking procedure. The resulting docked structures were grouped in clusters by assigning a minimum cluster size of 4 and an RMSD (root-mean-square-distance) lower than 2.5 Å using the program ProFit (<http://www.bioinf.org.uk/software/profit/>). All the clusters selected for each sequence based on visual inspection of the lowest energy docked solution were analyzed according to twelve scoring functions grouped in three families, dComplex, XScore (HPScore, HMScore, HSScore, $-\log(K_d)$, and Δ_iG), and FireDock (global, attractive VdW, repulsive VdW, ACE, and hydrogen bond) [42-45,47]. The twelve rankings were thus compiled, each listing the sequences ordered based on the scoring value obtained according to the respective function. These rankings were finally totaled and averaged to obtain a final list of sequences, where lower score indicates higher affinity.

7.2.3. Solid-phase synthesis of selected peptides

Each of the selected sequences were synthesized on 200mg of Toyopearl AF-Amino-

650M resins ($d = 75\text{-}150$ micron, amino group density = 0.4 mmol/g). Each amino acid coupling step was conducted for 25 min in a polypropylene tube fitted with a Teflon frit under continuous nitrogen flow and at a temperature of 35°C . After rinsing the resin in DMF for 10 min, one coupling was performed with Fmoc-Ala-OH (3 eq. molar excess as compared to the base resin functional density), HCTU (3 eq.) and DIPEA (6 eq.) in 3 mL of dry DMF. An acetylation step with acetic anhydride and DIPEA (50eq.) in 4 mL of DMF was carried out for 30 min at room temperature. The Fmoc protection was then removed by incubating with 5 mL of 20% piperidine in DMF for 20 min. The peptide sequences were synthesized via conventional Fmoc/tBu strategy. For each amino acid, an anhydrous DMF solution (2.5 mL) of Fmoc-amino acid (2 eq.), HCTU (2 eq.) and DIPEA (4 eq.) was added to the resin. Two couplings were performed for each amino acid to saturate all the available amino groups, as monitored by Kaiser test. The Fmoc protection on the last amino acid was removed with 5 mL of 20% piperidine in DMF for 20 min and each batch of resin was split in two aliquots, of which one was acetylated as indicated above. After rinsing the resins with DMF and DCM, peptide deprotection was performed using a cleavage cocktail containing TFA/DCM/indole (70/28/2) for 1.5 hours. Resins were then copiously rinsed with DCM and DMF and finally dried under vacuum.

7.2.4. Chromatographic evaluation of IgG binding and resistance to proteolytic enzymes of the peptide ligands

All resins (35 mg each) were packed in a 30 mm x 2.1 mm I.D. Microbore column

(0.1mL) (Alltech-Applied Science, Somerset, PA, USA) and swollen with 20%v/v methanol. After equilibration with PBS, pH 7.4, three IgG binding tests were performed using a 10 mg/mL solution of hIgG in PBS. Between each binding test, the resin was contacted with a 0.15 mg/mL solution of either trypsin or α -chymotrypsin in Tris HCl buffer, pH 8.5. The chromatographic protocol employed for all five injections was as follows. One hundred microliters of feed sample was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). After a washing step with 2 mL of equilibration buffer at a flow rate of 0.2mL/min (348 cm/h), elution was performed with 4 mL of 0.2M acetate buffer pH 4.0 at a flow rate of 0.4 mL/min (696 cm/h). Finally, the adsorbent was regenerated with 4 mL of 0.85% phosphoric acid. The adsorbents HWRGWV-, HYFKFD-, and HFRRHL- Toyopearl resin were used as controls. The effluent was monitored by absorbance at 280nm.

7.2.5. Purification of IVIG from Cohn fraction II + III of human plasma using the adsorbents HW_{Met}CitGW_{Met}V-, HY_{Met}F_{Met}K_{(Met)2}F_{Met}D- and HF_{Met}CitCitHL-Toyopearl resin

Cohn fraction II + III was dissolved in PBS, pH 7.4 to obtain an approximate IgG concentration of 5 mg/mL and filtered sequentially using a 0.44 μ m and a 0.22 μ m filter from Pall Corporation (Port Washington, NY, USA). Each peptide resin was packed and swollen as described in Section 7.2.4. After equilibration with PBS buffer containing 0.25M NaCl, 100 μ L of feed sample were loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). After washing the column with 2 mL of equilibration buffer at a flow rate of 0.2mL/min (348

cm/h), elution was performed with 4 mL of 0.2M acetate buffer pH 5.0 at a flow rate of 0.4 mL/min (696 cm/h). Cleaning and regeneration were performed using 4 mL of 0.85% phosphoric acid followed by a wash with 4 mL of acetate buffer (pH 4.0). Toyopearl AF-rProtein A-650F resin was used as a positive control. As per manufacturer's instructions, the chromatographic protocol comprised binding with PBS, pH 7.4 (at a flow rate of 0.05mL/min) and elution with 0.1M Glycine buffer pH 2.5 (at a flow rate of 0.4mL/min). The effluent was monitored by absorbance at 280nm. Fractions were collected and used for analysis of IgG purity and yield as described in Section 7.2.7.

7.2.6. Effect of conductivity on the IgG purification from Cohn fraction II + III of human plasma using the adsorbents Ac-HWRGWV- and Ac-HW_{Met}CitGW_{Met}V- Toyopearl resins

The resins were packed and swollen as described in Section 7.2.4, while the Cohn fraction II + III for the injection was prepared as described in Section 7.2.5. The effect of conductivity of the binding buffer was studied at 0, 0.135 and 0.25 M NaCl added to PBS. After equilibration with binding buffer, 100 μ L of feed was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). Chromatographic protocol and fraction collection were done as described in Section 7.2.5.

7.2.7. Sample analysis for yields and purities

The amount of IgG in the collected fractions was quantified by HPLC using 1 mL

HiTrap Protein G column. The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE[®] Novex 4–12% Bis-Tris gels in a Xcell SuperLock[™] Mini-Cell system (LifeSciences, Carlsbad, CA, USA). Sample preparation was done by adding 5 L of NuPAGE[®] LDS buffer and 2 μ L of NuPAGE[®] reducing agent to 13 μ L of sample and boiling the resulting mixture for 10 min. Gels were Coomassie-stained by using SimpleBlue[™] SafeStain. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institutes of Health, Bethesda, MD, USA). The purity of the product was calculated as the fraction of the total area equivalent to the IgG bands at 25 and 50 KDa.

7.3. Results

7.3.1. Molecular modeling

Seven non-natural amino acids were chosen for this study, namely N_{in}-methyl-tryptophan (where N_{in} indicates the tryptophan indole nitrogen), N_{in}-formyl-tryptophan, 4-methyl-phenylalanine, 4-carbamoyl-phenylalanine, O-methyl-tyrosine, ϵ -methyl-lysine, ϵ,ϵ -dimethyl-lysine, and citrulline (Figure 7.1).

The parameter and topology files of these residues were created using the files available for each corresponding standard amino acid as base structures. The closest matching moieties on standard residues were used to describe modifications on the side chain

functional groups. To ensure electrical neutrality, adjustments were made to the charge distribution within the functional group of each residue. The resulting parameter and topology file modifications were checked against submission to the PRODRG server, a standard verification process for parameterization of amino acid modifications. Hence, a virtual library of peptide sequences was created and screened against hIgG using HADDOCK 2.1 [40,41].

In order to perform a physically meaningful docking, a few constraints based on previous findings by Yang *et al.* were introduced in the simulations. First, MS analysis of protease digests of the Fc fragment of hIgG revealed a putative binding sequence for HWRGWV on the pFc segment, comprising the loop Ser383-Asn 389 (SNGQPEN), which was found to be distinct from the Protein A binding site (residues 341-443 on hIgG) [23]. This result was consistent with the observation that the peptide HWRGWV does not compete with Protein A for hIgG binding. Second, the basic motif comprising the first three amino acids of the peptide sequence, that is, histidine followed by an aromatic and a basic residue, is crucial in IgG binding. This has been evidenced by the consensus found in the sequences HWRGWV, HYFKFD, and HFRRHL identified by screening a solid phase library of hexapeptides [21]. Based on this homology, it is reasonable to assume that the two sequences HYFKFD and HFRRHL interact with the same binding site of hIgG as HWRGWV. Finally, since the C-terminus of the peptide is tethered with the surface of the chromatographic resin, it is rather likely that residues 5 and 6 of the hexapeptide ligands play only a modest role in targeting IgG. Based on this information, the residues Ser383-Asn389 on hIgG were defined as “active” and used as targets for ligand docking. All active residues exhibit a relative

solvent accessibility higher than 40%, as defined by the program NACCESS [48]. Further, on each peptide variant, residues 1-2 were targeted to residues 389-387 of hIgG, residues 3-4 were targeted to residues 386-383, while residues 5 and 6 were left unassigned.

To minimize bias in the validation, the following set of general criteria was devised for selecting the complexes resulting from docking simulations: 1) All the docked structures determined for each sequence in the final stage of molecular docking were clustered based on a stringent RMSD (root-mean-square-distance) cutoff of 2.5Å, whereas default clustering RMSD cutoff is usually set at 7.5Å, and a minimal cluster size of 4 structures. Herein, a cluster is defined as a group of docked peptide structures that share similar conformation and orientation within a certain window (2.5Å). Figure 7.2 shows examples of clusters of the original sequences HWRGWV and HFRRHL, and their variants HW_{Met}CitDW_{Met}V and HF_{Met}CitCitHL, docked on hIgG. 2) the structures used for the analysis were the most energetically favored docked structures from each cluster; 3) Each cluster was analyzed using the scoring methods dComplex, XScore, and FireDock, each comprising empirical scoring functions that estimate the binding affinity of a given protein-ligand complex of known three-dimensional structure. These functions account for van der Waals interactions, hydrogen bonding, deformation penalty, and hydrophobic effects, atomic contact energy, softened van der Waals interactions, partial electrostatics, additional estimations of the binding free energy and dipole-dipole interactions [42-45,47]. The hybrid approach of using multiple scoring methods was adopted so as not to bias the results to one particular method. Each cluster was ranked according to its individual score in the respective scoring method and the individual rankings thus produced were totaled and averaged. The final ranking as well as the estimated

free energy of interaction (Δ_iG) for the original sequences and several selected variants is reported in Table 7.1.

Although docking simulations generated multiple clusters per sequence, many sequences had their cluster #1 showing the highest number of structures, as well as lowest Δ_iG and highest XScore values. Such reproducibility is indicative of well-performed docking simulations and helps directing the choice of the best performing candidate variants. By comparing HWRGWV and HFRRHL with their variants HWCitGWV and HFCitCitHL, it was noted that the former have the most contacts with the target antibody, while the latter have the most hydrogen bonds. This indicates a different binding mechanism of the variants as compared to the original sequences that contain arginine, particularly with respect to the electrostatic component. In fact, by replacing positively charged (at pH 7.4) arginine with electrically neutral citrulline, the electrostatic component of binding is considerably reduced. On the one hand, this causes the predicted free energy of binding of the peptide variants to the target antibody to be lower when compared to the original sequences, which in turn suggests that the former might have a lower binding capacity than the latter. On the other hand, it makes the variant potentially less prone to nonspecific electrostatic binding of negatively charged proteins, albumin in particular, which lowers the product purity. These differences have direct implications on the chromatographic protocol, especially on the effect of conductivity of the binding buffer on the IgG yield and purity, as discussed in detail in Section 7.3.3. Other variants of HWRGWV and HFRRHL were designed using formyl-tryptophan and carbamoyl-phenylalanine, neither of which, however, obtained a good docking score. One more variant, HW_{Met}CitDW_{Met}V, was created by replacing glycine with

aspartic acid for the purpose of increasing affinity by potentially forming hydrogen bonds between the aspartic acid and the residues 383-386 (SNGQ) on IgG. Against expectations, however, this sequence obtained a lower score. HYFKFD and its two variants $\text{HY}_{\text{Met}}\text{F}_{\text{Met}}\text{K}_{\text{Met}}\text{F}_{\text{Met}}\text{D}$ and $\text{HY}_{\text{Met}}\text{F}_{\text{Met}}\text{K}_{(\text{Met})2}\text{F}_{\text{Met}}\text{D}$ were also run, but obtained, on average, worse scores, indicative of lower affinity as compared to HWRGWV, HFRRHL, and their variants.

The sequences listed in Table 7.1 were synthesized on Toyopearl resin, all at the same peptide density of 0.12 meq/g. Each adsorbent was packed into a chromatographic column (0.1 mL) and tested for IgG binding. Flowthrough and elution fractions were collected and analyzed by Protein G chromatography to determine IgG yield (Table 1). The comparison between the predicted free energy of binding, the average rank, and the yield for each sequence indicates good agreement between the affinity predicted by the docking simulation and the experimental results of antibody binding. This confirms that the design of the virtual library, the assignment of docking constraints, and the analysis of the simulation results were well performed and form an effective strategy for the selection of peptide variants.

7.3.2. Chromatographic evaluation of the peptide ligands by IgG binding and resistance to proteolytic enzymes

Based on the results reported in Table 7.1, the original sequences HWRGWV, HFRRHL, and HYFKFD, and their variants $\text{HW}_{\text{Met}}\text{CitGW}_{\text{Met}}\text{V}$, $\text{HF}_{\text{Met}}\text{CitCitHL}$, and $\text{HY}_{\text{Met}}\text{F}_{\text{Met}}\text{K}_{(\text{Met})2}\text{F}_{\text{Met}}\text{D}$ were tested for their resistance to enzymatic digestion. The sequences

HW_{Met}CitGW_{Met}V, HF_{Met}CitCitHL were selected as the best variants of their respective original peptides, while HY_{Met}F_{Met}K_{Met2}F_{Met}D was used as a negative control. Finally, two more sequences, HWCitGWV and HW_{Met}RGW_{Met}V, were chosen as intermediate variants, hence expected to show resistance to one enzyme only. Each adsorbent was subjected to five consecutive chromatographic runs. First, a solution of pure human polyclonal IgG at 10 mg/mL in PBS was injected to determine IgG yield for each adsorbent prior to contact with any enzyme. All four peptide variants gave a yield above 91% for this initial run. The resin was then contacted with a solution of α -chymotrypsin in Tris buffer pH 8.5 for 10 minutes. The amount of enzyme loaded onto the column was in a mass ratio of 1:100 peptide:enzyme, as done by Verdoliva *et al.* [35]. After rinsing the resin, a second injection of IgG was then performed to estimate the loss of binding capacity due to the digestion of the peptide ligand by α -chymotrypsin. The resin was then contacted with the second enzyme solution, *i.e.* trypsin in Tris buffer pH 8.5, at the same peptide:enzyme ratio. A third IgG injection was finally performed to estimate the residual binding capacity of each resin after the second enzyme treatment. Figure 7.3 shows the chromatograms of the three IgG injections for the adsorbents HW_{Met}CitGW_{Met}V-Toyopearl, HW_{Met}RGW_{Met}V-Toyopearl, HWCitGWV-Toyopearl, and HWRGWV-Toyopearl resins. The values of IgG yield before and after exposure to enzymes are summarized in Table 7.2.

While HWRGWV was evidently degraded by both trypsin and α -chymotrypsin, as indicated by the loss of binding capacity after both enzyme treatments (Figure 7.3A), its variant HW_{Met}CitGW_{Met}V is completely unaffected by either (Figure 7.3D). As expected, the intermediate variants HW_{Met}RGW_{Met}V and HWCitGWV show resistance towards one

enzyme only, α -chymotrypsin and trypsin respectively (Figure 7.3B and C). The results obtained with the other adsorbents are summarized in Table 7.2, which reports the values of IgG yield before (1st run) and after treatment with α -chymotrypsin (2nd IgG injection) and trypsin (3rd IgG injection).

The results obtained with the ligand HFRRHL and its derivative HF_{Met}CitCitHL closely resemble those of HWRGWV and HW_{Met}CitGW_{Met}V. The sequence HWRGWV is an ideal substrate for trypsin, most likely because the glycine in the C-position with respect to arginine sterically favors the attack of the enzyme onto the peptide. The peptide HFRRHL is also a good substrate for trypsin, although the contiguity of the arginines on the sequence slightly reduces the enzymatic attack. HYFKFD, instead, is almost immune to the attack of trypsin, likely due to the steric hindrance of the residues flanking lysine, which can impede the effective anchoring of the enzyme active site on the peptide. Its variant HY_{Met}F_{Met}K_{(Met)2}F_{Met}D shows high resistance to proteolysis as well, although the low yield values indicate that the sequence is unfit for protein recovery.

While sequence dependent, these results clearly demonstrate that the replacement of natural amino acids with similar synthetic residues, while maintaining similar antibody binding properties as the original sequences, as predicted by the docking simulations, confers high resistance to enzymatic digestion. The methylation of aromatic amino acids significantly reduces the proteolytic attack by α -chymotrypsin, while the use of citrulline and methylated lysine seems to completely prevent the action of trypsin. Despite seeming the most critical of the proposed substitutions, insofar as it reduces the electrostatic component of binding,

citrulline proved to be an excellent replacement under both aspects of target binding and biochemical resistance.

7.3.3. Purification of IVIG from Cohn fraction II + III of human plasma using the adsorbents $HW_{Met}CitGW_{Met}V-$, $HF_{Met}CitCitHL-$, and $HY_{Met}F_{Met}K_{(Met)2}F_{Met}D-$ Toyopearl resin

To determine the applicability of the proposed ligand variants for IVIG purification, the sequences $HW_{Met}CitGW_{Met}V$, $HF_{Met}CitCitHL$, and $HY_{Met}F_{Met}K_{(Met)2}F_{Met}D$ were used for purifying polyclonal antibodies from Cohn fraction II + III of human plasma. The original peptide ligands were employed as positive controls. The crude stock of Cohn II + III paste was diluted in PBS to prepare the feed sample and solid particles were removed by filtration prior to injection into the column. The chromatographic protocol adopted in this work was derived from previous optimizations and comprised the use of 0.25M NaCl in PBS as binding buffer and 0.2M acetate buffer pH 5.0 for elution [24]. The resulting chromatograms are presented in Figure 7.4. Fractions were collected and analyzed by Protein G chromatography and SDS-PAGE (Figure 7.5) to determine IgG yield and purity respectively. A summary of results is presented in Table 7.3.

Product yields and purities obtained with the variants $HW_{Met}CitGW_{Met}V$ and $HF_{Met}CitCitHL$ compare well with the results given by the original sequences and Protein A. Although small amounts of albumin can be detected in the eluted fractions (Figure 7.5), both ligands offered very high product yield and purity. Most of the observable contaminants

simply flow through the column and although some binding of other immunoglobulins, namely IgA and IgM, may occur, the elution conditions (pH 5.0) were chosen to minimize their presence in the eluted fraction [49]. The variant $\text{HY}_{\text{Met}}\text{F}_{\text{Met}}\text{K}_{(\text{Met})2}\text{F}_{\text{Met}}\text{D}$ gave high product purity, but performed poorly in terms of yield. While the latter was anticipated based upon the above results (Section 7.3.2), high IgG purity in the eluted fraction was not expected. It is surprising that despite the high sequence hydrophobicity due to the use of alkylated amino acids, there was little non-specific binding of impurities by hydrophobic interaction.

It is also interesting to note that the amount of albumin and other impurities bound by the peptide variants is consistently lower than observed with the original sequences. This can be explained in light of previous findings and the information provided by the docking simulations. The original sequence HWRGWV, for example, which bears at pH 7.4 a net charge of approximately 1.4 due to arginine and the peptide N-terminus, was found to capture albumin (pI = 4.7), the most abundant negatively charged protein present in plasma, by electrostatic interaction. To avoid this nonspecific binding of albumin and similar protein impurities, the conductivity of the binding buffer was increased by adding sodium chloride up to an optimum level that gives the best compromise in terms of product yield and purity [25]. The use of salt, however, translates in additional costs to the purification process. The replacement of positively charged residues with electrically neutral amino acids, like citrulline and dimethylated lysine, intrinsically reduces the extent of electrostatic binding regardless of the amount of salt present in the binding buffer. These findings, while explaining the higher purity given by the ligand variants (Table 7.3), call for a more in depth

study on the effect of salt on yields and purity, and this is presented in the Section that follows.

7.3.4. Effect of conductivity on IgG purification from Cohn fraction II + III of human plasma using the adsorbents HWRGWV-Toyopearl, Ac-HWRGWV-Toyopearl, HW_{Met}CitGW_{Met}V-Toyopearl, and Ac-HW_{Met}CitGW_{Met}V-Toyopearl resins

To determine the extent of the electrostatic component of binding, the effect of conductivity of the binding buffer on product yield and purity was studied using four peptide ligands with different charge value and distribution: a) the original HWRGWV, b) its acetylated version Ac-HWRGWV, c) the variant HW_{Met}CitGW_{Met}V, and d) its acetylated version Ac-HW_{Met}CitGW_{Met}V. The four sequences were used for purifying IVIG from Cohn II + III fraction. As mentioned above, in previous studies of IVIG purification using HWRGWV, PBS + 0.25M NaCl was chosen as the optimal binding buffer. The results obtained in the previous Section led to the hypothesis that the replacement of positively charged amino acids with neutral residues would reduce the electrostatic behavior of the ligands and hence increase product purity. To verify this hypothesis, binding studies were repeated using the above listed sequences and three binding buffers, comprising 0M, 0.13M, and 0.25M NaCl in PBS. Figure 7.6 shows the SDS-PAGE results obtained at different conductivities with each of the four adsorbents, while Table 7.5 reports the resulting values of product yield and purity.

As Table 7.4 indicates, lowering the number of positive charges on the peptide leads

to higher IgG purity. As expected, the effect of conductivity of the binding buffer on product purity is very evident for HWRGWV, which bears the highest net charge (+ 1.4) at pH 7.4 and is hence the most susceptible to the shielding of electrostatic forces. For Ac-HW_{Met}CitGW_{Met}V, instead, the effect of conductivity is nearly negligible. A comparison between the latter and HW_{Met}CitGW_{Met}V, as well as the original sequence and its acetylated form, show that the acetylation of the peptide N-terminus is less influential on product yield and purity than the replacement of arginine with citrulline. Our estimations indicate in fact that the decrease of the peptide net charge caused by acetylation (-0.5) is lower than that due to citrulline (-0.9). The IgG purity (93%) obtained with Ac-HW_{Met}CitGW_{Met}V using a low conductivity binding buffer is higher than any value obtained using HWRGWV (81% - 83%). Notably, high purity has not been achieved at the expense of yield, which remained stably above 90%, even though some decrease was expected based upon the results of the docking calculations, which predicted for the variant a Δ_iG of binding slightly lower than that of the original sequence. The sequence Ac-HW_{Met}CitGW_{Met}V possesses many required features for an affordable and robust process of antibody purification based on small peptide ligand affinity chromatography.

7.4. Conclusions

This study offers a strategy for the design of small peptide ligands comprising non-natural amino acids with excellent characteristics of target affinity and selectivity, and biochemical stability. Based on the information available for known peptide sequences, in

particular the binding site on the target biomolecule, and the use of state-of-the-art modeling tools, this method directs the replacement of key amino acid residues with non-natural variants to conveniently modify the binding mechanism or to confer stability against chemical and biological agents, such as strong acids and bases, and proteolytic enzymes. Three antibody binding peptides, HWRGWV, HYFKFD, and HFRRHL, were utilized as models to develop ligand variants that show higher proteolytic resistance and maintain high target affinity and specificity. Due to the high value of antibodies recovered from plasma-based fluids, like Cohn fractions and hybridoma cell culture, this work aimed to confer the peptides with biochemical stability against the major plasma proteases, trypsin and α -chymotrypsin. To this end, a virtual library of variants was designed by replacing aromatic and basic amino acids with methylated variants and citrulline, and then screened *in-silico* against the peptide binding site on IgG (Ser383 – Asn389) using the molecular docking software HADDOCK. The peptide variants selected based on the results of docking calculations were synthesized on chromatographic resins and tested for resistance to proteolysis and purification of IVIG from Cohn II+III fraction of human plasma. These variants possess target affinity comparable to their parental sequences and a much higher biochemical resistance. Furthermore, an in-depth study on the electrostatic component of the IgG binding mechanism of HWRGWV-related variants resulted in the identification of the sequence Ac-HW_{Met}CitGW_{Met}V, which exhibits higher selectivity than the original HWRGWV. The adsorbent Ac-HW_{Met}CitGW_{Met}V-Toyopearl resin demonstrated intrinsically lower binding of albumin and other impurities, which translates into lower amount of salt needed in the binding buffer to attain high IgG purity and hence lower purification costs.

The approach used here is generally valid for any small synthetic or natural peptide ligand targeting a biomolecule. Once the binding site is known with good approximation, it is possible to design and screen large libraries, quickly and inexpensively using reliable programs for molecular docking. These tools, in addition to providing good estimations of the binding strength, also offer insights regarding the nature of ligand-target interactions. The judicious choice of amino acid substitutions enables fine-tuning of the biochemical properties of the peptide ligands. In particular, by modifying the distribution of charge as well as hydrophobic and hydrophilic groups, it is possible to enhance, affinity and selectivity in addition to biochemical stability. The use of synthetic variants in place of amino acids that are prone to chemical degradation, *e.g.* asparagine and glutamine which undergo deamidation in alkaline conditions, is particularly suited for designing peptide ligands for affinity chromatography, where harsh chemical agents are used for protein elution and column cleaning and sanitization. Reducing the extent of chemical degradation translates into longer adsorbent lifetime.

The approach presented herein is also amenable for fundamental studies of the non-covalent interactions that underlie the mechanisms of protein activity. By silencing or activating specific components of binding using suitable amino acids, it is possible to study the phenomena of biorecognition and design small biomolecules that control the specific interactions between target and ligand. This work offers an example in this direction by presenting small peptide variants that, in several respects, outperform Protein A in binding target antibodies. These findings represent a further step towards optimal synthetic protein mimetics with great potential for bioseparations and, more generally, a variety of applications

in biotechnology.

7.5. References

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Table 7.1. Predicted free energy of binding, docking rank, and IgG yield obtained for the original peptide sequences and their variants.

Sequence	Δ_iG (kcal/mol)	Average rank	IgG yield
HFRRHL	- 6.96	2.00	93 %
HF _{Met} CitCitHL	- 6.57	2.00	90 %
HWCitGWV	- 6.64	4.00	91 %
HWRGWV	- 6.37	5.00	95 %
HW _{Met} CitGW _{Met} V	- 6.71	9.00	90 %
HW _{Met} RGW _{Met} V	- 6.38	10.00	91 %
HYFKFD	- 5.66	14.00	78 %
HW _{For} CitGW _{For} V	- 5.41	16.17	75 %
HY _{Met} F _{Met} K _{Met} F _{Met} D	- 5.17	18.67	49 %
HF _{carb} CitCitHL	- 5.03	21.50	53 %
HW _{Met} CitDW _{Met} V	- 5.50	26.50	47 %
HY _{Met} F _{Met} K _{Met2} F _{Met} D	- 4.69	26.67	42 %

Table 7.2. Values of IgG yield before and after contacting the resin with enzyme solutions.

Peptide sequence	IgG Yield		
	1 st run	2 nd run (after α -chymotrypsin)	3 rd run (after trypsin)
HWRGWV	95 %	72 %	36 %
HW _{Met} RGW _{Met} V	91 %	91 %	82 %
HWCitGWV	91 %	73 %	71 %
HW _{Met} CitGW _{Met} V	90 %	91 %	90 %
HW _{For} CitGW _{For} V	75 %	69 %	69 %
HFRRHL	93 %	78 %	54 %
HF _{Met} CitCitHL	90 %	92 %	91 %
HYFKFD	78 %	76 %	71 %
HY _{Met} F _{Met} K _{(Met)2} F _{Met} D	52 %	49 %	53 %

Table 7.3. Yields and purity of IgG purified from Cohn fraction II + III of human plasma. IgG purity is determined by densitometric analysis of the Coomassie-stained SDS-PAGE reported in Figure 7.2.

Sequence	IgG Yield	IgG Purity
HWRGWV	85 %	83 %
HW _{Met} CitGW _{Met} V	91 %	92 %
HFRRHL	91 %	86 %
HF _{Met} CitCitHL	89 %	91 %
HYFKFD	54 %	87 %
HY _{Met} F _{Met} K _{(Met)2} F _{Met} D	48 %	90 %
Protein A	94 %	75 %

Table 7.4. Yields and purity of IgG purified from Cohn fraction II + III of human plasma using binding buffers at different salt concentration. IgG purity is determined by densitometric analysis of the Coomassie-stained SDS-PAGE reported in Figure 7.3.

Sequence	0 M NaCl		0.13 M NaCl		0.25 M NaCl	
	IgG Yield	IgG Purity	IgG Yield	IgG Purity	IgG Yield	IgG Purity
HWRGWV	90 %	81 %	85 %	83 %	85 %	83 %
Ac-HWRGWV	91 %	80 %	84 %	80 %	83 %	84 %
HW _{Met} CitGW _{Met} V	89 %	91 %	90 %	91 %	91 %	92 %
Ac-HW _{Met} CitGW _{Met} V	91 %	93 %	93 %	90 %	94 %	92 %

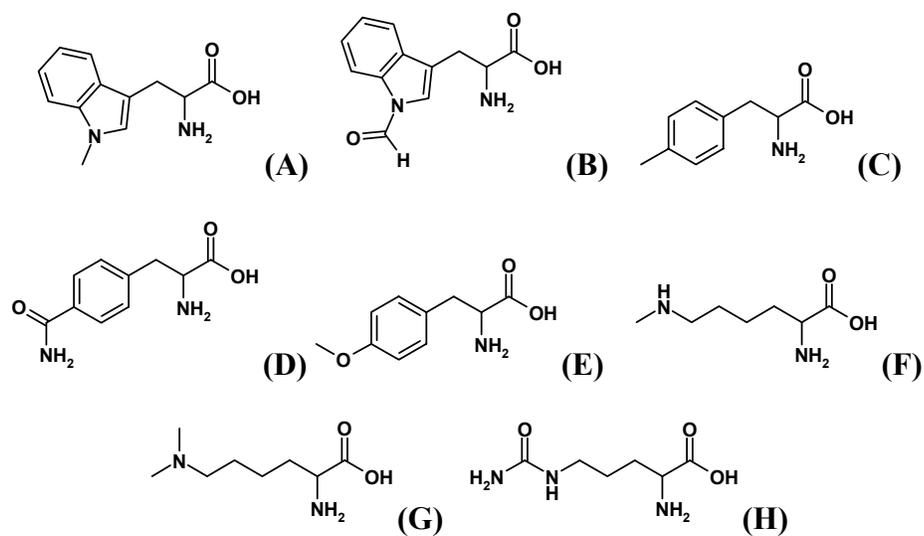


Figure 7.1. Non-natural amino acids used for building peptide variants: (A) N_{in} -methyl-tryptophan, (B) N_{in} -formyl-tryptophan, (C) 4-methyl-phenylalanine, (D) 4-carbamoyl-phenylalanine, (E) O-methyl-tyrosine, (F) ϵ -methyl-lysine, (G) ϵ,ϵ -dimethyl-lysine, and (H) citrulline.

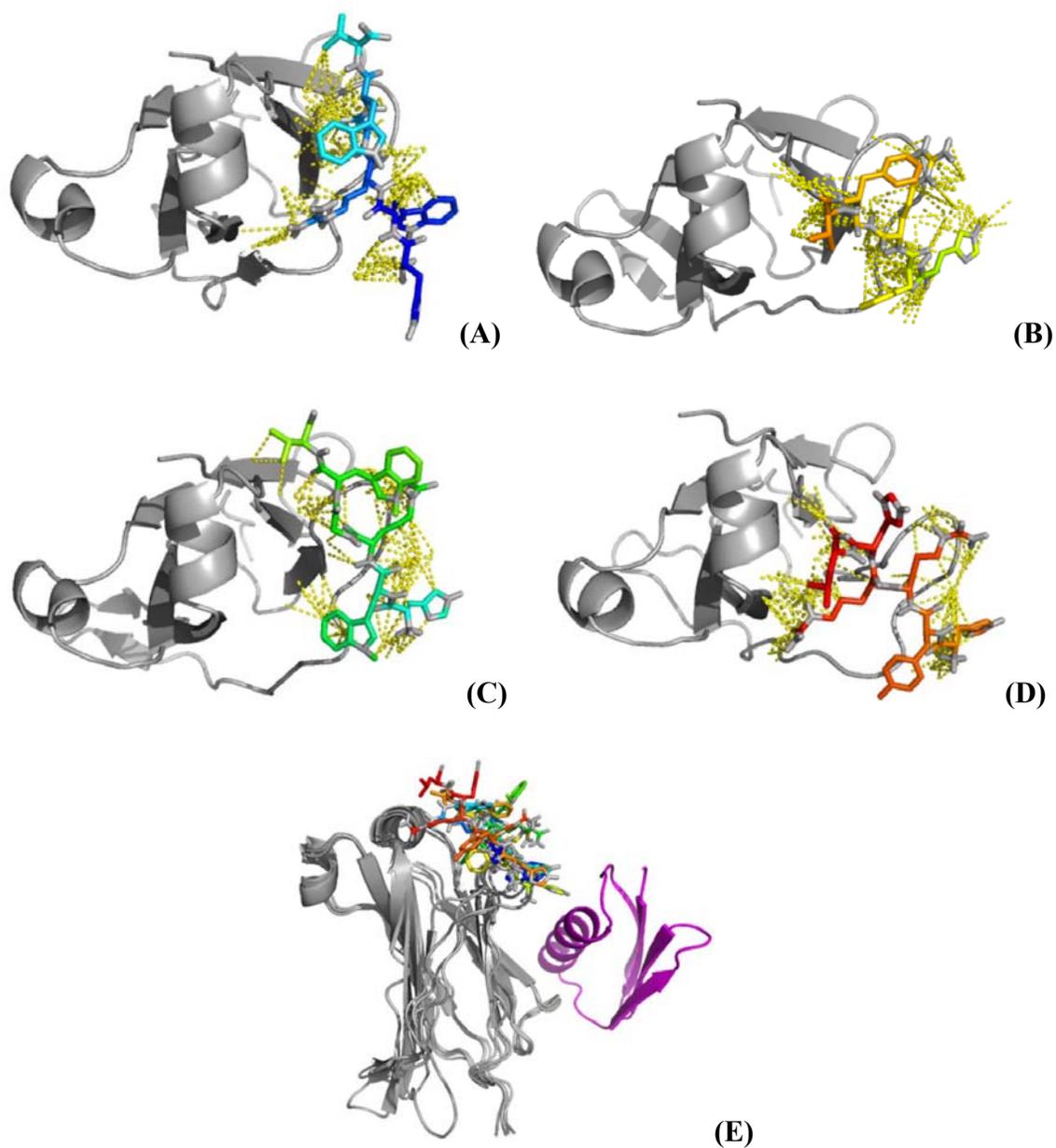


Figure 7.2. Lowest energy structures from best scoring clusters of docked peptide structures. Shown in grey cartoon format is IgG (PBD code: 1FCC) with the peptide structures shown in stick format: (A) HWRGWV, (B) HWMetCitGWMetV, (C) HFRRHL, and (D) HFMetCitCitHL, and (E) overlay of the lowest energy structures of the docked peptide clusters. In (E), protein A molecule is shown in purple cartoon format to highlight the absence of interactions between Protein A and the peptides. Yellow dashed lines denote atom contacts between atoms in IgG and the peptides that are less than 4Å apart.

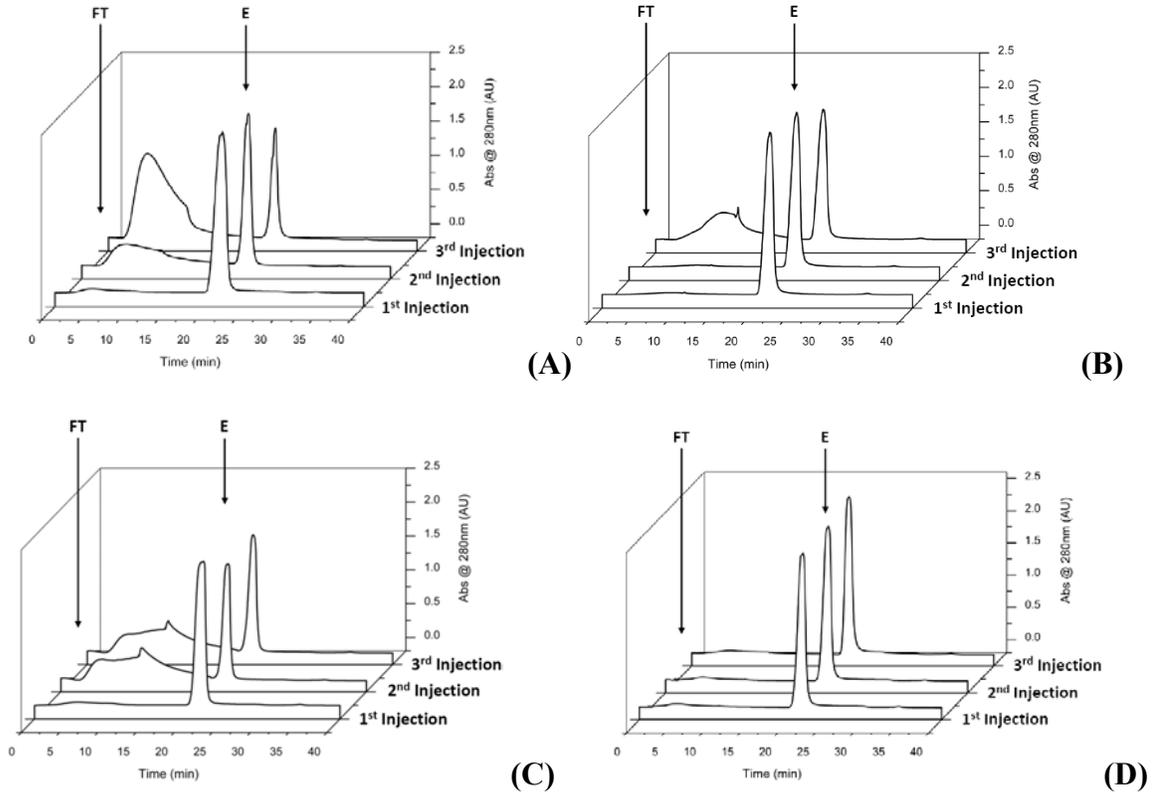


Figure 7.3. Chromatograms of IgG binding with intermediate enzyme treatment using the adsorbents: (A) HWRGWV-Toyopearl, (B) HW_{Met}RGW_{Met}V-Toyopearl, (C) HWCitGWV-Toyopearl, and (D) HW_{Met}CitGW_{Met}V-Toyopearl resin.

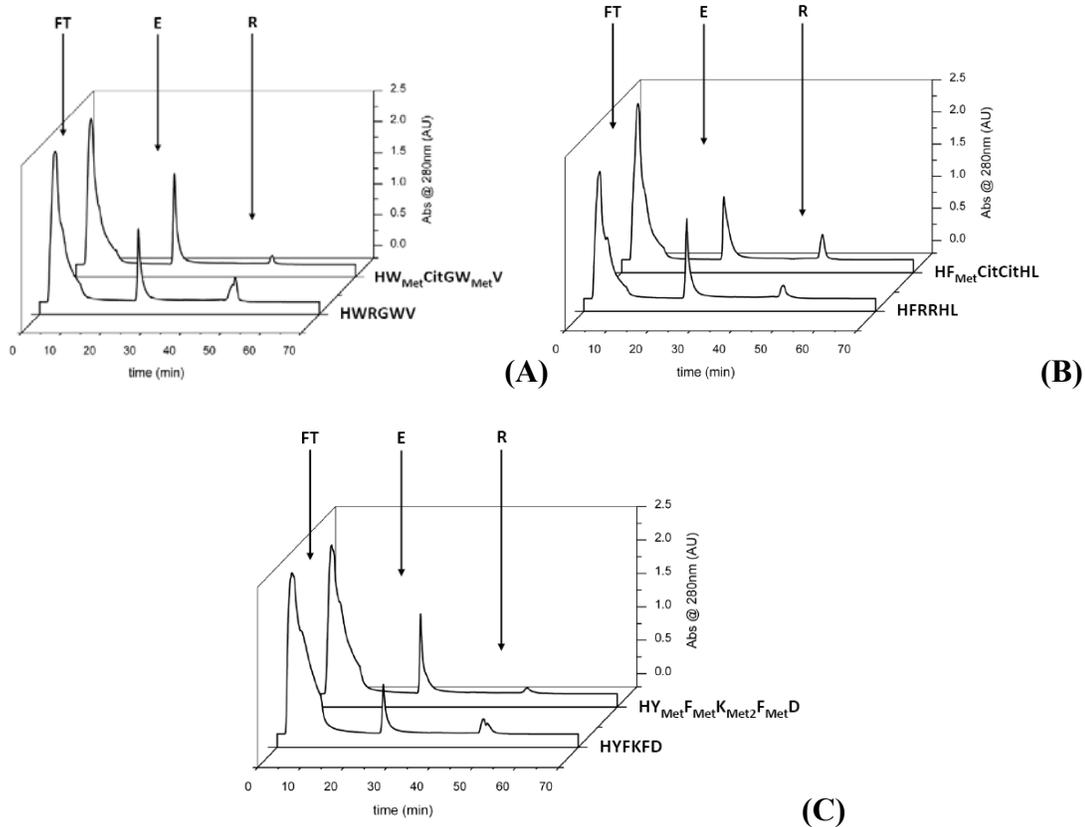


Figure 7.4. Chromatographic purification of IgG from Cohn fraction II + III of human plasma using the adsorbents: (A) HWRGWV-Toyopearl resin and $HW_{Met}CitGW_{Met}V$ -Toyopearl resin; (B) HFRRHL-Toyopearl resin and $HF_{Met}CitCitHL$ -Toyopearl resin; and (C) HYFKFD-Toyopearl resin and $HY_{Met}F_{Met}K_{(Met)2}F_{Met}D$ -Toyopearl resin. Labels: FT – flow-through; W – washing; EL – elution; R: regeneration.

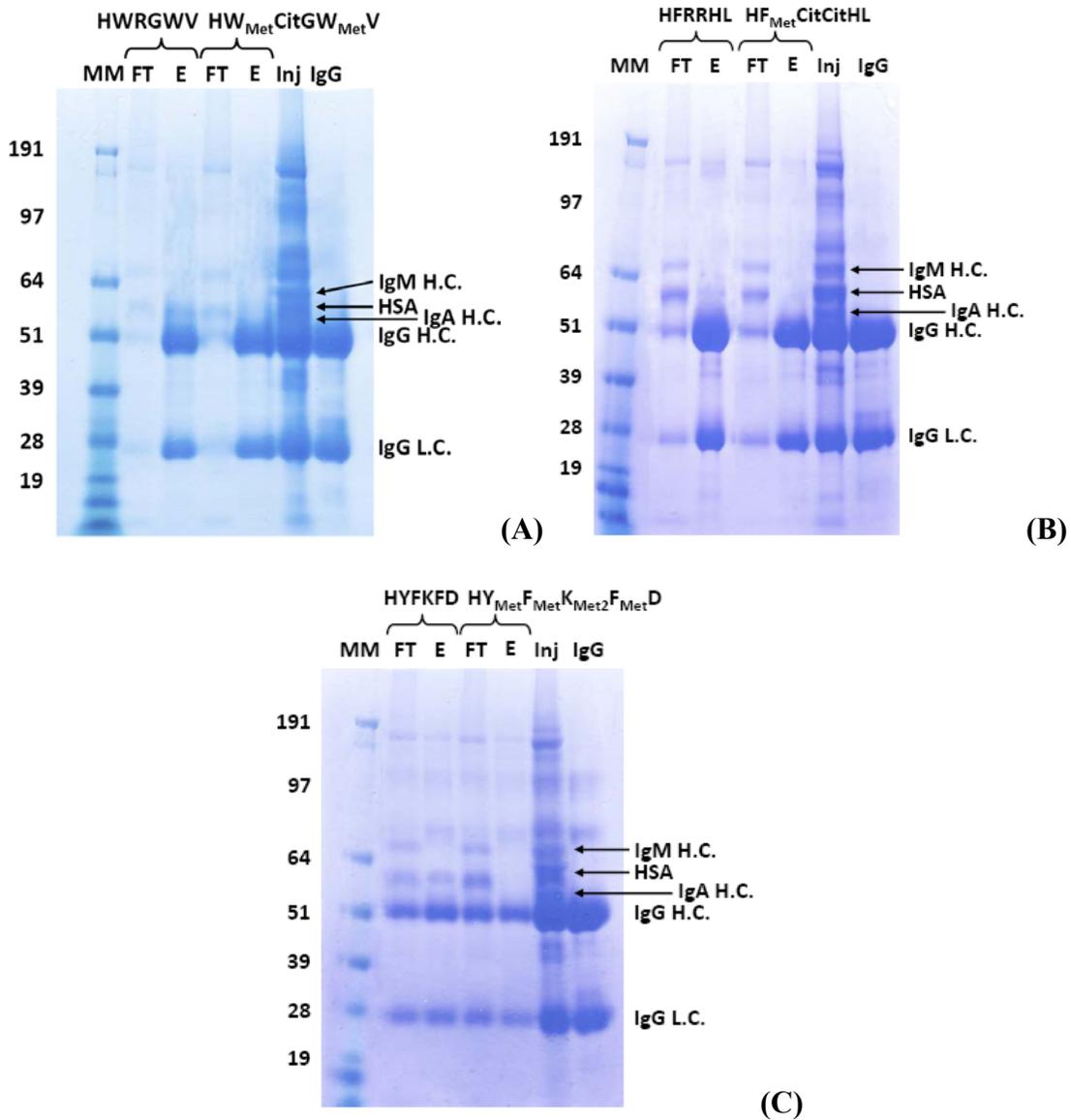


Figure 7.5. SDS-PAGE (reducing conditions) of chromatographic purification of IgG from Cohn fraction II + III of human plasma using the adsorbents: (A) HWRGWV-Toyopearl resin and HW_{Met}CitGW_{Met}V-Toyopearl resin; (B) HFRRHL-Toyopearl resin and HF_{Met}CitCitHL-Toyopearl resin; and (C) HYFKFD-Toyopearl resin and HY_{Met}F_{Met}K_{(Met)2}F_{Met}D-Toyopearl resin. Labels: FT – flow-through fraction; EL – elution fraction.

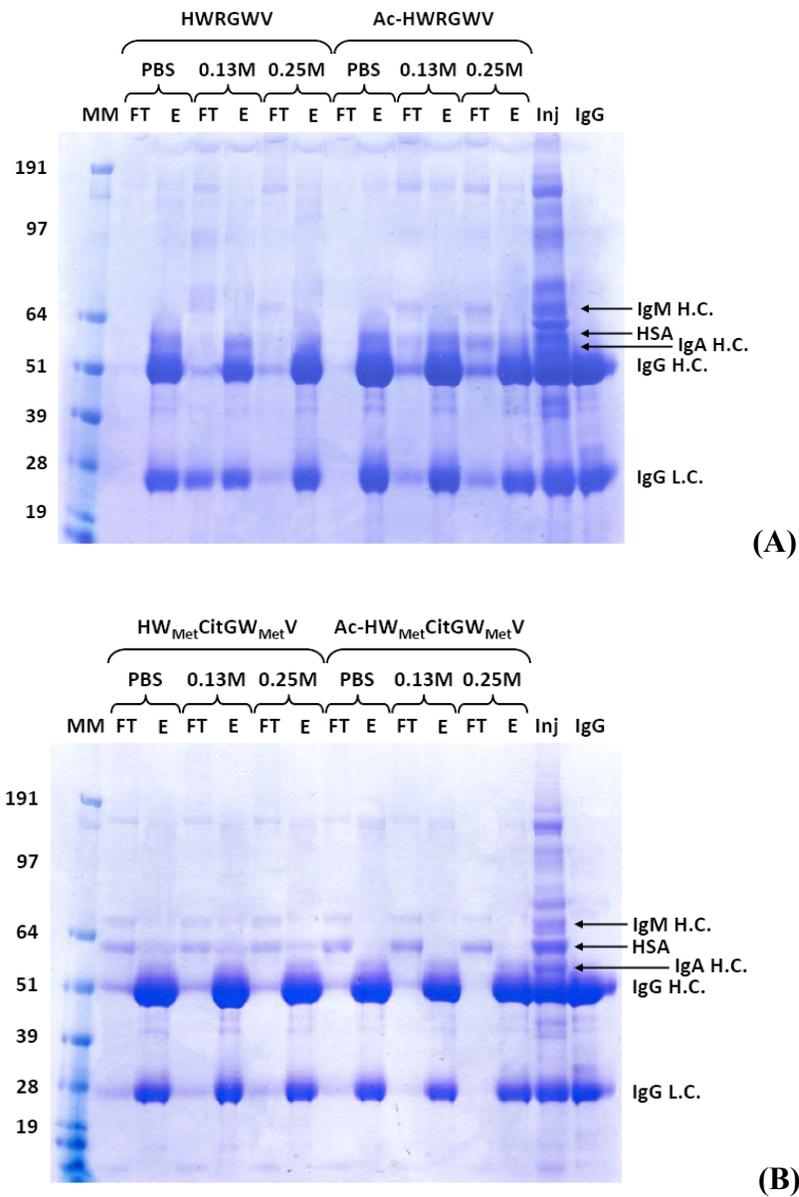


Figure 7.6. SDS-PAGE (reducing conditions) of chromatographic purification of IgG from Cohn fraction II + III of human plasma performed at different salt concentration in the binding buffer: (A) HWRGWV and Ac-HWRGWV, (B) HW_{Met}CitGW_{Met}V and Ac-HW_{Met}CitGW_{Met}V. Labels: FT – flow-through fraction; EL – elution fraction.

Chapter 8. mRNA display selection and solid-phase synthesis of

Fc-binding cyclic peptide affinity ligands

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A version of this chapter has been published in

Biotechnology and Bioengineering 2012, 110(3), 857-870.

Abstract

Cyclic peptides are attractive candidates for synthetic affinity ligands due to their favorable properties, such as resistance to proteolysis, and higher affinity and specificity relative to linear peptides. Here we describe the discovery, synthesis and characterization of novel cyclic peptide affinity ligands that bind the Fc portion of human Immunoglobulin G (IgG) (hFc). We generated an mRNA display library of cyclic pentapeptides wherein peptide cyclization was achieved with high yield and selectivity, using a solid-phase crosslinking reaction between two primary amine groups, mediated by a homobifunctional linker. Subsequently, a pool of cyclic peptide binders to hFc was isolated from this library and chromatographic resins incorporating the selected cyclic peptides were prepared by on-resin solid-phase peptide synthesis and cyclization. Significantly, this approach results in resins that are resistant to harsh basic conditions of column cleaning and regeneration. Further studies identified a specific cyclic peptide – cyclo[*Link*-M-WFRHY-K] – as a robust affinity ligand for purification of IgG from complex mixtures. The cyclo[*Link*-M-WFRHY-K] resin bound selectively to the Fc fragment of IgG, with no binding to the Fab fragment, and also bound immunoglobulins from a variety of mammalian species. Notably, while the recovery of IgG using the cyclo[*Link*-M-WFRHY-K] resin was comparable to a Protein A resin, elution of IgG could be achieved under milder conditions (pH 4 vs. pH 2.5). Thus, cyclo[*Link*-M-WFRHY-K] is an attractive candidate for developing a cost-effective and robust chromatographic resin to purify monoclonal antibodies (mAbs). Finally, our approach can be extended to efficiently generate and evaluate cyclic peptide affinity ligands for other targets of interest.

Keywords: Cyclic peptide; mRNA display; Fc binding; Affinity ligand; Library.

8.1 Introduction

The use of monoclonal antibody (mAb)-based biopharmaceuticals has increased exponentially over the last three decades [1-5]. Yet, the manufacturing costs of mAbs, particularly the cost of downstream processing, remain high. Consequently, replacement of the highly expensive Protein A/G media for mAb purification is an important goal [6,7]. To this end, several novel and robust synthetic affinity ligands have been proposed [8-13] and among these, peptides are a growing class [14-17]. In particular, recent studies have drawn attention to cyclic peptides as a new class of molecules with great potential [4,18-27]. Due to their conformational rigidity, cyclic peptides exhibit superior properties over linear counterparts, such as higher target specificity and affinity, and resistance to proteolysis [18,28-30]. Therefore, cyclic peptides are attractive candidates for the next generation of synthetic affinity ligands [31-35]. However, a robust and well-characterized cyclic peptide ligand for mAb purification has not yet been reported. Here, we present the discovery of novel cyclic peptide binders for the Fc portion of human Immunoglobulin G (IgG) (hFc), the synthesis of resins functionalized with these cyclic peptides, and their rigorous evaluation in the context of mAb purification.

Cyclic peptides have been previously isolated from large combinatorial libraries using library screening tools, such as phage display and mRNA display [24,36-41]. In particular, mRNA display is attractive due to the completely *in vitro* library synthesis and selection process that enables the inclusion of non-natural amino acids and ease of peptide cyclization [41-44]. Here, we generated an mRNA display library of cyclic pentapeptides using a novel

approach wherein peptide cyclization was achieved, with high yield and selectivity, using a solid-phase reaction; crosslinking between two primary amine groups was mediated by a homobifunctional linker. Subsequently, we screened this library to identify a pool of cyclic pentapeptides that bind to hFc. Further, we developed a novel strategy to synthesize the selected pentapeptides directly on a chromatographic support. Using this approach, we prepared and evaluated resins containing several different cyclic peptides selected by mRNA display, and identified a robust cyclic peptide affinity ligand for hFc. Notably, a chromatographic resin based on this ligand is highly selective to the Fc portion of IgG, binds IgG from a variety of mammalian species, and can be used to purify mAbs from complex cell culture supernatants. Furthermore, our studies show that the recovery of IgG using the cyclic peptide resin is comparable to that obtained with a Protein A resin, while allowing elution of IgG under mild conditions (pH 4). Importantly, our approach of on-resin peptide synthesis also results in resins that resist harsh basic conditions of column cleaning and regeneration [45]. Binders to other targets of interest may be isolated using the mRNA display library of cyclic peptides. Therefore, we expect that our overall approach can be extended to develop robust cyclic peptide affinity ligands in general.

8.2. Experimental

8.2.1. Synthesis of the model mRNA-peptide fusion (MVVFVVK) and mRNA-display library of linear pentapeptides

The oligonucleotides 5'- *GGA CAA TTA CTA TTT ACA ATT ACA ATG GTG GTG TTT GTG GTG AAA GGC GGC AGC GGC GGC AGC CAT CAC CAC CAT CAC CAT ATG GGA ATG* - 3' and 5'- *GGA CAA TTA CTA TTT ACA ATT ACA ATG NNN NNN NNN NNN NNN AAA GGC GGC AGC GGC GGC AGC CAT CAC CAC CAT CAC CAT ATG GGA ATG* - 3', encoding the sequence MVVFVVK and the pentapeptide library MX₁X₂X₃X₄X₅K (where X is one of the 20 amino acids) were PCR-amplified using the forward primer 5'- *GCA AAT TTC TAA TAC GAC TCA CTA TAG GGA CAA TTA CTA TTT ACA ATT AC*-3' and the reverse primer 5'-*ATA GCC GGT GCC AGA TCC AGA CAT TCC CAT ATG GTG ATG GT*-3'. All oligonucleotides were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA). The DNA obtained from the PCRs was used to generate mRNA-peptide fusions using *in vitro* transcription, ligation of the puromycin linker and *in vitro* translation, as previously described [46].

8.2.2. Studies of peptide cyclization of the model sequence MVVFVVK by crosslinking reaction in fed-batch mode

Oligo-dT cellulose (Life Technologies, Carlsbad, CA) was first swollen in elution buffer (10 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS) and then equilibrated with binding buffer (0.5 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS, 1 mM DTT) for 1 hour

at room temperature. 800 μ L of solution containing the mRNA-peptide fusions ($\sim 10^{-7}$ M) was incubated with oligo-dT cellulose (100 mg cellulose per mL of solution) for 2 hours at 4 $^{\circ}$ C. The cellulose was then washed with dT washing buffer (0.2 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.05% SDS, 1 mM DTT; 3 x 10 min) and preconditioned with crosslinking buffer (0.2 M NaCl, 1 mM EDTA, 0.05% SDS, 1 mM DTT; 3 x 10 min) before the crosslinking reaction. The oligo-dT cellulose was filtered and split in four aliquots, each resuspended in 0.3mL of crosslinking buffer. Fifty microliters of 2.3 mg/mL solution of crosslinker disuccinimidyl glutarate (DSG; Fisher Scientific, Pittsburgh, PA) in N,N-dimethylformamide (extra dry) (DMF; Sigma-Aldrich, St. Louis, MO) were added to the first aliquot (a) and incubated with 50 μ L of 2.3 mg/mL solution of crosslinker disuccinimidyl glutarate (DSG; Fisher Scientific, Pittsburgh, PA) in N,N-dimethylformamide (extra dry) (DMF; Sigma-Aldrich, St. Louis, MO) for 2 h at 4 $^{\circ}$ C. The second aliquot (b) was incubated two times with 50 μ L of a 1.2mg/mL solution of DSG in DMF for 2 hours at 4 $^{\circ}$ C, with an intermediate wash with reaction buffer (3 x 10 min). The third aliquot (c) was incubated three times with 50 μ L of a 0.6 mg/mL solution of DSG in DMF for 2 h at 4 $^{\circ}$ C, with two intermediate washes with fresh reaction buffer (3 x 10 min). The fourth aliquot (d) was used as a control. After reaction, the three aliquots (a), (b), and (c) were washed with washing buffer. After elution with 0.6 mL of 0.1% diethylpropylcarbonate (DEPC; Sigma-Aldrich) water containing 1mM DTT, the mRNA-peptide fusions were incubated with RNase (Life Technologies) (5% of reaction volume) for 3 h at room temperature, with DNase (Life Technologies) (5% of reaction volume) for 3 h at room temperature, and then purified using ProBondTM Ni-NTA resin (Life Technologies). The peptide-puromycin linker fusions were

then analyzed and purified by RP-HPLC using a Waters 626 LC system integrated with 2487 UV detectors (Milford, MA, USA). A volume of 50 μ L of each sample was injected into a C18 column (Alltech Atlantis® C18 3 μ m, 2 x 150 mm column) and run over a linear gradient from 10:90 to 90:10 MeCN:Water. The absorbance was monitored at 254 nm. Finally, mass spectrometry (MS) analysis of the samples was finally performed using a Q-ToF Premier mass spectrometer (Waters).

8.2.3. Peptide cyclization on the mRNA-display library via fed-batch crosslinking reaction on “solid-phase format”

The equilibration and washing of the oligo-dT cellulose was performed as described earlier. A volume of 0.75 mL of mRNA-display library of linear peptides was incubated with oligo-dT cellulose (100 mg cellulose per mL of solution) for 2 hours at 4°C. After rinsing, the cellulose was resuspended in 0.8 mL of reaction buffer and incubated for two times with 50 μ L of a 3.0 mg/mL solution of DSG in DMF for 2 hours at 4°C, with an intermediate wash with fresh reaction buffer. After reaction, the cellulose was washed with dT washing buffer (3 x 10 min). The library was then eluted with 0.6 mL of 0.1% DEPC water containing 1mM DTT. Next, the library was incubated with 25 μ L of 100 μ M reverse transcription primer (5'-TTT TTT TTT TNN CCA GAT CCA GAC ATT CCC AT-3'; IDT) for 15 min at room temperature. After addition of 200 μ l 5X first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂), 50 μ l 10 mM dNTPs, 100 μ l 0.1 M DTT, and 0.1% DEPC water to adjust the volume to 995 μ l, reverse transcription was performed by incubating with

5 μl SuperscriptTM reverse transcriptase (200 U/ μL ; Life Technologies) for 50 min at 42 °C. EDTA to a final 6 mM concentration was added to the reaction mixture and incubated for 5 min at room temperature. The cDNA-mRNA-cyclic peptide library was desalted using a Nap-10 desalting column (GE Healthcare, Piscataway, NJ) and finally purified using a Ni-NTA agarose beads (Qiagen, Valencia, CA) followed by desalting with a Nap-5 column (GE healthcare). The final volume of the mRNA display library was 880 μL . The estimated number of mRNA-cDNA-peptide fusions based on A_{260} measurement was 4×10^{12} .

8.2.4. Library screening and sequence identification

DynalTM biotin binder magnetic beads (Life Technologies) were incubated with biotinylated hFc (Jackson ImmunoResearch, Westgrove, PA) in PBS-BSA-sssDNA (Phosphate Buffered Saline with additional NaCl to get a final NaCl concentration of 0.2 M, pH 7.4, 0.1% Bovine Serum Albumin (BSA; Sigma-Aldrich), 0.1% sheared salmon sperm DNA (sssDNA, Ambion, Austin, TX), 0.1% Tween-20), overnight at 4°C to generate hFc-coated beads. First, a negative selection with plain DynalTM beads was performed by incubating the entire mRNA-display library with 10^8 magnetic beads for 1 hour at 4°C under gentle rotation. Supernatant from the negative selection step was mixed with hFc-coated magnetic beads and the mixture was incubated for 1 hour at 4°C with gentle rotation. Subsequently, beads were separated from the supernatant by a magnetic particle concentrator (Life Technologies) followed by a wash with 500 μl PBS-BSA-sssDNA. cDNA was eluted using 2x200 μl 0.15 M KOH at room temperature with shaking for 1 hour and the eluate was

neutralized by 5 N HCl. cDNA was precipitated using linearized acrylamide as previously described [46] and amplified by PCR. The PCR product was then used as a template to make a second mRNA display library of cyclic peptides (round 2), as described before. The round 2 mRNA display library was subjected to a negative selection step with Dynal™ biotin binder beads by incubating beads in a mixture of the library, 1 mL cMEM (complete mammalian cell culture medium prepared by combining essential MEM medium with 10% fetal calf serum and 5% tryptose broth phosphate) and 1ml PBS-BSA-sssDNA. Supernatant from the negative selection was mixed and incubated hFc-coated beads for 1 hour at 4°C with gentle rotation in a 15 ml conical tube. Subsequently, the beads were separated from the supernatant, washed twice with 1ml PBS-BSA-sssDNA, twice with 1 ml 0.2M potassium acetate, pH 5.0 followed by a wash with 1ml PBS-BSA-sssDNA. cDNA was isolated as described before and amplified by PCR. The PCR product was cloned into NovaBlue *E. coli* cells (EMD Biosciences, Merck, Germany) using CloneJet™ PCR cloning kit (Fermentas, Glen Burnie, MD) and DNA sequencing was carried out to identify the selected peptides.

8.2.5. Solid-phase peptide synthesis and cyclization on chromatographic resin

The selected peptides, namely MHGPRGK, MHGWRGK, MKGSFNK, MWFPHYK, MWFRHYK, and MWFKHYK were synthesized on 100-150 micron diameter Toyopearl AF-Amino-650M resin (substitution level of 0.4 meq/g) (Tosoh Bioscience, King of Prussia, PA). The reagents for peptide synthesis, Fmoc-protected amino acids, and the coupling agents were purchased from ChemPep Inc. (Wellington, FL, USA). DMF, dichloromethane

(DCM), 4-dimethylaminopyridine (DMAP), N,N-diisopropylethylamine (DIPEA), ethanedithiol (EDT), triisopropylsilane (TIPS), piperidine (99%), acetic anhydride, acetonitrile (HPLC grade), trifluoroacetic acid (TFA; HPLC grade) and indole were obtained from Sigma-Aldrich.

Each coupling step was conducted for 25 min in a polypropylene tube fitted with a Teflon frit under continuous nitrogen flow and the temperature was maintained at 35°C. The synthesis of each sequence was started with 250 mg of resin and consisted of the following steps: (i) two couplings were performed with 3 eq. (molar excess as compared to the density of amino groups) of Fmoc-Lys(Alloc)-OH, 3 eq. of O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) and 6 eq. of DIPEA in 2.5 mL of dry DMF. The Fmoc protection was then removed by incubating the resins with 5mL of 20% piperidine in DMF solution for 30 min. The linear peptide sequences were synthesized via conventional Fmoc/tBu strategy. An anhydrous DMF solution (2.5 mL) of 3 eq. protected amino acid, 3 eq. HCTU and 6eq. DIPEA was added to the resin. Two couplings were performed for each amino acid to saturate all the available amino groups, as monitored by a Kaiser test. Mono-allyl protected glutaric acid, prepared by reacting allyl alcohol with glutaric anhydride in presence of DMAP, was coupled on the peptide N-terminus by two couplings using 3 eq. HCTU and 6 eq. DIPEA in dry DMF for 45 minutes. The allyloxycarbonyl protection on the ϵ -amino group of lysine and the allyl ester protection on the carboxyl group on glutaric acid were removed with 0.1 eq. of Pd(PPh₃)₄ and 10 eq. phenylsilane as a scavenger in DCM. To remove the residual palladium catalyst, the resin was rinsed with 0.02 eq. of sodium diethyldithiocarbamate in DMF (3 x 15 min). A Kaiser

test was carried out to confirm the presence of free amino groups. The peptide cyclization was performed by coupling the carboxyl group of glutaric acid to the ϵ -amino group of lysine using 3 eq. HATU and 6 eq. DIPEA in dry DMF for 45 minutes. A second coupling was performed to ensure completion of reaction. A second Kaiser test was carried out to confirm the absence of free amino groups. Finally, the peptides were deprotected using a cleavage cocktail containing TFA/TIPS/H₂O/EDT (94/3/2/1) for 1.5 hours.

8.2.6. Chromatographic characterization of the selected sequences in non-competitive conditions

All chromatographic runs were performed on a Waters 626 LC system integrated with 2487 UV detectors. 35 mg of cyclic peptide resin were dry-packed in 30 mm x 2.1 mm I.D. microbore columns (0.1 mL) (Altech-Applied Science, Somerset, PA). The resin was swollen with 20% v/v methanol and then equilibrated with PBS, pH 7.4. A 100 μ L of 5mg/mL IgG in PBS was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 4 mL of 0.2M NaCl in PBS at flow rate of 0.2 mL/min (346 cm/h). Elution was then performed with 4 mL of 0.2 M acetate buffer pH 4 at a flow rate of 0.4 mL/min (692 cm/h). The column was finally regenerated with 0.85% v/v phosphoric acid at the flow rate of 0.2 mL/min (346 cm/h) for 10 min. The effluent was monitored by UV absorbance at 280 nm.

8.2.7. Binding of mammalian IgG and human IgG fragments

35 mg of cyclo[*Link*-M-WFRHY-K]-Toyopearl resin were dry packed in a 0.1 mL column, swollen and washed as described above. After washing, the resins were equilibrated with PBS at flow rate of 0.2 mL/min (346 cm/h). Samples of 100 μ L of Fc and Fab fragments of human IgG, as well as whole IgG from human (Equitech-Bio, Kernville, TX), mouse, rabbit, chicken, and cow (Sigma-Aldrich), all at the concentration of 1 mg/mL, were loaded onto the column at flow rate of 0.05 mL/min (87 cm/h). Chromatographic cycles were performed as previously described.

8.2.8. Determination of the binding capacity and K_D of cyclic peptide adsorbent

The adsorption isotherm of the adsorption cyclo[*Link*-M-WFRHY-K]-Toyopearl resin (peptide density of 0.09 meq/g) was measured in a batch mode at room temperature. Six aliquots of 10 mg of resin each were taken in 1.5 mL microcentrifuge tubes, swollen in 20% v/v methanol and equilibrated with PBS at pH 7.4. Human IgG solutions (400 μ L) with concentrations ranging from 0.5 to 8 mg/mL in PBS were added separately to the resin aliquots and incubated with gentle rotation for 30 min. The samples were centrifuged, and the supernatants were collected and analyzed by UV absorbance at 280 nm to determine the protein concentration. The amount of bound hIgG was calculated by mass balance. The data were fit to a Langmuir isotherm model $q = \frac{q_m C}{K_D + C}$ 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.

8.2.9. Purification of monoclonal antibodies from cell culture supernatants

Two CHO cell culture supernatants containing monoclonal antibodies (MAb), a chimeric IgG₁ and a humanized IgG₄, were obtained from two different biopharmaceutical manufacturers that asked for confidentiality. 35 mg of cyclo[*Link*-M-WFRHY-K]-Toyopearl resin were dry packed in a 0.1 mL column, swollen and washed as described above. The effect of salt concentration in the equilibration and binding buffers was studied at 0 M, 0.1 M, 0.2 M, 0.3 M, and 0.5 M NaCl in PBS. After equilibrating the column with the binding buffer at flow rate of 0.2 mL/min (346 cm/h), 100 µL of CHO cell culture supernatant containing IgG₁ or IgG₄ was loaded at flow rate of 0.05 mL/min (87 cm/h). The unbound proteins were washed from the column by using 4 mL of equilibration buffer. Elution was carried out by using 4 mL of 0.2 M sodium acetate buffer, pH 4. Cleaning was performed by 4 mL of 0.85% (v/v) phosphoric acid and regeneration was done by 4 mL of 2 M urea in sodium acetate pH 4 buffer. Elution, cleaning and regeneration were all performed at a flow rate of 0.4 mL/min (692 cm/h). Fractions were collected and concentrated five times by centrifugation at 4°C, 20817 x g for 30 min using Amicon Ultra centrifugal filter (3000 MWCO) (Millipore, King of Prussia, PA), and then used for analysis of IgG purity and yield.

8.2.10. Quantification of IgG yield and purity

The amount of IgG in the collected fractions was quantified by HPLC using a 1 mL HiTrap Protein G column (GE Healthcare). The yield of IgG was calculated as the ratio of IgG eluted to total IgG loaded. The purity of IgG in the eluted fractions was determined by SDS-PAGE under reducing conditions, as described by Yang et al. [47]. The IgG purity was determined by densitometric analysis of Coomassie-stained gels by means of ImageJ 1.32j software (National Institute of Health, Bethesda, MD).

8.3. Results

8.3.1. Synthesis of an mRNA display library of cyclic pentapeptides

Millward *et al.* have previously described the cyclization of peptides in an mRNA display format by a liquid-phase crosslinking reaction between two primary amine groups using a homobifunctional linker [43]. However, this approach suffers from low yields (30-50%) of cyclic peptides, and can result in undesired byproducts due to poly-modification and intermolecular crosslinking [42,43]. To overcome these limitations, we developed an alternative “solid-phase format” protocol. The cyclization of the peptide moiety on each fusion was performed after adsorbing the mRNA-peptide fusions on solid phase, hence mimicking the cyclization of peptides performed on solid-phase via organic synthesis (Figure 8.1).

Since the concentration of mRNA-peptide fusions in solution is in the range of 10^{-7} M, the adsorption of 0.2 mL of fusion solution to 20 mg of oligo-dT cellulose leads to an

approximate peptide density of $\sim 10^{-4}$ meq/g. Note that this value is much lower than the functional density characteristic of the resins employed for peptide synthesis. We hypothesized that such spatial segregation on the solid phase will prevent the intermolecular contact between the fusions and consequently exclude the formation of intermolecular crosslinking byproducts, thereby leading to high yield and selectivity of the cyclization reaction. To rigorously test this hypothesis and identify optimal conditions for solid-phase peptide cyclization, we conducted studies on cyclization of a model peptide with the sequence MVVFVVK. We evaluated three different strategies for peptide cyclization – a single step reaction and fed-batch reactions using two or three steps. The single step cyclization reaction was carried out for 2 h at 4°C by reacting the peptide on the solid phase with 50 μ L of 2.3 mg/mL DSG in DMF. Two fed-batch reactions, one in two steps with an intermediate wash step, and one in three steps with two intermediate wash steps, were performed with the same volume (50 μ L) of 1.2 mg/mL and 0.6mg/mL DSG in DMF respectively. The intermediate washes were performed with fresh crosslinking buffer, free of crosslinker. All reactions were terminated by washing the solid phase with a Tris containing buffer, which both quenches the reaction and washes the final products. After elution, the nucleotide portions of the fusions were digested sequentially with RNase and DNase and finally, the cyclic peptides were purified by Ni-NTA chromatography.

The products of the single step reaction and the two fed-batch reactions as well as the control linear peptide were analyzed by RP-HPLC. The corresponding chromatograms are presented in Figure 8.2. Fractions collected during the chromatographic runs were further analyzed by ESI-MS for molecular identification; the resulting spectra are shown in Figure

8.3. Note that removal of the nucleotide portions from both linear and cyclic peptides was necessary to ensure clarity of RP-HPLC and ESI-MS analysis. The comparison between the chromatograms in Figure 8.2A (single-step cyclization) and Figure 8.2D (unreacted peptide MVVFFVVK) indicates that the single-step conversion of the linear product was very high. Peak 2a-1, which corresponds to the unreacted peptide in the single step reaction, is much smaller than peak 2d-1 associated with the control linear peptide. However, the plurality of peaks appearing in Figure 8.2A also suggests that this reaction leads to the formation of undesired by-products. The MS analysis of the collected peaks showed that peak 2a-2 corresponds to the desired cyclic peptide (predicted MW = 2671 Da; measured MW = 2680.3 Da); peak 2a-3 is due to a bis-modified product resulting from the coupling of two DSG molecules with the ϵ -amino group on lysine and the N-terminus (predicted MW = 2803 Da; estimated MW = 2812.2 Da). While the formation of the bis-modified product was clear, the presence of non-cyclic mono-acylated product was not detected. As clearly indicated by Figures 8.2B and 2C, selectivity of the two-step and three-step fed batch reactions is much higher than that of the single step reaction. Bis-modified product was considerably lower in the two-step reaction (peak 2b-2) and could not be detected in the product of the three-step reaction. However, cyclic peptide yield is the lowest in case of the three-step reaction. Although low crosslinker concentration is beneficial for product purity, it does not provide a satisfactory cyclic peptide yield. Approximate values of reaction yield and selectivity are presented in Table 8.1.

Taken together, our results show that solid-phase peptide cyclization provides improved yield and selectivity of mRNA-cyclic peptide fusions over the previously reported

liquid-phase cyclization scheme [43]. The two-step fed-batch reaction offers the best compromise between cyclic peptide yield (87.2 %) and purity (94.4 %).

8.3.2. Library screening to identify binders to hFc

We constructed an mRNA display library of cyclic pentapeptides using solid-phase cyclization with a two-step fed batch reaction, as discussed earlier. Binders to hFc were isolated from this library using biotinylated hFc immobilized on streptavidin-coated magnetic beads as the target. Note that the selection was carried out in the presence of excess DNA (sssDNA), in cell culture medium (cMEM) containing a wide spectrum of serum proteins. Also, a negative selection step with naked magnetic beads was included to eliminate streptavidin-binding peptides, prior to selection of hFc binders.

Sequences of hFc-binding peptides isolated were identified by DNA sequencing (Table 8.2). Strikingly, candidate sequences are enriched in histidine, aromatic (phenylalanine, tyrosine and tryptophan), and basic (arginine and lysine) amino acids. These findings are in agreement with our previous studies that identified a family of highly homologous linear peptide ligands for hFc containing histidine, aromatic and basic amino acids [47].

8.3.3. Chromatographic characterization of cyclic peptide adsorbents generated by on-resin solid-phase peptide synthesis and cyclization

We sought to evaluate the binding of human IgG to resins containing immobilized cyclic peptides in order to confirm their hFc-binding activity, and to identify the best candidate ligand for further chromatographic analysis. The strategy that is generally adopted for the preparation of cyclic peptide adsorbents involves several steps: synthesis and purification of the precursor linear peptide, its cyclization in liquid phase, purification of the cyclic peptide and finally its coupling on a solid-phase support [43]. In contrast, we developed a method wherein peptide synthesis and cyclization is performed directly on the chromatographic matrix; we adopted the polymethacrylate-based Toyopearl AF-Amino-650M resin. This resin is chemically stable to the solvents and reagents employed in peptide synthesis, mechanically resistant to the flow conditions employed in chromatography, and provides low non-specific binding of proteins and high functional density [48]. It is important to note that resins prepared using our on-resin peptide synthesis approach can withstand harsh alkaline conditions that are typical of column cleaning and regeneration processes [45].

Our approach is summarized in Figure 8.4A. The synthesis of the linear precursor begins with the coupling of N_{α} -Fmoc- N_{ϵ} -Alloc protected Lysine. No spacer arm was introduced before coupling Lysine, as the resin already has a built-in (PEG)_n spacer. The special protecting group on the ϵ -amino group of Lysine, i.e. allyloxycarbonyl, is orthogonal to the cleavage of Fmoc and the acid labile protecting groups on the following amino acid residues [49]. The peptide sequences are then synthesized on Toyopearl amino resin by

conventional Fmoc/tBu coupling chemistry. In place of DSG, a structurally equivalent linker, mono-allyl glutarate, is prepared by reaction of allyl alcohol with glutaric anhydride in DMAP and used for peptide cyclization. The linker is coupled directly on the N-terminus of the peptide sequence. Then, both the allyloxycarbonyl protection on the ϵ -amino group of lysine and the allyl ester protection on the glutaric acid linker are removed in minutes with a Pd-based catalyst. The peptide is finally cyclized and deprotected in acidolytic conditions. The use of mono-allyl ester protected glutaric acid allows us to exactly reproduce the sequence and structure of each cyclic peptide, as it was on the mRNA-peptide fusion (Figure 8.4B).

We evaluated cyclic peptide resins prepared using the approach described above, corresponding to peptide sequences in Table 8.2, in the context of binding to human IgG under non-competitive conditions. 100 μ L of 5 mg/mL IgG in PBS was injected at low linear velocity (87 cm/h) in a column packed with the cyclic peptide resin. Toyopearl resin with immobilized Protein A was used as a control. The chromatograms for these experiments are shown in Figure 8.4C. The IgG recovery, calculated as the ratio between the mass of protein eluted and the mass of protein loaded, is reported in Table 8.3. All cyclic peptide resins evaluated showed binding to IgG. However, the fractional recovery of IgG varied significantly (17-94%). Based on our studies, we identified cyclo[*Link*-M-WFRHY-K] as the best cyclic peptide ligand. The fractional recovery of IgG by cyclo[*Link*-M-WFRHY-K] was comparable to that of Protein A in our studies. However, IgG was eluted from the cyclo[*Link*-M-WFRHY-K] resin at pH 4.0, in much milder conditions than that required for Protein A media (pH 2.5). The use of gentle conditions is a significant advantage of the

peptide adsorbent, as it avoids the risk of protein aggregation. Cyclo[*Link-M-WFRHY-K*]-Toyopearl was selected for further characterization.

8.3.4. Characterization of binding affinity, capacity and specificity for cyclo[*Link-M-WFRHY-K*]-Toyopearl

We obtained the adsorption isotherm for binding of cyclo[*Link-M-WFRHY-K*]-Toyopearl to human IgG at room temperature (Figure 8.5A). The apparent equilibrium dissociation constant (K_D) and maximum equilibrium binding capacity (q_m) were determined as 7.6×10^{-6} M and 19.7 mg/mL respectively, by fitting the data to the Langmuir adsorption isotherm. By comparison, in previous studies, the K_D and q_m values for resins containing linear hexamer peptides with similar ligand densities (1.0 meq/g vs. 0.09 meq/g for the cyclic peptide in this study) are in the range 1.0×10^{-5} - 2.6×10^{-5} M and 27.0 - 33.6 mg/mL respectively [50]. The K_D of the cyclic peptide adsorbent lies in the normal range of synthetic ligands for affinity chromatography [51-54]. Notably, this value is higher than that of Protein A (10^{-7} - 10^{-8} M) [55]; the higher K_D may explain the elution of IgG from cyclo[*Link-M-WFRHY-K*]-Toyopearl at pH 4.0, as opposed to pH 2.5 for Protein A media. Also, the binding capacity may be increased by optimizing the density of immobilized cyclic peptide.

To evaluate the specificity of cyclo[*Link-M-WFRHY-K*]-Toyopearl, we first tested the selectivity for the Fc and Fab fragments of human IgG. After column equilibration, 100 μ L of 1 mg/mL IgG fragment solution in PBS was loaded onto the column at low linear velocity (87 cm/h) and eluted at pH 4.0. For comparison, 100 μ L of 1 mg/mL whole human

IgG solution in PBS was loaded and eluted in the same conditions. The chromatograms in Figure 8.5B clearly show that cyclo[*Link-M-WFRHY-K*] is highly specific for the Fc fragment of IgG. While the Fc fragment and the whole IgG were completely bound (~ 97%), the Fab fraction showed almost no binding (< 1%).

We also tested cyclo[*Link-M-WFRHY-K*]-Toyopearl for binding of IgG from other mammalian species, namely mouse, rabbit, chicken, goat, and cow. 100 µL of a 10 mg/mL solution of IgG in PBS was loaded at low linear velocity and eluted with 0.2 M acetate buffer pH 4. The chromatograms in Figure 8.5C show that the ligand has affinity for IgG from all the listed species. This is a significant advantage over Protein A, which shows weak binding to mouse IgG₁ and bovine IgG₁, and does not bind goat and chicken IgG.

8.3.5. Purification of monoclonal antibodies from CHO cell culture supernatants using cyclo[*Link-M-WFRHY-K*]-Toyopearl

The cyclo[*Link-M-WFRHY-K*]-Toyopearl resin was used for the purification of two mAbs – a chimeric antibody of IgG₁ subclass and a humanized antibody of IgG₄ subclass – from clarified CHO cell culture fluids used in a previous study [56]. The concentrations of the two mAbs were 2.3 mg/mL and 1.5 mg/mL respectively. The supernatants contain antifoam agents (pluronic acid), amino acids and peptides, sugars, host cell proteins and residual nucleic acids, and other cellular components; they were directly loaded onto the column without any pre-treatment.

Several cycles of antibody purification from these cell culture supernatants were performed using different salt concentration in the equilibration and binding buffer (0M, 0.1M, 0.2M, 0.3M and 0.5M NaCl in PBS). The chromatograms and the corresponding SDS-PAGE analyses of the collected fractions are shown in Figure 8.6 and Figure 8.7. Both MAbs were eluted from the cyclo[*Link*-M-WFRHY-K]-Toyopearl column with high yield using 0.2M acetate buffer at pH 4. It is also evident that the presence of salt in the equilibration and binding buffer has only a minor influence on product yield and purity, which was $96\% \pm 2\%$ and $93\% \pm 3\%$ respectively for both IgG₁ and IgG₄. Also, Figure 8.6B, shows the presence of the light chain in the flow through fractions. This is most likely due to an overexpression of light chain over heavy chain of the IgG₁ by the CHO cells. As discussed earlier, the peptide ligand is highly specific for the Fc fragment comprising only the heavy chain; the light chain does not bind the column.

These results are comparable with those previously obtained with Protein G [56]. It should be noted that, unlike MAbsorbent A2P [57], the binding of the cyclic peptide ligand is not affected by the presence of pluronic acid and other surfactants. Taken together, our results show that cyclo[*Link*-MWFRHY-K]-Toyopearl is an effective adsorbent for the purification of MAbs from complex cell culture supernatants.

8.4. Conclusions

In this study, we have demonstrated the discovery, synthesis and characterization of novel cyclic peptide affinity ligands for hFc. We developed a strategy for solid-phase

cyclization of mRNA-peptide fusions that results in high yield (87.2%) and purity (94.4%). Using this approach, we constructed an mRNA display library of cyclic pentapeptides and screened the library to isolate binders to hFc. Subsequently, we prepared polymethacrylate-based resins containing specific cyclic peptides identified by mRNA display. Notably, we developed a novel approach wherein peptide synthesis and cyclization are carried out directly on the chromatographic resin, without the need for separate purification, cyclization and coupling. We have previously shown that resins prepared by on-resin peptide synthesis as described here are alkaline-stable [45].

Studies with non-competitive IgG-binding on a set of cyclic peptide resins allowed us to identify the peptide cyclo[*Link*-M-WFRHY-K] as the best candidate for further characterization.

Further studies on the cyclo[*Link*-M-WFRHY-K] resin showed that cyclo[*Link*-M-WFRHY-K] binds specifically to the Fc portion of IgG. Additionally, unlike Protein A, cyclo[*Link*-M-WFRHY-K] shows binding activity to immunoglobulins from various mammalian species. Importantly, while the recovery of IgG using the cyclo[*Link*-M-WFRHY-K] resin was comparable to a Protein A conjugated resin, elution in case of the peptide resin could be achieved under mild conditions (pH 4). By contrast, Protein A media require significantly harsher conditions for elution (pH 2.5) that may result in aggregation of IgG or loss of bioactivity. Equilibrium adsorption measurements determined the adsorbent maximum capacity and the K_D as 19.7 mg/mL and 7.6×10^{-6} M respectively. The ability to elute IgG from the peptide resin under mild conditions may be attributed to the relatively

high K_D ; by comparison, the corresponding K_D for Protein A is in the range of $10^{-7} - 10^{-8}$ M [58]. Finally, the cyclo[*Link*-M-WFRHY-K] resin was successfully used for the purification of two monoclonal antibodies – a chimeric IgG₁ and a humanized IgG₄ – from industrial CHO cell culture supernatants, with high product yield ($96\% \pm 2\%$) and purity ($93\% \pm 3\%$). Therefore, cyclo[*Link*-M-WFRHY-K] is a promising candidate for development of a cost-effective and robust chromatographic resin to purify mAbs from complex mixtures. Cyclic peptide binders to other targets of interest can be easily isolated using our overall approach. Thus, in summary, our work presents a quick and efficient procedure for the discovery and characterization of cyclic peptide affinity ligands.

8.5. Acknowledgments

The authors thank Dr. Robert K. Blackburn for his help with the MS analysis of the samples and gratefully acknowledge funding from the National Science Foundation (NSF; grant CBET-0853771) and Biotechnology Training and Education Center (BTEC) at North Carolina State University.

8.6. References

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Table 8.1. Cyclization reaction yield and selectivity.

Reaction mode	Yield	Selectivity
Single step	92.8 %	64 %
Two step fed batch	87.2 %	94.4 %
Three step fed batch	60.5 %	~ 100 %

Table 8.2. Peptide leads identified from screening of the mRNA display library of cyclic peptides.

#	Sequence
1	MHGPRGK
2	MHGFRGK
3	MKGSFNK
4	MWFPHYK
5	MWFRHYK
6	MWFKHYK

Table 8.3. IgG binding in non-competitive conditions by cyclic peptide resins (linear sequences only are listed) and Protein A-Toyopearl resin.

Sequence	IgG recovery^a
MHGPRGK	18%
MHGFRGK	82%
MKGSFNK	17%
MWFPHYK	89%
MWFRHYK	94%
MWFKHYK	67%
Protein A	95%

^aIgG recovery is defined as the ratio of the amount of IgG eluted from the column to the amount loaded on the column.

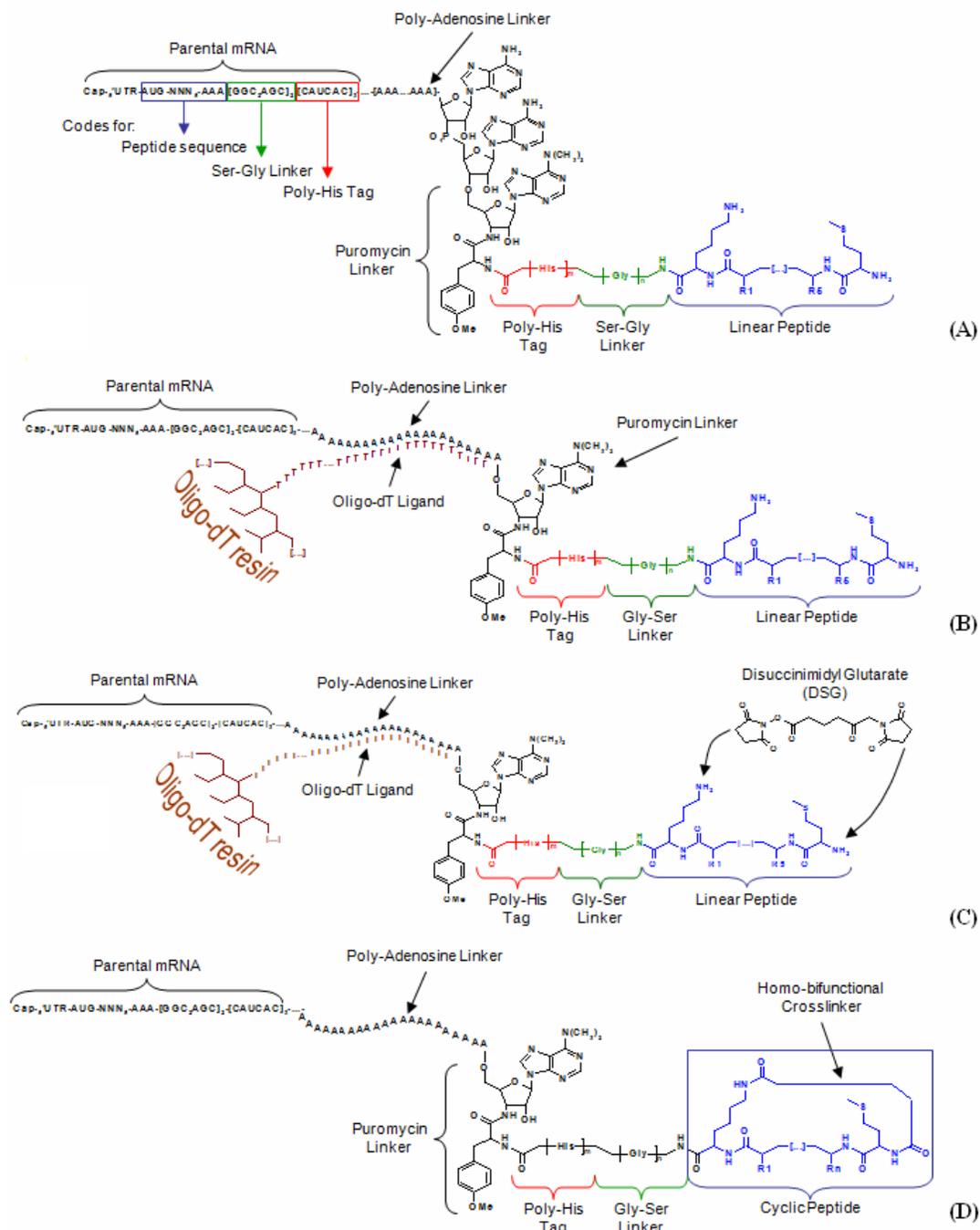


Figure 8.1. Synthesis of the mRNA-display library of cyclic peptides: (A) mRNA-linear peptide hybrid resulting from *in vitro* translation; (B) adsorption of the mRNA-peptide hybrid through its poly-A linker onto oligo-dT solid phase; (C) solid-phase peptide cyclization by crosslinking reaction with disuccinimidyl glutarate (DSG); (D) mRNA-cyclic peptide hybrid obtained after desorption.

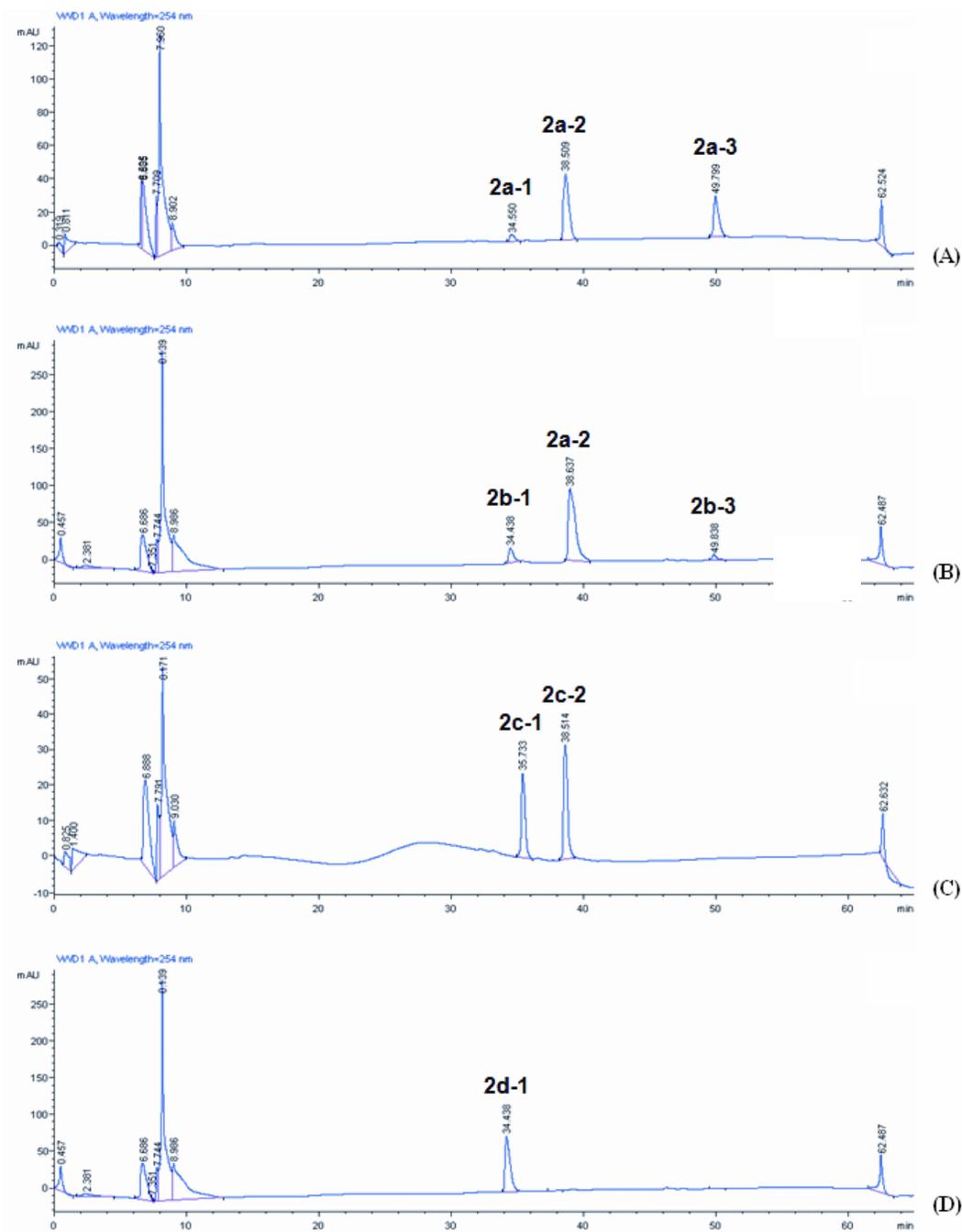


Figure 8.2. RP-HPLC (C18) analyses of the products of the solid-phase crosslinking reactions performed with DSG on the mRNA-peptide hybrids carrying the sequence MVVFVVK: (A) single-step reaction with 50mL of 2.3mg/mL DSG in DMF; (B) two-step reaction with 50mL of 1.2mg/mL DSG in DMF; (C) three-step reaction with 50mL of 0.6mg/mL DSG in DMF; (D) Linear peptide. All reactions were carried out for 2h at 4°C. For clarity of results, the nucleotides were removed enzymatically before RP-HPLC analysis.

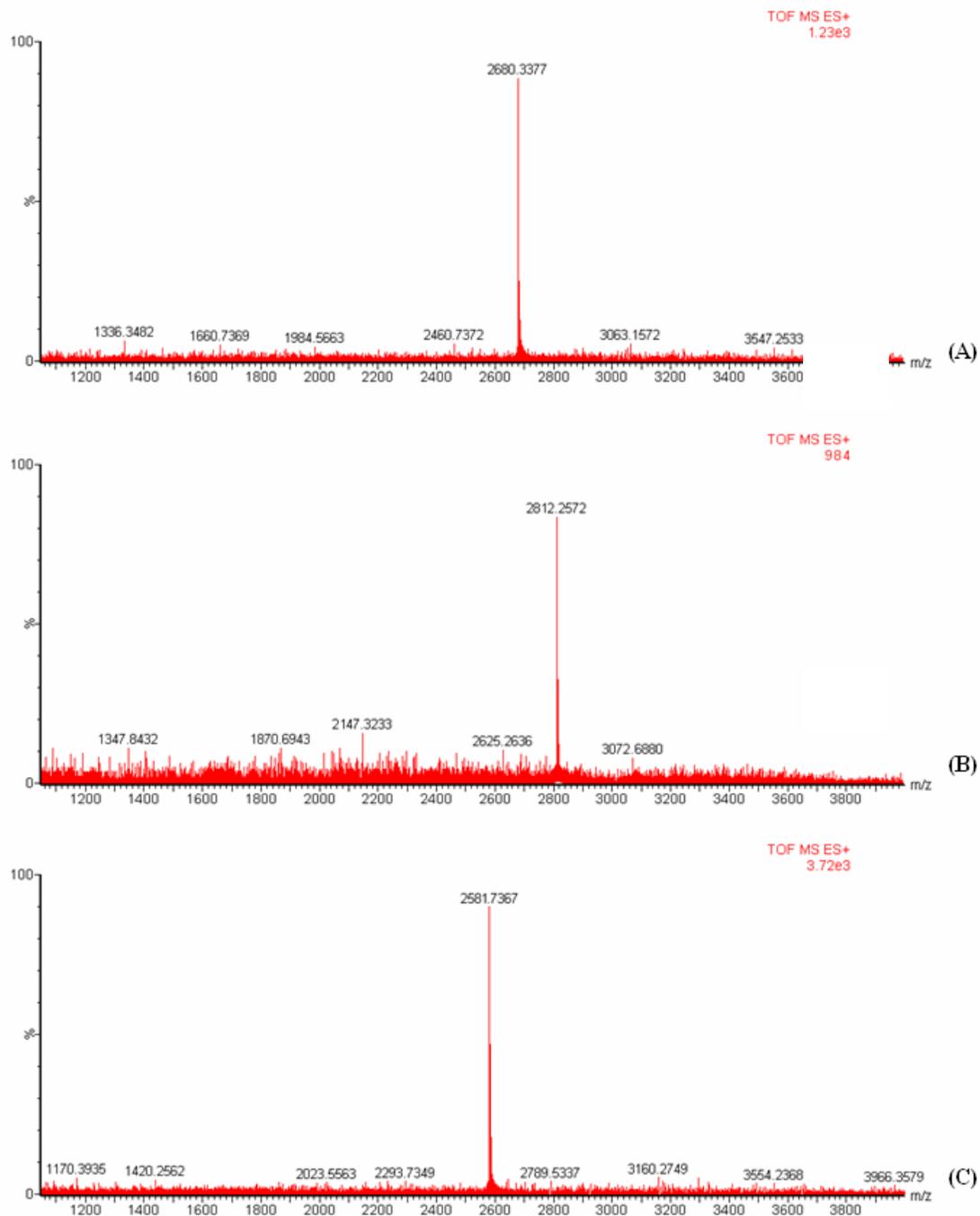


Figure 8.3. ESI-MS analyses of the peaks collected from RP-HPLC of the products of the solid-phase crosslinking reactions: a) cyclic peptide (peak 2a-2 in Figure 2; predicted Mw = 2671, measured Mw = 2680.3); (B) bis-modified peptide (peak 2a-3 in Figure 2; predicted Mw = 2803, measured Mw = 2812.2); c) linear peptide (peak 2d-1 in Figure 2; predicted Mw = 2575, measured Mw = 2581.7).

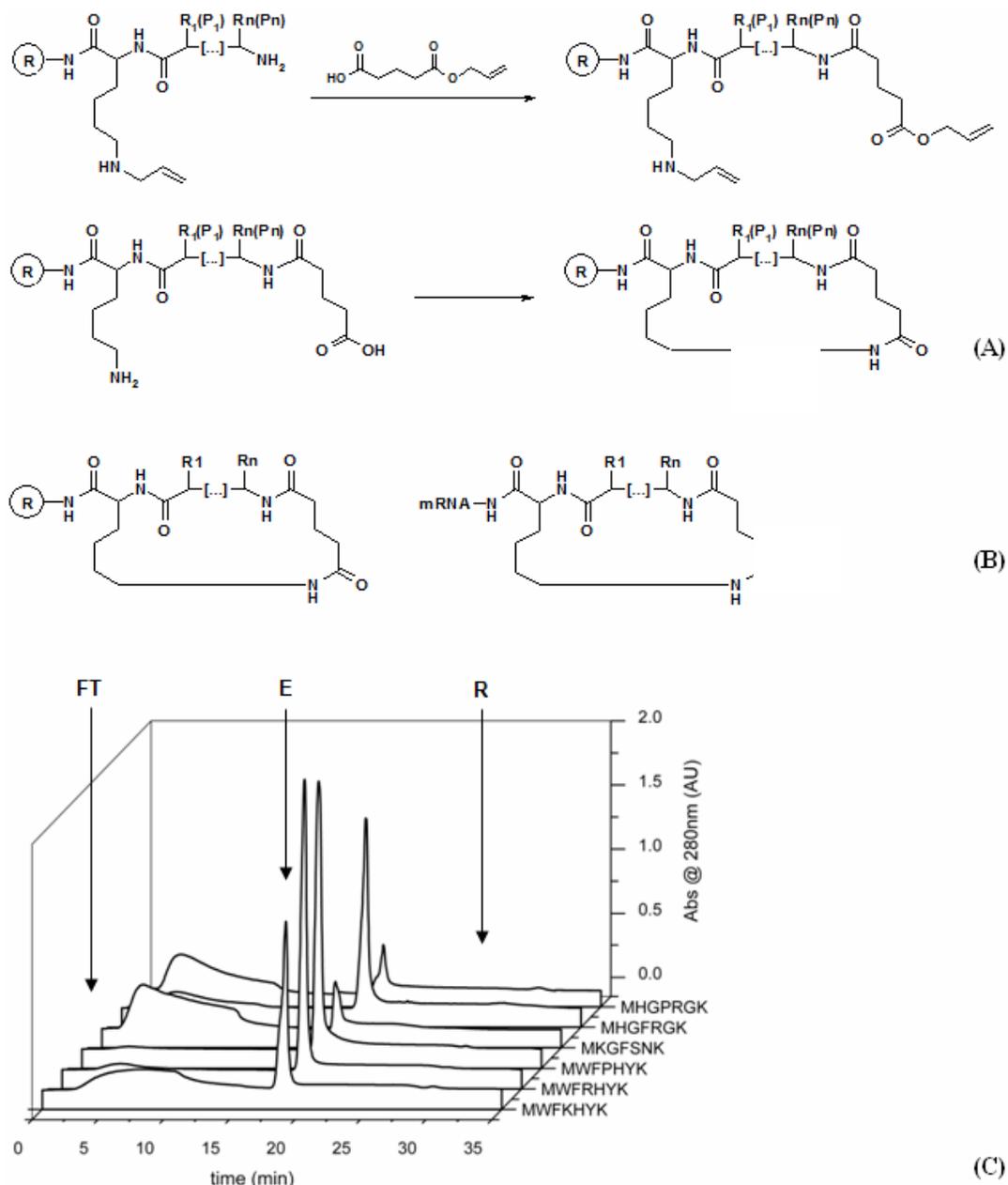


Figure 8.4. (A) Protocol of peptide synthesis and cyclization on a chromatographic resin. Allyl glutarate is coupled on the N-terminus of the peptide Lys(Alloc)-X₁-X₂-X₃-X₄-X₅. Subsequently, the allyl protection is removed and peptide cyclization is carried out. (B) comparison between the structures of the cyclic peptide synthesized on the chromatographic resin and the cyclic peptide hybridized with its parental mRNA; (C) chromatographic comparison of the selected sequences by IgG binding. FT – flowthrough, E - elution, R – regeneration.

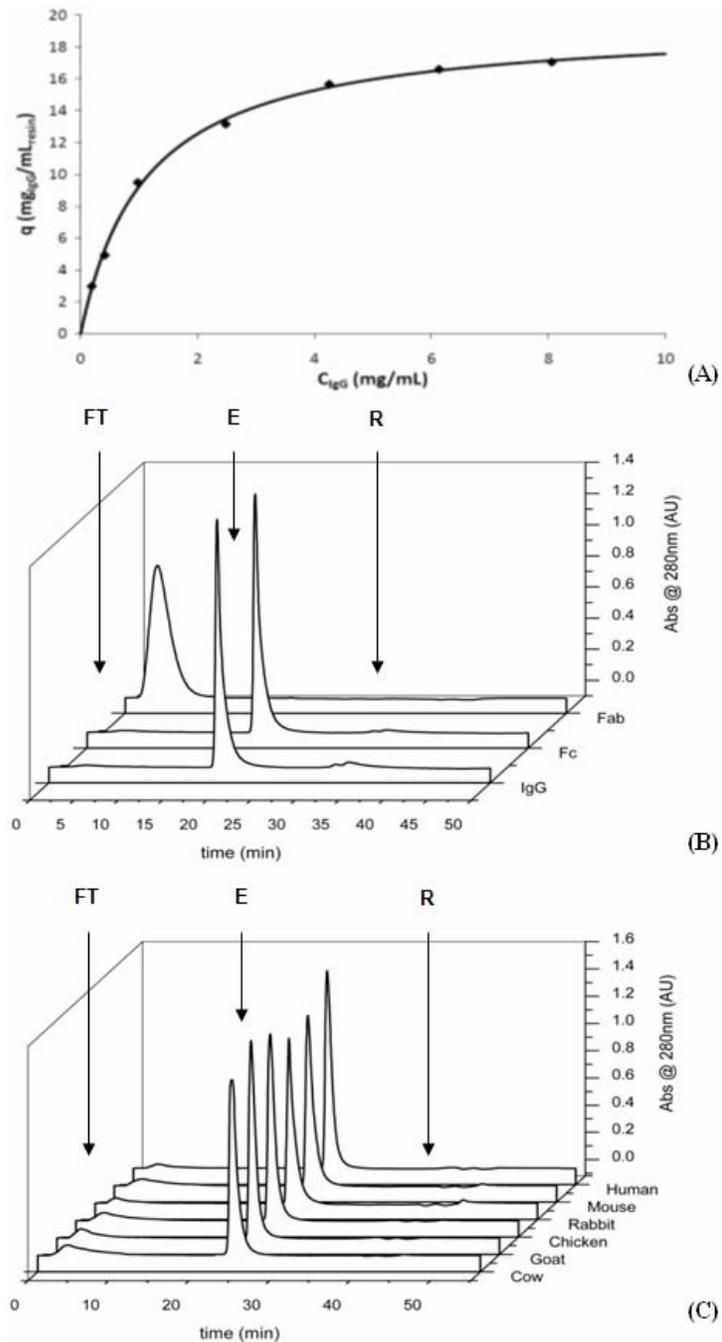


Figure 8.5. Characterization of the adsorbent cyclo[Link-M-WFRHY-K]-resin: (A) adsorption isotherm (resulting $K_D = 7.6 \times 10^{-6}$ M and $q_m = 19.7$ mg/mL); (B) binding specificity towards the Fc (97%) and Fab (~1%) fragments of IgG; (C) binding of different mammalian IgGs (human, mouse, rabbit, chicken, goat, and cow). Labels: FT – flowthrough, E - elution, R – regeneration.

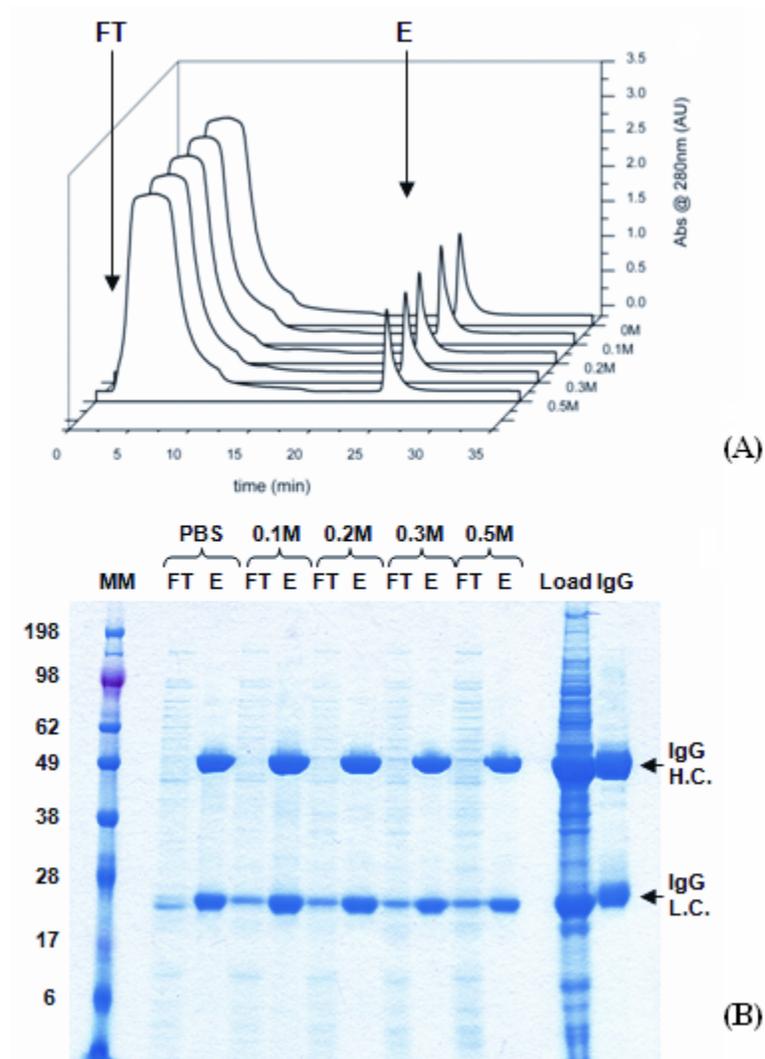


Figure 8.6. (A) Chromatograms of purification of IgG₁ from CHO cell culture supernatant (2.3 mg/mL) under different concentration of sodium chloride in the equilibration and binding buffer using cyclo[Link-MWFRHYK]-Toyopearl resin; (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM – molecular weight marker, FT – flowthrough, E – elution fraction.

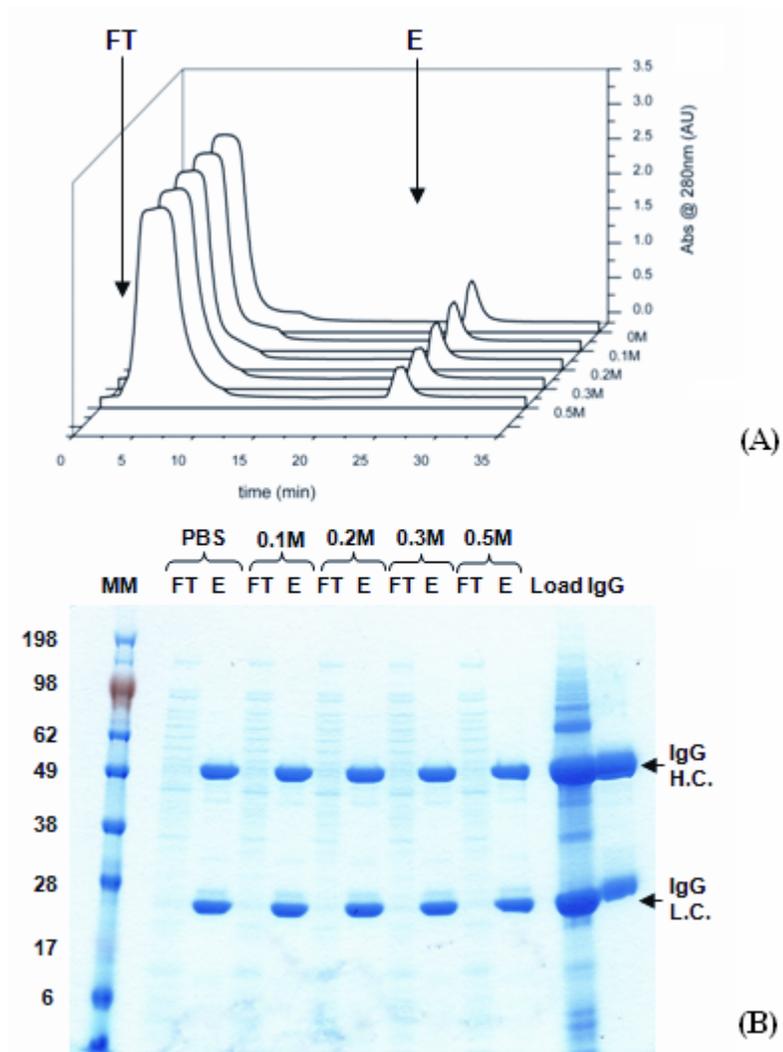


Figure 8.7. (A) Chromatograms of purification of IgG₄ from CHO cell culture supernatant (1.5 mg/mL) under different concentration of sodium chloride in the equilibration and binding buffer using cyclo[Link-MWFRHYK]-Toyopearl resin; (B) SDS-PAGE (reducing conditions) of flowthrough and elution fractions. Labels: MM – molecular weight marker, FT – flowthrough, E – elution fraction.

**Chapter 9. Reversible cyclic peptide libraries for the discovery
of novel affinity ligands**

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*A version of this chapter has been prepared for submission to
Analytical Chemistry*

Abstract

A model strategy is presented for the identification of cyclic peptide ligands from combinatorial libraries of reversible cyclic depsipeptides. A method for the solid-phase synthesis of individual cyclic depsipeptides and combinatorial libraries of these compounds is proposed, which employs lactic acid (Lact) and the dipeptide ester (N -Ac)-Ser(Ala)- as linkers for dilactonization. Upon alkaline treatment of the beads selected by screening a model library, the cyclic depsipeptides are linearized and released from the solid support to the liquid phase, to be sequenced via single step MS/MS. The protocol presented for library synthesis provides for wide structural diversity. Two model sequences, VVWVVK and AAWAAR, were chosen to present different structural examples for depsipeptide libraries and to demonstrate the process of sequence determination by mass spectrometry. Further, a case study using the IgG binding cyclic depsipeptide cyclo[(N -Ac)-S(G)-RWHYFK-Lact-E] is presented to demonstrate the process of library screening and sequence determination on the selected beads. Finally, a method is shown for the synthesis of the irreversible cyclic peptide corresponding to the proposed depsipeptide structure, to make the ligand stable to the aqueous acid and alkaline conditions encountered in affinity chromatographic applications. The cyclic peptide ligand was synthesized on a polymethacrylate resin and used for chromatographic binding of the target IgG.

Keywords: Reversible cyclic peptides; Cyclic peptide ligands; Solid-phase combinatorial libraries; Peptide sequencing; Affinity chromatography; Antibody purification.

9.1. Introduction

Small synthetic compounds with high affinity for biomolecules have great potential in a broad range of applications, from cell biology, to medicine, to protein purification. Peptides in particular are a very promising class of compounds [1-8]. Owing to their high selectivity, chemical stability, ease of synthesis, and wide structural and chemical diversity, peptides have found vast application as both diagnostic tools and therapeutics [9-11]. Many small peptides have been proposed as affinity ligands for the purification of biomolecules [12-15]. A number of properties, such as chemical stability, mild elution conditions, low immunogenicity, and relatively low production costs, indicate that these compounds have potential as replacements for biological ligands currently used in downstream bioprocessing [15-26]. Several linear peptides have been proposed as alternatives to costly Protein A and Protein G ligands for the purification of immunoglobulins and others have been reported for the recovery of blood factors and therapeutic enzymes from biological fluids and cell culture [27-44].

However, many studies indicate that cyclic peptides have an even larger potential as a new generation of affinity ligands. Due to their conformational rigidity, these compounds show superior properties compared to linear peptides, in particular: 1) higher specificity and avidity towards the target, 2) higher enzymatic stability, and 3) higher conformational integrity. Cyclic peptides are known and employed as highly potent drugs for controlling protein expression, enzyme inhibition and viral activity, and some have already been proposed as affinity ligands for the purification of biomolecules [45-48]. The screening of

solid-phase combinatorial libraries of cyclic peptides is a powerful technique for the identification of novel cyclic peptide ligands and biomimetics. However, the process of ligand discovery is hindered at the stage of sequence identification of the selected resin beads. Techniques routinely used for the sequencing of linear peptides, like Edman degradation and single stage MS/MS, are not feasible for cyclic peptides sequencing. Edman degradation cannot be used with cyclic peptides due to the absence of the peptide N_α-terminus, while MS-based techniques for cyclic peptides entail considerable effort and a high level of uncertainty [49-54]. In a mass spectrometer, the cyclic peptide undergoes ring opening at multiple positions to produce a complex mixture of shorter peptides, making spectral interpretation difficult and highly uncertain. Whilst a few solutions have been proposed [47,50,55-57], there is still much room to develop better techniques for the high throughput identification of cyclic peptides from small beads selected through library screening.

This study presents a strategy for the easy and high throughput discovery of cyclic peptides with high affinity and selectivity for biomolecules. The key element of this method consists of reversible cyclic depsipeptides, specifically cyclic dilactones comprising a binding peptide sequence framed between two ester bonds. In this work, lactic acid (Lact) and the dipeptide ester (N_α-Ac)-Ser(Ala)- were adopted as linkers for dilactonization. The two ester linkages can be readily hydrolyzed by base, thereby returning the peptide to its linear structure and removing it from the solid phase. Solid-phase combinatorial libraries of these cyclic depsipeptides cyclic depsipeptides can be screened for the selection of beads carrying candidate binders. While during the process of library screening the depsipeptides

retain the cyclic structure, a treatment of the selected beads in alkaline conditions is performed to linearize the peptide sequences and release them in solution, where they are finally sequenced with commonly used techniques, such as single step ESI-MS/MS. This represents a fast and inexpensive method for sequencing a large number of candidate binders, which in turns enables the identification of binding sequence homology. Selected sequences can then be tested for target binding by affinity chromatography.

To be used in affinity purification, a ligand must demonstrate chemical stability towards the acid conditions required for protein elution and the alkaline conditions used for periodic cleaning and sanitization of the adsorbent. Since cyclic depsipeptides cannot withstand these conditions, a method is presented for the synthesis of alkaline-stable homodetic cyclic peptides structurally equivalent to the selected sequences. The method basically consists in replacing the ester bonds formed by the Lact and (N_α-Ac)-Ser(Ala)- linkers with amide bonds formed with Alanine and (N_α-Ac)-Dap(Ala)- (Dap: 2,3-diaminopropionic acid).

In this work, the model sequences VVWVVK and AAWAAR were used to illustrate the protocol for the solid-phase synthesis of the proposed cyclic depsipeptides as well as peptide linearization and sequence determination by mass spectrometry. Combinations of the two model sequences were also synthesized to provide examples of the structural diversity that combinatorial libraries of these compounds can explore. To simulate the process of library screening and post-screening hit identification, the immunoglobulin G – binding cyclic depsipeptide cyclo[(N_α-Ac)-S(G)-RWHYFK-Lact-E] was adopted as a positive

control. A bioinformatic approach employing the database search algorithm MASCOT 2.4 was also integrated in the screening method at the step of sequence determination. Analyses indicated that all cyclic depsipeptides were synthesized at high density and purity, which are critical requirements towards the success of library screening. Finally, the cyclic homodetic peptide cyclo[(N_α-Ac)-Dap(G)-RWHYFK-Lact-E] was synthesized on chromatographic resin and the resulting adsorbent was tested in chromatographic format for IgG binding.

9.2. Experimental

9.2.1. Materials

Protected amino acids and coupling agents for peptide synthesis, and palladium(0) tetrakis(triphenylphosphine) [Pd(PPh₃)₄] were purchased from ChemImpex Inc. (Wood Dale, IL, USA). Diisopropylethylamine (DIPEA), piperidine, trifluoroacetic acid (TFA), triisopropylsilylamine (TIPS), ethanedithiol (EDT), phenylsilane, thioanisole, sodium diethyldithiocarbamate, indole, Tween 20, sodium cyanoborohydride, aqueous 1N NaOH, phosphate buffer saline (PBS) pH 7.4, and Kaiser test kit were from Sigma Aldrich (Saint Louis, MO, USA). N,N'-dimethylformamide (DMF), dichloromethane (DCM), HPLC grade acetonitrile and water, sodium acetate, sodium chloride, acetic acid glacial, glycine, hydrochloric acid, 85%v/v phosphoric acid, BCA assay kit, Kodak Biomax MR autoradiography films, an Alltech® Alltima™ C18 HPLC column, and ZipTip® pipette tips were purchased from Fisher Scientific (Pittsburgh, PA, USA). PEG based HMBA ChemMatrix resin (functional density of 0.6meq/g) was purchased from PCAS

Biomatrix Inc. (Saint-Jean-sur-Richelieu, Quebec, Canada). Toyopearl AF-Amino-650M resins were a kind gift from Tosoh Bioscience (King of Prussia, PA, USA). Human polyclonal immunoglobulin G (IgG) in lyophilized form was purchased from Equitech-Bio, Inc. (Kernville, TX, USA). Cell culture medium EMEM (Eagle Minimum Essential Medium) was from Quality Biologicals (Gaithersburg, MD, USA), while fetal calf serum (FCS) and tryptose phosphate broth (TPB) were obtained from Hyclone (Logan, UT, USA). The complete mammalian cell culture medium (cMEM) was prepared by combining EMEM with 10% FCS and 5% TPB. ^{14}C -formaldehyde for protein radiolabeling was from Perkin Elmer (Wellesley, MA, USA). 1% low melt agarose electrophoresis grade and EconoPac 10DG Desalting Column were purchased from BioRad (Hercules, CA, USA). 160-180 mm GelBond films were from BioWhittaker Molecular Applications (Rockland, ME, USA). A Waters 626 LC system integrated with 2487 UV detectors (Waters, MA, USA) was used for all chromatography runs. Microbore stainless steel columns 30 mm long x 2.1 mm I.D. were obtained from Alltech-Applied Science (Somerset, PA, USA). All chromatographic runs were carried out at room temperature.

9.2.2. Synthesis of cyclo[(N α -Ac)S(A)-VWVVK-Lact-E], cyclo[(N α -Ac)S(A)-AAWAAR-Lact-E] on aminomethyl ChemMatrix resin

The cyclic depsipeptides cyclo[(N α -Ac)S(A)-VWVVK-Lact-E] and cyclo[(N α -Ac)S(A)-AAWAAR-Lact-E] were synthesised on 100 mg of HMBA ChemMatrix resin (d = 75-150 micron, substitution = 0.6 mmol/g). Each coupling step was conducted for 25 min

in a polypropylene tube fitted with a Teflon frit under continuous nitrogen flow. To enhance the reaction rate, sonication was carried out using a Branson ultrasonic bath (Model 1510; sonicating frequency 40 kHz) while the temperature was maintained at 35°C. Two couplings were performed with Fmoc-Glu(OAll)-OH (3 eq. molar excess as compared to HMBA density), HATU (3 eq.) and DIPEA (6 eq.) in 3 mL of dry DMF. An acetylation step with acetic anhydride and DIPEA (50eq.) in 4 mL of DMF was carried out for 30 min at room temperature. The Fmoc protection was then removed by incubating with 5 mL of 20% piperidine in DMF for 20 min. Lactic acid (Lact) was then coupled on glutamic acid by reacting with lactic anhydride (50 eq.) and DIPEA (50 eq.) in 4mL of DMF for 30 min at room temperature. The sequences Val-Val-Trp-Val-Val-Lys and Ala-Ala-Tyr-Ala-Ala-Arg were synthesized on lactic acid via conventional Fmoc/tBu strategy. For each amino acid, an anhydrous DMF solution (2.5 mL) of Fmoc-amino acid (3 eq.), HCTU (3 eq.) and DIPEA (6 eq.) was added to the resin. Two couplings were performed for each amino acid to saturate all the available amino groups, as monitored by Kaiser test. The Fmoc protection on the last amino acid was removed with 5 mL of 20% piperidine in DMF for 20 min. Two couplings were performed with Ac-Ser(Trt)-OH (3 eq.), HATU (3 eq.) and DIPEA (6 eq.) in 2.5 mL of dry DMF. A Kaiser test was performed to monitor the absence of free primary amines. After rinsing the resin in DCM, the trityl protection on the hydroxyl group of serine was removed by treatment with 2% TFA in DCM (5% TIPS as scavenger), with a 10 min prewash followed by incubation for 1h under gentle rotation. After rinsing the resin with DCM and DMF, Fmoc-Ala-OH was coupled on the hydroxyl group of Ser by performing three couplings with Fmoc-Ala-OH (3 eq.), HATU (3 eq.) and DIPEA (6 eq.) in 2.5 mL of dry

DMF. The allyl ester protection on the γ -carboxyl group of glutamic acid was removed in minutes by treatment with Pd(PPh₃)₄ (0.1 eq.) and phenylsilane (10 eq.) as scavenger in DCM. The resin was then rinsed with sodium diethyldithiocarbamate (0.2 eq., 3 x 15 min) in DMF. The Fmoc group on Alanine was removed with 5 mL of 20% piperidine in DMF for 20 min. The peptide cyclization was performed by reacting the resin with a solution of HATU (4 eq.) and DIPEA (8 eq.) in dry DMF. Two couplings were performed for 30 min. A Kaiser test was performed on resin before and after cyclization. Finally, peptide deprotection was performed using a cleavage cocktail containing TFA/DCM/indole (70/28/2) for 1.5 hours.

9.2.3. Structural diversification of the model sequence VVWVK and AAWAAR

The cyclic depsipeptides Ac-AYAR-cyclo[A-S-VWVK-Lact-E] and cyclo[AYARA-(N _{α} -Ac)S-VWVK-Lact-E] were synthesized on ChemMatrix resins following the protocols described above. The synthesis was performed on 200 mg of HMBA-ChemMatrix resin. The coupling of Fmoc-Glu(OAll)-OH, lactic acid, and the synthesis of the sequence Val-Trp-Val-Lys were performed using conventional Fmoc/tBu strategy as described in Section 9.2.2. The batch of resin was then split in two aliquots. On the first aliquot, Fmoc-Ser(Trt)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, and Ac-Ala-OH were sequentially coupled via HCTU chemistry. The cleavage of the trityl protecting group from serine, the coupling of Fmoc-Ala-OH on the β -hydroxyl group of Ser with HATU, the removal of the allyl ester protection from the γ -carboxylic group on Glu, and the final cyclization were

performed as explained in Section 9.2.2. On the second aliquot, Ac-Ser(Trt)-OH was coupled by HCTU chemistry and the trityl protection was removed as explained above. Fmoc-Ala-OH was coupled on the β -hydroxyl group of Ser with HATU. After the cleavage of Fmoc from Ala with 20% piperidine in DMF, Fmoc-Arg(Pbf)-OH, Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Ala-OH were sequentially coupled via HCTU chemistry. Hence, the removal of the allyl ester protection from the γ -carboxylic group of Glu, the final cyclization and deprotection were performed as in Section 9.2.2.

9.2.4. Synthesis of the IgG-binding cyclic depsipeptide cyclo[(N $_{\alpha}$ -Ac)S(A)-RWHYFK-Lact-E] on aminomethyl ChemMatrix and Toyopearl AF-Amino-650M

The cyclic depsipeptide cyclo[(N $_{\alpha}$ -Ac)S(A)-RWHYFK-Lact-E] was synthesized on Aminomethyl ChemMatrix resin (substitution level of 0.6 mmol/g) and Toyopearl AF-Amino-650M resin (substitution level of 0.12 mmol/g) following the protocols described in Section 9.2.2. All couplings on Toyopearl resin were performed without sonication. Final deprotection was performed using a cleavage cocktail containing TFA/thioanisole/EDT/anisole (90/5/3/2) for 1.5 hours. Five milligrams indole was added to the cleavage cocktail.

9.2.5. Synthesis of cyclic (homodetic) peptides cyclo[(N_α-Ac)Dap(A)-RWHYFK-A-E] on Toyopearl AF-Amino-650M

The cyclic peptide cyclo[(N_α-Ac)Dap(A)-RWHYFK-A-E] was synthesized on 100mg of Toyopearl AF-Amino-650M resins. The amino acids Fmoc-Gly(OAll)-OH, Fmoc-Ala-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Dap(Mtt)-OH were sequentially coupled using HCTU coupling agent as explained in Section 9.2.2. The terminal Fmoc protection was removed and the peptide N-terminus was acetylated with acetic anhydride (50 eq.) and DIPEA (50 eq.) in 4 mL of DMF for 30 min at room temperature. After rinsing with DMF and DCM, the methyltrityl from diaminopropionic acid was removed with 1% TFA in DCM for 30 min at room temperature. After rinsing with DCM and DMF, Fmoc-Ala-OH was coupled on the β-amino group of diaminopropionic acid with HCTU. The allyl ester protection from glutamic acid was removed as previously explained. Peptide cyclization and deprotection were performed as described in Section 9.2.4.

9.2.6. Linearization, cleavage and analysis of the linearized peptides for sequence determination

A 5 mg aliquot of ChemMatrix resin carrying a cyclic depsipeptide was rinsed with 20:80 acetonitrile:water and then treated with 0.1mL of 0.1M NaOH in 20:80 acetonitrile:water for 20 min at room temperature. An appropriate volume of pure TFA was added to the cleavage solution to neutralize the pH. The solution pH was monitored with pH

paper (Whatman). RP-HPLC analysis of 20 μ L of cleavage samples was performed with an Atlantis® dC18 (3 μ m) column by running a linear elution gradient from 95/5 to 50/50 (water + 0.1% TFA) / (acetonitrile + 0.1% TFA) over 30 min. The UV absorbance was monitored at 280nm. For sequence determination from a single bead, each resin bead was rinsed with 20/80 acetonitrile/water and then treated with 50 μ L of 0.1M NaOH in 20/80 acetonitrile/water for 20 min at 4C. After acidification with an appropriate amount of 0.2% formic acid in 50/50 water / acetonitrile aqueous 1% TFA, the sample was desalted with ZipTip (Millipore). The sample was adjusted to the final volume of 100 μ L with 0.2% formic acid in 50/50 water / acetonitrile and finally analyzed by infusion ESI-MS and ESI-MS/MS.

Mass spectrometry analysis was performed using a Q-ToF API-US mass spectrometer (Waters), running MassLynx 4.0 acquisition software. The instrument was calibrated using product ions from MSMS glu-fibrinopeptide (Sigma). Infusion was performed by on-board syringe pump at the flow rate of 10 – 20 μ L/min. The MS was operated at a resolution of 6000. Source parameters, including cone voltage and ESI capillary voltage were adjusted as necessary to provide maximum intensity for the precursors of interest. Collision energy for specific peptides was adjusted manually to provide optimal fragmentation of the selected precursors. For manual or automated analysis, product ion spectra were smoothed (Savitzky-Golay, window = 3, number smooth = 2) and centered. For automated bioinformatic analysis, the spectra were exported as .dta files from MassLynx. Exported .dta files were searched against a peptide virtual library using MASCOT 2.4 (Matrix Science). Parameters were used

were as follows: 1) no enzyme specificity, 2) no variable or fixed modifications, 3) peptide ion tolerance ~ 50 ppm, 4) product ion tolerance ~ 100ppm.

9.2.7. Simulation of library screening

Immunoglobulin G (IgG) was labeled by reductive methylation using ^{14}C -formaldehyde as reported by Yang *et al.* [58]. The concentration of labeled protein was measured by BCA assay. Fifty milligram aliquots of cyclo[(N $_{\alpha}$ -Ac)Dap(A)-RWHYFK-A-E]-Toyopearl resin were washed in 20% methanol and equilibrated in PBS pH 7.4. Beads were then incubated with 1% w/v casein. ^{14}C -labeled IgG was then added to the resin to reach a concentration of 0.5 mg/mL. After 2h of incubation at room temperature under gentle agitation, resins were rinsed with PBS, 0.2M NaCl and 0.1% Tween 20 in PBS, 0.2M Acetate buffer, pH 5.0, and finally PBS. Resins were then suspended in 1% low melting agarose and poured onto GelBond film. The gels were allowed to dry overnight, followed by exposure to a Kodak Biomax MR autoradiography film for 7 days. The films were developed with a Konica Medical Film Processor (Tokyo, Japan).

9.2.8. Chromatographic comparison of the binding properties of reversible with irreversible cyclic peptides synthesized on polymethacrylate resins

Thirty-five milligrams of cyclo[(N $_{\alpha}$ -Ac)Dap(A)-RWHYFK-A-E]-Toyopearl resin and cyclo[(N $_{\alpha}$ -Ac)Dap(A)-RWHYFK-A-E]-Toyopearl resin were packed in a 30 mm x 2.1 mm

I.D. Microbore column (0.1 mL) (Alltech-Applied Science, Somerset, PA, USA) and swollen with 20% v/v methanol. One hundred microliters of feed sample, 5mg/mL IgG in PBS pH 7.4, was loaded onto the column at a flow rate of 0.05 mL/min (87 cm/h). The column was washed with 2 mL of equilibration buffer at a flow rate of 0.2 mL/min (348 cm/h). Elution was then performed with 4 mL of 0.2M glycine buffer pH 2.5 at the flow rate of 0.4 mL/min (696 cm/h). Cleaning and regeneration were performed by 4 mL of 0.85% phosphoric acid. The effluent was monitored by absorbance at 280 nm.

9.3. Results

9.3.1. Synthesis of cyclo[(N_α-Ac)S(A)-VWVVK-Lact-E], cyclo[(N_α-Ac)S(A)-AAWAAR-Lact-E], and structural analogues

This study proposes a basic structure of cyclic depsipeptides as peptide dilactones, that is, cyclic peptides containing two ester bonds in place of amide bonds. The general structure cyclo[(N_α-Ac)S(A)-X₁X₂X₃X₄X₅X₆-Lact-E], portrayed in Figure 9.1, comprises two main features: a “keystone” trifunctional amino acid (in blue) and two cleavable linkers that form the corresponding ester (depside) bonds (in red). The “keystone” amino acid is a Y-shaped amino acid upon which the peptide cycle is structured, glutamic acid (E) in this work, while the linkers are lactic acid (Lact) and the dipeptide ester (N_α-Ac)Ser(Ala)-. Since the peptide sequence Ac-S-X₁X₂X₃X₄X₅X₆ is framed between two ester bonds, the treatment in alkaline conditions affords the simultaneous opening of the cyclic structure and the cleavage of the linearized peptide in solution (Figure 9.1).

PEG-based HMBA-ChemMatrix resin was chosen as a solid support for peptide synthesis, owing to its excellent swelling properties and high functional group density (~ 0.6 meq/g). The model sequences VVWVVK and AAWAAR have been adopted for optimizing and testing the proposed protocol of solid-phase synthesis. A solution comprising 0.1M NaOH in 20:80 acetonitrile:water was employed for linearizing and extracting the peptides from the solid phase. The resulting extracts were analyzed by C18 RP-HPLC to estimate the purity of the depsipeptides on the solid-phase. Further, the cleavage mixtures obtained from a single bead were desalted and analyzed by ESI-MS and MS/MS to confirm the molecular weight and the sequence of the linearized peptides respectively. The RP-HPLC analysis of the cleaved fractions indicate that both cyclic depsipeptides were synthesized on solid phase with high purity, 92% and 84% for VVWVVK and AAWAAR respectively. This is critical for the success of library screening, as high peptide purity lowers the risk of identifying false positives and significantly facilitates the sequencing. ESI-MS analysis of the desalted cleavage mixtures obtained from single beads confirmed these findings. The spectra shown in Figure 9.2 indicate that the peak related to the linearized peptide is by far the most prominent one in the whole m/z range for both sequences. This in turn enables the use of automated peak recognition and interrogation for successive sequence determination by MS/MS, which is in turn an important step towards high throughput screening. The MS/MS spectra of the two linearized peptides (Figure 9.3) show a very regular fragmentation predominantly according to the b series, which allows manual confirmation of the sequences. A Gaussian distribution of PEG oligomers can be noted in Figure 9.2A , which derives from the alkaline treatment of the single ChemMatrix bead. While not affecting the outcome of the

MS/MS analysis, these impurities can be removed by performing a second desalting of the same sample using a ZipTip C18 pipette tip. This additional step was performed for the peptide Ac-SAAWAAR, which gave a cleaner spectrum (Figure 9.2B).

The proposed chemistry is also amenable to a wide range of structural diversity, which can be obtained by varying the position and number of amino acids in the loop or by producing different combinations of linear and cyclic portions within the same molecule. A model structure is reported in Figure 9.4, wherein $X_1...X_n$ and $Z_1...Z_n$ are respectively the variable and the constant regions within the loop, while $Y_1...Y_n$ is a variable region outside the loop.

The sequences Ac-AYAR-cyclo[A-S-VWVK-Lact-E] and cyclo[AYARA-(N -Ac)S-VWVK-Lact-E] are proposed as examples of structural diversity corresponding to the structures suggested in Figure 9.4. The linearized sequence corresponding to the first structure is Ac-AYAR-S-VWVK, while that corresponding to the second structure is only Ac-SVWVK, since the additional sequence AYARA within the loop represents a portion shared by all peptides in the library and therefore does not need to be sequenced. The HPLC analysis of the linearized sequences indicated high purity (> 90% for both peptides). ESI-MS/MS results are reported in Figure 9.5 and allow manual identification of the sequences.

These results demonstrate the amenability of the proposed strategy towards a variety of structural variations, which ultimately broadens the conformational ability of peptide ligands to bind targets and enhances the “affinity” property of these molecules.

9.3.2. Library screening and sequence determination via bioinformatic approach

To simulate the process of library screening, the IgG-binding dilactone ligand cyclo[A-S(N_α-Ac)-RWHYFK-Lact-E] was synthesized on ChemMatrix resin. Other research groups have used this PEG-based substrate for the synthesis and screening of solid-phase libraries [59,60]. In fact, its high pore diameter and the low non-specific protein binding related to its hydrophilic character make it an ideal support for library screening against biomolecules. To mimic a library aliquot, 10 mg of a 1:1:1 mixture of cyclo[(N_α-Ac)S(A)-RWHYFK-Lact-E]-ChemMatrix, cyclo[(N_α-Ac)S(A)-VWVVK-Lact-E]-ChemMatrix, and cyclo[(N_α-Ac)S(A)-AAWAAR-Lact-E]-ChemMatrix resins was prepared. The sequences VWVVK and AAWAAR simulate the non-binding sequences. A radiological approach that has been successfully employed by our group for the discovery of peptide ligands was adopted for the library screening [61]. rProtein A – Toyopearl and acetylated aminomethyl ChemMatrix were used as positive and negative controls respectively. After equilibration, resin aliquots of library model and controls were separately incubated with the ¹⁴C-radiolabeled target protein in the presence of cMEM, a complex cell culture medium containing albumin and other serum proteins. After incubation, resins were rinsed with PBS and then washed with a sequence of 0.2M NaCl and 0.1% Tween 20 in PBS, 0.2M acetate buffer, pH 5.0, and finally PBS until the radioactivity reached the baseline level. As in previous studies [58], the presence of NaCl and Tween 20 in the washing buffer reduces the non-specific binding due to weak electrostatic and hydrophobic interactions, while low pH wash ensures the selection of peptides with stronger target binding. Beads were then

suspended in agarose and plated on a GelBond film. After drying the gel, beads were contacted with a photographic film for five days. The developed film shown in Figure 9.6 indicates that the cyclo[A-S(N α -Ac)-RWHYFK-Lact-E]-ChemMatrix resin bound a sufficient amount of radiolabeled protein to result in a dark spot on the radiographic film.

The difference between the levels of radioactivity carried by the three mixed resins was enough to discriminate the ligand-carrying beads and the positive control from the other beads in the mixture and the negative control. By overlapping the developed X-ray and the agarose films, ten positive and ten negative beads were selected, excised and washed in water at 75C to remove the layer of agarose. The washed beads were then treated in alkaline conditions (0.1M NaOH in 20:80 MeCN:water) at low temperature (4°C) in order to linearize the cyclic depsipeptides and cleave the resulting sequences from the resins. The resulting samples were desalted and analyzed by Q-ToF ESI-MS (Figure 9.7.a) to determine the mass of the linearized peptides. Eight out of the ten positive beads selected from the screening simulation against IgG were found to contain the ligand cyclo[A-S(N α -Ac)-RWHYFK-Lact-E] based on the value of molecular weight of the linearized sequence (N α -Ac)SRWHYFK (Mw [M+H]⁺ =1065.18 amu, [M+H]⁺² = 533.27). All the negative beads were found to carry either the (N α -Ac)SVVWVVK or (N α -Ac)SAAWAAR sequences. Notably, the peptides extracted from single beads were highly pure (Figure 9.7.a), which allowed unequivocal selection of the precursor to be interrogated for sequence determination by MS/MS. However, the spectrum resulting from the fragmentation of the linearized sequence Ac-S-RWHYFK (Figure 9.7B) appears rather complex. The variety of charged species, which

results from the presence of the two positively charged amino acids arginine and lysine, makes the spectral interpretation more difficult. Therefore, in place of manual sequencing, we have designed a bioinformatic approach to perform unbiased and accurate sequence identification.

A virtual library comprising all possible linearized structures $(N_{\alpha}\text{-Ac})S\text{-}X_1X_2X_3X_4X_5X_6$ was created in FASTA format. The data base search algorithm MASCOT was then used to screen the .edt file containing the smoothed and centered MS/MS spectrum against the library of theoretical spectra generated according to model fragmentation patterns [62]. The search parameters were assigned as follows:

Type of search: MS/MS Ion Search

Enzyme: None

Fixed modifications: Acetyl (N-term)

Mass values: Monoisotopic

Peptide Mass Tolerance : ± 0.8 Da

Fragment Mass Tolerance: ± 0.8 Da

Max Missed Cleavages: 0

The peptide sequence Ac-SRWHYFK was ranked as the first among the top ten scoring peptide matches, with a Score of 27.9 and an Expect value of 0.013 (where a Score greater of 22 indicates identity, or real match, and an Expect value of 0.05 is the default significance threshold), whereas the second sequence had a Score of 16.2 and an Expect

value of 0.2. Such a significant difference in scoring parameters between the first and the following matches indicates that the spectral interpretation and sequence assignment is unambiguous [63]. It is also worth mentioning that we also performed a search against a randomized “decoy” database, which returned a 0% false positive rate. These results demonstrate that the sequence was determined with very high certitude. A similar bioinformatic approach would be particularly valuable for setting up an automated process for sequencing tens of beads selected from library screening.

9.3.3. Chromatographic comparison of the binding properties of reversible with irreversible cyclic peptides synthesized on polymethacrylate resins

The final step of the screening procedure presented is the validation of the binding of the selected cyclic peptide by chromatographic to the target IgG. A dilactone peptide ligand, as selected through library screening, cannot be directly employed in affinity chromatography, as the acidic and alkaline conditions employed for elution and resin regeneration are likely to hydrolyse the peptide ligand and compromise the resin’s performance. It is hence necessary to turn the dilactone peptide into the corresponding lactam, that is, a homodetic cyclic peptide, by replacing the two ester bonds with amide bonds resulting in structurally equivalent molecules (Figure 9.9). In the present case, lactic acid and the dimer Ac-Ser(Gly)-OH were respectively replaced with alanine and Ac-Dap(Gly)-OH. To prove that this modification does not alter the affinity of cyclic peptide

ligand, the dilactone and the homodetic lactam versions of the ligand were compared side-by-side on a single IgG binding study.

The ligands cyclo[A-S(N_α-Ac)-RWHYFK-Lact-E] and cyclo[A-Dap(N_α-Ac)-RWHYFK-A-E] (Figure 9.9) were synthesised on Toyopearl AF-Amino-650M resin and compared for IgG binding on a chromatographic format. Toyopearl resin was chosen as a chromatographic support for its high hydrophilicity and high mechanical strength. PBS pH 7.4 and 0.2M Glycine pH 3.5 were chosen as binding and elution buffers respectively.

The chromatograms shown in Figure 9.10 indicate no difference in the binding ability between the peptide dilactone and the corresponding irreversible cyclic peptide. This indicates that the ester (depside) bonds do not play a major role in target binding. This is likely because in the proposed structures of the cyclic depsipeptides on solid phase the depside bond is located away from the exposed region of the ligand and hence inaccessible to the target during the screening procedure. Therefore the selected cyclic ligands are likely to show the same binding ability, independently of heterodetic cyclic depsipeptide form (lactones) or homodetic cyclic peptide form (lactam), provided that the linkers for making the ester or amide bonds are structurally equivalent.

9.4. Conclusions

This study presents a strategy for the identification of cyclic peptide ligands with high affinity and specificity for target biomolecules. First, a method was introduced for the synthesis of a solid-phase library of cyclic depsipeptides. These compounds, which comprise

the binding sequence framed between two ester linkages according to the general formula $\text{cyclo}[(\text{N}_\alpha\text{-Ac})\text{S}(\text{A})\text{-X}_1\text{X}_2\text{X}_3\text{X}_4\text{X}_5\text{X}_6\text{-Lact-E}]$, maintain the cyclic structure during the process of library screening to enable the selection of sequences with high target affinity and selectivity. After screening, a rapid treatment in alkaline conditions of the selected beads affords the linearized peptide sequences decoupled from the solid phase, which can be easily analyzed using common MS techniques. In the first part of this study, the model sequences VVWVVK and AAWAAR were used to demonstrate how the dilactone peptides and their structural variations are easily construed at high purity on hydrophilic PEG-based resins ChemMatrix® and later sequenced by electrospray ionization mass spectrometry (ESI-MS/MS). The exploration of the structural diversity and the interpretation of the spectral results were presented and discussed as critical parameters for the design of a high throughput method for discovery of novel affinity ligands.

A case study was then presented to illustrate in detail the process of ligand discovery using the proposed library of reversible cyclic peptide. A model library was produced by mixing aliquots of ChemMatrix® beads carrying the IgG-binding sequence RWHYFK and the sequences VVWVVK and AAWAAR as non-binding peptides. A radiological method used in previous work was adopted for selecting the positive leads. Notably, nine out of ten selected positive beads were carrying the positive peptide, as confirmed by the MS analysis. To interpret the rather complex spectrum resulting from peptide fragmentation, a bioinformatic approach was utilized. To implement this method, a virtual library $(\text{N}_\alpha\text{-Ac})\text{S-X}_1\text{X}_2\text{X}_3\text{X}_4\text{X}_5\text{X}_6$ was created in FASTA format and mounted on the MASCOT 2.4 server. The library search against the experimental .dta spectrum resulted in the unequivocal

identification of the correct matching sequence. This approach, extremely simple and robust, can be easily implemented for the sequencing of a high number of leads selected from the screening of one or even multiple libraries. In the latter case, it is in fact sufficient to concatenate multiple virtual libraries in a single FASTA file and combine the sequencing of differently structured linearized peptides in a single automated process.

In the latter part of this study, a method is presented for applying the identified sequences to the chromatographic purification of the target biomolecules. To ensure the stability of the cyclic ligands towards the acidic and alkaline conditions respectively employed for protein elution and column regeneration, stable amide bonds replaced the cleavable ester links. As an example, the cyclic peptide cyclo[A-Dap(N α -Ac)-RWHYFK-A-E] was synthesized on the Toyopearl AF-Amino-650M resin and the resulting adsorbent was successfully tested for IgG binding.

In conclusion, this paper introduces a simple and robust protocol for identifying highly selective cyclic peptide ligands using solid phase combinatorial libraries. By overcoming the difficulties that still hinder the process of discovery of these compounds, this method demonstrates great applicability in those wide areas, like drug and diagnostic research, and bioseparations, where the introduction of synthetic affinity ligands could be extremely beneficial in terms of both cost and safety.

9.5. References

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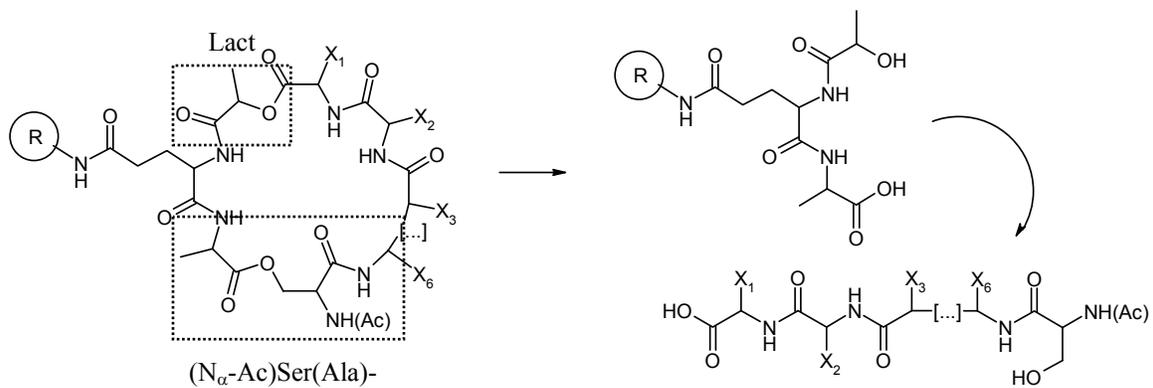


Figure 9.1. Model structures of the cyclic dilactone cyclo[(N_α-Ac)S(A)-X₁X₂X₃X₄X₅X₆-Lact-E] and linearized structure Ac-S-X₁X₂X₃X₄X₅X₆.

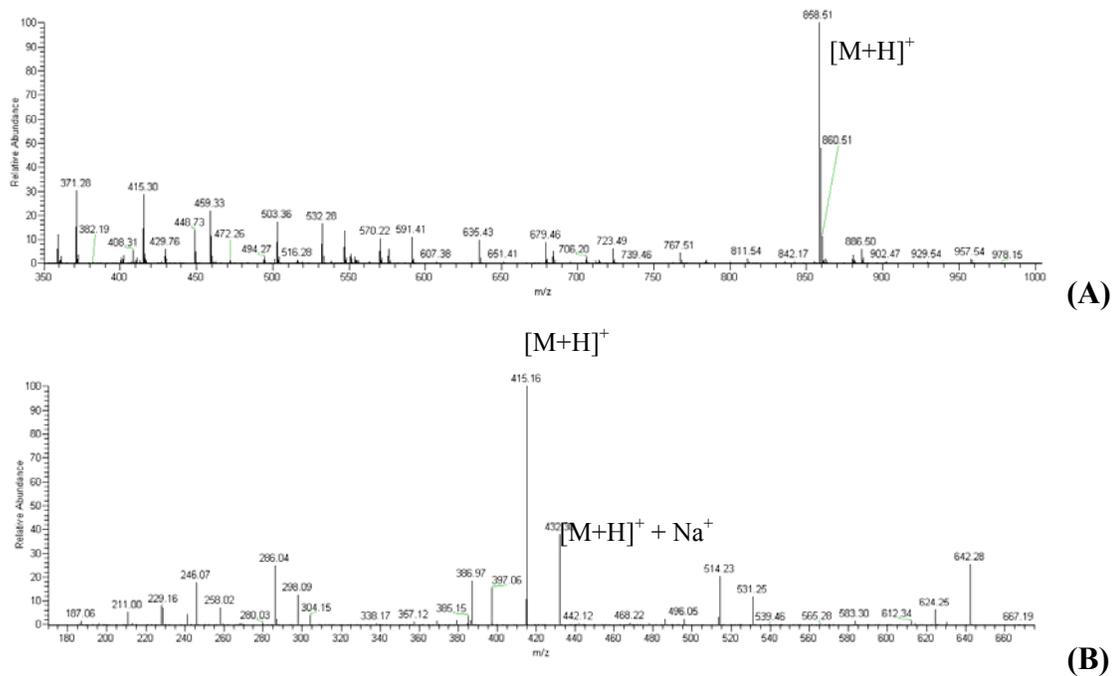


Figure 9.2. ESI-MS analysis of the linearized peptides: (A) Ac-SVWVVK (M_w $[M+H]^+ = 858.51$ amu) and (B) Ac-SAAWAAR (M_w $[M+H]^+ = 774.39$ amu).

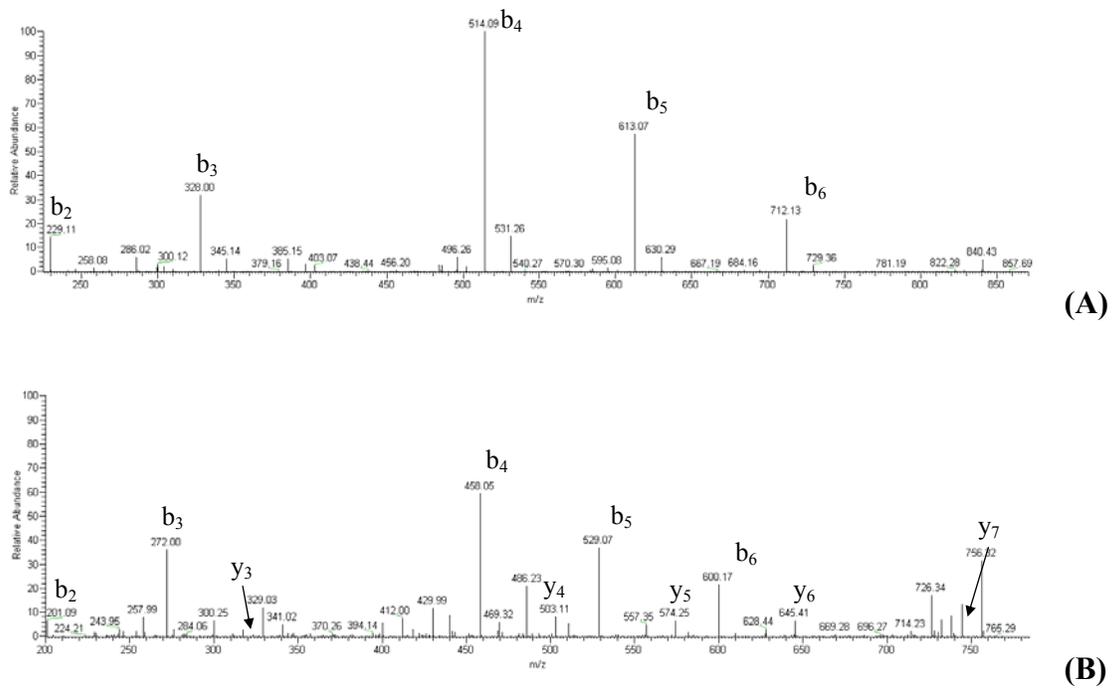


Figure 9.3. Sequence determination by ESI-MS/MS of the linearized peptides: (A) Ac-SVWVVK and (B) Ac-SAAWAAR.

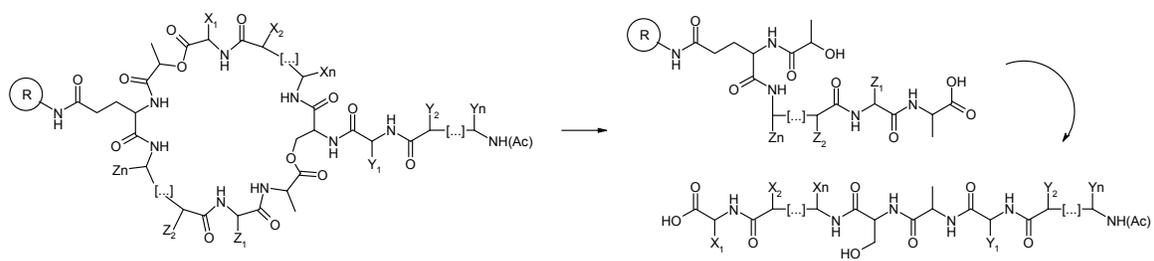


Figure 9.4. General model of structural diversity for library design.

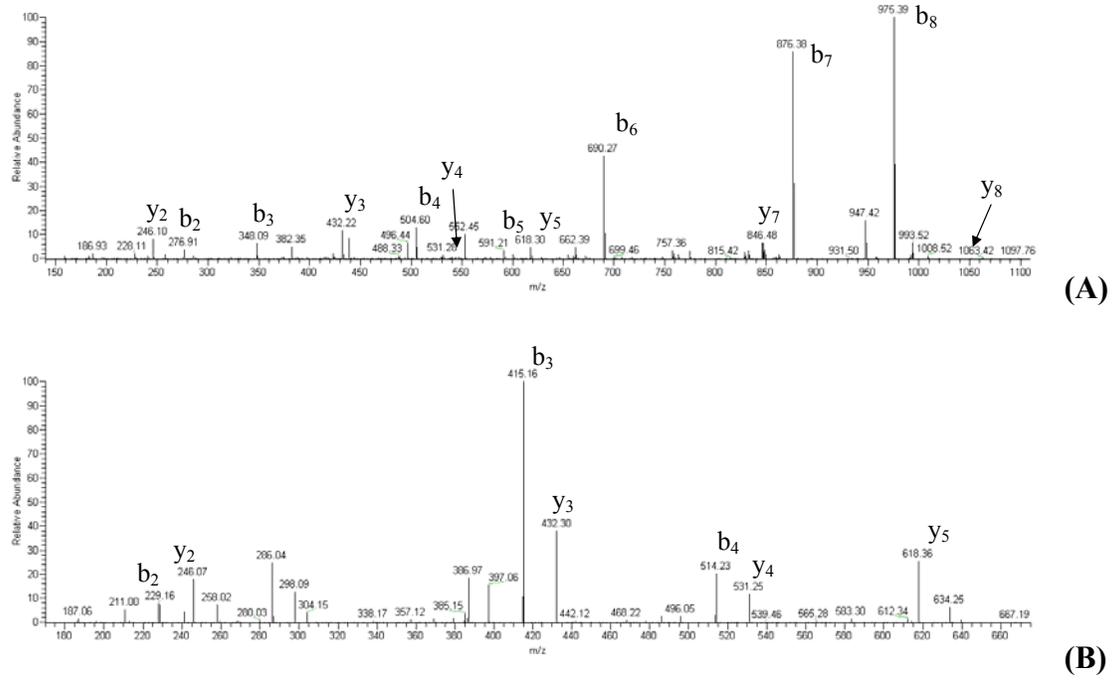


Figure 9.5. Sequence determination by ESI-MS/MS of the linearized peptides: (A) Ac-AYAR-S-VWVK and (B) Ac-SVWVK.



Figure 9.6. Developed photographic film. Positive control (rProtein A- Toyopearl resin) is located in the lower left corner and the negative control (acetylated aminomethyl ChemMatrix resin) is located in the lower right corner.

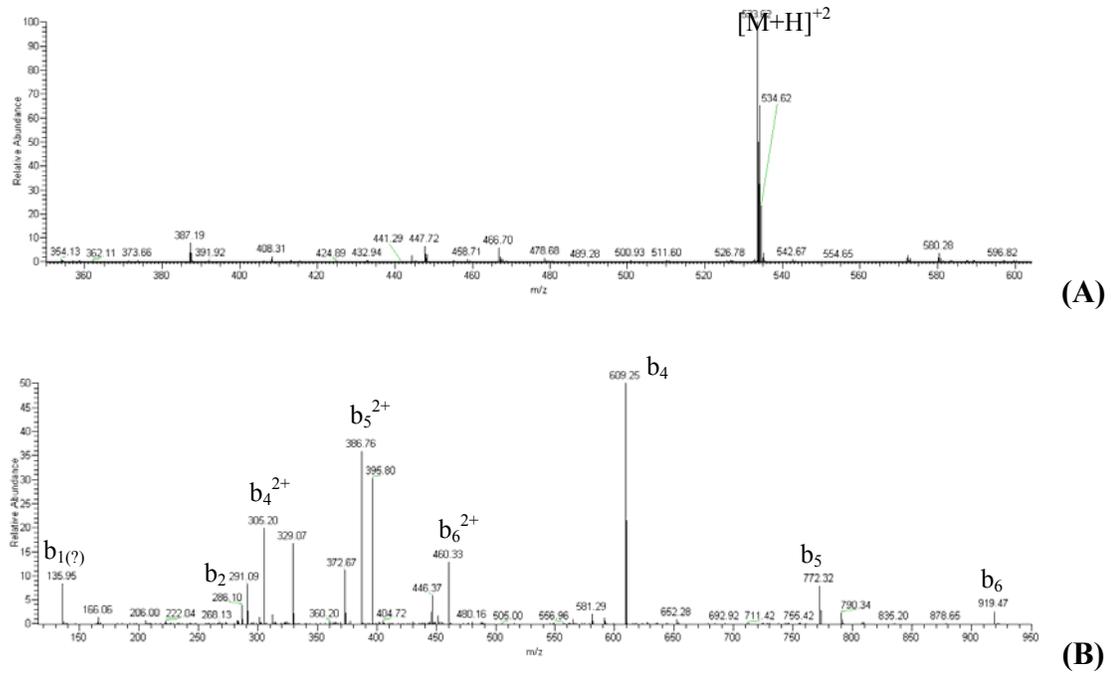


Figure 9.7. (B) ESI-MS and (A) ESI-MS/MS analysis of the linearized peptide Ac-SRWHYFK (M_w $[M+H]^+ = 1065.53$ amu) extracted from a positive bead.

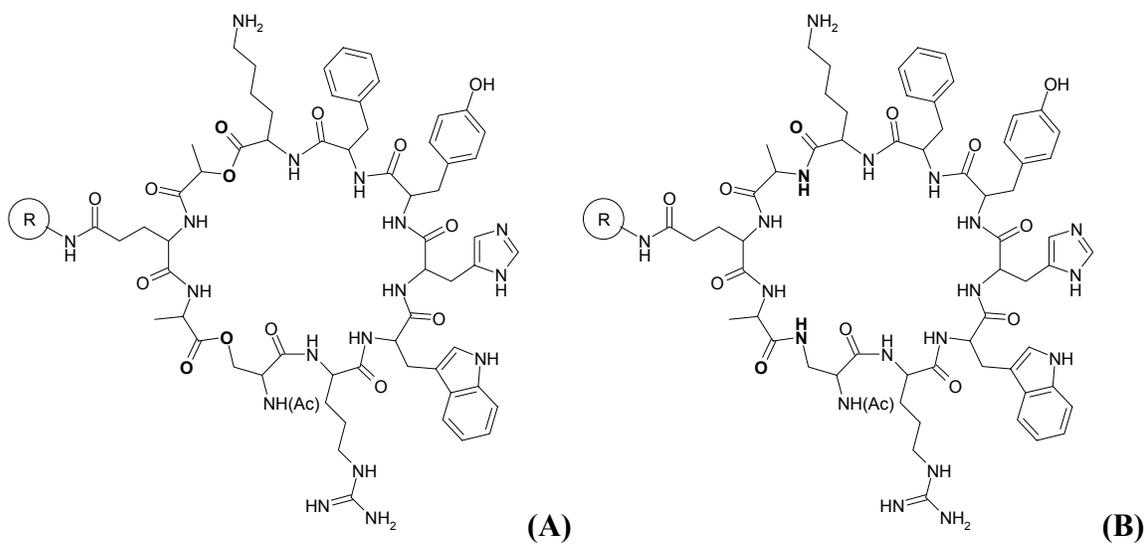


Figure 9.8. Structures of (A) cyclo[A-S(N α -Ac)-RWHYFK-Lact-E] and (B) cyclo[A-Dap(N α -Ac)-RWHYFK-A-E].

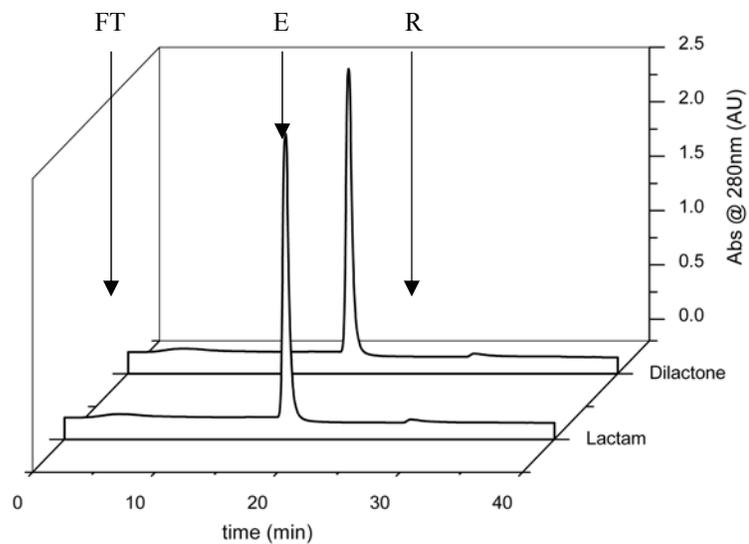


Figure 9.9. Comparison between the dilactone and the lactam versions of the cyclic ligand. Labels: FT, flow-through; E, elution; R, regeneration.

Chapter 10. Conclusions and Future work

10.1. Summary and conclusions

This work focuses on the identification and development of small, robust, synthetic peptide ligands for bioseparations. The studies presented in the previous eight chapters of this thesis address two main topics, that is, the development of linear peptide ligands for the purification of antibodies (chapters 2 through 7) and the design of novel methods for the discovery of cyclic peptide ligands (chapters 8 and 9).

The linear peptide ligands utilized in this work have been discovered by screening a solid-phase library of linear hexapeptides and characterized in terms of binding and elution conditions, product yield and purity, and binding site on the target antibody molecule [1-3]. Initial studies have revealed that, unlike Protein A, these peptide ligands bind all IgG subclasses and allow elution at much milder conditions (pH 4.0 – 5.0). Several aspects, however, needed more in depth investigation and optimization. First, the ligands were to be tested for their capacity of purifying IgG from different sources. Second, the binding capacity as well as the chemical and biochemical stability of the peptide-based adsorbents needed substantial improvement.

The peptide ligands, HWRGWV in particular, were tested against a variety of antibody sources, namely commercial CHO cell culture supernatants, Cohn II+III fraction of human plasma, milk, and extract of transgenic plants [4-6]. In all these studies, the ligands performed on par with Protein A, in terms of yield, purity and removal of contaminants, such as the host cell proteins and DNA from cell culture supernatant.

Substantial work has also been carried out in order to improve the dynamic binding capacity (DBC) of the peptide-based adsorbents [7]. The protocol for coupling the whole peptide on the chromatographic resin was thoroughly studied in order to optimize the ligand density on the resin surface. This allowed increasing the DBC from about 20 mg/mL to about 50 – 60 mg/mL, which compares well with Protein A media and is a remarkable value for a small synthetic ligand. Higher DBC translates into lower column volume and higher productivity, and hence lower capital and process costs.

Further studies were performed to increase the adsorbent's chemical and biochemical stability. A method of modification of the resin's surface chemistry was developed to enable the production of alkaline-stable peptide-based adsorbents either by solid-phase peptide synthesis or peptide coupling (*e.g.* by HCTU or HATU chemistry) [8]. The resulting adsorbents were used for up to 200 cycles of IgG binding, each one followed by sanitization with aqueous 0.1M NaOH, without showing substantial decrease of binding capacity. These results are better than ordinary Protein A resin and compare well with MabSelect Sure resin (\$ 20,000 / liter), which is designed for high resistance to alkaline cleaning. Finally, a strategy has been developed for endowing the peptide ligands with high stability against proteolytic enzymes and consists in replacing natural amino acids with non-natural analogs [9]. The resulting sequences, while maintaining good target affinity and specificity, show much higher resistance to proteolysis. Further, it was observed that the substitution of charged amino acids with electrically neutral analogs decreased non-specific binding of contaminants by electrostatic interactions, ultimately increasing product purity.

The second part of this work presents novel techniques for the design and identification of cyclic peptide ligands. The choice of cyclic peptides for ligand discovery is motivated by the inherently better characteristics that these compounds possess as compared to their linear counterparts. Owing to their higher affinity and specificity, cyclic peptides are suitable candidates for applications that require high binding strength selectivity. Combinatorial libraries of cyclic peptides are a valuable tool for ligand discovery, and as the current technology enables the use of biological as well as synthetic libraries, this work presents an example of both, respectively a mRNA-display library of cyclic peptides and a solid-phase library of “reversible” cyclic peptides.

The mRNA-display library was generated using a novel protocol, called “peptide cyclization on reversible solid-phase format” [10]. This method affords higher cyclization yield and hence high library purity as compared to the techniques reported in the literature [11,12]. Library screening was performed following the common mRNA-display procedure and resulted in the identification of several candidate sequences. These peptides showed remarkable similarity in terms of amino acid composition with the linear hexapeptides identified from the solid-phase library. One sequence was synthesized on a chromatographic resin and successfully used for purifying IgG from two commercial cell culture supernatants. The ligand strength as well as the product yield and purity resulted slightly higher than those of linear peptides with similar composition, hence confirming that cyclic structure possess better binding.

Finally, a method was presented for the synthesis of solid-phase libraries of “reversible cyclic” peptides [13]. These compounds retain a cyclic structure during the process of library screening and selection, but, before the sequencing step, they are linearized and eluted from the solid support into the liquid phase. These novel peptide structures were designed to overcome the issues associated with the sequencing of cyclic peptides by mass spectrometry (MS) and expedite the process of post-screening lead identification. The MS-based sequencing of linear peptides is, in fact, much simpler than that of stable cyclic peptides. Reversible cyclic peptides, on the other hand, cannot be directly applied for chromatographic use, due to their low chemical stability. A protocol was hence developed for the synthesis of the corresponding irreversible cyclic form of the selected sequences. These structures are fully stable and can be reused multiple times without loss of capacity.

The methods presented herein form a complete set of tools for the discovery and development of robust, selective and affordable synthetic peptide ligands, which show a great deal of promise for replacing protein ligands for bioseparations. Unlike advanced protein-based affinity media, which show a direct proportionality between performance and cost, peptide-based adsorbents tend to be more affordable the more they are developed. The optimization of both sequence composition and ligand density, in fact, lowers the synthetic costs and promotes better usage of the peptide and longer lifetime, which higher the convenience of peptide-based adsorbents.

10.2. Future work

10.2.1. Design and development of Protein L – mimetic peptide ligand (PLMP) for the purification of antibody fragments

10.2.1.1. Introduction and motivation

Most of the therapeutic antibodies employed today for therapeutic and diagnostic purposes are full-length IgG molecules [14,15]. These biomolecules possess high structural stability, long *in vivo* half-life, and important Fc-mediated functions. Due to their large size, however, they also suffer from poor tissue penetration, biodistribution, and clearance [16-18]. To overcome these limitations, antibody fragments Fab (fragment antigen-binding) and scFv (single chain variable fragment) have been proposed as the new generation of antibodies for therapeutic and diagnostic applications [18,19]. Fab consists of one constant and one variable domain of each of the heavy and the light chain of IgGs (Figure 10.1). There are two types of light chains, kappa (κ) and lambda (λ), the former being more abundant than the latter (60:40), hence two types of Fab, Fab κ and Fab λ . scFv is a fusion protein of the variable regions of the heavy and of the light chain of IgGs, connected with a short peptide linker made of 10 up to 25 amino acids. While retaining the target specificity of parental mAbs, these molecules are produced more easily and possess remarkable features for diagnostic and therapeutic applications [20,21]. While full-length antibodies can only be expressed by costly mammalian cell lines that possess the cell machinery for the glycosylation of the Fc fragment, antibody fragments can be produced relatively inexpensively by yeasts and

bacteria [22]. This translates into lower cost-of-goods and contributes to a wider application of these therapeutics. Further, owing to their smaller size, Fab (50kDa) and scFv (30kDa) fragments show higher tissue penetration and clearance as compared to whole antibodies [23]. Due to these properties, they have been found to be ideal carriers for a range of molecules that require a fast delivery to the target tissue, such as toxins for cancer treatments, viruses for gene therapy, cationic tails for DNA delivery and liposomes, as well as to have high potential as diagnostic reagents, and as non-immunogenic biosensors [20].

The purification of antibody fragments is an issue of great interest in biomanufacturing. A few ligands only are currently available for the affinity purification of these species. Protein L, an 86 kDa cell wall protein of *Peptostreptococcus magnus*, binds the light chains of IgGs and is the first ligand to have been used for Fab purification. This ligand, however, binds only to $\kappa 1$, $\kappa 2$ and $\kappa 4$ light chains, but not to $\kappa 3$ and λ subgroups [24]. Recently, variable domains (13 kDa) of Llama antibodies have been engineered as affinity ligands for the purification of Fab and scFv. CaptureSelect LC-kappa and and CaptureSelect LC-lambda adsorbents specifically recognize kappa and lambda light chains of human IgG, IgA, IgM, and IgE and can be used for purifying Fab from plasma, serum and cell culture supernatants with DBC of about 11 mg/mL of sorbent (at the linear flowrate of 150 cm/h, as specified by the manufacturer).

These protein-based adsorbents, however, suffer from issues at all similar to Protein A, such as the high cost, and the low chemical and biochemical stability, in particular toward the high alkaline conditions required in the industry for periodical cleaning and sanitization.

The identification of synthetic affinity ligands for Fab would overcome these issues and have a positive effect on the cost-of-good of these biopharmaceuticals. Unlike Protein A, however, little research has been done towards finding mimetic of Protein L. At the best of our knowledge, only one synthetic ligand has been identified for Fab purification. This ligand, a triazinic compound called Ligand 8/7, has been discovered by screening a solid-phase triazine-based tri-substituted peptoidal scaffold library against whole hIgG, Fc and Fab [25,26]. The agarose-based 8/7 adsorbent, while binding Fab with good affinity ($K_d = 2.6 \times 10^{-6}$ M) and selectivity, shows low binding capacity (approx. 2 mg/mL).

10.2.1.2. Design and development of Protein L – derived affinity ligands for the purification of antibody fragments

Thanks to their chemical and structural versatility, peptides are ideal candidates for the identification of small synthetic ligands for the specific targeting of biomolecules. The identification of peptide ligands can follow two routes, combinatorial and rational. The latter, with the aid of software for molecular dynamics and docking simulations, aims to tailor a peptide ligand that mimics the structure and the binding properties of a protein ligand.

Protein L is a particularly ideal model for the design of a protein mimetic peptide ligand. The Fab binding site on Protein L, contained in the protein C3 domain, is a continuous and compact structure comprising a pair of antiparallel β -sheets. The analysis of the crystal structure available on Protein Data Bank (PDB code: 1HEZ) also indicates that the binding unit is independent of the surrounding protein framework. This suggests the possibility of abstracting the binding site of Protein L to obtain a self-assembled peptide

“hairpin” that possesses a biorecognition power similar to that of the parental protein. Further optimization studies can be performed to endow the abstracted sequence with the desired chemical and biological properties, so as to produce a stable, highly selective affinity ligand for large scale production.

A method was hence proposed for abstracting a Fab-binding peptide sequence from Protein L, which comprises: 1) design and modeling of sequences by molecular dynamic simulations to identify small peptides that resemble the structure of the protein binding unit; 2) molecular docking of the modeled structures to select suitable ligand candidates; 3) synthesis and testing of the selected sequences for protein binding by affinity chromatography to narrow the selection to a peptide ligand; 4) optimization of the selected ligand and development of an affinity adsorbent with high binding capacity.

A peptide sequence was chosen based on the solved structure of the complex Protein L – Fab (PDB code: 1HEZ). The 19-mer TIKVNLIFACGKIQTAEFK, comprising the residues 822-840 of the C3 domain of Protein L, was abstracted and modeled using PEP-FOLD 1.5, a *de novo* peptide structure prediction available online at Mobylye@RPBS, to determine the most thermodynamically favorable conformation. The aspartic acid on residue 381 of the original sequence was replaced with a cysteine to enable coupling the peptide on an iodoacetic acid activated chromatographic resin. All five most favorable structures predicted by molecular dynamics were antiparallel β -sheets, showing a high degree of structural overlapping with the native binding site of Protein L (Figure 10.1).

The structure of the Protein L – mimetic peptide (PLMP) as obtained from molecular dynamic simulations was docked against the Fab fragment of human IgG using the docking program HADDOCK 2.1. The coordinate file for the kappa light and heavy chain of Fab was obtained from the PDB file 1HEZ. The binding energy of the complex PLMP – Fab was estimated with the two empirical function Δ^iG and XScore, which account for van der Waals interactions, hydrogen bonding, deformation penalty, electrostatic and hydrophobic effects, atomic contact energy, dipole-dipole interactions, and the presence of water [27-31].

The most energetically favored docked structures were selected as the best representation of the Fab – PLMP complex. As an additional condition, only the docked structures that contain more than 70% of native Protein L – Fab contacts were considered as successful. The docking calculations returned multiple peptide structures docked on the Fab binding site, which were collected in clusters based on geometrical similarity. The three best clusters are reported in Table 10.1.

The docking calculations of the candidate PLMP returned impressive results in terms of both reproduction of native contacts (percentage of conserved native contacts > 70%) and free energy of binding, the latter being found to be quite consistent with the value of the Fab – Protein L complex. The comparison between clusters 1 and 2 and the solved Protein-L complex reported in Figure 10.2 indicates very good overlay between PLMP and Protein L. These results confirm that the selected peptide sequence is a good candidate for binding the Fab fragment of IgG.

The peptide TIKVNLIFACGKIQTAEFK was synthesized and coupled on iodoacetic acid – activated Toyopearl AF-Amino-650M resin. The sequence bore two modifications, namely the amidation of the peptide C-terminus and the acetylation of the N-terminus, to simulate the binding sequence as originally present within the framework of Protein L. The adsorbent was tested for the binding of Fab and Fc fragments of human IgG by affinity chromatography. The chromatograms shown in Figure 10.3 indicate that the PLMP ligand is very selective for Fab.

On the other hand, the adsorbent's binding capacity is not very high, being in the order of about 1.5 – 2 mg/mL, and call for future optimization studies.

10.2.1.3. Future directions

While the Protein L – mimetic peptide ligand (PLML) shows very high selectivity towards the Fab fragment of human IgG, the adsorbent's binding capacity needs improvement. To this end, it is necessary to optimize the peptide density on the resin surface. This can be done by performing ligand coupling with higher peptide density in solution or by trying other coupling methods, such as “click chemistry”, which has been already used for conjugating peptides on solid surfaces. The goal is to approach the binding capacity of commercial protein-based adsorbents, namely 8 – 10 mg/mL.

Further developments will comprise modification of the peptide sequence to tune the ligand selectivity and increase its chemical and biochemical stability. Similarly to what done in previous studies [9], non natural residues will be used in place of natural amino acids in order to endow the PLML with resistance towards harsh chemical agents and proteases. Also,

after determining the ligand selectivity for Fab κ and λ , site-specific amino acid mutations will be tested to design two sequence variants specifically targeting each of the two subgroups.

10.2.2. Design of polycyclic peptides for the identification of ultra-high affinity ligands

Recent advancements in chemical and biochemical synthesis have opened the way to a new generation of highly complex peptide architectures [32-34]. Multi-domain ligands, that is, molecules capable of binding a target by multisite interactions, are promising candidates for applications, such as difficult bioseparations as well as therapeutics or diagnostics, where very high affinity and selectivity are required. The higher structural complexity promotes in fact an “avidity effect” within the single ligand, which results in high binding strength and binding specificity [35].

Bicyclic and tricyclic peptides are ideal structures for creating ligands that mimic the binding mechanism typical of natural protein-protein biorecognition. The synthesis and screening of biological libraries of compounds labelled as “byclic peptides” have been reported in literature [36-40]. The proposed structure, however, resembles more closely that of a mono-cyclic peptide internally connected with a linker and, in fact, the level of affinity achieved by the selected ligands ($K_d \sim 10^{-8} - 10^{-9}M$) is not higher than the values reported for other cyclic peptides [11].

Our group has proposed a method for the synthesis of bicyclic and tricyclic peptides on solid phases. These structures comprise independent peptide cycles connected by a spacer

arm (Figure 10.4). This conformation is expected to allow better access of the cyclic binding units into neighbour binding sites, thus promoting higher ligand affinity for the target biomolecule. These peptides have the potential for becoming the next generation of ligands, anticancer agents, antibiotics, receptor antagonists and models for studying protein structural motifs.

Owing to their high affinity and selectivity, polycyclic peptides could replace antibodies for therapeutic applications as well as for the design of *in vitro* and *in vivo* diagnostics. Highly specific monoclonal antibodies, in fact, while being currently employed in a variety of therapeutic and diagnostic applications, suffer from important drawbacks, in particular the high cost and the batch to batch variations. Polycyclic peptides, on the other hand, are more chemically robust and can be produced synthetically in large amounts, hence reducing cost and variability. The use of small synthetic ligands with high specificity for tumor antigens and biomarkers in place of antibodies would therefore enable the production of more affordable bioassays characterized by higher accuracy and reproducibility.

10.2.3. Design of peptide scaffolds for the design of affinity ligands and drugs

10.2.3.1. Introduction and motivation

Ordinary solid-phase peptide libraries, while being a valuable tool for discovery of therapeutic agents and affinity binding ligands, suffer from one main limitation, that is, the difficulty and the high cost associated with the introduction of functional groups other than those carried by natural amino acids [41]. While challenging under both technical and

economic aspects, exploring the chemical space outside that defined by the 20 natural amino acids can be extremely rewarding in terms of ligand affinity and selectivity [42]. Chemical diversity, or chemical space, designates the range of functional groups and backbone properties comprised in the sequence. The inclusion of groups other than amino acids, *e.g.* sugars, lipids, nucleobases, and non-natural functionalities, can significantly improve the biophysical and biochemical of a library and of the ligands identified by library screening [43-47]. In glycopeptides, for example, the combination of amino acids and sugars enhances the affinity and selectivity as well as the pharmacokinetics of the ligand or drug [46]. Lipopeptides and lipoglycopeptides, owing to their aliphatic or aromatic, often halogenated, moieties, are among the most potent antibiotics [48]. Fully synthetic functional groups and reactive moieties can also be introduced to impart other properties or allow sequence modifications, for example coupling a small drug or a fluorescent label.

10.2.3.2. Poly-amino and poly-carboxyl peptide scaffolds

In order to benefit from the potential of a library with the widest possible chemical diversity and, at the same time, reduce the cost and the synthetic effort inherent to building such massive collection of compounds, we have proposed a method for the synthesis of a solid-phase combinatorial library of compounds functionalized with natural and non natural groups displayed on a peptide scaffold, along with compounds useful for carrying out such methods. The general structure of the peptide scaffold is presented in Figure 10.5.

Where R^{\prime} is a solid support, each R^{\prime} is an independently selected functional group, $\text{R}^{\prime\prime}$ is a functional group, each X is a linking group, each n is 0, 1 or 2, each n' is 0 or an integer of 1, 2, 3, 4 or 5, and n'' is an integer of from 1, 2, 3 or 4 to 18 or 20.

The structure portrayed in Figure 10.5 basically comprises a peptide frame decorated with a variety of functional groups. Prior to appending these functionalities, the peptide frame is synthesized in a protected form, which can be either polyamine or polycarboxyl. The former consists of a protected poly-diaminoacid, *e.g.* poly-DAP (diaminopropionic acid), poly-DAB (diaminobutyric acid), poly-DAP (poly-diaminopentanoic acid, else called poly-ornithine), poly-lysine, and the like. The latter consists of a protected poly-carboxyl structure, *e.g.* poly-aspartic or poly-glutamic acid. The two are shown in Figure 10.6.

In Figure 10.6, each Y is an orthogonal protecting group, *i.e.* a guard that can be selectively removed from a single functional group, in this case either an amino or a carboxyl group, while leaving all other functional groups protected. A wide variety of protecting groups are known and reported in literature [49].

The method for functionalizing a peptide scaffold comprises an iteration of two basic steps: 1) removing a protecting group under specific cleavage conditions from a single functional group on the peptide scaffold, either an amino or a carboxyl group, and 2) coupling the desired functionality onto the free amino or carboxyl group. The functionalities appended on the peptide scaffold can be either natural biomonomers, such as the side chain of natural amino acids, sugars, lipids, or nucleobases, or non natural moieties. In order to “decorate” a polyamine scaffold, these functionalities need to be in the form of functional

aldehydes (R-COH, where R is the desired functionality). The aldehyde is coupled to the free amino group by reductive amination. In order to “decorate” a polycarboxyl scaffold, instead, the functionalities need to be in the form of functional alcohols (R-OH). The alcohol is coupled to the carboxyl group, following the activation of the latter with an appropriate reagent, *e.g.* HCTU, by formation of an ester bond. The resulting poly-secondary-amine and poly-ester structures are shown in Figure 10.7.

These structures offer three main advantages over ordinary peptide molecules, namely higher chemical diversity, structural flexibility, and synthetic efficiency and scalability. The variety of groups available for functionalizing the peptide scaffolds is practically unlimited and the spacing between the functional groups, in terms of both distance between side chains and side chain length, can be varied very easily. This diversity is expected to enable the fine tuning of the affinity and specificity of the ligands based on these structures.

10.2.3.3. Examples of poly-amino scaffolds: mimetics of the IgG-binding tripeptides HWK and HFK

Two poly-amino peptide scaffolds were created (Figure 10.8), which mimick the IgG-binding tripeptides HWK and HFK. These structures were called K and K respectively. These ligands were synthesized on chromatographic resin Toyopearl AF-Amino-650M resin and the resulting adsorbents were tested for antibody binding following the chromatographic protocol employed for HWRGWV [5].

The chromatograms reported in Figure 10.9 indicate that the scaffold ligands can bind the antibody target. This in turn suggests that these structures could be effective ligands for a variety of applications in biotechnology.

In the future, libraries of these compounds will be designed, synthesised and screened for the identification of ligands for bioseparations or drugs.

10.3. References

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Table 10.1. Results of the molecular docking simulations of the Fab – PLMP complex. Fab-Protein L complex is used as a reference.

Peptide	Cluster	# of structures	Δ^iG (kcal/mol)	XScore	Conserved native contacts
PLMP	1	102	-3.5	-5.36	71.42 %
	2	40	-4.3	-3.09	92.85 %
	3	16	-0.8	-5.43	71.42 %
1HEZ A:E	N/A	N/A	-3.2	-3.64	N/A

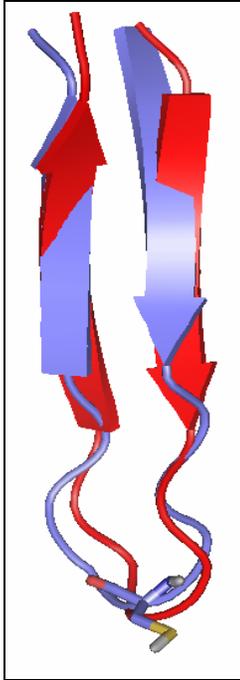


Figure 10.1. Comparison of the native Fab binding site of Protein L (red) with the structure of the Protein L – mimetic peptide (PLMP) as predicted by Pepfold (violet).

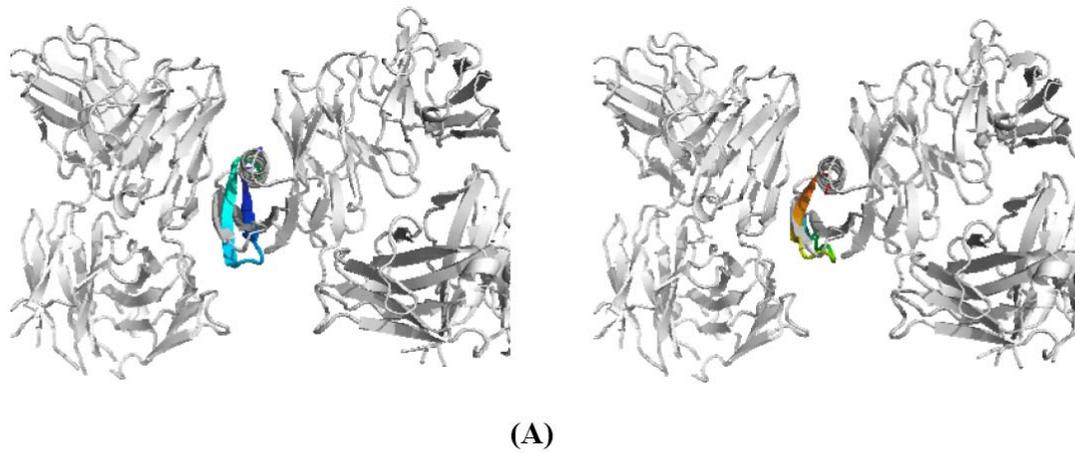


Figure 10.2. Overlay of clusters #1 (A) and #2 (B) of PLMP with solved structure of the Protein L – Fab complex.

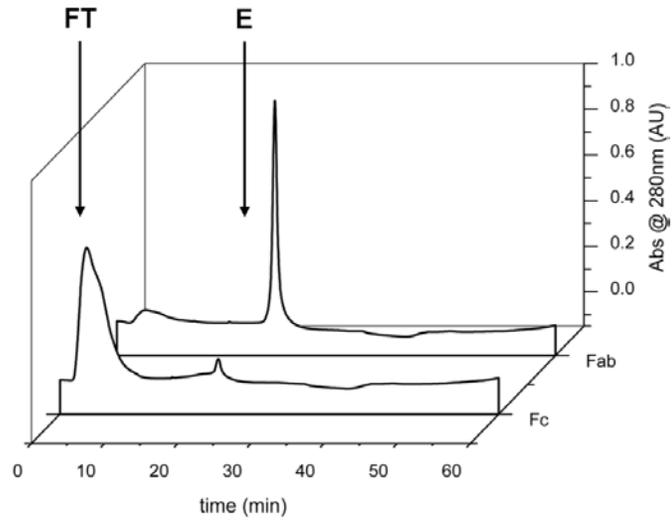


Figure 10.3. Chromatograms of Fab and Fc binding on the Protein L – mimetic peptide resin.

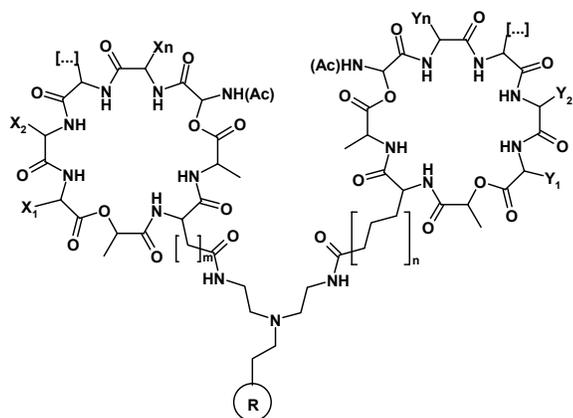


Figure 10.4. Structure of a bicyclic peptide.

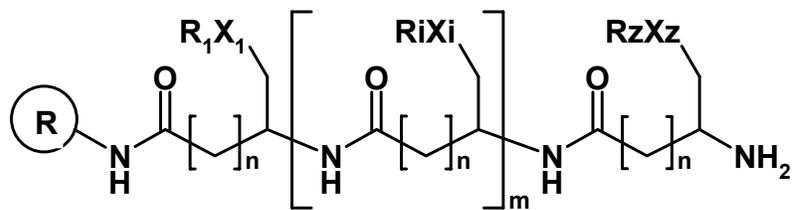


Figure 10.5. General structure of the functionalized peptide scaffold.

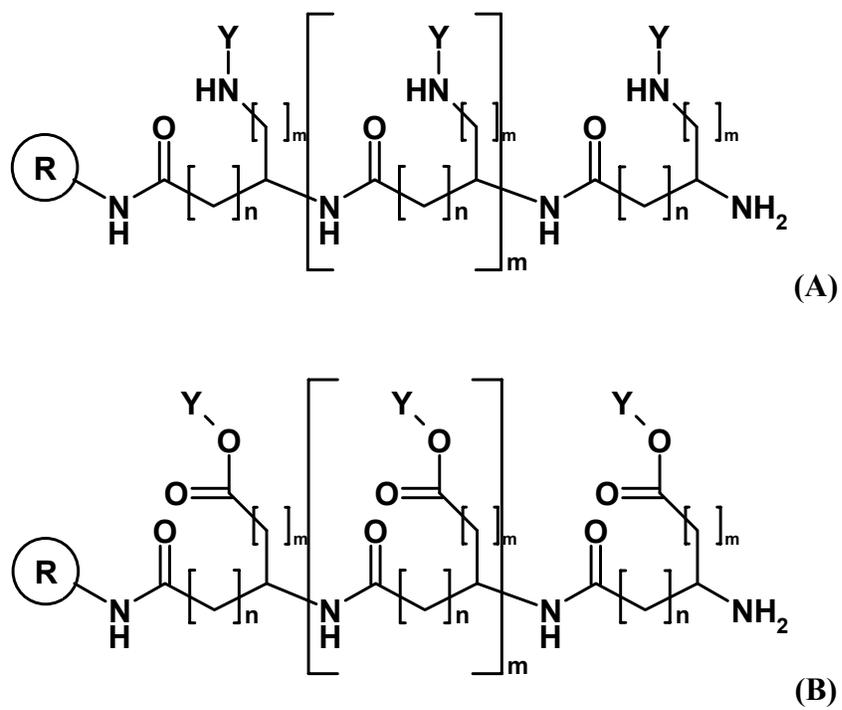


Figure 10.6. General structure of the protected (A) polyamino or (B) polycarboxyl peptide scaffold.

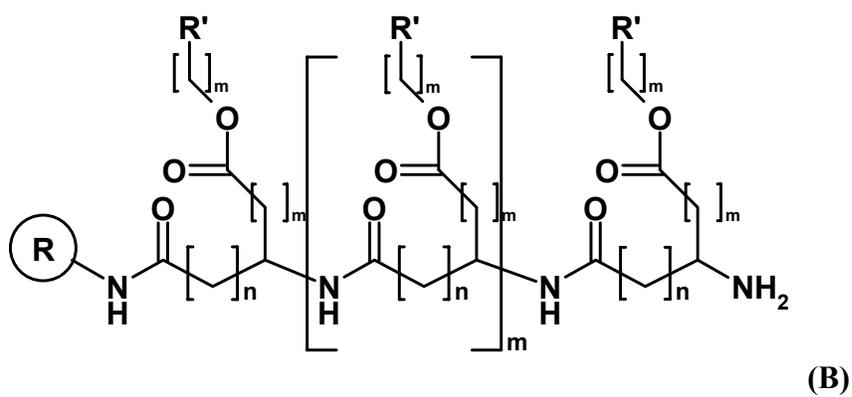
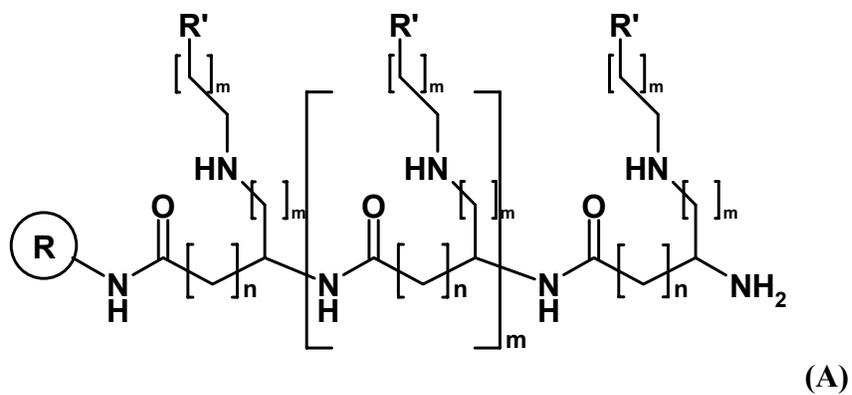


Figure 10.7. General structure of the functionalized (A) polyamino or (B) polycarboxyl scaffold.

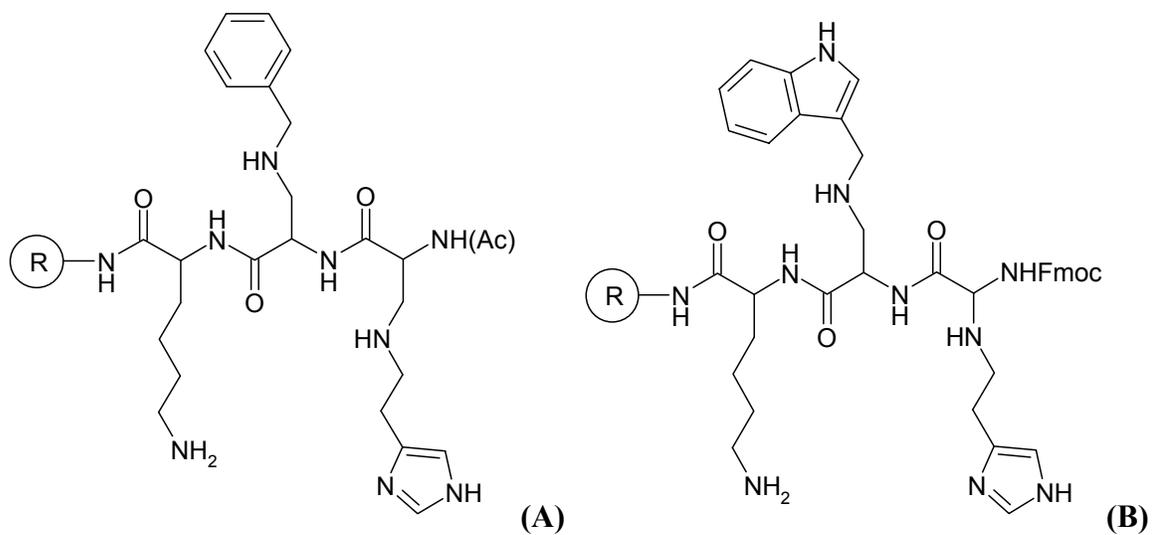


Figure 10.8. Polyamino based mimetics of the IgG-binding trimers HWK and HFK: (A) HΩK and (B) HΦK.

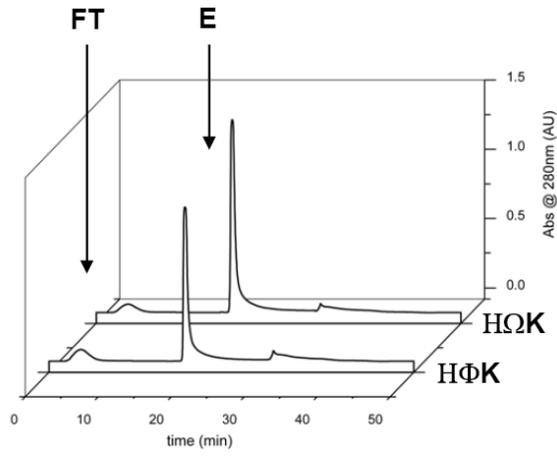


Figure 10.9. Chromatograms of IgG binding using the adsorbents (A) HΩK-Toyopearl resin and (B) HΦK-Toyopearl resin.