ABSTRACT

LIU, ZHUO. Affinity Chromatographic Purification of Human Immunoglobulins by Hexamer Peptide Ligands. (Under the direction of Dr. Ruben G. Carbonell.)

Antibodies are highly specific, naturally evolved molecules that recognize and eliminate pathogenic and disease antigens. The past 30 years of research have proven their potential as therapeutic agents to fight cancer, autoimmune diseases and infections. Today, antibody-based therapeutics account for one third of all new treatments in USA. The world's therapeutic monoclonal antibodies market exceeded $40 billion in 2010 and is expected to reach $70 billion by 2015. Although most of antibodies in the market and in the clinical development are immunoglobulin-G-based drugs, more and more attention has been paid to immunoglobulins A (IgA) and M (IgM) for their potential as new biotherapeutics for the treatment of mucosal vaccines, autoimmune diseases and cancer therapies.

A family of linear hexamer peptides, exhibiting the ability to recognize and bind immunoglobulin G (IgG) through its Fc portion, has been previously identified through a three-step screening of a synthetic solid phase combinatorial peptide library. As a family, all three peptide ligands HWRGWV, HYFKFD and HFRRHL have the ability to recognize both human IgA and IgM and have the potential for large-scale purification of hIgA and hIgM.

Hexamer peptide ligand HWRGWV demonstrates the strongest binding affinity to hIgM, followed by hIgA and hIgG, respectively. Relatively high recovery (> 90%) can be achieved for hIgG by using an elution pH larger than 4.0; however, high recovery (> 90%) can only be achieved for hIgA and hIgM by using elution pH lower than 4.0. N-terminal acetylation of the ligand would decrease the binding of hIgA and hIgM since electrostatic interactions play a role in hIgA and hIgM binding to the peptide. At low peptide density,
salting-in salts such as MgCl$_2$ and CaCl$_2$ and non-electrolytes such as ethylene glycol, urea and arginine can be used as elution additives to facilitate hIgA and hIgM elution.

Hexamer peptide ligands demonstrate the ability to purify hIgA and hIgM from complex media, mammalian cell culture supernatants as well as Cohn fraction II/III. For antibody purification from cMEM with peptide ligand HWRGWV at four different peptide densities (0.04, 0.11, 0.22 and 0.55 mequiv./g), the improved recovery at higher peptide density due to increased binding affinity was compensated by the decrease in purity for all three antibodies. Over 80% recovery and 90% purity were achieved for hIgG and hIgA at peptide densities of 0.11 and 0.22 mequiv./g. For hIgM, 75.7% recovery and 86% purity were achieved at peptide density of 0.04 mequiv./g. For antibody purification from cMEM with other ligands HYFKFD and HFRRHL at peptide density of 0.11 mequiv./g, higher purities but lower recoveries were achieved with HYFKFD compared to HFRRHL, suggesting better binding specificity and possible lower dynamic binding capacity. About 95% recoveries and 90% purities were achieved for human IgA and secretory IgA purified from spiked Chinese hamster ovary cell culture supernatants using peptide ligand HWRGWV at peptide density of 0.11 mequiv./g. For purification of hIgM from spiked human B lymphocyte cell culture supernatants using peptide ligand HWRGWV at peptide density of 0.04 mequiv./g, the final recovery and purity of the antibody is feedstock dependent, but can reach levels of both recovery and purity as high as 95%. Because of the binding affinity difference, one-column purification of hIgA, hIgG and hIgM from Cohn fraction II/III was achieved. After pretreatment with caprylic acid precipitation or the combination of caprylic acid and polyethylene glycol precipitation, highly purified (> 95%) hIgG was obtained with hIgA enriched fraction and hIgM enriched fraction as by-products.
This work demonstrates hexamer peptide ligands, initially screened from a solid phase combinatorial peptide library for IgG purification; also have the potential for large-scale purification of human IgA and IgM.
Affinity Chromatographic Purification of Human Immunoglobulins by Hexamer Peptide Ligands

by
Zhuo Liu

A dissertation submitted to 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

2012

APPROVED BY:

Dr. Ruben G. Carbonell
Chair of Advisory Committee

Dr. Michael C. Flickinger

Dr. Patrick V. Gurgel

Dr. Jason Haugh

Dr. Balaji Rao
DEDICATION

To my parents

and Jingjing Zhang
BIOGRAPHY

Zhuo Liu was born on November 27th, 1981 in Changsha, Hunan Province, the only child of Zhenkui Liu and Jingsai Yang. He went to Affiliated Elementary School of Hunan University at Changsha and Affiliated Middle School of Hunan Normal University at Changsha. After four years of study, he obtained his B.S. degree in Chemistry from Hunan University at the age of twenty. In August 2003, the author came to the United States and studied in the Department of Chemistry at Kent University in Ohio. Two years later, he obtained his M.A. degree in Analytical Chemistry under the direction of Dr. Arne Gericke. In the same year of 2005, he was enrolled in the Department of Chemistry at University of Pittsburgh to pursue his Ph.D. degree. After one-year of course study, he transferred to the Department of Chemical and Biomolecular Engineering at North Carolina State University in August 2006. The author joined the Bioseparation Group under the direction of Dr. Ruben G. Carbonell in September 2007.
ACKNOWLEDGEMENTS

I would like to express my gratitude to those who positively affected my life with their support, wisdom, love and care.

First, I would like to thank my advisor, Dr. Ruben G. Carbonell, for his endless guidance, support and patience. He is a true mentor to me and his passion for science and technology has inspired me to continue learning with an open and positive mind.

I would like to thank Dr. Patrick V. Gurgel for his encouragement, advisory discussions, support and help in all aspects throughout my Ph.D. study. He is not only a great advisor, but also a good friend I have been benefited from.

I would like to thank Dr. Michael Flickinger, Dr. Jason Haugh, and Dr. Balaji Rao for being on my advisory committee. I also like to thank Dr. Sherie L. Morrison at UCLA for her generosity for providing us the CHO cell line for human IgA production.

I would like to thank the members of the Bioseparations Group members for their kindness, friendship, generous help, invaluable discussion, and the happy collaborative working environment they provided.

Finally, I would like to give my deepest thanks to my close family members, my parents, my fiancee Jingjing Zhang and my dog Gabriel for their endless support, care, and unconditional love.
TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................................. x

LIST OF FIGURES ................................................................................................................................. xii

Chapter 1. Introduction .......................................................................................................................... 1
  1.1. Motivation ..................................................................................................................................... 2
  1.2. Goals ............................................................................................................................................ 5
  1.3. Overview of this dissertation ....................................................................................................... 5
  1.4. References .................................................................................................................................... 7

Chapter 2. Literature Review ............................................................................................................... 10
  2.1. Immunoglobulins ....................................................................................................................... 11
      2.1.1. Overview ............................................................................................................................. 11
      2.1.2. Structure and properties of human immunoglobulins ......................................................... 12
      2.1.3. Functions and roles of human immunoglobulins ................................................................. 14
      2.1.4. Therapeutic application and potential usage of human immunoglobulins ...................... 16
  2.2. Purification of immunoglobulins ................................................................................................. 20
      2.2.1. Overview ............................................................................................................................. 20
      2.2.2. Biospecific ligands for antibody purification ......................................................................... 22
      2.2.3. Pseudo-biospecific ligands for antibody purification ........................................................... 25
      2.2.4. Bioengineered and synthetic mimic ligands for antibody purification ............................... 29
      2.2.5. Hexamer peptide ligands for antibody purification ............................................................ 33
  2.3. References .................................................................................................................................... 35

Chapter 3. Effects of Peptide Density and Elution pH on Affinity Chromatographic Purification of Human Immunoglobulins A and M ............................................................................. 51
  3.1. Introduction .................................................................................................................................. 53
3.2. Experimental ................................................................. 58
  3.2.1. Materials .................................................................. 58
  3.2.2. Equipment .............................................................. 59
  3.2.3. Chromatographic capture and elution of human IgG, IgA and IgM .......................................................... 59
  3.2.4. pH gradient elution for captured human IgG, IgA and IgM .......................................................... 60
  3.2.5. Chromatographic isolation of human IgG, IgA, and IgM from cMEM ................................................. 60
3.3. Results and discussion ......................................................... 62
  3.3.1. Effect of elution solution pH on recovery of human IgG, IgA and IgM at different peptide densities .......................................................... 62
  3.3.2. Elution profiles of human IgG, IgA and IgM using pH gradients .......................................................... 63
  3.3.3. Effect of peptide density on human IgG, IgA and IgM isolation from cMEM ........................................ 65
  3.3.4. Human IgG, IgA and IgM isolation from cMEM using peptide ligands HYFKFD and HFRRHL .......................................................... 68
3.4. Conclusions .................................................................... 70
3.5. References ........................................................................ 71

Chapter 4. Purification of Human Immunoglobulins A, G and M from Cohn Fraction II/III by Small Peptide Affinity Chromatography .......................................................... 86

  4.1. Introduction .................................................................... 88
  4.2. Experimental ................................................................. 91
    4.2.1. Materials .................................................................. 91
    4.2.2. Equipment .............................................................. 92
    4.2.3. Chromatographic capture and recovery of human IgG, IgA and IgM .......................................................... 92
    4.2.4. Preliminary elution condition study for the isolation of human IgG, IgA and IgM from a 10:2:1 mg/mL IgG, IgA, IgM mixture .......................................................... 93
    4.2.5. Cohn fraction II/III sample preparation and pretreatments .......................................................... 93
    4.2.6. Purification of human IgG, IgA and IgM from Cohn fraction II/III .......................................................... 95
    4.2.7. Sample analysis for yields and purities .......................................................... 96
4.3. Results and discussion ................................................................. 97

4.3.1. Effects of elution additives on the recovery of human IgG, IgA and IgM at different peptide densities ................................................................. 97

4.3.2. Preliminary elution condition study for the isolation of human IgG, IgA and IgM from a 10:2:1 mg/mL IgG, IgA, IgM mixture .................................................. 102

4.3.3. Cohn fraction II/III sample preparation and pretreatments .................. 104

4.3.4. Purification of human IgG, IgA and IgM from Cohn fraction II/III .......... 107

4.4. Conclusions ................................................................................. 110

4.5. References .................................................................................. 112

Chapter 5. Affinity Chromatographic Purification of Human Immunoglobulin A from Chinese Hamster Ovary Cell Culture Supernatant ........................................ 137

5.1. Introduction ................................................................................... 139

5.2. Materials and Methods ................................................................. 143

5.2.1. Materials ................................................................................ 143

5.2.2. Equipment ............................................................................... 144

5.2.3. Adsorption isotherm measurements ........................................... 144

5.2.4. Generation of breakthrough curves ............................................. 145

5.2.5. Cell culture .............................................................................. 146

5.2.6. Sample preparation ................................................................... 146

5.2.7. Purification of human IgA from different feedstocks ..................... 147

5.2.8. Sample analysis for recoveries and purities ................................... 147

5.3. Results and discussion ................................................................. 148

5.3.1. Adsorption isotherms ............................................................... 148

5.3.2. Dynamic binding capacity ......................................................... 150

5.3.3. Affinity chromatography purification of human IgA and SIgA from spiked CHO cell culture supernatant .............................................................. 151

5.3.4. Affinity chromatography purification of recombinant human IgA from 100-fold concentrated CHO cell culture supernatant ........................................ 154
Chapter 6. Affinity Chromatographic Purification of Human Immunoglobulin M from Human B Lymphocyte Cell Culture Supernatant .................................................. 168

6.1. Introduction ........................................................................................................... 170
6.2. Material and Methods ............................................................................................ 173
   6.2.1. Material ............................................................................................................. 173
   6.2.2. Equipment ........................................................................................................ 174
   6.2.3. Generation of breakthrough curves ................................................................. 174
   6.2.4. Cell culture ....................................................................................................... 175
   6.2.5. Sample preparation ............................................................................................ 176
   6.2.6. Purification of human IgM from different feedstocks ........................................ 176
   6.2.7. Sample analysis for recoveries and purities ...................................................... 177
   6.2.8. Analytical size-exclusion chromatography ...................................................... 178
6.3. Results and discussion ............................................................................................ 178
   6.3.1. Dynamic binding capacity .............................................................................. 178
   6.3.2. Preliminary running condition study ................................................................. 180
   6.3.3. Affinity chromatography purification of human IgM from 100-fold concentrated B lymphocyte cell culture supernatant ............................................................. 183
   6.3.4. Affinity chromatography purification of human IgM from IgM spiked B lymphocyte cell culture supernatant .......................................................... 185
6.4. Conclusions ............................................................................................................. 187
6.5. References ............................................................................................................. 188

Chapter 7. Conclusions and Recommendations for Future Work ................................. 202

7.1. Conclusions ............................................................................................................. 203
7.1.1. Potential binding sites for HWRGWV on human immunoglobulins A and M 203
7.1.2. Effect of elution solution pH on recovery of human IgG, IgA and IgM at different peptide densities ................................................................. 203
7.1.3. Effect of N-terminal acetylation on capture and elution of human IgG, IgA and IgM ................................................................. 204
7.1.4. Effect of elution additives on recovery of human IgG, IgA and IgM at different peptide densities ................................................................. 204
7.1.5. Chromatographic isolation of human IgG, IgA and IgM from cMEM ........ 205
7.1.6. Purification of human immunoglobulins A, G and M from Cohn fraction II/III ................................................................................................. 206
7.1.7. Affinity chromatographic purification of human immunoglobulin A from Chinese hamster ovary cell culture supernatant ........................................ 207
7.1.8. Affinity chromatographic purification of human immunoglobulin M from human B lymphocyte cell culture supernatant ........................................ 207
7.2. Recommendations for future work .......................................................... 208
7.2.1. Mass transfer properties for hexamer peptide ligand HWRGWV adsorbents. 208
7.2.2. Purification of SIgA and IgM by affinity membrane and monolith with immobilized hexamer peptide ligand HWRGWV ................................. 209
7.3. References ................................................................................................ 211
LIST OF TABLES

Table 2.1. Structural and biological properties of human immunoglobulins. ..................... 48
Table 2.2. Human immunoglobulin chain characteristics. ...................................................... 49
Table 2.3. General comparison of biospecific and pseudobiospecific affinity ligands for antibody purification .................................................................................................................. 50
Table 3.1. Recovery (R) and purity (P) of isolated human IgG, IgA and IgM from cMEM by HWRGWV resins at different peptide densities ........................................................................................................ 74
Table 3.2. Recovery (R) and purity (P) of isolated human IgG, IgA and IgM from cMEM by HYFKFD and HFRRHL resins at peptide density of 0.11 mequiv./g. ............................................ 75
Table 4.1. Elution conditions for the isolation of human IgG, IgA and IgM (10:2:1) from their mixture with HWRGWV at peptide density of 0.23 mequiv./g. .............................................. 115
Table 4.2. Elution conditions for the isolation of human IgG, IgA and IgM (10:2:1) from their mixture with HWRGWV at peptide density of 0.11 mequiv./g. .............................................. 116
Table 4.3. Yield and purity for isolated human IgG, IgA and IgM from their mixture with HWRGWV at peptide density of 0.23 mequiv./g. ................................................................. 117
Table 4.4. Yield and purity for isolated human IgG, IgA and IgM from their mixture with HWRGWV at peptide density of 0.11 mequiv./g ................................................................. 118
Table 4.5. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III prepared in H2O and PBS w/o pretreatment ................................................................. 119
Table 4.6. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (H2O) with pretreatment ........................................................................................................ 120
Table 4.7. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (H2O) with pretreatment (continued) .................................................................................. 120
Table 4.8. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (PBS) with pretreatment ................................................................. 121
Table 4.9. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (PBS) with pretreatment (continued) ................................................................. 121
Table 5.1. Running conditions for hIgA purification from different feedstocks. ................. 161

Table 5.2. Apparent dissociation constant ($K_d$) and maximum binding capacity ($q_m$) for hIgA with peptide ligand HWRGWV at different ligand densities. ........................................ 161

Table 5.3. Recovery and purity for hIgA and hSIgA purification from spiked CHO cell culture supernatant. ........................................................................................................ 162

Table 5.4. Sample preparation for CHO cell culture supernatants after 100-fold concentration by ultrafiltration membrane. ........................................................................................................ 162

Table 5.5. Recovery, percentage and enrichment factor for hIgA purification from 100-fold concentrated CHO cell culture supernatant. ........................................................................................................ 163

Table 6.1. Running conditions for hIgM purification from different feedstocks. ................. 192

Table 6.2. Recovery, purity and enrichment factor for IgM capture from 5.7-fold concentrated human B lymphocyte cell culture supernatant. ......................................................... 193

Table 6.3. Sample preparation for human B lymphocyte cell culture supernatant after 100-fold concentration by ultrafiltration membrane. ........................................................................................................ 194

Table 6.4. Recovery and purity for hIgM purification from 100-fold concentrated B lymphocyte cell culture supernatant. ........................................................................................................ 194

Table 6.5. Recovery and purity for hIgM purification from spiked human B lymphocyte cell culture supernatant. ........................................................................................................ 195
LIST OF FIGURES

Figure 3.1. Amino acid sequence alignments of the predicted HWRGWV binding site on human IgG1 with possible binding sites on human IgA1 and IgM. .................................................. 76

Figure 3.2. Structural diagram of human IgG1, IgA1 and IgM monomer. ................................. 77

Figure 3.3. Recoveries for chromatographic capture and elution of human (A) IgG, (B) IgA and (C) IgM with HWRGWV resins at different peptide densities .................................................. 78

Figure 3.4. Chromatograms for human (A) IgG, (B) IgA and (C) IgM recovery by pH gradient elution with peptide resin HWRGWV at 0.11 mequiv./g and peptide resin Ac-HWRGWV at 0.08 mequiv./g. .................................................................................. 79

Figure 3.5. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM using HWRGWV resins at different peptide densities using pH 5.0 elution. .............. 80

Figure 3.6. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from cMEM using HWRGWV resins at different peptide densities using pH 3.0 elution. .............. 81

Figure 3.7. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from cMEM using HWRGWV resins at different peptide densities using pH 3.0 elution. .............. 82

Figure 3.8. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 5.0 elution. ........................................................................................................ 83

Figure 3.9. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 3.0 elution. ........................................................................................................ 84

Figure 3.10. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 3.0 elution. ........................................................................................................ 85

Figure 4.1. NaCl, MgCl2 and CaCl2 elution effects on human immunoglobulins A(■), G(▲) and M(■) with peptide ligand HWRGWV at peptide densities (A) 0.04 mequiv./g and (B) 0.55 mequiv./g. ........................................................................................................ 122
Figure 4.2. Ethylene glycol, urea and arginine elution effects on human immunoglobulins A(○), G(■) and M(▲) with peptide ligand HWRGWV at peptide densities (A) 0.04 mequiv./g and (B) 0.55 mequiv./g. ................................................................. 122

Figure 4.3. Separation scheme for purification of hIgG, hIgA and hIgM .................... 123

Figure 4.4. Total protein and antibody concentrations in Cohn II/III dissolved in water (A) and PBS (B) after each preparation or pretreatment step. ......................................................... 124

Figure 4.5. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H2O) without pretreatment using HWGWV resins at a peptide density of 0.11 mequiv./g. ......................... 125

Figure 4.6. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H2O) with caprylic acid precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. ......................... 126

Figure 4.7. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H2O) with caprylic acid and polyethylene glycol precipitations using HWGWV resins at a peptide density of 0.11 mequiv./g. ................................................................. 127

Figure 4.8. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H2O) with polyethylene glycol precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. ................................................................. 128

Figure 4.9. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H2O) with saturated ammonium sulfate precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. ................................................................. 129

Figure 4.10. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) without pretreatment using HWGWV resins at a peptide density of 0.11 mequiv./g. ......................... 130

Figure 4.11. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with caprylic acid precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. ......................... 131
Figure 4.12. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with caprylic acid and polyethylene glycol precipitations using HWGWV resins at a peptide density of 0.11 mequiv/g. ................................................................. 132

Figure 4.13. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with polyethylene glycol precipitation using HWGWV resins at a peptide density of 0.11 mequiv/g. ................................................................. 133

Figure 4.14. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with saturated ammonium sulfate precipitation using HWGWV resins at a peptide density of 0.11 mequiv/g. ................................................................. 134

Figure 4.15. Chromatograms of purification of human IgG, IgA and IgM from Cohn II/III in (A) HPLC grade water and (B) PBS with or w/o pretreatment using HWGWV resins at a peptide density of 0.11 mequiv/g. ................................................................. 135

Figure 4.16. SDS-PAGE of the flow through (FT) purification of human IgG, IgA and IgM from Cohn II/III in HPLC grade water or PBS with or w/o pretreatment. ......................... 136

Figure 5.1. Langmuir fits (lines) of isotherms for hIgA adsorption to HWRGWV resins at three different peptide densities of 0.11, 0.22 and 0.55 mequiv/g. ........................... 164

Figure 5.2. Breakthrough curves for hIgA on HWRGWV resins at peptide density of 0.11 mequiv/g at flow rate of 0.02 mL/min, 0.05 mL/min, and 0.1 mL/min. Column loading expressed in terms of (A) mg protein/mL of resin and (B) mg protein/g of resin. .......... 165

Figure 5.3. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from 1.5 mg/mL hIgA spiked CHO cell culture supernatant using HWRGWV resin at peptide density of 0.11 mequiv/g using five different running conditions........................................ 166

Figure 5.4. (A) Chromatograms and (B) corresponding SDS-PAGE of SIgA separation from 1.5 mg/mL SIgA spiked CHO cell culture supernatant using HWRGWV resin at peptide density of 0.11 mequiv/g using five different running conditions........................................ 167

Figure 6.1. Breakthrough curves for hIgM on HWRGWV resins at peptide density of 0.04 mequiv/g at flow rate of 0.02 mL/min, 0.05 mL/min, and 0.1 mL/min. Column loading expressed in terms of (A) mg protein/mL of resin and (B) mg protein/g of resin. ........... 196
Figure 6.2. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from 100-fold concentrated B lymphocyte cell culture supernatant using HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions. 

Figure 6.3. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from 1.5 mg/mL hIgM spiked B lymphocyte cell culture supernatant using HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions.

Figure 6.4. Analytical size-exclusion chromatograms on Ultrahydrogel 500 (7.8 mm I.D. × 300 mm) of IgM spiked human B lymphocyte cell culture supernatant and affinity purified IgM from the HWRGWV-Toyopearl column. IgM and bovine serum albumin are indicated using arrows.

Figure 6.6. (A) SDS-PAGEs of hIgM separation from spiked B lymphocyte cell culture supernatant with 0.1 mg/mL hIgM 1000 µL load by HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions.
Chapter 1. Introduction
1.1. Motivation

Immunoglobulins are among the best-studied proteins of the human body. They are highly specific, naturally evolved molecules that recognize and eliminate pathogenic and disease antigens. The past 30 years of research have proven their potential as therapeutic agents to fight cancer, autoimmune diseases and infections [1, 2]. Antibody-based therapeutics account for one third of all new biotherapeutic treatments in the USA and the size of the therapeutic antibody market exceeded $40 billion in 2010 and is expected to reach $75 billion by 2015 [3, 4]. Most antibody therapeutics in the market and in clinical development are immunoglobulin-G-based drugs because of their extended plasma half life, effectiveness in activating human complement and recruiting natural killer cells for complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity pathways [3, 5, 6]. Nevertheless, more and more attention has been paid to immunoglobulins A (IgA) and M (IgM) for their potential as mucosal vaccines and therapeutic agents. IgA is regarded as the first line of defense for human mucosal surfaces for its ability against challenging pathogens such as bacteria and viruses [7]. As therapeutic agents for cancer treatment, IgA has several advantages over IgG such as effectiveness in translocation across a monolayer of epithelium for improved targeting, and in recruiting neutrophils, the most prevalent cytotoxic cells in humans, for antibody-dependent cellular cytotoxicity [8, 9]. IgM has been shown to not only be involved in early recognition of challenging pathogens, but also to play an important role in immune surveillance mechanisms against malignant cells [10]. As a result, there is an increasing demand for therapeutic IgA and IgM antibodies and better methods for their isolation from various sources.
Downstream processing and purification accounts for a significant percentage (50-80%) of the total manufacturing costs of therapeutic antibodies and their derivatives [11]. Traditionally, the purification process for antibodies involves at least three steps: initial capture, intermediate purification, and final polishing steps. Because of the highly specific recognition between the antibody molecule and a complementary ligand, affinity chromatography reduces non-specific interactions, increases operational yields and facilitates the elimination of undesirable contaminants, often allowing purification and concentration of antibodies in one single step. As a result, affinity chromatography has been traditionally the method of choice for capture and purification of native and genetically engineered antibodies both in laboratories as well as in large scale biomanufacturing [12].

Compared to the most commonly used affinity ligands for antibody purification, Staphylococcus aureus Protein A and Streptococcus Protein G, small peptide ligands offer some advantages for these applications. These include low immunogenicity, relatively low cost, high stability, and moderate affinity allowing moderate elution conditions. Since small peptides can also exhibit good selectivity and capture efficiency for target proteins, these properties make them potential alternatives for large-scale antibody purification. The attributes of small peptides as potential ligands have been gaining significant attention due to their unique advantages of being more stable, less immunogenic, and less expensive than protein ligands [13-16]. A small peptide ligand may also exhibit less strong binding to the target protein than antibodies or other proteins, like Protein A, allowing for milder elution conditions to preserve the activity of target proteins.
Solid phase one-bead-one-peptide (OBOP) combinatorial libraries have been successfully employed to search for affinity ligands for large proteins [17-21]. The “couple-divide-recombine” technique [22, 23] makes it possible to rapidly synthesize millions of compounds for a library and the high-throughput screening technique enables the screening of the library for a desirable ligand.

A family of linear hexamer peptides, exhibiting ability to recognize and bind human immunoglobulin G through its Fc portion, has been previously identified in our group through a three-step screening of a synthetic solid-phase combinatorial peptide library, and it has been found that the selectivity of these peptides to the Fc portion of IgG is comparable to that of Protein A [24]. Peptide ligand HWRGWV exhibited the ability to bind all human IgG subclasses as well as IgGs from bovine, mouse, goat and rabbit. After direct synthesis on Toyopearl AF Amino 650M resins, HWRGWV was able to purify hIgG from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth with both purity and recovery as high as 95% under optimized conditions [25]. In addition, HWRGWV demonstrated its ability to purify two different commercial CHO cell culture media with purities and recoveries higher than 85% and 94%, respectively, again matching very well results found with Protein G using the same cell culture fluids [26]. Because of the homology between human immunoglobulins classes in their heavy chain constant regions [27], the peptide ligand demonstrated its ability to bind human IgA and IgM as well [24]. Although the mechanism of interaction between HWRGWV with IgA and IgM was not clear, the peptide ligand could still have the potential for IgA and IgM capture and purification.
1.2. **Goals**

The primary goal of this project was to investigate the possibility of using hexamer peptide ligands to purify IgA and IgM and to try and understand the binding mechanism between the peptides and IgA and IgM. The hexamer peptide ligands were characterized for affinity chromatographic purification of human IgA and IgM in terms of static binding capacity, dynamic binding capacity, antibody adsorption kinetics, peptide density effect as well as chromatographic binding and elution conditions. The hexamer peptide ligands were packed into chromatographic columns to examine their ability to purify human IgA or IgM from complete minimum essential medium containing 10% fetal calf serum and 5% tryptose phosphate broth as well as more complicated media such as B Lymphocyte and Chinese Hamster Ovary (CHO) cell culture media. The possibility to achieve one-step purification of human IgA, IgG and IgM from Cohn fraction II/III was also explored for broader application.

1.3. **Overview of this dissertation**

This dissertation focuses on the affinity chromatographic purification of human immunoglobulins A, G and M by small hexamer peptide ligands.

Chapter 2 describes structure, properties, functions and potential therapeutic applications of human immunoglobulins, especially human immunoglobulin A and M (hIgA and hIgM). It also reviews the current technologies for antibody purification and compares the advantages and disadvantages for each technology. Lastly, small hexamer peptide ligands are introduced as potential affinity ligands for IgA and IgM purification.
Chapter 3 presents an investigation of the potential of hexamer peptide ligands for hIgA and hIgM purification. The possible binding sites for hIgA and hIgM to hexamer peptide ligand HWRGWV are explored. The effects of N-terminal acetylation of the peptide as well as elution buffer pH on chromatographic elution of human IgG, IgA and IgM from HWRGWV resins at various peptide densities are investigated. The results shown in this chapter show that the tested hexamer peptide ligands exhibit potential to separate hIgG, hIgA and hIgM from a complex medium (complete Eagle's minimum essential medium (cMEM)) supplemented with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB).

Chapter 4 describes the efforts to purify human IgG, IgA and IgM using one affinity column chromatography step from Cohn fraction II/III paste using the HWRGWV peptide resin. The effects of different elution additives on recovery of three antibodies at two different peptide densities are investigated. Based on the results, the best elution condition and peptide density were chosen based on the recoveries and purities of three antibodies when a 10:2:1 mg/mL of IgG, IgA and IgM is employed as the starting material. Finally, Cohn fraction II/III is used as the starting material for one-column affinity purification of human IgG, IgA and IgM using HWRGWV peptide resin. Different pretreatment methods are also employed in order to achieve better purity and yield for three antibodies.

Chapters 5 and 6 further examine the ability of the hexamer peptide ligand HWRGWV for purification of IgA (Chapter 5) and IgM (Chapter 6) from mammalian cell culture supernatants. Two different mammalian cell lines were cultured for antibody production and different running condition strategies based on previous experiments are employed to improve the purities of antibodies.
All results above are summarized in Chapter 7, with recommended future work.

1.4. References


Chapter 2. Literature Review
2.1. Immunoglobulins

2.1.1. Overview

Immunoglobulins (Igs, also referred to as gamma-globulins) are a family of proteins that function as antibodies, the antigen-binding proteins that are present on the B-cell membranes and also produced by plasma cells into a variety of secretions such as saliva, sweat, nasal secretions, breast milk, and colostrums. Membrane-bound antibodies confer antigenic specificity on B cells; antigen-specific proliferation of B-cell clones is elicited by the interaction of membrane antibodies with antigens. Secreted antibodies circulate in the bodily fluids and serve as the effectors of humoral immunity by searching out and neutralizing antigens or marking them for elimination. All immunoglobulins share certain structural features, bind to antigen, and participate in a limited number of effector functions [1].

The serum antibodies produced in response to a particular antigen are heterogeneous and polyclonal antibodies that are composed of different monoclonal antibodies binding to different antigenic determinants. Although polyclonal antibodies produced in vivo are beneficial to the organism, they have numerous disadvantages for immunologic research and clinical applications that demand precise control of antibody quantity, properties and specificity. Since the hybridoma technique was developed in 1975 [2], monoclonal antibodies (mAbs) can be produced by fusing an antibody-producing cell to a B lymphocyte tumor cell. The mAbs produced are homogeneous in specificity, affinity, and isotype; each monoclonal product is specific to a single antigenic determinant. Both polyclonal and
monoclonal antibodies have been generally used for identification, measurement, and purification of biological molecules, and for therapeutic applications [3].

2.1.2. Structure and properties of human immunoglobulins

Human immunoglobulins account for approximately 20% of all proteins in human plasma. Five distinct classes or isotypes of immunoglobulins have been identified in human serum based on their structure, biological and antigenic differences as shown in Table 2.1 [1]. Immunoglobulin A, the most abundant immunoglobulin in a body, plays the most important role in mucosal immunity. Indeed, the daily IgA produced exceeds the combined production of all other immunoglobulin classes, with a production of up to 66 mg/kg of body weight, compared with 34 mg of IgG and 7.9 mg of IgM [4]. Immunoglobulin G constitutes approximately 75% of the total serum immunoglobulins. Immunoglobulin M is a high molecular weight protein consisting of five or rarely six subunits (IgM monomers) and accounts for about 10% of serum Igs in healthy humans. [5].

The immunoglobulin structure is well adapted to the functional role that an antibody molecule has to play [6]. As a family, human immunoglobulins share a basic four-chain structure composed of two identical heavy (H) chains of 50-70 kDa, and two identical light (L) chains of 25 kDa which are held together by both noncovalent forces and covalent disulfide bonds between the two heavy chains (Table 2.2). Each polypeptide chain folds up into domains comprised of 100-110 amino acids formed by intra-chain disulfide bonds. The light (L) chains have two domains: a variable region domain (V_L) and a constant region domain (C_L) and the heavy chains have more domains, with the exact number depending on
the class of immunoglobulins. Five distinct heavy chains termed alpha (α), gamma (γ), mu
(μ), delta (δ) and epsilon (ε) correspond to the five different classes of immunoglobulins: IgA,
IgG, IgM, IgD and IgE, respectively. The IgA and IgG classes are further subdivided into
two subclasses (IgA1 and IgA2) and four subclasses (IgG1, IgG2, IgG3 and IgG4),
respectively. The N-terminal domains of the light and heavy chains are the variable (V)
domains that exhibit an enormous diversity between different immunoglobulins. Each
variable domain comprises a beta-sheet “framework” to support the three hypervariable loops
or complementary determining regions (CDRs), which are spatially close to each other and
constitute the antigen-binding site. The COOH-domains, defined as constant domains, are
identical between chains from the same class, subclass, and with the same allotypes [1, 5].

Of all of the Ig classes, only IgA and IgM share the ability to polymerize through the
linkage of multiple monomer units. IgA predominantly polymerizes into dimers, which are
stabilized through covalent interaction with the joining (J) chain, a 15-16 kDa polypeptide
(137 amino acid residues) also present in pentameric IgM. The J chains are synthesized in
plasma cells and are incorporated into polymeric IgA (pIgA) or IgM (pIgM) shortly before or
at the time of secretion. The J chain, rich in acidic amino acids, does not resemble any other
known protein. It comprises a single polypeptide containing eight cysteine residues, six of
them forming intrachain disulfide bridges, and it is highly conserved (~70%) between species.
Another feature shared by secreted polymeric IgA and IgM is that they all have an 18 amino
acid long extension called secretory tailpiece at the C-terminal of the α and μ heavy chains.
In both IgA and IgM, the tailpiece interacts with the J chain; however, the presence of the
tailpiece is not the only factor for incorporation of J chain into the polymeric
immunoglobulins [1, 5]. The polymeric secretory IgA (SIgA) comprises not only IgA and J chain, but also a 70 kDa heavily glycosylated protein called secretory component (SC), which is complexed with the IgA during the secretion process, stabilizes the structure of SIgA and increases its resistance to proteolysis. It is part of a cell surface polymeric immunoglobulin receptor (pIgR) that mediates the transcytosis of polymeric IgA and IgM across the epithelial cell barrier. SC and J chain are both disulfide-linked to the Fc region of the IgA, although not to each other [1, 5, 7].

2.1.3. Functions and roles of human immunoglobulins

IgA represents the most abundant antibody class in the mucosal surface and the second most abundant antibody class in human serum. Mucosal epithelia in the human body include the linings of digestive, respiratory and urogenital tracts and comprise a vast surface area (~400 m²) which is continuously exposed to attack by potentially harmful agents such as bacteria, virus, fungi, and parasites, in addition to soluble dietary and environmental substances. The first line of protection of the mucosal surfaces against colonization and possible entry and invasion by microbes mainly depend on the specific immune recognition from secretory IgA (SIgA) and, to some extent, secretory IgM (SIgM) as well [4]. Inhibition of adherence and penetration is a major mechanism for SIgA-mediated defense of mucosal surfaces. SIgA is a hydrophilic, negatively charged molecule because of the predominance of hydrophilic amino acids in the Fc region and abundant glycosylation of both IgA and SC. As a result, SIgA can surround pathogenic microorganisms with a ‘hydrophilic shell’ that is repelled by mucosal surfaces. However, because of the abundance of food components and
microbial flora in the digestive tract, antigens can still continuously reach the lamina propria by diffusion or transcytosis. The immune complexes formed by absorbed antigen and pIgA could be either transcytosed back to the lumen by pIgR-mediated transport or taken up by M-cells of Peyer’s patches. Other defensive mechanisms include its ability to agglutinate microbes and inhibit bacterial motility by binding to their flagella [8]. SIgA can also interact with viruses or bacteria products such as enzymes and toxins, and neutralize their actions [9, 10].

Although the importance of SIgA in mucosal secretions is well established, the functions of serum IgA are incompletely understood. In human serum, 10% of IgA is polymeric, while 90% is monomeric. Two subclasses of IgA (IgA1 and IgA2) have been identified, and 90% of them in serum are IgA1 [1]. Unlike SIgA’s direct effector mechanism, the functions of serum IgA in immunity result from indirect killing mechanisms activated by their constant domains. The indirect mechanisms include complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), cell phagocytosis, and potentially antibody-mediated antigen presentation [11].

A relevant percentage of the blood-circulating immunoglobulins are pentameric IgM, and they are also present in the gastrointestinal tracts, lymphatic vessels, mucosal tissues, bone marrow and others [12]. IgM differs from other classes of immunoglobulins because it is produced predominantly by B1 cells in the absence of exogenous antigen stimulation. Consequently, IgM is the major component of the natural antibodies and is also the first class of immunoglobulins produced during a primary antibody response [13]. IgM can bind to invading pathogens as they enter and result in complement activation as a first line of defense;
IgM also provides protection to *Listeria, Vaccinia* and vesicular stomatitis virus infection by playing a protective role in early trapping of viral and bacterial particles in the spleen.

2.1.4. **Therapeutic application and potential usage of human immunoglobulins**

Currently, immunoglobulin-G-based drugs are dominant in the market and in clinical development because of their extended plasma half life and their effectiveness in interaction with the human immune system [14-16]. However, more than 95% of the infections are initiated at the mucosal surfaces, where IgA is the primary immune effector antibody [8]. IgA-based antibodies against pathogenic bacteria including *Streptococcus pneumonia* [17], *Neisseria meningitides* [18], *Bordetella pertussis* [19], and *Shigella flexneri* [20] have shown efficacy in laboratory-based studies. In a human trial, the plant-derived recombinant monoclonal SIgA against *Streptococcus mutans* was shown to offer protection against oral streptococcal recolonization for at least 4 months, suggesting its potential in microbial colonization control [21]. A chimeric murine-human IgA1 against a major ragweed pollen antigen was shown to prevent airway hyper-responsiveness and reduce eosinophil influx into the lung [22]. Interestingly, serum IgA enabled efficient phagocytosis *in vivo* of bacteria by interactions between IgA and FcαRI expressed on liver Kupffer cells induced by inflammatory mediators, suggesting these interactions may provide a second line of defense in mucosal immunity [23]. Consequently, application of IgA antibodies to mucosal surfaces has significant potential for preventing and treating infections.

About 76% of human immunodeficiency virus (HIV) resistant women expressed HIV-1-specific IgA in their genital tract as opposed to 26% of infected women [24]. IgA
appeared to play an important role in preventing sexual transmission of HIV-1 because the IgA from these highly exposed, persistently seronegative individuals not only neutralized HIV-1, but also prevented its transcytosis across tight epithelial cell layers [25, 26]. Recombinant IgA also showed promise in the treatment of parasitic diseases such as malaria and corneal infections. The high titers of *Plasmodium*-specific IgA in serum and breast milk have been found in humans living in endemic area, suggesting that IgA plays a crucial role in immunity against malaria [27, 28]. Two monoclonal IgA antibodies specific for *Acanthamoeba* antigens were generated and tested, showing protection against *Acanthamoeba keratitis* by inhibiting adhesion of the parasite to the corneal epithelium [29].

IgA has also shown some potential in cancer therapeutics and diagnostics. Comparing to IgG, IgA may have additional advantages of forming natural dimers with improved signaling capacity on tumor cells, and being actively transported into mucosal secretions with the potential for improved targeting of certain carcinomas from the luminal surface [30]. For example, carcinoembryonic antigen (CEA) has been shown to be one of the best markers for *in vivo* tumor targeting of radiolabeled antibodies. However, a large proportion of CEA molecules may remain inaccessible to intravenously injected radiolabeled anti-CEA IgG antibodies because of its particular histological localization. A chimeric dimeric IgA generated by the specific anti-CEA hybridomas (CE-25) showed the ability to translocate efficiently across a monolayer of epithelial cells expressing the pIgR and retain full CEA binding activity, suggesting a more effective agent than IgG for tumor localization [31]. Epidermal growth factor receptor (EGF-R) IgG antibodies are currently approved for treatment of colon or head and neck cancer, and chimeric IgA antibodies against EGF-R
were proved as effective as IgG in mediating direct effector mechanism such as blockage of EGF binding, inhibition of EGF-R phosphorylation, and induction of growth inhibition. Furthermore, IgA was significantly more effective than IgG1 in recruiting neutrophils, the most common cytotoxic cells in humans, for effective cell-mediated tumor killing, resulting in improved tumor cell killing in whole blood assays [32, 33]. Similarly, recombinant human IgA1 produced in BHK cells against the tumor-associated antigen Ep-CAM efficiently recruited immune effector cells and mediated the killing of the tumor cells by isolated polymorphonuclear cells (PMNs) [34]. In clinical trials, two murine IgA antibodies were systemically administered to humans, one directed against CD3, the other against the transferring receptor. IgA against CD3 was as effective as IgG2a in depleting CD3-positive cells from peripheral blood with diminished clinical side effects because of lower activation of complement and fibrinolytic systems as well as cytokine release [35]. The IgA transferring receptor antibody was well tolerated without reaching dose-limiting toxicity up to 300 mg/m², although the transferring receptor is widely expressed by normal tissue [36].

IgM has been shown to not only be involved in early recognition of challenging pathogens such as bacteria and viruses, but also to play an important role in immunosurveillance mechanisms against malignant cells [37, 38]. An interesting phenomenon of immunity against malignant cells is that all tumor-specific human monoclonal antibodies isolated were of the IgM isotype, no tumor-specific IgAs or IgGs and no affinity-maturated tumor-specific antibodies were detected. IgM has the ability to bind to carbohydrate epitopes on post-transcriptionally modified surface receptors and induce a cancer-specific apoptosis by triggering the intrinsic apoptotic pathway [39].
One apoptosis-inducing IgM is the human monoclonal antibody SC-1, which binds to a new variant of CD55/DAF (decay accelerating factor), specifically expressed on the membrane of stomach cancer cells. The human antibody SC-1 induces apoptosis of gastric cancer cells in vitro and in vivo and is being used successfully in clinical trials [40, 41]. Another example is the human germ-line coded monoclonal IgM antibody PAM-1 which binds to a membrane receptor which is present on almost all epithelial cancers of every type and origin and all tested precursor stages, but not on healthy tissue. The receptor was identified as a 130 kDa integral membrane glycoprotein, homologous to CFR-1 (cysteine-rich fibroblast growth factor receptor). A study shows that PAM-1 could inhibit cell growth and induce apoptosis, in vitro and in vivo by binding and blocking the CFR-1/PAM-1 receptor [42]. Both the unique tumor-specific expression of the CFR-1/PAM-1 receptor and the growth inhibitory effect of the PAM-1 antibody make this combination an optimal diagnostic and therapeutic tool for all kinds of epithelial cancer and precursor lesions. Human monoclonal IgM antibody SAM-6 isolated from a gastric cancer patient could bind specifically to malignant cells to induce an over-accumulation of intracellular neutral lipids, resulting in cellular lipotoxicity [43, 44].

Monoclonal antibodies have been one of the most anticipated drugs for melanoma treatment because melanoma cells express tumor-associated ganglioside antigens on their cell surfaces, and monoclonal antibodies can mediate tumor cell lysis via CDC and/or ADCC. In studies of active immunotherapy, patients who received a melanoma cell vaccine for treatment of metastatic melanoma had survival rates that correlated with serum concentration of IgM but not IgG anti-ganglioside antibody [45, 46]. Human monoclonal IgM L72 and
L612 binds specifically to ganglioside GD2 and GM3, respectively, and have a strong cytotoxic effect on human melanoma cells in the presence of complement [47, 48]. Also in melanoma, naturally occurring IgM to the ganglioside GM2 has been shown to correlate with improved survival rate [49]. Therefore, IgM antibodies have a great potential in cancer therapeutics and diagnostics.

2.2. Purification of immunoglobulins

2.2.1. Overview

In the industry, mammalian expression systems such as Chinese hamster ovary (CHO) cells and the murine myeloma cell lines (SP2/0 and NS0) have been extensively used for the production of recombinant antibodies. Two types of cell culture medium are usually used: cMEM, complete mammalian cell culture media containing 10% fetal calf serum, and chemically-defined, protein-free and serum-free cell culture media. Intravenous immunoglobulins (IVIG) can also be purified directly from pooled human plasma for the treatment of immune deficiencies, inflammatory, autoimmune diseases and acute infections. Fraction II + III paste from the Cohn process is the usual starting material containing hIgG, hIgA and hIgM concentration in a 10:2:1 ratio with human serum albumin (HSA) as the major contaminant. Efficient recovery of native or engineered antibodies from human plasma or cell culture media is a critical part of minimizing manufacturing costs. As a rule, the downstream processing and purification accounts for a significant percentage (50-80%) of the total manufacturing cost of therapeutic antibodies and their derivatives [50]. Downstream processing is highly dependent on the starting material that contains the protein of interest.
together with impurities including host cell proteins, DNA, antibody variants, cell culture medium additives, endotoxins, prions and viral particles, which must be removed to produce a safe, pure, and consistent product. Consequently, different strategies of downstream processing would be used for different starting materials and protein of interest. Traditionally, the antibody purification process involves an initial capture (removing water and other main contaminants), intermediary purification (reaching 40-90% of the final degree of purity) and a final polishing step (~100% purity). The initial capture step usually follows precipitation or filtration techniques whereas one affinity-based or several classical chromatographic steps are typically employed.

Ion-exchange chromatography (IEC), while less selective than affinity chromatography, represents a useful technique to isolate antibodies either by anion exchange (pH > pI) or cation exchange (pH < pI) [51, 52]. In industry, a anion exchange chromatography step (AEX) is usually accompanied by an cation exchange (CEX) step since they complementally bind proteins with different charges [53]. Newly developed IEC resins have been reported to exhibit protein capacities as high as 180 mg/mL media [52] or to be capable of direct binding of antibodies from media of physiological ionic strength (150-200 mM NaCl) [54]. However, the isoelectric point variation in antibodies makes it necessary to explore and determine the binding to an IEC resin on an individual basis. As a result, the contaminant spectrum with which an antibody is associated on resins is usually different from one binding condition to another, involving numerous iterations since the lack of harmonization in antibody purification, leading to more time and cost consumed in downstream development.
Gel filtration or size exclusion chromatography (SEC) has been frequently utilized and is limited as a polishing step to reach antibodies preparations with purities greater than 95% due to the high cost and long separation time [55].

Affinity-chromatography (AC), introduced by Cuatrecasas and co-workers in 1968 [56], has been traditionally the method of choice for the purification of native and genetically engineered antibodies. The affinity chromatography relies on the specific recognition between the antibody molecule and a complementary ligand. Since the binding is highly specific, affinity chromatography reduces non-specific interactions, increases operational yields and facilitates the elimination of undesirable contaminants, which allows the purification of antibodies in fewer steps possible [57]. In affinity chromatography, the specific ligand is covalently immobilized onto a solid support, and then the affinity adsorbent is packed on a chromatographic column. First, the target antibody is absorbed on the column, followed by washing step to wash off unbound species. Finally, the antibody is recovered by elution with either a non-specific (e.g. change of pH, ionic strength, temperature) or specific eluant (competitive molecule), and the solid support is regenerated for the next cycle.

### 2.2.2. Biospecific ligands for antibody purification

Natural antibody-binding molecules include the antigen, anti-antibodies, lectins or bacterial immunoglobulin-binding proteins. Biospecific ligands usually are derived from natural sources, presenting high affinity constants \( K_a \approx 10^5 \text{ - } 10^8 \text{ M}^{-1} \) and selectivity for immunoglobulins. As a result, these ligands have been regarded as valuable tools for antibody purification [56].
Affinity purification with immobilized antigen as affinity ligand is widely used because there is specific antigen recognition by the antibodies, which enables the purification of antibodies with desired specificities from a mixture of other immunoglobulins and contaminants. The purification of monospecific antibodies from antisera is a typical example of such a challenge. Immobilized antigen has also been used for the selection of antibodies with desired affinities [58]. However, this method is restricted in its use because of the properties of the antigen such as high price and difficulty in handling.

The coupling of anti-antibodies is another option since the constant domains of both the H and L chains of antibodies are potential targets for the affinity purification of antibodies [59]. Because of the high binding capacity and equilibrium association constant, a considerable saving in the operating costs of an affinity chromatographic process could be achieved [60]. However, the costs associated with producing pure human antibodies as ligands for affinity separations would prohibit their implementation. This is the reason why this is not done in large scale commercial applications.

Since immunoglobulins are glycoproteins, it is also possible to use immobilized lectin adsorbents for their purification [61]. For example, Concanavalin A (ConA) was used to bind dog immunoglobulins A, G, E, and M. All IgE and IgM, 60% IgG and 58% IgA bound to the Con A-Sepharose column. The separation of antibodies could be achieved based on carbohydrate content with different elution buffers [62]. Jacalin, the major protein from jackfruit seeds, is highly specific for the \(\alpha-O\)-glycoside of the disaccharide Thomsen-Friedenreich antigen. This property makes the jacalin-agarose suitable for affinity chromatographic purification of O-linked glycoproteins, particularly human IgA1 [63]. All
binding, washing, and elution steps can be performed at neutral pH; however, elution is achieved with a sugar, usually galactose or mellobiose which is costly and impractical for large-scale operations [64, 65]. Immobilized mannose binding protein (MBP), from the family of animal lectins, was shown to bind IgM antibodies from a variety of species; however, the binding capacity is limited (1–2 mg/mL) [66, 67]. Other MBPs, such as snowdrop bulb lectin (GNA) have been used specifically for murine IgM isolation, but with the drawback of requiring elution of adsorbed immunoglobulins by addition of 0.1 M methyl-α-D-mannoside [68].

Numerous cells and viruses have proteins on their surfaces that bind to the Fc portion of immunoglobulins. So, immobilized bacterial surface proteins can be used as affinity ligands for antibody purification. Protein A is a cell wall protein of *Staphylococcus aureus* that binds selectively to the Fc region of IgG but not to IgG3 [69, 70]. Protein G is a surface IgG-binding protein produced by *Streptococci* groups C and G, which has a special affinity for the Fc region of IgG [71]. Protein G exhibits binding sites not only for antibodies but also for albumin and α2-macroglobulin, which limits the protein-G-based affinity purification. Although Protein A and Protein G share no sequence homologies, they bind to the same IgG constant regions, indicating a convergent evolution of such proteins [72]. Protein A affinity chromatography is the most common method for antibody purification because of its high affinity for the Fc region of immunoglobulins, stability over wide pH (2-11), and ability to refold after urea or guanidinium salts treatment [59]. Protein L, isolated from *Peptostreptococcus magnus* strains, binds with high affinity ($K_d \approx 10^{-9}$ M) to a large number of immunoglobulins with κ1, κ2 and κ4 light chains, but not to κ3 and λ subgroups, and thus
recognizes 50% of human and more than 75% of murine immunoglobulins [73]. As a result, Protein L is suitable for purification of scFV, Fab and F(ab’)$_2$ fragments. *Streptococcus pyogenes* protein Arp4 and Sir22, members of the M protein family, bind to Fc portion of human IgA and IgG with high affinity ($K_d \approx 10^{-8}$ M); however, immobilized protein M-based affinity adsorbents for antibody purification are still not commercially available [74, 75].

Despite their general use as effective ligands for antibody purification, biospecific ligands are biologicals that have to be produced and isolated for further applications, which is expensive and laborious. In addition, ligand immobilization onto a solid matrix is an important issue that needs to be considered to assure the availability and bioactivity of immobilized ligands. Because of the high affinity between the biospecific ligands and antibodies, more drastic dissociation conditions (low pH) are usually required for the elution step, which could alter the conformation of immunoglobulins, and cause ligand leakage and contamination of the final product [76].

2.2.3. Pseudo-biospecific ligands for antibody purification

Pseudo-biospecific affinity ligands have some advantages over natural antibody-binding ligands such as reduced costs of production, increased resistance to chemical and biochemical degradation, and lower toxicity and immunogenicity; which make them cost-effective alternatives to conventional Protein A/G adsorbents [77, 78]. The association constants ($K_a \approx 10^3$-$10^5$ M$^{-1}$) are generally lower when compared to natural ligands, but sufficiently high to ensure a good selectivity to immunoglobulin molecules under optimized conditions. Recent studies have demonstrated that process parameters such as binding
capacity, selectivity, yield and purity are highly dependent on the source and isotype of target antibody, and on the optimization of operational conditions when comparing Protein A and different pseudo-biospecific ligands [79, 80].

Histidine-ligand-affinity chromatography [81, 82] uses immobilized histidine as affinity ligand for antibody purification. The immobilized histidine can interact with antibodies through its carboxyl, amino or imidazole groups by charge-induced and hydrophobic interactions. The purification of intact antibodies with peptidase activities was successful with a recovery of high-purity IgG directly from the sera of autoimmune disease patients [83]. The preservation of catalytic activities of the antibodies was attributed to the mild elution conditions. The addition of sodium chloride to the binding buffer was sufficient for elution, and this is considered a major advantage of this type of affinity chromatography.

Hydrophobic ligands take advantage of the fact that most antibodies have a stronger hydrophobicity than most of their contaminants. Antibodies are bound or “precipitated” onto an hydrophobic medium at high salt concentration, and then eluted with progressively lower salt concentrations [77]. However, there are several limitations in using hydrophobic interaction chromatography such as possible denaturation of antibodies on excessively hydrophobic supports, the insolubility of antibodies at high salt concentration required to support retention on weakly hydrophobic media, and the necessity to add a lyotropic salt (water structure-forming salts such as ammonium or sodium sulfate) to the raw material which increases processing costs to large scale users.

Thiophilic affinity separation, first reported by Porath and co-workers in 1985 [84], is similar to hydrophobic interaction chromatography in adsorption and elution steps. The
adsorption is promoted at neutral pH and at high concentration of lyotropic salts, while elution is achieved by lowering the salt concentration [85]. T-gels, resulted from the reaction of divinylsulfone and 2-mercaptoethanol, are the most common thiophilic adsorbents. These gels carry linear ligands with two sulfur atoms, presenting a predominant hydrophilic property. Thiophilic adsorption chromatography has been described for the purification of a large number of antibodies from different sources such as murine monoclonal antibodies from hybridoma cell culture [86], polyclonal antibodies from colostrums and bovine milk whey [87, 88], and human immunoglobulins from blood [89]. However, lack of specificity for different antibody classes was found even with variations in the structure of the original thiophilic spacers.

Hydrophobic charge induction chromatography (HCIC) is based on the pH-dependent behavior of dual-mode ionizable hydrophobic ligands as described by Burton and Harding [90, 91]. The ligand contains structural features to support hydrophobic binding in almost neutral conditions without modification of ionic strength. When pH of the mobile phase is reduced, both the ionizable ligand and the target molecule take on net positive electric charge. The resulting electrostatic charge repulsion overcomes hydrophobic binding interactions, and the desorption of the molecule occurs [92]. Although a large variety of ligands correspond to this mechanism of action, 4-mercapto-ethyl-pyridine (MEP) attached to a hydrophilic matrix via a hydrophobic spacer arm provides enhanced selectivity for antibody adsorption. MEP is a pH-sensitive weak binder with a dynamic binding capacity for IgG ranging from 25-35 mg/mL at pH 7-8 and at 10% breakthrough [93]. HCIC using MEP is an effective method for the separation of antibodies from a variety of feed stocks, and the final purity of the antibody
is feedstock-dependent. Purity and recovery of 44% and 75% were obtained when feedstock solution was from protein-free cell culture supernatant containing 0.0474 mg/mL IgG [94]. Purities as high as 98% were achieved when IgG concentration in the feedstock was 0.1 mg/mL, and the hIgG purity and recovery of 76% and 69% were obtained when dealing with cell culture supernatant containing 5% fetal bovine serum (FBS) at an IgG concentration of 0.1 mg/mL [95]. Although an effective ligand for antibody separation, the washing salt concentration and elution pH needed to be experimentally decided for each antibody in order to separate it from other contaminant proteins.

Hydroxyapatite crystals $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ with a structured arrangement of interaction points can be a alternative for antibody purification [96]. Positively charged proteins are bound to the hydroxyapatite solid supports due to the electrostatic interaction between protein amino groups and matrix-phosphate sites. Negatively charged proteins are retained by coordination bonds with the calcium sites on the adsorbents. The standard binding buffer is a low concentration phosphate buffer with neutral pH. The elution could be achieved by gradient of increase ionic strength (either $\text{PO}_4^{3-}$ and $\text{Cl}^-$ or $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$). Antibody separations on hydroxyapatite remain difficult to predict and are not very widespread due to the complex mechanisms. Also hydroxyapatite suffers from some limitations for routine use and scale up such as low capacity, high back pressure, low flow rate and short column lifetimes [97].

Immobilized metal affinity chromatography (IMAC), introduced by Porath and co-workers in 1975 [98], uses covalently bound chelating compounds on solid supports to entrap metal ions which act as affinity ligands for antibody purification. Although amino acid
residues such as Glu, Asp, Tyr, Arg, Lys and Met may participate in binding to IMAC resins, the retention of native protein molecules is mainly dependent on the availability of exposed histidyl residues. All metal ions that interact with proteins can be used in IMAC, and the mostly studied ions are Ni$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Co$^{2+}$. Ni$^{2+}$ and Zn$^{2+}$ are electrochemically stable under the conditions used in IMAC, but the Cu$^{2+}$ and Co$^{2+}$ can be easily reduced or oxidized by redox-active solutions [99, 100]. Antibodies and their proteolytic/recombinant fragments have been purified on IMAC resins based on their surface available histidyl residues [101-103]. IDA Sepharose immobilized Ni$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Co$^{2+}$ demonstrated that Fc and digested human IgG have higher affinity for transition metal ions than F(ab)$_2$, which could be efficiently separated in one step by IMAC [104]. A rapid, single step purification of goat serum immunoglobulins was achieved by IMAC on a new high capacity gel. Under selected conditions, over 95% homogeneity could be obtained [105]. There are several advantages of IMAC such as ligand stability, low cost, high protein loading, mild elution condition and ease in regeneration, which makes IMAC a potential alternative for antibody purification [106]. However, several disadvantages are also associated with the use of IMAC, which include extensive process optimization, oxidative reduction conditions inside the column, protein backbone damage caused by metal-induced cleavage, toxicity from metal ion leakage, and extra purification steps for removing histidine-tags for engineered proteins [99].

2.2.4. Bioengineered and synthetic mimic ligands for antibody purification

The progresses in molecular modeling, genetic engineering, and both natural and synthetic combinational methodologies, have made it possible for the design and
development of engineered pseudo-biospecific affinity ligands with desired and improved properties [57]. This class of ligands includes biological (engineered protein domains and peptides) and fully synthetic molecules. The designed ligands are generally intended to improve the performance of natural binding proteins with respect to chemical resistance and tailored specificity.

Combinational engineering of immunoglobulin-binding Protein A analogue domain (domain Z) was first introduced by Nilsson and co-workers in 1987 [107] to provide protein-based affinity adsorbents for antibody purification to overcome sensitivity to the conventional NaOH cleaning-in-place step and be able to allow milder pH elution conditions [108, 109]. Affinity proteins (affibodies), selected from protein libraries constructed by combinational mutagenesis of a 58-amino-acid, three-helix bundle domain derived from the IgG-binding protein A, showed specific affinity to human IgA subclasses as well as secretory IgA without cross-reactivity to other classes [110]. As a result, efficient affinity recovery of IgA from unconditioned human plasma was achieved.

Selected peptides from peptide libraries have been extensively studied as affinity ligands for purification of antibodies. After coupling to Sepharose, peptide PDTRPAP proved to be an efficient adsorbent for anti-epithelial murine monoclonal antibodies recovery from either hybridoma tissue cultures or ascites fluids [111, 112]. A linear peptide sequence EPIHRSTLTALL was localized within the Fc-Protein A binding site sequences with 42% amino acid homology. The immobilized peptide showed the ability to bind humanized anti-Tac IgG1 antibody (HAT) [113]. A 50-residue synthetic peptide, comprising the 29-residue region, designated as Sap (Streptococcal IgA-binging peptide), was successfully used for single-
step affinity chromatography of human IgA from serum and saliva and also for the specific
detection of IgA bound to antigen [114].

Peptide H, a cyclic dimeric peptide of formula (CFHH)$_2$KG with the two N-terminal
cystein residues covalently linked through a disulfide bridge, is an option for mouse and rat
IgG affinity purification, but less selective for human and rabbit IgG [115]. Kaptiv-GY
(Interchim, Montlucon, France) based on the tripeptide tetramer (Arg-Thr-Tyr)$_4$-Lys$_2$-Lys-
Gly, denoted as PAM (protein A mimetic) or TG19318, was first identified by Fassina and co-workers [116]. After covalent immobilization on epoxy-activated affinity support
(Eupergit C30N), PAM has proved to be efficient for the one-step purification of IgG directly
from crude sera with both purity and yield about 90%. The selectivity for IgG from different
sources was similar to that of Protein A [117]. TG19318 affinity columns were also used for
the one-step purification of IgM directly from crude sources [118], IgA and IgE from cell
culture supernatants [119, 120], and IgY from chicken egg yolk [121]. The affinity columns
with immobilized TG19318 are not affected by the presence of denaturants, detergents or
other sanitation reagents used for pathogen removal and are stable in repeated use [122].
Since the binding of the ligand with all human IgG subclasses has not been reported, whether
TG19318 binds to human IgG3 is not known. Since the only indication that the ligand binds
the Fc portion of IgG is based on an indirect observation that the ligand inhibits the binding
of IgG with Protein A, direct evidence should be demonstrated for further approval. In
addition, the mechanism of interaction between TG19318 and IgA, IgM, IgE, and IgY needs
to be ascertained.
Artificial Protein A ligand 22/8, the basis for Mabsorbent A2P (ProMetic BioSciences Inc., Wayne, NJ), was first identified by Lowe and co-workers [123, 124]. The ligand 22/8, possessing a triazine ring system which allows the introduction of natural or unnatural amino acid side chain functional residues, mimicked the traditional Protein A adsorbents in binding IgG ($K_a = 1.4 \times 10^5 \text{ M}^{-1}$) from various sources, separating IgG from human plasma with recoveries of 67-69% and purities of 98-99% [124]. Ligand 22/8 showed affinity for all human IgG subclasses including IgG3, and the adsorbed human IgG can be eluted with 0.1M glycine-HCl at pH 2.9. Another advantage of ligand 22/8 is its resistance to cleaning-in-place procedure because of its stability in 1 M NaOH for over 140 h. However, ligand 22/8 was shown to bind both Fab and Fc fragments. In addition, ligand 22/8 binds IgA and IgM avidly, these Igs would be lost when serum sample is passed unless a basic, less denaturing elution buffer could be used to recover them from the affinity matrix. As a result, no application for IgA and IgM purification was achieved by the ligand 22/8 [125]. Another ligand 8/7 [126], mimicking Protein L, was also developed by the same group and showed the ability to bind uniquely to the Fab portion of immunoglobulins. Under non-optimized conditions, ligand 22/8 adsorbents were able to isolate immunoglobulins from crude samples with purification factors of seven-fold and purities up to 95% [127].

The bioaffinity company BAC (Naarden, The Netherlands) in collaboration with GE Healthcare (Uppsala, Sweden) has developed an IgG affinity ligand (Igselect) based on camelid-derived single domain antibody fragments. The ligand is a 12-kDa single domain fragment (VHH fragment) that comprises the 3 CDRs that form the antigen-binding domain, efficiently produced by the yeast Saccharomyces cerevisiae. This affinity ligand enables the
purification of all subclasses of IgG from any sources due to its human specificity, making it suitable for the purification of monoclonal antibodies from transgenic sources or IVIG from plasma. Besides this specificity, the Igselect ligand can as well be used with caustic cleaning and have milder elution conditions. However; unlike Protein A, VHH fragments are monomeric and do not have the capability to bind multiple IgG molecules, which cause a limitation on capacity. A lower capacity of polyclonal IgG with 26 mg/mL was found, compared to approximately 70 mg/mL of MabSelectXtra SpA media [128]. Human IgA and IgM affinity ligands were also developed with the same method; however, they are only for research purposes, and no further data could be found from the literature.

Compared to traditional biospecific affinity ligands, synthetic mimic ligands have some advantages such as high ligand capacity, low toxicity, low cost, high sterilizability and stability, and moderate affinity and, hence, mild in elution. With all these advantages, synthetic mimic ligands are great alternatives to the traditional biospecific affinity ligands for large scale antibody purification. Consequently, the search for new synthetic mimic ligands for antibody purification is still highly active in both industry and academia [129-133].

2.2.5. Hexamer peptide ligands for antibody purification

A family of linear hexamer peptides, exhibiting the ability to recognize and bind immunoglobulin G (IgG) through its Fc portion, has been previously identified in our group through a three-step screening of a synthetic solid phase combinatorial peptide library, and it has been found that the selectivity of these peptides to the Fc portion of IgG is comparable to that of Protein A [134]. Among them, peptide ligand HWRGWV exhibited the ability to bind
all human IgG (hIgG) subclasses as well as IgGs from bovine, mouse, goat and rabbit. After direct synthesis on Toyopearl AF Amino 650M resins, HWRGWV was able to purify hIgG from complete mammalian cell culture medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth with both purity and recovery as high as 95% under optimized conditions [135]. Temperature had little effect on the chromatographic isolation of hIgG from cMEM and the N-terminal acetylation of HWRGWV had no influence on adsorption and isolation of hIgG from cMEM, suggesting that the charge of the N-terminal histidine played no major role on the retention of hIgG to the ligand. In addition, HWRGWV demonstrated its ability to purify monoclonal antibodies from two different commercial CHO cell culture media with purities and recoveries higher than 85% and 94%, respectively, again matching very well the results found with Protein G using the same cell culture fluids [136]. Based on mass spectrometry data and dock simulation, it was concluded that HWRGWV binds to the pFc portion of hIgG and interacts with the amino acids in the loop Ser383-Asn389 (SNGQPEN) located in the C\textsubscript{H}3 domain. The binding of hIgG to HWRGWV is specific, apparently involving hydrogen bonding, hydrophobic, and electrostatic interactions [137]. As a result, this Fc-specific hexamer peptide ligand offers a potential alternative to the Protein A or Protein G for large-scale affinity purification of IgG. Since the HWRGWV ligand was initially screened against the Fc-portion of IgG and homology is present between human immunoglobulin classes in their heavy chain constant regions [138], it is reasonable to expect that HWRGWV binds to the heavy chain constant regions of human IgA and IgM as well. The binding of HWRGWV to human IgA and IgM was confirmed by the preliminary results [134]. Although the mechanism of interaction between HWRGWV with IgA and IgM
was not investigated in detail, the results suggest that the peptide ligand could have the potential for IgA and IgM capture and purification.

The objectives of this dissertation focus on the characterization in more details of the peptide ligands for IgA and IgM capture and purification.

2.3. References


[34] G. Huls, I.A.F.M. Heijnen, E. Cuomo, J. van der Linden, E. Boel, J.G.J. van de Winkel and T. Logtenberg, Antitumor immune effector mechanisms recruited by


S. Gulich, M. Uhlen and S. Hober, Protein engineering of an IgG-binding domain allows milder elution conditions during affinity chromatography, J Biotechnol 76 (2000) 233-244.


Table 2.1. Structural and biological properties of human immunoglobulins [1].

<table>
<thead>
<tr>
<th></th>
<th>IgA</th>
<th>IgG</th>
<th>IgM</th>
<th>IgD</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgA1</td>
<td>IgA2</td>
<td>IgG1</td>
<td>IgG2</td>
<td>IgG3</td>
</tr>
<tr>
<td>Molecular weight of</td>
<td>160 (m) 300 (d)</td>
<td>160 (m) 300 (d)</td>
<td>150</td>
<td>150</td>
<td>155-165</td>
</tr>
<tr>
<td>secreted form (kDa)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavy chain class</td>
<td>α1</td>
<td>α1</td>
<td>γ1</td>
<td>γ2</td>
<td>γ3</td>
</tr>
<tr>
<td>Light chain class</td>
<td>κ and λ</td>
<td>κ and λ</td>
<td>κ and λ</td>
<td>κ and λ</td>
<td>κ and λ</td>
</tr>
<tr>
<td>Functional valency</td>
<td>2 or 4</td>
<td>2 or 4</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Structure **</td>
<td>monomer (mb,s) dimer (sec)</td>
<td>monomer</td>
<td>monomer (mb) pentamer (s)</td>
<td>monomer</td>
<td>monomer</td>
</tr>
<tr>
<td>Interheavy disulfide</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>bonds per monomer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other chain</td>
<td>J chain (16 kDa) secretory component (70 kDa)</td>
<td>None</td>
<td>J chain (16 kDa)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Tail piece</td>
<td>Yes</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hinge (amino acids)</td>
<td>18</td>
<td>5</td>
<td>15</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td>Carbohydrate average (%)</td>
<td>7-11</td>
<td>2-3</td>
<td>10-12</td>
<td>9-14</td>
<td>12-13</td>
</tr>
<tr>
<td>Adult level range (age 16-60) in serum (g/L)</td>
<td>1.4-4.2</td>
<td>0.2-0.5</td>
<td>5-12</td>
<td>2-6</td>
<td>0.5-1</td>
</tr>
<tr>
<td>Approximate % total Ig in adult serum</td>
<td>11-14</td>
<td>1-4</td>
<td>45-53</td>
<td>11-15</td>
<td>3-6</td>
</tr>
<tr>
<td>Synthetic rate (mg/kg weight day)</td>
<td>19-29</td>
<td>3.5-5.3</td>
<td>33</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>Biological half life (day)</td>
<td>5-7</td>
<td>4-6</td>
<td>21-24</td>
<td>21-24</td>
<td>7-8</td>
</tr>
<tr>
<td>Transplacental transfer</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Complement activation classical pathway (C1q)</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>
### Table 2.1. Continued

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>0</th>
<th>+++</th>
<th>+/-</th>
<th>+++</th>
<th>+/-</th>
<th>0</th>
<th>+</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding macrophages and other phagocytic cells (FcγR) ***</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding mast cells and basophils (FcεR)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>Binding to epithelial poly-Ig receptor</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Reactivity with protein A</td>
<td>0</td>
<td>0</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

m=monomer, d=dimer, p=pentamer  
**mb=membrane, s=secreted, sec=secretory  
*** +/-: binding depends on the FcγR isoform and on the type of cell  
0:null, +/-low,++:mean, +++:high for the biological properties

### Table 2.2. Human immunoglobulin chain characteristics [1].

<table>
<thead>
<tr>
<th></th>
<th>α1</th>
<th>α2</th>
<th>γ1</th>
<th>γ2</th>
<th>γ3</th>
<th>γ4</th>
<th>μ</th>
<th>δ</th>
<th>ε</th>
<th>κ</th>
<th>λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (kDa)</td>
<td>55</td>
<td>55</td>
<td>50</td>
<td>50</td>
<td>53-57</td>
<td>50</td>
<td>70</td>
<td>60</td>
<td>70</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Number of variable domains</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Number of constant domains</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N-glycosylation sites</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>O-glycosylation sites</td>
<td>8</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>7</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

49
Table 2.3. General comparison of biospecific and pseudobiospecific affinity ligands for antibody purification [77].

<table>
<thead>
<tr>
<th></th>
<th>Biospecific</th>
<th>Pseudobiospecific</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-mimic</td>
</tr>
<tr>
<td>Specificity</td>
<td>High</td>
<td>Low to medium</td>
</tr>
<tr>
<td>((K_a \approx 10^5)-(10^8)M(^{-1}))</td>
<td>((K_a \approx 10^3)-(10^5)M(^{-1}))</td>
<td>((K_a \approx 10^4)-(10^6)M(^{-1}))</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Very high</td>
<td>Low to medium</td>
</tr>
<tr>
<td>Capacity</td>
<td>Low(^a)</td>
<td>High(^b)</td>
</tr>
<tr>
<td>Product elution</td>
<td>Difficult</td>
<td>Mild</td>
</tr>
<tr>
<td>Stability(^c)</td>
<td>Low</td>
<td>Very high</td>
</tr>
<tr>
<td>Toxicity(^d)</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Cost</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

\(^a\) 0.01-1% ligand utilization  
\(^b\) >10% ligand utilization  
\(^c\) Stability evaluation concerns easiness of leakage and sterilizability  
\(^d\) Toxicity is related to potential immunogenicity when ligand contaminates the final product
Chapter 3. Effects of Peptide Density and Elution pH on Affinity Chromatographic Purification of Human Immunoglobulins A and M

Zhuo Liu, Patrick V. Gurgel, Ruben G. Carbonell

A version of this chapter has been published in
Journal of Chromatography A, 1218 (2011) 8344-8352
Abstract

A family of linear hexamer peptide ligands HWRGWV, HYFKFD and HFRRHL, initially identified for their affinity to the Fc portion of human immunoglobulin G (hIgG), also have potential for use in the purification of human immunoglobulins A (hIgA) and M (hIgM). HWRGWV demonstrated the strongest binding affinity to hIgM, followed by hIgA and hIgG respectively. The effects of N-terminal acetylation of the peptide, as well as elution buffer pH, on the chromatographic elution of human IgG, IgA and IgM from HWRGWV resins at various peptide densities (0.04-0.55 mequiv./g) were investigated. Over 80% recovery and 90% purity were achieved for human IgG and IgA isolation from complete minimum essential medium (cMEM) using HWRGWV resin at optimum peptide densities. For human IgM, 75.7% recovery and 86.0% purity were achieved by using HWRGWV at a low peptide density of 0.04 mequiv./g. Although HYFKFD and HFRRHL exhibited their ability for isolation of human IgG, IgA and IgM from cMEM as well, HWRGWV is the best option among them for large-scale purification of human IgG, IgA and IgM based on conditions tested.

Keywords: human immunoglobulin; IgG; IgA; IgM; affinity chromatography; binding site.
3.1. Introduction

Immunoglobulins are highly specific therapeutic agents used to fight cancer, cardiovascular and autoimmune diseases, infections and inflammations [1-4]. Antibody-based therapeutics account for one-third of all new biotherapeutics treatments in the USA and the size of the therapeutic antibody market in the world was projected to reach over 26 billion by 2010 [5]. Most of the antibody therapeutics in the market and in clinical development are immunoglobulin-G-based drugs because of their extended plasma half life, and their effectiveness in activating human complement and in recruiting natural killer cells for complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity pathways [3,5-7]. Nevertheless, more and more attention is being paid to immunoglobulins A (IgA) and M (IgM) for their potential as mucosal vaccines and therapeutic agents [8-11].

IgA is regarded as the first line of defense for human mucosal surfaces for its ability to fight challenging pathogens such as bacteria and viruses [12]. As a therapeutic agent for cancer treatment, IgA has several advantages over IgG. IgA elicits a stronger response on tumor cells because of its ability to form dimers, and it is more effective than IgG in translocating across a monolayer of epithelial cells. These factors lead to improved targeting and recruitment of neutrophils, the most common cytotoxic cells in humans for antibody-dependent cellular cytotoxicity (ADCC) [13-17]. IgM has been shown to be involved in early recognition of challenging pathogens, and to play an important role in immunosurveillance mechanisms against malignant cells [18]. Human monoclonal IgM antibodies have demonstrated ability to induce apoptosis of gastric and epithelial cancer cells in vitro and in vivo and were used successfully in clinical trials [19-21]. Anti-ganglioside IgM antibodies
have great potential in melanoma cancer therapy and diagnostics because of their ability to mediate tumor cell lysis via complement-dependent cytotoxicity (CDC) and/or ADCC through melanoma cell-expressed ganglioside antigens [22-24]. As a result, there is an increasing demand for therapeutic IgA and IgM antibodies and better methods for their isolation from various sources.

Downstream processing and purification accounts for a significant percentage (50-80%) of the total manufacturing cost of therapeutic antibodies and their derivatives [25]. Traditionally, the purification process for antibodies involves at least three steps: initial capture, intermediate purification, and final polishing step. Because of the highly specific recognition between the antibody molecule and a complementary ligand, affinity chromatography reduces non-specific interactions, increases operational yields and facilitates the elimination of undesirable contaminants, which allows the purification and concentration of antibodies in one single step possible. As a result, since its introduction by Cuatrecasas and co-workers in 1968, affinity chromatography has been traditionally the method of choice for the capture and purification of native and genetically engineered antibodies both in laboratories as well as in large scale biomanufacturing [26].

Affinity ligands for antibody purification can be distinguished into biospecific, pseudobiospecific, and bioengineered or synthetic mimic ligands. Biospecific ligands such as antigens, anti-antibodies, lectins or bacterial immunoglobulin-binding proteins have advantages such as high specificity and selectivity [27]. Protein A and Protein G affinity adsorbents are the most commonly used biospecific ligands for industrial purification of whole immunoglobulin G. Nevertheless, due to their biological origin, chemical nature, and
production methods, these ligands tend to be expensive and unstable to the sterilization and column washing and regeneration conditions used in the manufacturing of antibodies. In addition, there is a potential risk of contamination of the final product from ligand leakage resulting from harsh elution conditions, which requires additional polishing and purification steps, and increases processing and validation costs [28]. Pseudobiospecific ligands including hydrophobic, thiophilic, hydroxyapatite, chelating metal ions and mixed-mode ligands have some advantages over natural antibody-binding ligands such as reduced cost of production, increased resistance to chemical and biochemical degradation, high binding capacity and lower toxicity and immunogenicity. These generally make them cost-effective alternatives to conventional Protein A/G adsorbents. Nevertheless, the specificity and selectivity are relatively low comparing to biospecific ligands [27]. On the other hand, bioengineered and synthetic mimic ligands generated by the rational, the combinatorial and the combined methods not only surmount some of the drawbacks of biospecific ligands, but also preserve their advantages such as high specificity and selectivity [28]. With these advantages, bioengineered and synthetic mimic ligands are great potential alternatives to traditional biospecific affinity ligands for large-scale antibody purification. Consequently, the search for new synthetic mimic ligands for antibody purification is still highly active in both industry and academia [29-33].

A family of linear hexamer peptides, exhibiting the ability to recognize and bind human immunoglobulin G through its Fc portion, has been previously identified in our group through a three-step screening of a synthetic solid phase combinatorial peptide library, and it has been found that the selectivity of these peptides to the Fc portion of IgG is comparable to
that of Protein A [34]. Peptide ligand HWRGWV exhibited the ability to bind all human IgG subclasses as well as IgGs from bovine, mouse, goat and rabbit. After direct synthesis on Toyopearl AF Amino 650M resins, HWRGWV was able to purify hIgG from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth with both purity and recovery as high as 95% under optimized conditions [35]. In addition, HWRGWV demonstrated its ability to purify monoclonal antibodies from two different commercial CHO cell culture media with the purities and recoveries higher than 85% and 94%, respectively, again matching very well results found with Protein G using the same cell culture fluids [36]. Based on mass spectrometry data and docking simulation, it was concluded that HWRGWV binds to the pFc portion of hIgG and interacts with the amino acids in the loop Ser383-Asn389 (SNGQPEN) located in the C\(\text{H}_3\) domain. The binding of this loop to HWRGWV is specific, apparently involving hydrogen bonding, hydrophobic, and electrostatic interactions [37]. This Fc-specific hexamer peptide ligand offers a potential alternative to the Protein A or Protein G for large scale affinity purification of IgG.

Because of the homology between human immunoglobulins classes in their heavy chain constant regions [38], it is reasonable to expect that HWRGWV also binds to the heavy chain constant regions of human IgA and IgM as well. Therefore, potential binding sites in human IgA and IgM were searched by using the amino acid sequence SNGQPEN as a reference in heavy chain constant regions of human IgA and IgM. As a result, several possible binding sites, five for hIgA (B1-B5) and three for hIgM (B6-B8) were found based on comparison with the binding amino acid sequence in hIgG1. Figure 3.1 displays the
sequence alignment of the 20 amino acid segment of 261-280 from hIgG1 heavy chain constant region compared to possible binding regions for human IgA1 and IgM with the same amino acids, the same type amino acids group or the same charge amino acids highlighted. Column 2 in Figure 3.1 indicates the beginning and ending amino acid in all the sequences being compared, and the total number of amino acids in each heavy chain constant region. For better illustration, the possible binding site sequences B1-B8 are shown in the human IgA1 and IgM structures in Figure 3.2.

Based on a simple amino acid sequence comparison, for human IgA1, four out of five possible binding sites are located in the C_H2 and C_H3 domains, which are similar to the location of the binding site in human IgG1, while two out of three possible binding sites for human IgM are located in the C_H1 domain. This domain of IgM should have higher accessibility to the peptide ligand HWRGWV when compared to the C_H3 and C_H4 domains, since hIgM exists mainly as a pentamer with the J chain in the center of the molecule [39]. As a result, possible binding sites located in the C_H1 domain might lead to stronger binding affinity for hIgM to peptide ligand HWRGWV compared to hIgG because of the multiple accessible binding sites.

Preliminary experiments indicate that IgA and IgM bound very well to HWRGWV resin and that these proteins eluted more slowly from the column than IgG, indicating a stronger binding interaction to the resin. Although the mechanism of interaction between HWRGWV with IgA and IgM was not investigated in detail, the results suggest that this peptide ligand had the potential for IgA and IgM capture and purification. The present work focuses on the exploration of the potential of HWRGWV for the capture and purification of
human IgA and IgM from different sources such as recombinant cell culture media. The effects of peptide density and elution solution pH on the recovery of captured human IgG, IgA and IgM with different peptide ligands were investigated.

3.2. Experimental

3.2.1. Materials

The peptides, HWRGWV, N-terminal acetylated HWRGWV (Ac-HWRGWV), HYFKFFD and HFRRHL were synthesized directly on Toyopearl AF-Amino-650M (particle size 65μm) (Tosoh BioScience, Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International, Inc (Louisville, KY, USA). The HWRGWV resins were synthesized at different peptide densities from 0.04 to 0.55 mequiv./g, while the Ac-HWRGWV resin was synthesized at a peptide density of 0.08 mequiv./g. The HYFKFD and HFRRHL resins were synthesized at a peptide density of 0.11 mequiv./g. Phosphate-buffered saline (PBS) pH 7.4 (containing 0.01 M phosphate, 0.138 M sodium chloride, and 2.7 mM potassium chloride) was obtained from Sigma (St. Louis, MO, USA). Sodium acetate, glacial acetic acid, guanidine hydrochloride, tris hydrochloride and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). NuPAGE Novex 4-12% Bis-Tris gels, NuPAGE reducing agent, NuPAGE MOPS SDS running buffer, SeeBlue Plus2 pre-stained molecular weight marker and SimplyBlue Safestain were all from Invitrogen (Carlsbad, CA, USA). Human IgG lyophilized power was purchased from Equitech-Bio (Kerrville, TX, USA). Human IgA and IgM were from Fitzgerald (Concord, MA, USA). Human IgG enzyme-linked immunosorbent assay (ELISA) kit was purchased
from Alpha Diagnostic International (San Antonio, TX, USA) and IgA, IgM ELISA kits were from Bethyl Laboratories (Montgomery, TX, USA). Cell culture medium Eagles Minimum Essential Media (EMEM) was from Quality Biologicals (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. Microcon Ultracel YM-3 centrifugal filters (regenerated cellulose, 3000 MWCO) were purchased from Millipore (Billerica, MA, USA). Microbore columns (30 mm × 2.1 mm) for all chromatographic experiments were from Alltech (Deerfield, IL, USA).

3.2.2. Equipment

All chromatographic experiments were carried out on a Waters 626LC system. Absorbance of protein samples was measured at wavelength 280 nm using a built-in 2487 dual wave-length UV detector from Waters (Milford, MA, USA). ELISA protein concentration was determined by μQuant Spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT, USA).

3.2.3. Chromatographic capture and elution of human IgG, IgA and IgM

Peptide resins were dry packed in 30 mm × 2.1 mm I.D. microbore columns (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 hours followed by HPLC grade water and PBS washes. Before sample loading, columns were equilibrated with pH 7.4 equilibration buffer consisting of PBS + 1 M NaCl. Samples were manually injected at room temperature using a 100 μL sample loop (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 minutes, and then the flow rate was increased to 0.3 mL/min for the
remainder of the run. PBS + 1M NaCl was also used as the loading buffer, the column was washed sequentially with 3 mL loading buffer, 6 mL elution buffer, and 6 mL 6M guanidine-HCl to regenerate the column, followed by PBS + 1M NaCl for equilibration. Different elution buffers were used including 0.2 M acetate buffer (AB) at pH values ranging from 3.0 to 6.0. The recoveries of hIgG, hIgA, hIgM were calculated based on the peak areas for elution and regeneration assuming all injected proteins bound to the resin since only small flow through peaks appeared for all injections.

3.2.4. pH gradient elution for captured human IgG, IgA and IgM

Columns with peptide resins HWRGWV (0.11 mequiv./g) and Ac-HWRGWV (0.08 mequiv./g) were prepared and equilibrated by the same methods described in section 3.2.3. The samples were loaded with PBS + 1M NaCl at 0.05 mL/min for 10 minutes, followed by 10 minutes wash with the same loading buffer at flow rate 0.3 mL/min, which remained until the last 20 minutes, when the flow rate was increased to 0.4 mL/min for equilibration. After 20 minutes, the column was eluted with 0.2 M acetate buffer pH 6.5 for 10 minutes, pH gradient elution starting from 0.2 M acetate buffer pH 6.5 to 0.2 M acetate buffer pH 3.0 with 3 sections of linear gradient in order to assure a linear decrease of pH, followed by 10 minutes constant elution with 0.2 M acetate buffer pH 3.0, 20 minutes regeneration with 6 M guanidine-HCl, and 24 minutes equilibration with PBS + 1M NaCl.

3.2.5. Chromatographic isolation of human IgG, IgA, and IgM from cMEM

Columns with peptide HWRGWV resins at four different peptide densities (0.04, 0.11, 0.22 and 0.55 mequiv./g) as well as peptide HYFKFD and HFRRHL resins at peptide density
of 0.11 mequiv./g were prepared and equilibrated by the same methods described in section 3.2.3. Human IgG, IgA, IgM were individually spiked into cMEM to form a complex starting material with the desired antibody concentration of 1 mg/mL. The cMEM was formulated by combining Eagle’s minimum essential medium (EMEM) with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth (TPB). The salt concentration and pH of the starting materials at this state were similar to PBS pH 7.4. The same HPLC running protocol was used as the one described in the chromatographic capture and elution analysis except that 0.2 M acetate buffer pH 5.0 was used for hIgG elution and 0.2 M acetate buffer pH 3.0 or 3.5 were used for hIgA and hIgM elution. All chromatographic runs were performed reproducibly in duplicate and the second run was used for plotting. The pH of the elution fractions was immediately adjusted to neutral by adding an appropriate volume of 1 M Tris buffer pH 8.0. All collected fractions were directly subjected to ELISA assays.

3.2.6. Sample analysis for recoveries and purities

The recoveries of human IgG, IgA and IgM purification were calculated according to the hIgG, hIgA, and hIgM concentration determined by ELISA using human IgG ELISA kits from Alpha Diagnostic International, and IgA and IgM ELISA kits from Bethyl Laboratories. All collected fractions were concentrated 4-5 times by centrifugation at 4°C, 14,000 × g for 99 min using Microcon YM-3 filters before the electrophoresis analysis. Concentrated fractions of chromatography peaks were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE Novex 4-12% Bis-Tris gels with MOPS running buffer on an X-cell SuperLock™ Mini-Cell system from
Invitrogen. The gels were stained by SimpleBlue SafeStain. The purities of human IgG, IgA, and IgM elution peaks were determined by densitometric measurement using software ImageJ 1.32j (National Institute of Health, Bethesda, MD, USA).

3.3. Results and discussion

3.3.1. Effect of elution solution pH on recovery of human IgG, IgA and IgM at different peptide densities

The effect of pH of the elution solution on the recovery of captured immunoglobulins from resins with ligand HWRGWV at four different peptide densities (0.04, 0.11, 0.22 and 0.55 mequiv./g) was determined by using 0.2 M acetate buffer at elution pH values ranging from 3.0 to 6.0. The recoveries for human IgG, IgA and IgM at different peptide densities as a function of the elution pH are shown in Figure 3.3.

As the pH value of the elution solution decreased, the resins with different peptide densities behaved, in general, in a similar way. For all three immunoglobulins, the recoveries increased with decreases of elution solution pH at all peptide densities. Elution at pH 3.0 leads to recoveries of more than 95% for all three immunoglobulins. The recovery of hIgG remained very high (> 90%) until pH 5.0, dropping to around 80% (60% for 0.55 mequiv./g resin) at pH 6.0, suggesting that this protein is not as tightly bound to the resin as hIgA and hIgM. These proteins start to display a drop in recovery at pH 4.0 instead of pH 6.0. Furthermore, IgM seems to be more strongly bound to the resins, as very small amounts of this protein are eluted at pH 4.5, while about 50% of the IgA is recovered in the elution fraction at lower peptide densities, with recovery dropping to about 25% at 0.22 mequiv./g, and around 10% at the highest peptide density tested (0.55 mequiv./g). For each
immunoglobulin, lower elution pH was required to achieve the same recovery level than at higher peptide density, indicating higher peptide density resins associate with higher binding affinity.

These results indicate that elution at pH 5.0 might be used for hIgG isolation from complex media with high purity together with reasonable recovery. Elution at pH 3.0 or 3.5 could be used for hIgA and IgM isolation from complex media. For situation such as human plasma where all three immunoglobulins exist together, the results obtained suggest that IgG, IgA and IgM could be separated by using a pH gradient during elution, with IgG being eluted at relatively high pH values of 5-6, followed by IgA in the 4-5 range, and finally IgM.

3.3.2. Elution profiles of human IgG, IgA and IgM using pH gradients

In order to investigate the effect of elution pH for captured human IgG, IgA and IgM, and the effect of N-terminal acetylation of peptide ligand HWRGWV, N-terminal-acetylated peptide ligand Ac-HWRGWV, as well as un-acetylated HWRGWV were used for chromatographic capture and pH gradient elution of human IgG, IgA and IgM. The acetylated version of the peptide ligand was investigated to determine if this prominent functional group has an effect on the chromatographic profile obtained for the target proteins. Solutions of each protein were subjected to the same chromatographic methodology, and the elution profiles were determined.

As shown in Figure 3.4A for hIgG, the elution profiles for peptide ligands HWRGWV and Ac-HWRGWV overlapped with each other, suggesting that N-terminal acetylation does not influence the binding of hIgG, in good agreement with previous results.
For hIgA, as shown in Figure 3.4B, the captured protein with HWRGWV started to be eluted at pH 5.0, which is as expected based on previous single-pH elution experiment. The elution peak is very broad, starting at pH 5.0 and extending to the end of the elution (pH 3.0). The elution profile of the captured hIgA using Ac-HWRGWV resin was different from the one obtained with HWRGWV. A small fraction of hIgA was eluted at pH 6.5, suggesting that the N-terminal acetylation does effect the binding of hIgA. For hIgM, as shown in Figure 3.4C, the elution profiles for both peptide ligands are similar; two relatively sharp and symmetrical peaks were observed for hIgM elution. However, the elution peak of hIgM with Ac-HWRGWV was slightly shifted to a higher elution pH, suggesting N-terminal acetylation decreased the binding of hIgM to the resin to some level.

In order to exclude the possibility that these elution pattern differences for hIgA and hIgM were due to different peptide densities, peptide resin HWRGWV with a peptide density of 0.04 mequiv./g was also used with the same protocol for capture and elution of human IgG, IgA and IgM. Elution patterns similar to the ones obtained using HWRGWV at 0.11 mequiv./g were observed for all three immunoglobulins suggesting that the differences were mostly due to the acetylation of the terminal amino group.

The majority of hIgG subclasses have isoelectric points in the range between 7 and 9.5. Human IgA subclasses have isoelectric points in the range between 4 and 7.1, while the isoelectric point of hIgM is in the range between 4 and 9.1, with a pI peak between pH 5.5 and 6.7 [40]. Therefore, the majority of the hIgG molecules are positively charged at the binding condition used (pH 7.4), while hIgA and hIgM molecules are negatively charged, and the HWRGWV ligand (pI=9.85) is positively charged at this pH [41]. The opposite
charges on the ligand and the immunoglobulins suggest that these electrostatic interactions play a role in hIgA and hIgM binding to the peptide, and this can help explain the apparent higher affinity of the ligand to hIgA and hIgM compared to hIgG. The N-terminal acetylation of HWRGWV decreased the binding of hIgA and hIgM, and this resulted in early partial elution of hIgA and a shift in the elution peak of hIgM. Although the charge of the N-terminal histidine might play an important role, it does not seem to be the dominant force in hIgA and hIgM binding since stronger affinities of hIgA and hIgM to the ligand were still shown compared to hIgG after the N-terminal acetylation. The other sources for electrostatic interactions could come from the histidine side chain in the peptide that becomes positively charged when the pH decreases below 6.0, while arginine is always positively charged at acidic pH.

3.3.3. Effect of peptide density on human IgG, IgA and IgM isolation from cMEM

In order to determine the optimum peptide density for monoclonal antibody purification, cMEM spiked with human IgG, IgA or IgM was used as a model complex starting material to study the effect of peptide density on the capture and elution of human IgG, IgA and IgM. Representative chromatograms and corresponding SDS-PAGE gels are shown in Figures 3.5, 3.6, and 3.7 for human IgG, IgA and IgM, respectively. The average recoveries and purities of eluted immunoglobulins using pH 5.0 for hIgG, and pH 3.0 and 3.5 for hIgA and hIgM are listed in Table 3.1. When antibody-spiked cMEM was applied to the HWRGWV columns at different densities, three peaks appeared in the chromatogram: a flow through peak (FT), pH acetate buffer elution peak (P1) and the 6M guanidine HCl wash and
regeneration peak (W). The protein profiles of the three chromatographic peaks from HWRGWV resins with different peptide densities of 0.04, 0.11, 0.22 and 0.55 mequiv./g are shown in lanes 4-6, 7-9, 10-2, and 13-15 respectively on the appropriate SDS-PAGE gels.

As seen from the chromatograms for all three immunoglobulins, with increased peptide density, more contaminant proteins were adsorbed on the column and co-eluted in the antibody elution fractions. Similar results were also observed by Yang, et al [35] when purifying IgG using HWRGWV. Compared to the wash peaks in chromatograms for hIgG isolation, the wash peaks for hIgA and hIgM were much smaller, which suggests that 0.2 M acetate buffer at pH 3.0 is strong enough to wash almost everything off the affinity matrix. As shown in the SDS-PAGE gels, the P1 lanes in Figure 3.5B, 3.6B and 3.7B contained mostly human IgG, IgA and IgM, respectively, in their different isoforms, as can be seen by comparing the respective bands in the standard human IgG, IgA and IgM (lane 2 of each gel). Although the main contaminant protein in P1 was albumin in its monomer form with MW about 65 kDa, a large amount of the albumin present in the starting material seemed to flow through the column without binding. The relatively low binding of albumin was achieved by adding 1M NaCl to the loading buffer (PBS), which decreased electrostatic interaction between the resin and the protein [35]. Similar effect could also be achieved with addition of sodium caprylate [36].

By comparing the recoveries and purities achieved with different ligand densities (Table 3.1), it can be observed that the improved recovery at higher peptide density due to increased binding affinity was compensated by a decrease in purity for all three immunoglobulins, indicating that the increased peptide density elevated not only the specific
binding to antibodies but also the non-specific binding of contaminant proteins. For hIgG, both 0.11 and 0.22 mequiv./g HWRGWV resins achieved over 80% and 95% in recovery and purity, respectively. According to Yang et al. [35], better recoveries and purities could be achieved if the concentration of hIgG in the starting material is higher. In addition, instead of acetate buffer pH 4.0, acetate buffer pH 5.0 would be a better choice for hIgG elution to prevent coelution of contaminant proteins since only slight differences in recoveries are seen for both elution conditions. For hIgA, about 80% recovery and over 90% purity were obtained by 0.04 and 0.11 mequiv./g HWRGWV resins, which make them potential candidates for large-scale purification of hIgA. For hIgM, 75.7% and 86.0% recovery and purity were achieved using 0.04 mequiv./g HWRGWV resins. The recoveries of isolated hIgM by different peptide densities are similar to those of hIgA and hIgG, but the purity is much lower than that of hIgA and hIgG at the same peptide density, especially at high densities. Consequently, different strategies should be used in order to achieve better recovery and purity for hIgM isolation. For example, different additives could be used in the loading buffer to decrease competitive protein binding due to nonspecific protein-protein or protein-surface interactions [42]. Since the pI for albumin is between 4.7 and 5.5 [35], positively charged albumin could be washed at pH 5.0 before the final IgM elution. It is also noticeable that there is a trend between the optimum peptide densities and the molecular weights of the target proteins. The larger the antibody needs to be purified, the lower the peptide density that should be used based on purity.
3.3.4. Human IgG, IgA and IgM isolation from cMEM using peptide ligands HYFKFD and HFRRHL

Since peptide ligands HYFKD and HFRRHL are from the same family of HWRGWV, with the three amino acid closest to the N-terminal showing strong homology (His + aromatic amino acid + positively charged amino acid [34]), it is reasonable to believe that they can bind hIgA and hIgM.

In order to test this hypothesis, peptide ligands HYFKFD and HFRRHL at a peptide density of 0.11 meq/g were used for human IgG, IgA and IgM isolation from cMEM. Representative chromatograms and corresponding SDS-PAGE gels are shown in Figures 3.8, 3.9, and 3.10 for human IgG, IgA and IgM, respectively. The average recoveries and purities of eluted immunoglobulins using pH 5.0 for hIgG, and pH 3.0 for hIgA and hIgM are listed in Table 3.2. The protein profiles of the three chromatographic peaks from HYFKFD and HFRRHL resins are shown in lanes 4-6 and 7-9 respectively on the appropriate SDS-PAGE gels.

The data in Table 3.2 shows that ligand HYFKDF had recoveries of IgG, IgA and IgM in the range of 72-75% using a peptide density of 0.11 mequiv./g, while peptide ligand HFRRHL displayed recoveries in the range of about 82-88%. As mentioned earlier, peptide HWRGWV had recoveries ranging from 80% to 84% under similar conditions (Table 3.1). In terms of purity, IgM was consistently the protein with the lowest values, independent of the peptide ligand used in the purification, with values ranging from about 68% (HWRGWV, Table 3.1) to 75% (HYFKFD, Table 3.2). All ligands tested at 0.11 mequiv./g showed high purity values for IgG, with intermediate purities for IgA, and lower purities for IgM.
As seen from the chromatograms for all three immunoglobulins, similar elution profiles were observed as those with peptide ligand HWRGWV at a peptide density of 0.11 mequiv./g. Comparing HYFKFD to HFRRHL, HFRRHL showed higher binding capacity, resulting from higher binding affinity, to all three immunoglobulins since relatively smaller amounts of targeting proteins were lost in the flow through (FT), resulting in higher recoveries for all three immunoglobulins. About 10% differences in recoveries were confirmed by ELISA analysis. However, HYFKFD exhibited better specificity to targeting proteins compared to HFRRHL, resulting in better purities for all three immunoglobulins. As shown in the SDS-PAGE gels, the P1 lanes in Figure 3.8B, 3.9B and 3.10B contained mostly human IgG, IgA and IgM, respectively, in their different isoforms, as it can be seen by comparing the respective bands in the standard human IgG, IgA and IgM (lane 2 of each gel). Over 93% purity was achieved for hIgG with both peptide ligands, and 94.3% and 75.0% purity were achieved for hIgA and hIgM with HYFKFD. The results confirm the hypothesis that the binding ability of HYFKFD and HFRRHL to all three immunoglobulins, making them potential candidates for human IgA and IgM purification.

In terms of recovery and purity for all three immunoglobulins, HWRGWV at low peptide densities was the best option among the conditions tested. The homology displayed by the peptide ligands (terminal histidine, followed by aromatic residues and positively charged residues) suggests that HYFKFD and HFRRHL could have a similar behavior at low peptide densities.
3.4. Conclusions

Hexamer peptide ligands HWRGWV, HYFKFD and HFRRHL initially screened against the Fc portion of hIgG exhibit the ability to capture and isolate human IgA and IgM as well. The strongest binding affinity is to hIgM, followed by hIgA and hIgG, respectively. As a result, relatively high recovery can be achieved for hIgG by using an elution pH larger than 4.0. However, high recovery can only be achieved for hIgA and hIgM by using an elution pH lower than 4.0. An elution buffer of pH 5.0 might be suitable for separating hIgG from hIgA and hIgM in a mixture.

Electrostatic interactions play an important role in human IgA and IgM binding with the ligand since human IgA and IgM are negatively charged at binding conditions, which is opposite to the net charge on HWRGWV. N-terminal acetylation of HWRGWV had an effect on the pH-based elution of adsorbed hIgA and hIgM, suggesting the charge of the N-terminal histidine plays a role in hIgA and hIgM binding. Nevertheless, the dominant electrostatic interactions result from the histidine and arginine residues.

Peptide ligand HWRGWV showed great potential for human IgA and IgM purification from complex mixtures. The peptide density of HWRGWV had a significant influence on the recovery and purity of human IgG, IgA and IgM isolated from cMEM. The improved recovery at higher density due to increased binding affinity was compensated by the decrease in purity for all three immunoglobulins. Over 80% recovery and 90% purity were achieved for human IgG and IgA isolation from cMEM using HWRGWV resin at optimum peptide densities. For human IgM, 75.7% recovery and 86.0% purity were achieved by using HWRGWV at a peptide density of 0.04 mequiv./g.
3.5. References


Table 3.1. Recovery (R) and purity (P) of isolated human IgG, IgA and IgM from cMEM by HWRGWV resins at different peptide densities.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Elution pH</th>
<th>Recovery and purity</th>
<th>Peptide density (mequiv./g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Human IgG</td>
<td>5.0</td>
<td>R (%) 73.7 ± 3.6</td>
<td>80.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%) 99.0</td>
<td>98.6</td>
</tr>
<tr>
<td>Human IgA</td>
<td>3.0</td>
<td>R (%) 77.7 ± 11.8</td>
<td>83.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%) 93.9</td>
<td>91.6</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>R (%) 75.0 ± 7.7</td>
<td>81.4 ± 11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%) 93.4</td>
<td>92.4</td>
</tr>
<tr>
<td>Human IgM</td>
<td>3.0</td>
<td>R (%) 75.7 ± 4.6</td>
<td>84.4 ± 5.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%) 86.0</td>
<td>69.0</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>R (%) 73.5 ± 8.1</td>
<td>81.6 ± 6.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%) 76.7</td>
<td>68.2</td>
</tr>
</tbody>
</table>
Table 3.2. Recovery (R) and purity (P) of isolated human IgG, IgA and IgM from cMEM by HYFKFD and HFRRHL resins at peptide density of 0.11 mequiv./g.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Elution pH</th>
<th>Recovery and purity</th>
<th>Peptide Ligand at 0.11 mequiv./g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R (%)</td>
<td>HYFKFD</td>
</tr>
<tr>
<td>Human IgG</td>
<td>5.0</td>
<td>72.1 ± 8.3</td>
<td>81.8 ± 14.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P (%)</td>
<td>99.5</td>
</tr>
<tr>
<td>Human IgA</td>
<td>3.0</td>
<td>71.8 ± 0.2</td>
<td>84.4 ± 3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94.3</td>
<td>78.9</td>
</tr>
<tr>
<td>Human IgM</td>
<td>3.0</td>
<td>74.9 ± 12.7</td>
<td>87.5 ± 14.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75.0</td>
<td>61.5</td>
</tr>
</tbody>
</table>
Figure 3.1. Amino acid sequence alignments of the predicted HWRGWV binding site on human IgG1 with possible binding sites on human IgA1 and IgM. Red amino acids indicate the loop on hIgG1 predicated to bind HWRGWV. The sequences are from UniProt (http://www.uniprot.org/): P01857 (hIgG1), P01876 (hIgA1), and P01871 (hIgM). All amino acid sequences are of the constant region of the heavy chain.
Figure 3.2. Structural diagram of human IgG1, IgA1 and IgM monomer. “Y” shape structure is composed of two identical light (L) chains and two heavy (H) chains. The light chains have two domains: variable region domain (\(V_L\)) and constant region domain (\(C_L\)). The heavy chains are composed of variable region domain (\(V_H\)) and multiple constant region domains (\(C_H\)) based on the class of immunoglobulins.
Figure 3.3. Recoveries for chromatographic capture and elution of human (A) IgG, (B) IgA and (C) IgM with HWRGWV resins at different peptide densities.
Figure 3.4. Chromatograms for human (A) IgG, (B) IgA and (C) IgM recovery by pH gradient elution with peptide resin HWRGWV at 0.11 mequiv./g and peptide resin Ac-HWRGWV at 0.08 mequiv./g.
Figure 3.5. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM using HWRGWV resins at different peptide densities using pH 5.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 5.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the reminder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG) and loading material (L).
Figure 3.6. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from cMEM using HWRGWV resins at different peptide densities using pH 3.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 3.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the remainder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgA (IgA) and loading material (L).
Figure 3.7. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from cMEM using HWRGWV resins at different peptide densities using pH 3.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 3.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the reminder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgM (IgM) and loading material (L).
Figure 3.8. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgG separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 5.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 3.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the remainder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG) and loading material (L).
Figure 3.9. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 3.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 3.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the reminder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgA (IgA) and loading material (L).
Figure 3.10. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from cMEM using HYFKFD and HFRRHL resins at a peptide density of 0.11 mequiv./g using pH 3.0 elution. A: Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of acetate buffer pH 3.0 for elution and 6M guanidine HCl for washing. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the remainder of the time. Injection volume was 100 μL. The detected peaks were flow through (FT), elution peak (P1) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgM (IgM) and loading material (L).
Chapter 4. Purification of Human Immunoglobulins A, G and M from Cohn Fraction II/III by Small Peptide Affinity Chromatography

Zhuo Liu, Patrick V. Gurgel, Ruben G. Carbonell

A version of this chapter has been published in

Abstract

This work describes attempts to purify human IgG, IgA and IgM from Cohn fraction II/III using HWRGWV affinity peptide resin. The effects of peptide density and different elution additives on recovery of the three antibodies were investigated. At low peptide density, salting-in salts such as magnesium chloride and calcium chloride facilitated antibody elution. Ethylene glycol, urea and arginine also facilitated elution because of their ability to decrease hydrophobic interactions, hydrogen bonding and electrostatic interactions. However, at high peptide density, no recovery improvements were observed because of increased non-specific hydrophobic interactions. The final elution conditions for each antibody were chosen based on the resulting yields and purities when a 10:2:1 mg/mL mixture of human IgG, IgA and IgM was used as starting material. Different pretreatment methods were employed in order to improve the purity of antibodies from Cohn fraction II/III. After pretreatment with caprylic acid precipitation or combination of caprylic acid and polyethylene glycol precipitation, purities over 95% and yields of about 60% were obtained for IgG, which are comparable to current chromatographic purification methods involving two chromatography steps when IgG is isolated from plasma fractions. A IgA-enriched fraction with 42% hIgA and 56% hIgG, as well as a IgM enriched fraction with 46% hIgM, 28% hIgA and 24% hIgG, were obtained as the by-products.

Keywords: hexamer peptide; IVIG; Cohn fraction; elution additive; PEG; ammonium sulfate.
4.1. Introduction

Antibodies have been used as therapeutics in various forms for over a century since their first administration in the form of sera in the 1890s [1-5]. With more than 20 molecules in clinical use, the world's therapeutic monoclonal antibodies market exceeded $40 billion in 2010 and is expected to reach $70 billion by 2015 [6,7]. Apart from the therapeutic monoclonal antibodies, the importance of polyclonal antibodies obtained from human plasma has grown over the last 30 years. For more than 20 years, polyclonal antibodies derived from plasma have been used in the treatment of a wide range of primary and secondary immunodeficiencies. As one of the leading products of the human plasma fractionation industry, intravenous immunoglobulin (IVIg) is a sterile, highly purified immunoglobulin G (IgG) product manufactured from pooled human plasma [8]. Typically, IVIg contains more than 95% unmodified IgG and only trace amounts of immunoglobulin A (IgA) and immunoglobulin M (IgM) [9]. Intravenous immunoglobulin (IVIg) is not only standard therapy for most primary immunodeficiencies (PID), but also is extensively used in the treatment of an increasing number of autoimmune and inflammatory diseases [10-13].

Immunoglobulin M enriched IVIg (IVIgM) and Immunoglobulin A and M enriched IVIg (IVIgAM) are powerful therapeutic agents, which can be used to prevent inflammation [14-16], combat bacterial infections [17], in the treatment of severe sepsis and septic shock [18,19], to inhibit alloantigen-induced proliferation in mixed lymphocyte reaction in vitro [20] as well as reduce endotoxaemia and its consequences in bone marrow transplant patients [21].

Traditional plasma fractionation is currently carried out using either the Cohn-Oncley [22,23] or the Kistler-Nitschmann [24] methods, which are processes that involve cold
ethanol precipitation with tightly controlled conditions including temperature, pH, ionic strength and ethanol concentration. Recently, chromatographic purification methods have been introduced which usually start with plasma or intermediates derived from the Cohn-Oncley or Kistler-Nitschmann process and involve at least two chromatography steps. For example, Gamunex® (Talecris Biotherapeutics, RTP, NC, USA) uses Cohn-Oncley fraction II/III as starting material; after caprylate pre-treatment to precipitate non-IgG proteins and to inactivate enveloped viruses, two successive anion-exchange columns are used to purify IgG and remove caprylate [25]. Both Baxter’s Gammagard SD® and IGIV 10% (Baxter International Inc, Deerfield, IL, USA) are also manufactured in a similar manner, involving two ion-exchange chromatography steps after solvent-detergent treatment of the ethanol fraction [26]. These processes do not use affinity chromatography due to limitations such as high cost, low chemical and physical stability of ligands such as Protein A and Protein G, leachability and toxicity-related issues. Other valuable immunoglobulins such as IgA and IgM are typically treated as contaminant proteins that need to be removed during the purification process.

Hexamer peptide ligand HWRGWV exhibits the ability to recognize and bind hIgG through its Fc portion. This peptide has been previously identified by our group through the screening of a synthetic solid phase combinatorial peptide library, and it has been found that its selectivity to the Fc portion of IgG is comparable to that of Protein A [27-29]. Previous studies showed that the peptide ligand binds to hIgA and hIgM as well, having the strongest binding affinity to hIgM, followed by hIgA and hIgG, respectively [30]. Ligand density had a marked effect on the purities and recoveries obtained for the different proteins when being
purified from cMEM, with lower peptide densities (0.04 mequiv./g) showing lower recoveries and higher purities, while higher peptide densities (up to 0.55 mequiv./g) showing progressively higher recoveries, and progressively lower purities. It was found that a pH gradient elution was not able to separate the captured antibodies from the affinity column [30]. However, results also suggested that the three antibodies could be separated from a mixture by using different elution solutions, due to their different binding affinities to the peptide resin.

Experiments comparing the performance of the HWRGWV peptide resin and its terminal amine acetylated equivalent (Ac-HWRGWV) showed that the terminal charge had an effect on the capture and the pH-based elution of antibodies. However, the results also suggest that the dominant electrostatic component of binding was due to the histidine and arginine residues in the ligand.

In this work, the effects of different elution additives on the separation of IgG, IgA and IgM were studied, in order to determine the optimum conditions for maximizing the yields and purities for each of the proteins. Based on the results, the best elution conditions and peptide density were chosen based on the yields and purities of the three antibodies from a starting solution consisting of 10:2:1 mg/mL of IgG, IgA and IgM, respectively. Finally, Cohn fraction II/III was used as the starting material for the purification of human IgG, IgA and IgM using HWRGWV peptide resin. Different pretreatment methods were used in order to achieve enhanced purities and yields for the three antibodies. To our knowledge, this is the first time in the literature that an affinity chromatography column was employed to separate IgG, IgA and IgM from Cohn fraction II/III in a single column.
4.2. Experimental

4.2.1. Materials

Peptides International (Louisville, KY, USA) synthesized HWRGWV directly on the resin Toyopearl AF-Amino-650M (particle size 65μm) (Tosoh BioScience, Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry. The HWRGWV resins were synthesized at different peptide densities from 0.04 to 0.55 mequiv./g. Phosphate-buffered saline (PBS) pH 7.4 (0.01 M phosphate, 0.138 M sodium chloride, and 2.7 mM potassium chloride), urea, arginine and polyethylene glycol (MW4600) were obtained from Sigma (St. Louis, MO, USA). Sodium chloride, sodium hydroxide, magnesium chloride, calcium chloride, sodium acetate, glacial acetic acid, phosphoric acid, ammonium sulfate, ammonium hydroxide, guanidine hydrochloride, tris hydrochloride, ethylene glycol and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). NuPAGE Novex 4-12% Bis-Tris gels, NuPAGE reducing agent, NuPAGE MOPS SDS running buffer, SeeBlue Plus2 pre-stained molecular weight marker and SimplyBlue Safestain were all from Invitrogen (Carlsbad, CA, USA). Human IgG lyophilized power was purchased from Equitech-Bio (Kerrville, TX, USA). Human IgA and IgM were from Fitzgerald (Concord, MA, USA). Human IgG, IgA and IgM enzyme-linked immunosorbent assay (ELISA) kit were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Microcon Ultracel YM-3 centrifugal filters (regenerated cellulose, 3000 MWCO) were purchased from Millipore (Billerica, MA, USA). 0.2 μm and 0.45 μm Acrodisc Syringe Filter were purchased from Pall Corporation (Port Washington, NY, USA). BCA (bicinchoninic acid) protein assay was from
Thermo Scientific (Rockford, IL USA). Microbore columns (30 mm × 2.1 mm) for all chromatographic experiments were from Alltech (Deerfield, IL, USA).

4.2.2. Equipment

All chromatographic experiments were carried out on a Waters 626LC system. Absorbance of protein samples was measured at wavelength 280 nm using a built-in 2487 dual wave-length UV detector from Waters (Milford, MA, USA). ELISA wash step was performed on ELx50 Microplate Strip Washer from Bio-Tek Instruments Inc. (Winooski, VT, USA), and ELISA protein concentration was determined by μQuant Spectrophotometer from Bio-Tek Instruments Inc. as well.

4.2.3. Chromatographic capture and recovery of human IgG, IgA and IgM

Peptide resins were dry packed in 30 mm × 2.1 mm I.D. microbore columns (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 h followed by HPLC grade water and PBS washes. Before sample loading, columns were equilibrated with pH 7.4 equilibration buffer consisting of PBS + 1 M NaCl. Samples were manually injected at room temperature using a 100 μL sample loop (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 minutes, and then the flow rate was increased to 0.3 mL/min for the remainder of the run. PBS + 1M NaCl was also used as the binding buffer, the column was washed sequentially with 3 mL binding buffer, 6 mL elution buffer, and 6 mL 6M guanidine-HCl to regenerate the column, followed by PBS + 1M NaCl for equilibration. Acetate buffer (0.2 M) at pH 5.0 with different elution additives including NaCl, MgCl₂, CaCl₂, ethylene glycol, urea and arginine were used as elution buffers. The recoveries of hIgG, hIgA, hIgM
were calculated based on the peak areas for elution and regeneration assuming all injected proteins bound to the resin since only small flow through peaks appeared for all injections.

### 4.2.4. Preliminary elution condition study for the isolation of human IgG, IgA and IgM from a 10:2:1 mg/mL IgG, IgA, IgM mixture

Columns with peptide resin HWRGWV at the densities of 0.11 mequiv./g and 0.23 mequiv./g were prepared and equilibrated by the same methods described in section 4.2.3. A mixture of human IgG, IgA and IgM in PBS at concentrations of 10, 2 and 1 mg/mL respectively was manually injected at room temperature using a 50 μL sample loop (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 minutes, and then the flow rate was increased to 0.3 mL/min for the rest of the run. PBS + 1M NaCl was used as the binding buffer, the column was washed sequentially with 3 mL binding buffer, 6 mL hIgG elution buffer, 6 mL hIgA elution buffer, 6 mL hIgM elution buffer and 6 mL 6M guanidine-HCl to regenerate the column. The selections of elution buffers for human IgG, IgA and IgM are listed in Tables 4.1 and 4.2 for HWRGWV at 0.23 mequiv./g and 0.11 mequiv./g, respectively. All elution fractions were collected and the pH was immediately adjusted to neutral by adding an appropriate volume of 1 M Tris buffer pH 8.0. All collected fractions were directly subjected to ELISA assays.

### 4.2.5. Cohn fraction II/III sample preparation and pretreatments

#### 4.2.5.1. Cohn fraction II/III sample preparation

Fifty mg/mL Cohn fraction II/III containing 60-85% protein purchased from Sigma (St. Louis, MO, USA) was dissolved in HPLC grade water or PBS and stirred overnight at
4°C. This original Cohn fraction was named stock solution I. After centrifugation for 15 min at 6,000 × g to remove any un-dissolved material, stock solution II was obtained. The stock solution II was passed through a 0.45 μm followed by 0.2 μm Acrodisc Syringe Filter from Pall Corporation (Port Washington, NY, USA) to achieve stock solutions III and IV, respectively.

4.2.5.2. Cohn fraction II/III pretreatments

4.2.5.2.1. Caprylic acid and PEG pretreatment

The pH of stock solution IV was adjusted to pH 5.0 at room temperature with 0.2 M acetic acid. Caprylic acid was added to a concentration of 50 mM over a 5-min time-period. The suspension was mixed for 1 h and the precipitate was removed by centrifugation at 14,000 × g. In the pretreatment without polyethylene glycol (PEG) precipitation, the supernatant solution pH was raised to pH 7.0 with 0.2 M sodium hydroxide and filtered through a 0.2 μm syringe filter, while in the pretreatment with PEG precipitation, the supernatant solution pH was adjusted to pH 5.5 with 0.2 M sodium hydroxide, PEG 4600 was added to reach a concentration of 3% (w/v). The solution was mixed for 1 h and the precipitate was removed by centrifugation at 14,000 × g. Finally, the supernatant solution pH was raised to pH 7.0 with 0.2 M sodium hydroxide and filtered through a 0.2 μm syringe filter.

4.2.5.2.2. PEG only pretreatment

The stock solution IV pH was adjusted to 5.5 with 0.2 M acetic acid and PEG 4600 was added to achieve concentration of 3% (w/v). The suspension was mixed for 1 h and the
precipitate was removed by centrifugation. After the pH was raised to pH 7.0 with 0.2 M sodium hydroxide, the solution was filtered through a 0.2 μm syringe filter.

### 4.2.5.2.3. SAS pretreatment

A saturated ammonium sulfate (SAS) solution was prepared at room temperature. The pH was adjusted to pH 7.0 by addition of ammonium hydroxide, and the solution was filtered through 0.2 μm membrane to remove particulate material. Two milliliters of SAS was added over 5 min with gentle stirring to 4.8 mL stock solution IV. Stirring was continued at room temperature for 20 min, and the mixture was centrifuged for 10 min at 14,000 × g in 2 mL centrifugal tubes. The pellets were discarded, and 2.4 mL of SAS was added to the combined supernatant over a period of 5 min. After stirring for 30 min at room temperature, the mixture was centrifuged for 15 min at 14,000 × g. The supernatants were decanted as mush solution as possible by inverting the tubes for 5 min. Finally, 4.0 mL of HPLC grade water or PBS pH 7.4 was added to the combined pellets to dissolve all and the clear solution was filtered through 0.2 μm syringe filter.

### 4.2.6. Purification of human IgG, IgA and IgM from Cohn fraction II/III

A column with peptide resin HWRGWV at the density of 0.11 mequiv./g was prepared and equilibrated by the same methods described in section 4.2.3. Cohn fraction II/III after preparation with or without pretreatments was manually injected at room temperature using a 50 μL sample loop (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 min, and then the flow rate was increased to 0.3 mL/min for the rest of the run. PBS + 1M NaCl was used as the binding buffer, the column was
washed sequentially with 3 mL binding buffer, 6 mL hIgG elution buffer, 6 mL hIgA elution buffer, 6 mL hIgM elution buffer and 6 mL 6 M guanidine-HCl to regenerate the column. The selection of elution buffers for human IgG, IgA and IgM was based on the preliminary elution condition study. All elution fractions were collected and the pH was immediately adjusted to neutral by adding an appropriate volume of 1 M Tris buffer pH 8.0. All collected fractions were directly subjected to ELISA assays.

4.2.7. Sample analysis for yields and purities

The yields of purified human IgG, IgA and IgM were calculated according to the hIgG, hIgA, and hIgM concentration determined by ELISA using human IgG, IgA and IgM ELISA kits all from Alpha Diagnostic International. The flow through and hIgG elution fractions were concentrated about 4 times and hIgA and IgM elution fractions were concentrated about 20 times by centrifugation at 20°C, 14,000 × g for 10 and 75 min, respectively, using Microcon YM-3 filters before the electrophoresis analysis. Concentrated fractions of chromatography peaks were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE Novex 4-12% Bis-Tris gels with MOPS running buffer on an X-cell SuperLock™ Mini-Cell system from Invitrogen. The gels were stained by SimpleBlue SafeStain. The purities of human IgG, IgA, and IgM elution peaks were determined by densitometric measurement using software ImageJ 1.32j (National Institute of Health, Bethesda, MD, USA).
4.3. Results and discussion

4.3.1. Effects of elution additives on the recovery of human IgG, IgA and IgM at different peptide densities

In affinity chromatography, the interactions of bound protein and the complementary ligand commonly involve electrostatic, hydrophobic, van der Waals and hydrogen bonding interactions, among others. Therefore, the dissociation and subsequent recovery of bound protein are typically dependent on perturbing all or a majority of the physical interactions [31]. Different elution buffer systems have been employed in affinity chromatography for the recovery of antibodies as well as other proteins [32-35].

In a previous study [30], we have shown that IgG can be eluted from the HWRG WV resin and its acetylated form at higher pH values than IgA and IgM, and that ligand density has an effect on the final purity and recovery values. We started the present work by investigating the effect of adding different elution additives to the elution buffer, using peptide resins at two different ligand densities.

4.3.1.1. Effects of electrolytes

Salts, whether inorganic or organic, play an important role in the majority of chromatography processes for protein purification. Salts can be used to facilitate binding and elution of proteins, or to suppress non-specific protein-protein or protein-surface interactions [36]. There are two types of salt effects: nonspecific and specific effects. Nonspecific salt effects are simply due to their ionic properties and are independent of the type of the salts. However, at higher salt concentrations, salts can exert specific effects that depend on the type of salt being used. Such salt effects are often described by the Hofmeister series and a
mechanistic explanation of action has been provided based on the surface tension of salt solutions and the preferential interaction of salts with the proteins [37].

In order to investigate the effect of salts on the dynamic elution of hIgG, hIgA and hIgM, 0.2 M acetate buffer pH 5.0 was used as elution buffer, with the addition of different salts, including NaCl, MgCl₂, and CaCl₂ at concentrations ranging from 0 to 2.0 M. All salts were used in their chloride ion form to eliminate the salt effect due to anions since Cl⁻ is a “neutral” anion in the Hofmeister series [38].

As shown in Figure 4.1, the recoveries for all three antibodies decreased or remained low with increases in the NaCl concentration. At low peptide density (0.04 mequiv./g), the weak salting-out salt NaCl might have increased the specific binding affinity of the antibodies to the peptide ligand, resulting in lower recoveries with increasing salt concentration. At high peptide density (0.55 mequiv./g), not only the elution peaks decreased, but also the 6 M guanidine HCl regeneration peaks became shorter and broader (data not shown). These effects might have resulted from either increased specific interaction or increased non-specific hydrophobic interactions at high peptide density [36]. The increased binding affinity with the addition of NaCl might explain the previously observed increased yield and purity during hIgG isolation from cMEM with the addition of NaCl in the loading buffer [28].

With the addition of MgCl₂ or CaCl₂ (salting-in salts), no significant change in recovery was observed for hIgG at low peptide density (Figure 4.1A), suggesting the possibility of counteracting effects of salting in and increased specific binding affinity from the salts. At high peptide density (Figure 4.1B), the recovery of hIgG decreased with
increased salt concentration most likely because of dominant non-specific hydrophobic interactions. For hIgA and hIgM at low peptide density (Figure 4.1A), the addition of both MgCl₂ and CaCl₂ in the elution buffer seems to have facilitated the antibody elution resulting in better recoveries with increased salt concentration. This can be explained by the favorable interaction between the divalent ions and antibodies at high salt concentration [37]. In addition, the same level of recovery was achieved by a lower concentration of CaCl₂ compared to MgCl₂ (0.2 M CaCl₂ vs. 0.5 M MgCl₂) showing a stronger effect from CaCl₂. This was expected since CaCl₂ is a stronger salting-in salt from the Hofmeister series. However, high concentrations of CaCl₂ should be avoided to prevent protein aggregation since multiple peaks were observed in the 6M guanidine HCl regeneration step after elution with 1 M CaCl₂ (data not shown). At high peptide density, the salting in effect of MgCl₂ and CaCl₂ could not overcome the increased non-specific hydrophobic interactions. Therefore, no increase in recoveries was observed.

Overall, the results obtained from this set of experiments suggest that salting-in electrolytes such as MgCl₂ and CaCl₂ could be used to elute antibodies from an affinity resin containing the ligand HWRGWV at low ligand densities.

4.3.1.2. Effects of non-electrolytes

Non-electrolytes such as water-miscible organic solvents, denaturing agents and amino acids have been employed as solvent additives for protein purification by affinity chromatography because of their ability to suppress nonspecific protein-protein or protein-surface interactions [31,37,39]. In order to investigate the effect of non-electrolyte additives
on the dynamic elution of hIgG, hIgA and hIgM, recovery of bound proteins was achieved by using 0.2 M acetate buffer pH 5.0 containing different elution additives including ethylene glycol, urea, and arginine at different concentrations.

The effect of ethylene glycol at concentrations from 0 to 40% (v/v) was examined. As shown in Figure 4.2, at both peptide densities, ethylene glycol had no effect on the recovery of hIgG and hIgM elution, with the recoveries for both proteins being virtually constant in the ethylene glycol concentration range used. The difference for the two proteins was that in the case of hIgG the recovery remained around 90-95%, while for hIgM the values for recovery were in the 0-5% range. For hIgA, the recovery increased with the increase in the ethylene glycol concentration at low peptide density (Figure 4.2A, left panel), while they remained low at high peptide density (Figure 4.2B, left panel). Ethylene glycol has the ability to suppress hydrophobic interactions and bind to the protein surface. Therefore, the elution mechanism of ethylene glycol is similar to MgCl$_2$ except that ethylene glycol enhances electrostatic interactions by lowering the dielectric constant [37,40]. As a result, the increased recovery at low peptide density might have been achieved by decreased hydrophobic interactions exceeding increased electrostatic interactions. However, at high peptide density, the decrease in hydrophobic interactions could not overcome the increased non-specific interactions and electrostatic interactions leading to unimproved recovery.

Urea at concentrations ranging from 0 to 2.0 M was tested (Figure 4.2, center panels), since higher urea concentration might affect the protein structure and stability. Similar behavior was observed for the effect of urea compared to ethylene glycol at both peptide densities. Compared to ethylene glycol, urea exhibits affinity for all side chains of amino
acids instead of only non-polar side chains, resulting in decreased hydrophobic interactions. At the same time, urea can reduce polar interactions by forming more stable hydrogen bonds with peptide bonds than with water and slightly increasing the dielectric constant of water [35]. Therefore, at low peptide density, urea weakened the hydrophobic and polar interactions to a sufficient extent that caused the elution of hIgA. However, at high peptide density, the dominant non-specific hydrophobic interactions could not be overcome by decreased hydrophobic and polar interactions.

Arginine has been used for elution of antibodies from Protein A affinity chromatography because of its ability to suppress aggregation and protein-protein or protein-surface interactions without denaturing proteins [34,41,42]. The recovery of hIgG did not change with the addition of arginine in the elution buffer at both peptide densities (Figure 4.2, right panels), remaining relatively high for all conditions. At low peptide density, the recovery of both hIgA and hIgM increased to over 95% at an arginine concentration of 1.0 M. Nevertheless, arginine seems to have had a stronger effect on hIgA elution because higher recovery was achieved with hIgA compared to hIgM at lower arginine concentration, which probably resulted from the weaker binding affinity of HWRGWV peptide ligand to hIgA. At high peptide density, because of the dominant increased non-specific hydrophobic interaction, the recovery of hIgA only improved from 1.8% to 38.9% and hIgM recovery remained almost constant.

Based on the results described above, arginine seems to be the best elution additive among the three tested in terms of perturbing the physical interactions of the bound proteins
and the resins. However, ethylene glycol and urea might also be useful for hIgA purification from hIgA and hIgM mixture at low peptide density.

4.3.2. Preliminary elution condition study for the isolation of human IgG, IgA and IgM from a 10:2:1 mg/mL IgG, IgA, IgM mixture

Since the three target molecules behave differently at different elution conditions, it should be possible to achieve purification of hIgG, hIgA and hIgM from their mixture using the general scheme shown in Figure 4.3. The selection of elution buffers was based on the experiments listed in Tables 4.1 and 4.2 for HWRGWV at peptide densities of 0.23 and 0.11 mequiv./g. The resulting yields and purities for isolated human IgG, IgA and IgM from their mixture by HWRGWV peptide resins at two different peptide densities are listed in Tables 4.3 and 4.4. For 0.23 mequiv./g HWRGWV peptide resin (Tables 4.1 and 4.3), both 0.2 M acetate buffer at pH 5.0 and pH 5.2 can be used to achieve hIgG with over 90% in yield and 95% in purity. In Runs 1 and 2 the elution conditions of for IgG and IgA were kept constant (0.2 M acetate buffer (AB) pH 5.0, and 0.2 M AB pH 5.0 + 0.2 M CaCl$_2$, respectively), while the elution conditions for IgM varied. In Run 1 a salt gradient (0-2.0 M MgCl$_2$ in 0.2 M AB pH5.0) was tested, while run 2 used 0.2 M AB pH5.0 + 1 M MgCl$_2$. In Runs 3-5 the elution condition for IgG was changed to 0.2 M AB pH 5.2, while IgA was eluted using the same buffer as in Runs 1 and 2. IgM conditions tested used 0.2 M AB pH 5.0 with 0.5 M MgCl$_2$ (Run 3), 1.0 M MgCl$_2$ (Run 4), and 2.0 M MgCl$_2$ (Run 5). The results obtained (Table 4.3) confirmed that 2 M MgCl$_2$ would be needed to achieve over 90% both in yield and purity of hIgM. Considering the convenience and similar performance of step elution compared to gradient elution, hIgM step elution with 0.2 M acetate buffer pH 5.0 with 2 M MgCl$_2$ was
used to search for optimum hIgA elution buffer (Runs 6-9). For hIgA, 0.2 M acetate buffer pH 5.0 with 1 M arginine (Table 4.1, Run 9) is the best option to elute hIgA with 75.8% and 63.3% in yield and purity, respectively (Table 4.3, Run 9). However, the combination of optimum elution buffers for both hIgA and hIgM did not work well since 0.2 M acetate buffer pH 5.0 with 1 M arginine was so strong that coelution of hIgM happened. Therefore, the resulting yield for hIgM elution decreased from over 90% (Table 4.3, Runs 1, 5-7) to only 14.2% (Table 4.3, Run 9).

For 0.11 mequiv./g HWRGWV peptide resin (Table 4.4), relatively lower yields for hIgG were obtained compared to the 0.23 mequiv./g HWRGWV peptide resin, which could be a result from lower dynamic binding capacities. At the lower peptide density, 0.2 M acetate buffer pH 5.0 needed to be used for hIgG elution in order to achieve yields with an average above 90% (Tables 4.2 and 4.4, Runs 1, 2, 6, 7). When 0.2 M acetate buffer pH 5.0 with 0.2 M CaCl$_2$ was used as hIgA elution buffer after IgG was eluted using 0.2 M AB pH 5.2 (Table 4.4, Runs 3-5), relatively higher yields were achieved compared to those when 0.23 mequiv./g HWRGWV peptide resin was used (Table 4.3, Runs 3-5), suggesting a lower binding affinity with lower peptide density. Although the same hIgA elution buffer was used, different yields for hIgA were achieved when different hIgG elution buffers were used (Table 4.4, Runs 1 and 2 compared to 3-5). These results suggest that the yield of an eluted antibody is dependent not only on the elution buffer but also on the previous buffer used. The use of 0.2 M acetate buffer pH 5.0 with 2 M MgCl$_2$ (Runs 4 and 7) is still the best option for hIgM elution. Without coelution of hIgM, 0.2 M acetate buffer pH 5.0 with 0.5 M arginine is the optimum selection for hIgA elution based on yield and purity results (Runs 8 and 9).
Based on the overall results of the elution experiments, HWRGWV peptide resin at density of 0.11 mequiv./g was selected for further studies on the purification of human IgG, IgA and IgM from Cohn II/III. The elution buffers selected for each antibody were 0.2 M acetate buffer pH 5.0 for hIgG, 0.2 M acetate buffer pH 5.0 with 0.5 M arginine for hIgA and 0.2 M acetate buffer pH 5.0 with 2 M MgCl$_2$ for hIgM.

4.3.3. Cohn fraction II/III sample preparation and pretreatments

Since the original 50 mg/mL Cohn Fraction II/III (60-85% pure protein) stock solution contained about 15-40% non-protein material, centrifugation and two filtration steps were used to remove non-dissolved contaminants. BCA protein assay and ELISA kits were used to determine the total protein concentration and each antibody concentration after each step.

Different pretreatment methods were used to remove some major contaminant proteins such as human serum albumin (HSA), HSA dimer, transferrin and α-1 antitrypsin before the chromatographic step in order to improve the purity of purified antibodies. The pretreatment methods used were modified based on previous research from literature [43,44].

The total protein as well as human IgG, IgA and IgM concentration during the sample preparation and after each pretreatment are shown in Figure 4.4. The volume factor for each pretreatment was taken into account when the protein concentration was calculated. The total protein concentration from Cohn fraction II/III after dissolving in HPLC grade water and PBS were 33.2 and 34.9 mg/mL with slightly higher concentration achieved by PBS as the solvent. Therefore, higher concentrations were observed in PBS solvent than in HPLC grade
water for human IgG, IgA and IgM after each preparation and pretreatment step. After one
centrifugation and two filtration steps, yields of about 90% were obtained for total proteins,
of which about 50% were antibodies, with IgG, IgA and IgM present at a ratio of about
10:2:1.

Caprylic acid can be used as free acid to inactivate enveloped viruses and precipitate
contaminating proteins [25]. PEG precipitation alone was able to remove contaminating
proteins and the combination of PEG precipitation with caprylic acid treatment was found
not only for the removal of polymers but also for the removal of non-enveloped viruses [44].
SAS precipitation has been employed to remove lipoproteins and fibrinogen without
denaturation of IgM to achieve large scale recovery of IgM from human plasma [43].

After the caprylic acid pretreatment, about 20 mg/mL of proteins were recovered,
which was about 67% of the total proteins. The average recovery for hIgG in water and PBS
was 86%, a value that was higher than the average recoveries of hIgA and hIgM of 71% and
46%, respectively. As a result, the percentage of hIgG in the solution increased from 40% to
50% or more after the pretreatment. Therefore, caprylic acid treatment seems to be a
relatively good pretreatment method to remove contaminating proteins for hIgG recovery.
However, more than 50% of hIgM was lost during the precipitation which might be caused
not only by the lower solubility of hIgM (pI: 5.5-6.7) at the caprylic acid precipitation pH of
5.0, but also by the large molecular structure as a pentamer with molecular weight of 950 kDa.

With the combination of caprylic acid and PEG precipitation, the total protein
concentration decreased to about 55% of the stock Cohn fraction II/III. The hIgG
concentrations remained about the same as the ones with only caprylic acid treatment, which further increased the hIgG percentage to around 60% and confirmed that the combination of caprylic acid and PEG precipitation works well for removing contaminating proteins from Cohn fraction II/III for hIgG purification. However, only about 62% of hIgA and 30% of hIgM were recovered after caprylic acid and PEG precipitations.

With PEG precipitation alone, for Cohn fraction II/III dissolved in PBS, the recoveries for total protein as well as three antibodies were about the same at around 91%, except for hIgM with a 72% recovery, further confirming that precipitation is not a good method to recover large molecules such as hIgM. For Cohn fraction II/III dissolved in water, a 78% recovery was obtained for total protein compared to around 90% recoveries for both hIgG and hIgA, and 61% recovery for hIgM. Therefore, reasonably good recoveries were achieved for all three antibodies with poorer contaminant removal efficiency when using PEG pretreatment alone.

SAS pretreatment, employed for large scale recovery of IgM from human plasma [43], showed good recovery for hIgM, when compared to the other three pretreatment methods tested, with values of 77% and 62% achieved for Cohn fraction II/III dissolved in water and PBS, respectively. The recoveries for total protein, hIgG and hIgA from Cohn fraction II/III dissolved in water and PBS are about the same with averages of 66%, 73% and 76%, respectively. Therefore, even with good efficiency for contaminants removal and high recovery for hIgM, the hIgG recovery was low compared to other three pretreatment methods.
4.3.4. Purification of human IgG, IgA and IgM from Cohn fraction II/III

Chromatographic purification of the antibodies from Cohn fraction II/III was performed using a resin with ligand density of 0.11 mequiv./g. The columns were washed consecutively with PBS + 1M NaCl (binding and wash buffer), 0.2 M AB pH 5.0 (IgG elution buffer), 0.2 M AB pH 5.0 + 0.5 M arginine (hIgA elution buffer), 0.2 M AB pH 5.0 + 2 M MgCl₂ (hIgM elution buffer), and 6 M guanidine HCl (regeneration solution).

The chromatograms and corresponding SDS-PAGEs for one column purification of human IgG, IgA and IgM from Cohn fraction II/III (prepared in HPLC grade water or PBS) without or with different pretreatment methods are shown in figures from Figure 4.5 to Figure 4.14. Without any pretreatment for the loading material, a large flow through peak was observed in the chromatograms as shown in Figure 4.15. The results obtained by SDS-PAGE analysis (Figure 4.16) showed that the flow through peaks contained HSA and HSA dimer as the main contaminants. Since a large amount of contaminant proteins was present in those runs, it lead to a lower dynamic binding capacity, causing human IgG as well as IgA and IgM to be detected in the flow through (unbound) fractions albeit at low concentrations. Therefore, the yields for purified hIgG were 60.5% and 76.3% for Cohn fraction II/III dissolved in water and PBS, respectively (Table 4.5). Higher yields for runs using PBS as solvent are likely to be a result of the lower content of competing proteins with non-specific hydrophobic interactions prevented by the presence of salt. Purities of around 90% were obtained for IgG, which are somewhat lower than what was obtained with the use of pretreatments (Table 4.6), possibly due to coelution of hIgA, HSA and HSA dimer.
For hIgA and hIgM elution, enriched hIgA fractions were achieved with solutions containing around 44% hIgA and 35% hIgG, for both diluents tested, while for hIgM values of around 36% hIgM, 21% hIgA, and 4% hIgG were achieved for both diluents (Table 4.5). Although the purities were similar for the hIgG fractions, hIgA-enriched fractions and hIgM-enriched fractions for both water and PBS as solvents, the recoveries for hIgA and hIgM were about 10% higher for PBS compared to water as solvent. This result further suggests that the presence of salts enhances antibody binding to the HWRGWV affinity resin.

After pretreatment with caprylic acid, much smaller flow through peaks (Figures 4.15A and 4.15B) were observed, compared to those without pretreatment, likely because of the partial removal of contaminating proteins by the pretreatment. This observation is supported by the results shown in Figure 4.16, lanes 2 and 7, showing the SDS-PAGE protein profile of the material after pre-treatment with caprylic acid. Over 98% in purity for hIgG was achieved for two different solvents (Tables 4.6 and 4.8). The overall yields achieved with caprylate pretreatment were comparable to the one achieved using Cohn fraction without any pretreatment (Table 4.5), at about 60%. hIgA-enriched fractions containing an average of 43.4% hIgA and 54.3% of hIgG were obtained when using caprylic acid (Tables 4.6 and 4.8), as well as hIgM-enriched fractions containing an average 44.8% hIgM, 28.5% hIgA and 21.6% hIgG. Since significant losses of hIgA and hIgM during the pretreatment step were observed, the overall yields for hIgA and hIgM were only about 20%.

For the combination of caprylic acid and PEG pretreatment using water and buffer (Tables 4.6 and 4.8, respectively), similar results were obtained: over 98% in purity for hIgG for the two different solvents, with overall yields of about 60%. For hIgA and hIgM elution,
a hIgA-enriched fraction containing on average 40.8% hIgA and 58.4% hIgG, as well as a hIgM-enriched fraction containing average 46.6% hIgM, 27.6% hIgA and 25.8% hIgG were achieved. Caprylic acid pretreatment as well as the combination of caprylic acid and PEG pretreatment can achieve highly purified hIgG (over 95% purity), with overall yields comparable to reported industrial methods for manufacturing of IgG from human plasma [25,44]. Although the overall process yield was generally low for hIgA and hIgM, enriched fractions containing those antibodies can be achieved as by-products of IgG purification from Cohn fraction II/III.

Pretreatment using PEG precipitation showed the highest total protein and antibody recoveries among the tested pretreatments as shown in Figure 4.4, when tested against the Cohn fraction II/III. Better results were achieved for purified human IgG, IgA and IgM in terms of purity and overall recovery when using PBS as solvent (Table 4.9) for the Cohn fraction, compared with water (Table 4.8), except for the overall recovery of purified hIgG from Cohn fraction. The reason for this exception is that after the PEG pretreatment, the Cohn fraction in water was buffered by acetate buffer, which has a higher PEGmidpt value (the weight percentage of PEG in solution required to decrease the protein concentration by 50%) than phosphate buffer in PBS [45]. As a result, more bound hIgG would be eluted by the elution buffer due to higher solubility at the same level of PEG under the two different buffer conditions. The buffer effect became seems to be less effective for hIgA and hIgM elution because, as previously mentioned, the yield of an eluted antibody seems to be dependent not only on the elution buffer but also on the previous buffer used. The resulting purities and yields for hIgA and hIgM have comparable values to those from Cohn fraction without
pretreatment, with the PEG pretreatment achieving better results for IgG content in the hIgA-
hIgM-enriched fractions (Tables 4.7 and 4.9) when water was used as diluents.

Human IgG had the lowest observed recovery after the SAS pretreatment step, resulting in the lowest overall yields with 45.6% with water (Table 4.7) and 48.6% in PBS. Although the hIgA and hIgM concentrations were relatively high after the pretreatment, the resulting overall yields (~23.6%) for both hIgA and hIgM were not very high. This might have been caused by ammonium sulfate residue left in the Cohn fractions, suggesting that SAS would not be a good pretreatment method for hIgG, hIgA and hIgM purification from Cohn fraction II/III.

4.4. Conclusions

This study has demonstrated the effects of elution additives on the recovery of human IgG, IgA and IgM with small peptide ligand HWRGWV. Unlike salting-out salts, salting-in salts MgCl₂ and CaCl₂ can be employed in the elution buffer to facilitate the elution of human IgA and IgM at low peptide density because of favorable interactions between the divalent ions and bound antibodies. At high peptide density, none of these salts facilitate elution as a result of increased non-specific hydrophobic interactions. Among the three non-electrolyte elution additives examined, arginine showed the best results for improving recovery of human IgA and IgM at low peptide density, resulting from its ability to suppress hydrophobic, H-bonding and electrostatic interactions. Still, at high peptide density, the recovery of human IgA and IgM did not improve or only improved to a small extent because of the increased non-specific hydrophobic interactions. Based on these results, we can
conclude that at low peptide density, the binding of human IgA and IgM with HWRGWV involves comparable electrostatic and specific hydrophobic interactions together with possible H-bonding. At high peptide density, the dominant binding force is non-specific hydrophobic interactions.

Based on the preliminary elution condition study, 0.2 M acetate buffer at pH 5.0 for hIgG, 0.2 M acetate buffer at pH 5.0 with 0.5 M arginine for hIgA and 0.2 M acetate buffer at pH 5.0 with 2 M MgCl$_2$ for hIgM are the optimum elution buffers for purification of human IgG, IgA and IgM from their mixture. The yield results for hIgA and IgM also suggested that the yield of eluted antibody from HWRGWV peptide resin is affected not only by the elution buffer but by also the previous elution condition.

The applicability of small peptide ligand HWRGWV to achieve purification of human IgG, IgA and IgM from Cohn fraction II/III using one column was demonstrated. Without pretreatment, better results were achieved for Cohn fraction using PBS instead of water as solvent because of the decreased non-specific hydrophobic interactions in the presence of salts. With the similar purities as 90% for hIgG, 44% enriched hIgA with 35% hIgG and 36% enriched hIgM with 21% hIgA and 4% hIgM, higher yields (10% or more) were obtained for all three purified antibodies from Cohn fraction in PBS. After the caprylic acid pretreatment or the combination of caprylic acid and PEG pretreatment, highly purified hIgG (over 95% purity) was achieved with about 60% in yield for both solvents, which is comparable to industrial chromatographic purification of IgG isolated from plasma fractions. A hIgA-enriched fraction with average about 42% hIgA and 56% hIgG as well as a hIgM enriched fraction with average about 46% hIgM, 28% hIgA and 24% hIgG were obtained as
the by-products of the purification of hIgG from Cohn fraction II/III. To our knowledge, it is the first time that an affinity chromatography column was used for the simultaneous capture and purification of human IgG, IgA and IgM from Cohn fraction II/III.

4.5. References


Table 4.1. Elution conditions for the isolation of human IgG, IgA and IgM (10:2:1) from their mixture with HWRGWV at peptide density of 0.23 mequiv./g.

<table>
<thead>
<tr>
<th>Run</th>
<th>hIgG elution</th>
<th>hIgA elution</th>
<th>hIgM elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂ (g*)</td>
</tr>
<tr>
<td>2</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>3</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 0.5 M MgCl₂</td>
</tr>
<tr>
<td>4</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>5</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>6</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 40% EG</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>7</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 2 M urea</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>8</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 0.5 M Arginine</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>9</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 1 M Arginine</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
</tbody>
</table>

g* : gradient elution
Table 4.2. Elution conditions for the isolation of human IgG, IgA and IgM (10:2:1) from their mixture with HWRGWV at peptide density of 0.11 mequiv./g.

<table>
<thead>
<tr>
<th>Run</th>
<th>hIgG elution</th>
<th>hIgA elution</th>
<th>hIgM elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 0.5 M MgCl₂</td>
</tr>
<tr>
<td>2</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>3</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>4</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>5</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>6</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 40% EG</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>7</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 5.0 + 2 M urea</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
<tr>
<td>8</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.5 M Arginine</td>
<td>0.2 M AB pH 5.0 + 1 M MgCl₂</td>
</tr>
<tr>
<td>9</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 0.5 M Arginine</td>
<td>0.2 M AB pH 5.0 + 1 M Arginine</td>
</tr>
<tr>
<td>10</td>
<td>0.2 M AB pH 5.2</td>
<td>0.2 M AB pH 5.0 + 1 M Arginine</td>
<td>0.2 M AB pH 5.0 + 2 M MgCl₂</td>
</tr>
</tbody>
</table>
Table 4.3. Yield and purity for isolated human IgG, IgA and IgM from their mixture with HWRGWV at peptide density of 0.23 mequiv./g.

<table>
<thead>
<tr>
<th>Run</th>
<th>hIgG</th>
<th>hIgA</th>
<th>hIgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield</td>
<td>Purity</td>
<td>Yield</td>
</tr>
<tr>
<td>1</td>
<td>95.3 ± 7.4</td>
<td>97.4 ± 1.8</td>
<td>16.9 ± 8.2</td>
</tr>
<tr>
<td>2</td>
<td>54.9 ± 5.3</td>
<td>98.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>91.5 ± 5.6</td>
<td>98.3 ± 1.6</td>
<td>16.4 ± 3.2</td>
</tr>
<tr>
<td>4</td>
<td>3.1 ± 0.5</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>94.5 ± 3.9</td>
<td>92.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>95.4 ± 3.3</td>
<td>98.8 ± 0.9</td>
<td>8.0 ± 3.0</td>
</tr>
<tr>
<td>7</td>
<td>2.3 ± 1.3</td>
<td>n/a</td>
<td>101.1 ± 10.1</td>
</tr>
<tr>
<td>8</td>
<td>28.3 ± 10.0</td>
<td>90.5</td>
<td>81.3 ± 10.3</td>
</tr>
<tr>
<td>9</td>
<td>75.8 ± 13.0</td>
<td>63.3</td>
<td>14.2 ± 2.6</td>
</tr>
</tbody>
</table>
Table 4.4. Yield and purity for isolated human IgG, IgA and IgM from their mixture with HWRGWV at peptide density of 0.11 mequiv./g

<table>
<thead>
<tr>
<th>Run</th>
<th>hIgG</th>
<th></th>
<th>hIgA</th>
<th></th>
<th>hIgM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield</td>
<td>Purity</td>
<td>Yield</td>
<td>Purity</td>
<td>Yield</td>
<td>Purity</td>
</tr>
<tr>
<td>1</td>
<td>93.9 ± 8.0</td>
<td>99.1 ± 0.4</td>
<td>12.0 ± 0.8</td>
<td>98.9 ± 0.4</td>
<td>17.6 ± 0.1</td>
<td>99.4</td>
</tr>
<tr>
<td>2</td>
<td>57.8 ± 3.0</td>
<td>99.6 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>87.6 ± 10.3</td>
<td>99.3 ± 0.6</td>
<td>29.5 ± 6.0</td>
<td>98.5 ± 0.1</td>
<td>80.3 ± 1.4</td>
<td>94.9</td>
</tr>
<tr>
<td>4</td>
<td>93.5 ± 3.0</td>
<td>99.3 ± 0.1</td>
<td>13.5 ± 1.0</td>
<td>60.3</td>
<td>85.4 ± 13.2</td>
<td>59.2</td>
</tr>
<tr>
<td>5</td>
<td>24.4 ± 1.7</td>
<td>59.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3.1 ± 0.4</td>
<td>62.6</td>
<td>86.3 ± 7.6</td>
<td>49.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>88.9 ± 3.8</td>
<td>99.4 ± 0.1</td>
<td>60.4 ± 4.0</td>
<td>79.4 ± 0.6</td>
<td>57.5 ± 1.0</td>
<td>98.1</td>
</tr>
<tr>
<td>8</td>
<td>52.9 ± 1.2</td>
<td>92.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>95.3 ± 2.8</td>
<td>48.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1.2 ± 1.7</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III prepared in H$_2$O and PBS w/o pretreatment.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>H$_2$O</th>
<th></th>
<th>PBS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purity (%)</td>
<td>Yield (%)</td>
<td>Purity (%)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>hIgG</td>
<td>90.5</td>
<td>60.5 ± 4.1</td>
<td>89.8</td>
<td>76.3 ± 3.3</td>
</tr>
<tr>
<td>hIgA / hIgG</td>
<td>43.9/35.1</td>
<td>28.1 ± 0.3</td>
<td>43.6/35.6</td>
<td>39.2 ± 3.8</td>
</tr>
<tr>
<td>hIgM / hIgA / hIgG</td>
<td>37.3/20.0/5.1</td>
<td>27.8 ± 0.5</td>
<td>35.2/22.6/3.6</td>
<td>36.4 ± 3.4</td>
</tr>
</tbody>
</table>
Table 4.6. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (H₂O) with pretreatment.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Antibody</th>
<th>Caprylic acid</th>
<th>Caprylic acid + PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Purity (%)</td>
<td>Yield (%)</td>
<td>Overall Yield (%)</td>
<td>Purity (%)</td>
</tr>
<tr>
<td>hIgG</td>
<td>98.5</td>
<td>67.4 ± 6.2</td>
<td>59.4 ± 5.5</td>
</tr>
<tr>
<td>hIgA / hIgG</td>
<td>41.9/57.9</td>
<td>26.0 ± 2.2</td>
<td>16.7 ± 1.4</td>
</tr>
<tr>
<td>hIgM / hIgA / hIgG</td>
<td>47.1/28.1/20.0</td>
<td>33.1 ± 1.4</td>
<td>16.3 ± 0.7</td>
</tr>
</tbody>
</table>

Table 4.7. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (H₂O) with pretreatment (continued).

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Antibody</th>
<th>PEG</th>
<th>SAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Purity (%)</td>
<td>Yield (%)</td>
<td>Overall Yield (%)</td>
<td>Purity (%)</td>
</tr>
<tr>
<td>hIgG</td>
<td>93.9</td>
<td>77.1 ± 1.2</td>
<td>69.5 ± 1.1</td>
</tr>
<tr>
<td>hIgA / hIgG</td>
<td>43.0/37.9</td>
<td>35.3 ± 4.6</td>
<td>31.5 ± 4.1</td>
</tr>
<tr>
<td>hIgM / hIgA / hIgG</td>
<td>37.8/17.5/5.6</td>
<td>38.4 ± 3.2</td>
<td>23.3 ± 1.9</td>
</tr>
</tbody>
</table>
Table 4.8. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (PBS) with pretreatment.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Caprylic acid</th>
<th></th>
<th>Caprylic acid + PEG</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody</td>
<td>Purity (%)</td>
<td>Yield (%)</td>
<td>Overall Yield (%)</td>
</tr>
<tr>
<td>hIgG</td>
<td>98.4</td>
<td>72.2 ± 3.5</td>
<td>60.6 ± 2.9</td>
<td>98.2</td>
</tr>
<tr>
<td>hlIgA / hlIgG</td>
<td>44.9/50.7</td>
<td>29.2 ± 7.8</td>
<td>23.1 ± 6.2</td>
<td>42.1/56.3</td>
</tr>
<tr>
<td>hIgM / hlIgA / hlIgG</td>
<td>42.5/28.9/23.1</td>
<td>42.2 ± 3.1</td>
<td>18.2 ± 1.3</td>
<td>50.6/27.2/22.2</td>
</tr>
</tbody>
</table>

Table 4.9. Purity and yield for purified human IgG, IgA and IgM from Cohn fraction II/III (PBS) with pretreatment (continued).

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>PEG</th>
<th></th>
<th>SAS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody</td>
<td>Purity (%)</td>
<td>Yield (%)</td>
<td>Overall Yield (%)</td>
</tr>
<tr>
<td>hIgG</td>
<td>94.5</td>
<td>66.6 ± 1.8</td>
<td>60.7 ± 1.6</td>
<td>94.6</td>
</tr>
<tr>
<td>hlIgA / hlIgG</td>
<td>41.7/40.3</td>
<td>38.7 ± 5.6</td>
<td>35.2 ± 5.1</td>
<td>39.0/44.3</td>
</tr>
<tr>
<td>hIgM / hlIgA / hlIgG</td>
<td>31.6/46.7/0</td>
<td>34.4 ± 1.8</td>
<td>31.3 ± 1.6</td>
<td>44.7/39.6/8.1</td>
</tr>
</tbody>
</table>
Figure 4.1. NaCl, MgCl\(_2\) and CaCl\(_2\) elution effects on human immunoglobulins A(●), G(■) and M(△) with peptide ligand HWRGWV at peptide densities (A) 0.04 mequiv./g and (B) 0.55 mequiv./g.

Figure 4.2. Ethylene glycol, urea and arginine elution effects on human immunoglobulins A(●), G(■) and M(△) with peptide ligand HWRGWV at peptide densities (A) 0.04 mequiv./g and (B) 0.55 mequiv./g.
Figure 4.3. Separation scheme for purification of hIgG, hIgA and hIgM
Figure 4.4. Total protein and antibody concentrations in Cohn II/III dissolved in water (A) and PBS (B) after each preparation or pretreatment step. The concentration standard deviations are shown by error bar.
Figure 4.5. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H₂O) without pretreatment using HWGWW resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.6. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H_2O) with caprylic acid precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.7. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H₂O) with caprylic acid and polyethylene glycol precipitations using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.8. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H₂O) with polyethylene glycol precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.9. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (H_2O) with saturated ammonium sulfate precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g.

A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.10. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) without pretreatment using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.11. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with caprylic acid precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2'), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.12. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with caprylic acid and polyethylene glycol precipitations using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2’), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.13. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with polyethylene glycol precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2’), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.14. (A) Chromatograms and (B) corresponding SDS-PAGE of one column purification of human IgG, IgA and IgM from Cohn fraction II/III (PBS) with saturated ammonium sulfate precipitation using HWGWV resins at a peptide density of 0.11 mequiv./g. A: The detected peaks were flow through (FT), hIgG elution peak (P1), hIgA elution peaks (P2 and P2’), hIgM elution peak (P3) and wash peak (wash). B: SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), standard hIgG (IgG), hIgA (IgA), hIgM (IgM) and loading material (L).
Figure 4.15. Chromatograms of purification of human IgG, IgA and IgM from Cohn II/III in (A) HPLC grade water and (B) PBS with or w/o pretreatment using HWGWV resins at a peptide density of 0.11 mequiv./g. Columns were loaded in PBS + 1M NaCl at pH 7.4, and sequentially passed through 20 min each of 0.2 M acetate buffer pH 5.0 for hIgG elution, 0.2 M acetate buffer pH 5.0 with 0.5 M arginine for hIgA elution, 0.2 M acetate buffer pH 5.0 with 2 M MgCl₂ for hIgM elution and 6 M guanidine HCl for washing and regeneration. The flow rate was 0.05 mL/min for the first 10 min and was increased to 0.3 mL/min for the reminder of the time. Injection volume was 50 μL.
Figure 4.16. SDS-PAGE of the flow through (FT) purification of human IgG, IgA and IgM from Cohn II/III in HPLC grade water or PBS with or w/o pretreatment: lane 1, 6: Cohn II/III w/o pretreatment, lane 2, 7: after caprylic acid pretreatment, lane 3, 8: after caprylic acid and PEG pretreatment, lane 4, 9: after PEG pretreatment, lane 5, 10: after SAS pretreatment.
Chapter 5. Affinity Chromatographic Purification of Human Immunoglobulin A from Chinese Hamster Ovary Cell Culture Supernatant

Zhuo Liu, Patrick V. Gurgel, Ruben G. Carbonell

A version of this chapter has been accepted by

Biotechnology Progress
Abstract

Hexamer peptide ligand HWRGWV, initially screened from a solid phase combinatorial peptide library for immunoglobulin G (IgG) purification, is shown to also have potential for immunoglobulin A (IgA) purification. The determined dissociation constants for hIgA on HWRGWV resins at three different peptide densities from 0.11 to 0.55 mequiv./g fall in the range of $10^{-6}$ to $10^{-7}$ M, which are somewhat lower than those for hIgG. Although relatively low dynamic binding capacity (DBC) in the range of 9.2 to 16.8 mg IgA/mL resin at linear flow rates from 173 to 35 cm/h were obtained for IgA compared to IgG, the DBC value of HWRGWV for IgA is much greater than current commercially available affinity ligands. Although relatively lower binding affinity to secretory IgA compared to monomeric IgA was observed, the peptide ligand resins exhibit great potential for large-scale purification of both human IgA and secretory IgA. Recoveries of 96.0% and 94.3%, and purities of 90.3% and 91.7% were achieved for human IgA and secretory IgA purification respectively from spiked Chinese hamster ovary cell culture supernatants without an extra afterwash step. Over 95% in purities were achieved for IgA and secretory IgA with an extra afterwash step; however, the recoveries would decrease at least 15% and 40% for IgA and secretory IgA, respectively.

Keywords: affinity chromatography; hexamer peptide; CHO; human immunoglobulin; secretory IgA.
5.1. Introduction

Although most antibody therapeutics on the market or in clinical trials are immunoglobulin-G-based drugs, increasing attention is being given to immunoglobulins A (IgA) as biotherapeutics for the treatment of infectious and malignant diseases, mucosal vaccines and cancer therapies.\(^1-^4\) Secretory IgA, the dimeric form of IgA, is regarded as the first line of immune defense for human mucosal surfaces for its ability to fight challenging pathogens such as bacteria, viruses, fungi and parasites.\(^5,^6\) Serum IgA (monomeric) can provide a second line of defense in mucosal immunity by eliminating pathogens that have entered through the mucosal surfaces.\(^7,^8\) As a therapeutic agent for cancer therapy, IgA has several advantages over IgG. Polymorphonuclear neutrophils (PMNs), the most abundant effector cell population in humans for antibody-dependent cellular cytotoxicity (ADCC) against tumor cells, are more effectively recruited by human IgA than by IgG antibodies.\(^9-^11\) Compared to IgG, IgA antibodies have the ability to form natural dimers with improved signaling capacity on tumor cells, and to undergo active transport into mucosal secretions with the potential for improved targeting of certain carcinomas from the luminal surface.\(^12,^13\)

IgA is the most abundant immunoglobulin in secretions, and the second most abundant immunoglobulin in plasma. In humans, two different IgA subclasses have been identified, IgA1 and IgA2, differentiated by the presence of a proline-rich 13-amino-acid sequence in the hinge region on IgA1.\(^14\) Both subclasses are heavily glycosylated with a number of \(N\)-linked carbohydrates, consisting of 6-7% of the molecular mass in IgA1 and 8-10% in IgA2. The longer hinge region of IgA1 also bears three to six short \(O\)-linked carbohydrates.\(^4\) Human serum IgA is predominantly monomeric with an IgA1 to IgA2 ratio
of 9 to 1. A small portion of serum IgA is in dimeric form, linked by disulphide bonds and an additional cysteine-rich polypeptide joining (J) chain (16 kDa). Polymeric secretory IgA (SIgA), existing in mucosal secretions, comprises not only IgA and J chain, but also a 50-90 kDa heavily glycosylated protein called secretory component (SC). SC forms a complex with the IgA during the secretion process. It stabilizes the structure of SIgA and increases its resistance to proteolysis.15

Unlike the large scale purification of IgG with extensively studied affinity ligands such as protein A from Staphyococcus aureus, protein G from group C and G Streptococci or protein A/G (a recombinant fusion protein combining IgG binding domains of both protein A and G), no particular affinity purification method for IgA has achieved broad acceptance. The classical chromatographic methods involves time-consuming and laborious steps such as ammonium sulphate precipitation,16 ion exchange chromatography,17 18 hydrophobic interaction chromatography19 and size exclusion chromatography.18,19

Jacalin, a major lectin protein isolated from jackfruit seeds,20 demonstrates specific binding to IgA and can be used for the affinity purification of IgA from colostrums or serum.21,22 However, it only binds to IgA1 through the D-galactose moiety but not to IgA2.23 Therefore, galactose is required for IgA elution from the affinity columns, which is costly and impractical for large-scale purification of IgA. In addition, careful control is required to prevent ligand leakage from the chromatographic resins since jacalin is a biologically active lectin, a potent T cell mitogen and a strong B cell polyclonal activator.24 Protein L, a cell wall molecule of some strains of the anaerobic bacteria species Peptostreptococcus magnus, demonstrates binding affinity for IgA as well as IgG and IgM. However, it only binds to
variable domains of antibodies with specific κ-light chain families. As members of the M protein family, cell surface proteins such as protein Sir22 and protein Arp4, isolated from Streptococcus pyogenes, exhibit binding to both IgA1 and IgA2 and recognize the CH₂-CH₃ domain on the IgA Fc fragment. However, no purification applications using this protein-based affinity ligand for IgA were found. Immobilized streptococcal IgA-binding peptide (Sap), a 50-residue synthetic peptide derived from protein Sir22, was used for single-step purification of secretory IgA from human saliva with 45% recovery, and highly purified serum IgA was achieved with over 99% recovery after passage through a Sap column. This synthetic peptide for affinity purification of human IgA purification is commercialized for research purposes only with the name Peptide M from InvivoGen (San Diego, CA, USA). Artificial protein A (ApA) as well as artificial protein L (PpL), developed by Lowe and collaborators initially targeting the Fc or Fab domains of IgG, also demonstrated the ability to bind IgA. However, no further characterization and application was found for IgA capture and purification, potentially due to a low binding capacity. Fassina et al. developed a tetrameric tripeptide, denoted PAM (protein A mimetic, TG19318), which was successfully applied for the purification of IgG, IgA, IgM, IgE and IgY. Affinity purification of IgAs using this ligand was achieved from cell culture supernatants as well as IgG-deprived murine serum with recoveries up to 80%. The column binding capacity for IgA was close to 7 mg of IgA/mL of support.

Hexamer peptide ligand HWRGWV, previously identified in our group through a three-step screening process of a synthetic solid phase combinatorial peptide library, demonstrated the ability to bind the Fc domain of immunoglobulin G, allowing its use for
affinity chromatographic purification of antibodies. The selectivity of the ligand to the Fc portion of IgG is comparable to that of protein A and the ligand exhibited the ability to bind all human IgG subclass as well as IgGs from different species.\textsuperscript{33, 34} After direct synthesis onto Toyopearl AF Amino 650M resin, HWRGWV was able to purify monoclonal and polyclonal IgGs from different sources.\textsuperscript{35, 36} as well as human IgA and IgM.\textsuperscript{37-39} A study on the binding site of the ligand on the protein suggested that the peptide ligand binds to the Fc portion of IgA\textsuperscript{37} and previous experiments showed that the peptide density of HWRGWV had a significant influence on the recovery and purity of isolated IgA. The improved recoveries at high peptide density, due to increased binding affinity, were compensated by a decrease in purity. The optimum peptide density for IgA purification is 0.11 mequiv./g in terms of recovery and purity. As a result, 83.8\% recovery and 91.6\% purity were achieved for IgA purification from complete minimum essential medium (cMEM) containing 10\% fetal calf serum and 5\% tryptose phosphate broth.\textsuperscript{37}

The present work extends the characterization of HWRGWV for IgA purification. Dissociation constants and maximum binding capacities for IgA at three different peptide densities were measured by adsorption isotherm. The dynamic binding capacities of the ligand for IgA at the optimum peptide density with different flow rates were determined. Chinese hamster ovary (CHO) cell culture supernatants with or without spiked IgA and SIgA were used as starting materials for affinity chromatographic purification of human IgA and SIgA. In order to achieve high recovery and purity, different running conditions were employed.
5.2. Materials and Methods

5.2.1. Materials

The peptide HWRGWV was synthesized directly on Toyopearl AF-Amino-650M (mean particle diameter of 65 μm) (Tosoh BioScience, Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International, Inc (Louisville, KY, USA). The HWRGWV resins were synthesized at three different peptide densities of 0.11, 0.22 and 0.55 mequiv./g. Phosphate-buffered saline (PBS) pH 7.4 (containing 10 mM phosphate, 138 mM sodium chloride, and 2.7 mM potassium chloride), urea, and arginine were obtained from Sigma (St. Louis, MO, USA). Sodium chloride, sodium acetate, glacial acetic acid, guanidine hydrochloride, tris hydrochloride, ethylene glycol and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). NuPAGE Novex 4-12% Bis-Tris gels, NuPAGE reducing agent, NuPAGE MOPS SDS running buffer, SeeBlue Plus2 pre-stained molecular weight marker and SimplyBlue Safestain were all from Invitrogen (Carlsbad, CA, USA). Purified human IgA and SIgA were purchased from Fitzgerald (Concord, MA, USA). Human IgA enzyme-linked immunosorbent assay (ELISA) kits were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Microcon Ultracel YM-3 centrifugal filters (regenerated cellulose, 3 kDa MWCO), Durapore 0.45 μm centrifugal filters, ultrafiltration membranes (regenerated cellulose, 100 kDa MWCO) and Millex-GV 0.22 μm Syringe Driven Filter Unit (low protein binding Durapore PVDF membrane) were purchased from Millipore (Billerica, MA, USA). Bicinchoninic acid (BCA) protein assay was from Thermo Scientific (Rockford, IL USA). Microbore columns (30 mm × 2.1 mm) for all chromatographic experiments were from Alltech (Deerfield, IL, USA).
5.2.2. Equipment

All chromatographic experiments were carried out on a Waters 626LC system. The UV absorbance of protein samples was measured at a wavelength of 280 nm using a built-in 2487 dual-wave-length UV detector from Waters (Milford, MA, USA). Centrifugal concentration of samples was performed using a Centrifuge 5417R from Eppendorf (Hauppauge, NY, USA). ELISA wash steps were performed on an ELx50 Microplate Strip Washer from Bio-Tek Instruments Inc. (Winooski, VT, USA). BCA and ELISA protein concentrations were determined using a μQuant Spectrophotometer from Bio-Tek Instruments Inc.

5.2.3. Adsorption isotherm measurements

Adsorption isotherms of resins with HWRGWV peptide ligand at three different peptide densities were measured in a set of batch experiments at room temperature. Resins were weighed as a dry powder, swollen in 20% methanol for 2 h, washed thoroughly using high performance liquid chromatography (HPLC) water and equilibrated with PBS at pH 7.4. Centrifugal filters (0.5 mL) with 0.45 μm Durapore membranes were used as adsorption vessels. Five hundred microliters of hIgA solutions with concentrations ranging from 0.04 to 4 mg/mL in PBS were added separately to the adsorption vessels containing 5 mg of resin and incubated on an orbital shaker for 4 h. The unbound hIgA fractions were collected by centrifugation and the protein concentrations were determined by UV-absorbance at 280 nm. The amount of bound hIgA was calculated by mass balance and the data were fit to a Langmuir isotherm model.
\[ q = q_m \frac{C}{(K_d + C)} \]  

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

5.2.4. Generation of breakthrough curves

For all frontal analysis experiments, peptide resins with a peptide density of 0.11 mequiv./g were dry packed into a 30 mm × 2.1 mm I.D. microbore column (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 h followed by wash with HPLC grade water and equilibration with PBS. Purified human IgA in the equilibration buffer of PBS pH 7.4 (containing 10 mM phosphate, 138 mM sodium chloride, and 2.7 mM potassium chloride) at a concentration of 2 mg/mL was used as a feed material, and a 1 mL sample loop was used to ensure saturation of the column. Column effluent fractions were collected during the loading step and the protein concentration in each fraction was determined by BCA protein assay using IgA standards to generate a calibration curve. Protein concentration at the column outlet as a fraction of the inlet feed concentration was plotted against column loading to obtain the breakthrough curve. The breakthrough volume at 10% of the feed concentration was multiplied by the inlet feed concentration and normalized with respect to the packed bed volume or packed resin weight to express the x-axis of the breakthrough curves in the same units as the dynamic binding capacity. For all these chromatographic experiments, the column was equilibrated and loaded with PBS pH 7.4, and the bound protein was eluted by 6 M guanidine HCl, that also served as an agent for washing and regeneration. The flow rates
used were 0.02, 0.05 and 0.1 mL/min, which corresponded to linear flow rates of 35, 87 and 173 cm/h with residence times of 5, 2 and 1 min, respectively.

5.2.5. Cell culture

A Chinese hamster ovary (CHO) cell line (TECA 2.2.2.3) for human monoclonal IgA (IgA2m(2)) production was a gift from Professor Sherie L. Morrison's group at UCLA. The cells were grown at 37°C under 5% CO₂ in Iscove's modified Dulbecco's medium (IMDM) from Irvine Scientific (Irvine, CA, USA) supplemented with 5% fetal calf serum from Hyclone Laboratories (Logan, UT, USA). The cells were maintained in 75 cm² cell culture flasks from Corning Incorporated (Corning, NY, USA).

5.2.6. Sample preparation

Collected cell culture was clarified by centrifugation using an IEC Centra CL2 Centrifuge at 2500 × g for 10 min followed by filtration using a 0.22 μm Millex-GV syringe filter in order to remove any cells or cell debris. After filtration, the filtrate was concentrated about 100-fold by using a membrane filter holder with 47 mm diameter fritted glass support from Whatman Inc. (Piscataway, NJ, USA) with a 100 kDa molecular weight cutoff regenerated cellulose membrane. Total protein as well as antibody concentrations in the original and concentrated cell culture supernatant were determined by BCA protein assay and ELISA, respectively, based on suppliers' instructions. The 1.5 mg/mL human IgA and SIgA spiked CHO cell culture supernatants were achieved by adding purified human IgA and SIgA at a stock concentration of 5.4 mg/mL into the original CHO cell culture supernatant, which had a total protein concentration (before spiking) of 2.66 mg/mL.
5.2.7. **Purification of human IgA from different feedstocks**

Peptide resins were dry packed in 30 mm × 2.1 mm I.D. microbore columns (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 h followed by HPLC grade water and PBS washes. Before sample loading, columns were equilibrated with the loading buffer based on running conditions. Samples were manually injected at room temperature using a 100 μL sample loop (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 minutes, and then the flow rate was increased to 0.3 mL/min for the remainder of the run. The column was washed sequentially with 3 mL loading buffer, 6mL afterwash if necessary, 6 mL elution buffer, and 6 mL 6 M guanidine-HCl to clean and regenerate the column, followed by loading buffer for equilibration until baseline was reached. Afterwash step was used in order to elute weakly bound contaminant protein resulting from non-specific interactions. Acetate buffer (AB) pH 5.2 with different elution additives such as ethylene glycol, urea and arginine were used as afterwash step before IgA elution step. The predetermined running conditions for chromatographic purification of IgA are listed in Table 5.1. All elution fractions were collected and the pH was immediately neutralized by 1 M Tris buffer pH 8.0 with predetermined volume.

5.2.8. **Sample analysis for recoveries and purities**

The recoveries of purified human IgA and SIgA from different feedstocks were calculated according to the hIgA and hSIgA concentrations determined by ELISA using a human IgA ELISA kit according to the supplier's instructions. The protein concentration of HIgA flow-through and elution fractions from different feedstocks were adjusted by
concentration using Microcon Ultracel YM-3 centrifugal filters (regenerated cellulose, 3 kDa MWCO) in a 5417R centrifuge at 14,000 × g, 20°C. The treated samples were mixed with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent according to the supplier’s instructions before being incubated at 70 °C for 10 min and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE Novex 4-12% Bis-Tris gels with MOPS running buffer on an X-cell SuperLock™ Mini-Cell system from Invitrogen at 200 V for 50 min. The gels were stained using SimpleBlue SafeStain. The purities of human IgA and SIgA elution peaks were determined by densitometric measurement using software ImageJ 1.32j (National Institute of Health, Bethesda, MD, USA).

5.3. Results and discussion

5.3.1. Adsorption isotherms

Adsorption isotherms of monomeric hIgA in PBS using resins with peptide ligand HWRGWV at different peptide densities of 0.11, 0.22 and 0.55 mequiv./g were determined (Figure 5.1) with hIgA concentrations in the range of 0.04 to 4 mg/mL in PBS at room temperature. The isotherm adsorption data were directly fitted to a Langmuir model and the obtained dissociation constant (\(K_d\)) and maximum binding capacity (\(q_m\)) for each peptide density are listed in Table 5.2. The dissociation constants for resin with peptide densities of 0.11, 0.22 and 0.55 mequiv./g are \(1.05 \times 10^{-6}\), \(0.646 \times 10^{-6}\) and \(0.509 \times 10^{-6}\) M, respectively, while the maximum binding capacities are 66.8, 86.4 and 93.8 mg/g for peptide densities from 0.11 to 0.55 mequiv./g. The dissociation constants of hIgA adsorption decreased with
increasing HWRGWV peptide density, suggesting a more intense and stronger contact between the protein and the sorbent at higher ligand densities. As a result, the maximum binding capacity of the resins increased at higher peptide densities because of the stronger binding affinity. These results are in good agreement with a previous study showing that at increased peptide densities, lower elution pH were required to elute bound hIgA from the resins. The dissociation constants and maximum binding capacities of hIgA adsorption are compared to those obtained for hIgG adsorption in Table 5.2. The dissociation constants and maximum binding capacities of the peptide resins for hIgA are shown to be lower than those for hIgG at all three different peptide densities. These data further confirm the conclusion from earlier work that the peptide ligand HWRGWV demonstrates higher binding affinity to hIgA than hIgG.

The observed dissociation constants for hIgA on HWRGWV resin can be compared to those with other IgA binding ligands such as protein Sir22 and the 50-residue synthetic peptide, streptococcal IgA-binding peptide (Sap), derived from protein Sir22. Much stronger binding affinity with dissociation constants in the range of $10^{-8}$ to $10^{-9}$ M was achieved, $1.4 \times 10^{-9}$ M for Sir22 and $2 \times 10^{-8}$ M for Sap, which are smaller than those for most affinity ligands used in affinity chromatography with dissociation constant within the normal range of $10^{-4}$ to $10^{-8}$ M. This might be the reason that these ligands are not suitable for large scale IgA purification since low elution pH is required resulting in possible protein denaturation. Falling in the range of $10^{-5}$ to $10^{-7}$ M usually found with short peptides as affinity ligand for proteins, the dissociation constants for hIgA on HWRGWV are comparable to the hIgA-specific ligands (affibodies) from combinatorial engineering of
protein A with $K_d$ in the range of 0.5 to 3 $\mu$M.\textsuperscript{41} However, lower purification and production cost is the advantage for HWRGWV with only 6 amino-acid residues compared to the affibodies with 58 amino-acids.

### 5.3.2. Dynamic binding capacity

As mentioned in the introduction, the binding capacity of IgA affinity ligand directly determines its potential application for large-scale purification of the protein. To further characterize the peptide ligand HWRGWV for IgA purification, breakthrough curves for human IgA in PBS to the HWRGWV resin at peptide density of 0.11 mequiv./g were generated at different flow rates as shown in Figure 5.2. Dynamic binding capacity (DBC) values in mg/mL resin as well as mg/g resin at the 10% breakthrough point shown in Figure 5.1 were compared. At the flow rates of 0.02, 0.05 and 0.1 mL/min corresponding to linear flow rates of 35, 87 and 173 cm/h, the DBC values are 16.8, 13.9 and 9.2 mg IgA/mL resin, corresponding to 46.7, 38.7 and 25.6 mg IgA/g resin, respectively. The DBC values for IgA are dependent on the flow rate as a result of diffusional as well as binding kinetic limitations on these affinity chromatography resins.

The resulting dynamic binding capacities of IgA (9.2-16.8 mg IgA/mL resin) were lower than those obtained for hIgG (20 mg IgG/mL resin at linear flow rate of 87 cm/hr),\textsuperscript{36} but they are much greater than the binding capacities of affinity ligands for IgA found in the literature. For example, artificial protein L (PpL)\textsuperscript{30} has a reported DBC of 0.4 mg of IgA/g of support and PAM (protein A mimetic, TG19318) has a DBC of approximately 7 mg of IgA/mL of support.\textsuperscript{32} The measured DBC value for the HWRGWV resin is larger than that of
commercially available affinity ligands such as Peptide M from InvivoGen (San Diego, CA, USA) with a binding capacity of 4-6 mg IgA/mL, CaptureSelect IgA from BAC (Naarden, Netherlands) with a DBC larger than 8 mg IgA/mL support and Immobilized Jacalin from Thermo Scientific (Rockford, IL USA) with binding capacity about 1-3 mg hIgA1/mL resin.

5.3.3. Affinity chromatography purification of human IgA and SIgA from spiked CHO cell culture supernatant

From previous studies on the purification of IgA from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth, a recovery of 83.8% and 91.6% purity were obtained with hexamer peptide ligand at the optimum peptide density of 0.11 mequiv./g. To date, murine myeloma and CHO cell lines have been the expression systems of choice for the production of IgA and SIgA. In order to test the ability of the peptide ligand for IgA purification from mammalian expression systems such as murine myeloma and CHO cell lines, and further improve the purity; different running conditions were used for hIgA purification from IgA spiked CHO cell culture supernatant as shown in Table 5.1. As the major contaminant, bovine serum albumin (BSA) has the isoelectric points in the range between 4.5 and 5.5 compared the isoelectric point of IgA in the range between 4 and 7.1, with a pI peak between pH 4.7 and 5.9. Based on previous studies, bound IgA could only be eluted from the column by solutions with a pH lower than 5.0 and over 90% recovery was achieved by elution at pH 3.0 or 3.5. In addition, the effects of elution additives provided the opportunity of decreasing non-specific interactions between the peptide ligand and contaminant proteins without disturbing the IgA binding. PBS pH 7.4 with 1 M NaCl was used in loading and wash steps in order to
eliminate BSA binding from decreased electrostatic interaction between the resin and the protein, and to wash any unbound proteins. After that, acetate buffers (AB) with pH 5.2 with or without different elution additives such as ethylene glycol (EG), urea and arginine were used as afterwash step in order to wash weakly bound contaminants before IgA elution.

The resulting recoveries and purities are listed in Table 5.3. The chromatograms for five different running conditions and the corresponding SDS-PAGE gel with collected flow-through and IgA elution fractions are shown in Figure 5.3. As expected, most contaminant proteins including BSA passed through the affinity column without binding as shown in the flow-through in the chromatograms (Figure 5.3A) and the SDS-PAGE gel (Figure 5.3B). Very good results with 96.0% recovery and 90.3% purity were achieved for running condition 1 with 0.2 M acetate buffer pH 3.0 elution without the afterwash step. For the other four different running conditions using an extra afterwash step, the isolated IgA purities increased about 5% with over 95% in purity. However, the extra afterwash step resulted in an over 15% decrease in recovery from 96.0% to below 80%, and in the range of 55.7% to 78.5%. The decrease in recovery resulted from the partial elution of bound IgA by the afterwash step, which was confirmed by ELISA. Since the isoelectric points for BSA and IgA overlap with each other in a broad range, the buffer pH for afterwash step is hard to be determined to achieve complete BSA elution without coelution of IgA. The elution additives chosen were based on effects on IgA recovery when peptide ligand HWRGWV at ligand density of 0.04 mequiv./g was used. As a result, the determined elution additives at certain concentration might not perform the same as with peptide ligand at ligand density of 0.11
mequiv./g. Therefore, more controlled conditions for the afterwash step need to be employed to maintain high recovery.

The same running conditions were also employed to purify SIgA from spiked CHO cell culture supernatant to examine the potential of peptide ligand HWRGWV for SIgA purification. The resulting recoveries and purities are listed in Table 5.3. The chromatograms for five different running conditions and the corresponding SDS-PAGE gel with collected flow-through and SIgA elution fractions are shown in Figure 5.4. As expected, most contaminant proteins including BSA passed through the affinity column without binding as shown in the flow through in the chromatograms (Figure 5.4A) and the SDS-PAGE gel (Figure 5.4B). With 0.2 M acetate buffer pH 3.0 elution without the afterwash step (Run 1), resulted in a 94.3% recovery and 91.7% purity, indicating that the hexamer peptide ligand HWRGWV has the potential not only for IgA purification but also for SIgA purification from cell culture supernatants.

Compared to the structure of monomeric IgA, SIgA is in dimeric form with joining (J) chain and a secretory component as shown in Figure 5.4B (SIgA lane) with bands corresponding to IgA heavy chain, light chain, J chain and secretory component. In a previous study, it was pointed out that four out of five possible binding sites in IgA for peptide ligand HWRGWV are located in the C_{H}2 and C_{H}3 domains, which are similar to the location of the binding site for HWRGWV in human IgG.\textsuperscript{37} Therefore, a relatively lower binding affinity for SIgA is suspected compared to IgA because of the hindered accessible binding site resulting from the presence of J chain and secretory component. This was confirmed by the results for chromatographic purification of SIgA as shown in Table 5.3.
Although similar high purities (>95%) were achieved for both IgA and SIgA, much lower recoveries in the range of 30.3% to 49.6% were obtained for SIgA compared to IgA recoveries in the range of 55.7% to 78.5% because of the elution of bound SIgA in the afterwash step. With 0.1 M arginine as the elution additive in running condition 5, less than 10% recovery was obtained and the purity could not be determined by SDS-PAGE stained with Coomassie Blue. Since the running conditions used were determined based on the effects of elution additives on recovery of IgA purification, better running conditions specific for SIgA purification need to be investigated in order to achieve better recovery.

5.3.4. Affinity chromatography purification of recombinant human IgA from 100-fold concentrated CHO cell culture supernatant

The original CHO cell culture supernatant with recombinant human IgA was also used as the starting material for IgA purification in an attempt to use a real cell culture medium containing a low titer of IgA. This challenging material gives a good idea of the performance of the resin under extreme conditions. It has a low recombinant human IgA titer, making the purification of the target material more difficult, as the level of contaminants is higher in relation to the concentration of IgA. Since the rhIgA titer was very low (0.18 µg/mL), an ultrafiltration step with 100 kDa molecular weight cut-off regenerated cellulose membrane was used to process the cell culture supernatant prior to injection onto the affinity column. The same running conditions used for IgA and SIgA purification from spiked CHO cell culture supernatants were employed. As shown in Table 5.4, after the 100-fold volume decrease, IgA concentration increased to 11.0 µg/mL with a 61.1% recovery. Although the ultrafiltration successfully concentrated IgA about 60 times, it was not able to completely
remove contaminant such as BSA with molecular weight of 66 kDa. Therefore, even with a total protein recovery of only 10%, the IgA percentage (0.042%) in the feed material was still low compared to the total protein concentration. The purification results were not good compared to previous spiked experiments as shown in Table 5.5. The IgA percentages in elution fractions were calculated by IgA concentrations versus total protein concentrations determined by micro BCA. Although the final IgA percentages in the elution fractions were low (2.3-4.6%), enrichment factor in the range of 55 to 111 was achieved with the IgA recoveries in the range of 54.8% to 77.2% after one step of affinity chromatography.

5.4. Conclusions

Unlike the large-scale purification of IgG with extensively studied affinity ligands such as protein A, protein G or protein A/G, no particular affinity purification method for IgA has achieved broad acceptance. Hexamer peptide ligand HWRGWV initially screened against the Fc domain of human IgG also exhibits the ability to capture and purify both human monomeric IgA and secretory IgA from spiked CHO cell culture supernatants. Stronger binding affinity of HWRGWV to hIgA compared to hIgG was confirmed by adsorption isotherm study with the dissociation constants for hIgA in the range of $10^{-6}$ to $10^{-7}$. With lower maximum binding capacities for hIgA compared to hIgG at three different peptide densities, the dynamic binding capacities for hIgA at peptide density of 0.11 mequiv./g are in the range of 9.2 to 16.9 mg/mL at linear flow rates from 173 to 35 cm/h, which is also lower than those obtained for hIgG. However, they are much greater than the binding capacity of affinity ligands found in the literature and are competitive to current
commercially available affinity ligands such as PAM, Peptide M, CaptureSelect IgA and Immobilized Jacalin.

Weaker binding affinity was observed for secretory IgA compared to monomeric IgA because of the dimeric structure of SIgA with joining chain and secretory component, which confirmed our previous prediction that the potential binding sites of IgA to peptide ligand HWRGWV located in CH$_2$ and CH$_3$ domain. Peptide ligand HWRGWV resin exhibits the ability to achieve high recovery and purity of IgA from complex mixtures, which makes it attractive for large-scale purification of human IgA as well as secretory IgA. Recoveries in 96.0% and 94.3%, and purities in 90.3% and 91.7% were achieved for human IgA and SIgA purification respectively from spiked CHO cell culture supernatants without the extra afterwash step. With the afterwash step, although the resulting purities are over 95% for both IgA and SIgA, the recoveries for IgA and SIgA decreased at least 15% and 40%, respectively. Therefore, more controlled buffer conditions with different additives as afterwash step for either IgA or SIgA purification need to be determined in order to improve both recovery and purity.

5.5. Acknowledgement

The authors would like to thank Professor Sherie L. Morrison from Department of Microbiology, Immunology and Molecular Genetics at UCLA for providing the recombinant human IgA producing Chinese hamster ovary cell line.

5.6. References


8. Woof JM, Kerr MA. IgA function - variations on a theme. *Immunology* 2004; 113: 175-177.


40. Yoo EM, Yu LJ, Wims LA, Goldberg D, Morrison SL. Differences in N-glycan structures found on recombinant IgA1 and IgA2 produced in murine myeloma and CHO cell lines. *mAbs* 2010; 2: 320-334.


Table 5.1. Running conditions for hIgA purification from different feedstocks.

<table>
<thead>
<tr>
<th>No.</th>
<th>Loading and Wash</th>
<th>After-wash</th>
<th>Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS + 1M NaCl</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>2</td>
<td>PBS + 1M NaCl</td>
<td>0.2 M AB pH 5.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PBS + 1M NaCl + 10% EG</td>
<td>0.2 M AB pH 5.2 + 10% EG</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>4</td>
<td>PBS + 1M NaCl + 0.2M urea</td>
<td>0.2 M AB pH 5.2 + 0.2M urea</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PBS + 1M NaCl + 0.1M arginine</td>
<td>0.2 M AB pH 5.2 + 0.1M arginine</td>
<td></td>
</tr>
</tbody>
</table>

AB: acetate buffer

Table 5.2. Apparent dissociation constant ($K_d$) and maximum binding capacity ($q_m$) for hIgA with peptide ligand HWRGWV at different ligand densities.

<table>
<thead>
<tr>
<th>Peptide Density (mequiv./g)</th>
<th>hIgA</th>
<th>hIgG $^{35}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ ($\times 10^{-6}$ M)</td>
<td>$q_m$ (mg/g)</td>
</tr>
<tr>
<td>0.11*</td>
<td>1.05</td>
<td>66.8</td>
</tr>
<tr>
<td>0.22</td>
<td>0.646</td>
<td>86.4</td>
</tr>
<tr>
<td>0.55</td>
<td>0.509</td>
<td>93.8</td>
</tr>
</tbody>
</table>

* peptide density of 0.10 mequiv./g for hIgG
Table 5.3. Recovery and purity for hIgA and hSIgA purification from spiked CHO cell culture supernatant. All experiments were run in duplicate (n=2).

<table>
<thead>
<tr>
<th>Running Condition</th>
<th>hIgA</th>
<th>hSIgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>Purity (%)</td>
</tr>
<tr>
<td>1</td>
<td>96.0 ± 5.9</td>
<td>90.3 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>78.5 ± 1.3</td>
<td>96.8 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>65.5 ± 2.2</td>
<td>97.3 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>69.0 ± 1.6</td>
<td>94.4 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>55.7 ± 8.9</td>
<td>98.9 ± 0.3</td>
</tr>
</tbody>
</table>

Table 5.4. Sample preparation for CHO cell culture supernatants after 100-fold concentration by ultrafiltration membrane.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Protein (mg/mL)</th>
<th>Recovery (%)</th>
<th>hIgA (µg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO supernatant</td>
<td>2.66 ± 0.04</td>
<td>100</td>
<td>0.18 ± 0.04</td>
<td>100</td>
</tr>
<tr>
<td>100x CHO supernatant</td>
<td>26.5 ± 0.6</td>
<td>10.0 ± 0.2</td>
<td>11.0 ± 2.7</td>
<td>61.1 ± 15.0</td>
</tr>
</tbody>
</table>
Table 5.5. Recovery, percentage and enrichment factor for hIgA purification from 100-fold concentrated CHO cell culture supernatant.

<table>
<thead>
<tr>
<th>Running Condition</th>
<th>Recovery (%)</th>
<th>Percentage (%)</th>
<th>Enrichment Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>77.2 ± 2.1</td>
<td>4.6 ± 0.2</td>
<td>111</td>
</tr>
<tr>
<td>2</td>
<td>76.0 ± 1.3</td>
<td>3.2 ± 0.1</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>74.2 ± 2.5</td>
<td>3.1 ± 0.2</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>73.9 ± 1.0</td>
<td>2.9 ± 0.1</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>54.8 ± 1.1</td>
<td>2.3 ± 0.0</td>
<td>55</td>
</tr>
</tbody>
</table>
Figure 5.1. Langmuir fits (lines) of isotherms for hIgA adsorption to HWRGWW resins at three different peptide densities of 0.11, 0.22 and 0.55 mequiv./g.
Figure 5.2. Breakthrough curves for hIgA on HWRGWV resins at peptide density of 0.11 mequiv./g at flow rate of 0.02 mL/min, 0.05 mL/min, and 0.1 mL/min. Column loading expressed in terms of (A) mg protein/mL of resin and (B) mg protein/g of resin.
Figure 5.3. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgA separation from 1.5 mg/mL hIgA spiked CHO cell culture supernatant using HWRGWV resin at peptide density of 0.11 mequiv./g using five different running conditions. Run 1, 2, 3, 4 and 5 correspond to running condition 1, 2, 3, 4 and 5, respectively. (A) Flow through (FT) and hIgA elution peak (P) were collected and neutralized by 8M tris HCl pH 8.0. (B) SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), loading material (L), standard BSA and hIgA (IgA) with IgA light chain (LC), heavy chain (HC), light chain + heavy chain (LC + HC), two heavy chains (2x HC), BSA and BSA dimer pointed out.
Figure 5.4. (A) Chromatograms and (B) corresponding SDS-PAGE of SIgA separation from 1.5 mg/mL SIgA spiked CHO cell culture supernatant using HWRGWV resin at peptide density of 0.11 mequiv./g using five different running conditions. Run 1, 2, 3, 4 and 5 correspond to running condition 1, 2, 3, 4 and 5, respectively. (A) Flow through (FT) and SIgA elution peak (P) were collected and neutralized by 8M tris HCl pH 8.0. (B) SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), loading material (L), standard BSA and secretory IgA (SIgA) with IgA light chain (LC), heavy chain (HC), joining (J) chain, secretory component (SC), BSA and BSA dimer pointed out.
Chapter 6. Affinity Chromatographic Purification of Human Immunoglobulin M from Human B Lymphocyte Cell Culture Supernatant

Zhuo Liu, Patrick V. Gurgel, Ruben G. Carbonell

A version of this chapter has been published in
Biochemical Engineering Journal, 70 (2013) 63-70
Abstract

Compared to immunoglobulin G purification with extensively studied affinity ligands such as protein A and protein G, little work has been done on affinity chromatographic purification of immunoglobulin M. Hexamer peptide ligand HWRGWV, previously shown to bind specifically to the Fc fragment of IgG, also demonstrated potential for IgM purification. This study presents further characterization and investigation of this ligand for its potential for purification of IgM. Different running conditions were employed in order to improve the recovery and purity of IgM. The final recovery and purity of the antibody is feedstock dependent, but can reach levels of both recovery and purity as high as 95%. The dependence of the recovery and purity on total loading amount and initial IgM concentration were investigated and discussed. Although relatively low dynamic binding capacities (DBC) in the range of 4.6 to 13.1 mg IgM/mL resin at linear flow rates from 173 to 35 cm/h were obtained for IgM compared to IgG because of the large molecular weight of IgM, the DBC value of HWRGWV for IgM is much greater than protein-based IgM affinity ligands found in the literature and is competitive with current commercially available affinity ligands, such as KAPTIVE-M, CaptureSelect IgM and Ultralink Immobilized Mannan Binding Protein.

Keywords: affinity chromatography; hexamer peptide; human immunoglobulin; IgM; dynamic binding capacity; elution additive.
6.1. Introduction

Immunoglobulin M (IgM) has been shown not only to be involved in early recognition of challenging pathogens, but also to play an important role in immunosurveillance mechanisms against malignant cells [1]. In the meantime, IgM monoclonal antibodies offer promising anticancer activity, which have created a strong interest in their diagnostic and therapeutic applications [2-5]. Compared to immunoglobulin G (IgG) in the form of monomer with molecular weight of about 150 kDa, IgM occurs naturally in two different forms; dominantly in pentamers with a molecular weight of about 950 kDa and rarely in hexamers with a molecular weight of about 1.15 MDa. Therefore, the diffusion constant of IgM is about half of that of IgG, with $2.6 \times 10^{-7}$ and $4.9 \times 10^{-7}$ cm$^2$/s, respectively. In addition, IgM is a more heavily glycosylated protein than IgG, with a range of 10-12% carbohydrate [6, 7].

Because of the large and unstable structure, it has been much more difficult to purify IgM than IgG antibodies [7]. Compared to IgG purification, the major challenge for IgM purification is that IgMs tend to be soluble in a narrower range of conditions and they are more susceptible to denaturation. Traditional methods used for the purification of IgM have been based on combinations of non-chromatographic and chromatographic techniques including precipitation [8-10], hydrophobic interaction chromatography [11, 12], size exclusion chromatography [8-11, 13-15], ion-exchange chromatography [8, 10, 12, 14, 15] and hydroxyapatite chromatography [12, 13]. Because of the highly specific recognition between the antibody molecule and a complementary ligand, affinity chromatography commonly reduces non-specific interactions, increases operational yields and facilitates the
elimination of undesirable contaminants, which allows the purification and concentration of antibodies in one single step possible. As a result, since its introduction by Cuatrecasas and co-workers in 1968, affinity chromatography has been the method of choice for the capture and purification of native and genetically engineered antibodies both in laboratories as well as in large scale biomanufacturing [16].

Protein A from *Staphylococcus aureus*, protein G from group C and G *Streptococci* or protein A/G (a recombinant fusion protein combining IgG binding domains of both protein A and G), widely used for industrial affinity purification of IgG antibodies, do not recognize immunoglobulins of M class well and are not used for capture and purification of IgM [17]. Protein L, a cell wall molecule of some strains of the anaerobic bacteria species *Peptostreptococcus magnus*, demonstrates binding affinity for IgM as well as IgG and IgA. However, it only binds to variable domains of antibodies with κ light chain subgroup I, III and IV; but not to antibodies with λ light chain or κ light chain subgroup II [18]. Other protein-based affinity ligands including mannose binding protein (MBP) [19], snow bulb lectin (GNA) [20] and complement protein C1q [21] suffer from several drawbacks, such as limited binding capacity, temperature-dependent binding or elution, specificity only for murine IgM, susceptibility to proteolysis and low stability to sanitization by sodium hydroxide. Artificial protein A (ApA) as well as artificial protein L (ligand 8/7) developed by Lowe and collaborators initially targeting the Fc or Fab domains of IgG also demonstrated the ability to bind IgM [22, 23]. However, no further characterization and application was found for IgM capture and purification. Fassina with collaborators developed a tetrameric tripeptide, denoted PAM (protein A mimetic, TG19318), which was successfully applied for
the purification of IgG, IgA, IgM, IgE and IgY [24]. Affinity purification of IgMs using this ligand was achieved from different sources including serum, ascitic fluid and cell culture supernatants with purities in the range of 85-95%. The column binding capacity for IgM ranged from 2 to 8 mg of IgM/mL of support which was related to the type of support used for ligand immobilization [17].

Hexamer peptide ligand HWRGWV, previously identified in our research group through a three-step screening of a synthetic solid phase combinatorial peptide library, belongs to a family of linear hexamer peptide ligands with specific composition from the N-terminal. It demonstrated the ability to bind the Fc domain of immunoglobulins, which could be used for affinity chromatographic purification of antibodies. The selectivity of the ligand to the Fc portion of IgG is comparable to that of protein A and the ligand exhibited the ability to bind all human IgG subclass as well as IgGs from different species [25, 26]. After direct synthesis onto Toyopearl AF Amino 650M resin, HWRGWV was able to purify monoclonal and polyclonal IgGs from different sources [27-29], as well as human IgA and IgM [30-32]. Previous experiments showed that the peptide density of HWRGWV had a significant influence on the recovery and purity of isolated IgM. The improved recoveries at high density, due to increased binding affinity, were compensated by a loss in purity. At the optimum peptide density of 0.04 mequiv./g of resin, 75.7% recovery and 86% purity were achieved for IgM purification from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth.

In the present work, we extended the characterization of HWRGWV for IgM purification. The dynamic binding capacities of the resin for IgM at the optimum peptide
density with different flow rates were determined. Human B lymphocyte cell culture supernatant without or with spiked hIgM was used as starting material for affinity chromatographic purification of human IgM. In order to achieve high recovery and purity, different running conditions were employed. The effect of total loading amount as well as initial IgM concentration on recovery and purity was investigated.

6.2. Material and Methods

6.2.1. Material

The peptide HWRGWV was synthesized directly on Toyopearl AF-Amino-650M (particle size 65μm) (Tosoh BioScience, Montgomeryville, PA, USA) using fluorenylmethyloxycarbonyl (Fmoc) chemistry by Peptides International, Inc (Louisville, KY, USA). The HWRGWV resins were synthesized at a peptide ligand density of 0.04 mequiv/g. Phosphate-buffered saline (PBS) pH 7.4 (containing 10 mM phosphate, 138 mM sodium chloride, and 2.7 mM potassium chloride), urea, and arginine were obtained from Sigma (St. Louis, MO, USA). Sodium chloride, magnesium chloride, calcium chloride, sodium acetate, sodium caprylate, glacial acetic acid, guanidine hydrochloride, tris hydrochloride, ethylene glycol and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). NuPAGE Novex 4-12% Bis-Tris gels, NuPAGE reducing agent, NuPAGE MOPS SDS running buffer, SeeBlue Plus2 pre-stained molecular weight marker and SimplyBlue Safestain were all from Invitrogen (Carlsbad, CA, USA). Purified human IgM were purchased from Fitzgerald (Concord, MA, USA). Human IgM enzyme-linked immunosorbent assay (ELISA) kits were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Microcon Ultracel
YM-3 centrifugal filters (regenerated cellulose, 3 kDa MWCO), Amicon Centricon centrifugal filters (regenerated cellulose, 50 kDa MWCO), ultrafiltration membranes (regenerated cellulose, 300 kDa MWCO) and Millex-GV 0.22 μm Syringe Driven Filter Unit (low protein binding Durapore PVDF membrane) were purchased from Millipore (Billerica, MA, USA). Bicinchoninic acid (BCA) protein assay was from Thermo Scientific (Rockford, IL USA). Microbore columns (30 mm × 2.1 mm) for all chromatographic experiments were from Alltech (Deerfield, IL, USA).

6.2.2. Equipment

All chromatographic experiments were carried out on a Waters 626LC system. Absorbance of protein samples was measured at wavelength 280 nm using a built-in 2487 dual wave-length UV detector from Waters (Milford, MA, USA). Centrifugal concentration was achieved on IEC Centra CL2 Centrifuge from Thermo Scientific (Asheville, NC, USA) and Centrifuge 5417R from Eppendorf (Hauppauge, NY, USA). ELISA wash step was performed on ELx50 Microplate Strip Washer from Bio-Tek Instruments Inc. (Winooski, VT, USA). BCA and ELISA protein concentrations were determined by μQuant Spectrophotometer from Bio-Tek Instruments Inc. as well.

6.2.3. Generation of breakthrough curves

For all frontal analysis experiments, peptide resins at a ligand density of 0.04 mequiv./g were dry packed into a 30 mm × 2.1 mm I.D. microbore column (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 hours followed by HPLC grade water and PBS equilibration. The purified human IgM in the equilibration buffer at a concentration of 2
mg/mL was used as a feeding material, and a 1-mL sample loop was used to ensure the saturation of the affinity column. Column effluent fractions were collected during the loading step and the protein concentration in each fraction was determined by BCA protein assay using IgM as standards for calibration curve. Protein concentration as a fraction of the inlet feed concentration was plotted against column loading to obtain the breakthrough curve. Breakthrough volume was multiplied by the inlet feed concentration and normalized with respect to the packed bed volume or packed resin weight to express the x-axis of the breakthrough curves in the same units as the dynamic binding capacity. For all these chromatographic experiments, the column was equilibrated and loaded with PBS pH 7.4, and the bound protein was eluted by 6 M guanidine HCl, which also served as wash and regeneration agent. The flow rates used (based on column performances in past experiments [30]) were 0.02, 0.05 and 0.1 mL/min, which corresponded to linear flow rates of 35, 87 and 173 cm/h with residence time of 5, 2 and 1 min, respectively.

6.2.4. Cell culture

Human B. lymphocyte MC 116 cell line (ATCC # CRL-1649) for human monoclonal IgM production was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in suspension at 37°C under 5% CO₂ in RPMI-1640 medium from Invitrogen (Carlsbad, CA, USA) supplemented with 10% fetal bovine serum from HyClone Laboratories (Logan, UT, USA). With the doubling time about 26 to 28 h, cell density was maintained at or below 3 × 10⁶ cells/mL in 75 cm² cell culture flasks from Corning Incorporated (Corning, NY, USA).
6.2.5. Sample preparation

Collected cell culture was clarified by centrifugation using IEC Centra CL2 Centrifuge at 2500 × g for 10 min followed by filtration using 0.22 μm Millex-GV syringe filter in order to remove any cells or cell debris left. After filtration, the filtrate was concentrated about 5.7-fold or 100-fold by using Amicon Centricon centrifugal filters (50 kDa MWCO regenerated cellulose) or membrane filter holder with 47 mm fritted glass support from Whatman Inc. (Piscataway, NJ, USA) and 300 kDa molecular weight cutoff regenerated cellulose membrane. Total protein as well as antibody concentrations in the original and concentrated cell culture supernatant were determined by BCA protein assay and ELISA, respectively, based on suppliers’ instructions. The 1.5 mg/mL human IgM spiked cell culture supernatant was achieved by adding purified human IgM at stock concentration of 7.27 mg/mL into original B lymphocyte cell culture supernatant with a resulting IgM percentage of 34%. The same IgM percentage was kept to obtain 0.75 mg/mL human IgM spiked cell culture supernatant by 1:2 dilution with PBS pH 7.4. The 0.1 mg/mL human IgM spiked cell culture supernatant was achieved by adding purified human IgM at stock concentration of 7.27 mg/mL into 1:2 diluted original B lymphocyte cell culture supernatant with resulting IgM percentage at 5%.

6.2.6. Purification of human IgM from different feedstocks

Peptide resins at peptide density of 0.04 mequiv./g were dry packed in 30 mm × 2.1 mm I.D. microbore columns (0.1 mL) and swollen in 20% methanol at 0.05 mL/min for 2 h followed by HPLC grade water and PBS washes. Before sample loading, columns were
equilibrated with the loading buffer based on running conditions. Samples were manually injected at room temperature using different size of sample loop from 50 μL to 1 mL (Thomson, Springfield, VA, USA). The samples were loaded at 0.05 mL/min for 10 min or 40 min with 1 mL sample loop, and then the flow rate was increased to 0.3 mL/min for the remainder of the run. The column was washed sequentially with 3 mL loading buffer, 6 mL afterwash if necessary, 6 mL elution buffer, and 6 mL 6M guanidine-HCl to clean and regenerate the column, followed by loading buffer for equilibration until baseline was reached. The buffers used for the preliminary running condition study are listed in Table 6.1. All elution fractions were collected and their pH was immediately neutralized by 1 M Tris buffer pH 8.0 with predetermined volume.

6.2.7. Sample analysis for recoveries and purities

The recoveries of purified human IgM from different feedstocks were calculated according to the hIgM concentrations determined by ELISA using a human IgM ELISA kit from Alpha Diagnostic International (San Antonio, TX, USA). Human IgM flow through and elution fractions from different feedstocks were concentrated by different factors based on the total protein and IgM concentrations by centrifugation using Centrifuge 5417R at 20°C, 14,000 × g with Microcon Ultracel YM-3 centrifugal filters (regenerated cellulose, 3 kDa MWCO). Different feedstocks were diluted different times based on total protein and IgM concentrations for gel loading. The treated samples were mixed with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent according to the supplier's instructions before being incubated at 70 °C for 10 min and then analyzed by sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using NuPAGE Novex 4-12% Bis-Tris gels with MOPS running buffer on an X-cell SuperLock™ Mini-Cell system from Invitrogen at 200 V for 50 min. The gels were stained by SimpleBlue SafeStain. The purities of human IgM elution peaks were determined by densitometric measurement using software ImageJ 1.32j (National Institute of Health, Bethesda, MD, USA).

6.2.8. Analytical size-exclusion chromatography

Analytical size-exclusion chromatography (SEC) was conducted using a 7.8 mm I.D. × 300 mm Ultrahydrogel 500 column from Waters (Milford, MA, USA) under isocratic conditions with a mobile phase of 100 mM sodium phosphate, 200 mM sodium chloride, pH 7.0 and a flow rate of 1.5 mL/min. Samples were injected into the column and eluted with running buffer, and the protein was detected by measuring absorbance at 280 nm. The collected IgM fractions from spiked cell culture supernatant were concentrated about 5 or 10 times before applying to the column.

6.3. Results and discussion

6.3.1. Dynamic binding capacity

As mentioned in the introduction, binding capacity of an affinity medium is one of the factors that directly determine its potential application for large scale purification of IgM. To further characterize the peptide ligand HWRGWV for IgM purification, breakthrough curves for human IgM on HWRGWV resin at peptide density of 0.04 mequiv./g were generated at different flow rates, and are shown in Figure 6.1. Dynamic binding capacity (DBC) values in mg/mL resin as well as mg/g resin at the 10% breakthrough point were
compared. At the flow rates of 0.02, 0.05 and 0.1 mL/min, corresponding to linear flow rate 35, 87 and 173 cm/h, the DBC values were 13.1, 7.2 and 4.6 mg IgM/mL resin, respectively. The DBC values for IgM are highly dependent on the flow rate and decreased about 65% at a linear flow rate changed from 35 cm/h to 173 cm/h. The effect is as expected because of the large molecular weight (950 kDa) of IgM and the property of the column resin with diffusion limitation.

The lower dynamic binding capacity for IgM compared to IgG resulted from the restricted diffusion of the large IgM molecules into the chromatographic resin pores. For non-hindered diffusive transport into pores, a pore to molecule diameter ratio of 10:1 has been suggested [33]. Therefore, for IgM with a hydrodynamic diameter of 24 nm, pores of 240 nm would be required, which are approximately two and half times larger than the pore size of Toyopearl AF-Amino-650M resin with 100 nm pores. In order to overcome the drawback of chromatographic resins, other chromatographic media such as membranes [34] or monoliths [35] can be used for affinity purification of IgM because of the advantages such as high dynamic binding capacity for large molecules, flow independent performance and low pressure drop at high flow rates [36]. Therefore, peptide ligand HWRGWV with alternative chromatographic media such as membranes and monoliths might be the better solution for large scale affinity purification of IgM.

Although the DBC value of HWRGWV resin at peptide density of 0.04 mequiv./g for IgM is relatively low compared to that for IgG (20 mg IgG/mL resin at linear flow rate of 87 cm/hr) [28], they are much greater than the binding capacity of protein-based affinity resins using ligands such as mannose binding protein (MBP) [19] and complement protein C1q [21].
with 0.4 to 2 mg of IgM/mL of support. The DBC value is also competitive to previous and current commercially available affinity media such as KAPTIVE-M (PAM, TG19318) from Tecnogen (Piana di Monte Verna, Italy) with binding capacity about 2-8 mg/mL of support [17], CaptureSelect IgM from BAC (Naarden, Netherlands) with DBC about 2.5 mg IgM per mL matrix and Ultralink Immobilized Mannan Binding Protein from Thermo Scientific (Rockford, IL USA) with binding capacity about 0.75 mg mouse IgM/mL settled gel [37].

6.3.2. Preliminary running condition study

From a previous study, 75.7% recovery and 86% purity were achieved for IgM purification from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth [30]. In order to further improve the purity, different running conditions needed to be investigated for most effective contaminant removal. As the major contaminants, bovine serum albumin (BSA) and α1-antitrypsin have the isoelectric point in the range between 4.5 and 5.5 compared to the isoelectric point of IgM in the range between 4 and 9.1 with a pI peak between pH 5.5 and 6.7 [38]. Therefore, washing weakly bound BSA and α1-antitrypsin before IgM elution or decreasing BSA and α1-antitrypsin binding during loading step would be the best strategies to achieve high purity of IgM from the cell culture supernatant. Based on a previous study, HWRGWV demonstrated the strongest binding affinity to IgM, followed by IgA and IgG. The study of elution pH showed that the bound IgM could only be eluted by solution with pH lower than 4.5 and over 90% recovery could be achieved by elution at pH 3.0 or 3.5 [30]. In addition, the effects of elution additives provided the opportunity of decreasing non-specific interactions between the
peptide ligand and contaminant proteins without disturbing the IgM binding [31]. Therefore, a total of ten different running conditions for IgM capture and elution were selected for investigation (Table 6.1). In order to further improve the isolated IgM purity, different strategies were used. Acetate buffer (AB) pH 5.0 was used as afterwash in order to wash weakly bound contaminant proteins before IgM elution. Different additives were added in acetate buffer at pH 5.0 as the loading and wash buffer in order to eliminate contaminant protein binding to the HWRGWV resin in the first place. Low concentration of sodium caprylate was used in order to check for the possibility of decreasing the amount of sodium chloride usage in the loading buffer which was used for decrease non-specific interaction of BSA.

One hundred microliters of 5.7-fold concentrated cell culture supernatant was injected without further treatment. The resulting recoveries, purities and enrichment factors for isolated human IgM for different running conditions are listed in Table 6.2. The determined hIgM concentration in the 5.7-fold concentrated human B lymphocyte cell culture supernatant was 12.8 μg/mL. The value is as expected from the cell line MC116 which produces 0.3-3.8 μg/10^6 cells/48 h. The initial percentage of hIgM in the concentrated cell culture supernatant is 0.086% based on the total protein concentration of 14.9 mg/mL determined by BCA protein assay. The resulting recoveries of hIgM were in the range between 26.5% and 88.4%, and the purities determined by SDS-PAGE densitometric analysis were in the range between 0.3% and 32.3%. Afterwash using acetate buffer pH 5.0 seems to have improved the purity of the recovered IgM, as evidenced by comparing the results from running conditions 1 and 2, which have similar recoveries, but different purities.
Acetate buffer pH 5.0 with different additives as loading and wash buffer resulted in different combinations of recoveries and purities spanning a large range. Weak additives [31] such as ethylene glycol, urea and magnesium chloride (running conditions 4, 5 and 10) in general led to high recoveries but low purities, possibly because of the binding of contaminants resulting from non-specific hydrophobic interactions. Sodium caprylate, a strong additive, resulted in low recovery (running condition 9), possibly because of a decreased IgM binding during loading. When 50 mM of sodium caprylate added to 200 mM sodium chloride were used instead of 1 M of sodium chloride in the phosphate buffer as the loading and wash buffer as in running conditions 2 and 8, the resulting recovery increased from 75.6% to 90.4% but the purity decreased from 23.9% to 17.0%. Although low concentration of sodium caprylate can be used to replace sodium chloride to depress non-specific interaction of BSA, as observed in a previous study [28], the specific concentration need to be carefully chosen for better purity.

Compared to the IgM purity in the loading material, the purities of IgM in the elution fractions increased dramatically at different levels among the ten different running conditions with a maximum purity of 32.2% using calcium chloride as additive in acetate buffer pH 5.0 for loading and wash steps (running condition 7). Conditions 2 and 6, which employed acetate buffer pH 5.0 as afterwash or arginine as additive in acetate buffer pH 5.0 for loading and wash steps, achieved high recoveries (>75%) with good purities (~24%) which improved the percentage of hIgM about 280-fold from initial 0.086%. Therefore, affinity chromatography with hexamer peptide ligand HWRGWV has the potential for capture and enrichment step in early stage of IgM purification process because of the high enrichment factor.
6.3.3. Affinity chromatography purification of human IgM from 100-fold concentrated B lymphocyte cell culture supernatant

In order to improve the purity of isolated IgM, it is important to increase the IgM concentration while partially removing contaminant proteins such as BSA and α1-antitrypsin. Ultrafiltration step using 300 kDa molecular weight cut-off regenerated cellulose membrane was used to process the cell culture supernatant prior to injection onto the affinity column. As shown in Table 6.3, after the 100-fold concentration (measured by volume), the IgM concentration increased from 4.04 µg/mL to 318.7 µg/mL, with a 78.9% recovery of the target protein. Although the ultrafiltration successfully concentrated IgM about 80-fold, it was not able to completely remove smaller molecules such as albumin and α1-antitrypsin. This is surprising, considering that the molecular weight cut-off of the membrane (300 kDa) is relatively large, about 5 to 6 times larger than albumin (66 kDa) and α1-antitrypsin (53 kDa). Because of the property of the ultrafiltration apparatus as a dead end filtration device, the retention of albumin and α1-antitrypsin could result from the blockage of the membrane by large molecular weight molecule IgM. As a result, with the final total protein concentration of 32.7 mg/mL, the concentrated IgM percentage was still very low about 0.97% compared to the total protein concentration.

Three different running conditions (2, 6 and 7, Table 6.1) were chosen based on results from the preliminary running condition study (section 6.3.2). The chromatograms for the three different running conditions and the corresponding SDS-PAGE gel with collected flow through and IgM elution fractions are shown in Figure 6.2. Because of the low titer of IgM in the 100-fold concentrated cell culture supernatant, it is difficult to see corresponding
bands for that protein in the 1/50 diluted loading material (lane L Figure 6.2B). It is worth noticing that the BSA dimer percentage increased compared to original cell culture supernatant without concentration. Therefore, BSA dimer could form during the ultrafiltration step which might be the second explanation for the retention of BSA during the ultrafiltration. Most contaminant proteins including BSA and α1-antitrypsin passed through the affinity column without binding, as shown in the flow through (FT) peaks in the chromatograms and the corresponding lanes in the SDS-PAGE gel. However, because of the increased percentage of BSA dimer, it became the major contaminant protein together with BSA monomer and α1-antitrypsin shown in the IgM elution fractions. As a result, poor purities were achieved for IgM, as listed in Table 6.4. Even with an amount IgM loaded onto the column far below the resin's capacity, recoveries ranging only from 63% to 45% were achieved for three tested running conditions.

The results obtained match the literature for purification of IgM from serum-containing cell culture supernatants achieved by Lee et al. using a three-step purification procedure for IgM from hybridoma supernatant with serum [39]. After the first tangential flow filtration (TFF) step, 149 µg/mL mAb 84 in 10.8 mg/mL total protein was achieved with initial IgM percentage about 1.4% and used for the following two chromatographic-steps including anion-exchange and size-exclusion chromatography. Low recovery (42%) and purity (52%) were obtained after the two steps of chromatography. However, much better results were achieved with the same purification process after adaptation of the hybridoma culture to serum-free media. With 152 µg/mL mAb 84 in 0.80 mg/mL total protein after the first TFF step, 62% recovery and 96% purity were obtained after the two
chromatographic steps. Therefore, in order to improve the recovery and purity in our case, adaptation of the cell culture to chemically defined serum-free or protein-free media and increase of the antibody titer would possibly be the best solution of process development.

6.3.4. Affinity chromatography purification of human IgM from IgM spiked B lymphocyte cell culture supernatant

Since it is unrealistic for us to adapt the cell line to chemically defined serum-free or protein-free media in a short period of time, we decided to spike the original cell culture supernatant to increase the human IgM titer as we mentioned above. Different loading amount of human B lymphocyte cell culture supernatant with different spiked hIgM concentrations were used to load the affinity column with HWRGWV resins at peptide density of 0.04 mequiv./g. The resulting recoveries and purities were listed in Table 6.5. For cell culture supernatant spiked with 1.5 mg/mL hIgM with total 100 µL loading, as we expected, most contaminant proteins including BSA and α1-antitrypsin passed through the column without binding as shown in the flow through in the chromatograms and the SDS-PAGE gel shown in Figure 6.3. Since no ultrafiltration step was used, BSA dimer percentage remained low. Therefore, high purity around 95% was achieved for all three conditions. These results were also confirmed by the analytical size-exclusion chromatography as shown in Figure 6.4. In terms of recovery, employing acetate buffer pH 5.0 as afterwash or arginine as additive in acetate buffer pH 5.0 for loading and wash steps achieved similar outcomes of over 95% recovery. However, relatively lower recovery (57.9%) was obtained when calcium chloride was used as additive in acetate buffer pH 5.0 since the strong salting-in effect of calcium chloride leads to decreased binding capacity for IgM in loading [31].

185
For the same total loading amount of hIgM with the same initial hIgM percentage, although the total loading amount is 50% of the previous experiment, over 95% purities were achieved for all three running condition at two different situations similar results as shown in Figure 6.5 with the corresponding SDS-PAGE gel analysis of the purified hIgM fractions. However, lower recoveries were obtained compared to previous experiment suggesting that the hIgM recovery is dependent on the total loading amount. In the meantime, relatively high recoveries obtained for loading sample with the high hIgM initial concentration suggest that the hIgM recovery is not only dependent on the total loading amount but also in the hIgM initial concentration.

As we further decreased the initial hIgM percentage to 5% and used 1000 µL loading volume, almost no visible hIgM bands were observed in the loading sample lane as shown in corresponding SDS-PAGE gel analysis (Figure 6.6A). Although relatively low purities from 67.1% to 77.5% were obtained for three running conditions suggesting that hIgM purity is dependent on the initial percentage in the loading, about 14-fold hIgM percentage increase was achieved after one step of affinity chromatography. As you can see from the size-exclusion chromatography analysis shown in Figure 6.6B, after the affinity chromatography step, the hIgM percentage compared to BSA and other contaminant proteins increase dramatically. In industry, the total loading amount about 80-90% of the dynamic binding capacity is normally used to challenge the affinity column. Therefore, high purity is expected when high loading amount is used.
6.4. Conclusions

Because of the large and unstable structure, IgM is often considered much more difficult to purify than IgG antibodies. In the meantime, compared to IgG purification with extensively studied affinity ligands such as protein A and protein G, limited work has been done in the literature about affinity chromatographic purification of IgM. Hexamer peptide ligand HWRGWV initially screened against Fc doamin of human IgG also exhibits the ability to capture and isolate human IgM. Further characterization demonstrated that the dynamic binding capacity of this affinity resin at peptide density of 0.04 mequiv./g is much higher than those of protein-based affinity media found in literature. HWRGWV is also competitive to previous and current commercially available affinity resins for IgM purification in terms of dynamic binding capacity. Although lower dynamic binding capacity for IgM was obtained compared to IgG because of the large molecular weight of IgM, alternative chromatographic media such as membranes and monoliths can be employed in order to overcome this limitation. Poor results were obtained when concentrated B lymphocyte cell culture supernatants were used as starting material since the initial IgM concentration was too low (< 1%) compared to total protein concentration. However, high recovery (~95%) and purity (~95%) were achieved when IgM titer in the original cell culture supernatant was reasonable compared to other contaminants. The recovery is dependent on not only the total loading amount but also the initial IgM concentration, and the purity is dependent on the initial IgM percentage in the loading sample. As a result, peptide ligand HWRGWV might have potential for use in industrial scale purification of IgM.
6.5. References


Table 6.1. Running conditions for hIgM purification from different feedstocks.

<table>
<thead>
<tr>
<th>No.</th>
<th>Loading and wash</th>
<th>After-wash</th>
<th>Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS + 1M NaCl</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>2</td>
<td>PBS + 1M NaCl</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>3</td>
<td>0.2 M AB pH 5.0</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>4</td>
<td>0.2 M AB pH 5.0 + 40% EG</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>5</td>
<td>0.2 M AB pH 5.0 + 2 M Urea</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>6</td>
<td>0.2 M AB pH 5.0 + 0.2 M Arginine</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>7</td>
<td>0.2 M AB pH 5.0 + 0.2 M CaCl₂</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>8</td>
<td>PBS + 0.2M NaCl + 0.05 M NaCaprylate</td>
<td>0.2 M AB pH 5.0</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>9</td>
<td>0.2 M AB pH 5.0 + 0.05 M NaCaprylate</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
<tr>
<td>10</td>
<td>0.2 M AB pH 5.0 + 0.2 M MgCl₂</td>
<td>n/a</td>
<td>0.2 M AB pH 3.0</td>
</tr>
</tbody>
</table>

AB: acetate buffer, EG: ethylene glycol
Table 6.2. Recovery, purity and enrichment factor for IgM capture from 5.7-fold concentrated human B lymphocyte cell culture supernatant.

<table>
<thead>
<tr>
<th>Condition No.</th>
<th>Recovery (%)</th>
<th>Purity (%)</th>
<th>Enrichment Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74.4 ± 2.0</td>
<td>18.5 ± 1.3</td>
<td>215</td>
</tr>
<tr>
<td>2</td>
<td>75.6 ± 1.9</td>
<td>23.9 ± 2.1</td>
<td>278</td>
</tr>
<tr>
<td>3</td>
<td>88.0 ± 3.0</td>
<td>10.4 ± 0.1</td>
<td>121</td>
</tr>
<tr>
<td>4</td>
<td>74.4 ± 13.5</td>
<td>0.3 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>91.3 ± 6.7</td>
<td>8.1 ± 0.5</td>
<td>94</td>
</tr>
<tr>
<td>6</td>
<td>82.6 ± 5.0</td>
<td>23.7 ± 0.8</td>
<td>276</td>
</tr>
<tr>
<td>7</td>
<td>56.4 ± 5.3</td>
<td>32.2 ± 1.1</td>
<td>374</td>
</tr>
<tr>
<td>8</td>
<td>90.4 ± 0.4</td>
<td>17.0 ± 0.6</td>
<td>123</td>
</tr>
<tr>
<td>9</td>
<td>26.5 ± 2.4</td>
<td>7.5 ± 1.2</td>
<td>54</td>
</tr>
</tbody>
</table>
**Table 6.3.** Sample preparation for human B lymphocyte cell culture supernatant after 100-fold concentration by ultrafiltration membrane.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (mg/mL)</th>
<th>Recovery (%)</th>
<th>hIgM (µg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B lymphocyte supernatant</td>
<td>3.71 ± 0.13</td>
<td>100</td>
<td>4.04 ± 0.20</td>
<td>100</td>
</tr>
<tr>
<td>100x B lymphocyte supernatant</td>
<td>32.7 ± 0.7</td>
<td>8.83 ± 0.19</td>
<td>318.7 ± 8.6</td>
<td>78.9 ± 2.1</td>
</tr>
</tbody>
</table>

**Table 6.4.** Recovery and purity for hIgM purification from 100-fold concentrated B lymphocyte cell culture supernatant.

<table>
<thead>
<tr>
<th>Running Condition</th>
<th>Recovery (%)</th>
<th>Purity (%)</th>
<th>Enrichment Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>63.0 ± 0.2</td>
<td>15.5 ± 1.1</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>63.7 ± 4.1</td>
<td>16.2 ± 2.5</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>45.1 ± 7.8</td>
<td>20.7 ± 2.4</td>
<td>21</td>
</tr>
</tbody>
</table>
Table 6.5. Recovery and purity for hIgM purification from spiked human B lymphocyte cell culture supernatant.

<table>
<thead>
<tr>
<th>Sample condition</th>
<th>Running Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>$V_0$ (µL)</td>
<td>$C_0$ (mg/mL)</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>50</td>
<td>1.5</td>
</tr>
<tr>
<td>100</td>
<td>0.75</td>
</tr>
<tr>
<td>1000</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 6.1. Breakthrough curves for hIgM on HWRGWV resins at peptide density of 0.04 mequiv./g at flow rate of 0.02 mL/min, 0.05 mL/min, and 0.1 mL/min. Column loading expressed in terms of (A) mg protein/mL of resin and (B) mg protein/g of resin.
Figure 6.2. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from 100-fold concentrated B lymphocyte cell culture supernatant using HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions. Run 1, 2 and 3 correspond to running condition 2, 6 and 7, respectively. (A) Flow through (FT) and hIgM elution peak (P) were collected and neutralized by 8 M tris HCl pH 8.0. (B) SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), loading material (L), standard BSA and hIgM (IgM) with IgM light chain (LC), heavy chain (HC), BSA, BSA dimer and α1-antitrypsin pointed out. Loading material (L) with 1:50 dilution, flow through (FT) and hIgM elution peak (P) with 5-fold concentration.
Figure 6.3. (A) Chromatograms and (B) corresponding SDS-PAGE of hIgM separation from 1.5 mg/mL hIgM spiked B lymphocyte cell culture supernatant using HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions. Run 1, 2 and 3 correspond to running condition 2, 6 and 7, respectively. (A) Flow through (FT) and hIgM elution peak (P) were collected and neutralized by 8M tris HCl pH 8.0. (B) SDS-PAGE gel of the peaks denoted in panel A, as well as molecular weight marker (M), loading material (L), standard BSA and hIgM( IgM) with IgM light chain (LC), heavy chain (HC), BSA, BSA dimer and α1-antitrypsin pointed out. Loading material (L) with 1:5 dilution, flow through (FT) and hIgM elution peak (P) with 5-fold concentration.
Figure 6.4. Analytical size-exclusion chromatograms on Ultrahydrogel 500 (7.8 mm I.D. × 300 mm) of IgM spiked human B lymphocyte cell culture supernatant and affinity purified IgM from the HWRGWV-Toyopearl column. IgM and bovine serum albumin are indicated using arrows.
Figure 6.5. SDS-PAGEs of hIgM separation from spiked B lymphocyte cell culture supernatant with 1.5 mg/mL hIgM 50 µL load (A) and 0.75 mg/mL hIgM 100 µL load (B) using HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions. Run 1, 2 and 3 correspond to running condition 2, 6 and 7, respectively. SDS-PAGE gels with molecular weight marker (M), loading material (L), standard BSA and hIgM(IgM) with IgM light chain (LC), heavy chain (HC), BSA, BSA dimer and α1-antitrypsin pointed out. (A) Loading material (L) with 1:5 dilution, flow through (FT) and hIgM elution peak (P) with 10-fold concentration. (B) Loading material (L) with 1:2.5 dilution, flow through (FT) and hIgM elution peak (P) with 10-fold concentration.
Figure 6.6. (A) SDS-PAGEs of hIgM separation from spiked B lymphocyte cell culture supernatant with 0.1 mg/mL hIgM 1000 µL load by HWRGWV resin at peptide density of 0.04 mequiv./g using three different running conditions. Run 1, 2 and 3 correspond to running condition 2, 6 and 7, respectively. SDS-PAGE gels with molecular weight marker (M), loading material (L), standard BSA and hIgM(IgM) with IgM light chain (LC), heavy chain (HC), BSA, BSA dimer and α1-antitrypsin pointed out. Loading material (L) with 1:2 dilution, flow through (FT) and hIgM elution peak (P) with 10-fold concentration. (B) Analytical size-exclusion chromatograms on Ultrahydrogel 500 (7.8 mm I.D. × 300 mm) of IgM spiked human B lymphocyte cell culture supernatant and affinity purified IgM from the HWRGWV-Toyopearl column. IgM and bovine serum albumin are indicated using arrows.
Chapter 7. Conclusions and Recommendations for Future Work
7.1. Conclusions

7.1.1. Potential binding sites for HWRGWV on human immunoglobulins A and M

Because of the homology between human immunoglobulins classes in heavy chain constant region, it is reasonable to expect the hexamer peptide ligand HWRGWV, initially screened for targeting the Fc region of human immunoglobulin G (hIgG), also binds to heavy chain constant regions of human immunoglobulins A and M as well. Using the amino acid sequence SNGQPEN found in hIgG as a reference, five and three possible binding sites were found for hIgA and hIgM, respectively, based on comparison with the binding amino acid sequence. For hIgA, four out of five possible binding sites are located in the C_H2 and C_H3 domains, which are similar to the location of the binding site in human IgG. For hIgM, two out of three possible binding sites are located in the C_H1 domain, which leads to stronger binding affinity for hIgM to HWRGWV compared to hIgG and hIgA because of the multiple accessible binding sites.

7.1.2. Effect of elution solution pH on recovery of human IgG, IgA and IgM at different peptide densities

Hexamer peptide ligand HWRGWV, initially screened against the Fc portion of hIgG, exhibits the ability to capture and isolated human IgA and IgM as well. For this ligand, capture of the three antibodies is not very sensitive to the peptide density. HWRGWV demonstrated the strongest binding affinity to hIgM, followed by hIgA and hIgG, respectively. Relatively high recovery (> 90%) can be achieved for hIgG by using an elution pH larger than 4.0; however, high recovery (> 90%) can only be achieved for hIgA and hIgM.
by using elution pH lower than 4.0. Because of the binding affinity difference, an elution buffer of pH 5.0 might be suitable for separating hIgG from hIgA and hIgM in a mixture.

7.1.3. **Effect of N-terminal acetylation on capture and elution of human IgG, IgA and IgM**

N-terminal acetylation of the peptide ligand HWRGWV has no effect on hIgG capture and elution. However, N-terminal acetylation decreased the binding of hIgA and hIgM, which resulted in early partial elution of hIgA and a shift in the elution peak of hIgM. The opposite charges on the ligand and the immunoglobulins at neutral pH suggest that electrostatic interactions play a role in hIgA and hIgM binding to the peptide. Although the charge of the N-terminal histidine might play an important role, it does not seem to be the dominant force in hIgA and hIgM binding since stronger affinities of hIgA and hIgM to the ligand were still observed compared to hIgG after the N-terminal acetylation. The other sources for electrostatic interactions could come from the histidine side chain in the peptide that becomes positively charged when pH decreases below 6.0, while arginine is always positively charged at acidic pH.

7.1.4. **Effect of elution additives on recovery of human IgG, IgA and IgM at different peptide densities**

The recoveries for three antibodies decreased or remained low with the increase of NaCl concentration at low (0.04 mequiv./g) and high (0.55 mequiv./g) peptide densities because of either the increased specific interactions or increased non-specific hydrophobic interactions. At low peptide density, addition of both MgCl₂ and CaCl₂ in the elution buffer facilitated the hIgA and hIgM elution resulting in better recoveries with increased salt
concentration because of the favorable interactions between divalent ions and the antibodies. At high peptide density, no increase in recoveries was observed because the salting in effect of MgCl$_2$ and CaCl$_2$ could not overcome the increased non-specific hydrophobic interactions. Therefore, salting-in salts can be used as elution additives for hIgA and hIgM elution at low peptide density.

The hIgG recovery remained high and was not affected by the increase concentrations of non-electrolyes at both low and high peptide densities. At low peptide density, hIgA recovery increased with the increase concentrations of ethylene glycol, urea and arginine because of the decreased hydrophobic interactions. However, hIgM recovery can only be increased by arginine at concentration larger than 0.2 M. At high peptide density, only hIgA recovery increased with the increase concentration of arginine. Therefore, ethylene glycol, urea and arginine can be used as elution additives for hIgA or hIgM elution at low peptide density.

7.1.5. Chromatographic isolation of human IgG, IgA and IgM from cMEM

Hexamer peptide ligand HWRGWV at four different peptide densities (0.04, 0.11, 0.22 and 0.55 mequiv./g) were used for antibody purification from cMEM spiked with 1 mg/mL of human IgG, IgA and IgM. The improved recovery at higher peptide density due to increased binding affinity was compensated by the decrease in purity for all three antibodies. Over 80% recovery and 90% purity were achieved for human IgG and IgA at optimum peptide densities. For hIgM, 75.7% recovery and 86% purity were achieved using HWRGWV at peptide density of 0.04 mequiv./g. The other two ligands HYFKFD and
HFRRHL from the same family of HWRGWV were also used for chromatographic isolation of human IgG, IgA and IgM. Higher purities but lower recoveries were achieved with HYFKFD for three antibodies compared to HFRRHL, suggesting better binding specificity and possible lower dynamic binding capacity. Although the performances of these two ligands are different, they are all comparable to that of peptide ligand HWRGWV. Therefore, as a family of linear hexamer peptide ligands, HWRGWV, HYFKFD and HFRRHL all have the potential for large-scale purification of hIgA and hIgM.

7.1.6. Purification of human immunoglobulins A, G and M from Cohn fraction II/III

Because of the binding affinity difference, we have achieved one-column purification of human immunoglobulins A, G and M from Cohn fraction II/III. Different pretreatment methods were used for contaminant removal before the chromatography step. After pretreatment with caprylic acid precipitation or the combination of caprylic acid and polyethylene glycol precipitation, highly purified (> 95%) hIgG was obtained with about 60% recovery, which is comparable to current chromatographic purification methods involving two chromatography steps when IgG is isolated from plasma fractions. H1gA enriched fraction with 42% hIgA and 56% hIgG as well as hIgM enriched fraction with 46% hIgM, 28% hIgA and 24% hIgG were obtained as the by-products. To our knowledge, it is the first time that an affinity chromatography column was used for the simultaneous capture and purification of human IgG, IgA and IgM from Cohn fraction II/III.
7.1.7. Affinity chromatographic purification of human immunoglobulin A from Chinese hamster ovary cell culture supernatant

The determined dissociation constants for hIgA with HWRGWV at three different peptide densities (0.11, 0.22 and 0.55 mequiv./g) fall in the range of $10^{-6}$ to $10^{-7}$ M, which are relatively smaller than those for hIgG suggesting stronger binding affinity. The maximum binding capacity determined by adsorption isotherms are 66.8, 86.4 and 93.8 mg/g for three different peptide densities, which are relatively smaller than those for hIgG leading to lower dynamic binding capacity as well. At the peptide ligand density of 0.11 mequiv./g, the dynamic binding capacities at 10% breakthrough are 16.8, 13.9 and 9.2 mg/mL at linear flow rates of 35, 87 and 173 cm/h, respectively. Although relatively lower DBC was obtained for IgA compared to IgG, the DBC value of HWRGWV for IgA is much greater than current commercially available affinity ligands such as Peptide M, CaptureSelect IgA and Immobilized Jacalin. About 95% recoveries and 90% purities were achieved for human IgA and secretory IgA purified from spiked Chinese hamster ovary cell culture supernatants suggesting that hexamer peptide ligand HWRGWV has the potential for large scale purification of IgA as well as secretory IgA.

7.1.8. Affinity chromatographic purification of human immunoglobulin M from human B lymphocyte cell culture supernatant

Hexamer peptide ligand HWRGWV, previously screened from a solid phase combinatorial peptide library for IgG purification, also demonstrated potential for large scale IgM purification. Different running conditions were employed in order to further improve the recovery and purity of IgM from spiked human B lymphocyte cell culture supernatant. The
final recovery and purity of the antibody is feedstock dependent, but can reach levels of both recovery and purity as high as 95%. The recovery is dependent on not only the total loading amount but also the initial IgM concentration, and the purity is dependent on the initial IgM percentage in the loading sample. At the peptide ligand density of 0.04 mequiv./g, the dynamic binding capacities at 10% breakthrough are 13.1, 7.2 and 4.6 mg IgM/mL resin at linear flow rates of 35, 87 and 173 cm/h, respectively. Although relatively lower dynamic binding capacity (DBC) was obtained for IgM compared to IgG because of the large molecular weight of IgM, the DBC value of HWRGWV for IgM is much greater than protein based affinity ligands found in the literature and competitive to previous and current commercially available affinity ligands such as KAPTIVE-M, CaptureSelect IgM and Ultralink Immobilized Mannan Binding Protein.

7.2. Recommendations for future work

7.2.1. Mass transfer properties for hexamer peptide ligand HWRGWV adsorbents

Hexamer peptide ligand HWRGWV has the potential to be attached to a large variety of different base matrices such as cross-linked agarose, surface modified porous glass, coated polystyrene, hydrogel filled into a ceramic shell and other materials based on organic polymers. Different adsorbents need to be compared for large-scale purification of antibody in terms of their binding capacities, mass transfer properties, process performance, and lifetime. Mass transfer properties are especially important in these comparisons since they often limit the overall rate of antibody adsorption and the productivity in capture applications [1].
Mass transfer properties of chromatography adsorbents can be studied by macroscopic methods. Isocratic pulse experiments are readily implemented using standard chromatographic equipment assuming that the adsorption isotherm is linear and the injected pulse is infinitesimal [2]. Batch adsorption methods are suitable for measuring mass transfer rates with nonlinear isotherms and at high adsorbate loadings. They generally require small amounts of adsorbent and protein and can yield data directly for the design of batch capture processes. The uptake kinetics can be measured in a finite [3] or infinite bath [4]. The first is typically a batch adsorption in an agitated vessel or in an rotated tube. The latter can be simulated in shallow bed reactor, a micro-column with only approximately 5-20 µL stationary phase [5]. Breakthrough curves and dynamic binding capacity (DBC) measurements are also applicable at high protein loadings and when the isotherm is nonlinear. Since the measurement is analogous to the operation of an actual capture column, the results can be used directly for design and scale-up [2]. Parameters characterizing the morphology of the stationary phase including particle diameter, intraparticle porosity, interstitial porosity as well as the pore size are required to fit the uptake kinetics, breakthrough and pulse curves by appropriate models assuming various mass transfer and kinetics resistance [1, 3, 5, 6]. In addition, the peptide ligand density is also an important parameter to help study the adsorption process especially in case of affinity chromatography [6].

7.2.2. Purification of SIgA and IgM by affinity membrane and monolith with immobilized hexamer peptide ligand HWRGWV

In membrane chromatography, transport of solutes to their binding sites takes place predominant by convection instead of diffusion happened in conventional column
chromatography, therefore reducing both process time and recovery liquid volume. The binding capacity is generally independent of the feed flow rate over a wide range and therefore very high flow rates may be used. The pressure drop is also significantly lower than column chromatography. Another major advantage of membrane adsorbers is the relative ease of scale up when compared with packed beds [7-9]. Also, membrane chromatography is particularly suitable for large proteins with molecular weight larger than 250 kDa since the surface area available for binding is larger for membranes than that of chromatographic media because these molecules rarely enter the small bead pores and only bind to the external surface area [8, 10]. As a result, the limitations in both capacity and resolution on traditional porous-particle-based chromatography media for large antibodies such as SIgA (300-350 kDa) and IgM (950 kDa) could be solved by membrane chromatography.

Another alternative to packed bed chromatography, which has certain similarities with membrane chromatography, is based on the use of monolith columns. These columns are prepared using rod-shaped porous structures through which convective flow of mobile phase can take place. The main advantages of monolith columns are similar to those for membrane chromatography. However, monoliths differ from membranes in terms of material of construction and morphology [11]. The combined use of monolithic supports with selective affinity as stationary phases has given rise to a new method know as affinity monolith chromatography (AMC) which has been used for affinity purification of a variety of proteins such as antibodies, coagulation factor VIII, lysozyme, lactoferrin, protein G, and others. [12, 13].

210
7.3. References


