

Antibody Purification from Tobacco by Protein A Affinity Chromatography

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## ABSTRACT

Antibodies represent the largest group of biopharmaceuticals. Due to the nature of their clinical applications, they often need to be produced in large quantities. Plants have distinct advantages of producing large quantities of recombinant proteins, and tobacco is arguably the most promising plant for plant-made-pharmaceuticals (PMP) due to its high biomass yields and robust transformation technology. However, to produce proteins using transgenic tobacco for human applications, purification of the proteins is challenging. On the other hand, Protein A, a bacterial cell wall protein isolated from *Staphylococcus aureus* that binds to the Fc regions of immunoglobulins, is useful to the isolation and purification of antibodies. An affinity chromatography purification step utilizing Protein A resin introduced early in the purification process can reduce successive unit operations, thereby reducing the overall process cost. However, directly applying tobacco extract to Protein A chromatography columns may be problematic due to the non-specific binding of native tobacco proteins (NTP). In this project, three different Protein A resins, ProSep-vA High Capacity, ProSep-vA Ultra, and ProSep Ultra Plus, marketed by Millipore, were studied to provide valuable information for future downstream processes for antibody purification from transgenic tobacco. The efficiency of the post load wash buffer to reduce non-specific binding of NTP to the ProSep A resins were evaluated by altering the ionic strength and pH. Lower salt concentrations of sodium chloride (NaCl) in the post load wash performed best at reducing the non-specific binding of NTP to the ProSep A resins, while higher salt concentrations were more effective at reducing the amount of NTP contaminants present during elution of the columns. Using a post load wash buffer with an intermediate pH between the binding buffer and the elution buffer was more efficient at eluting our model antibody, human IgG. However, lowering the ionic strength and the pH of the post load wash buffer resulted in a greater presence of IgG prematurely eluting from the ProSep A resins. The non-specific binding of NTP to the resins reduced the dynamic binding capacity (DBC) of the resins after repeated cycles of tobacco extract samples were loaded onto the column. Nevertheless, cleaning the columns with

denaturing solutions, such as urea or guanidine hydrochloride, every 8-10 cycles was effective in regenerating the DBC of the resins and prolonging the life cycle of the resins. This is important to evaluating the economic feasibility of directly using Protein A chromatography to recover antibodies from tobacco extract. Of the three Protein A resins studied, ProSep Ultra Plus performed best for antibody purification from tobacco using a PBS wash buffer with a lower ionic strength of 140mM NaCl and an intermediate pH of 5.

## **Dedication**

I dedicate this thesis to my loving parents, Drs. Flynn and Gloria Auchey. They have provided me with the mental and financial support necessary for me to complete my degree.

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## **Chapter One: Introduction**

### 1.1 Molecular Farming

The use of genetically modified plants or animals for the production of pharmaceuticals has been termed molecular farming. For over a decade, therapeutic proteins have been produced through the use of transgenic plants and animals as ‘bioreactors’, many of which are now in clinical trials [1]. The purpose of molecular farming is to produce safe and inexpensive biopharmaceutical proteins [2]. In order to produce large amount of active, safe, and inexpensive pharmaceutical proteins, there are two stages to molecular farming: 1) optimization of the expression system and 2) scale-up to desired production level to meet demands [2].

Advances in technology have established proof of concept for production of a wide range of therapeutic proteins, including antibodies, in plants. Therefore, research is now shifting toward the commercial exploitation of these products. To purify the metric tons of plant antibodies to meet demands, the processing of large quantities of biomass is required, even with high expression levels [3]. With the increasing demand for antibodies worldwide, one of the potential bottlenecks is in the downstream purification process. This project aims to tackle some practical issues associated with antibody purification from transgenic plants.

### 1.2 Objectives of the Research

The objectives of this project are to: (1) determine if native tobacco protein reduces the purification efficiency of three Protein A resins (ProSep-vA High Capacity, ProSep-vA Ultra, and ProSep Ultra Plus); (2) examine how different purification conditions effect the purification efficiency of the three Protein A resins; and (3) collect information regarding the efficiency/effectiveness on ProSep Protein A Resins for future downstream process applications.

## Chapter 2: Review of Literature

### 2.1 Antibodies

#### *2.1.1 Physical and Chemical Properties of IgG*

Antibodies are members of the immunoglobulin family [4]. Immunoglobulins are proteins composed of four polypeptide chains: two identical heavy chains and two identical light chains. The heavy chains carry covalently attached oligosaccharides group, while the light chains are non-glycosylated. However, all four chains contain a single variable (V) region. The variable regions of the light and heavy chains combine to form two identical antigen-binding sites. The variable region of each antibody binds specifically to one antigen. It is estimated the human body can produce  $10^6$  to  $10^8$  types of antibodies, each with a different binding affinity and specificity to a foreign antigen that enter the body [5]. The light chains contain a single constant (C) region, and the heavy chains contain three C regions [4]. The immunoglobulin family has five major classes, IgA, IgD, IgE, IgG, and IgM, which are divided according to the components of the heavy chain. The antibody IgG is monomeric and, in humans, this class of antibody is further divided into four subclasses: IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub>. The pI of IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub> range from 5.0-9.5, 5.0-8.5, 8.2-9.0, and 5.0-6.0, respectively [6]. The molecular weight of IgG is approximately 150 kDa with the molecular weight of the heavy chains approximately 50 kDa each and the molecular weight of the light chains approximately 25 kDa each. Disulfide bonds between the heavy and light chains and between the heavy chains stabilize the IgG antibody molecules.

#### *2.1.2 Types and Uses of Antibodies*

Antibodies represent the largest group of biopharmaceuticals and are useful for the prevention, detection, and treatment of a variety of diseases [3]. There are many different types of antibodies, such as monoclonal antibodies, polyclonal antibodies, and recombinant antibodies.

Due to the advances in medical research and the sequencing of the human genome, the amount of medical and biological knowledge has increased and resulted in the development of novel pharmaceuticals with increased demand for them. Many of these therapeutic proteins, from antibodies that help prevent dental caries to antibodies

that assist in the treatment of many types of cancer including non-Hodgkin's lymphoma, are now approaching commercialization[7].

Monoclonal antibodies are one of the most widely studied therapeutic proteins in plants for passive immunotherapy [1]. Monoclonal antibodies are the collection of a single type of well-defined antibodies produced by hybridoma cell that can only recognize a specific antigen [5]. Hybridoma cells are a hybrid cell produced from myeloma cell, for the immortal property, and a single antibody-producing cell. Monoclonal antibodies are beneficial because they have well-defined specificity. This specificity is especially advantageous for therapeutic applications. Currently, monoclonal antibodies (mAb) are in production for the treatment of asthma, Crohn's disease, rheumatoid arthritis, lupus, and various types of cancer [8]. One of the important characteristics of monoclonal antibodies is their long circulating half-life, which can provide the patient with therapeutic effects over several days. However, the monospecific nature of monoclonal antibodies is ineffective not only against antigen mutations but also against diseases caused by a virus with multiple strains [8]. Also, the production of monoclonal antibodies is expensive, time consuming, and labor intensive [9].

On the other hand, polyclonal antibodies are the collection of antibodies produced by the host, each independently recognizing a specific antigen [10]. The production of polyclonal antibodies is relatively easy and contains antibodies directed to a larger variety of antigenic determinants. Recombinant polyclonal antibodies represent a new emerging generation of therapeutic antibodies capable of responding to infectious diseases and maintain activity against mutating antigens [11]. Recombinant polyclonal antibodies are more effective at retaining therapeutic activity than monoclonal antibodies against viruses that undergo random mutation to evade the immune system. Therefore, polyclonal antibodies are expected to become more prominent in the antibody therapeutic market in the future.

### *2.1.3 Technological Advancements with Engineered Recombinant Antibodies*

The first monoclonal antibodies developed for human therapeutics were murine antibodies. One of the first approved murine antibodies for human use was Orthoclone OKT3 for the prevention of kidney transplant rejection [10]. During clinical trials,

patients required large, repetitive doses of antibodies. The immunogenicity of the murine antibodies in humans during clinical trial resulted in 86% of the patients producing human antimurine antibodies (HAMA), and the presence of HAMA resulted in rapid clearance of the murine antibodies limiting the efficacy of the OKT3 treatment.

The immunogenicity of the therapeutic murine antibodies promoted the development of more human-like antibodies, chimeric antibodies, which contain the murine antigen-binding portion and the human constant region. Although the new chimeric antibodies were approximately 75% human, the murine antigen-binding portion of the antibodies was still able to cause an HAMA response in patients. To reduce the immunogenicity of therapeutic antibodies, technological advancements for the production of more humanized or fully humanized antibodies were developed. According to Jones *et al.*, most future therapeutic antibodies will be humanized or fully human antibodies [12].

#### *2.1.4 Increased Demand for Antibodies and Scalability*

Monoclonal antibodies are one of the dominant products in the biopharmaceutical market, generating revenues of several billion dollars per year [10]. In 2006 monoclonal antibodies represented approximately 20% of biologic products on the biopharmaceutical market, generating over 20 billion dollars in revenues [10]. According to Datamonitor, in 2007 total global monoclonal antibodies sale reached \$26 billion and is expected to reach \$49 billion by 2013 [13].

According to a 2008 biotechnology report, 633 medicines were in development, 192 of which are monoclonal antibodies [8]. Due to the wide range of commercial use of antibodies, one of the most important aspects for the commercialization of biopharmaceuticals is product yield. Many treatment processes using antibodies, such as in cancer treatments, require repeated high dose application of the product. The production of monoclonal antibodies for oncology applications is one of the main driving forces behind the antibody market and its projected growth [14].

Significant cost is associated with the production of monoclonal antibodies, such as IgG, due to their complex structure. According to Li *et al.* the most popular expression system for the production of monoclonal antibodies are recombinant mammalian cell culture, such as Chinese hamster ovary cells (CHO) and murine myeloma (NSO) [15]. For full biological activity, IgG is often produced in mammalian cell cultures, such as

CHO or NSO cells, because mammalian cells are capable of complex post-translational modifications and the formation of disulfide bonds [14]. The production of IgG in mammalian cell cultures is an expensive and time-consuming process. The amount of monoclonal antibodies entering clinical trials and being approved for manufacturing are increasing. As the demand for therapeutic proteins increase, the manufacturing capacity for antibody production becomes increasingly challenging. Therefore, as more therapeutic proteins are required in large quantities, the demand cannot be attained economically in cell cultures [1].

As of 2002, it was estimated that the mammalian cell manufacturing capacity of monoclonal antibodies could produce approximately 2500 kg per year, and it is unlikely for mammalian culture facilities to meet the future market demands [16]. Improving the facilities to increase mammalian manufacturing capacity would cost companies time and money. Therefore, using transgenic plants is an alternative to cell culture-based antibody production. One of the appealing aspects to plant-made pharmaceuticals is that growing transgenic plants in a field has ‘unparalleled potential for scalability’, resulting in unlimited production [17]. The production of the recombinant proteins on an agricultural scale will generate even greater savings, providing a lower cost of production and flexibility to easily scale up or down to meet changes in market demands. According to Hood *et al.*, the “estimated costs of goods sold (COGS) in mammalian cell cultures would range from \$106 to \$650 per gram”, while production in plants would cost from \$0.10 to \$1 per gram, representing a 1000-fold reduction in manufacturing cost [16].

New advances in the biotechnology industry are expanding the original boundaries by producing recombinant protein in plants for diagnostic and therapeutic applications in health care and life sciences [2]. One such breakthrough was the expression of functional antibodies in tobacco leaves in 1989. Haitt *et al.* successfully expressed functional, full size IgG antibodies in tobacco plants, reportedly accumulating up to 1.3% of the total soluble protein in tobacco leaves [18]. While the improvements in the upstream processing have increased, the expression levels of antibodies, optimization of the downstream process capacity, and ability to handle these high quantities are still required.

## 2.2 Expression Systems

### *2.2.1 Types of Expression Systems*

The production of plant-made pharmaceuticals has reached a point where it could challenge previously established bacteria, yeast, and mammalian expression systems. Bacterial fermentation is a cost effective alternative; however, bacteria do not have complex processing capability, i.e. post-translational modifications (PTM), required for many human proteins, such as antibodies [16]. On the other hand, mammalian expression systems can perform post-translational modifications; however, expression in mammalian cells require not only long lead time and high cost for facility and process design but also has limited scale-up potential and the possibly of contamination by viruses and prions. For instance, the use of human tissue cells as a source of human proteins is in limited supply and is constrained by the potential of contamination by infectious agents, such as HIV and Hepatitis B [19]. Therefore, the development of cost-effective and scalable systems with complex processing for PTMs is essential for the production of therapeutic proteins, such as antibodies. Plant expression systems are a cost effective alternative to both bacterial and mammalian expression systems that can perform complex PTMs similar to mammalian expressions.

### *2.2.2 Advantages and Disadvantages of Plant Expression Systems*

Genetically-engineered plants have many advantages in the production of antibodies: (1) large scale biomass production at low cost, (2) ease, efficiency, and low cost of producing stably transformed plants, (3) similarities of PTMs in plants and animals, (4) increased safety because plants do not serve as host for human and animal infectious agents, and (5) the absence of animal rights concerns [12]. Plants offer a reduction in capitalization cost, especially when producing the raw material on an agricultural scale [1]. According to Fischer and Emans, the production costs for recombinant proteins in plants is 10 to 50 times lower than the cost of production for the same protein in *E. coli* [2]. While the scale-up of plant expression systems to agricultural level is relative easy and inexpensive, one of the main concerns with plant expression systems is the cross-contamination of transgenic crops with non-transgenic crops, especially feed or food crops. Therefore, one solution is the production of these biopharmaceutical products in non-feed, non-food crop, such as tobacco.

Another concern with plant expression systems is differences between plants and humans PTMs, such as N-linked glycosylation patterns, eliciting an immune response or even an allergic reaction by the patient [20]. N-linked glycosylation (N-glycans) is the covalent attachment of oligosaccharides side chains to the amide nitrogen on specific asparagines (Asn) residues [20]. More than one-third of protein biopharmaceuticals' function and biological activity are affected by the N-glycans. According to Fischer *et al.*, studies have shown that even though recombinant IgG produced in plants differ in glycosylation patterns, neither the antibody nor the glycans were immunogenic when administered to mice [21]. On the other hand, according to Saint-Jore-Dupas *et al.*, immunogenic N-glycans have elicited glycan-specific antibodies in human [20]. Therefore, the administration of plant-made pharmaceuticals, especially glycoproteins, to humans is questionable and it is important to eliminate or minimize the plant specific PTMs to obtain a more humanized plant-made pharmaceutical.

In the production of recombinant glycoprotein many new strategies are being developed to produce more humanized glycans, which lower potential immunogenicity and improve biological activity. First, retention of the recombinant glycoprotein in the ER will lack plant-specific core modifications, such as xylosylation and fucosylation, often present on glycoproteins that pass into the Golgi of plant cells. Second, reducing the activity of plant-specific glycosyltransferases by the inactivation or silencing of genes coding for plant glycosylation enzymes. However, this method requires extensive genomic information of the host expression system. Thus, a third method, i.e. introducing genes coding for enzymes involved with the mammalian N-glycosylation pathway, has proven to be successful in plants cells. These three methods have been shown to reduce, to some degree, plant-specific modifications. Other methods that are being explored include *in vitro* remodeling of the N-glycans, changing the N-glycosylation through medium supplements, and the production of unglycosylated proteins [22].

### 2.2.3 Common Plant Expression Systems Used for Biopharmaceutical Production

Plants used for medicinal purposes are not a recent revelation [2]. In fact, close to one quarter of prescription drugs have plant origins. As more treatments for diseases are being identified, more novel proteins are being developed and manufactured on a large scale. Through higher expression levels of target proteins in plants and high intensity

agriculture, large amount of biomass can be produced. This results in a more economical large-scale production of therapeutic proteins.

There are many different plant species that can be used in the production of biopharmaceutical proteins. The composition of the material of plant hosts are vastly different from each other, and it is important to choose a plant host that will correctly produce the protein of interest while reducing the final costs by optimization of the purification process [23]. Leafy crops, such as tobacco, lettuce, and alfalfa, have a high biomass yield but have low protein stability in harvested material [21]. For instance, tobacco can produce 170 metric tons of biomass per hectare through high intensity agriculture [2]. In 1990, one of the first recombinant pharmaceutical proteins to be derived from plants was human serum albumin in tobacco [17]. According to Fischer and Emans, in 2000 the world wide demand of human serum albumin (HAS) was approaching 550 tons purified protein per year [2]. Assuming a 1% TSP in tobacco, it is estimated that the cultivation of 30,000 hectares can meet the worldwide demand.

Cereal crops, such as maize, rice, wheat, and barley, have higher protein stability during storage. While maize and rice have high yields and are easy to transform, wheat and barley have low yields and are difficult to transform [21]. Legumes, such as soybeans, have high protein content but are difficult to transform and have low expression levels. For example, the protein content of soybeans is 4 times higher than corn, yet corn will produce roughly the same amount of protein per acre due to its abundant seed production [23]. According to Fisher *et al.*, an industry leader in the production of commercial proteins by cereal crops, Prodigene Inc., used maize for the production of avidin and  $\beta$ -glucuronidase (GUS) [21]. However, in 2002, Prodigene Inc. was involved in two contamination incidents due to cross-pollination, and has since been taken over by Stine Seed Company [24]. Another company, Monsanto, was able to create a corn line that could produce 1.5 kg of human antibodies per acre of corn [2]. Monsanto has also been able to cultivate transgenic soybeans that produced humanized antibodies against herpes simplex virus 2 (HSV-2).

The utilization of plants for the production of therapeutic proteins not only are easily scaled to meet worldwide demand but also can reduce the cost of proteins produced through conventional methods such as CHO cell cultures. One of the most

expensive drugs is human glucocerebrosidase (hGC), a therapeutic protein used for the treatment of Gauche disease [22]. The treatment is estimated to cost approximately \$200,000 per year per patient. According to Cramer *et al.*, 8 hours after tobacco leaves were induced by agrobacterium-mediated transformation to produce hGC, the resultant hGC represented 10% of the total soluble proteins of the tobacco leaves. At this expression level, one plant could yield at least one standard therapeutic dose, significantly reducing the production costs [19].

#### *2.2.4 Improving Protein Yield When Using Plant Expression Systems*

Advances in the expression of antibodies in plants are making plant expression systems a more viable option, thus increasing the importance of researching downstream purification processes of antibodies from plants. Strategies to improve the expression of recombinant proteins in plants include: 1) the development of novel promoters, 2) the addition of signal sequences to target protein to the correct intercellular compartment, and 3) improvement in protein stability including the expression of multimeric proteins. For example, current methods to minimize the impact of these PTM of the plant-made antibodies while increasing expression levels include the targeting of recombinant antibodies to the secretory pathway, specifically the endoplasmic reticulum (ER) and Golgi apparatus. According to Giddings, retention of recombinant antibodies in the ER can typically increase the level of protein expression 10 to 100 fold [25]. This results in expression levels varying from 0.35 % to 2% of the total soluble proteins [25].

As the expression level of the recombinant proteins within the plant increases and the quantity of plants to be processed to meet market demand increases, improvements of downstream processing technology to efficiently recovery more protein is necessary [17]. Efficiency of protein recovery after plant harvest depends on plant material storage/plant fractionation, protein extraction and solid/liquid clarification, and protein purification [23]. Where the protein is targeted for accumulation within the cell greatly affects the unit operations used in the purification process and the storage of the material. For example, proteins expressed in plant seeds have a longer shelf life than proteins expressed within the plant leaves.

The purification processes can be affected by the size, solubility, and charge characteristics of the protein within the protein extract [23]. High levels of recombinant

protein can be recovered through wet-gridding or homogenization of the plant biomass in an aqueous extraction buffer. Due to the variations of native components within the different plant species, the addition of salts, detergents, and protease inhibitors to the extraction buffer or changes of the pH of the buffer can influence the amount of proteins extracted from the leaves. According to Balasubramaniam *et al.*, the pH of the extraction buffer greatly influences the amount of total protein extracted from tobacco leaves [26]. Once extracted, the desired proteins can be purified through chromatographic or non-chromatographic methods. For instance, an initial step of Protein A and Protein G affinity chromatography can yield product purity greater than 99%, greatly reducing the number of successive unit operations. Non-chromatographic methods such as isoelectric or polyelectrolyte precipitation, or aqueous phase partitioning are also unit operations used in the purification process.

### 2.3 Tobacco Expression System

One of the most promising plants for the production of recombinant proteins is tobacco due to the high biomass yields and robust transformation technology. Also, tobacco is one of the easiest plants to be genetically engineered, is highly reproductive, and is capable of facilitating rapid scale up. Currently, tobacco is a crop at risk due to the increase in smoke-related health concerns, federal regulations, and proposed taxation [19]. Since the infrastructure for crop cultivation, harvesting, distribution, and processing already exists, using a portion of the current tobacco fields for the production of biopharmaceuticals is economically viable. Tobacco leaves are the main sites for harvesting protein [23]. Due to the variations of the chemical components within the leaves of different tobacco species, the total protein content in the leaves of the tobacco plant can vary from 4-10% of the leaves dry mass. In stably transformed plants, the average expression levels of recombinant antibodies are approximately 1 to 2% of the total soluble proteins [12].

The protein content of tobacco is divided into two fractions: Fraction I and II. These fractions are expressed equally in the leaf extract at approximately 6.4 mg protein/g fresh leaf [23]. Fraction I is mainly comprised of a chloroplast enzyme ribulose 1,5 biphosphate carboxylase oxygenase (RuBisCo). Rubisco is an 18S molecule with a molecular weight of approximately 560 kDa. It consists of 8 small and 8 larger subunits

with a molecular weight of approximately 12.5 and 55 kDa, respectively. Also, the pI's of the small and large subunits are 5.3 and 6 respectively and are soluble in high salt (NaCl) solutions. The Rubisco molecule is shaped like a barrel, with a height along the barrel axis of 10.5 nm and a diameter of approximately 13.2 nm [27]. Fraction II consists of a mixture of smaller molecular weight protein that has yet to be characterized. Fraction II proteins have an overall acidic nature [23]. According to Balasubramaniam *et al.*, the maximum of total protein extracted is at a buffer pH of 7 [26].

One of the major concerns for the production of recombinant proteins in tobacco is the presence of phenolic compounds or toxic metabolites, such as nicotine. These phenolic compounds are a major concern for protein extraction from the stem and leaves and are best removed early in the purification process [3]. These phenolic compounds can interact with proteins causing dramatic and irreversible alterations within the target protein [3]. The fouling of the resin adsorbent due to phenolic compounds and their derivatives can produce high concentration of hydrophobic aryl compounds [28]. This is especially significant when adsorptions are used early in the purification process. Protein contaminants can become attached to these fouled areas of the resin adsorbents through hydrophobic and electrostatic interactions. Thus, it is important to develop methods to remove these compounds to improve the efficiency of the chromatographic methods for protein purification.

## 2.4 Protein Purification

### *2.4.1 Downstream Processing*

Downstream processing of a target protein can be divided into two stages, initial recovery stage and the high-resolution purification stage [29]. The objective of the initial recovery is to isolate the protein extract from the cell debris and particulate matter to obtain a well-clarified aqueous solution suitable for high-resolution purification. The protein purification protocols for animal and microbial expression systems are well established; however, purification strategies from plants are still being researched [2]. Furthermore, the cost for purifying recombinant protein from tobacco is mainly determined by the initial recovery and purification of the protein [29]. Therefore, research and improvement to downstream processing, especially in the initial stage of purification, could reduce the overall costs for product production. For instance, the introduction of an

affinity purification step early in the purification process can reduce the successive unit operations, thereby reducing purification costs [30].

The purification of proteins for therapeutic purposes must be extremely high (>98%) [31]. The purity of the IgG can be affected by the co-elution of host cell proteins (HSP) non-specifically binding to the chromatography media and leakage of the affinity ligand of the chromatography media [32]. Therefore, further purification techniques may be required to minimize the risk of unwanted side effects, such as an immunogenic response, due to the presence of these impurities. In addition, the optimization of the downstream processing, such as improvements to extend the life and reusability of expensive chromatography media, is required for cost efficient processes.

#### *2.4.2 Protein Extraction*

The purpose of protein extraction is to isolate the recombinant protein from the plant biomass into an aqueous environment [23]. Homogenization of the harvested leaves requires no pre-grinding and is capable of extracting high levels of recombinant proteins in a short period of time. Most often the pH value is the most important aspect of the extraction buffer; however, the additions of salts, detergents, and protease inhibitors can also enhance the amount of protein extracted. As mentioned above, (Balasubramaniam *et al.*) the total native protein extracted from the tobacco biomass increases as the pH increasing, maximizes at pH 7, and decreases at pH greater than 7 [26].

Polyvinylpyrrolidone (PVPP) is a binding agent used during the extraction process to adsorb and remove phenolic compounds from the leaf [28]. It is an insoluble form of polyvinylpyrrolidones (PVP) that can be centrifuged out of the extract before clarification of the supernatant. The extraction of protein from the tobacco biomass is best at a pH of 7. During the extraction process PVPP works satisfactorily over the pH range 6.5-7.2, and has weak adsorption at alkaline conditions (pH >8) [28]. The addition of a reducing agent,  $\beta$ -2-mercaptoethanol (BME), may also alleviate the interference of phenols with the target proteins [33]. The chelating agent EDTA may be used as a protease inhibitor by binding metal ions, deactivating metal-dependent enzymes, and suppressing damage to proteins [34].

## 2.5 Protein A Affinity Chromatography

### 2.5.1 Affinity Chromatography

Affinity chromatography has high selectivity for effective removal of native plant proteins, subsequently reducing the number of successive unit operations required for protein purification [30]. The first step to remove contaminating plant proteins efficiently in affinity chromatography for the purification of antibodies is by the usage of Protein A matrices [2]. The first adsorption stage accounts for 28% of the annual operating cost for purification [23]. Therefore, optimization of the Protein A affinity chromatography can significantly reduce the overall production costs. However, the utilization of Protein A affinity chromatography for the downstream purification process has problems, including the processing of large volumes and higher requirements for the cleaning-in-place protocols [30].

The chromatography process is divided into four stages: 1) adsorption of the product, 2) washing of loosely bound non-specific components, 3) elution of target protein, and 4) regeneration and re-equilibration of the column [34]. To increase the interaction between Protein A and IgG, parameters, such as pH, ionic strength, temperature, and chemical composition, are optimized. Protein A affinity chromatography normally generates up to 80% recovery of the target protein with greater than 95% purity [31].

For effective maintenance of the columns for large-scale purification, it is important to maintain the longevity of the media [35]. The main contributors to the fouling of the column bed are particulate matter in the sample, non-specific binding of native proteins, and microbial contamination. Filtering the samples prior to loading onto columns will reduce particulate matter. However, the removal of non-specifically bound proteins can be more difficult.

### 2.5.2 Protein A

Protein A is a polypeptide found in the cell wall of the bacteria *Staphylococcus aureus* [4]. The carboxyl-terminus of this polypeptide contains the region anchored to the cell wall. The amino-terminus contains 5 homologous antibody-binding domains, which share 65-90% sequence identity. However, only 4 of the 5 domains bind specifically to the Fc region of IgG [35]. The binding domains of Protein A have a molecular weight of

approximately 7 kDa each. Each binding domain has approximately the same ability to bind antibodies and consists of an anti-parallel triple helix bundle motif (Figure 2.1) [35]. When Protein A is immobilized, only 2 of the 4 binding domains are available for binding to IgG [35]. The molecular weight of Protein A is approximately 54 kDa [4]. However, if the cell wall region of the polypeptide is deleted, the molecular weight is approximately 42.5 kDa. Protein A is resistant to physiochemical stress and has high conformational stability. It is stable over a wide pH range; however, the Protein A ligands are sensitive to alkaline conditions. Also, Protein A is able to refold after being treated with denaturing solutions, such as urea or guanidine hydrochloride. Protein A has a greater stability than Protein G and is, therefore, better suited for industrial purification.

### 2.5.3 Comparison Protein A and Protein G

Protein G is a bacterial cell wall protein from group C and group G *Streptococcus aureus* capable of binding to both immunoglobulins and albumin [5]. Protein G is approximately the same molecular weight of Protein A consisting of three binding domains. Similar to Protein A, Protein G binds specifically to the Fc region of IgG. For the affinity purification of immunoglobulins, the recombinant form of Protein G contains two Fc-binding regions (B1 and B2), eliminates the albumin binding domain, and has a molecular weight of approximately 23 kDa [35]. The Fc binding domain consists of an alpha-helix packed along one face of a mixed beta-sheet (Figure 2.1) [36]. Protein G is a more versatile binding reagent and can bind a more diverse range of antibody molecules than Protein A. Protein G can bind strongly to all four IgG subclasses, while Protein A binds strongly to IgG subclasses 1 and 2, weakly to subclasses 3 and 4, and very weakly to IgA, IgM, IgE, and IgD. Protein G often has a higher binding affinity to immunoglobulins making it more difficult to elute and recover the immunoglobulins than Protein A. Also, Protein A typically tends to be cheaper than Protein G and is able to withstand harsher conditions that are required for the clean-in-place (CIP) steps [37].

### 2.5.4 Protein A Chromatography

Protein A affinity chromatography, which utilizes immobilized Protein A ligands to bind to the human Fc region of the antibody, is used for the isolation of IgG antibodies, especially IgG subclasses IgG<sub>1</sub>, IgG<sub>2</sub>, and IgG<sub>4</sub> [30]. The interaction between IgG and Protein A primarily consists of hydrophobic interaction, with some hydrogen bonding and

salt bridges. The Protein A and IgG binding site both contain highly conserved histidyl residues [4]. At alkaline pH, these residues are uncharged with no restriction of interfacial contact. However, at low pH these histidyl residues are fully charged and mutually repellent. Therefore, the purification process requires the binding conditions be at an alkaline pH and a low pH to elute the IgG from the Protein A media. A majority of the purification process occurs during Protein A affinity chromatography by the removal of host cell proteins (HCP) and small molecules [30]. However, Protein A affinity chromatography does not remove aggregates and introduces small amounts of Protein A into the sample due to leaching of the molecule from the column [38]. Protein A costs nearly one-quarter of the consumable material used in the downstream processing [4] and is nearly an order of magnitude more expensive than conventional chromatography resins [39]. Therefore, the use of Protein A for downstream purification requires a smaller column, which can be re-cycled several times to purify a single batch rather than processing a batch of antibodies in a single cycle [38]. Sodium hydroxide (NaOH) is a commonly used cleaning agent for equipment as well as resin. It is capable of removing tightly bound residual molecules for the column as well as inactivation of microorganisms [30]. Protein A affinity columns are not always stable toward high pH; therefore, NaOH solutions are not recommended for cleaning these columns. Alternatively, chaotropic solutions such as 6 M urea and 6 M guanidine HCl solutions are often used for cleaning. For instance, the silica backbone of the ProSep-vA resin family is unstable at alkaline conditions; therefore, a chaotropic solution is better suited for these resins than NaOH solutions. The Protein A ligand after treatment with the denaturing solution is able to refold during re-equilibration of the column [40]. However, chaotropic solutions are costly and have special disposal requirement compared to NaOH solutions. For instance, the cost of preparing a 6M guanidine HCl solution was approximately 10-fold more expensive than the cost of preparing a 6M urea solution with chemicals supplied by Sigma-Adlrch.

### 2.6 Commercial Protein A Resin

A wide variety of Protein A resins are currently available on the market (Table 2.1). These resins vary according to whether the source of the Protein A ligand is natural or recombinant, the immobilization chemistries, and the bead characteristics [4]. Natural

Protein A derives from *Staphylococcus aureus*; while the recombinant form is often produced in *Escherichia coli*. The ProSep-vA High capacity and the ProSep-vA Ultra media are both manufactured using a natural, animal-free Protein A derived from *S. aureus* [41], while ProSep Ultra Plus media is manufactured using recombinant Protein A expressed in *E. coli* [42].

**Table 2.1. Comparison of Commercially available Protein A resins**

Sorbent	Manufacturer	Matrix	Binding Capacity human IgG(mg/ml)	Particle size (um)	Ligand Density (mg/ml)	Ref #
Hi Trap rProtein A FF	GE Healthcare	cross-linked Agarose	~50	90	n.a.	[43]
rProtein A Sepharose Fast Flow		cross-linked Agarose	~50	60-165	~6	[44]
rmp Protein A Sepharose Fast Flow		cross-linked Agarose	~35	60-165	n.a.	[45]
HiTrap Protein A HP		Cross-linked agarose	~20	34	n.a.	[46]
Protein A HP Spin Trap		Cross-linked agarose	~ 1	34	~3	[47]
Protein A HP Multi Trap		Cross-linked agarose	~1 mg/well	34	~3	[48]
nProtein A Sepharose 4 FF		Cross-linked agarose	~35	45-165	~6	[49]
Protein A Sepharose CL-4B		Cross-linked agarose	~20	90	~3	[50]
MabSelect		Cross-linked agarose	~30	40-130	n.a.	[51]
HiTrap MabSelect		Rigid cross-linked agarose	~30	85	n.a	[51-53]
		Rigid cross-	~30	85	n.a	[54]

HiScreen  
MabSelect

		linked agarose				
MabSelect SuRe		Rigid cross-linked agarose	~35	85	n.a	[55]
HiTrap MabSelect SuRe		Rigid cross-linked agarose	~30	85	n.a.	[52]
HiScreen MabSelect SuRe		Rigid cross-linked agarose	~30	85	n.a	[54]
MabSelect Xtra		Rigid cross-linked agarose	~40	75	n.a.	[51]
HiTrap MabSelect Xtra		Rigid cross-linked agarose	~40	75	n.a.	[53]
HiScreen MabSelect Xtra		Rigid cross-linked agarose	~40	75	n.a	[54]
Streamline rProtein A		macroporous cross-linked agarose	~50	80-165	1.3 g/ml	[56]
Immobilized Protein A	RepliGen	Cross-linked agarose	18-43	n.a.	n.a.	[57]
ProSep-A High Capacity	Millipore	Control Porous Glass	15-30	75-125	1.3g/ml	[58]
ProSep-rA High Capacity		Control Porous Glass	15-30	75-125	1.3g/ml	[58]
ProSep vA High Capacity		Control Porous Glass	15-30	75-125	1.3g/ml	[58]
ProSep vA Ultra		Control Porous Glass	20-45	75-125	1.3g/ml	[58]
ProSep Ultra		Control	50	60	n.a.	[42]

Plus		Porous Glass				
Protein A Ceramic HyperD F Resin	Pall	Polyacrylamide in ceramic macrobead	30	50	3-5	[59]
POROS A 50	Applied BioScience	Polystyrene divinylbenzene beads	<20	45-50	5-6	[60]
POROS A LP		Polystyrene divinylbenzene beads	<20	55-60	5-6	[61]
POROS MabCapture A		Polystyrene divinylbenzene beads	>45	45	n.a.	[62]
Pierce Protein A Agarose	Pierce	Cross-linked agarose	12-19	n.a.	n.a.	[63]
Pierce Protein A Plus Agarose		Cross-linked agarose	>34	n.a.	n.a.	[64]
Protein A Trisacryl		Trisacryl GF-2000	15	n.a.	n.a.	[64]
Protein A UltraLink		UltraLink Biosupport	>16	50-80	n.a.	[64]
Protein A Plus UltraLink		UltraLink Biosupport	>30	50-80	n.a.	[65]
Protein A Ultraflow	Sterogene Bioseparations	Agarose	50	40-160	n.a.	[66]
Protein A Cellthru 300 Plus		Agarose	35	200-300	n.a.	[67]
Affi-Gel Protein A Gel	Bio-Rad	Cross-linked agarose	10	n.a.	2	[68]
Affi-Prep Protein A Support		Polymeric	10	n.a.	2	[68]

### *2.6.1 Characteristics of Protein A Affinity Chromatography Support Material*

Silica-based support material, such as controlled porous glass of the ProSep media family, are hydrophilic due to the silanol group that cover the surface of silica [5]. The silanol groups are weak acids at neutral pH. This gives the silica surface a strong negative charge, resulting in the irreversible adsorption of positively charged proteins to the silica-based material. Therefore, polymer-coating techniques have been used to render the silica surface inert toward solutes such as proteins. According to US Patent 6127526, the ProSep media family immobilizes Protein A on a glycerol coated-controlled porous glass column, which reduces but does not eliminate the non-specific interactions with native feed contaminants [69]. Due to pH cycling during the affinity chromatography purification process silica is susceptible to hydrolytic damages causing dissolution of the support matrix at alkaline pH [5]. Cross-linked agarose consists of polymeric chains of disaccharide agarobiose clustered together to form porous, hydrophilic networks, where the groups facing the solvent are unlikely to attract sample components. Polystyrene support materials are highly hydrophobic and must be coated to render the support material hydrophilic [5].

Cross-linked agarose supports are ideal absorbent for chemical stability under operating conditions (pH range between 3-12) compared to silica supports (pH range should not exceed pH 8) [5]. However, silica and polystyrene supports are ideal absorbents for mechanical stability compared to cross-linked agarose. Silica and polystyrene support are stronger supports and less vulnerable to collapse when operated at elevated pressure, and, therefore, can be operated at faster flow rates which results in higher productivity.

The pore size of the affinity support should ideally provide unhindered access of the solute to the immobilized ligand. To allow unhindered access of the solute to the pores and avoid severe restriction of the rate of diffusion, the pore diameter should be at least five times the diameter of the solute [5]. Supports with larger pore size provide less hindered diffusion of solutes, but also provides less surface area for ligand attachment [5]. Therefore, medium with larger pore sizes reduce the binding capacity of the medium. Theoretically, smaller particle sizes promote faster mass transfer of the solutes from the

mobile phase into the interior of the support particle. This results in faster adsorption of the target solute, making the wash and elution steps more efficient [5]. Furthermore, smaller particle sizes increase the surface area for ligand attachment; thereby, increasing the dynamic binding capacity of the medium [5]. However, as the size of the packing material decreases, pressure drop increases and there is a greater tendency of contaminants in the feedstream to clog affinity columns.

The immobilization of the Protein A ligands to the support material is usually preformed through its amine groups [5]. ProSep media family uses immobilization chemistry that enables optimum orientation and distribution of the ligand [42]. ProSep media family also use multipoint attachment of the Protein A ligand to the support matrix. Through multipoint attachment of the ligand to the support matrix, Protein A leakage is minimized and the impact of feedstream protease activity is reduced, thereby maintaining the binding capacity of the media and increases the lifetime of the media [4].

#### *2.6.2 Non-Specific Binding of Feedstock Contaminates*

To some degree all chromatography resin experience non-specific binding of HCP, which can decrease the binding capacity of the resin if non-specific binding is not completely reversed. According to Millipore, non-specific binding usually occurs through ionic or hydrophobic interactions with base matrix or immobilized ligand coupling [42]. Also, the level of non-specific binding of HCP to controlled porous glass matrices is sometimes greater than the level exhibited with compressible, agarose-based matrices [70]. The degree of the non-specific binding of HCP depends on the protein composition of the feedstock. Therefore, it is important to determine the degree at which HCP non-specifically binds to the Protein A resin as well as how conditions of the intermediate wash step will reduce the amount of non-specifically bound HCP.

The reusability of the chromatography media is one of the most important criteria for minimizing manufacture cost, especially since Protein A chromatography media are generally more expensive than other types of media. Therefore, it is important to minimize the non-specific binding of HCP to the media being used [70]. This is done by altering the conditions of the post load wash buffer so as to disrupt the interaction between the HCP and the media. However, one must be cautious that the conditions do not prematurely elute the antibodies. According to Millipore, the purity of the eluate

often exceeds 96% with low levels of residual HCP [70]. An important aspect to optimizing the reusability of the media is cleaning the media properly to remove residual protein and other contaminants to maintain media performance.

### 2.6.3 *ProSep-vA High Capacity*

ProSep-vA High Capacity chromatography media was developed for the efficient capture step in the purification of polyclonal, monoclonal, and engineered antibodies [41]. The static binding capacity of this resin for human monoclonal antibodies is greater than 30 mg/ml and greater than 40 mg/ml for human polyclonal antibodies. The biopharmaceutical industry is now being encouraged to remove mammalian-derived material from the manufacturing process. Therefore, this resin is made from natural Protein A derived from *S. aureus* and was designed for the high throughput capabilities at initial capture due to the higher demand for therapeutic antibodies.

The media is based on a fully incompressible porous glass matrix with a particle size 75-125  $\mu\text{m}$  and a pore size of 1000  $\text{\AA}$  [41]. The packed density of this media is 1.3 g/ml and has a pH range from 1 to 9. However, high alkaline conditions should be avoided. The incompressible nature of the media allow for very rapid mass transfer, which results in high dynamic binding capacity and sharper breakthrough curves [41]. Because of the incompressible characteristic of the matrix, there is a linear relationship between the backpressure and flow rate [71]. Therefore, based on the column length and diameter, the response of the packed column to increase flow rates can be determined. The characteristic of the high capacity media results in high throughput and productivity. Also, the incompressible nature of the media provides greater flexibility with column selection and process design. As of 2006, the vA-high capacity media was used in the purification of more than 30 therapeutic products [71]. In terms of reusability, research has shown the ProSep-vA media family has over a 300-cycle lifetime without loss in performance, which reduces media costs 5-fold compared to media with 50-cycle lifetime [72].

### 2.6.4 *ProSep vA-Ultra Resin*

The technological developments, which increase the expression level of IgG in the host organism, require a more efficient purification process. Increasing the binding capacity of the resins is one of the most efficient ways to reduce the manufacturing costs.

The ProSep-vA Ultra chromatography media was developed based on the ProSep-vA High Capacity media, but with binding capacity optimized to meet the needs of the biopharmaceutical industry [73]. It has the same immobilization chemistry and Protein A as the High Capacity media; however, the pore size of the controlled porous glass is smaller, approximately 700 Å. This results in more Protein A immobilized on to the matrix increasing the number of IgG binding sites. The pore structure maintains the rapid mass transfer and facilitates higher binding percentages before risk of premature breakthrough. The static binding capacity of this resin for human polyclonal antibodies is greater than 56 mg/ml.

This chromatography media also maintains the operation flexibility of the high capacity resin [73]. Also, the low backpressure and rigidity allows high operational flow rates, resulting in high throughput and greater productivity. This media utilizes multipoint to minimize ligand leakage. However, due to the decrease in pore size, the high capacity media may be a more appropriate medium when purifying antibody conjugates exceeding 150 kDa. This is because larger pore size of the high capacity media provides less diffusion resistance to larger molecules.

#### *2.6.5 ProSep Ultra Plus Resin*

The ProSep Ultra Plus chromatography media was manufactured based on the ProSep-vA Ultra media but further optimized binding capacity [42]. This media has been shown to provide the highest dynamic binding capacity and flow rates when compared to other comparable resins available on the market. The increased binding capacity purifies samples with higher expression levels in a shorter process time. The Ultra Plus media utilizes a smaller particle size of 60 µm than the high capacity and ultra media. The pore size of the Ultra Plus resin is 800 Å [74]. The static binding capacity of human polyclonal antibodies for this media is greater than 67 mg/ml. While the recommended mobile phase velocity for the High Capacity and Ultra media can exceed 1000 cm/h, the recommended mobile phase velocity for the Ultra Plus should not exceeded 800 cm/h. Although this is still a high linear velocity, this decrease in mobile phase velocity is a result of the smaller particle size utilized by this media. Also, since the Ultra Plus resin is developed based on the -vA High Capacity and -vA Ultra media, it maintains the re-usability. The Ultra Plus resins also maintains the mass transfer characteristic of the -vA High Capacity and -vA

Ultra, which allows more antibodies to bind to the resins before the risk of premature breakthrough.

### 2.7 Protein A Leakage

Protein A leakage is the leaching of Protein A molecules from the commercial media. Protein A often co-elutes with the desired IgG during the elution phase of the process [32]. According to Vunnum *et al.*, Protein A chromatography can leach intact as well as fragments of Protein A molecules with molecular weights ranging from 6 to 40 kDa [4]. This is caused by proteolytic cleavage of the inter-domain sequences by proteases present in the protein extract. Although the addition of EDTA to protein extraction samples from the host cell inhibits, to some extent, proteases from proteolytic cleavage of the ligand, other proteases may still occur. In addition, controlled porous glass matrixes have increased solubility at basic pH; therefore, the variation in pH during chromatography cycling may cause small portions of the base-matrix as well as the Protein A attached to dissolve. This causes the free Protein A that is bound to IgG to co-elute with the antibody. Protein A co-eluting with pharmaceutical antibodies for humans presents a problem that requires further processing steps to remove the Protein A. For instance, the Protein A chromatography to capture the antibodies has been coupled with cation-exchange chromatography (CEX) to remove aggregates of leached Protein A [39].

### 2.8 Conclusion

Technological advancements in molecular farming have shifted the production of therapeutic protein towards the commercial exploitation of these products. However, the increasing demand for these products, especially antibodies, requires the processing of larger quantities of biomass with higher expression levels of the product. Tobacco is a promising expression system due to its high biomass yield, robust transformation technology, and ease of scale up. Therefore, downstream processing is now being improved to accommodate the higher capacity required by the biotechnology industry for therapeutic protein production. Protein A affinity chromatography, if introduced early in the purification process, can reduce successive unit operations, thereby reducing purification costs. However, using affinity chromatography early in the process can lead to fouling of the column as well as loss in media performance due to non-specific binding of HCP and Protein A leakage. Characteristics of the commercial ProSep media family

have been improved for high throughput and productivity. Established cleaning protocols have shown the Prosep media family to have 300 cycle lifetime without loss of performance. However, it is not known how tobacco protein extract will interact and/or interfere with antibody purification on these Prosep medias. Therefore, this project was conducted to:

- To determine if there is evidence of non-specific binding of native tobacco protein to the Protein A resins, and how different purification conditions affect the purification efficiency of the three Protein A resins
- To determine if native tobacco protein reduces the purification efficiency of three Protein A resins (ProSep-vA High Capacity, ProSep-vA Ultra, and ProSep Ultra Plus).
- To collect information regarding the efficiency/effectiveness on ProSep Protein A Resins over multiple cycles of tobacco protein extracts.

## Chapter 3: Experimental

### 3.1 Materials

Acetic acid (ACS grade, 99.7%), 2-mercaptoethanol, 0.22  $\mu\text{m}$  sterile mixed cellulose ester (MCE) syringe filters, acetone (histological grade, 99.5+%), ethanol (HPLC grade, 91%), ethylenediaminetetraacetic acid (EDTA) (ACS grade, 99+%), hydrochloric acid, purified human IgG, methanol (HPLC grade, 99.9%), sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ) (ACS grade), sodium hydroxide (NaOH), sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) (USP grade), phosphoric acid (ACS grade, 85%), polyvinylpyrrolidone cross-linked (PVPP), potassium chloride (KCl) (99.0%), potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (ACS grade, 99.7%), sodium chloride (NaCl) (Biological grade, 99.9%), were obtained from Fisher Scientific (Pittsburg, PA).

Guanidine HCl, magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) (minimum 98%), sodium acetate (ACS grade, 99+%), phytigel, phytatray II tissue culture boxes, potassium hydroxide (KOH), potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) (ACS grade, 100%), scalpel blades and handles, urea (ACS grade) were obtained from Sigma-Aldrich Company (St. Louis, MO). Murashige and Skoog basal salts, vitamins, and sucrose were obtained from Bioworld (Dublin, OH).

NuPAGE 20x MOPS SDS running buffer, NuPAGE antioxidants, 4x LDS (lithium dodecyl sulfate) sample loading buffer, NuPAGE 20x MES SDS running buffer (1 M 4-Morpholineethanesulfonic acid, 1 M Tris, 2% (w/v) SDS, 20 mM EDTA), 4-12% Bis-Tris NuPAGE gels, 10x NuPAGE sample reducing agent (500 mM stabilize dithiothreitol (DTT)), SilverXpress silver staining kit, SeeBlue Plus2 Pre-Stained Standard were obtained from Invitrogen Life Technologies (Carlsbad, CA).

YM-10 Microcon centrifugal filter devices (MWCO 10,000), YM-10 Amicon Ultra centrifugal filter devices (MWCO 10,000), ProSep -vA High Capacity resin, ProSep -vA Ultra resin, and ProSep Ultra Plus resin were obtained from Millipore Corporation (Billerica, Ma)

Concentrated protein assay reagent were obtained from Bio-Rad Laboratories, Incorporated (Hercules, CA). Bovine serum albumin (BSA) and Bovine gamma globulin (BGG) were obtained from pierce Biotechnology, Incorporated (Rockford, IL).

The 0.45  $\mu\text{m}$  polyvinylidene difluoride (PVDF) syringe filters and 0.45  $\mu\text{m}$  nylon membranes filters were obtained from Whatman Incorporated (Sanford, ME). Microcloth was obtained from EMD biosciences, Incorporated (La Jolla, CA). The 15 mL and 50 mL polystyrene tubes, flat bottom 96-well microtiter plate, and disposable syringes without needles were obtained from Becton Dickinson and Company (Franklin Lakes, NJ).

### 3.2 Method

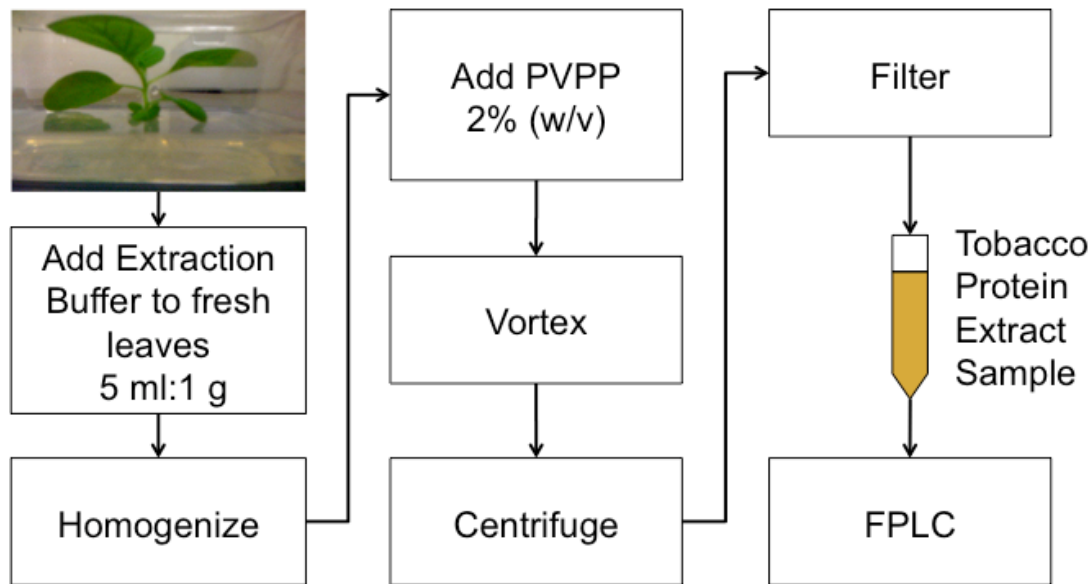
#### *3.2.1 Generation of Tobacco Plants*

*Nicotiana tabacum* cv. Xanthi seedlings, provided by Dr. Carol Wilkinson (Virginia Tech Southern Piedmont Agriculture Research and Extension Center), were surface sterilized by stirring continuously for 15 minutes in a 20% bleach solution. The seedlings were washed 4 times in sterilized  $\text{H}_2\text{O}$  and transferred into Phytatray II magenta boxes containing approximately 100 mL modified MS media. The modified MS media consists of Murashige and Skoog basal salts and vitamins, 3% (w/v) sucrose, 0.04% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.4 % (w/v) phytigel. The pH of the mMS media was adjusted to 5.7 with KOH and then autoclaved. Fresh plants were maintained by excising the spiral shoot of plants and transplanting into fresh mMS media approximately every 4 weeks. Seedling and plants were grown aseptically in a CU-32L growth chamber (Percival Scientific; Boone, IA) with a 16-hour light, 8-hour dark photoperiod at 24°C. To maintain sterile plants and seedlings, all tissue work was performed in the NuAire Laminar Air Flow Hood (Plymouth, MN).

#### *3.2.2 Protein Extraction from Tobacco Plants*

Fresh plant tissue was harvested and leaf tissue was weighed out and placed in a 50 ml conical tube containing a chilled extraction buffer at a ratio of 5 ml extraction buffer: 1 g leaf tissue. The extraction buffer consists of 50 mM sodium phosphate buffer (NaPi) at pH 7, 10mM BME, 1mM EDTA. The chilled samples were homogenized using a PowerGen 700 Homogenizer (Fisher Scientific, Pittsburg, PA) in 30 seconds increments until no large particulates remained. Immediately after homogenization, 2% (w/v) insoluble PVPP was added to each sample and vortexed vigorously. The samples were incubated at room temperature for 15 minutes. Then the samples were centrifuged for 20 minutes at 4°C and 17,000 x g using a 5810R Centrifuge (Eppendorf AG; Westbury, NY). The supernatant was decanted and filtered through a 0.22  $\mu\text{m}$  syringe

filter into a fresh 50 ml conical tube. Figure 3.1 presents the overall flowchart for the sample preparation procedure for the tobacco protein extract.



**Figure 3.1. Flowchart of overall procedure for sample preparation of tobacco protein extract.**

### *3.2.3 Quantification of Tobacco Protein Extract Concentration and IgG samples*

The protein concentration of the tobacco extract was quantified using a Bio-Rad protein assay. Using bovine serum albumin at 2 mg/ml (Promega Corp.; Madison, WI) as a protein standard, five dilutions were prepared. The BSA was diluted in 50 mM NaPi extraction buffer to produce final concentrations of 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, and 0.5 mg/ml. The dye reagent was prepared by diluting one part Bio-Rad protein dye reagent concentrate with four parts of de-ionized water. 10  $\mu$ L of tobacco protein extract was allocated into a 0.5 mL microcentrifuge tube and diluted 4-fold in extraction buffer. 10  $\mu$ L of protein standards and samples was transferred into separate wells of a flat bottom 96-well microtiter plate. The assay was performed in duplicate and 200  $\mu$ L of dilute dye reagent was added to each well. The samples were mixed using a clean pipette for each sample. The plate was incubated at room temperature for 5 minutes. The absorbants were measured using a BioTek Synergy HT multi-detection microplate reader (Winooski, VT) at 595 nm. The absorbency readings of protein standards were plotted against the known concentration of the standard. The equation and

regression of the trendline were determined and used to calculate the protein concentration of tobacco extracts. The protein concentration of the IgG was quantified using the Bio-Rad protein assay, but used bovine gamma globulin at 2 mg/ml (Promega Corp.; Madison, WI) as a IgG standard. The BGG was diluted in 50 mM NaPi extraction buffer to produce final concentrations of 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, and 0.5 mg/ml.

#### *3.2.4 Packing 1 ml ProSep Resin Columns*

ProSep media resin, stored at 4 °C in 0.1 M sodium acetate at pH 5.2 containing 1% benzyl alcohol, was warmed to room temperature and mixed well. Media was poured into a graduated cylinder and allowed to settle until the resin volume reached approximately 1 ml. The storage buffer was removed and replaced with an appropriate amount of PBS buffer to create an approximately 80% slurry. The bottom of a 15 ml conical tube was cut off and the slurry was added to the upside-down conical tube (cap on). A small magnetic stir bar was dropped into the slurry and placed onto a hotplate stirrer PC-351 (Corning; Lowell, MA). The tubing connected to the pump was attached to the open-ended conical tube. The stir plate was activated and the slurry was degassed for approximately 30 minutes.

An empty C 10/10 column (GE healthcare; Pittsburgh, PA) was assembled and mounted vertically at an elevated height. A 10 ml syringe was connected to the outlet and PBS buffer was pumped into the bottom of the column until the buffer reached approximately 2 cm from the bottom to wet the bottom bed support. The outlet was then closed. The slurry was poured down the column wall to avoid air entrapment and allowed to settle. While settling, the side of the column was tapped with a hard object for approximately 2 minutes to consolidate media.

The top AC 10 flow adapter (GE healthcare; Pittsburgh, PA) was assembled. Air bubbles were removed from the inlet tubing, and the adapter tip was wetted with PBS buffer. Once the air bubbles were removed from the assembled flow adaptor, the inlet tube was closed. The bottom outlet was opened and the flow adapter was inserted into the top of the column, pushing the buffer through the column slowly until a stable bed was formed and there was no liquid space above the packed bed. The outlet was closed and the flow adapter was fastened into place.

### 3.2.5 Column Hook-Up to Akta Explorer System

An Akta Explorer fast performance liquid chromatography (FPLC) system (GE healthcare; Pittsburgh, PA) was used for all chromatographic experiments. Pump A and B on the FPLC were purged using a 10 ml syringe. The system wash was performed using the PBS equilibration buffer at pH 7.4, the PBS wash buffer, the 100 mM acetate elution buffer at pH 3, and the 150 mM phosphoric acid regeneration buffer at pH 1.5. The four PBS wash buffers contained either 140 mM NaCl at pH 5, 140 mM NaCl at pH 7, 1 M NaCl at pH 5, or 1 M NaCl at pH 7. Using the equilibration buffer, the AKTA system was switched to column position 2 with an upward flow direction at 0.5 ml/min. The outlet tubing of the C 10/10 column containing ProSep Ultra Plus resin was connected to the column position 2 outlet of the AKTA system. Then a downward flow direction was executed and the inlet tubing of the column was connected to the column position 2 inlet of the AKTA system. This procedure was repeated for connecting C 10/10 columns containing the ProSep -vA high capacity and -vA Ultra media to column position 7 and 8, respectively, making sure not to introduce air bubbles into the column.

### 3.2.6 Testing efficiency of packed Prosep affinity column

After the packed columns were hooked up to the FPLC system, the columns were equilibrated with 5-8 CV PBS buffer at pH 7.4, 0.14 mM NaCl. A 1% acetone solution was injected onto the column to determine the peak retention volume ( $V_R$ ) and peak asymmetry ( $A_s$ ) for a non-binding solution. This was used to determining the efficiency of the column. The column was washed with 4-5 CV the equilibration buffer. Using the Unicorn system the peak retention volume ( $V_R$ ), the peak width at half height ( $w_R$ ) and asymmetry values were obtained. The number of theoretical plates, and HETP were calculated by the following equations.

$$N = 5.54 \cdot \left( \frac{V_r}{W_H} \right)^2 \quad (1)$$

where

$N$ = number of theoretical plates

$V_R$ = peak retention (elution) volume or time

$W_h$ = peak width at half height

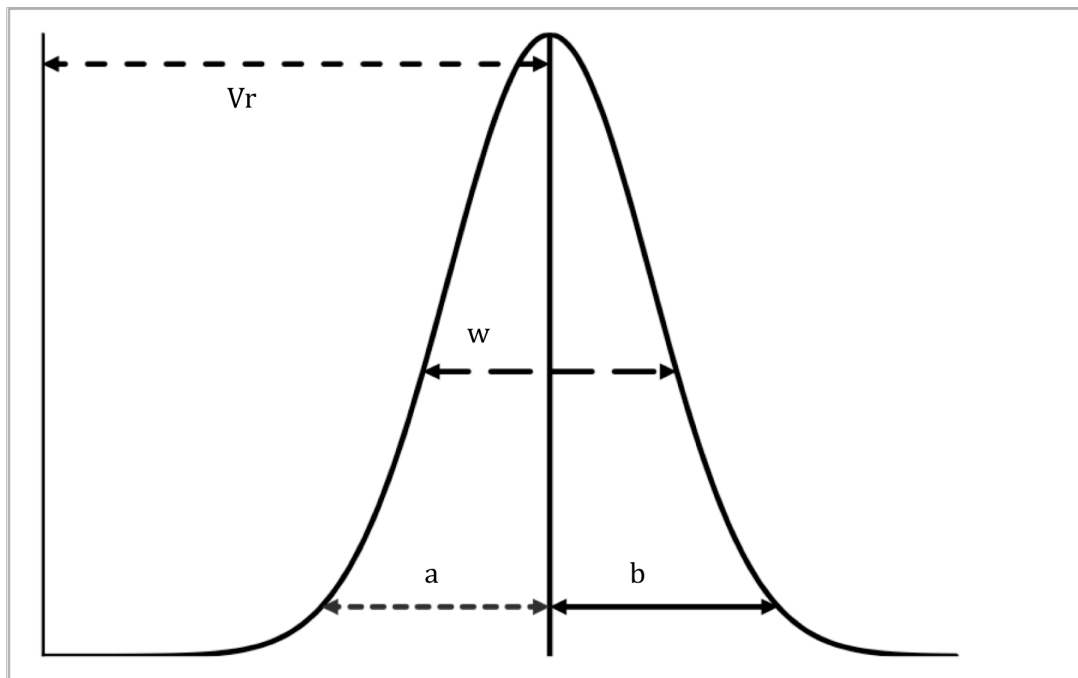
$$HETP = \left( \frac{L}{N} \right) \quad (2)$$

where  
HETP= height equivalent to a theoretical plate  
L= bed height (cm)

$$A_s = \left( \frac{b}{a} \right) \quad (3)$$

Where  
A<sub>s</sub>=asymmetry  
b= tailing peak width measured at 10% of peak height  
a= leading peak width measured at 10% of peak height

Figure 3.2 presents a chromatography peak labeled with the parameter used for the calculating the HETP and asymmetry. An acceptable Prosep Ultra Plus column requires the HETP be less than 0.1 cm and the asymmetry be between 0.8-1.6. These same values were assumed for the ProSep -vA High Capacity and PorSep -vA Ultra columns.



**Figure 3.2. Chromatography peak labeled with parameter required for determining height equivalent theoretical plates (HETP) and asymmetry (As).**

### 3.2.7 Operating Chromatography Cycle

Each column was equilibrated with 10 column volumes (CV) of phosphate buffered saline (PBS), pH 7.4 at a flowrate of 1.7 ml/min. Purified human IgG was solubilized in a NaPi buffer at a concentration of 2 mg/ml and clarified using a 0.22  $\mu$ m

syringe filter prior to loading. The human IgG sample was loaded onto the column with a residence time of 3 min until 10% of the breakthrough curve was reached. The column was then washed with 10 CV of PBS buffer at 1.7 ml/min. The IgG was then eluted in 10 CV of 100 mM acetate buffer, pH of 3.0. The eluted IgG was captured and re-concentrated using Amicon Ultra YM-10 at 10,000 x g for 25 minutes. The column was regenerated with 5 CV of 150 mM phosphoric acid, pH 1.5 at 1.7 ml/min and then re-equilibrated with 10 CV of PBS buffer at 1.7 ml/min. The same chromatography cycle was repeated when loading the tobacco extract onto the column; however, the tobacco extract was loaded until it reached 90-100% breakthrough. The tobacco flowthrough was captured and quantified using the Bio-Rad protein assay. The proteins washed during the elution and regeneration of the column were captured and concentrated for further analysis. For every 8-10 cycles of tobacco extract run through the column, the cleaning-in-place (CIP) method was applied to wash the column off tightly bound residual proteins with 6 M Guanidine hydrochloride for 5 CV. Table 3.1 summarizes a typical chromatography cycle.

**Table 3.1. Summary of chromatography cycle used when running tobacco protein extract or IgG samples through columns packed with ProSep-vA High Capacity, ProSep-vA Ultra, and ProSep Ultra Plus resins.** This protocol follows *ProSep Ultra Plus Affinity Chromatography Media* developed by Millipore.

Step	Buffer	Buffer pH	Column Volume (CV)	Flow Rate (mL/min)
Equilibration	PBS	7.4	10	1.7
Load	Sample (tobacco protein extract or IgG sample)		Dependent on concentration of sample and binding capacity of resin	0.3 (residence time ~3 min)
Wash	PBS	5 or 7.4	10	1.7
Elution	0.1 M acetate	3.0	10	1.7
Regeneration	150 mM Phosphoric acid	1.5	5	1.7
Re-equilibration	PBS	7.4	10	1.7
Cleaning (~ every 5-10 cycles of tobacco extract)	6 M Guanidine HCl or 6 M Urea		5	1.7

### *3.2.8 Testing Post Load Wash Buffer Conditions*

The columns were equilibrated using PBS buffer containing 140 mM NaCl at pH 7.4. Then, 1 mg of the IgG sample was loaded onto the column. The same PBS buffer used to equilibrate the buffer was then used to wash the unbound IgG from the column, collecting the flowthrough for quantification. The IgG was then eluted from the column and the IgG proteins were collected for quantification, and then concentrated using Amicon Ultra YM-10 filtration device for reuse. This procedure was repeated using 3 other post load wash buffer conditions: PBS with 140 mM NaCl @ pH5, PBS with 1M NaCl @ pH 7, and PBS 1M NaCl @ pH 5. These post load wash buffers were used to determine what conditions were optimal for the capture of IgG while avoiding premature elution of the IgG.

Once it was determined how the post load wash buffers would affect IgG samples loaded onto the columns, tobacco extract was then tested using the same procedure. Although tobacco proteins should not bind to the columns, a small portion of the tobacco proteins will non-specifically bind to the column. Therefore, in order to collect a large enough sample of tobacco protein that non-specifically bind to the column and elute during the elution step of the chromatography cycle, the tobacco samples were loaded onto the column until the breakthrough point was reached.

### *3.2.9 Mass Balance Analysis of Tobacco Extract and IgG Samples Loaded onto Column*

The tobacco proteins collected during the post load wash step were quantified using the Bio-Rad protein assay, as described above, but using the same post load wash buffer in which the samples were collected to prepare the standard. The IgG protein collected during the elution step were quantified using the Bio-Rad protein assay, as described above, but using the same elution buffer in which the samples were collected to prepare the standard.

The tobacco and IgG samples collected during the elution and post load wash step, respectively, were quantified using a Bio-Rad Protein microassay. Using bovine serum albumin at 2 mg/ml (Promega Corp.; Madison, WI) as a protein standard for tobacco proteins, five dilutions were prepared. The BSA was diluted in elution buffer to

produce final concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml. 160 µL of the protein samples were transferred into separate wells of a flat bottom 96-well microtiter plate. The assay was performed in duplicate and 40 µl of dye reagent concentrate was added to each well. The samples were mixed using a clean pipette for each sample. The plate was incubated at room temperature for 5 minutes. The absorbents were measured using a BioTek Synergy HT multi-detection microplate reader (Winooski, VT) at 595 nm. The absorbency readings of protein standards were plotted against the known concentration of the standard. The equation and regression of the trendline were determined and used to calculate the protein concentration of the samples. The same procedure was repeated to quantify the IgG samples except using bovine gamma globulin at 2 mg/ml (Promega Corp.; Madison, WI) as a protein standard. The BGG was diluted in the corresponding post load wash buffer to produce final concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 60 µg/ml, and 80 µg/ml.

### *3.2.10 Analysis of Proteins via SDS-PAGE Gel*

The tobacco protein extract and the flowthrough were diluted 20 fold. The fractions collected during the elution and regeneration of the column were concentrated using Amicon Ultra YM-10 filtration device. Samples were concentrated according to the operation manual at 10,000 x g for 20 minutes. The protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The samples were diluted with 4x NuPAGE LDS sample running buffer and 10x NuPAGE reducing agent to 1x. All samples were heated for 10 minutes in a 70°C water bath (Precision; Winchester, VA). A 4-12% Bis-Tris NuPAGE gel was mounted into an XCell SureLockMini-Cell (Invitrogen Life Technologies; Carlsbad, CA). The inner chamber was filled with 200 ml of 1 x MES SDS running buffer containing 500 µL NuPAGE antioxidants, and the outer chamber was filled with approximately 600 ml of 1x MES SDS running buffer. The samples were loaded onto the gel and, by applying 200V for 40 minutes, the proteins within the samples migrated based on size down the gel. This separated the proteins within the sample based on size with the smaller proteins migrating farther down the gel.

The gels were silver-stained using the SilverXpress silver staining protocols. The samples were fixed to the gel by lightly shaking in 200 ml of fixing buffer for 10 minutes.

The fixing buffer was removed and the gels were incubated in 100 ml of sensitizing buffer for 30 minutes twice. The gels were then washed twice in 200 ml of ultra pure water for 10 minutes and stained for 15 minutes in 100 ml of staining buffer. The gels were washed in 200 ml of ultra pure water for 5 minutes twice. The stained gels were developed with 100 ml of developing buffer for 3-5 minutes. Once the gels were visibly developed, 5 ml of stopper was added directly to the developing buffer and shaken for 10 minutes. Finally, the gels were washed three times with 200 ml of ultra pure water for 10 minutes. The gel image was captured under epi-white light with the ChemiDoc XRS molecular imager (Bio-Rad Laboratories Inc.; Hercules, CA).

### *3.2.11 Dynamic Breakthrough Curves*

Purified human IgG was added to NaPi extraction buffer to a final concentration of 2 mg/ml. The IgG sample was run through the bypass of AKTA explorer system, and the UV absorbency at 280 nm was monitored to determine the value of 100% breakthrough point. Once 100% breakthrough point was known, the IgG sample was loaded onto the column until 10% breakthrough point was reached. The IgG was recaptured during the flowthrough and elution phase of the chromatography cycle and re-concentrated using YM-10 Amicon centrifugal filter devices for re-use. Once the IgG cycle was complete, the samples of tobacco protein extract were repeatedly loaded onto the column. Periodically, the dynamic breakthrough point of the column was retested by loading an IgG sample (2mg/ml) onto the column until 10% breakthrough point was reached. The column was cleaned using 6M Guanidine HCl or 6M Urea every 8-10 cycles of tobacco extract run.

## Chapter 4: Results and Discussion

### 4.1 Column Efficiency

The columns were packed with 1 mL of ProSep-vA High Capacity, ProSep-vA Ultra, and ProSep Ultra Plus resin and the efficiency of the column were tested with 1% acetone. Figure 4.1 presents the chromatogram of the efficiency test for the 1 mL ProSep Ultra Plus column. The asymmetry and the HETP were calculated for each 1 mL column (Table 4.1). Once the asymmetry was within 0.8-1.6 the tobacco extract or IgG samples were loaded onto the column.

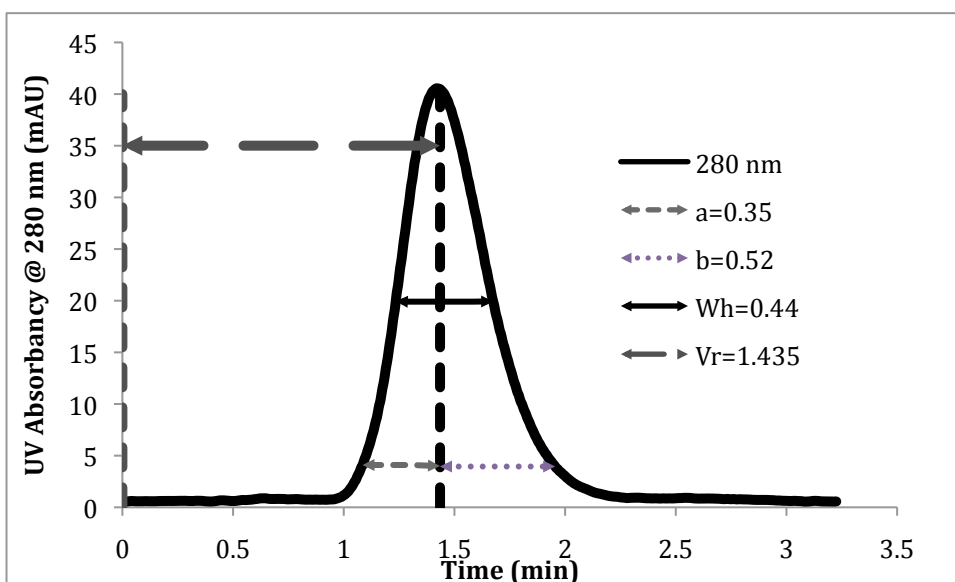


Figure 4.1. Efficiency test for 1 ml ProSep Ultra Plus column.

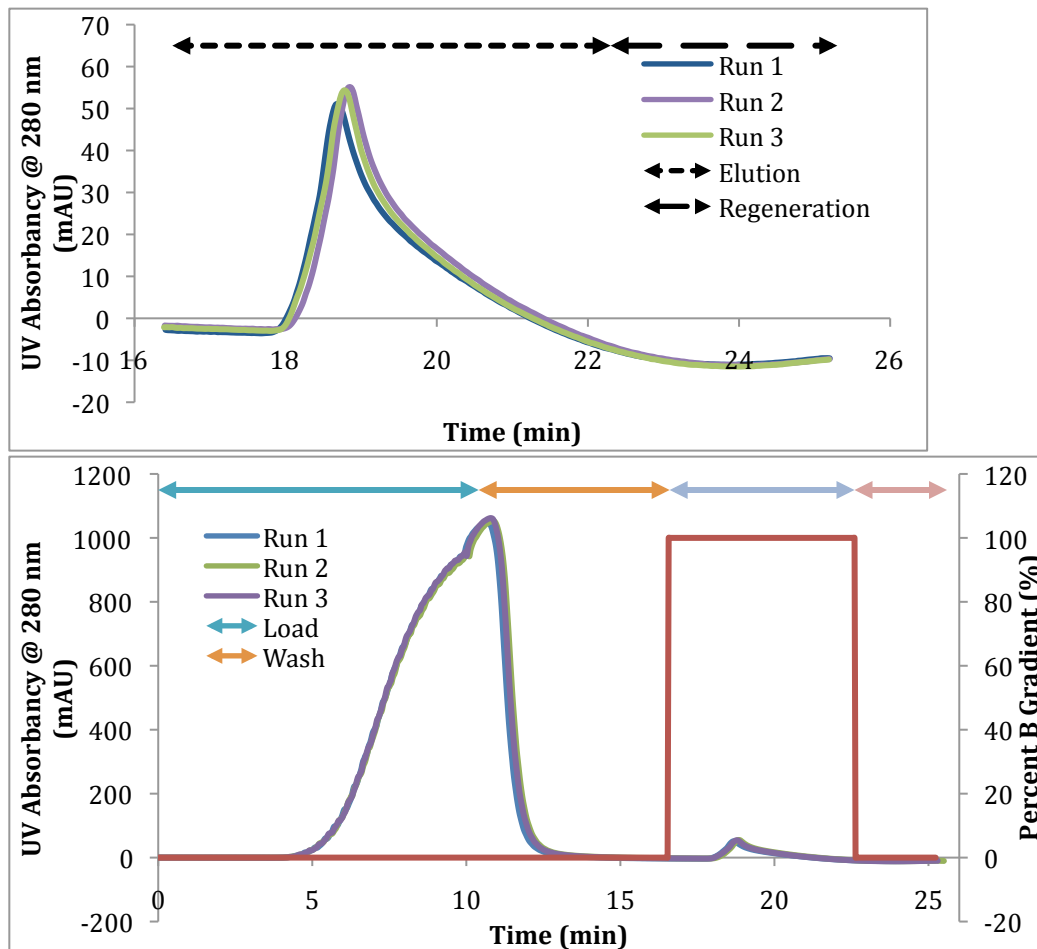
Table 4.1. Summary of asymmetry ( $A_s$ ), number of theoretical plates ( $N$ ), and height equivalent theoretical plates (HETP) for ProSep-vA High Capacity, vA Ultra, and Ultra Plus.

Resin	Asymmetry ( $A_s$ )	Number of Theoretical Plates ( $N$ )	Height equivalent theoretical plates (HETP) (cm)
vA High Capacity	1.51	66.7	0.015
vA Ultra	1.52	36.1	0.019
Ultra Plus	1.32	84.3	0.011

### 4.2 Wash Buffer Conditions

Tobacco extract was loaded onto the column to determine whether, and to what degree, tobacco proteins were non-specifically binding to the ProSep A resins. As shown in Figure 4.2, the chromatogram shows 3 runs of tobacco protein extract loaded onto the ProSep-vA Ultra column and an enlarged view of the elution and regeneration steps of

the chromatography cycle. During the elution step, a spike in the absorbency at 280 nm was detected indicating the presence of native tobacco proteins non-specifically binding to the ProSep A resin. In order to reduce the amount of tobacco proteins non-specifically binding to the ProSep A resin, four different post load wash buffer conditions were examined. The column was equilibrated with a PBS buffer containing 140mM NaCl at pH 7 to promote the binding of IgG to Protein A. Therefore, the efficiency of the post load wash buffer was tested under 4 conditions: 140mM NaCl at pH 7 (same as equilibration buffer), 140mM NaCl at pH 5, 1M NaCl at pH 7, and 1 M NaCl at pH 5, on columns packed with 1 mL of the three different ProSep A resins used for this project.

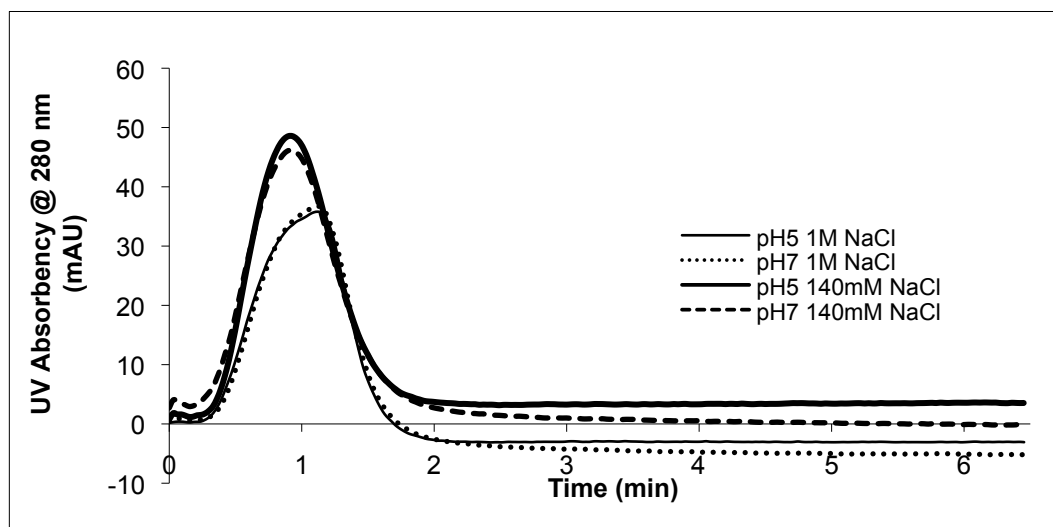


**Figure 4.2. Chromatogram of 3 runs of tobacco cv. xanthi protein extract on 1 mL ProSep vA-Ultra column.** After xanthi extract was loaded onto the column, the column was washed with PBS buffer containing 1 M NaCl at pH 7. (Bottom) complete chromatogram cycle; (Top) Elution and regeneration steps of chromatogram cycle.

#### 4.2.1 Testing Wash Buffers for IgG Loaded onto ProSep-vA High Capacity

##### Column

Prior to examining how four different wash buffer conditions affect the binding of tobacco proteins to the ProSep A resins, the four wash buffers were tested to determine how this conditions will affect the premature elution of Human IgG. First, the conditions of the post load wash buffer were tested when loading IgG samples on the column packed with 1 mL of the ProSep-vA High Capacity resin. Figure 4.3 presents an enlarged view of the post load wash step of the chromatography cycle for the various buffer conditions tested. Increasing the salt concentration of the mobile phase can disrupt ionic interactions; however, the Fc region of IgG mainly interacts to the Protein A ligand through hydrophobic interactions. Therefore, as Figure 4.3 indicates, increasing the salt concentration of the wash buffer decreased the amount of IgG in the post load wash mobile phase prior to elution. At low pH, the surface of the Protein A and IgG Fc region that face each other have highly conserved ionizable amino acid residues that become poistively charged. These charges repel each other weakening the hydrophobic interaction between the Portein A and human IgG, resulting in the elution of the antibody from the Protein A Ligand. Figure 4.3 indicates that when the post load wash buffer had a pH of 5, there was a slight increase of IgG present in the mobile phase of the post load wash buffer than the wash buffer with pH 7.

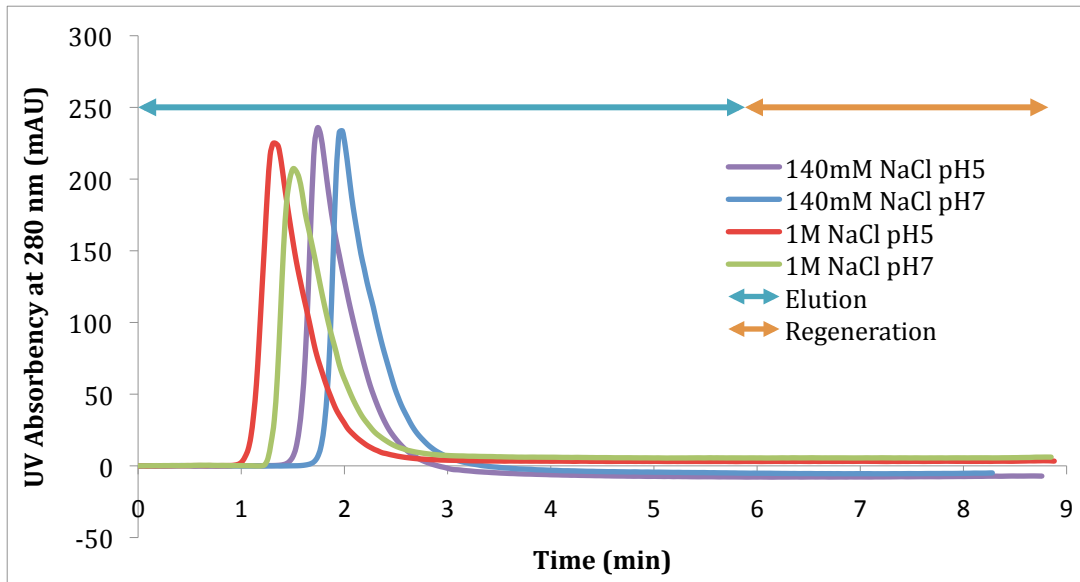


**Figure 4.3. Enlarged view of post load wash step of chromatography cycle for human IgG loaded onto ProSep- vA High Capacity resin with various buffer coniditions.** The column was packed with 1 mL of ProSep-vA High Capacity resin.

Table 4.2 summarizes the mass balance analysis of the IgG samples loaded onto the ProSep-vA High Capacity resin with various post load wash buffer conditions. Although increasing the salt concentration of the post load wash buffer did result in less human IgG prematurely eluting from the resin, it did result in a greater amount of IgG protein remaining bound to the vA High Capacity resin after elution. Figure 4.4 presents the IgG elution peak after the various post load wash buffer conditions were used to wash the column. Although the post load wash buffer containing 1M NaCl, at pH 7, was more efficient at preventing the premature elution of IgG from the column prior to elution, the post load wash contained 140mM NaCl, at pH 5, was more efficient at the capture of IgG during elution. The post load wash buffer conditions containing a higher salt concentration reinforces the hydrophobic bonds between IgG and Protein A, thereby delaying or preventing the elution of IgG from the Prosep-vA High Capacity resin. On the other hand, using an intermediate pH weaken the hydrophobic interaction between IgG and Protein A, promoting the elution of IgG from the ProSep A column.

**Table 4.2. Mass balance analysis of human IgG loaded onto ProSep vA High Capacity resin with various PBS post load wash buffers.** This data summarizes the mass balance calculations for the Human IgG samples loaded onto the ProSep-vA High Capacity column.

PBS Wash Buffer	Human IgG loaded onto column (mg)	IgG in post load wash ( $\mu$ g)	Percentage of Human IgG eluted (%)	Percentage of Human IgG still bound to column after elution (%)
140 mM NaCl @ pH 5	0.61 $\pm$ 0.0084	19.54 $\pm$ 1.76	89.90 $\pm$ 0.27	6.90 $\pm$ 0.06
140 mM NaCl @ pH 7	0.72 $\pm$ 0.13	37.17 $\pm$ 5.53	85.86 $\pm$ 1.71	8.94 $\pm$ 1.89
1 M NaCl @ pH 5	0.63 $\pm$ 0.017	14.12 $\pm$ 2.72	79.28 $\pm$ 1.15	18.50 $\pm$ 1.50
1 M NaCl @ pH 7	0.63 $\pm$ 0.021	12.04 $\pm$ 5.90	85.06 $\pm$ 2.40	13.04 $\pm$ 1.52

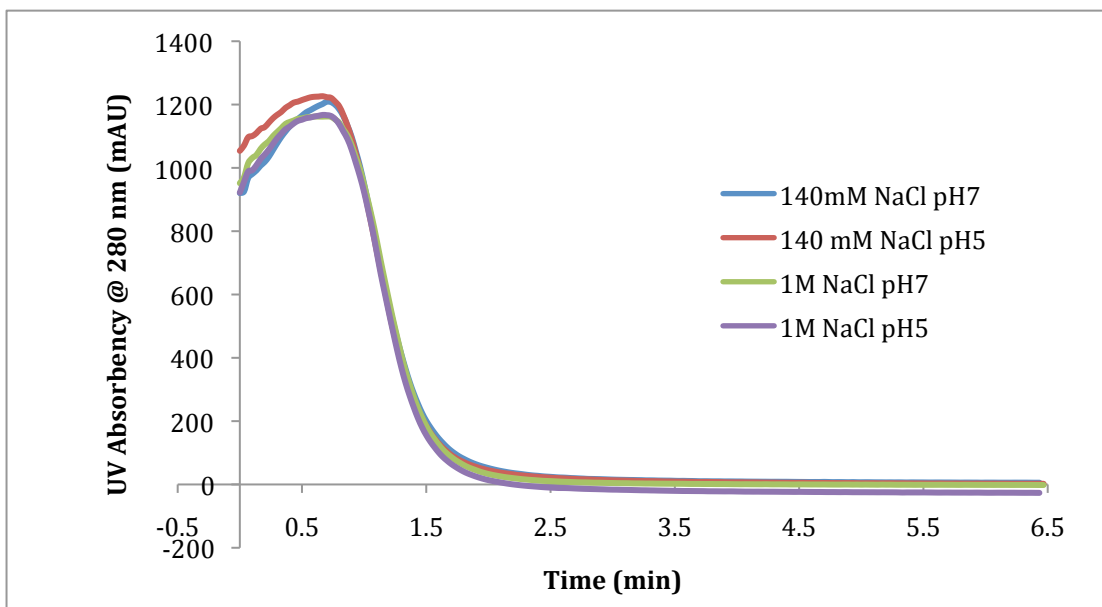


**Figure 4.4. Enlarged view of elution and regeneration step of chromatography cycle for human IgG loaded onto ProSep- vA High Capacity resin with various buffer conditions.** The figure presents data performed on the ProSep-vA High Capacity resin.

#### *4.2.2 Testing Wash Buffer Conditions for Tobacco Extract Loaded onto ProSep- vA High Capacity Column*

After testing the effects of the post load wash buffer conditions, the same conditions were tested when loading tobacco extract onto the ProSep-vA High Capacity resin. Figure 4.5 presents an isolated view of the wash step of the chromatography cycle after loading tobacco extract until breakthrough point was reached; therefore, the graph of the isolated view does not start at baseline. While it is recommended to wash the column with 10 column volumes (CV) of PBS buffer, the majority of the tobacco protein flowthrough was washed from column within 3 minutes or approximately 5 CV of wash buffer. All four wash buffer conditions tested washed the unbound tobacco proteins from the column in approximately the same amount of time. Therefore, during scale-up of this process, the amount of CV of wash buffer used for the removal of residual tobacco proteins from the column could be reduced, creating a more cost effective downstream process in terms of time and materials. Large volumes of wash buffer should be avoided to decrease weakly binding antibodies from prematurely eluting. In Affinity Chromatography Media Operating Instructions by Millipore, it is recommended that no

more than 10-15 CV be required to wash the ProSep-A resins [58].



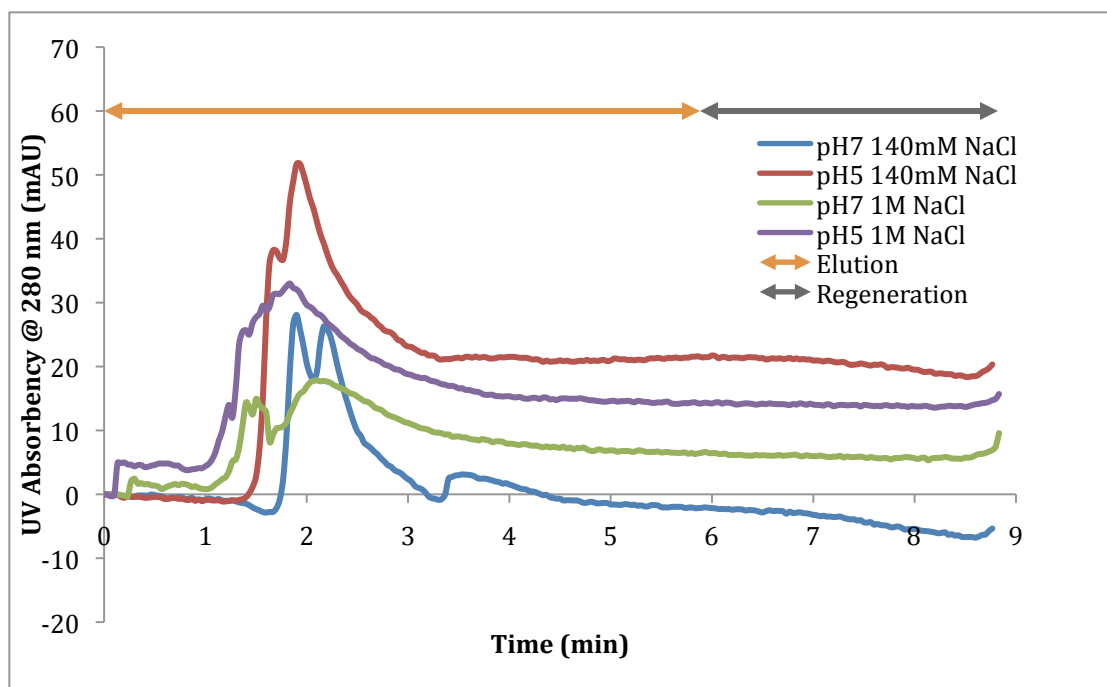
**Figure 4.5. Enlarged view of post load wash step of chromatography cycle for tobacco extract loaded onto ProSep- vA High Capacity resin with various buffer conditions.** The column was packed with 1 mL of ProSep-vA High Capacity resin. The graph presents the UV absorbency at 280 nm after loading tobacco extract until breakthrough point. Therefore, graph of isolate view does not start at baseline.

The efficiency of the post load wash buffer conditions were tested by conducting mass balance analysis of the protein loaded onto the column compared to the amount of protein in the post load wash buffer mobile phase and elution buffer mobile phase. Increasing the salt concentration of the mobile phase can disrupt ionic, or electrostatic, interactions between native tobacco proteins within the mobile phase and ProSep A resin. However, increasing the ionic strength of the mobile phase may lead to retention of other native tobacco protein through hydrophobic interaction. Table 4.3 summarizes this mass balance for the tobacco protein extract loaded onto the -vA High Capacity resin prior to elution. This table indicates that the PBS wash buffer containing 140 mM NaCl was more efficient at washing the tobacco proteins from the column prior to elution than the PBS wash buffer containing 1 M NaCl. Since the equilibration buffer contained 140 mM NaCl to promote the binding of IgG to Protein A, a lower salt concentration was not tested in these experiments.

**Table 4.3. Mass balance analysis of tobacco protein extract loaded onto a ProSep-vA High Capacity resin with various PBS post load wash buffer conditions.** This data summarizes the mass balance calculations for the tobacco protein extract samples loaded onto the ProSep-vA High Capacity column prior to elution.

PBS Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco protein in flowthrough (mg)	Tobacco protein bound to column after wash (mg)	Percentage of tobacco protein bound to column prior to elution (%)
140 mM NaCl @ pH 5	4.32 ± 0.04	3.65 ± 0.28	0.67 ± 0.32	15.4 ± 7.28
140 mM NaCl @ pH 7	3.99 ± 0.00	3.58 ± 0.14	0.41 ± 0.14	10.2 ± 3.43
1 M NaCl @ pH 5	4.01 ± 0.05	3.19 ± 0.18	0.77 ± 0.26	19.3 ± 6.66
1 M NaCl @ pH 7	4.03 ± 0.06	3.24 ± 0.31	0.84 ± 0.24	20.9 ± 5.64

According to Balasubramaniam *et al.*, there are more acidic proteins than basic proteins in tobacco and the overall cellular environment of tobacco is slightly acidic (pH~5) [26]. Therefore, decreasing the pH of the post load wash may affect the amount of native tobacco proteins bound to the Protein A columns through hydrophobic interactions. Ionic interactions between the resin and native tobacco proteins are disrupted by the addition of salts, such as sodium chloride (NaCl) to the post load wash buffer. The mass balance indicates that increasing ionic strength of the post load wash buffer resulted in an increase of proteins binding to the column. This suggests an increase in non-specific binding of native tobacco proteins to the Prosep A resin through hydrophobic interactions. Figure 4.6 presents an enlarged view of the elution and regeneration step of the chromatography cycle. Table 4.4 presents the amount of native tobacco proteins in the elution mobile phase. The presence of tobacco proteins during elution was more prevalent when the post load wash buffer contained 140 mM NaCl at pH 5. However, increasing the salt condition of the wash buffer decreased the presence of tobacco proteins during elution. The PBS wash buffer at pH 7 was more effective at the removal of tobacco proteins from the ProSep -vA High Capacity resin during the post load wash of the column, and resulted with less native tobacco proteins present in the eluent.



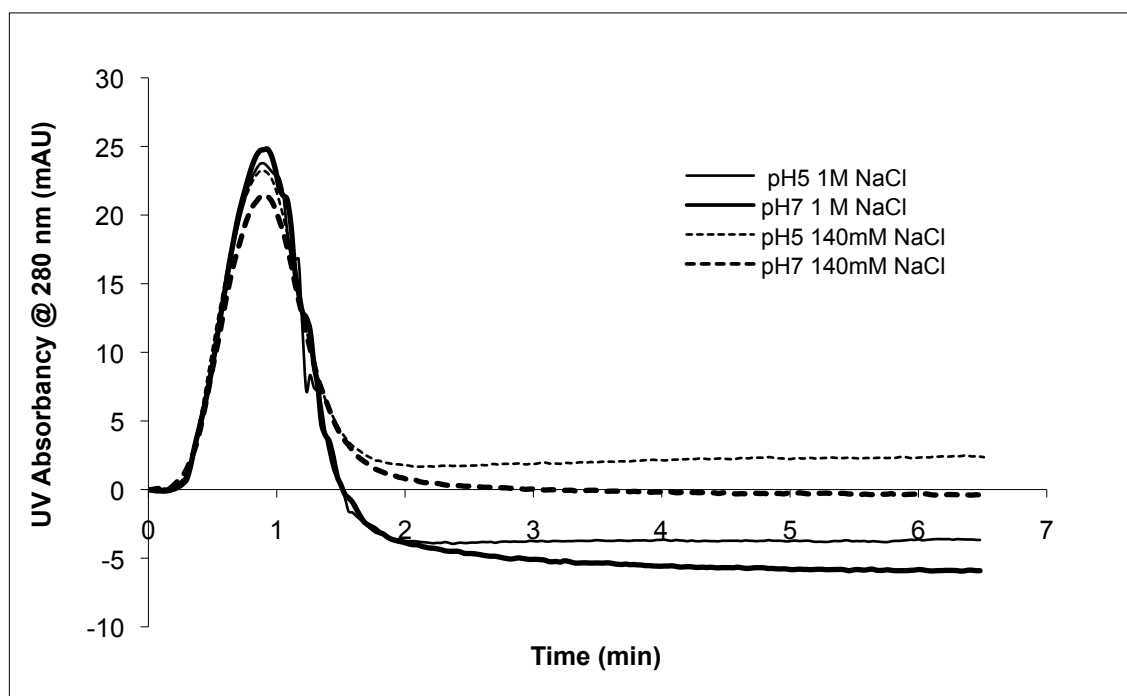
**Figure 4.6. Enlarged view of elution and regeneration step of chromatography cycle for tobacco extract after washed with various post load wash buffer conditions.** The figure presents data performed on the ProSep-vA High Capacity resin.

**Table 4.4. Native tobacco protein present during elution of ProSep vA High Capacity resin after washed with various PBS post load wash buffers.** This data summarizes mass balance of the tobacco protein present during elution step of chromatography cycle for ProSep-vA High Capacity column.

PBS Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco Present in elution ( $\mu\text{g}$ )	Percentage of tobacco protein in elution (%)	Total percentage of tobacco protein still bound to column after elution (%)
140 mM NaCl @ pH 5	$4.32 \pm 0.04$	$94.02 \pm 0.70$	$2.18 \pm 0.0017$	$13.2 \pm 7.28$
140 mM NaCl @ pH 7	$3.99 \pm 0.00$	$52.49 \pm 16.92$	$1.32 \pm 0.42$	$8.9 \pm 3.01$
1 M NaCl @ pH 5	$4.01 \pm 0.05$	$77.29 \pm 18.95$	$1.93 \pm 0.45$	$17.4 \pm 7.11$
1 M NaCl @ pH 7	$4.03 \pm 0.06$	$12.54 \pm 1.94$	$0.31 \pm 0.053$	$20.6 \pm 5.70$

#### 4.2.3 Testing Wash Buffers for IgG Loaded onto ProSep-vA Ultra Column

The efficiency of the ProSep-vA Ultra resin to capture human IgG was tested with the four post load wash buffer conditions. Similar to the -vA High Capacity resin, decreasing the pH of the post load wash buffer resulted in a greater volume of IgG prematurely eluting from the ProSep-vA Ultra resin. Figure 4.7 presents an enlarged view of the post load wash phase of the chromatogram cycle. As mentioned with the ProSep-vA high capacity resin, increasing the salt concentration of the mobile phase can disrupt electrostatic interaction; however, the Fc region of IgG mainly interacts to the Protein A through hydrophobic interaction. Therefore, changing the salt concentration of the post load wash was not observed to prematurely elute the IgG from the resin. However, increasing the salt concentration did increase the retention on IgG on to the ProSep-vA Ultra resin (Table 4.5), reducing the efficiency of the resin to capture IgG antibodies.

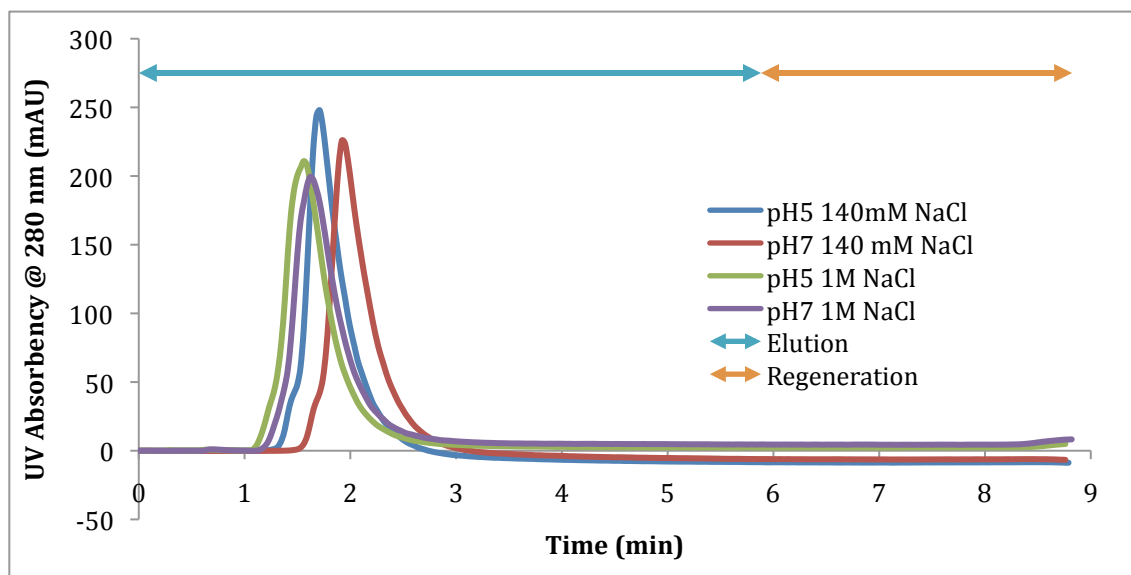


**Figure 4.7. Enlarged view of post load wash step of chromatography cycle for human IgG loaded onto ProSep-vA Ultra resin under various wash buffer conditions. The column was packed with 1 mL of ProSep-vA Ultra resin.**

**Table 4.5. Mass balance analysis of human IgG loaded onto ProSepvA ultra resin with various PBS wash buffer.** This data summarizes the mass balance calculations for the Human IgG samples loaded onto the ProSep-vA Ultra column.

PBS Wash Buffer	Human IgG loaded onto column (mg)	IgG in post load wash ( $\mu\text{g}$ )	Percentage of Human IgG eluted (%)	Percentage of Human IgG still bound to column after elution (%)
140 mM NaCl @ pH 5	$0.84 \pm 0.011$	$14.4 \pm 0.55$	$92.9 \pm 1.61$	$5.4 \pm 1.52$
140 mM NaCl @ pH 7	$0.83 \pm 0.011$	$13.2 \pm 10.02$	$88.1 \pm 10.4$	$10.3 \pm 11.65$
1 M NaCl @ pH 5	$0.83 \pm 0.00$	$19.5 \pm 0.36$	$84.9 \pm 2.48$	$12.8 \pm 2.43$
1 M NaCl @ pH 7	$0.81 \pm 0.028$	$10.8 \pm 1.08$	$80.7 \pm 8.53$	$18.0 \pm 8.72$

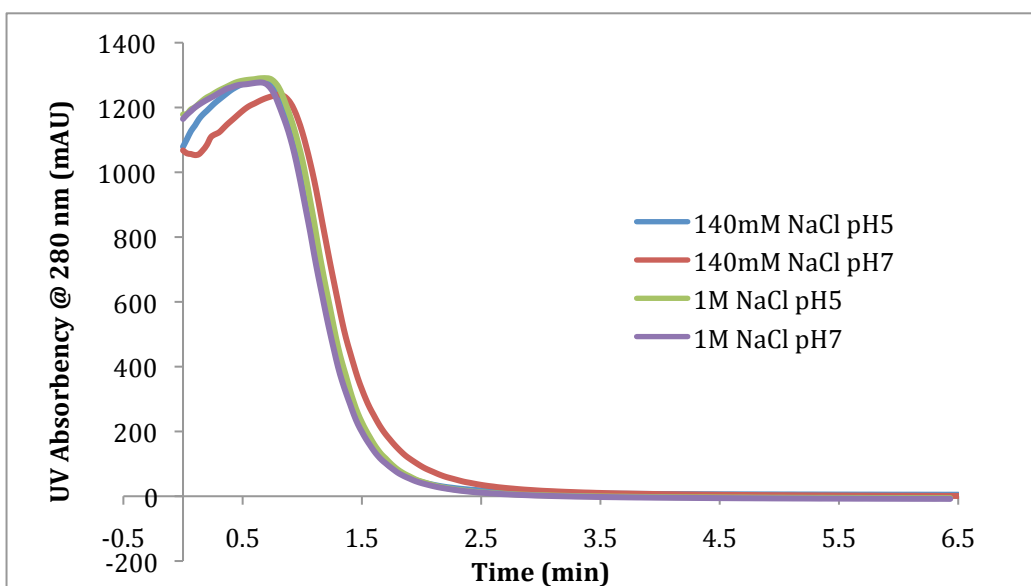
Although decreasing the pH of the post load wash did increase the amount of IgG prematurely eluted from the resin, it also resulted in a higher overall yield of the IgG samples (Table 4.5). As shown in Figure 4.8, the wash buffer containing 140mM NaCl at pH 5 had a larger elution peak than the other wash buffers tested. Therefore, the lower salt concentration of the post load wash buffer should be used for a more efficient capture of human IgG when using ProSep-vA Ultra resin.



**Figure 4.8. Enlarged view of elution and regeneration step of chromatography cycle for Human IgG loaded onto ProSep vA Ultra resin with various buffer conditions.** The figure presents data performed on the ProSep-vA Ultra resin.

#### 4.2.4 Testing Wash Buffers for Tobacco Extract Loaded onto ProSep-vA Ultra Column

The same four conditions of the PBS buffer used to wash the -vA High Capacity column after loading the tobacco extract and IgG samples were tested on the column packed with 1 mL of ProSep-vA Ultra resin. Figure 4.9 presents an isolated view of the different wash buffers used to wash the column prior to elution. As with the -vA High Capacity resin, the majority of the tobacco protein flowthrough was washed from the -vA Ultra resin after approximately 5 CV of PBS buffer with all four wash buffer conditions. Therefore, during scale-up of this process, the amount of CV of wash buffer used for the removal of residual tobacco proteins from the column could be reduced, creating a more cost effective downstream process in terms of time and materials.



**Figure 4.9: Enlarged view of post load wash step of chromatography cycle for tobacco extract loaded onto ProSep- vA Ultra resin under various post load wash buffer conditions.** The column was packed with 1 mL of ProSep-vA Ultra resin. The graph presents the UV absorbency at 280 nm after loading tobacco extract until breakthrough point. Therefore, graph of isolate view does not start at baseline.

The efficiency of the post load wash buffer to remove non-specifically binding native tobacco proteins from the Prosep -vA Ultra resin was evaluated by mass balance analysis of the tobacco protein extract samples loaded onto the resin (Table 4.6). Similar to the -vA High Capacity resin, lower salt concentrations in the post load wash buffer

were more effective as the removal of native tobacco proteins from non-specifically binding to the ProSep -vA Ultra resin. Increasing the salt concentration of the post load wash buffer indicates the retention of native tobacco proteins onto the column through hydrophobic interactions. Due to the slightly acidic cellular environment of tobacco, decreasing the pH of the post load wash buffer to 5 resulted in greater volume of native tobacco proteins non-specifically binding to the ProSep -vA Ultra resin.

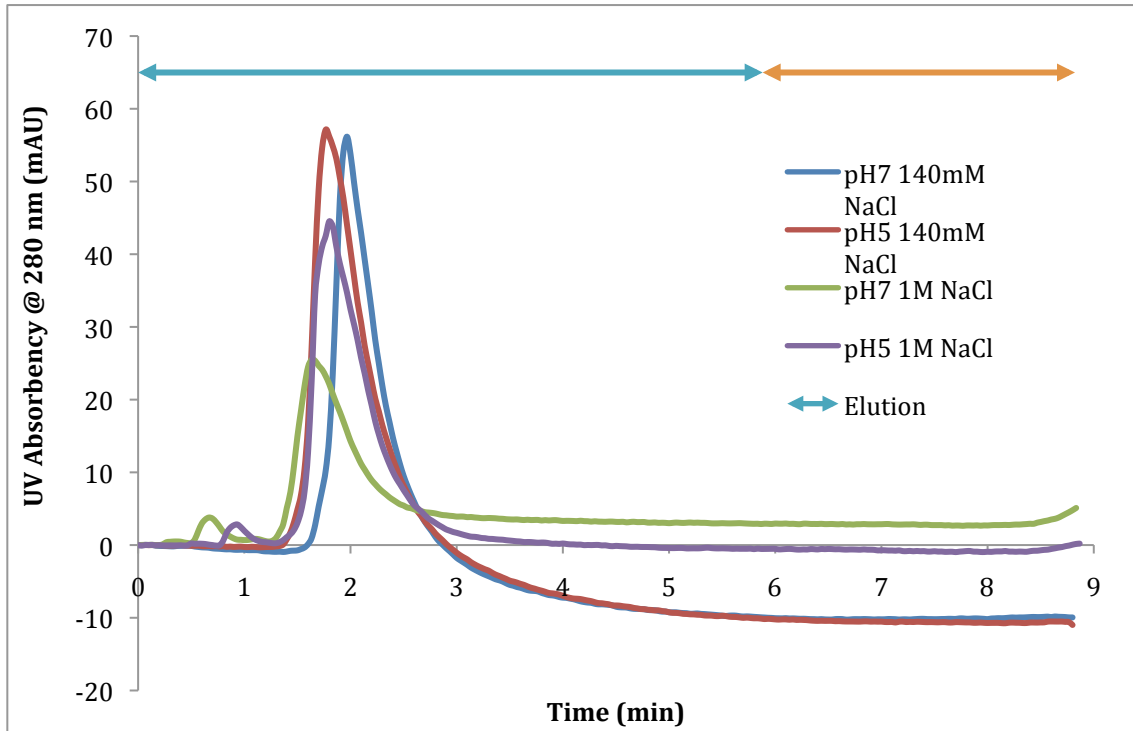
**Table 4.6. Mass balance analysis of tobacco protein extract loaded onto a ProSep-vA Ultra resin with various PBS post load wash buffer conditions.** This data summarizes the balance mass calculations for the tobacco protein extract samples loaded onto the ProSep-vA Ultra column.

PBS Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco protein in flowthrough (mg)	Tobacco protein bound to column after wash (mg)	Percentage of tobacco protein bound to column prior to elution (%)
140 mM NaCl @ pH 5	6.08 ± 0.0	5.20 ± 0.51	0.88 ± 0.51	14.5 ± 8.4
140 mM NaCl @ pH 7	6.07 ± 0.016	5.52 ± 0.60	0.57 ± 0.62	9.2 ± 10.18
1 M NaCl @ pH 5	6.06 ± 0.0	5.38 ± 0.70	0.68 ± 0.70	11.2 ± 11.48
1 M NaCl @ pH 7	6.05 ± 0.08	5.17 ± 0.43	0.88 ± 0.51	14.4 ± 8.20

Although the PBS wash buffer containing 140 mM NaCl at pH 7 was the most effective buffer at the removal of tobacco proteins from the ProSep -vA Ultra resin, lower salt concentrations resulted in a greater detection of native tobacco proteins during elution of the column (Figure 4.10). This indicates that native tobacco proteins non-specifically bind to the resin mostly through hydrophobic interactions. Similar to the -vA High Capacity column, a greater amount of tobacco proteins were detected during the elution of the column by decreasing the pH of the post wash buffer to 5. The post load wash buffer containing 140 mM NaCl at pH 7 was the most efficient post load wash buffer for the removal of native tobacco proteins from non-specifically binding to the ProSep A resin. This also resulted in a greater amount of native tobacco proteins present in the eluent than with the post load wash buffers at higher salt but the same pH (Table 4.7). Although the ProSep-vA Ultra resin was manufactured based on the ProSep-vA High Capacity resin, the ProSep-vA Ultra resin utilizes a smaller particle size. Therefore,

a smaller amount of large molecular weight molecules can enter the pores of the ProSep-vA Ultra resin compared to the Prosep-vA High Capacity resins.

Tobacco proteins were present in all post load wash buffers tested, and further purification of the samples would be required for all post load wash buffer conditions when purifying IgG to remove any residual tobacco proteins from the eluent. Protein A chromatography can clear a majority of the host cell proteins; however, it does not clear aggregate and also introduces Protein A into the eluent [38]. Therefore, cation exchange chromatography can be used to remove residual host cell proteins, aggregates, and leached Protein A, and anion exchange chromatography can then be used as a final polishing step to remove residual DNA, endotoxins, and host cell proteins to meet the purity restrictions required for antibody purification.



**Figure 4.10. Enlarged view of elution and regeneration step of the ProSep vA Ultra chromatography cycle for tobacco extract after washed with various post load wash buffer conditions.** The figure presents data performed on the ProSep-vA Ultra resin.

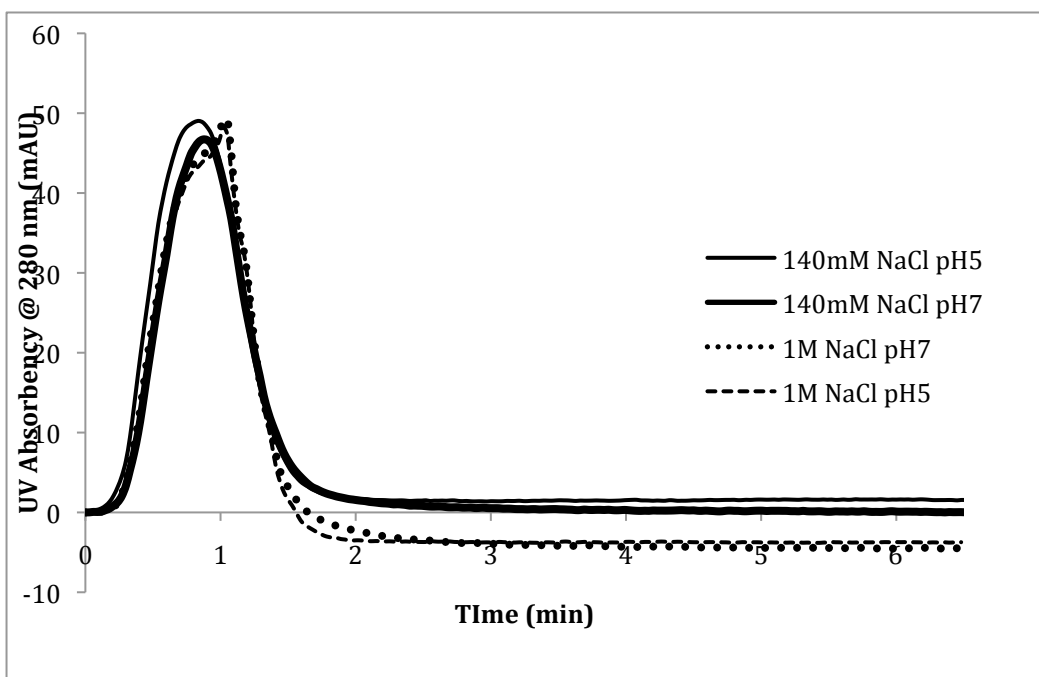
**Table 4.7. Native tobacco protein present during elution of the ProSep-vA Ultra resin after washed with various PBS wash buffer.** This data summarizes mass balance of the tobacco protein in extract samples eluted from ProSep-vA Ultra column.

PBS Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco Present in elution ( $\mu\text{g}$ )	Percentage of tobacco protein in Elution mobile phase (%)	Total percentage of tobacco protein still bound to column after elution (%)
140 mM NaCl @ pH 5	6.08 $\pm$ 0.0	175.7 $\pm$ 24.45	2.89 $\pm$ 0.41	11.6 $\pm$ 8.00
140 mM NaCl @ pH 7	6.07 $\pm$ 0.016	118.6 $\pm$ 3.66	1.95 $\pm$ 0.055	7.2 $\pm$ 10.1
1 M NaCl @ pH 5	6.06 $\pm$ 0.0	141.5 $\pm$ 33.58	2.83 $\pm$ 0.68	8.9 $\pm$ 10.92
1 M NaCl @ pH 7	6.05 $\pm$ 0.08	61.3 $\pm$ 25.01	1.05 $\pm$ 0.54	13.4 $\pm$ 7.80

#### 4.2.5 Testing Wash Buffers for IgG Loaded onto ProSep Ultra Plus Column

The efficiency of the ProSep Ultra Plus resin to capture human IgG was tested with the four post load wash buffer conditions. Figure 4.11 presents an enlarge view of the post load wash phase of the chromatogram cycle. According to Figure 4.11, decreasing the pH of the post load wash buffer resulted in a greater amount of IgG prematurely eluting from the ProSep Ultra Plus resin. However, changing the salt concentration or pH of the post load wash buffer had little effect on the premature elution of human IgG when the mass balance was conducted (Table 4.8). As mentioned with the ProSep-vA High Capacity resin, increasing the salt concentration of the mobile phase can disrupt electrostatic interactions; however, the Fc region of IgG mainly interacts to the Protein A through hydrophobic interaction. Therefore, increasing the salt concentration of the post load wash was not observed to prematurely elute the IgG from the resin. However, increasing the salt concentration did increase the retention on IgG onto the ProSep Ultra Plus resin (Table 4.8), reducing the efficiency of the resin to capture IgG antibodies. Furthermore, decreasing the pH of the post load wash in conjunction with a lower salt concentration resulted in a higher overall yield of the IgG samples. This is also shown in Figure 4.12 with the wash buffer containing 140mM NaCl at pH5 having a greater elution peak than the other wash buffers tested. Therefore, the lower salt concentration of

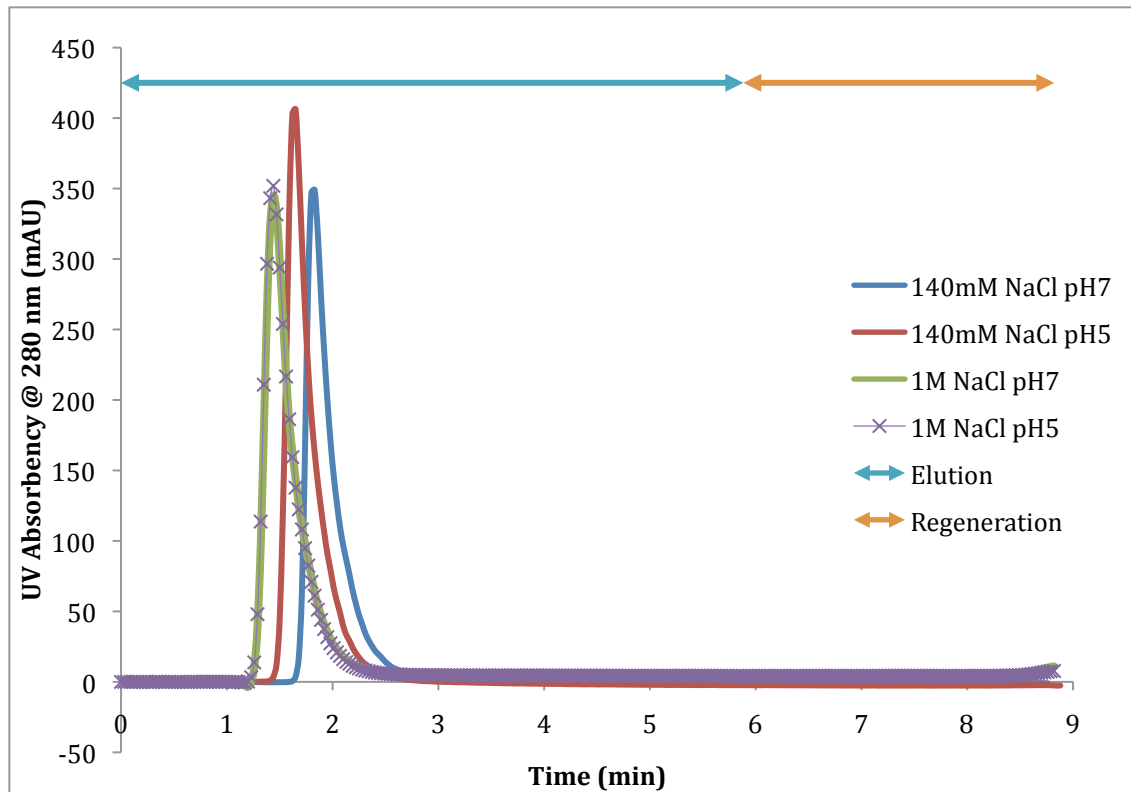
the post load wash buffer should be used for a more efficient capture of human IgG when using ProSep Ultra Plus resin.



**Figure 4.11. Enlarged view of elution and regeneration step of chromatography cycle for Human IgG loaded onto ProSep Ultra Plus resin with various buffer conditions.** The column was packed with 1 mL of ProSep Ultra Plus resin.

**Table 4.8. Mass balance analysis of human IgG loaded onto ProSep Ultra Plus resin with various PBS wash buffers.** This data summarizes the mass balance calculations for the Human IgG samples loaded onto the ProSep Ultra Plus column.

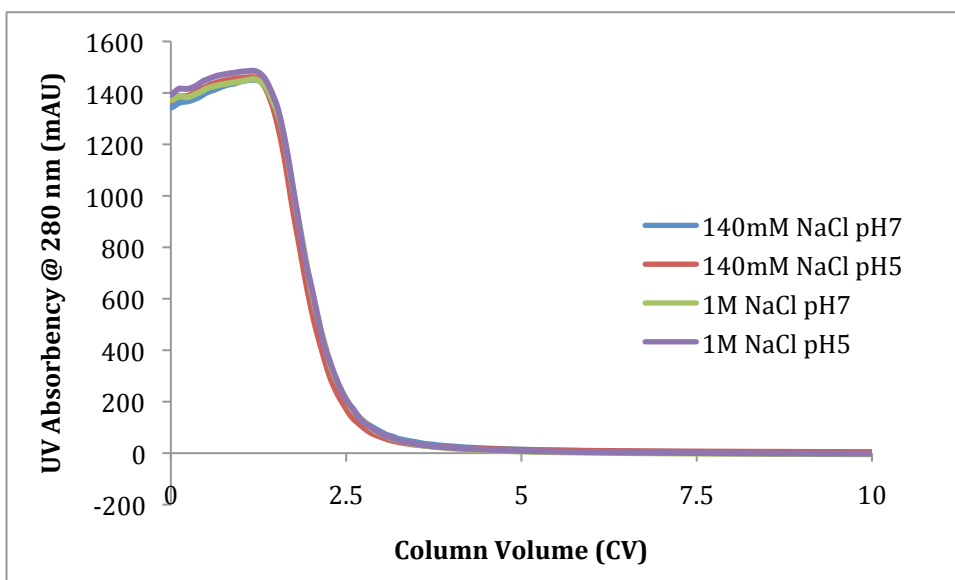
PBS Wash Buffer	Human IgG loaded onto column (mg)	IgG in post load wash ( $\mu\text{g}$ )	Percentage of human IgG eluted (%)	Percentage of human IgG still bound to column after elution (%)
140 mM NaCl @ pH 5	$0.85 \pm 0.061$	$19.4 \pm 11.16$	$93.9 \pm 2.16$	$2.9 \pm 2.40$
140 mM NaCl @ pH 7	$0.81 \pm 0.0056$	$26.7 \pm 3.85$	$78.0 \pm 20.0$	$19.7 \pm 21.34$
1 M NaCl @ pH 5	$0.82 \pm 0.0055$	$18.7 \pm 0.24$	$87.6 \pm 0.59$	$10.1 \pm 0.57$
1 M NaCl @ pH 7	$0.81 \pm 0.0056$	$14.0 \pm 2.10$	$87.9 \pm 4.03$	$10.4 \pm 4.30$



**Figure 4.12. Enlarged view of elution and regeneration step of chromatography cycle for human IgG loaded onto ProSep Ultra Plus resin with various buffer conditions.** The figure presents data performed on the 1 ml ProSep Ultra Plus column.

#### 4.2.6 Testing Wash Buffers for Tobacco Extract Loaded onto ProSep-vA Ultra Plus Column

As with the ProSep -vA High Capacity and -vA Ultra columns, the various wash conditions were tested on a 1 mL ProSep Ultra Plus column. Figure 4.13 indicates that, similar to the other two ProSep resins after approximately 5 CV of PBS wash buffer, a majority of the tobacco protein flowthrough was washed from the column. Therefore, during scale-up of this process, the amount of CV of wash buffer used for the removal residual tobacco proteins from the column could be reduced, creating a more cost effective downstream process in terms of time and materials.



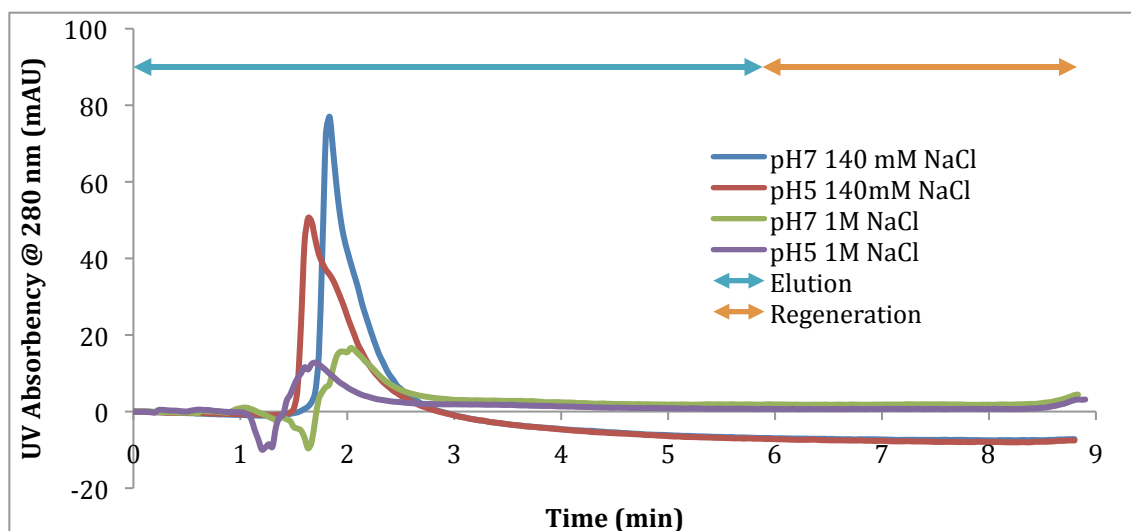
**Figure 4.13. Enlarged view of post load wash step of chromatography cycle for tobacco extract loaded onto ProSep Ultra Plus resin under various post load wash buffer conditions.** The column was packed with 1 mL of ProSep Ultra Plus resin. The graph presents the UV absorbency at 280 nm after loading tobacco extract until breakthrough point. Therefore, graph of isolate view does not start at baseline.

Unlike the other two ProSep A resins tested, higher salt concentrations in the post load wash buffer performed better at the removal of tobacco proteins from the ProSep Ultra Plus resin as indicated in Table 4.9. A small difference is noticeable between the different pH conditions tested. Also Table 4.9 indicates that a lower pH performed better at the removal of the tobacco proteins. Therefore, a PBS wash buffer containing 1 M NaCl at pH 5 was the most effective buffer for the removal of tobacco proteins from the ProSep Ultra Plus resin. The higher removal of tobacco proteins from the resin due to high salt concentration indicates that the native tobacco proteins bind to the resin mostly through electrostatic interactions. Furthermore, increasing the salt concentration of the post load wash resulted in a smaller amount of proteins detected during the elution of the column (Table 4.10). Although the ProSep Ultra Plus resin was manufactured based on the ProSep-vA High Capacity resin and ProSep-vA Ultra resin, the ProSep Ultra Plus resin utilizes a smaller particle size. Also, the ProSep Ultra Plus has a pore size bigger than the ProSep-vA Ultra but smaller than the ProSep-vA High Capacity. Large molecular weight molecules are more hindered from entering the pore and, therefore, less accessible to the Protein A ligand than with the -vA High Capacity resin. On the other

hand, large molecular weight molecules are less hindered from entering the pore and, therefore, more accessible to the ligand than the -vA Ultra resin. This results in a higher presence of tobacco proteins in the eluent for Ultra Plus resin than the -vA Ultra but lesser presence in the eluent for the -vA High Capacity.

**Table 4.9. Mass balance analysis of tobacco protein extract loaded onto ProSep Ultra Plus resin with various conditions PBS wash buffers.** This data summarizes the balance mass calculations for the tobacco protein extract samples loaded onto the ProSep Ultra Plus column.

PBS Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco protein in flowthrough (mg)	Tobacco protein bound to column prior to elution (mg)	Percentage of tobacco protein bound to column prior to elution (%)
140 mM NaCl @ pH 5	4.71 ± 0.025	4.46 ± 0.053	0.25 ± 0.079	5.24 ± 1.64
140 mM NaCl @ pH 7	4.72 ± 0.00	4.30 ± 0.063	0.42 ± 0.063	8.79 ± 1.33
1 M NaCl @ pH 5	4.77 ± 0.037	4.65 ± 0.017	0.116 ± 0.020	2.4 ± 0.40
1 M NaCl @ pH 7	4.74 ± 0.00	4.58 ± 0.035	0.17 ± 0.035	3.50 ± 0.75



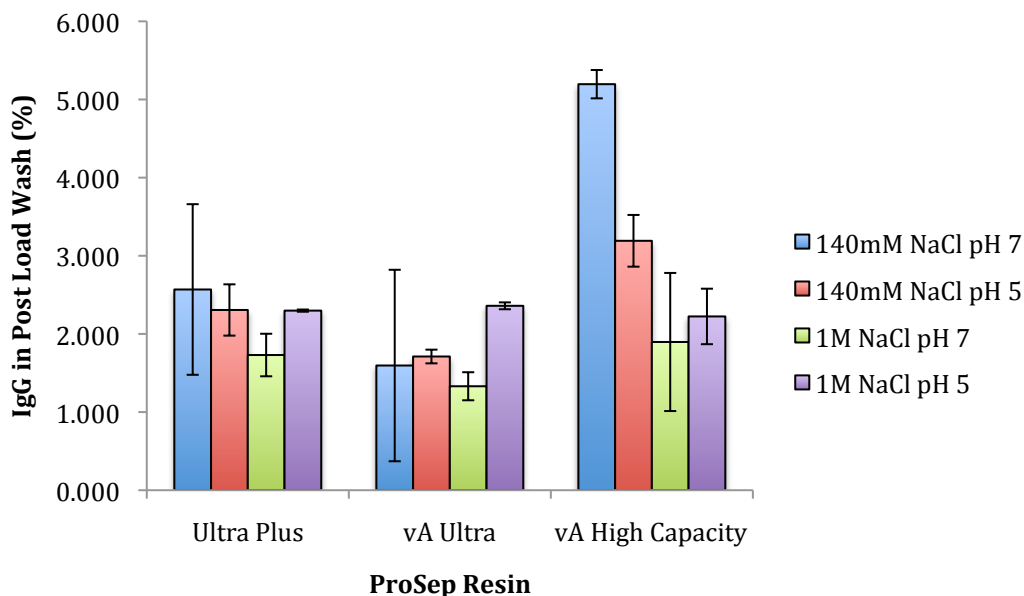
**Figure 4.14. Enlarged view of elution and regeneration step of chromatography cycle for tobacco proteins loaded onto ProSep Ultra Plus under various post load wash buffer conditions.** The figure presents data performed on the 1ml ProSep Ultra Plus column.

**Table 4.10. Native tobacco protein present in elution mobile phase of the ProSep Ultra Plus resin with various PBS post load wash buffers.** This data summarizes mass balance of the tobacco protein in extract samples eluted from ProSep-vA Ultra column.

PBS post load Wash Buffer	Tobacco protein loaded onto column (mg)	Tobacco Present in elution ( $\mu\text{g}$ )	Percentage of tobacco protein in elution (%)	Total percentage of tobacco protein still bound to column after elution (%)
140 mM NaCl @ pH 5	4.71 $\pm$ 0.025	77.5 $\pm$ 3.06	1.64 $\pm$ 0.056	3.6 $\pm$ 1.59
140 mM NaCl @ pH 7	4.72 $\pm$ 0.00	83.8 $\pm$ 15.61	1.77 $\pm$ 0.33	7.0 $\pm$ 1.00
1 M NaCl @ pH 5	4.77 $\pm$ 0.037	63.4 $\pm$ 5.71	1.3 $\pm$ 0.13	1.1 $\pm$ 0.53
1 M NaCl @ pH 7	4.74 $\pm$ 0.00	46.1 $\pm$ 7.11	1.0 $\pm$ 0.15	2.5 $\pm$ 0.60

#### 4.2.7 Comparison of Wash Buffer Conditions for Different ProSep Resins

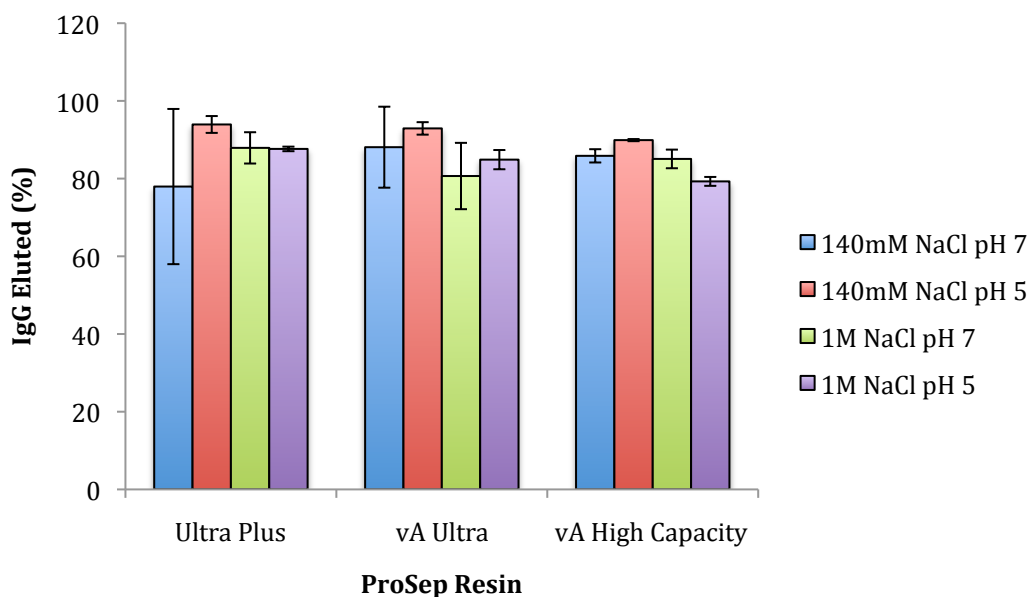
During downstream processing, it is important to optimize the recovery of the target protein. Figure 4.15 presents the amount of human IgG prematurely eluted with the four different post load wash buffer conditions tested on the three ProSep A resins. At low pH, the surface of the Protein A and IgG Fc region that face each other have highly conserved ionizable amino acid residues that become positively charged. These charges repel each other weakening the hydrophobic interaction between the Protein A and human IgG, resulting in the elution of the antibody from the Protein A ligand. Therefore, the use of post load wash buffer with intermediate pH can prematurely elute the protein of interest, human IgG. As the salt concentration of the post load wash buffer increased, the amount of human IgG prematurely eluted decreased. Increasing the salt concentration reduces electrostatic interactions thereby reducing the amount of IgG prematurely eluted from the columns. When the post load wash buffer contained 140 mM NaCl, the ProSep-vA High Capacity resin prematurely eluted the largest amount of IgG, followed by the ProSep Ultra Plus and the ProSep-vA Ultra prematurely eluted the smallest amount of IgG.



**Figure 4.15. Percentage of human IgG proteins prematurely eluted using different post load wash buffer for ProSep –vA High Capacity, ProSep –vA Ultra, and ProSep Ultra Plus resins.**

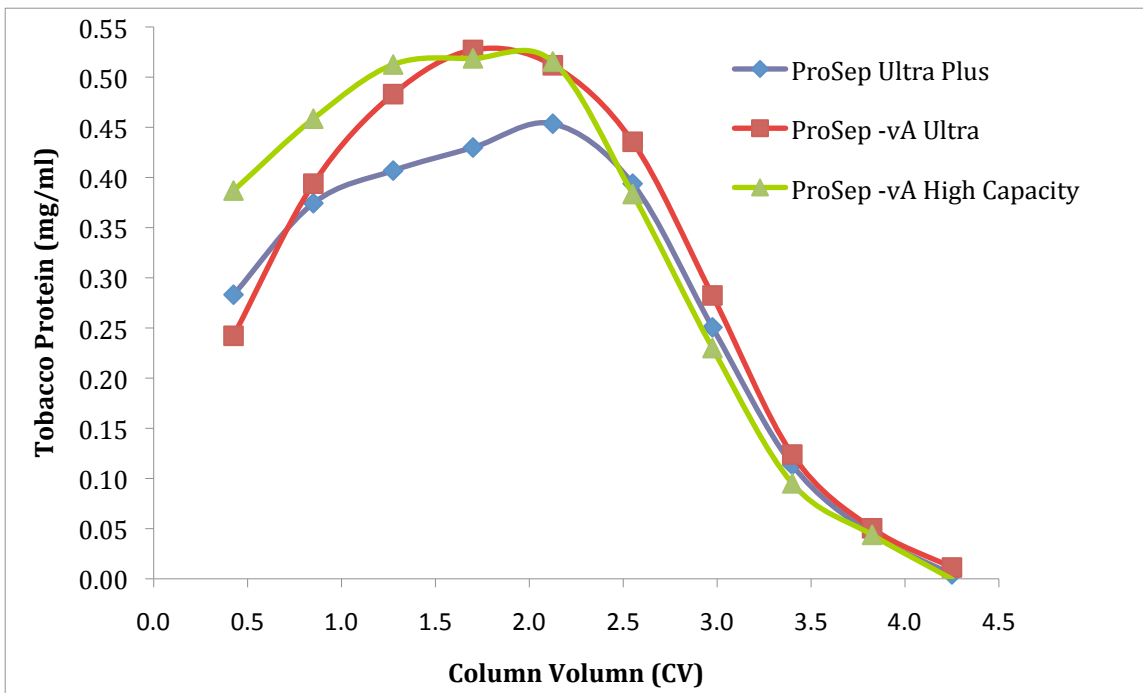
Of the three ProSep A resins tested, ProSep Ultra Plus performed the best for the capture of IgG, followed by ProSep-vA Ultra and finally by ProSep-vA High Capacity resin, which was to be expected. While the large pore size of the ProSep-vA High Capacity resin provides less hindered diffusion of solutes than the -vA Ultra and Ultra Plus, it also has a smaller surface area which diminishes the binding capacity. Therefore, as shown in Figure 4.16, ProSep-vA High Capacity was the least efficient at capturing IgG from the sample, especially at higher salt concentrations. The ProSep Ultra Plus utilizes a smaller particle size than the ProSep-vA High Capacity and ProSep-vA Ultra, which increases the rate of movement between the support and flow stream, thereby increasing the column efficiency. As shown in Figure 4.16, the ProSep Ultra Plus had the greatest efficiency of capturing IgG from the sample than the other ProSep A resins. In addition, using a high salt concentration and a neutral pH was more effective at retaining the IgG bound to the three ProSep A resins. However, these conditions also reduce the efficiency of the elution buffer to elute the IgG from the three ProSep A resins. Furthermore, an intermediate pH weakens the hydrophobic bonds between the Protein A and IgG Fc region, allowing for more efficient removal of IgG from the column during elution. Therefore, the post load wash buffer containing 140mM NaCl at

pH 5 weakened hydrophobic bonds and allowed for most efficient capture of IgG from all three ProSep A resins during the elution step of the chromatography cycle (Figure 4.16).



**Figure 4.16. Percentage of human IgG proteins in elution mobile phase using different post load wash buffer for ProSep –vA High Capacity, ProSep –vA Ultra, and ProSep Ultra Plus resins.**

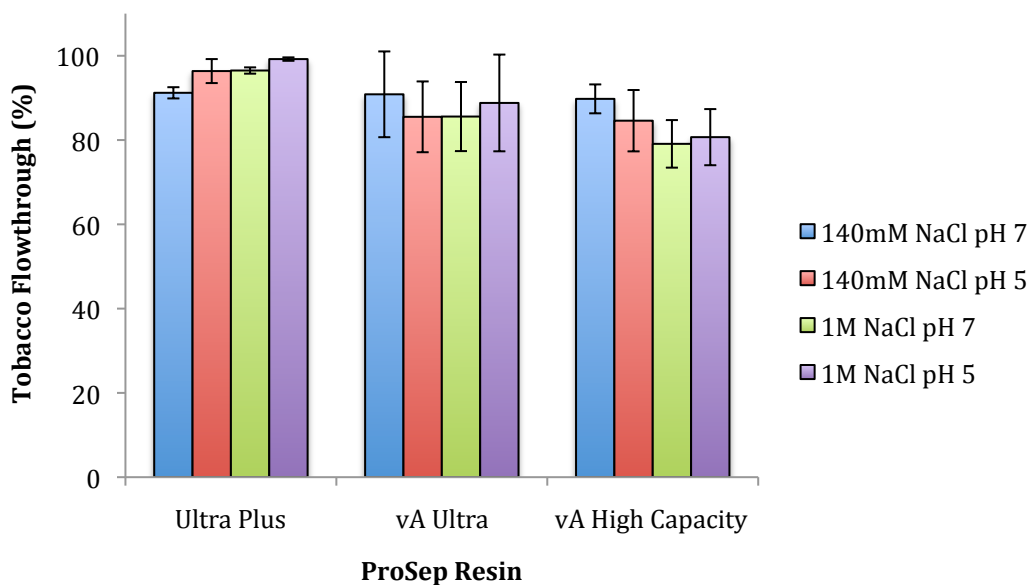
Downstream processing of the target protein make up 80% or more of the total production costs. Therefore, it is important to optimize both the time required to process the samples as well as the amount of materials used to process the samples. As Figures 4.5, 4.9, and 4.13 indicate, the conditions of the PBS buffer used to wash the column prior to elution had very little effect on the time required to wash the residual tobacco proteins from the column. Therefore, the concentration of the tobacco protein in the flowthrough was measured as a function of the column volume of PBS wash buffer (Figure 4.17). According to these results, after 4.25 CV of PBS wash buffer, the concentration of the tobacco present in the flowthrough was less than 50  $\mu\text{g/ml}$ . Therefore, if it is required to reduce the processing time of the target protein (IgG), the amount of PBS wash buffer used on the column should not be less than 5 CV of the packed column used in the purification process.



**Figure 4.17. Concentration of flowthrough as a function of the column volume of PBS wash buffer for ProSep vA High Capacity, ProSep vA Ultra, and ProSep Ultra Plus.** The PBS wash buffer contained 140 mM NaCl at pH 7 and each column contained 1 mL of resin.

The removal of tobacco protein is affected not only by the salt (NaCl) concentration and pH of the wash buffer but also by the resin being used in the purification process. Figure 4.18 compares the percent of tobacco proteins in flowthrough for the three ProSep A resins using wash buffers at the four different post load wash buffer conditions. The PBS wash buffer containing a lower salt concentration and a neutral pH was more efficient in the removal of tobacco proteins from columns prior to elution for the ProSep -vA High Capacity and -vA Ultra resin. For the ProSep Ultra Plus, all four buffer conditions tested removed greater than 90% of the native tobacco proteins, with the high salt concentration/intermediate pH buffer condition performing most efficiently. The ProSep Ultra Plus resin removed the greatest percentage of tobacco proteins from the column, followed by the -vA Ultra resin, and then the -vA High Capacity resin. The -vA High Capacity resin has the largest pore size followed by Ultra Plus resin and then by -vA Ultra resin. RuBisCo is a native tobacco protein that has a molecular weight of approximately 560 kDa and makes up approximately 50% of the total protein content. Although RuBisCo has a large molecular weight, it is only 13.2 nm in

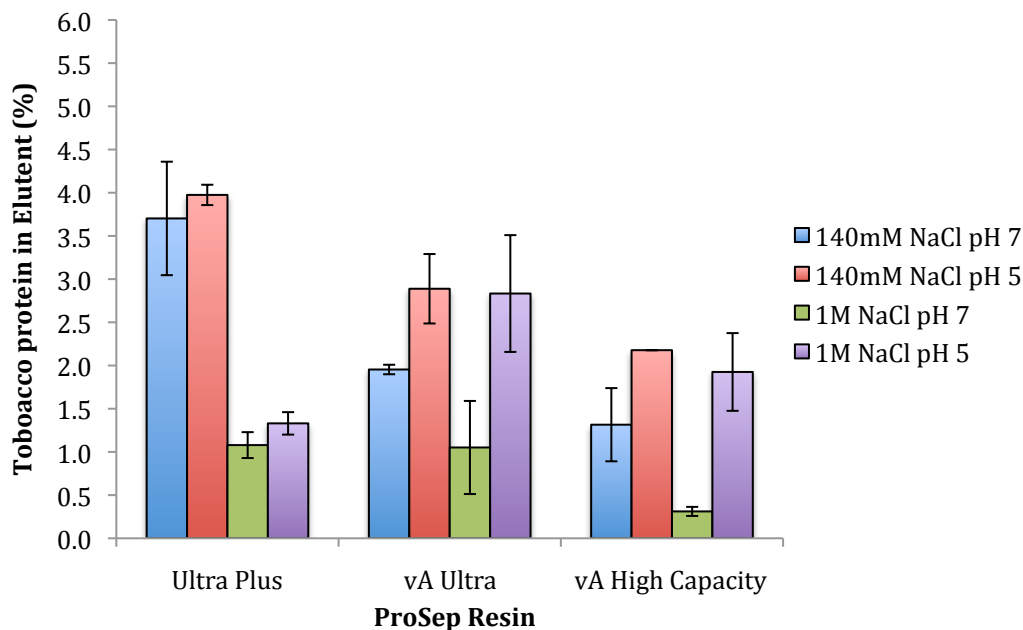
diameter and 10 nm in height. It is estimated that to avoid severe restriction of rate of diffusion, the pore size diameter should be five times the diameter of the solute. Therefore, the pore size of the ProSep resins must be smaller than  $660 \text{ \AA}$  to severely restrict the rate of diffusion of RuBisCo. However, the ProSep-vA Ultra resin, the resin with the smallest pore size, has a pore size of  $700 \text{ \AA}$ . Therefore, RuBisCo will not be severely restricted from entering the pores of the resins used in these experiments. These results could indicate that the larger pore size of the -vA High Capacity resin allows more RuBisCo proteins to enter the pore and bind to the column, while the smaller pore size of the vA Ultra and Ultra Plus resins results in more RuBisCo proteins flowing through the column.



**Figure 4.18. Percentage of tobacco proteins in flowthrough using different post load wash buffer for ProSep -vA High Capacity, ProSep -vA Ultra, and ProSep Ultra Plus resins.**

Figure 4.19 compares the percentage of tobacco proteins in the elution mobile phase of the chromatography cycle for the three ProSep A resins using wash buffers at the four different post load wash buffer conditions. When the post load wash buffer contained 140 mM NaCl, the ProSep Ultra Plus resin had the greatest percentage of tobacco proteins present during elution of the column, followed by the ProSep -vA Ultra resin, and finally the ProSep-vA High Capacity resin. High salt concentrations reduces electrostatic interactions, thereby increasing hydrophobic interactions and increasing the

retention of tobacco protein contaminants onto the column. When the post wash buffer contained 1M NaCl at pH 5 and 7, ProSep Ultra Plus had the smallest amount of tobacco proteins present in the elution mobile phase. When the post load wash buffer had an intermediate pH of 5, there was a larger presence of native tobacco proteins in the elution mobile phase for all three resins.

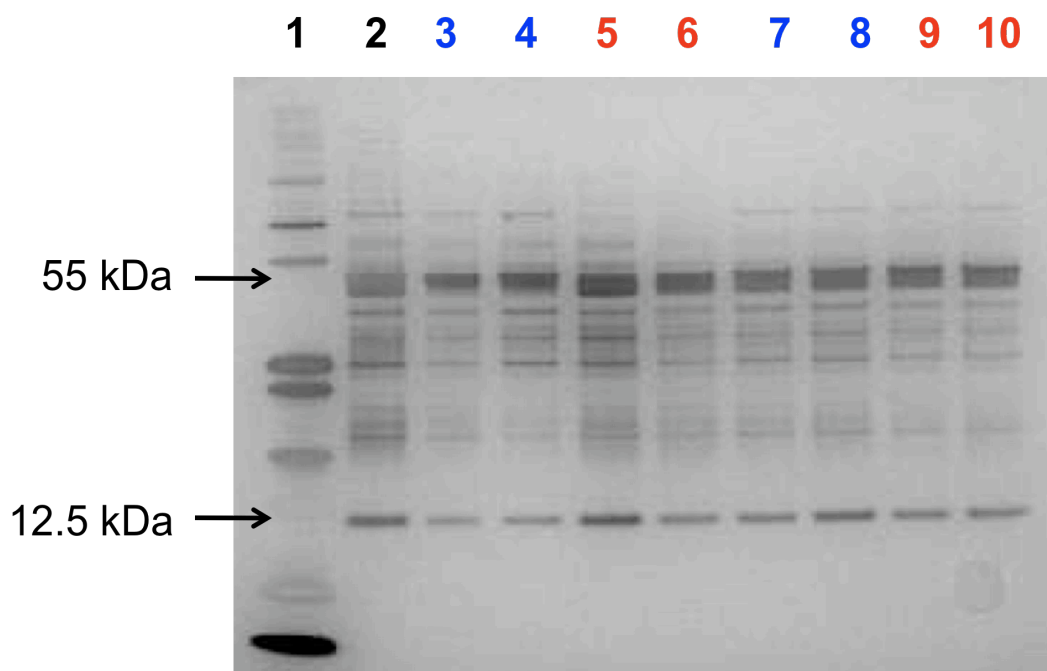


**Figure 4.19. Percentage of tobacco proteins in elution mobile phase using different post load wash buffer for ProSep –vA High Capacity, ProSep –vA Ultra, and ProSep Ultra Plus resins.**

Although increasing the salt concentration of the post load wash buffer decreased the amount of tobacco proteins in the elution, it also decreased the amount of IgG recovered during elution. Furthermore, as decreasing the pH of the post load wash buffer increased the efficiency of the ProSep A resins to capture and elute IgG from the resin, it also increased the amount of tobacco protein contaminants present in the elution mobile phase. Overall, ProSep Ultra Plus was most efficient at the capture and elution of IgG, while reducing the non-specific binding of native tobacco proteins to the resin and reducing the presence of native tobacco proteins during elution.

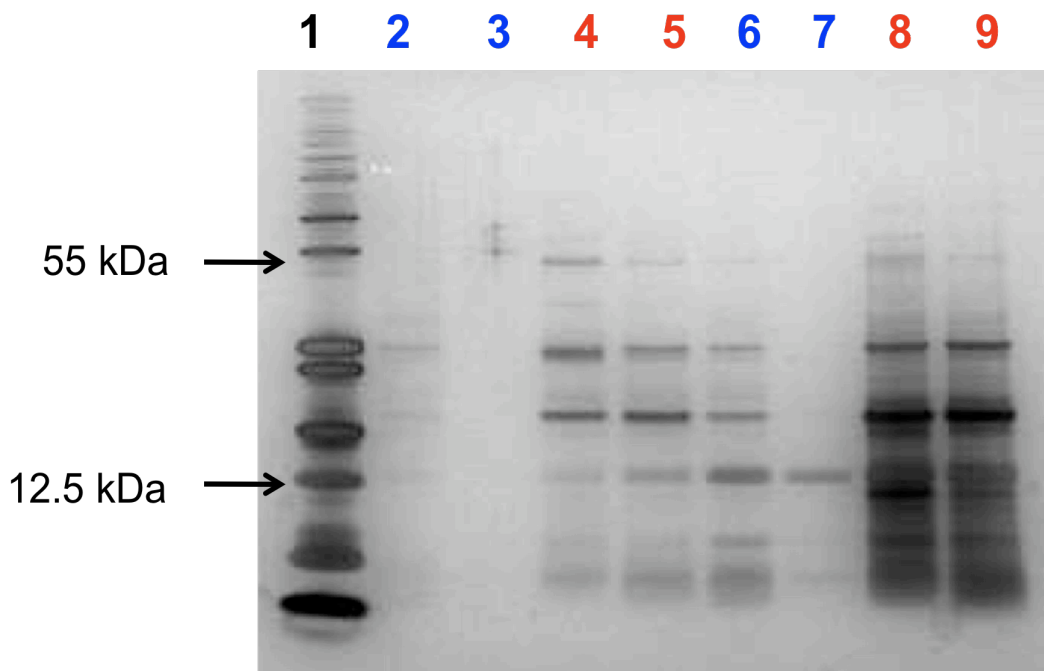
### 4.3 SDS-PAGE Analysis of Tobacco Proteins

Native tobacco proteins remaining on the column can be eluted during the elution and the regeneration phase of the column. These proteins are impurities that will need to be removed from the IgG with further purification. Therefore, it is important to determine what native tobacco proteins are present during these phases of the cycle through SDS-PAGE analysis. Of the three resins investigated throughout this project, The ProSep Ultra Plus resin performed the best at capturing IgG and removing residual tobacco proteins from the column. Figure 4.20 presents the SDS-PAGE gel of the tobacco proteins present in the flowthrough under the four different wash buffer conditions when run through the ProSep Ultra Plus Resin. This gel indicates high presence of proteins approximately 55kDa and 12.5 kDa in size. These protein bands correspond with the small and large subunits of the tobacco protein RuBisCo. Therefore, with each post load wash buffer condition tested, Rubisco was the prevalent protein present.



**Figure 4.20. SDS-PAGE gel of tobacco flowthrough washed from ProSep Ultra Plus column with various post load wash buffer conditions.** Samples were run on gel under reducing conditions and stained with silver stain. Lane 1 (L1): protein marker; L2: Tobacco extract; Flowthrough of tobacco protein washed with L3-4: PBS containing 1 M NaCl at pH 7; L5-6: PBS containing 1 M NaCl at pH 5; L7-8: PBS containing 140 mM NaCl at pH 7; L9-10: PBS containing 140 mM NaCl at pH 5.

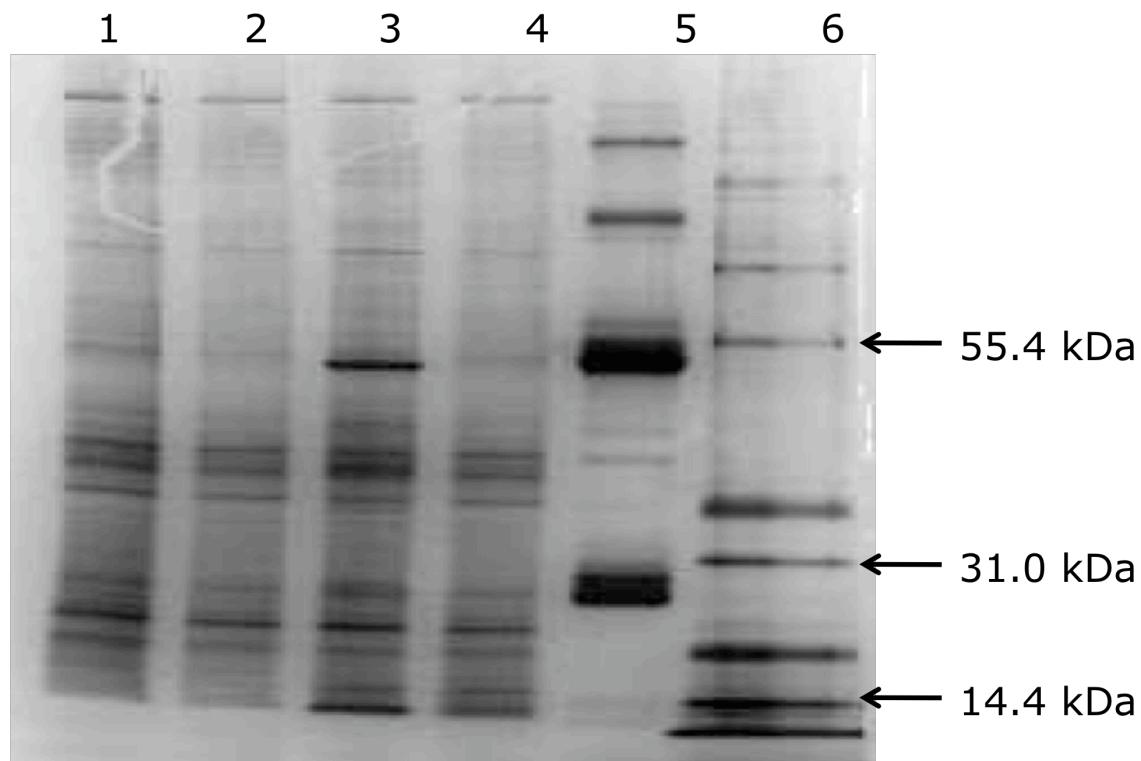
Figure 4.21 presents the SDS-PAGE gel of the tobacco proteins present during elution of the ProSep Ultra Plus column. Washing the ProSep Ultra Plus column with PBS buffer containing 1M NaCl at pH 7 had the least amount of tobacco proteins present during elution of the column; the PBS buffer containing 140 mM NaCl at pH 5 had the largest amount of tobacco proteins present. For each buffer conditions tested, little to no proteins approximately 55 kDa and 12.5 kDa, representing Rubisco, were present during elution of the column. Using a salt concentration of 140 mM and a pH 5 resulted in more smaller molecular weight proteins non-specifically binding to the column. Therefore, when washing the column with a low salt concentration at pH 5, more Fraction II tobacco proteins will be present during elution of the column.



**Figure 4.21. SDS-PAGE gel of tobacco proteins present during elution of the ProSep Ultra Plus column after washed under various post load wash buffer conditions.** Samples were run on gel under reducing conditions and stained with silver stain. Lane 1 (L1): protein marker; Tobacco protein present during elution of the column after washed with L2-3: PBS containing 1 M NaCl at pH 7; L4-5: PBS containing 1 M NaCl at pH 5; L6-7: PBS containing 140 mM NaCl at pH 7; L8-9: PBS containing 140 mM NaCl at pH 5.

Tobacco protein non-specifically bind to each of the ProSep A resins was investigated for this project. Figure 4.22 presents the SDS-PAGE gel of the tobacco proteins present in the flowthrough from each of the columns. As mentioned previously,

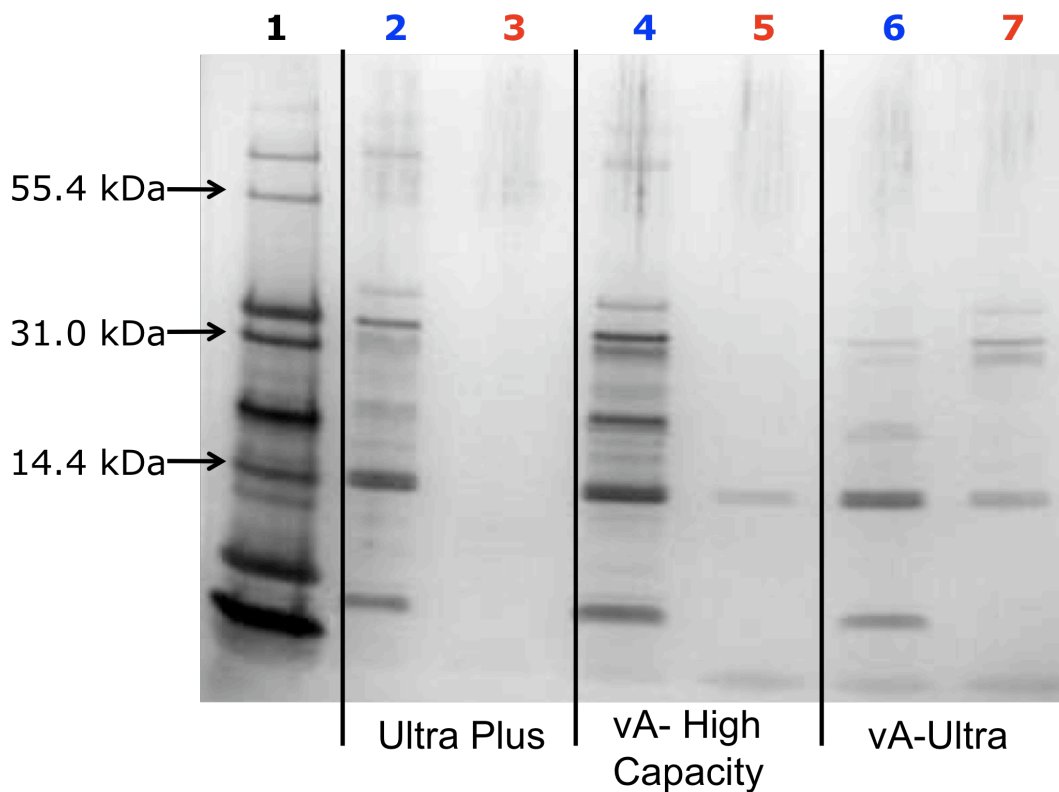
the ProSep-vA Ultra has the smallest pore size of the three resins, and, therefore, restricts the rate of diffusion of large molecular weight proteins, such as RuBisCo, more than the other ProSep A Resin. The ProSep-vA Ultra resin showed greater intensity protein bands at approximately 55 kDa and 12.5 kDa corresponding to the large and small subunit of RuBisCo respectively. Therefore, a larger presence of the RuBisCo was present in the flowthrough of the ProSep-vA Ultra during the post load wash step of the chromatography cycle than the other two ProSep resins (Figure 4.22).



**Figure 4.22. SDS-PAGE gel of tobacco flowthrough washed from ProSep vA-High, ProSep vA-Ultra, and ProSep Ultra Plus columns.** Samples were run on gel under reducing conditions and stained with silver stain. Lane 1 (L1): Tobacco extract; L2-4: Ultra Plus, vA-Ultra, and vA-High Capacity flowthrough of tobacco proteins, respectively; L5: IgG; L6: Protein Marker.

Figure 4.23 presents the SDS-PAGE gel of the proteins eluted during the elution and regeneration phases of the different ProSep resins. The samples were run under reducing condition. RuBisCo is a native tobacco protein that composes approximately 50% of the proteins present in tobacco leaves; therefore, there is a greater probability that RuBisCo is the protein binding to the column or present during the elution of the column. RuBisCo is composed of 8 small and 8 large subunits approximately 12.5 and 55 kDa in

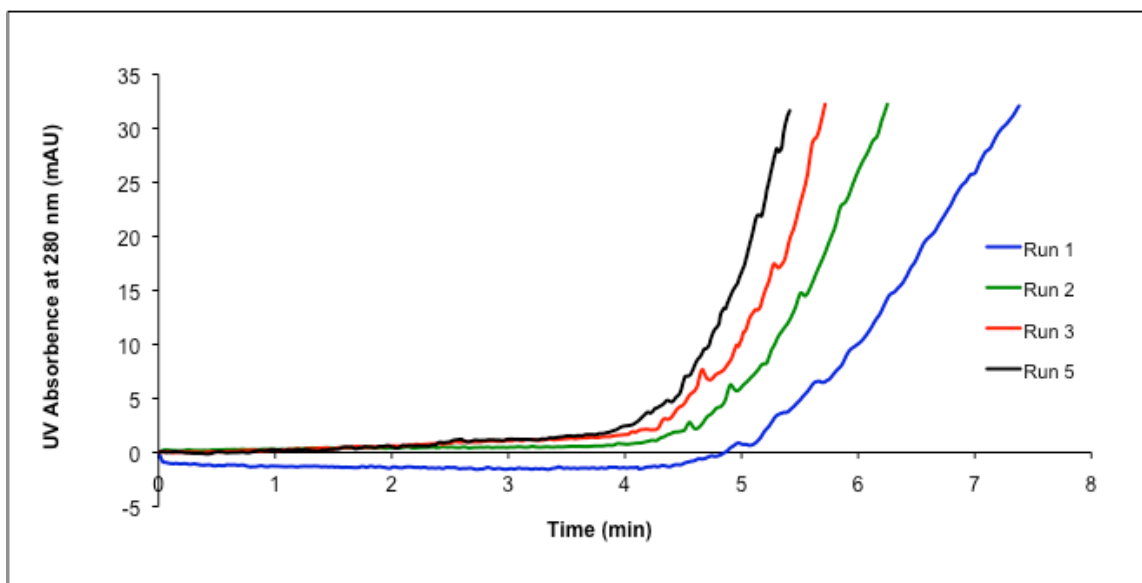
size respectively. Protein bands did appear at approximately 12.5 kDa which could indicate the RuBisCo small subunit; however, protein bands did not appear to be at approximately 55kDa. Therefore, the native tobacco proteins present in the eluent is not RuBisCo. This gel image indicates the majority of the proteins present in the samples range from approximately 6 to 42 kDa in size. This could indicate the presence of Fraction II tobacco proteins which consists of smaller molecular weight proteins, or intact Protein A and Protein A fragments leached from the column. Regardless of the nature of the protein impurities, further purification will be required to isolate IgG from the Protein A and other native tobacco proteins.



**Figure 4.23. SDS-Page gel of tobacco proteins during elution and regeneration of column from ProSep vA-High, ProSep vA-Ultra, ProSep Ultra Plus.** Gel stained with silver stain. L1: Protein Marker; L2-3 Ultra Plus elution and regeneration of tobacco proteins, respectively; L4-5: vA- High Capacity elution and regeneration of tobacco proteins, respectively; L6-7 vA-Ultra elution and regeneration of tobacco proteins, respectively.

#### 4.4 Dynamic Binding Capacity

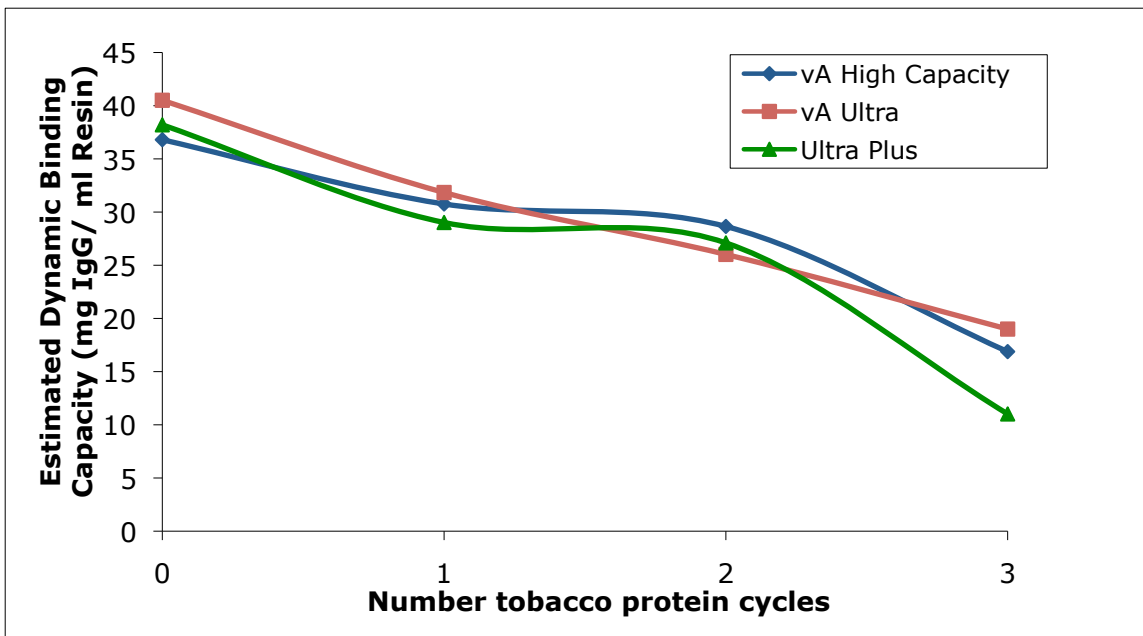
The binding of native tobacco proteins to the ProSep resins will progressively decrease the binding capacity of the Protein A resin. The previous section indicated that native tobacco proteins remain on the column after each cycle of tobacco sample loaded onto the column. Figure 4.24 shows the progressive decrease in the dynamic binding capacity of the High Capacity resin as the number of tobacco extract samples were run through the column.



**Figure 4.24. Time required for the dynamic binding capacity of the vA- High Capacity resin to reach 10% breakthrough as the number of tobacco extract cycle increase.** Chromatogram shows IgG samples run until 10% of the dynamic breakthrough was reached. PBS buffer used during equilibration and the post load wash contained 0.5 M NaCl.

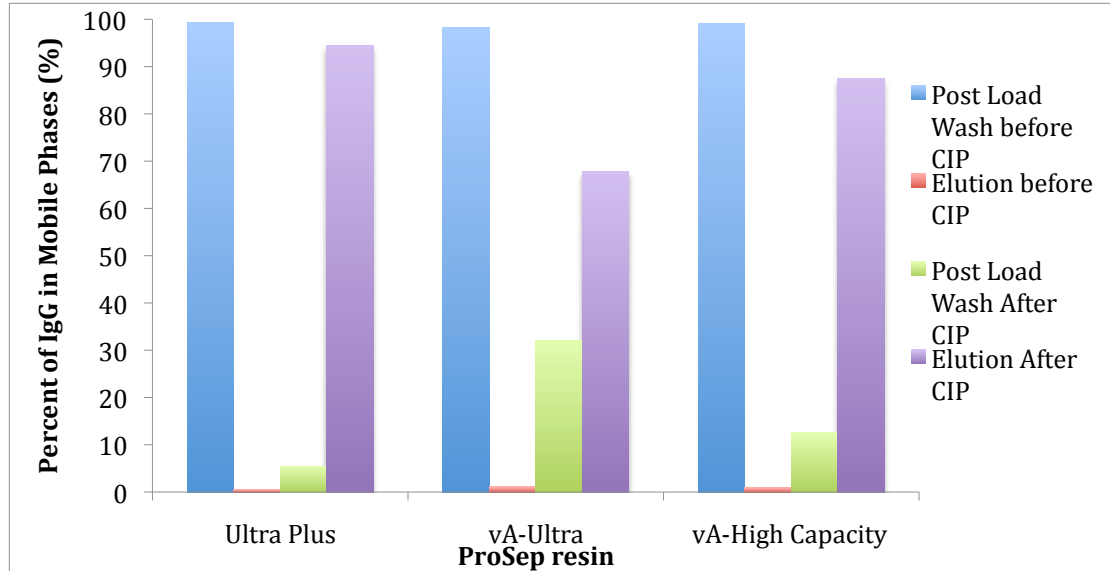
##### 4.4.1 Comparison of Dynamic Binding Capacity of the ProSep Resins

The amount of tobacco proteins that remains will increasingly decrease the dynamic capacity of the resin. Figure 4.25 presents how the dynamic binding capacity decreases for all three ProSep resins as the number of tobacco samples run through the column is increased.



**Figure 4.25. Estimated dynamic binding capacity for ProSep vA-High Capacity, ProSep vA-Ultra, and ProSep Ultra Plus resins as the number of tobacco extract cycle increase.** Binding capacity estimated from time to reach 10% breakthrough. PBS buffer used during equilibration and the post load wash contained 0.14 M NaCl at pH 7.

Native tobacco proteins, which strongly bind to the resin, can be eluted under denaturing conditions, such as the use of chaotropic solutions, when acidic solutions are insufficient at the removal of these proteins. Cleaning the column with 6 M of guanidine HCl or 6 M urea every 8-10 runs of tobacco extract removes tightly bound tobacco protein that is not removed by regeneration of the column at the end of each cycle. Figure 4.26 shows how the treatment of the column with 5 CV of 6 M guanidine HCl can regenerate the binding capacity of the resins after 8 cycles of tobacco extract were loaded onto the column. These results indicate that the treatment of the column with 6M guanidine HCl can greatly prolong the cycle-life of the columns. However, guanidine HCl is a very expensive chemical to use, especially on a large scale. Therefore, to reduce the total production cost, it may be better to use 6 M urea solution to clean the columns.

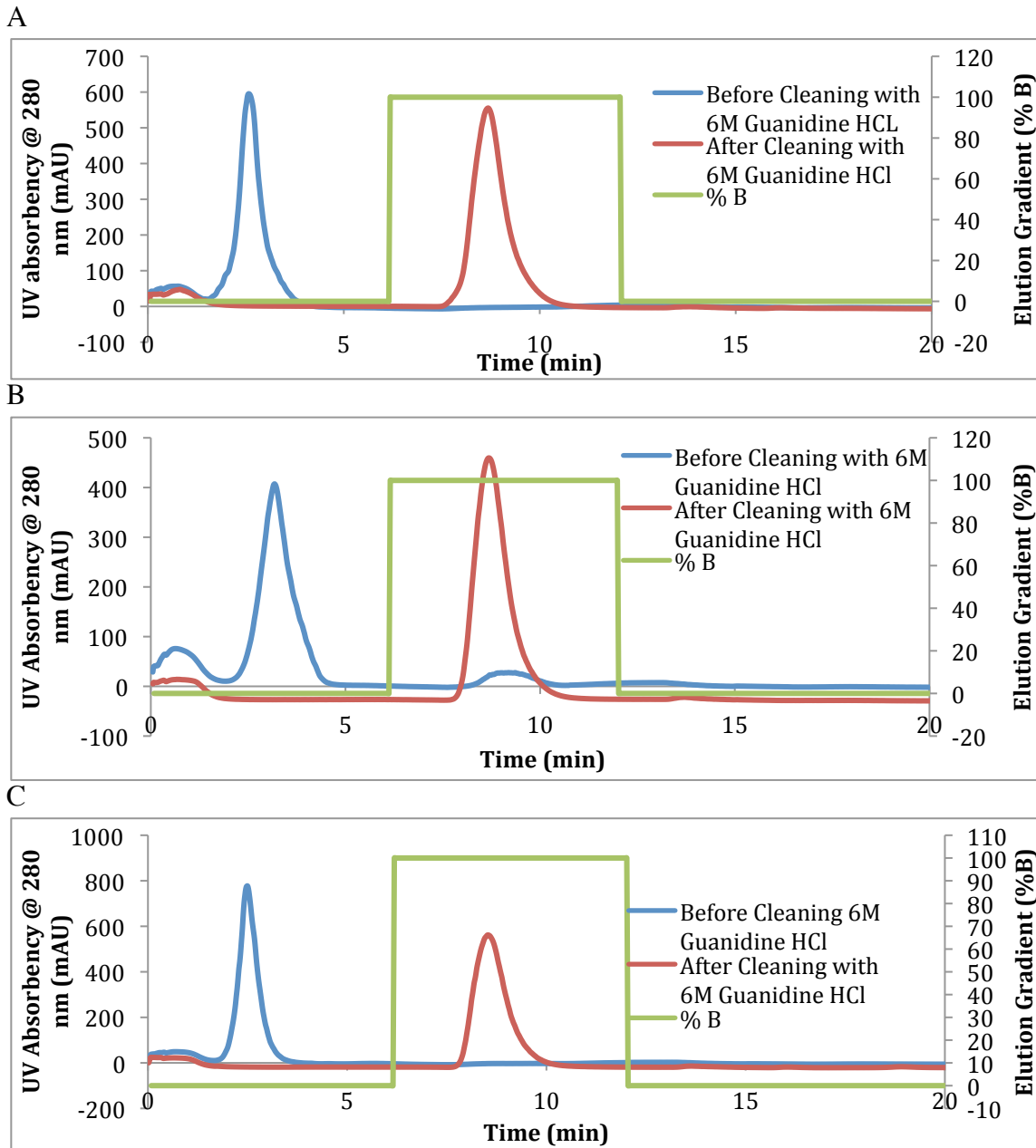


**Figure 4.26. Binding of human IgG to Ultra Plus, vA-Ultra, vA-High Capacity resin before and after cleaning (CIP) with 6 M guanidine hydrochloride.**

PBS buffer used during equilibration and the post load wash contained 140mM NaCl at pH 7. This cleaning was conducted after 8 cycles of tobacco protein samples were run through the columns.

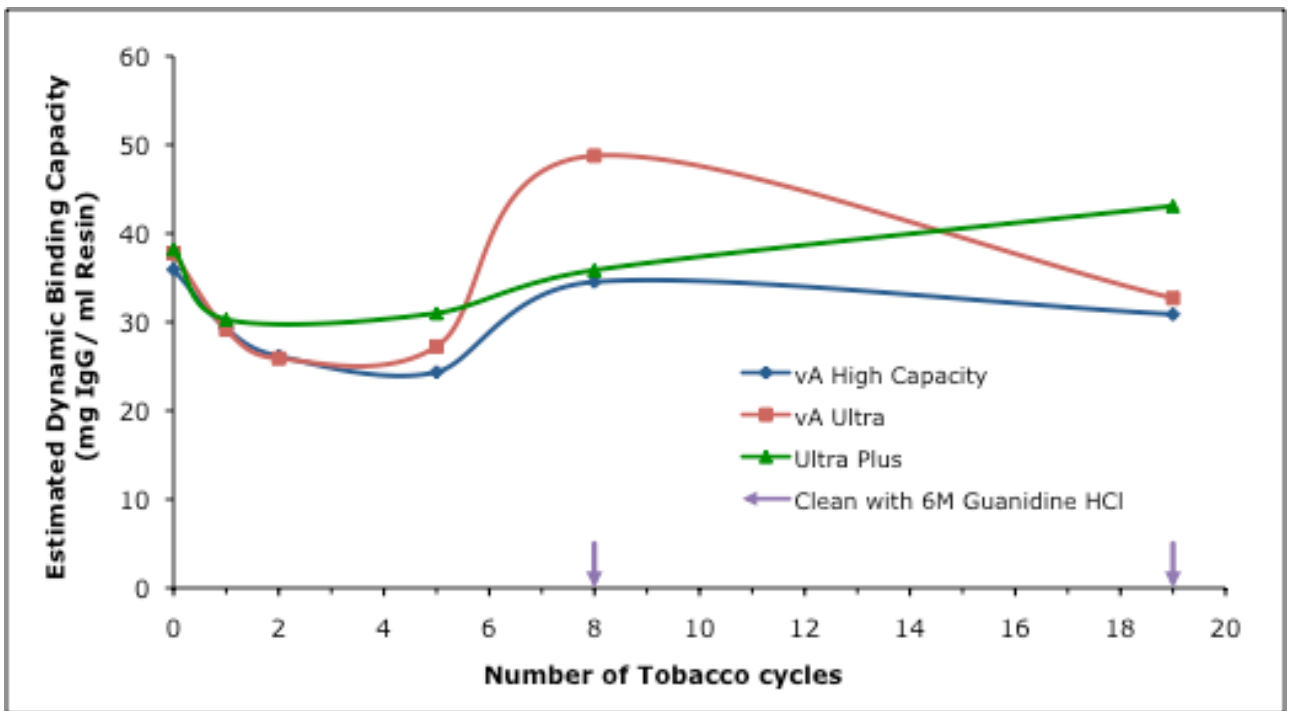
After 8 cycles of tobacco extract were loaded onto the resins, a sample of IgG was run through the column. As shown in Figure 4.26, the IgG was not binding to resins and a majority of the IgG was washed from the column during the post load wash step of the chromatography cycle. The resins were then treated with a denaturing solution, 6M guanidine hydrochloride, to remove the tightly binding proteins not removed from the column during the regeneration step of the chromatography cycle, thereby, regenerating the binding capacity of the resins. The chromatograms (Figure 4.27) indicate the ProSep A column's ability to capture IgG after 8 tobacco cycles, before and after cleaning the column with the denaturing solution of 6M guanidine HCl. Therefore, to maintain the dynamic binding capacity of the column, it is important to clean the column with 5 CV of 6M guanidine HCl or urea every 8-10 cycles. The chaotropic agents, urea and guanidine HCl, dissociate hydrogen bonds, causing protein denaturation. Therefore, proteins tightly bound to the ProSep A resins through hydrophobic interactions are eluted from the column when treated with these chaotropic agents. Since it was observed that native tobacco proteins non-specifically bind to the ProSep A resins through hydrophobic

interactions, the uses of one these chaotropic agents is important to remove residual tobacco proteins from the column and, thus, maintain the binding capacity of the resin.



**Figure 4.27. Regeneration of binding capacity for Ultra Plus, vA-Ultra, vA-High Capacity resin to capture human IgG for before and after cleaning (CIP) with 6 M guanidine hydrochloride.** PBS buffer was used during equilibration and the post load wash contained 140mM NaCl at pH 7. This cleaning was conducted after 8 cycles of tobacco protein samples were run through the columns. Columns were packed with 1 mL of: A: ProSep Ultra Plus resin; B: ProSep vA Ultra resin; C: ProSep vA High Capacity resin.

As more samples of tobacco extract were cycled through the column, the binding capacity of the resin decreased. Therefore, it is important to regenerate the binding capacity and, thus, extend the life of the Protein A resins. Figure 4.28 shows the dynamic binding capacity of the three different resins after 20 samples of tobacco proteins were run through the column. The dynamic binding capacity of the resins will oscillate even without the presence of tobacco samples being run through the column. Therefore, there was minimal, if any, decrease in dynamic binding capacity of the resins after 20 samples of tobacco extract are run through the column (Figure 4.28).



**Figure 4.28. Estimated dynamic binding capacity ProSep vA-High Capacity, ProSep vA-Ultra, and ProSep Ultra Plus resins as the number of tobacco extract cycle increase. Binding capacity estimated from time to reach 10% breakthrough. PBS buffer used during equilibration and the post load wash contained 1M NaCl.**

## Chapter 5: Conclusions

1. Mass balance analysis of the tobacco extract samples indicate that up to 20% of the proteins loaded onto column remain bound to column, thereby reducing the purification efficiency of the ProSep resins.
2. The PBS wash buffer containing the lower salt (NaCl) concentration removed more residual tobacco proteins than the higher salt concentration, indicating hydrophobic interactions between the native tobacco proteins and ProSep resins.
3. Using a PBS buffer with intermediate pH (pH 5) to wash the column prior to elution increases the amount of native tobacco proteins present during the elution phase of the column. This indicates that since more acidic proteins than basic proteins are present in tobacco and the overall cellular environment of tobacco is slightly acidic (pH~ 5), more hydrophobic interactions between tobacco proteins and ProSep resins are occurring.
4. Increased pore size of the -vA High Capacity resin reduced the removal of native tobacco proteins due to the -vA High Capacity resin providing less diffusion resistance to larger molecules than ProSep -vA Ultra and ProSep Ultra Plus resins.
5. The ProSep-vA Ultra resin had the smallest pore size of the three resins and, therefore, provided the highest diffusion resistance to large molecular weight molecules. This is shown by the increase presence of RuBisCo present in the flowthrough of the post load wash step of the chromatography cycle.
6. Increasing the salt concentration of the post load wash buffer decreased the amount of tobacco protein in the elution mobile phase. However, it also decreased the amount of IgG in the elution mobile phase. Furthermore, decreasing the pH of the post load wash buffer increased the efficiency of the ProSep A resins to capture and elute IgG from the resin, but it also increased the amount of tobacco protein present during elution. Thus, for efficient purification of IgG from tobacco, the post load wash buffer should have a low salt concentration at a neutral pH.

7. Overall, ProSep Ultra Plus was most efficient at the capture and elution of IgG, while reducing the non-specific binding of native tobacco proteins to the resin as well as reducing the presence of native tobacco proteins during elution.
8. SDS-PAGE gel does not indicate that RuBisCo is one of the tobacco proteins that is present during the elution of the column. On the other hand, the SDS-PAGE gel does indicate the possible presence of Fraction II tobacco proteins and Protein A (whole and fragments) during elution, which will require further purification during IgG processing.
9. Although the binding of native tobacco proteins to the ProSep resins will progressively decrease the binding capacity of the Protein A resins, cleaning the column with 6 M guanidine HCl or 6 M urea every 8-10 cycles of tobacco protein samples will regenerate the binding capacity of the ProSep resins, thereby prolonging the cycle-life of the resins and reducing total production costs. This increases the efficiency and effectiveness of the Protein A resins for the purification of IgG from tobacco.

## Chapter 6: Future recommendations and research

1. To prolong the cycle life of the ProSep Resin, the columns were cleaned with guanidine HCl, which is an expensive chemical, after 8-10 cycles of tobacco extract were run through the columns. The use of 6M urea solution instead to clean the column may be better suited in terms of reducing production costs. Therefore, determining if 6M urea solution is as effective as treating with 6M guanidine hydrochloride to regenerate the ProSep A columns should be investigated.
2. After Protein A affinity chromatography, it is important to remove host cell protein (HCP) contaminants, antibody-specific impurities, such as antibody aggregates, and leached protein A. Cation exchange (CEX) chromatography is useful in the removal of aggregates and HCP, and ceramic hydroxyapatite (CHT) is useful in the removal of leached protein A and aggregates [75]. Once the capture and purification of IgG from the tobacco has been achieved, one or both of the chromatography methods mentioned above could be used in polishing the target product. However, the CHT is difficult to pack for large-scale processes and the resin has a short lifetime.
3. All three Protein A resins used in this experiment are capable of capturing antibodies after multiple cycles of tobacco extract are loaded onto the column, which results in a cost efficient process. However, further research regarding the polishing of antibodies isolated from tobacco should be conducted.

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