ABSTRACT

EL OUAFIQ, SANAA. Running Conditions Optimization for Human IgA Chromatographic Purification Using HWRGWV Peptide Resin. (Under the direction of Dr. Ruben G. Carbonell.)

Human immunoglobulins A (hIgA) mediate a key role in mucosal immunity and are promising new immunotherapeutic candidates. Previous work has reported on the identification and characterization of the hexapeptide ligands HWRGWV at density 0.11 meq/g for the ability to purify human IgA.

In this thesis the running conditions were optimized for chromatographic purification of hIgA with HWRGWV resin in order to maximize the hIgA yield and purity. The influence of different equilibration buffers, intermediate wash buffers , as well as initial CHO supernatant concentration on the chromatographic purification of human IgA using HWRGWV resin were investigated. Experiments proved that phosphate buffer saline (PBS) with 1M NaCl equilibration buffer without any intermediate wash step resulted in high both recovery (96%) and purity (90.3%) when using spiked CHO supernatant with IgA 1.5mg/ml as starting material. The concentration of the starting material seemed to play an important role in human IgA purity.

The purity decreased from 90.3 % to 48.3% when using the spiked samples and the 100 times concentrated CHO supernatant, respectively. Furthermore the purity increased by 6.5% and recovery decreased by 7.5% with addition of an intermediate wash step using acetate buffer. More than 96% purity of hIgA was achieved when adding agents such ethylene glycol, urea

and arginine to the intermediate wash buffer, however, the improved purity was compensated by the decrease in recovery (<70%).

This study demonstrates that using PBS + 1M NaCl as equilibration buffer without a post wash step to purify human IgA from relatively high starting CHO supernatant IgA concentration is the optimal running conditions in HWRGWV peptide resin based chromatography purification of human IgA.

Running Conditions Optimization for Human IgA Chromatographic Purification Using HWRGWV Peptide Resin

by
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BIOGRAPHY

Sanaa El Ouafiq was born on February 26th, 1984. She was raised in Casablanca, Morocco, graduated in 2002 from High School. She entered Ben M'sik, Hassan II University that autumn for her BS degree in Organic Chemistry and attended the same University for Master degree in Quality Control. After graduation she worked as a quality assurance responsible at pharmaceutical industry. Two years later, the author moved to North Carolina, USA and joined Biomanufacturing Training and Education Center at Carolina State University to pursue her Master of Science degree in Biomanufacturing.

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I. INTRODUCTION

Over the past 20 years, therapeutic monoclonal antibodies (mAb) have become a significant addition to the pharmaceutical repertoire. Monoclonal antibodies now hold an established role in basic biomedical research, in diagnosis of disease, and in treatment of diseases, such as various forms of cancer. Monoclonal antibodies are important tools used by many investigators in their research, have led to many medical advances and promise to play an even more significant role in the future of pharmaceutical intervention in diseases [1,2-3].

The market for therapeutic monoclonal antibodies (mAbs) is one of the most dynamic sectors within the biotechnology industry. The global market for therapeutic monoclonal antibodies (mAbs) was estimated at $44.6 billion in 2010. With the rollout of at least eight new therapeutic mAb products and expanded indications for existing products expected during the forecast period, the global mAb market is expected to rise to nearly $62.3bn in 2015. The U.S. is projected to be the largest single market for therapeutic mAbs from 2011 to 2016. This particular market was nearly $19.8 billion in 2010 and reached $20.1 billion by 2011 this market will grow to $27.4 billion by 2015 [3-7].

Monoclonal antibodies are key elements of new treatment option. Therefore, many current research and development studies for new drugs are based on these molecules. Structurally, monoclonal antibodies (mAbs) are composed of two variable regions that contain a specific binding site and constant regions that control other functions, including complement

binding and antibody mediated cytotoxicity [4-5]. The diverse repertoire of immunoglobulin genes allows for production of various potential therapeutic agents [8].

The immunoglobulin can be divided into five different classes (IgA, IgM, IgG, IgE and IgD) based on differences in the amino acid sequences in the constant region of the heavy chains. The antibodies may have specific target binding and different functions depending on the immunoglobulin class [4-8]. The human IgG is the most widely used subclass, because it has well defined biochemical structure and an extended plasma half life, it activates human complement effectively and recruits Natural Killer NK cells for Antibody Dependent Cellular Cytotoxicity ADCC [6]. However IgA is the most abundantly generated antibody isotype and forms the first line of immune defense [16]. The human IgA exists in monomeric, dimeric and secretory isoforms (Figure 1-1) but this thesis is focused on the monomer hIgA isoform with molecular weight of 160 kDa.

The human IgA has a specific polymeric immunoglobulin receptor mediated transport mechanism for entry into the secretions and may inhibit attachment of microbes to the luminal surface of the mucosal epithelial lining [8]. In addition, experimental studies show that hIgA antibodies can neutralize viruses intracellularly if a virus is infecting an epithelial cell [6, 9-16]. Furthermore, human IgA antibodies can provide an internal mucosal barrier beneath the mucosal lining, bind to receptors on a variety of leukocytes and have been shown, in some experimental studies, to be capable of activating the alternative

complement pathway. Human IgA antibodies are a potential candidate in research and development of new mucosal vaccines and therapeutic drugs [6, 8-9].

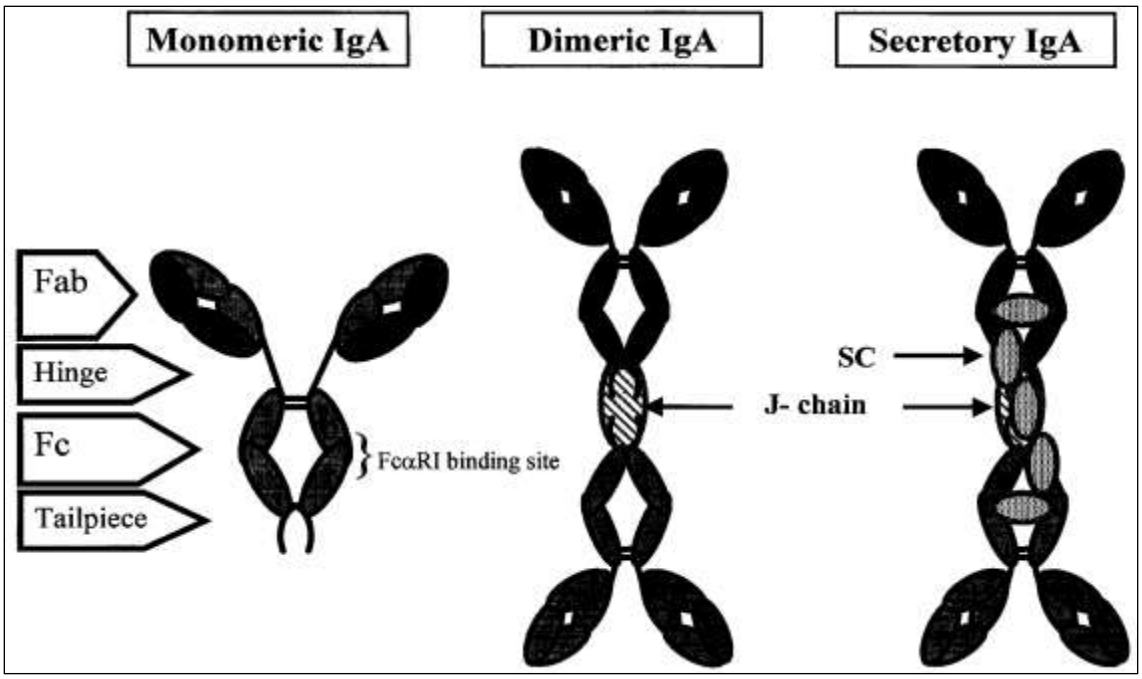

Figure 1-1 IgA monomeric, dimeric and secretory isoforms [8]

Downstream processing of biopharmaceuticals is the series of operations that take a feed stream from an upstream process and produce a purified bulk drug. It plays a major role in overall drug production and dictates a significant proportion of the total manufacturing costs. Downstream processing has often involved multiple steps with significant time and energy expended on maximizing product quality and yield. However the major concern of today's developer in this field is to minimize the increasing costs of downstream processing [10].

A variety of preparative modes of chromatography have been employed for the purification of mAbs based on their specific physical and chemical properties, such as size, solubility, charge, hydrophobicity and binding affinity [11]. However, affinity chromatography based purification continues to be the most efficient and commonly employed. Affinity chromatography is one of the simplest and most effective methods for purifying protein therapeutics, offering reduced process steps and therefore higher yields than non affinity methods can provide [12]. The effectiveness of affinity chromatography is largely based on the binding characteristics of the required antibody and the ligand used for antibody capture [12-13].

Most schemes have involved the use of Protein A affinity chromatography. Protein A chromatography is highly selective for mAbs where binding is usually achieved at physiological pH values. Acidic conditions of approximately pH 3 are usually needed for elution [11]. Protein A chromatography permits a generic approach that simplifies process development and supports purification platform development. However the demand for cost efficient production processes has led to the necessity of optimization of the downstream purification. Thus eliminating a protein A step from downstream process would overcome the known drawbacks of protein A chromatography such as high resin costs, protein A leaching and the formation of aggregates due to the acidic conditions needed for elution. Clearly, there is a significant incentive for the development of new alternative ligands to protein A. Ligands are essential components in affinity chromatography, as they play a major role in the specificity and stability of the system.

There are four ligands based affinity chromatography that can be used in antibodies purification:

Biospecific ligands, Pseudobiospecific ligands, Synthetic ligands and Affinity tags.

Biospecific ligands (Protein A and G) are a powerfully selective method for antibodies purification that represent a group of naturally derived substances such as antibody binding proteins, bacterially derived receptors, antigens, lectins or anti-antibodies directed to the antibody requiring purification [13,14-15].

Pseudobiospecific ligands exploit intrinsic properties such as hydrophobic and thiophilic properties of the immunoglobulin at the molecular level. They are promising candidates for antibodies purification, relatively inexpensive, more robust, structurally simple, less toxic and highly stable. However their affinity is generally lower compared to the biospecific ligands but it is sufficient to ensure selectivity towards antibody molecules [13, 14-15].

Synthetic ligands are developed affinity ligands specifically for an antibody which has high recycling options. Synthetic ligands are great potential alternatives to traditional biospecific affinity ligands for large-scale antibody purification. They can be tailored for specificity, affinity and tuned depending on the target [13-14].

Affinity tags can be short polypeptide sequences or whole proteins. Ligands specific for the tags are used for the purification of the protein tag enabling single step purification of the

target protein. Affinity tags also offer an attractive approach for monoclonal antibodies purification by ensuring proper orientation of the antibody [14-15].

Extensive effort has been spent on examining alternative synthetic peptide ligands with varying selectivity and complexity by bioseparation group of Dr. Ruben Carbonell (Chemical Engineering Department, North Carolina State University). HWRGWV synthetic peptide ligand has been reported to be selective for antibody purification, due to its unique interaction characteristics, allowing the reliable separation of antibodies from crude mixtures. Since the discovery of the effectiveness of HWRGWV peptide ligand based affinity chromatography for IgG purification, IgA purification was also studied by the same group using the same peptide. For IgA purification, various factors such as pH, ionic strength, temperature, presence of competing species and flow characteristics, have been exploited to improve the purification procedure. Basically, affinity chromatography relies on four essential steps, preparation of absorption media, loading, washing and elution to achieve maximum yield and purity. The aim of this study is to determine the optimal running conditions to purify human IgA from CHO cell supernatant by affinity chromatography with HWRGWV peptide ligand selected from the screening of multiple synthetic peptides library.

II. BACKGROUND OF STUDY

Currently the purification of monoclonal antibodies for therapeutic purposes is reliant on protein A affinity chromatography. The rapid growth of this class of therapeutic and their high value makes the screen and study of an alternative methods to protein A an urgent necessity. Multiple synthetic solid phase combinatorial linear hexamer peptide ligands were screened for their affinity to immunoglobulin subclasses by a radiolabeled-screening technique. HWRGWV demonstrated the strongest binding affinity to hIgM followed by hIgA and hIgG respectively. Furthermore this peptide ligand exhibits a protein A comparable affinity to bind to hIgG through its Fc portion and interacts with the amino acids SNGQPEN in the loop Ser383 – Asn389 [18].

A 85% purity and 94% recovery was achieved in IgG purification process from two different commercial CHO cell culture media using HWRGWV ligand under its optimal density (approximately 0.11 meq/g) [21]. HWRGWV was also able to purify hIgG with purity and recovery higher than 95% under the same conditions from complete minimum essential medium (cMEM) containing 10% fetal calf serum and 5% tryptose phosphate broth [19-20].

After demonstrating that the ability of HWRGWV ligand to purify IgG by binding through the Fc portion is comparable to protein A or G affinity chromatography and based on preliminary experiments which indicate that IgA bound very well to HWRGWV resin, a similar study was conducted on IgA purification using the same peptide resin. Five

potential binding sites for hIgA (B1–B5) were found in its heavy chain constant regions by using the amino acid sequence SNGQPEN as a reference. The mechanism of interaction between HWRGWV with IgA was investigated and, as a result, it has been determined that the electrostatic interactions and N-terminal histidine charge of HWRGWV ligand play important role in hIgA affinity chromatography purification.

The effects of different electrolytes and non electrolytes elution additives as well as the effects of peptide density and elution solution pH on human IgA purity and recovery were also investigated. High recovery and purity of hIgA were achieved at HWRGWV peptide resin density approximately 0.11meq/g and by using elution pH lower than 4 [17]. Although the present paper explores the effects of different running conditions on chromatographic purification of hIgA from concentrated CHO supernatant using HWRGWV peptide ligand in terms of hIgA purity and recovery.

III. PROJECT GOALS

The objective of this thesis is to propose the most suitable running conditions in terms of equilibration buffer, intermediate wash buffer and starting material concentration to be used during the affinity chromatography purification of immunoglobulin A with HWRGWV synthetic peptide ligand in order to achieve the desired purity and recovery of the Immunoglobulin A at the end of the purification process. Human IgA (hIgA) is a very important therapeutic antibody. In order to obtain low cost, high efficiency and less risk human IgA, affinity chromatography purification step using a synthetic peptide resin HWRGWV has been developed and need to be optimized.

In this thesis, a study of the performance of affinity chromatography purification of human IgA with HWRGWV peptide resin in terms of IgA purity and recovery will be conducted by using different equilibration and intermediate wash buffers. Multiple experiments will be performed using different equilibration buffers that consist of acetate buffer by itself or with addition of salt such as NaCl or some other agents such as urea, ethylene glycol and arginine. These equilibration buffers will be used for two different starting CHO supernatant concentrations. Based on this two first experiments results, the optimal equilibration buffers will be used to run a third experiment which includes the investigation of high concentration starting material by using spiked CHO supernatant with pure hIgA as well as the evaluation of the intermediate wash buffers in order to improve the purity of eluted hIgA.

At the end of the project, the generated results will be analyzed and discussed to identify the optimal loading and post washing conditions beside the optimal starting material concentration.

IV. EXPERIMENTAL METHODS

4.1. Chinese hamster ovary (CHO) cell culture

A Chinese hamster ovary (CHO) cell line from Dr. Sherine L. Morrison laboratory, department of Microbiology, Immunology and Molecular Genetics at the University of California, was used as the host cell for the expression of recombinant IgA. Human IgA containing supernatants were generated by cultivation of CHO cell lines at 37 °C and 5% CO_2 in culture flasks using Eagles Minimum Essential Media (EMEM) from Quality Biologicals (Gaithersburg, MD, USA) with 10% fetal calf serum (FCS) from Hyclone (Logan, UT, USA) and 5% tryptose phosphate broth (TPB) from Becton Dickinson (Sparks, MD, USA). Recombinant cell lines were cultured for at least 10 passages to monitor growth rate, stability of product expression and cell viability.

4.2. CHO cell supernatant samples preparation

The CHO cell supernatant samples were separated from the cells by centrifugation at 2000 ×g for 10 minutes. The supernatant was then filtered using 0.22 µm syringe filters from EMD Millipore Corporation (Billerica, MA, USA) before testing for total protein concentration. The CHO cell supernatant samples containing 0.149 ± 0.051 µg/ml of hIgA were concentrated 20 and 100 times using ultrafiltration filters with nominal molecular weight limit of 50K and 100K, respectively in experiment 1. In experiment 2 the samples were filtered using ultrafiltration membranes (100K NMWL, 44.5 mm). However, CHO supernatant samples were spiked with1.5mg/ml pure hIgA (>95%) purchased from Fitzgerald (Concord, MA, USA) to obtain high hIgA starting concentration in experiment

3. Filters and membranes were purchased from EMD Millipore Corporation (Billerica, MA, USA).

4.3. HWRGWV peptide ligand synthesis

The peptides, N-terminal acetylated HWRGWV was synthesized at a density of 0.11 meq/g from Toyopearl AF-Amino-650 M (particle size 65µm) by Tosoh BioScience (Montgomeryville, PA, USA) using Fluorenyl methyl oxycarbonyl (Fmoc) chemistry from Peptides International, Inc.(Louisville, KY, USA).

4.4. HWRGWV ligand based chromatographic capture and elution of human IgA

In all chromatography experiments, the peptide resin was dry packed in 30 mm × 2.1 mm I.D. microbore columns (0.1mL) from Alltech (Deerfield, IL, USA) and expanded in 20% methanol at 0.05 mL/min for 2 hours followed by HPLC grade water and Phosphate-buffered saline (PBS) washes. Before sample loading, columns were equilibrated with pH 7.4 equilibration buffer consisting of PBS + 1 M NaCl. PBS pH 7.4 contains 0.01 M phosphate, 0.138 M sodium chloride, and 2.7 mM potassium chloride were obtained from Sigma (St. Louis, MO, USA). Samples were manually injected and loaded at 0.05 mL/min for 10 min, 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 elution buffer, and 6 mL 6M guanidine–HCl to regenerate the column, followed by the equilibration buffer. Different running conditions were performed using multiple equilibration and intermediate wash buffers (Table 4-1 and 4-2). For all experiments 0.2M acetate buffer (AC) at pH 3 was used as the elution buffer. Human IgA samples were prepared as

described in section IV.2 and injected to equilibrated column. The HPLC running protocol was used as described above and the runs were performed reproducibly. The eluted fractions were collected and immediately adjusted to neutral pH by adding an appropriate volume of 1M Tris buffer pH 8.0 purchased from Fisher Scientific (Pittsburgh, PA, USA). All runs were performed on a Waters 626LC system with a 2487 UV detector and the effluent was monitored by absorbance at 280 nm. To compare the chromatographic properties of each condition, multiple HWRGWV peptide ligands based chromatography purification were carried out in each experiment, differing only with respect to the loading buffer, intermediate wash buffer or starting concentration used to load the samples.

4.5. Human IgA characterization assays

4.5.1. ELISA

Human IgA was quantified by sandwich ELISA using ELISA kit from Alpha Diagnostic International (San Antonio, TX, USA). A 200-300 µl of working wash solution was added to appropriate number of microwell strips for 5 minutes before discarding the liquid wash. The wells were then dried on a paper towel. In pre-determined wells, a 100 µl aliquot of standards, samples and controls was added and incubated for 60 minutes. An automatic plate washer was used to wash the wells 4 times before drying on fresh paper towel. After the wells were dried, a 100 µl aliquot of working Anti-Human IgA-HRP conjugate was added to each one and incubated for 30 minutes before being washed 5 times. A 100 µl of TMB substrate was added to each well and incubated for 15 minutes in the dark. A stop solution was added (100 µl) to stop the enzyme reaction and the plate was read using a microplate reader. Human IgA concentrations in the unknown samples and controls were

determined by interpolation from the standard curve that was generated using hIgA antibody samples at known concentrations.

4.5.2. SDS-PAGE

The collected fractions were concentrated 10 times by centrifugation at 14,000 ×g for about 10 min using Microcon YM-3 filters (regenerated cellulose, 3000 MWCO) purchased from Millipore (Billerica, MA, USA) before the electrophoresis analysis. Concentrated fractions 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 SuperLockTM Mini-Cell system from Invitrogen (Carlsbad, CA, USA). The gels were stained by SimpleBlue SafeStain and human IgA purity was determined by densitometric measurement using software ImageJ 1.32j (National Institute of Health, Bethesda, MD, USA).

4.5.3. BCA assay

Protein concentrations before and after chromatographic purification of hIgA were measured by the bicinchoninic acid (BCA) assay by using BCA Protein Assay Kit from Thermo Scientific (Rockford, USA) with a human BSA calibration curve.

4.5.4. Size Exclusion Chromatography (SEC)

A Shimadzu HPLC system was used to perform a size- exclusion chromatography analysis for hIgA eluted fractions from 100 times concentrated CHO supernatant samples generated in experiment 2. Samples diluted in phosphate buffer saline (PBS) were run in isocratic mode through an inert particles packed into a steel 150mm x 7.8mm column at 1ml/min flow rate. Absorbance was monitored at 280 nm.

Table 4-1 Running conditions of human IgA affinity chromatography purification with HWRGWV peptide ligand from 20 and 100 times concentrated CHO supernatant.

Running conditions	Equilibration buffers	Post wash	Elution
1	PBS + 1M NaCl	N/A	0.2M AB pH 3
2	PBS + 1M NaCl	0.2M AB pH 5.2	0.2M AB pH 3
3	0.2M AB pH 5.2	N/A	0.2M AB pH 3
4	0.2M AB pH 5.2 + 10% EG	N/A	0.2M AB pH 3
5	0.2M AB pH 5.2 + 0.2M urea	N/A	0.2M AB pH 3
6	0.2M AB pH 5.2 + 0.1M arginine	N/A	0.2M AB pH 3

AB: Acetate Buffer
EG: Ethylene Glycol

Table 4-2 Running conditions of human IgA affinity chromatography purification with HWRGWV peptide ligand from spiked CHO supernatant with 1.5mg/ml pure IgA.

Running conditions	Equilibration buffers	Post wash	Elution
1	PBS + 1M NaCl	N/A	0.2M AB pH 3
2	PBS + 1M NaCl	0.2M AB pH 5.2	0.2M AB pH 3
3	PBS + 1M NaCl	0.2M AB pH 5.2 + 10% EG	0.2M AB pH 3
4	PBS + 1M NaCl	0.2M AB pH 5.2 + 0.2M urea	0.2M AB pH 3
5	PBS + 1M NaCl	0.2M AB pH 5.2 + 0.1M arginine	0.2M AB pH 3

V. RESULTS AND DISCUSSION

5.1. Effect of running conditions on purity and recovery of human IgA purification from CHO supernatant

5.1.1. CHO supernatant samples filtered using ultrafiltration filters

The effects of different loading conditions on recovery and purity of human IgA purification using HWRGWV peptide ligand affinity chromatography from CHO supernatant was determined by using six different loading buffers (Table 4-1). The CHO supernatant samples were concentrated 20 times and 100 times by ultrafiltration filters with nominal molecular weight limit of 50K and 100K respectively. Human IgA subclasses have isoelectric points in the range between 4 and 7.1 and are negatively charged at all used binding conditions pH 7.4 except when the loading buffer pH is 5.2 (see condition 3 in Table 4-2) when the hIgA may be either negatively or positively charged.

HWRGWV peptide ligands (pI=9.85) are positively charged at all loading pH conditions, hence the opposite charges on HWRGWV ligands and human IgA (in most loading conditions) in turn suggesting that there are some electrostatic interactions between hIgA and the peptide which may explain the higher affinity of this ligand to hIgA.

The starting human IgA concentration in the CHO supernatant was 0.149 ± 0.051µg/ml making the concentration of hIgA in 20 times and 100 times concentrated samples to be approximately 2.267±0.639µg/ml and 11.22± 3.168µg/ml, respectively. Figures 5-1 and 5-2 show the chromatograms and SDS-PAGE results from the six different loading conditions runs using 20 times and 100 times concentrated CHO supernatant. It can be observed from the chromatograms that the flow through and eluted peaks differ from a

loading condition to another and from the SDS-PAGE results that the BSA band still existed in all purified hIgA samples using the six different running conditions.

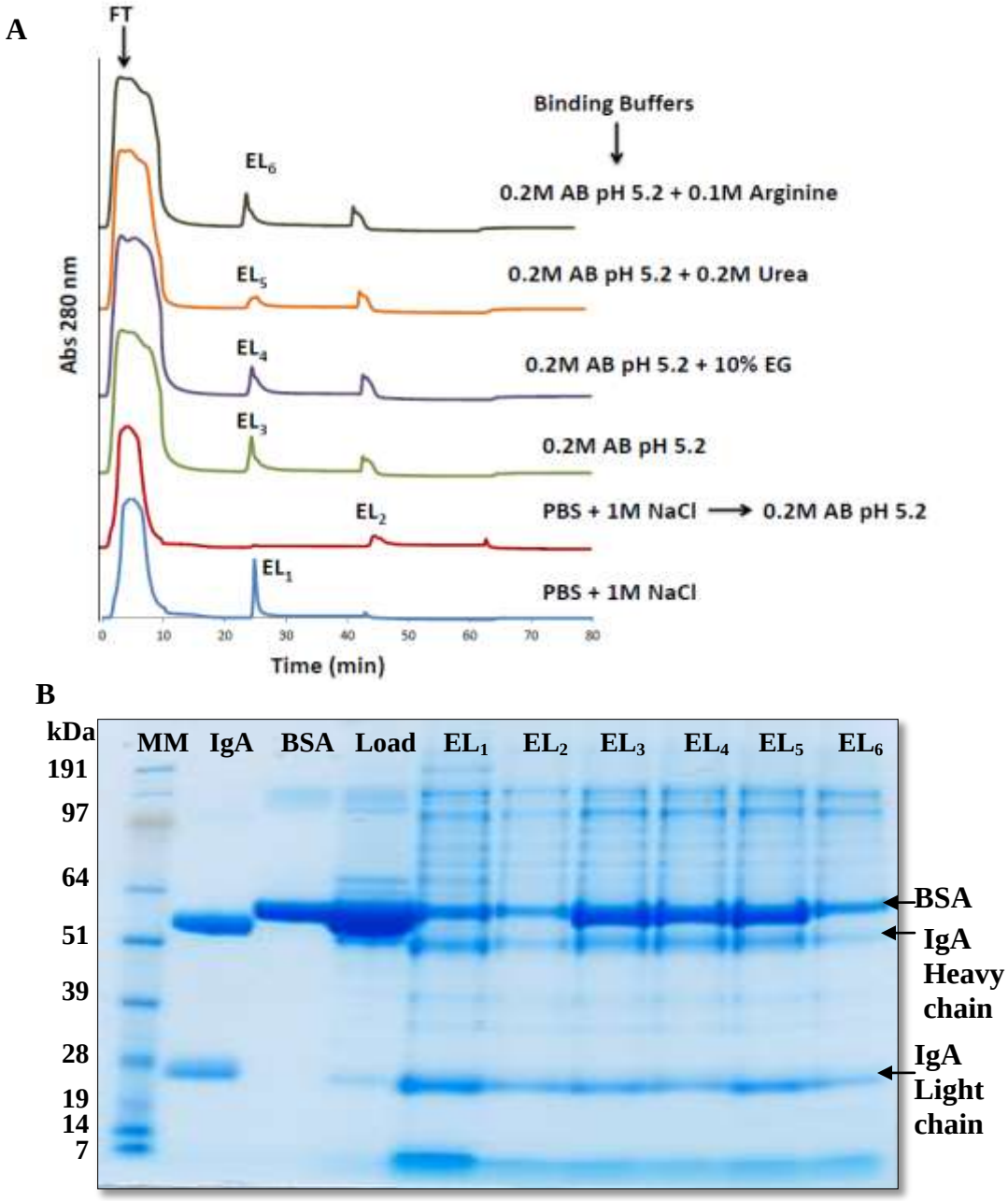

Figure 5-1 (A) Chromatograms of purification of human IgA from 20 times concentrated CHO supernatant using ultrafiltration filters with HWRGWV resin under different loading conditions. (B) SDS-PAGE of elution fractions under reducing conditions.MM-Molecular weight marker; EL-elution fraction.

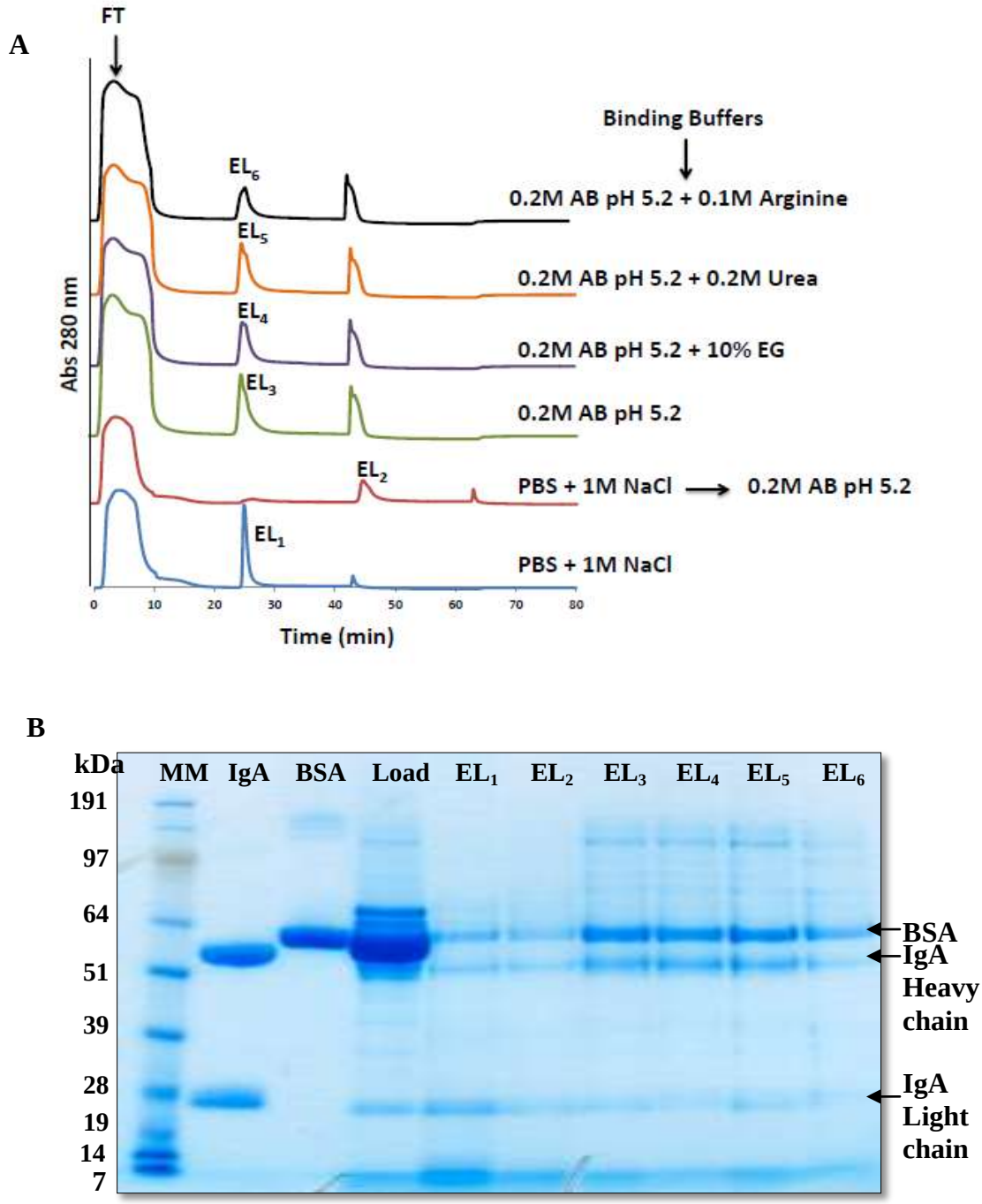

Figure 5-2 (A) Chromatograms of purification of human IgA from 100 times concentrated CHO supernatant using ultrafiltration filters with HWRGWV resin under different loading conditions. (B) SDS-PAGE of elution fractions under reducing conditions.MM-Molecular weight marker; EL-elution fraction.

The percentage recoveries and purities of eluted human IgA generated from each experiment are shown in figures 5-3 and 5-4. The results show that the yield and purity for both experiments (20 times and 100 times concentrated CHO supernatant) varied depending on the loading conditions have been used. The yield of hIgA was >70% for 20 times concentrated CHO supernatant and >90% for 100 times concentrated CHO supernatant under the following running conditions: 1) PBS + 1M NaCl without post wash step and 2) PBS + 1M NaCl with 0.2M AB pH 5.2 post wash step. NaCl plays an important role in the majority of column chromatography processes for protein purification and it can be used to facilitate binding and elution of proteins, or suppress non-specific protein-protein or protein-surface interactions [19]. In this experiment NaCl prevents the binding of BSA to the amino groups in the resin, making HWRGWV peptide groups available for hIgA binding, thereby increasing the binding capacity, in turn explaining the high recoveriy of hIgA. The non-specific binding of BSA is electrostatic, and is mostly due to presence of free amino groups that are formed during the solid phase synthesis of peptide on the polymethacrylate-based Toyopearl Amino resin. These results confirm the role of NaCl during the capture step of the hIgA purification.

The recoveries of hIgA using running conditions 4, 5 and 6 were less than the generated yield using the two previous conditions. The ethylene glycol is protein stabilizing and has the ability to suppress hydrophobic interactions by reducing the mobile phase dielectric constant. Urea weakens hydrophobic interactions and breaks hydrogen bonds [19, 20-21]. Arginine has the ability to suppress aggregation, hydrogen bonds and hydrophobic

interactions, but augments conductivity and thereby suppresses electrostatic interactions as well [22-23].

As results a similar effect of urea compared to ethylene glycol was observed on hIgA recoveries. The relatively high recoveries generated by using urea and ethylene glycol 70% (20 times samples) and 80% (100 times samples), might have been achieved by decreased hydrophobic interactions. However, when using arginine, the decrease in electrostatic interactions does not have the same impact on yield as the decrease of hydrophobic interactions leading to low recoveries 55% (20 times samples) and 65% (100 times samples) compared to recoveries generated from loading buffers containing urea and ethylene glycol.

A 0.2 M acetate buffer pH5.2 was used as equilibration buffer by itself in condition 3 without addition of any salt or agents. The recovery results in this experiment were approximately 65% for 20 times concentrated CHO supernatant and 80% for 100 times concentrated samples. It can be observed (Figures 5-3 and 5-4) that by adding NaCl, urea or EG the yield improved while adding arginine reduced it.

Purity results varied depending on the running conditions exactly as the yield but at low percentages. Running conditions 1 and 2 represent the highest purity achieved during those runs, approximately 45 % for 20 times concentrated CHO supernatant and 55% for 100 times concentrated CHO supernatant. However, the purity of hIgA samples generated from running conditions 3, 4, 5 and 6 was approximately 40%.

The BCA results (Table 5-1) demonstrated that the CHO supernatant has a relatively higher total protein concentration up to 62% in 20 times concentrated samples and 30.8% in 100

times concentrated samples while hIgA concentrations represent only 0.008% and 0.016%, respectively. These results confirmed the low purity of the purified samples from the runs and doubted the effectiveness of the ultrafiltration methods used to concentrate the CHO supernatant, since the BSA which is the major contaminant protein in this process was existed in high percentages after the ultrafiltration step.

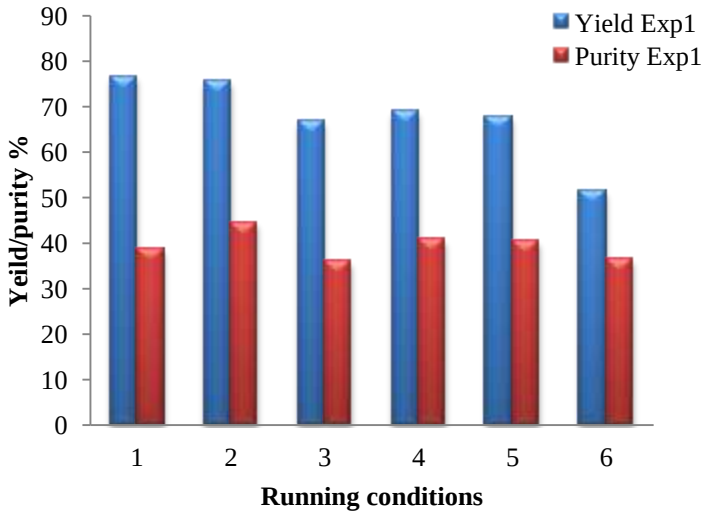

Figure 5-3 Yield and purity of purified IgA from 20 times concentrated CHO supernatant by ultrafiltration filters using affinity chromatography purification with HWRGWV resin under six different loading conditions

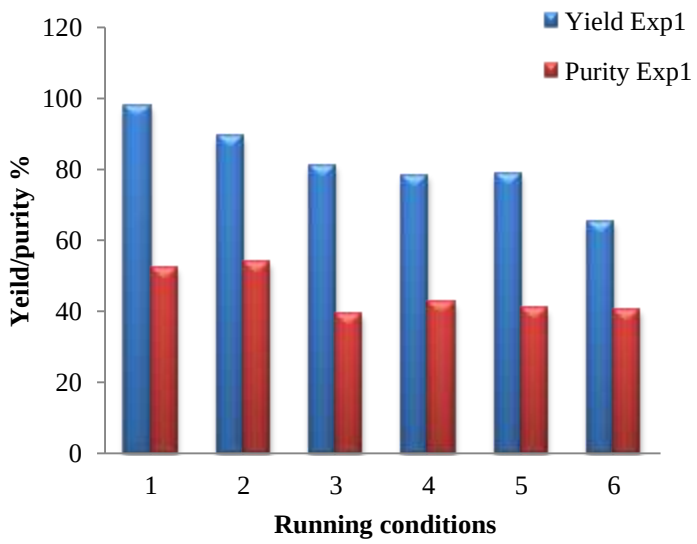

Figure 5-4 Yield and purity of purified IgA from 100 times concentrated CHO supernatant by ultrafiltration filters using affinity chromatography purification with HWRGWV resin under six different loading conditions

Table 5-1 Human IgA concentration /recovery and total protein concentration / recovery in different loading mixtures concentrated by ultrafiltration filters.

Loading Material	IgA (µg/ml)	Recovery% (IgA)	Total Protein (µg/ml)	Recovery % (Total protein)	IgA%
CHO supernatant 20 times	0.15 ± 0.051	100.0	2319	100.0	0.005
CHO supernatant 100 times	2.27 ± 0.639	76.1	28740	62.0	0.008
CHO supernatant	11.22 ± 3.168	75.3	71315	30.8	0.016

5.1.2. CHO supernatant samples filtered using ultrafiltration membrane

In order to improve the purity achieved from the previous runs, the CHO supernatant samples were concentrated using ultrafiltration membranes (100K NMWL, 44.5 mm) instead of ultrafiltration filters. The BCA results for this experiment (Table 5-2) show a decrease in the total protein concentration from 62.0% to 19.4% for the 20 times concentrated samples and from 30.8% to 9.9% for 100 times concentrated samples and increase in the hIgA concentration from 0.008% to 0.027% and from 0.016% to 0.041% for 20 times and 100 times concentrated samples, respectively.

The purity results for this experiment were not as expected, as there was no apparent improvement of purity for this set of running conditions comparing to the previous runs results (samples concentrated using ultrafiltration filters) even if the total protein concentration (BCA) decreased and the hIgA concentration increased (as per ELISA results) after using the ultrafiltration membrane. Figures 5-5 and 5-6 show the comparison of yield and purity results generated in the first and second experiments and it is clear that there was a minimal impact of using ultrafiltration membrane on the purity and yield of human IgA purification. This experiment confirms the results of previous running conditions in terms of the optimal loading conditions. The loading buffers containing NaCl used for hIgA purification generate higher relative yield and relative purity than the other four running conditions.

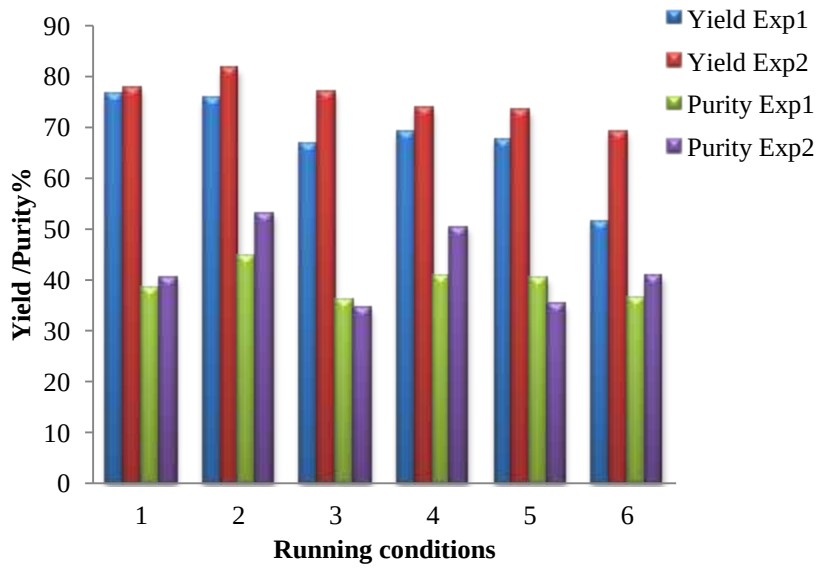

Figure 5-5 Comparison of human IgA yield and purity from 20 times concentrated CHO supernatant in experiments 1 and 2. Experiment1- CHO supernatant was concentrated 20 times using ultrafiltration filters; Experiment 2- CHO supernatant was concentrated 20 times using ultrafiltration membrane.

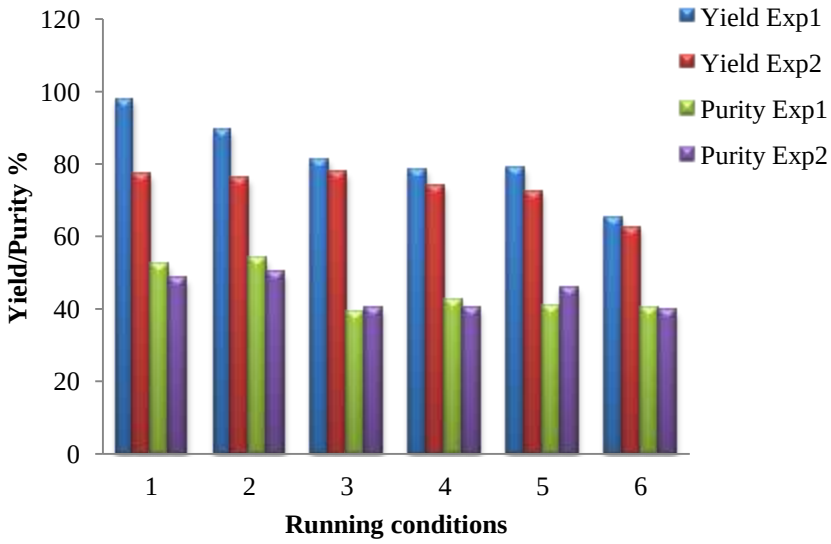

Figure 5-6 Comparison of human IgA yield and purity from 100 times concentrated CHO supernatant in experiments 1 and 2. Experiment1- CHO supernatant was concentrated 100 times using ultrafiltration filters; Experiment 2- CHO supernatant was concentrated 100 times using ultrafiltration membrane.

In order to assess and troubleshoot the purity problem in these runs, a SEC assay was conducted on purified fractions of hIgA from 100 times concentrated CHO supernatant. The purity of the eluted fractions was verified by analyzing the generated size-exclusion chromatograms. Figures 5-7, 5-8 and 5-9 illustrated the chromatograms from analyzing CHO supernatant, pure hIgA, BSA, sample 1 (purified sample using running condition 1) and sample 2 ((purified sample using condition 2). The chromatogram in figure 5-7 distinguishes the BSA peak at 8.5 min, the BSA dimer at 7.5 min and the CHO supernatant peaks at 8.5 min(BSA) ,at 7.5 min (BSA dimer), at 11.5 light chain human IgA and and other contaminants at 13 minutes elution time.Figures 5-8 and 5-9 show the generated chromatograms from analyzing the sample 1 and 2, respectively. Since the molecular weight of hIgA is 160kDa, its peak should shows up in the very beginning of the chromatogram but in all analyzed samples we did not see any hIgA peak, also the hIgA heavy chain (approximately 50kDa) should shows up right after the BSA (66kDa). This result may be explained by the amount of hIgA in the samples that was undetectable by the system. Therefore it was impossible to determine the purity based on this method. However both chromatograms generated from sample 1 and 2, were similar to the pure hIgA chromatogram, while the BSA and other contaminants peaks were smaller when compared to the CHO supernatant chromatogram. The purification of hIgA using this process has hence removed majority contaminants that remained after the ultrafiltration step. The SEC results confirms the comparable purity of the eluted hIgA to the pure hIgA (>95%).

Based on previous studies that have been done on the purification of hIgG using the same peptide ligand, the starting concentration of the paste can affect the yield and the purity of final product. The starting materials concentration used in previous experiments was ralatively low even after concentrated the samples 100 times, due to the low initial concentration of hIgA produced by the CHO cell lines used in this study. Therefore another experiment should be executed using relatively high starting materials concentration and evaluating different intermediate wash buffer to improve human IgA purity.

Table 5-2 Human IgA concentration /recovery and total protein concentration / recovery in different loading mixtures concentrated using ultrafiltration membrane.

Loading Material	**IgA (µg/ml)**	**Recovery% (IgA)**	**Total Protein (µg/ml)**	**Recovery % (Total Protein)**	**IgA%**
CHO supernatant	0.18 ± 0.039	100.0	2662	100.0	0.008
20 CHO supernatant	2.82 ± 0.60	78.3	10317	19.4	0.027
100 CHO supernatant	10.95 ± 2.68	60.8	26481	9.9	0.041

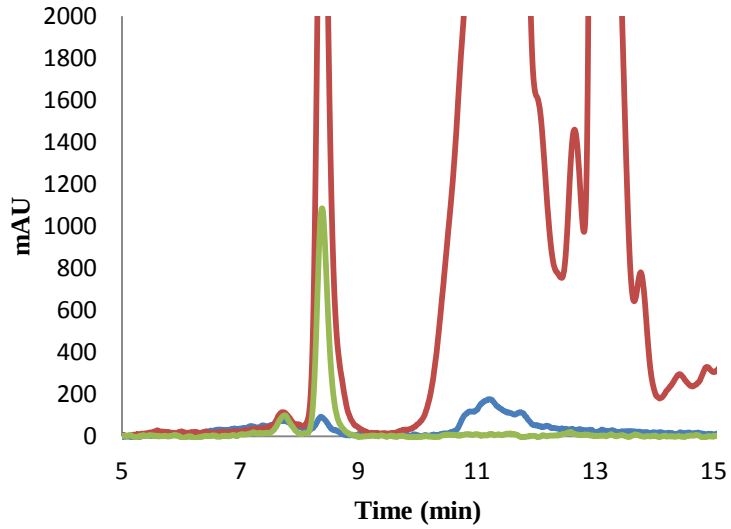

Figure 5-7 Size-exclusion chromatograms at 280nm of the CHO cell supernatant (——), pure 95% hIgA (——), and BSA (——).

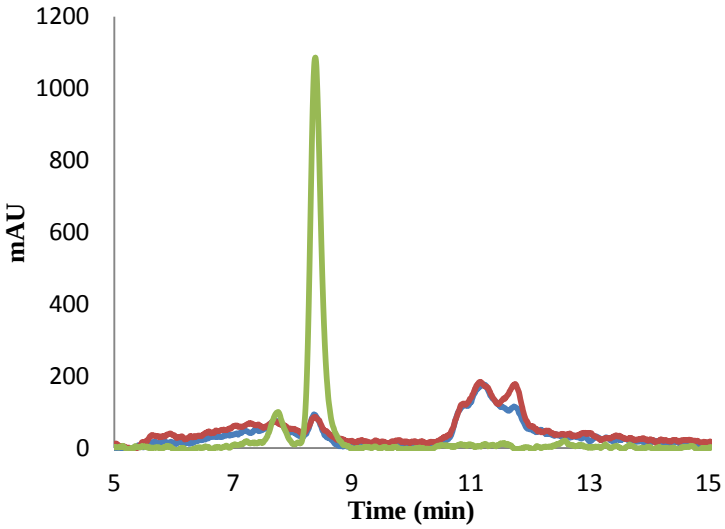

Figure 5-8 Size-exclusion chromatograms at 280nm of the purified sample1 (——), pure 95% hIgA (——), and BSA (——).

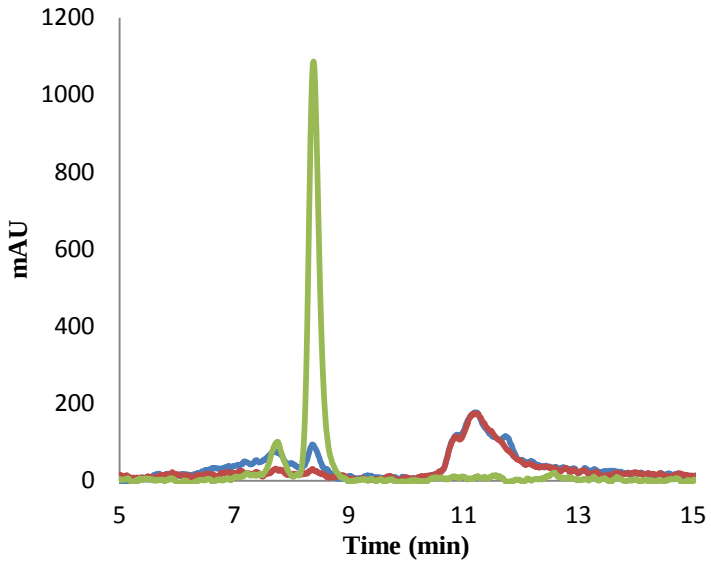

Figure 5-9 Size-exclusion chromatograms at 280nm of the purified sample 2 (—), Pure 95% hIgA (—), and BSA (—).

5.2. Effect of running conditions on purity and recovery of human IgA purification from CHO supernatant spiked with pure human IgA

Based on the previous experiments results, conditions using PBS+1M NaCl as equilibration buffer without a post wash step and PBS + 1M NaCl as equilibration buffer followed by 0.2M AB pH 5.2 as intermediate wash buffer were optimal for hIgA purification using HWRGWV peptide ligand. In this last experiment batch studies were carried out to investigate both the effect of high concentration of starting materials and different intermediate wash buffers (as described in Table 4-2) for human IgA affinity chromatography purification using HWRGWV peptide ligands. In order to check if the purity of hIgA containing fractions could be further increased, a post wash step was used in each running condition except the running condition 1.

The non-specific binding HWRGWV peptide ligand was already proven and it is due to either ionic or hydrophobic interactions. The aim of this experiment is to modify the post-load wash buffer in such a way as to disrupt these interactions, which will elute the non-specifically bound contaminants (BSA) without prematurely eluting the hIgA.

Acetate buffer at pH of 5.2 was used in all intermediate wash for maximum removal of non-specifically bound material; pH of 5.2 is an intermediate pH between the equilibration and the elution buffers pH, hence preventing a premature elution of the hIgA from the column. However an intermediate pH (5.2) wash may not be sufficient alone to attain desired hIgA purity and recovery. Therefore the addition of EG, urea and arginine to the intermediate wash buffer (acetate buffer) was also evaluated.

From Figure 5-10, it can be seen that all five tested conditions had effective results in terms of hIgA purity since the BSA band was not visualized in all five eluted samples analyzed. The recoveries purities and purification factor generated from running conditions 1 and 2 were calculated and compared to those from the 100 times concentrated CHO supernatant samples (Table 5-3). The equilibration buffer used in running condition 1 shows a difference in hIgA yields between the two used starting materials.

Human IgA yield achieved 96% when purified from the spiked samples and approximately 77% for the 100 times concentrated CHO supernatant under condition 1 while the yield generated from running condition 2 remained the same regardless of the starting material

concentration approximately 76%. Nonetheless with spiked samples, the purification factor decreased considerably from 1178.0 achieved in 100 times concentrated samples to 2.05 and from 1219.5 (100 times concentrated samples) to 2.2 in condition 1 and 2, respectively.

Table 5-4 summarizes the performance parameters, including yield, protein purity and purification factor for the different running conditions evaluated. The purification factor was between 2.05 and 2.24 for the five running conditions. Spiked the CHO supernatant with pure hIgA has considerably enhanced the final purity from 44.11 % to 98.9 %, with a removal of approximately all BSA (SDS-PAGE gel figure). Based on SDS-PAGE analysis and by comparing the density and intensity of the protein bands characteristic of the flow-through and elution pools, one can conclude that after purification of hIgA using HWRGWV peptide ligands most of impurities and specially BSA have been removed. However for the 100 times concentrated samples the BSA band was existed in all purified hIgA samples. These results can be explained by relatively high amount of hIgA in loading samples for the spiked CHO supernatant. This amount was high enough to compete effectively with BSA on binding to HWRGWV peptide resin which leads to high removal of BSA, hence high hIgA purity. In previous experiments the low concentration of hIgA allowed BSA to bind to the resin and affect the eluted hIgA samples purity.

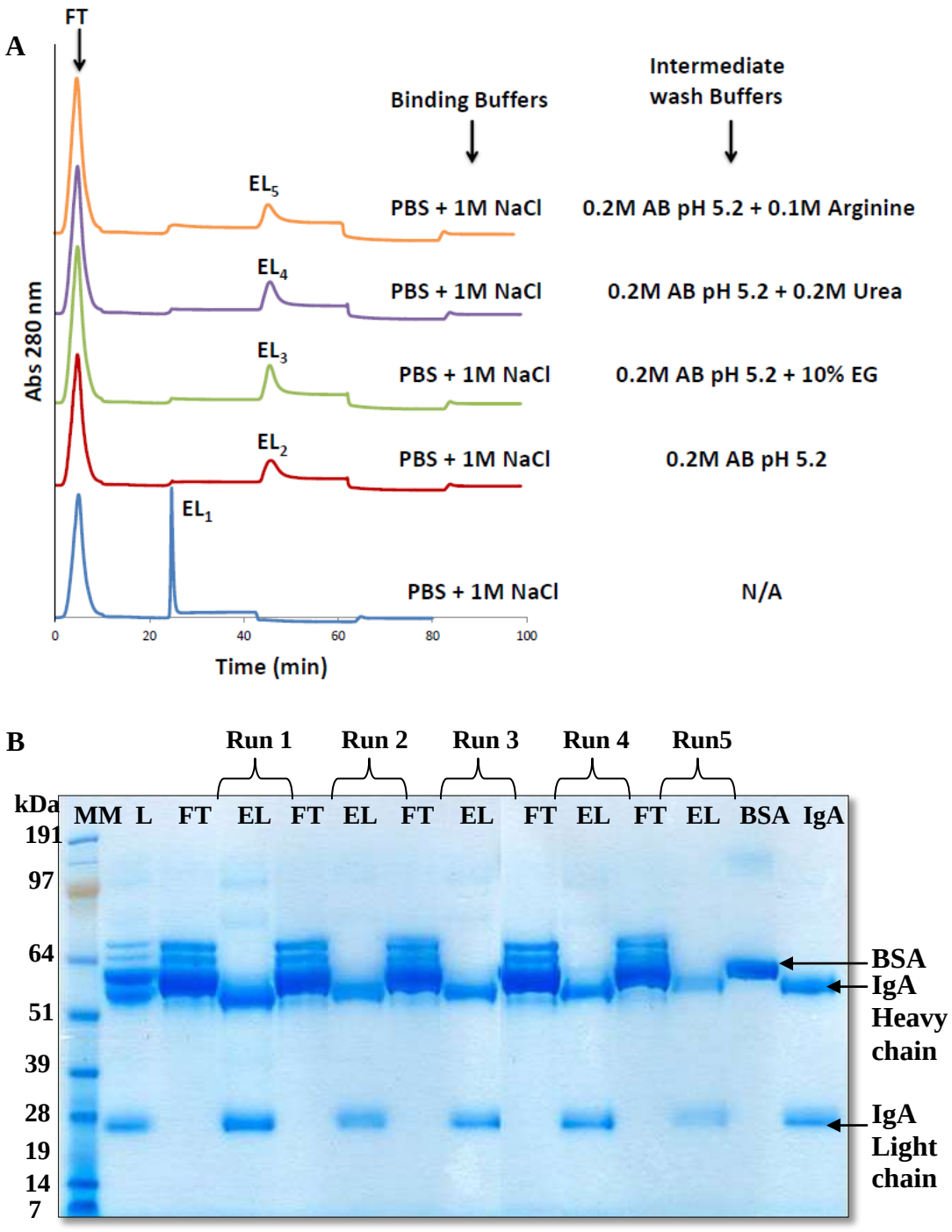

Figure 5-10 (A) Chromatograms of purification of human IgA from spiked samples with HWRGWV resin under different running conditions. (B) SDS-PAGE of elution fractions under reducing conditions.MM-Molecular weight marker; FT-flowthrough; EL-elution fraction.

These results indicate that the starting paste concentration bears an impact beside the equilibration and intermediate wash buffer used on the purity of the eluted hIgA. Therefore the screening of the optimal equilibration conditions and intermediate buffer was based on using a starting CHO supernatant with high hIgA concentration 1.5mg/ml. Starting hIgA concentration used in this experiment is comparable to normally generated hIgA concentration in CHO supernatant when using optimized CHO cell line. Table 5-4 compares the performance parameters generated from each of five running conditions using the spiked CHO supernatant samples with hIgA. There appears to be a reciprocal relationship between the yield and the purity. The running conditions 3, 4 and 5 represent high purity >94% and a lower relative yield (i.e. < 70%). These results might be explained by the addition of the reagents EG, urea and arginine to the intermediate wash buffer which strongly disrupt the non-specific binding interactions and initiate premature elution of the hIgA (loss of the target product) leading to high purity and low yield of hIgA. The yield of the hIgA sample loaded using running condition 5 was relatively low (i.e 55.7%) but with a higher relative purity (i.e. 98.9%). This results shows that arginine may have the highest capability to disrupt the non-specific binding interactions among all other reagents used, resulting in relatively high hIgA losses experienced during the post wash and elution steps.

The improved running conditions to be used for hIgA purification should be capable to generate an acceptable level of both yield and purity. Results demonstrated in Table 5-4, the running condition 1 (PBS + 1M NaCl equilibration buffer followed by elution step (no post wash step)) provided improved relative yield and purity combination with 96.0 %

recovery- 90.3 % purity. In addition the condition 2 that includes PBS +1M NaCl equilibration buffer followed by a post wash step using 0.2M AB pH 5.2 can be considered as optimal as well with 78.5% recovery and 96.8% purity. These results confirm previous conclusions from the previous experiments (low relative starting concentration of hIgA in the CHO supernatant) regarding the suitable running condition in terms of hIgA purity and recovery. Furthermore this experiment also proved that the purity of hIgA is highly dependent on the concentration of hIgA in the starting material that should be high enough to compete with BSA and bind the resin. A post wash step is not necessary in order to achieve a high purity and recovery of hIgA when using PBS + 1M NaCl as the equilibration buffer. However the addition of reagents such as EG, urea and arginine in the intermediate wash buffer was not considered as optimal running conditions even though they generated relatively high hIgA purity.

Table 5-3 Performance parameters obtained for the purification of hIgA from a 100 times concentrated CHO cell supernatant and spiked CHO supernatant with hIgA using two first running conditions.

- 100 times concentrated CHO supernatant: 0.041% hIgA purity
- Spiked CHO supernatant: 44% hIgA purity

	100 times concentrated CHO supernatant (ultrafiltration membrane)			**Spiked CHO supernatant with 1.5mg/ml hIgA**		
Conditions	**Yield %**	**Purity %**	**Purification Factor**	**Yield %**	**Purity %**	**Purification Factor**
1	77.2	48.3	1178.0	96.0	90.3	2.0
2	76.0	50.0	1219.5	78.5	96.8	2.2

Table 5-4 Performance parameters obtained for the purification of human IgA from spiked CHO supernatant with hIgA using five different running conditions.

Running Condition	Recovery (%)	Purity (%)	Purification Factor
1	96.0	90.3	2.0468
2	78.5	96.8	2.1941
3	65.5	97.3	2.2054
4	69.0	94.4	2.1397
5	55.7	98.9	2.2417

VI. CONCLUSION

The running conditions had an influence on the recovery and purity of human IgA. The work in this thesis focused on optimize reliable running conditions that can be used for human IgA chromatographic purification with HWRGWV peptide ligand and achieve high relative yield and relative purity. Multiple experiments were run to investigate and evaluate different running conditions with various equilibration buffers, intermediate buffers and starting CHO supernatant concentrations. High recovery and purity can be achieved for hIgA by using PBS with 1M NaCl as an equilibration buffer without a post wash step from spiked CHO supernatant with 1.5 mg/ml hIgA.

Electrostatic and hydrophobic interactions play an important role in the screening of the equilibration buffer used in human IgA purification with HWRGWV peptide ligand. Presence of the salt in the equilibration buffer was found to be an important parameter during the purification of hIgA by HWRGWV peptide resin. In low initial CHO supernatant concentrations (20 times concentration) of hIgA, the equilibration buffer containing NaCl increased the yield 75% whereas the purity was below 45%. Increasing the starting concentration by using 100 times concentrated CHO supernatant samples appeared to increase the yield 98% with a minimal effect on the purity below 50%.

The concentration of the CHO supernatant (20 and 100 times) using ultrafiltration membrane instead of ultrafiltration filters had relatively little meaningful influence on the human IgA purity and recovery even if the total protein concentration decreased. However,

starting with spiked CHO supernatant with 1.5 mg/ml hIgA, samples show to produce high relative recovery and relative purity for hIgA purification using NaCl salt along with PBS as equilibration buffer. The addition of NaCl prevents the binding of the impurities and allows the HWRGWV available for hIgA binding. In addition increasing the initial hIgA concentration increased the product purity.

After screening for the equilibration buffer, various intermediate wash buffers were evaluated using the spiked CHO supernatant with 1.5 mg/ml samples. For the human IgA purification using HWRGWV resin, PBS+1M NaCl equilibration buffer followed with no post wash step or just acetate buffer pH 5.2 were found to provide more efficient post washing conditions. The addition of other agents such as ethylene glycol, urea and arginine to the intermediate wash buffer (acetate buffer) increased the hIgA purity to greater than 96%. However the improved purity due to increased capability of the intermediate buffer to wash off the maximum of impurities was compensated by the decrease in recovery. The addition of the previously cited reagents to the intermediate wash buffer disrupt the non-specific binding interactions and initiate premature elution of the hIgA which lead to high purity and low yield.

Affinity chromatography using HWRGWV peptide ligand is a promising process for the purification of human IgA therapeutics. Compared with traditional protein A and G chromatography usage, this process is considerably simpler and relatively inexpensive. Human IgA, which will be used in many therapeutic products in the future, was purified

from CHO cell culture supernatant with a purity and yield above 90.3 % and 96% respectively. The yield and purity results obtained with the HWRGWV peptide resin were equal and comparable to the results obtained with a Protein A or Protein G column.

VII. FUTURE WORK

This thesis studied multiple running conditions during the purification of hIgA using HWRGWV peptide resin. The condition used PBS+1M NaCl as equilibration buffer without any post wash step and elution with acetate buffer at pH 3 has proved to be optimal. This condition gave a high relative purity and recovery of human IgA after the affinity chromatography purification step with HWRGWV peptide resin. This thesis also investigated the effects of the starting CHO supernatant concentration on purity and recovery of human IgA. However there are various other areas that need further consideration.

This section looks at some of these areas where further work is necessary to complete the study and determine the optimal processing conditions to be used to purify human IgA by affinity chromatography using HWRGWV peptide resin from CHO supernatant. In order to determine accurately the optimal running condition for hIgA affinity chromatography purification with HWRGWV peptide resin, an optimized CHO cell line for hIgA expression can be used to repeat the same study since the one used here had a very low hIgA yield.

HWRGWV peptide ligand can bind to BSA which is the major contaminant in the human IgA purification process. To optimize the running conditions; the binding mechanism between the two structures should be detailed and fully understood. It is important to fully understand the binding mechanisms between human IgA, the related contaminants, and the

HWRGWV peptide ligand. Previous studies demonstrated that four out of five binding sites may be localized in the C_H2 and C_H3 domains of human IgA that correlate to these activities [17]. These domains may be studied in more depth.

HWRGWV peptide resin has demonstrated a strong binding affinity to human IgA. Chromatography purification of human IgA from CHO cell culture supernatant materials using the HWRGWV resin obtained relative purity and yield of 90.3% and 96.0% respectively. These results indicate that HWRGWV affinity resin may be possibly used to purify secretory immunoglobulin (sIgA).

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