# Monoclonal Antibody Expression and Novel Purification in *Nicotiana benthamiana*

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Abstract

Over the past few decades researchers and industrial professionals alike have realized the vast potential of monoclonal antibodies to treat diseases ranging from arthritis, immune and infectious diseases to cancer. There are a number of antibodies on the market that constitute a large portion of the biopharmaceutical niche in the drug industry. Blockbuster drugs (selling greater than $1 billion/year), include antibodies such as Avastin (bevacizumab), Herceptin (trastuzumab), Rituxan (rituximab), Humira (adalimumab) and Remicade (infliximab), which are cornerstones in this type of sector. With the cost of development to market approval rising astronomically for a new drug, new ways to produce and process these molecules becomes a paramount objective to ultimately help both patients and drug developers.

Plants, such as *Nicotiana benthamiana*, offer a unique production platform due to their recently found ability to produce large amounts of therapeutic proteins in a quick manner. While production would be simple and cheap, purification would not be due to the presence of toxic compounds in ground plant tissue. The current methods to purify these molecules from plant extract include expensive affinity column steps (Protein A/G) that are difficult to scale-up to bed volumes that would be necessary for this technology.

In the following paper, a method to purify a monoclonal antibody by non-Protein A/G resins is accomplished and compared to purification by Protein A. The modified process involved an UF/DF step, a precipitation of native impurities step using a charged polymer, hydrophobic interaction chromatography and hydrophobic charge induction chromatography. The yield of this modified process was 19.0%. This process compared favorably with Protein A due to the fact that even with washing steps including NaCl and Tween-20, the Protein A elution fraction still contained a large portion of host cell impurities. A chromatography step would need to be included before Protein A to both protect the column resin and provide a more purified immunoglobulin.

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# Chapter 1

## Introduction and Objectives

The production of molecules from biological systems, specifically biopharmaceutical products, is a complicated process. It is one that requires planning, optimization, and years of clinical studies prior to approval. There are very important stages in the development of a drug candidate, and any of them may hinder the ability of a company or researcher to be able to present these products to the patient. These stages include drug development, clinical trials/FDA approval, and finally production. The costs associated with final production include everything from capital investments for tanks reactors, purification skids, to other resources for purification methods used. These costs alone may account for a large majority of a company's overhead and can often make developing new drugs restrictive. Therefore, one of the most important tasks a researcher has is to limit the costs of downstream processes in order to make drug production cheaper, so a company may divert assets directly. Developments of this manner may be responsible for a drug being produced that may not have under more restrictive circumstances. The goal of this research is to develop a process that will make further downstream operations more cost effective.

The vast majority of molecules in some bio-technology companies' pipelines are antibodies, immunoglobulin fragments, conjugations or fusions. These molecules have shown an efficacy in treating a wide variety of ailments from rheumatoid arthritis to cancers of multiple kinds. The majority of antibodies are being produced using mammalian cell culture. Some of the issues that surround industrial production of these molecules include the large capital investment it takes to culture and cultivate mammalian cell culture, as well as the regulatory issues that surround using mammalian cells since there are often issues with viral clearance. Using plants as an expression system has the potential to be more cost effective and safe due to the ease of growth and lack of mammalian pathogens. Of course, this thought is predicated on the idea that efficient, cost-effective purification strategies can be developed. The vast majority of purification schemes for antibodies involve using Protein A or G resins. Issues with this method include that these resins are expensive, cannot be cleaned in place with sodium hydroxide, and the functional ligands can leach into the product.

Commercial-scale purification schemes currently uses multiple chromatography steps for the purification of biopharmaceutical products. Polyelectrolyte precipitation followed by HIC and HCIC chromatography has the potential to replace expensive Protein A chromatography for the initial purification of recombinant immunoglobulin and immunoglobulin fragments from crude or partially purified extracts. The goal of this research was to develop an alternative purification scheme for the separation of an immunoglobulin against irradiated Ebola virus.

This thesis contains six chapters. Chapter one contains an introduction and the project objectives. Chapter two reviews the current biopharmaceutical industry including recent sales numbers and monoclonal antibodies available. It also presents different production platforms and purification methods used throughout the pharmaceutical industry. It also discusses the information used to improve the industry by using a novel purification system.

Chapter three explains the experimental procedures used during the course of the project to achieve the goals. Chapter four focuses on the results acquired from the experimental work. The results were obtained using an optimized process to purify a recombinant immunoglobulin from transgenic *N. benthamiana*. This chapter also compares the above process to the more commonly used process utilizing Protein A.

Chapter five gives the conclusions for this work. Chapter six discusses future work revolving around implementing this process at an industrial scale.

# Chapter 2

## Literature Review

### 2.1 Monoclonal Antibodies

Monoclonal antibodies (mAb), a typical IgG is shown in Figure 1, are molecules that react with very specific compounds known as antigens. Antigens have several epitopes that are recognized by a single antibody. Several antibodies attach to a single antigen through binding with its antigen binding region (Fab), pictured in Figure 1. When the antibody coats the foreign molecule, it helps stimulate effector functions against the pathogen in immune cells that recognize the fragment crystallizable (Fc) region. The interaction of the Fc region with the receptor on the immune cell triggers the effector function. Different cells lead to different actions that clear the molecule from the body. Antibodies for an infinite number of antigens can exist. Antigens can range from virus components to bacterial elements to proteins parts (Campbell and Farell, 2006). The applications of antibodies in the current therapeutic sector cover a wide variety of ailments such as cancer, arthritis, inflammation, and immune disorders and infectious diseases (Pavlou and Belsey, 2005).

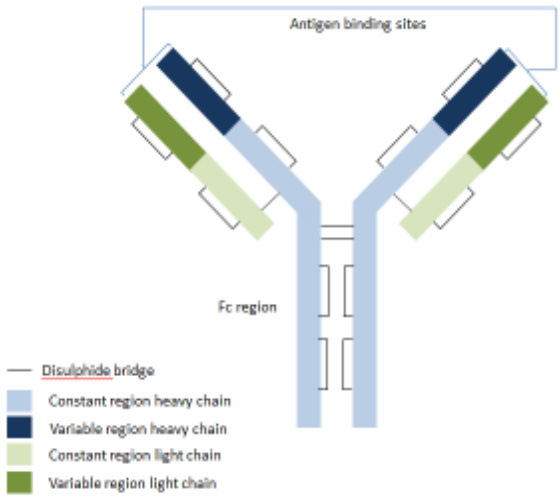


**Figure 1. Structure of an antibody**. The Fab region gives antibodies their specificity, while the Fc is responsible for the effector function.

There are several classifications of recombinant antibodies that are available. These are murine, chimeric, humanized, fully human, and conjugated. Murine antibodies are fully murine, in that, they are entirely formed of murine constant and variable regions. The obvious problems with this form as a therapeutic are that it cannot initiate human effector functions, will initiate an immunogenic response, and therefore has a short serum half-life. Chimeric antibodies have a murine variable region, which contains the very specific antigen binding site. The constant region has been replaced by the human constant region, which contains the crystallizable region that may elicit an immune response in the human body to remove the specific antigen. Humanized antibodies are created by replacing portions of a human antibody's antigen binding site with murine ones to gain specificity while minimizing the body's immunogenic response (Pavlou and Belsey, 2005). Replacing murine antibody genes with human genes will yield a fully human antibody (Trikha and Nakada, 2002). Conjugated antibodies are antibodies with attached agents for those used to treat cancer or other ailments. This conjugation optimizes the

specificity of the attached product (Pavlou and Belsey, 2005). The obvious implications of the antibody type are that the closer to human classification a drug is, the more effective and tolerated the drug will be.

## 2.2 Biopharmaceutical Market and Drug Development

### 2.2.1 Biotherapeutics and Antibody Market

Antibodies currently represent a fairly sizeable portion of the bio-therapeutics market. In 2008, biologics accounted for 80 billion USD of the estimated 256 billion USD that the world-spends on pharmaceuticals (Strohl and Knight 2009). It is also the fastest growing segment in pharmaceutical industry with 20% of the recently approved drugs in recent years and 40% of new entries in the pipeline (Karg and Kallio 2009). Chon and Zabis-Papastoitsis (2011) reported that sales of mAbs in 2010 exceeded $40 billion. A large portion of the sales of mAb is attributed to what is known as the "big five": Avastin (bevacizumab), Herceptin (trastuzumab), Rituxan (rituximab), Humira (adalimumab) and Remicade (infliximab) (Datamonitor 2008). There were 27 approved mAbs and Fc-fusion proteins available in 2009 and 30 were in advanced stages of clinical trials (Strohl and Knight, 2009).

One reason for the predicted rise can be attributed to a wider range of uses in the "big five" as the companies that market them are constantly searching for new clinical applications that the molecule can be used for. For instance, for the blockbuster drug Rituxan, the scope of treatment constantly increases. In 2009 Roche yearly reports, Rituxan was approved for the use of relapsed or refractory chronic lymphocytic leukemia in the European Union (EU) and Switzerland, first line chronic lymphatic leukemia in the EU, Rheumatoid Arthritis guidance on retreatment in patients with an inadequate response to anti-TNF therapy (Roche Finance Report

for 2009). In 2010 half year sales report, Rituxan was also approved for use in relapsed or refractory chronic lymphocytic leukemia in the USA and CLL-8 first-line chronic lymphocytic leukemia (Roche Finance Report for H1 2010). The effectiveness of the drug will also result in wider spread use and therefore greater sales. For instance the use of Herceptin for treating patients with breast cancer grew 82% in 2006 from 2005 (Lawrence, 2007). Not only are there developments in the scope of its application, new and also emerging markets for these applications are approved (Roche Finance Report for 2009 and H1 2010). Unfortunately, new cases of various diseases will also attribute to continued blockbuster sales of these drugs. The other major contribution to the rise of sales of monoclonal antibodies can be attributed to the use of new molecules. A few emerging antibodies will consist of a much larger portion of the market. The molecules that may become relevant in terms of large sales volume are Elan/Wyeth's bapineuzumab, Amgen and Eisai's denosumab, AstraZeneca/Abbott's Numax (motavizumab), Johnson & Johnson/Schering Plough's golimumab, Genentech/Novartis's Lucentis (ranibizumab), Roche/Chugai's Actemra (tocilizumab), Biogen Idec/Elan's Tysabri (natalizumab) and UCB's Cimzia (certolizumab pegol) (Datamonitor, 2008).

### 2.2.2 Drug Approval

Drug approval occurs through the use of clinical trials, which consists of three main divisions. Phase I is the first time that the drug is studied in humans. It is primarily concerned with safety of the drug's administration. It is usually given to volunteers or patients to evaluate the drug's effectiveness and pharmacology like drug metabolism, pharmacokinetics, and side effects related to the dose. It generally involves 20-80 patients, but this number can be different. Phase II is where the drug is evaluated as a drug for the specific ailment in a controlled clinical

study. It also helps to evaluate the risks associated with the dose. The number of individuals in this phase is no more than a few hundreds. Phase III looks at the drug at a much larger scope than Phase II. It looks at longer-term safety and effectiveness, dose-response analysis and mortality and morbidity outcomes (Temple 2000; Mackiewicz and Mackiewicz 2009).

Current estimates place the cost for approval between 1.2 and 1.7 billion USD (Strohl and Knight, 2009). Another estimate for the cost of an approved biopharmaceutical is approximately 1.318 billion USD (Redwan, 2007). The time line for producing a drug is approximately 10 years from discovery to market and the probability of success of a monoclonal antibody and Fc fusions, although relatively high, is merely around 17% (Strohl and Knight, 2009). A company developing a successful biopharmaceutical or antibody drug will have the burden of development and approval costs, as well as, the development and approval costs associated with failed and poor selling drugs. This makes the need to produce an antibody drug at a minimal cost even more important.

### 2.2.3 EBOLA Virus and Specific Antibody

Ebola virus infection causes Ebola hemorrhagic fever (EHF). There are a number of different strains, with some leading to fatality rates up to 90% (Takada et al., 2001). Moreover there are no effective ways to pre-emptively protect individuals with vaccines or treatments once the patient has been exposed to the virus (Sun et al., 2009). In one study, a protective IgG mAb 6D8 against Ebola virus GP1 was shown to be effective in treating Ebola Zaire in mice after exposure (Wilson et al., 2000). A research group was able to express a chimeric form of this antibody in *N. benthamiana* by replacing the mice gene sequences for the constant regions of the

heavy and light chains with human constant regions. The antibody retained its *in vitro* ability to interact with inactivated Ebola virus in this study (Huang et al., 2009).

### 2.3 Expression Systems

One way to help defray the cost associated with drug development is to choose an expression system that will reduce production costs. Various expression systems are used for the production of recombinant, full-sized monoclonal antibodies at the research level. While there are several benefits of the expression of antibody fragments in a wide variety of organisms, full sized, fully functional antibodies present challenges that up to this review have been unable to be overcome in some of the expression systems. Each model system has its own unique advantages and disadvantages, which will be discussed.

#### 2.3.1 Bacteria

Simple prokaryotes such as *Escherichia coli* have been used to express a wide range of therapeutics since the technology was discovered. They have also been used to express antibody fragments for therapeutic use, and this technology continues to be met with success since many fragments are currently in production and clinical trials from *E. coli* (Anderson and Reilly, 2004). Some of the major advantages of using a bacterial expression system are that there are no mammalian contaminants **(**Houdebine, 2009), rapid expression, high yield, simple fermentation and genomic modifications, and very inexpensive fermentation components (Demain and Vaishnav, 2009; Karg and Kallio 2009). Some of these advantages make bacterial hosts the most attractive expression system for the production of biopharmaceuticals; however, the

expression of full-sized monoclonal antibodies present several challenges that make using a bacterial system extremely challenging.

The obvious downside to using a bacterial expression system such as *E. coli* is that it lacks the intracellular components to properly perform mammalian post-translational modifications (PTM). One of the most important PTMs is glycosylation. If the mAb is not properly glycosylated, it will elicit an immunogenic response and be cleared from the body or it will fail to induce effector functions. Therefore, bacterial systems cannot produce a full sized antibody of sufficient quality for therapeutic use. Also, it cannot fold proteins properly or assemble subunits or form disulfide bonds (Houdebine, 2009; Karg and Kallio, 2009; Demain and Vaishnav, 2009). Some groups have attempted to improve expression of antibody fragments by co-expressing chaperones to improve protein folding (Ramm and Plückthun, 2000) or disulfide bridge formation (Humphreys et al., 1996).

In the past, there was an instance where a full-length antibody was produced in *E. coli*. The antibody obviously did not contain any glycosylation and could not bind to $Fc_\gamma$ receptors, making the antibody useless in terms of initiating effector functions. The antibody did interact with the neonatal Fc receptor (FcRn) and had a long half-life. The inability of the antibody to initiate effector functions controlled by $Fc_\gamma$ receptors made it ineffective in applications where activating the immune system in that manner was required; however, it did have the ability to interact with the antigen. This fact made it useful for applications that do not require effector functions (Anderson and Reilley, 2004).

### 2.3.2 Yeasts and Filamentous Fungi

Yeasts such as *Saccromyces cerevisiae* and *Pichia pastoris* have been used to express recombinant proteins since their inception. There are several reasons for their usage. Yeasts can

provide a vessel for high yields, are cost effective and robust, can grow to high densities, process proteins similarly to mammalian cells, express proteins with disulfide bonds, assist in protein folding and can glycosylate (Demain and Vaishnav, 2009). They grow rapidly and are generally regarded as safe, which allows them to be used as a production host (Karg and Kallio, 2009). They are also able to fold and secrete proteins into media, which simplifies purification strategy development (Houdebine, 2009).

A problem associated with recombinant protein expression in yeast is that N-linked glycosylation may be immunogenic or may lack the desired effector function, since *S. cerevisiae* often produces recombinant proteins that do not have ideal glycan structure for human use (Potgieter et al., 2009; Demain and Vaishnav, 2009). However, genetic engineering can alleviate some of those concerns. One group was able to express a functional mAb at 1 g/L with uniform N-linked glycan structure of type $Man_5GlcNAc_2$ (Potgieter et al., 2009).

Methyltrophic yeasts, such as *Pichia pastoris,* are used because they have strong, highly-regulated promoters, secrete proteins efficiently, often achieve higher yields than *S. cerevisiae*, avoid the hyper-glycosylation that plagues production in *S. cerevisiae*, and grow at reasonably strong methanol solutions that will kill other organisms. The more optimal glycosylation occurs due to shorter chain lengths of N-linked high mannose oligosaccharides up to 20 residues while *S. cerevisiae* is 50-150 residues. *P. pastoris* does not have alpha-1,3 linked mannosyl transferase which produces alpha-1,3 mannosyl terminal linkages in *S. cerevisiae.* This glycosylation pattern will lead to a recombinant protein with poor efficacy due to an immune response to the protein (Demain and Vaishnav, 2009). An issue with the technology is that in order to produce the protein in the necessary amounts, scaling up bioreactor facilities requires substantial and sometimes debilitating initial capital investment (Chen, 2008).

### 2.3.3 Mammalian Cells

The most successful and widely used system for expressing monoclonal antibodies for application in therapeutics involves the use of mammalian cell culture. About 60-70% of all the recombinant therapeutics are produced using mammalian cell lines (Redwan, 2007). All of the mAbs on the market are produced in mammalian cell lines. For instance, Rituxan, Herceptin, and Avastin are produced in Chinese Hamster Ovary cells (CHO), Remicade is made in NS0 cells, and Humira is made in Human Embryonic Kidney (HEK) 293 cells (Zhang and Robinson, 2005). There are major advantages to using mammalian cell culture as an expression system. mAbs expressed in these systems are of high therapeutic quality in terms of folding and glycosylation (Karg and Kallio, 2009**;** Demain and Vaishnav, 2009).

There are also several glaring disadvantages with using mammalian cell culture, which have left the need to develop other systems. The culture requirements are substantial, whether that is the large initial investment for bioreactors of appropriate size, facility building, and validation or the continuing investment of media and production support. Also, proof of consistent performance is something that needs to be achieved. There are viral clearance issues (Demain and Vaishnav, 2009). Plagued by low expression in the earlier stages of the technology, generic processes in fed-batch cell culture achieve titers from 3-5 g/L. Moreover much higher yields are common. One research group achieved titers in the realm of 25 g/L (Chon and Zabis-Papastoitsis, 2011). Further improvement can be made by designing anti-apoptosis additions. Apoptosis, programmed cell death, is triggered by hypoxia, nutrient depletion, waste-by product accumulation, and other factors during the cell culture process. When cells die, recombinant protein production suffers due to less cells available and proteolysis. Some of the studies to reduce apoptosis have been adding apoptosis inhibitors, re-supplying nutrients, and expression of

anti-apoptotic genes. For instance, in one study over-expression of *30Kc6,* a gene to prevent apoptosis, was able to improve expression 2.3 fold than the control in 5 days. Apoptosis was induced by moving the cells from a serum containing media to a medium without serum. When the gene product was expressed, it helped to maintain the mitochondrial membrane potential and helped to prevent apoptosis cascade (Wang et al., 2010).

### 2.3.4 Transgenic Animals

The first drug approved for therapeutic use in humans from a transgenic animal source was an anti-thrombin III from goat milk in 2006 (Karg and Kallio, 2009). Some of the advantages of using this technique are that transgenic animals are able to produce high quality proteins that are very similar to humans. Proteins can be produced in the milk, blood, egg white, seminal plasma, and urine. Milk, however, is the most mature system to produce therapeutics (Houdebine, 2009).

One of the major disadvantages is that it is extremely laborious and monitoring for mammalian pathogens is paramount (Houdebine, 2009). Also, there is a significant time investment, in that, the time required to assess production level can be quite long (Demain and Vaishnav, 2009). Other issues include cost and flexibility of scale-up (Karg and Kallio, 2009).

### 2.3.5 Transgenic Plants

Functional, full-sized monoclonal antibodies were first expressed in plants before the 1990s. The antibody represented 1.3% of the total soluble protein (TSP) (Hiatt, et al., 1989). The original idea of using plants to express recombinant proteins was appealing to both researchers and corporations alike. There are two methods of developing transgenic plants that express monoclonal antibodies: constitutive expression and transient expression. Each involves

the infection of plant tissue using *Agrobacterium tumefaciens*. *A. tumeafaciens* infects plants cells in a complicated process and can transfer and integrate a particular DNA segment known as T-DNA of its Ti plasmid into the host genome (Chilton et al, 1977).

The advantages of using plant systems are the low cost of growth, they have the ability to fold and glycosylate complex proteins, and their use minimizes the risk of mammalian/human pathogens (Houdebine, 2009; Demain and Vaishnav, 2009). The early technology prevented large scale industrial use of plant systems due to the cumbersome nature and time frame of the system, since developing transgenic plants had a long lead time; however, recently, monoclonal antibodies have been expressed in plant cells at extremely high levels in a matter of days (Giritch et al., 2006).

Constitutive expression involves the stable transformation of plants, the sexual crossing of the re-generated plants expressing certain subunits, and then the expression of the protein of interest by a fraction of the following progeny. However, this method of production of recombinant antibodies suffers from two major drawbacks. The first drawback is that the exogenous protein is usually expressed at extremely low levels (Chen, 2008). Yields of IgG and IgA by stable transformation are around 1-40 µg/ g biomass fresh weight. The other potential disadvantage occurs with respect to the long time frame required to generate stable progeny to express enough protein of therapeutic value (Giritch et al., 2006).

These two issues are solved by what is known as transient expression. In this production technology, *A. tumefaciens* is introduced into the plants, and in a matter of days, the protein (usually less than 10 days after infiltration) is expressed in the leaves. Protein and monoclonal antibody production is usually extremely high. In one study, a mAb against West Nile Virus

accumulated up to 800 μg/g fresh leaf tissue (Lai et al., 2010). Also the generation time to produce therapeutic quantities is reduced by incredible amounts.

Researchers were unable to use plants to produce proteins with multiple subunits with a viral-vector transient expression system for a long period of time. Earlier attempts to express mAbs transiently with viral-vectors failed because competition between two vectors resulted in the replication of one vector instead of the other (Dietrich and Maiss, 2003). The magnifection system helped to alleviate this problem. Two viral vectors derived from the tobacco mosaic virus and the potato virus X were found to be uncompetitive because they used different parts of the host cell. When the two were expressed together, both gene products would be expressed, allowing for proteins with multiple subunits to be made in plants. The system is extremely versatile and provides a high-throughput production platform (Gritich et al., 2006).

Another system used for replicons that is relevant in this paper is based on the bean yellow dwarf virus (BeYDV). It is a single stranded DNA virus that replicates efficiently. Magnifection systems that use RNA replicons are not as optimal as DNA replicon systems, since there will be a higher stability for DNA replication in the nucleus than for RNA replication in the cytosol (Huang et al., 2009).

As already stated, the major advantages of this system are that complex, multi-subunit proteins can be properly expressed and assembled in a plant system that requires very little capital cost to begin production. Therapeutic amounts for pre-clinical and early stages of clinical trials could be produced while the long-term production system (constitutive expression) was developed (Chen, 2008). This negates the lead time argument that detractors had used against this technology. There are still major drawbacks that will need to be addressed before plants become popular therapeutic expression systems. Some of the disadvantages of using plant

systems are that proteins are extremely difficult to purify from leaves due to presence of proteases and polyphenols (Houdebine, 2009). Fresh tobacco plants contain 80–90% of water and a large amount of impurities, such as sugars, amino acids, starch, cellulose, alkaloids, and polyphenols (Valdes et al., 2003). Also, there are uncertain regulatory issues associated with the system, in that it is difficult to conclude when or if a therapeutic will enter the market from this production platform (Houdebine, 2009). There have been, however, encouraging signs that a product from a plant may be approved. A drug known as CaroRx™ has been approved for topical use in Europe for the treatment of tooth decay, and it is produced in field-grown tobacco. Some molecules have entered varying stages of clinical trials such as Locteron (alpha-interferon) which is produced in a contained system of *L. minor* and a treatment for Gaucher's disease in phase III in carrot cells (Karg and Kallio, 2009).

## 2.4 Antibody Purification

### 2.4.1 Protein and Immunoglobulin Purification in Plants

Protein purification is one of the most important steps in the administration of drugs in terms of safety. Efficient downstream processing is paramount in production since up to 80% of manufacturing costs can be associated with purification costs (Roque et al., 2004). Column chromatography has been the most successful and widely used technique to purify antibodies regardless of the production system. Certain techniques have the ability to separate the target protein from native impurities by a multitude of characteristics including size, charge, solubility, hydrophobicity, affinity for specific ligands, and also similar characteristics that will target the native impurities (Chen, 2008). While chromatographic operations are absolutely paramount for safe drug dosage, non-chromatographic operations are intrinsically important in the purification process of pharmacological proteins produced in plants due to their ability to separate host

impurities from the target protein as well as other native molecules. Several studies show that due to the presence of native materials such as phenolics, alkaloids, and other unique products, direct loading of crude extract onto a Protein A or G column will result in column fouling and/or poor binding (Bai and Glatz, 2003; Menkhaus et al., 2004; Valdes et al., 2003). This fact is especially important when considering industrial processes that will require multiple robust chromatography processes that are able to continually produce consistent results. In Table 1, some of the mAbs expressed in plants with pharmacological targets, their method of expression, and their purification method are listed.

**Table 1. Antibodies produced in plants and their pharmacological targets.** The most commonly used method to separate immunoglobulins is with the biospecific resin Protein A.

| Antigen | Type | Expression type and Level | Source | Principle Separation Method | Reference |
|---|---|---|---|---|---|
| Streptococcal surface antigen SA I/II | S IgA/G | Stable 200-500 mcg/g tissue | *N. tabacum* | N/A | Ma et al., 1995 |
| Herpes Simplex virus 2 glycoprotein B | IgG | Stable; NA | *G. max* | Protein A, cation exchange, ion exchange | Zeitlin et al., 1998 |
| GA733-2 | IgG | Transient; NA | *N. benthamiana* | Protein A | Verch et al., 1998 |
| Human carcinoembryonic antigen CEA | Diabody | Transient ~1.5 mg/ kg fresh tissue to transformed plants ~0.5 mg/kg | *N. tabacum* | IMAC for $His_6$ tagged diabody | Vaquero et al., 2002 |

| | | | | | |
|---|---|---|---|---|---|
| Human rhesus D | IgG1 | Stable; 0.6% TSP | *A. thaliana* | 70% Ammonium Sulphate Precipitation, DEAE Sepharose, and Abx | Bouquin et al., 2002 |
| Human chorionic gonadotropin (HCG) | scFV, Diabody, IgG1 | Transient; 32 mg scFV, 40 mg diabody, 20 mg IgG per kg fresh tissue | *N. tabacum* | IMAC for His6 tagged scFV and diabody Protein A for Mab | Kathuria et al., 2002 |
| Rabies virus | IgG | Stable; 3 mcg g of fresh leaf weight (0.07% of total soluble protein), | *N. tabacum* | Protein A then Protein G | Ko et al., 2003 |
| Hepatitis B surface antigen | IgG | Suspension Cells; 0.1–2% of TSP | *BY-2 tobacco cells* | Ammonium sulphate Protein A | Yano et al., 2004 |
| Protective antigen PA of Bacillus anthracis | IgG | Transient; 1 mg/kg biomass purified antibody | *N. benthamiana* | Ammonium sulfate precipitation, Protein A, T-gel adsorbent | Hull et al., 2005 |
| Tumor-associated antigen GA733 | IgG | Stable; 310 mcg/kg biomass | *N. tabacum* | Ammonium Sulphate precipiation Protein G | Ko et al., 2005 |
| Tumor-associated antigen oligosaccharide Lewis Y | IgG2a | Stable; 30 mg/ kg tissue Purified 3 mg/ kg | *N. tabacum* | Ammonium sulphate, Protein A | Brodzik et al., 2006 |
| HIV p24 | p24-IgA Hc fusion | Stable; 0.88% of TSP (SD = 0.32), with the highest level of expression at 1.4% of TSP.purified 1 mg/ kg tissue | *N. tabacum* | Negative Isoelectric precipitation, Ammonium sulphate, Affinity chromatography with Goat anti-human polyvalent antiserum | Obregon et al., 2006 |

| | | | | | |
|---|---|---|---|---|---|
| CD30 | IgG | Transgenic stable | *Lemna minor* | Negative Isoelectric precipitation, Protein A, Aggregate removal with HAC | Cox et al., 2006 |
| Pseudomonas aeruginosa serotype O6ad PS O side chain | IgG | Stable; 4 mg/kg | *N. tabacum* | Immobilized metal affinity expanded bed, Protein G | McLean et al., 2007 |
| HIV gp 120 | IgG | Stable; 0.2% to 0.05% TSP | *A. thaliana* | Protein A | Schähs et al., 2007 |
| | | Stable; 75 mcg/g dry seed weight | *Z. mays* | Protein A Or Cation Exchange, IMAC on Zn2 -IDA-agarose | Ramessar et al., 2008 |
| | | Transient 110 mcg/g 0.5% TSP | N. benthamiana | Isoelectric negative precipitation, Protein A | Strasser et al., 2008 |
| Ebola GP1 | IgG | Transient; 500 mg/kg | *N. benthamiana* | Ammonium sulphate Protein G | Huang et al., 2010 |
| West Nile Virus | IgG | Transient; 800 mg/kg | *N. benthamiana* | Ammonium sulphate Protein A | Lai et al., 2010 |

### 2.4.2 Non-chromatographic and Pre-treatment Operations

Tangential flow filtration is an important ultrafiltration (UF) operation used in the bio-therapeutics industry. It allows for the concentration of the target molecule and the removal of low molecular weight, native impurities including cellular components, DNA, RNA, lipids, and polysaccharides. It provides cross-flow over the membrane to reduce membrane fouling and to maintain the flux through the membrane (Harrison et al., 2003). In terms of plant material, it can

be used to remove some of the high concentrations of phenolic compounds generated by plants like *Lemna minor* or from tissue grinding as in other plant species. Biolex Therapeutics uses a simple 5K MWCO UF operation to remove a majority of phenolic impurities in the beginning of their processes to purify a recombinant protein from *L. minor* (Scot Shepard, personal correspondence). In terms of *N. benthamiana*, it was determined that initial clarification experiments were unavoidable because the green components in the cell extract reacted strongly with Protein A chromatography components including the adsorbent. The lack of an initial clarification step resulted in the precipitation and blocking of the adsorbent, which caused poor recovery and purity. UF was unable to remove discolorations in the collected fractions, which undoubtedly represented remaining impurities (Valdes et al., 2003). Although it was unable to remove the impurities post- chromatography, UF may be able to improve earlier downstream processing. In a study by Yu et al. (2008), direct loading of the crude extract onto the Protein A media was found to yield significant backpressure and fouling. An ion-exchange step helped to alleviate this problem.

In a study by Balasubramaniam et al. (2003), it was determined that the majority of native tobacco proteins are of acidic nature. This fact was determined using isoelectric precipitation and measuring the amount of protein at each pH of extraction. More protein was extracted at pHs greater than pH 7. Such information has been used by researchers such as Platis et al. (2008) to remove native host impurities. By extracting proteins at an acidic pH, further purification steps will be more efficient as there are less initial impurities.

Another possible initial capture step can be derived from this information. Either precipitating the basic target protein or the acidic impurities would be beneficial as an initial capture and minor purification process. One of the ways to target a group of proteins based on

their charge is by their electrostatic interaction with a charged polymer. The polymer becomes charged based on its pKa and the pH of the system. The interaction can occur, and then separation would be completed via solid-liquid phase separation by centrifugation. After re-suspension, a high salt concentration can be used to over-compete with the electrostatic interaction, much like in the elution stage of ion exchange chromatography. Concentrations from 0.5-2.0 M NaCl may be used to interrupt this interaction according to Holler et al. (2007).

In order to precipitate out the target protein, in this case a basic monoclonal antibody, an anionic polymer could be used. In a previous study by Zhang et al. (2005), it was determined that the most effective polyelectrolyte in precipitating a basic target protein in the tobacco system was poly(acrylic) acid. Egg white lysozyme, a basic protein with an isoelectric point of around 10.5, was recovered at a yield of 85% of the soluble lysozyme, while almost no native proteins were co-precipitated. The precipitation protocol called for extraction at pH 5, since at pH 7 the procedure was shown to be ineffective. It would appear at this pH less native tobacco proteins were recovered, but the majority of lysozyme remained functional in the extract.

In a way to remove the native proteins without exposing the target protein to a harsh environment necessary to remove them based on isoelectric precipitation, a negative precipitation protocol may be employed. The target protein will remain in the supernatant after centrifugation, while the majority of the acidic impurities will be removed due to their interaction with a cationic polymer. Holler et al. (2007) utilized a procedure with a cationic polymer, polyethylenimine (PEI), to purify an acidic recombinant beta-glucuronidase from native tobacco proteins and compared those results to a normal industrial application of ion exchange chromatography as an initial capture step. This separation process compared favorably to anion exchange chromatography as an initial capture step. Also, there was a need to remove

DNA and RNA from the precipitated material to recover the acidic protein (Holler et al., 2007). This fact would benefit this procedure as a separation method for a basic protein since these impurities would be removed for the most part with negative precipitation.

Another separation technique that has been widely used is ammonium sulphate precipitation; this separation technique is common among researchers working with plants and is based on the principle that proteins will lose solubility when subjected to a high lyotropic salt environment. Proteins will "salt out" at different concentrations depending on their solubility. Ammonium sulphate is highly soluble even at very high concentrations (Harrison et al., 2003). Some studies have effectively used ammonium sulfate as an initial clarification method in the early parts of process development to purify antibodies from plant extracts (Bouquin et al., 2002; Hull et al., 2005; Ko et al., 2005; Brodzik et al., 2006; Huang et al., 2010; and Lai et al., 2010).

Aqueous two phase extraction (ATPE) is another system for the separation of target proteins and impurities. It involves two water soluble polymers or a polymer and salt in water above a critical concentration. The two materials that are chosen separate into two immiscible phases, one enriched in polymer and the other in salt or the other polymer. Proteins will partition in one of the phases based on several characteristics of the proteins and the interacting system including: protein molecular weight, protein surface properties and charge, polymer molecular weight, phase composition, salt effects, and affinity ligands attached to polymers. It is a non-denaturing, non-degrading separation technique (Harrison et al., 2003). In a study by Platis et al. (2008), phenolic materials were removed using an ATPE system that was a successful initial purification step that yielded 90% of an IgG at 1.5% purity. It would now be easier to apply the sample to a column and prevent fouling and loss in binding capacity. Although some of the advantages of this system are its robustness and scalability, there are significant disadvantages

such as the fact that the partitioning behavior is extremely complex and the only way to determine the partitioning is through time-consuming trial and error studies (Roque et al., 2007; Platis et al., 2008).

Another non-chromatographic technique involves affinity precipitation. In this method, an affinity ligand is covalently linked to the hydrophilic polymer. The bonding occurs in a single step in which the ligand interacts with the target molecule under aqueous conditions. The microenvironment is then manipulated in a certain way to precipitate the polymer (and the target molecule). Washing and elution would then occur similarly to affinity chromatography. The polymer would be kept insoluble during these steps so as to recover the target molecule in the supernatant after subsequent centrifuge steps. The polymer can then be recovered and readied for re-use by a simple re-solubilization, although the recovery of the polymer-ligand complex may be laborious (Roque et al., 2007).

### 2.4.3 Chromatographic Operations

Non-chromatographic separation processes are important due to their ability to separate the target molecule from other impurities, but they lack the resolving power of chromatographic operations. Initial pre-treatments can be important by increasing the longevity of resins by reducing fouling and help maintaining binding capacity. While these techniques can be essential to purifying antibodies to a therapeutic level in an economic manner, the real resolving of the target protein from natural impurities comes from chromatographic operations. This section will focus on affinity separation due to its high resolving power. There are several types of affinity resins, which will be discussed.

#### Bio-specific

Naturally occurring molecules, which have very high affinity constants, that can be used to bind antibodies include the antibody's antigen and bacterial immunoglobulin-binding proteins Using an antibody's antigen as an affinity separation method can be advantageous, but if the antigen is difficult to work with, its use is controlled, or its coupling to a matrix is expensive, this method for separation should not be instituted. Some examples of immunoglobulin binding proteins are Protein A and Protein G. These proteins are bacterial cell surface components that interact with the Fc portion of antibodies. Engineers have employed this technology to purify antibodies extremely effectively (Roque et al., 2007).

**Pseudobiospecific**

Due to some of the apparent issues involved with using naturally occurring molecules for affinity chromatography, resins were developed to replace Protein A and G. Some of these aforementioned issues are that the media is extremely expensive, it lacks the ability to be cleaned in place by sodium hydroxide, and toxic material can leach into the product (Roque et al., 2007). The alternative ligands, in general, have a lower affinity than the natural molecules, but are usually less costly and present other advantages. Some of these ligands are hydrophobic, thiophilic, hydroxyapatite, chelating metal-ions and mixed-mode affinity ligands and there are also mimic adsorbents that attempt to mimic interaction of Protein A (Chen, 2008).

**Hydrophobic and Thiophilic Ligands**

Hydrophobic interaction chromatography (HIC) uses the interaction of an immobilized hydrophobic adsorbent with the non-polar regions of a protein. These interactions increase with higher salt concentrations (Queiroz et al., 2001). This mechanism is extremely important when considering antibodies due to their hydrophobic properties, and HIC takes advantage of that fact

to bind immunoglobulins under high lyotropic salt conditions. Elution can occur with progressively lower salt concentrations (Roque et al., 2007). In terms of protein extraction from plants, Holler et al. (2007) found that a HIC step after a polyelectrolyte precipitation initial step was effective in removing some of the native impurities while still recovering around 78% of a recombinant beta-glucuronidase.

Thiophilic chromatography was discovered when Porath et al. (1985) determined that a chromatography process using an agarose based sorbent after the reaction of divinylsulfone with 2-mercaptoethanol was capable of fractionating plasma proteins. The most common type of sorbent used is called a T-gel, which carries linear ligands with two sulfur atoms. It shows good selectivity for immunoglobulins in high lyotropic salt concentrations. In this sense, it is similar to HIC and elution occurs at a lower ionic strength; however, different salts affect adsorption in different ways (sodium chloride promotes desorption from thiophilic sorbents) (Boschetti, 2001). Recoveries can be close to 100% for full-sized immunoglobulins (Hansen, 1998). In terms of mAbs recovered from plant extracts, in a study by Hull et al. (2005), thiophilic chromatography using T-gel was used as a later purification step after Protein A chromatography to remove impurities.

**Hydroxyapatite**

Hydroxyapatite chromatography (HAC) [$Ca_{10}(PO_4)_6(OH)_2$] utilizes a unique binding mechanism. Positively charged proteins interact with the phosphate portion of the resin through their amino groups by electrostatic attraction. Negatively charged groups interact with the matrixes calcium sites. Increasing the ionic strength gradually will elute the basic proteins, while using buffer components with high calcium affinity will elute negative proteins. The

mechanisms of interaction and elution are shown in Figure 2. Some issues with using this resin type involve a lower binding capacity than some other chromatographic materials typically used in antibody purification and short lifetimes (Roque et al., 2007). Holler et al. (2007) were able to resolve a negatively charged enzyme from a major native protein of *N. tabacum* using HAC, so it may have potential to resolve immunoglobulins from native materials. Also, HAC was used as a polishing step to remove aggregates from a *Lemna minor* expressed immunoglobulin in a study by Cox et al. (2006).

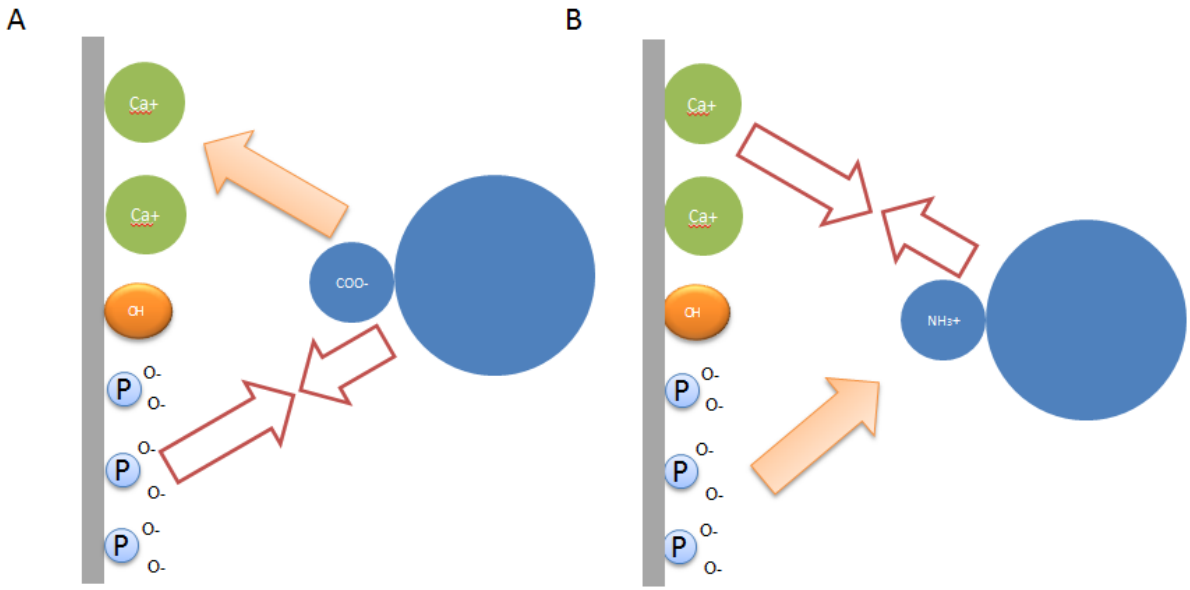


**Figure 2. Binding of proteins to HAC resin**. Positively charged proteins are electrostatically attracted to phosphate portions of the matrix, while negatively charged proteins covalently bind to the calcium sites.

**Chelating metal ions**

Chelating compounds are covalently linked to solid medium to trap metal ions in what is known as immobilized metal affinity chromatography (IMAC). The principle of IMAC revolves around polar bond formation between exposed amino acid residues and the matrix. The main

mode of interaction is through the immobilized element and exterior histidine residues on a protein, although other amino acids may have some effect. The number of exterior histidines determines the retention of the molecule, and the binding strength depends on the chelating agent (Roque et al., 2007).

Ligand stability, low cost, high protein loading, mild elution conditions and ease in regeneration are all advantages that make IMAC an attractive replacement for affinity separation of immunoglobulins at the industrial scale (Roque et al., 2007). The needs for extensive process optimization to achieve the necessary selectivity, toxic metals leaching into the product, and the addition of a purification step to remove the histidine-tag are disadvantages that IMAC users must overcome (Gaberc-Porekar and Menart, 2001).

In terms of antibody purifications from plant sources, there have been numerous applications of IMAC. Although some applications of IMAC employ the use of $His_6$ tag such as studies for diabodies completed by Vaquero et al. (2002) and Kathuria et al. (2002), antibodies will also bind to IMAC resins through the availability of surface histidines (Roque et al., 2007). IMAC has been used to purify antibodies that lack any sort of tag from plant sources by several groups (McLean et al., 2007; Ramessar et al. 2008). Platis et al. utilized IMAC to purify an antibody from *N. tabacum* to 97.2% purity in a spiked experiment (2008).

**Mixed-mode ligands**

Mixed-mode ligands have a hydrophobic core and are coupled with hydrophilic or ionic groups. Binding occurs when the attached group is uncharged at a neutral pH. Elution occurs when the pH is lowered in either a stepwise or gradient elution. Hydrophobic charge induction chromatography (HCIC) utilizes an ionizable pyridine ring coupled with the hydrophobic effects

of the rest of the resin (Roque et al., 2007). 4-Mercapto-ethyl-pyridine (MEP), shown in Figure 3, has a non-charged structure in neutral conditions (p*K*a 4.8), but becomes positively charged when the pH lowers to 4.8. Antibody adsorption occurs at neutral pH, when the resin is uncharged, and undergoes elution when the pH is reduced to near the pKa. This pH converts the matrix to the ionized form and promotes repulsion forces between the sorbent and the positively charged antibody (Arakawa et al., 2009).

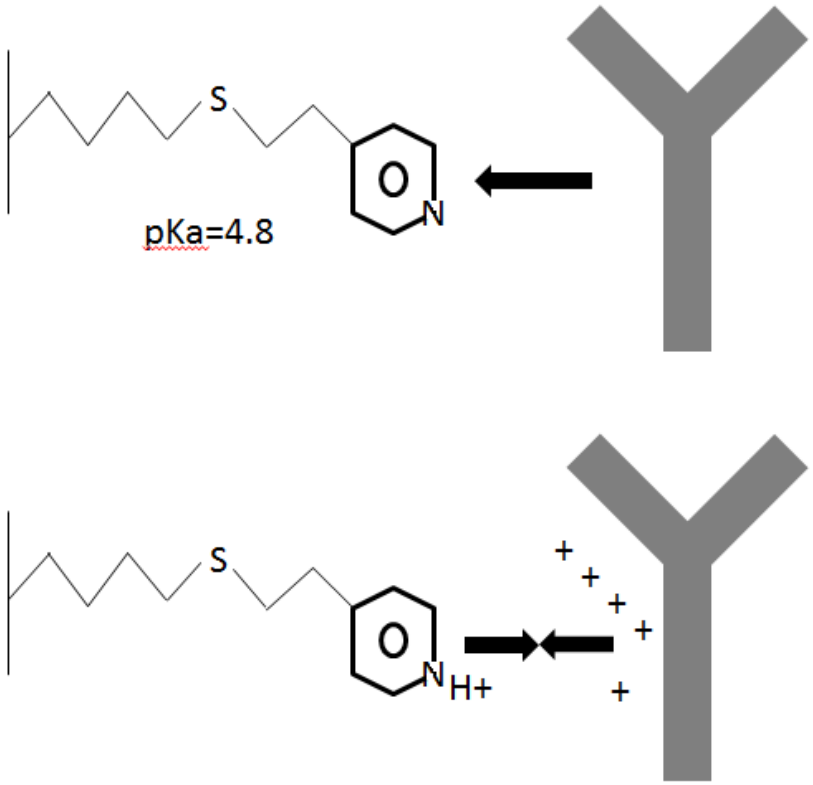


**Figure 3. Mode of operation of MEP HyperCel™.** Binding takes place under neutral pH conditions. Elution occurs by lowering the pH.

In terms of process development, HCIC has proven to be an effective replacement to Protein A chromatography (Roque et al., 2007). Immobilized histamine can also be used. In a study by Platis and Labrou (2008), immobilized histamine was used for the recovery of spiked immunoglobulins from maize extract. A single step salt elution was used, and process

performance was very good. A recovery of 90% and 95% purity from a spiked maize extract was achieved by the researchers.

**Bioengineered and Synthetic Mimic Ligands**

Through advanced engineering techniques, affinity ligands have been developed that have the binding ability of affinity resins, but do not have some of the negative characteristics that plague Protein A chromatography. It can include peptides and protein domains that once coupled to a solid matrix can make powerful affinity resins. Genetic engineering of the IgG binding domain of Protein G (C2) led to higher stability in alkaline conditions (Gülich et al., 2002). Another site directed mutagenesis experiment of the IgG-binding domain of Protein A, domain Z, allowed for elution to occur under more mild conditions (Gülich et al., 2000).

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# Chapter 3
# Materials and Methods

## 3.1 Materials

Non-transgenic *Nicotiana benthamiana* seeds and *Agrobacterium tumefaciens* capable of exogenous IgG expression were graciously provided by Dr. Qiang Chen of Arizona State University. Poly(acrylic) acid was obtained from Polysciences, Inc. (Warrington, PA). Commonly used laboratory reagents, such as ethylenediamine-tetraacetic acid (EDTA), magnesium sulfate, and 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) Solution, as well as polyethylenimine (PEI) were purchased from Sigma (St. Louis, MO). Other laboratory reagents like sodium phosphate monobasic and dibasic and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA, USA). Bio-Rad Protein Assay, Tween-20, 20X Transfer Buffer, chemiluminescent HRP substrate and PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin and 10K MWCO UF/DF devices were purchased from Pierce (Rockford, IL). Kanamycin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Bioworld (Dublin, OH). All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) products including 4-12% Bis-Tris gels, NuPAGE LDS (lithium dodecyl sulfate) sample buffer, 3-(N- morpholino)propane sulfonic acid (MOPS) SDS running buffer (50 mM MOPS, 50 mM Tris, 0.1% SDS, 1 mM EDTA, pH 7.7), SimplyBlue$^{TM}$ SafeStain, and antioxidant were purchased from Invitrogen (Carlsbad, CA). Microcon centrifugal filter devices and ProSep-vA High Capacity were purchased from Millipore (Bedford, MA). All antibodies and HRP substrate were obtained from Bethyl Laboratories (Montgomery, TX). Miracloth was purchased from Calbiochem. Yeast extract and Difco Nutrient Broth were purchased from MOBio Laboratories, Inc. (Carlsbad, CA) and Becton, Dickinson and Company (Franklin Lakes, NJ), respectively. Hydrophobic resins were

purchased from GE Healthcare (Pittsburgh, PA). MEP HyperCel™ was purchased from Pall Life Sciences (Exton, PA). An AKTA explorer with UNICORN software was used for all chromatography experiments and columns were purchased from GE.

### 3.2 *Agrobacterium tumefaciens* Infiltration

Working stocks of *A. tumefaciens,* containing the genetic code to produce 6D8 mAb, an antibody against GP1 in Ebola, were produced using an initial sample kindly sent by Dr. Qiang Chen of Arizona State University. Working stocks were produced by growing cells overnight at 30 °C on LB media with 1x Kanamycin produced from 1000x stocks in water. A colony was collected and cultured overnight again to an OD 1-1.8 in YenB media with the same antibiotics in a New Brunswick Scientific Excella E24 Shaker at 30°C. The culture was then diluted to an OD of 1.0 with YenB media containing antibiotics and diluted at a 1:1 ratio in 80% glycerol for freezing at -80 °C.

For infection, working stocks were placed in 7-10 mL of YenB media at 30°C until an $OD_{600nm}$ of 1-1.5 had been reached. These cells were grown in a larger culture for 16 hours to an $OD_{600nm}$ of 1-1.5. The cells were pelleted and re-suspended in a 10 mM MES, 10 mM $MgSO_4$ buffer at pH 5.5 to an OD of around 0.3. The plants were then infected by vacuum infiltration. Briefly, plants were inverted, and the stem was placed through a slotted plate. The plate was placed flush onto a plastic bowl containing the *A. tumefaciens* in a vacuum desiccator. The vessel was properly sealed and a vacuum was applied. The vessel was then placed under pressure for one minute. Then the vacuum was released, and the plants were placed back into the growth chamber.

### 3.3 Plant Proliferation and Protein Extraction

*N. benthamiana* plants used for experiments were grown in a Percival Scientific Growth Chamber at 25°C in soil. Leaves were removed with a scalpel and were either used immediately or frozen in liquid nitrogen and stored at -80°C. Plants were watered frequently. For infection, the plants were moved to a growth chamber at 25°C for four days between infection and harvest. This time frame was optimized by Dr. Chen (personal comminucation). The effluent from watering *A. tumefaciens* infected plants was collected and devitalized with bleach.

Protein extraction occurred at a ratio of 5:1 of buffer (mL) for every gram of fresh tissue. The buffer consisted of 25 mM sodium phosphate, varying amounts of NaCl, 10 mg/mL sodium ascorbate, 0.1% Triton X-100, 1mM EDTA, 0.3 mg/mL PMSF, 10 µg/mL leupeptin at pH 6.6. Leaf tissue was homogenized in a Fisher Scientific PowerGen 700. Samples were then centrifuged in an Eppendorf 5810R centrifuge at 17,000 X g for 20 minutes. The supernatant was filtered through Mircacloth, while the precipitate was discarded.

### 3.4 Polyelectrolyte Precipitation

For the studies to achieve the optimal conditions, samples were divided into 1 mL after extraction. After protein extraction, immunoglobulin G was added to the extract for initial purification studies using PAA. The extract was titrated to pH 5, incubated on ice for 10 minutes, and centrifuged for 10 minutes at 17,000 x g. Varying amounts of the anionic polyelectrolyte polyacrylic acid were added to the supernatant (0, 1, 5, and 10 mg/mg IgG). The sample was mixed vigorously using a vortex for 10 sec. The precipitation occurred on ice for 30 min. After precipitation, the samples were centrifuged in a Fisher Scientific Marathon 15KM centrifuge for 20 min at 17,000 x g. The supernatant was removed and saved for later analysis. 0.2 mL of extraction buffer with 1.5 M NaCl was used to re-suspend the precipitate and disrupt

the interaction of the polyelectrolyte and antibody. This mixture was centrifuged for 10 min at 17,000 x g. The supernatant was removed and saved for later analysis. The fractions were analyzed using ELISA. The concentration of immunoglobulin in each sample was estimated using the hardware ELISA developed by the CDC.

PEI was used to precipitate native proteins while leaving IgG in the supernatant after centrifugation. In early experiments dosages of 0, 50, 200, 400, 800 mg PEI/ mg TSP were added to the extract. The precipitation experiment occurred as above. The precipitate was re-suspended with the same re-suspension buffer as above. The precipitate required light sonication to re-suspend. This step often required multiple sonication cycles and sometimes did not fully re-suspend the material. The continued sonication may negatively impact recovery. Analysis occurred as above. In later experiments, UF/DF with a 10K MWCO membrane was used. Dosages were significantly reduced in order to be effective. Dosages of PEI after UF/DF were 0.009, 0.09, 0.18, and 0.36 mg PEI/ mg TSP.

### 3.5 Chromatography

Comparison of the two Processes

Two purification schemes were developed and compared. A commonality between the two processes was the product recovery and initial clarification steps. The divergence was in the column chromatography steps. One process involved hydrophobic interaction chromatography followed by hydrophobic charge induction chromatography, and the other included only Protein A chromatography. A process schematic is given in Figure 4.

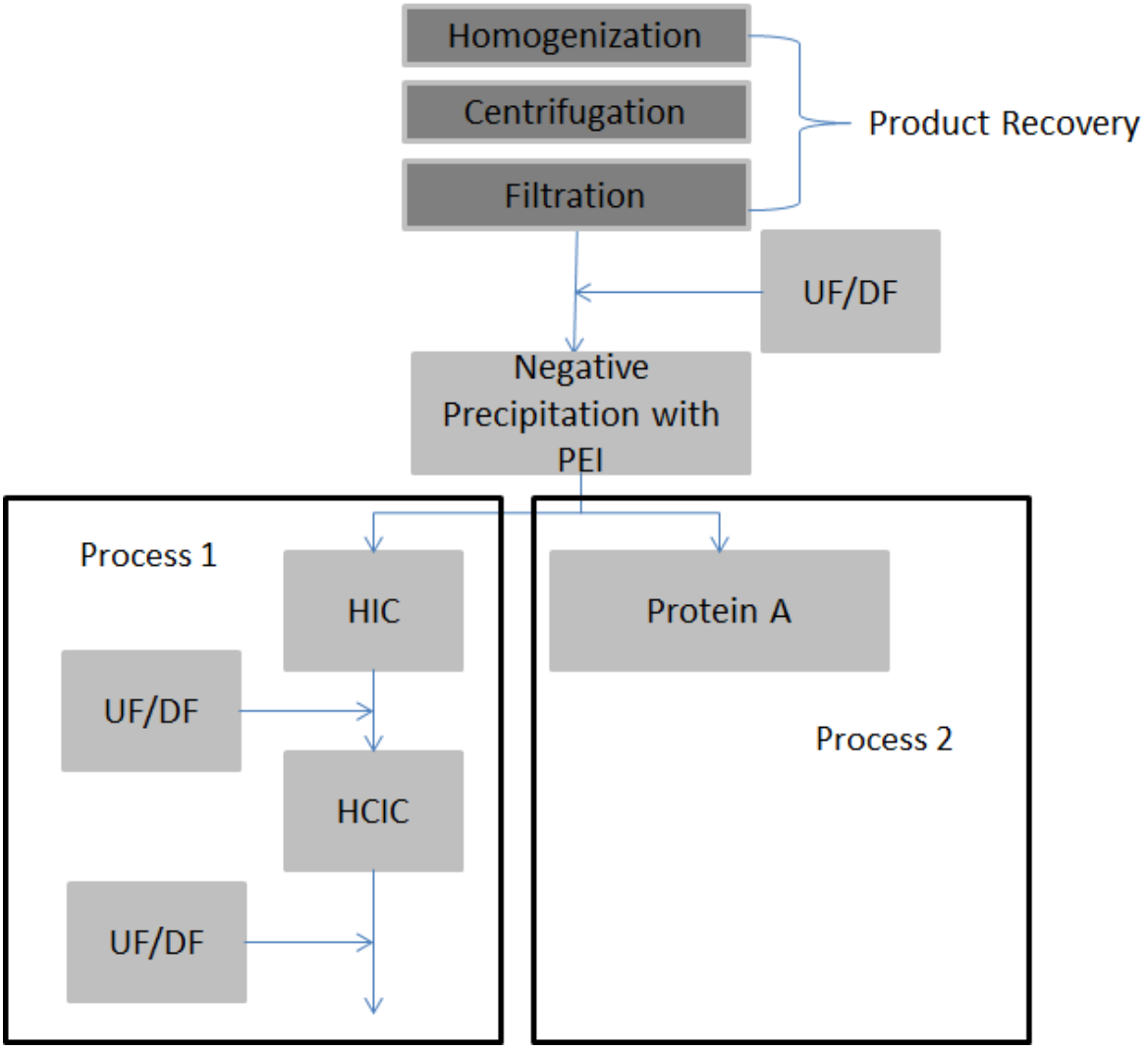



**Figure 4. Process schematic for the two processes used to purify 6D8 mAb from *N. benthamiana* leaves**. The initial steps are paramount to protect the chromatography resins as much as possible since this production platform is prone to fouling resins and leading to short column lifetimes.

HIC

Phenyl sepharose 6 fast flow high substitution was used. The columns were equilibrated with 25% ammonium sulphate in 50 mM sodium phosphate pH 7.0. Flow-rate was constant throughout all chromatography experiments (1 mL/min). An elution gradient of 20 min of 100% buffer A (25% ammonium sulphate in 50 mM sodium phosphate 7.0) to 100% buffer B (50 mM sodium phosphate 7.0) was used to optimize elution. After the linear gradient experiment was performed and analyzed to see which fractions contained antibodies, a wash step using a

combination of the equilibration buffer and 50 mM sodium phosphate pH 7.0 to yield a conductivity of ~80 mS/cm was included. Elution was performed using 50 mM sodium phosphate at pH 7.0. The column was cleaned in place with 1 M NaOH.

HCIC

Hydrophobic charge induction chromatography utilizes a mixed mode sorbent that binds proteins at neutral pH and low salt concentrations and elutes under a descending pH gradient. The proper elution fractions from HIC were pooled and concentrated. The pooled fractions were loaded on a MEP HyperCel$^{TM}$ column equilibrated with 50 mM Tris-HCl pH 8.0. A wash step at pH 5.6 and a gradient elution from 5.6 to 3.6 in 100 mM sodium acetate were applied to elute the proteins. This pH range was reduced due to the ability of the antibody to elute at pH 5.0. Later elution steps occurred at pH 5.0. Neutralization occurred immediately using 1.5 M Tris-HCl pH 7.0. The column was then regenerated using 1 M NaOH.

Protein A

Protein A chromatography was accomplished by treating the extract with PEI after dialysis. The centrifuge supernatant was applied directly to a ProSep-vA High Capacity. The loading buffer was 50 mM PBS with 150 mM NaCl pH 7.4. A wash of 25 mM sodium phosphate with 500 mM NaCl and Tween-20 at pH 5.0 was used. Elution was accomplished using 100 mM glycine at pH 2.5. Neutralization occurred immediately using 1.5 M Tris-HCl pH 7.0.

### 3.6 Analytical Methods

Bio-Rad Protein Assay

Total soluble protein concentration was determined by the Bio-Rad protein assay with bovine serum albumin (BSA) as the standard. All assays were carried out in 96-well clear, flat bottom Greiner microtiter plates and performed in duplicates while following the manufacturer's specifications. Absorbance measurements were read at 595 nm on a Bio-Tek Synergy microplate reader.

Enzyme-linked immunosorbent assay (ELISA)

An ELISA was utilized to determine IgG concentration in the samples. A goat-anti human IgG Fc region was used as a capture antibody, and a HRP-conjugated goat anti-human IgG Kappa Light Chain was used as a detection antibody. Absorbance measurements were performed as above at 450 nm. The protocol outlined by the manufacturer was followed. Briefly, 96-well plates were coated for one hour with capture antibody diluted in 50 mM carbonate-bicarbonate at pH 9.6, washed with 300 µL/well of TBST 4 times, blocked with 200 µL TBS containing 5% BSA for 30 min, washed, coated with 100 µL sample diluted in TBST for 1 hr, again washed, coated with 100 µL HRP diluted to about 1:40,000 in TBST, and washed again. The HRP reaction substrate was added, and the reaction was stopped using filtered 1 M HCl.

SDS-PAGE

Samples were non-reduced or reduced and run on 4-12% Bis-Tris gels with MOPS as the running buffer according to the manufacturer's specifications. Samples were either reduced or non-reduced and denatured for 10 min. at 70 °C. A voltage of 200 V was applied for 50 min. Staining protocols employed SimplyBlue$^{TM}$ SafeStain or the SilverXpress Silver Staining kit.

The gels were scanned with a Bio-Rad ChemiDoc XRS imager and analyzed using Quantity One Software.

Western Blot

Western blotting protocol given by the manufacturer was followed using PVDF membranes treated with methanol. Briefly, after SDS-PAGE the proteins were transferred onto a PVDF membrane at 30 V for 1 hr. Blocking with 5% milk in TBS with 0.1% Tween-20 was completed overnight. The membrane was washed three times with TBST for five minutes, and then an HRP conjugated antibody against either the heavy or light chain (diluted in TBST) was applied to the membrane for 1 hr. After incubation with the detection antibody, a second round of wash steps was performed to remove non-specific binding. Then the HRP substrate was added, and the reaction was allowed to occur for 5 min. Image analysis was completed as above.

# Chapter 4
# Results and Discussion

## 4.1 mAb Expression

The antibody was expressed in *N. benthamiana*. Typical titers achieved were 87.2 ± 25.2 µg/g fresh tissue weight, while total expression of soluble protein after extraction with a 1:5 leaf mass to buffer ratio was 1.94 ± 0.37 mg/mL. The immunoglobulin percentage of total soluble protein was 0.75 ± 0.35. These values were not typical when compared to the results achieved in other laboratories. Other groups have achieved 0.5 mg/g fresh tissue weight (Huang et al., 2009, Giritch et al., 2006). The reasoning behind this variation was that the exact soil components were not used and most likely the plants were not in optimal health to achieve high levels of mAb accumulation.

## 4.2 Polyelectrolyte Precipitation

The majority of the success in the PAA experiment was primarily due to extraction conditions. The low pH extraction was required to promote the interaction between the molecules, and the charged polymer precipitated the majority of the native impurities without disrupting a high percentage of the active immunoglobulin. Also, re-suspending the pellet containing the immunoglobulin proved to be very difficult to the point where bioactivity could not be recovered (or maintained) using the techniques in this paper (data not shown). Since the effectiveness of PAA came solely from isoelectric precipitation, the actual polyelectrolyte-protein complex was difficult to break efficiently, and poor clarification of the extract, PAA precipitation was determined to be a poor choice for initial clarification step. Therefore, negative precipitation was deemed an attractive method due to its ability to not interact with the antibody while still removing the vast majority of native impurities.

In the beginning stages of using negative precipitation with PEI, the dosage was applied immediately to the crude extract. As the dose increased, the amount of TSP in the supernatant decreased due to the sheer number of polyelectrolyte particles available to form complexes with the finite, primarily acidic proteins in tobacco extracts. Although this interaction was extremely effective in removing some of the remaining host proteins (Figure 5), applying the resultant to a chromatography column was disadvantageous, since there was a large portion of host cell material still remaining. One advantage of this procedure was that under all of the dosages explored, the antibody remained in the supernatant.

In terms of mass balance of the TSP, there are some considerations. It was difficult to fully recover all the protein from the re-suspended precipitated material. This difficulty seemed to have been primarily due to the strength of the interaction between the high number of charged polyelectrolytes and the finite amount of host cell protein. At higher dosages, it became harder and harder to re-solubilize the resulting pellet even with multiple sonication operations. Multiple sonication steps could prove detrimental in the recovery of precipitated proteins, due to the shear and heating effects. All of the above could have attributed to the somewhat poor recovery of precipitated proteins.

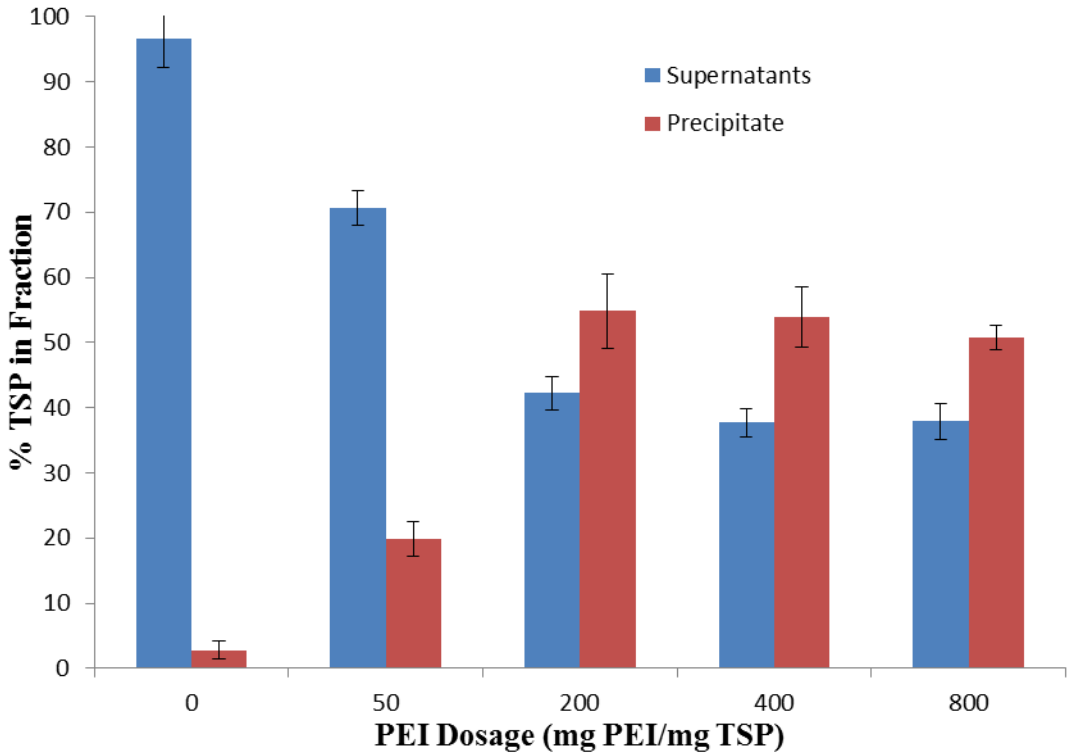
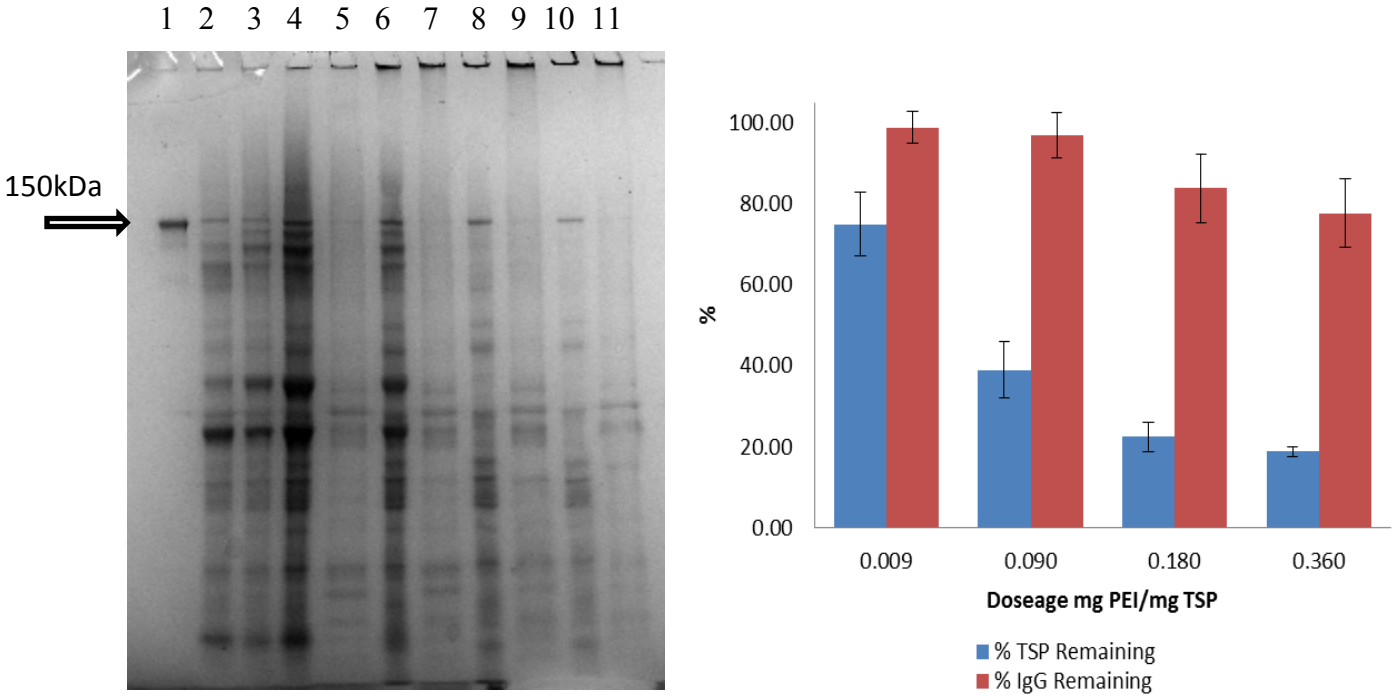



**Figure 5. PEI Dosage and protein in each fraction after centrifugation.** Immunoglobulin remained mostly untouched by this procedure.

The 10K MWCO UF operation that was introduced was effective in several ways. Although the analysis in this paper did not focus on this step, this step removed some of the smaller host cell impurities including small proteins. This conclusion was due to the slight purification factor of immunoglobulin achieved upon its introduction. Also, Holler et al. (2007) noted that different buffer components had different effects on the ability to form PEI-protein complexes. Removing some of the buffer components may also have increased the effectiveness of later PEI steps after UF.

The precipitation experiments became much more effective in two facets. One improvement was that the sample was almost completely clarified. The other advantage was that less polyelectrolyte was required to produce fantastic results, where 80% of the host cell proteins were removed at the highest dosage. One tradeoff was there was a greater chance of also co-

precipitating antibody. The results of these experiments are shown in Figure 6. The decrease in IgG recovery as the dosage increases is primarily due to a higher rate of interaction between the charged polymer and the antibody. Although the antibody has a basic pI, there are still portions that carry negative charge, and connection with the polymer may occur in these environments if the probability of their contact increases due to a higher dosage being administered.



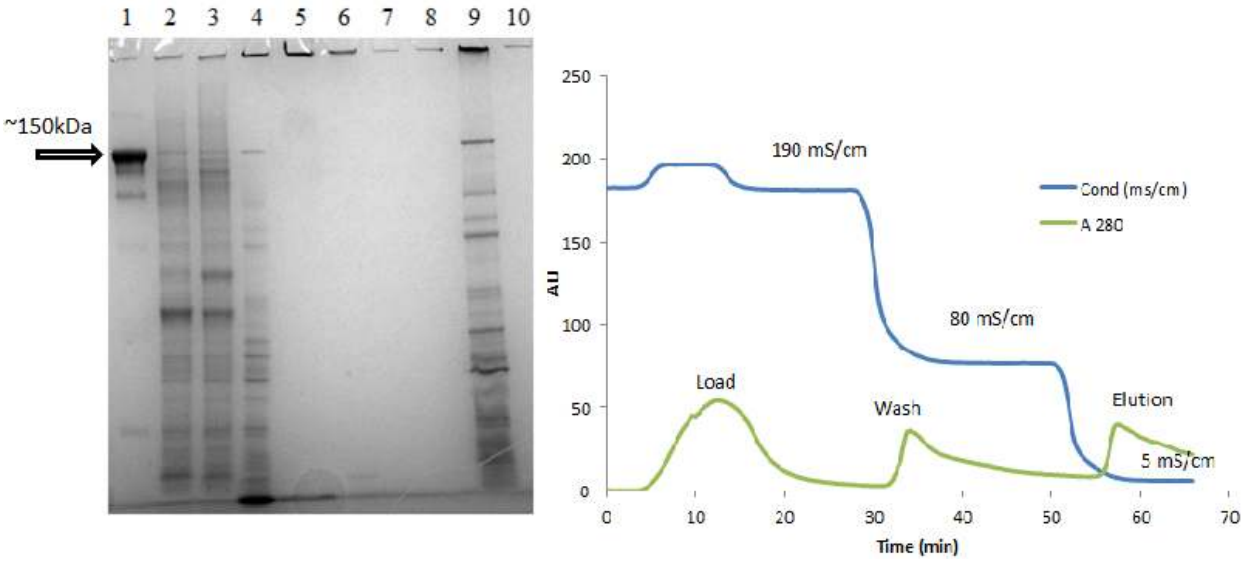

A) B)

**Figure 6. PEI Precipitation after an UF operation**. As dosage increased, the amount of TSP remaining in the supernatant decreased. The antibody is marked by its approximate size of 150 KDa. The dosage that was the most effective in removing the most host cell impurities was 0.36 mg PEI/ mg TSP; however, a tradeoff was that the probability of interacting with an antibody component was increased. This led to some product loss in this step. A) 1-Positive Control Human IgG; 2-Crude Extract; 3-Dialysis Result; 4-0.009 mg PEI/mg TSP Supernatant; 5-0.009 mg PEI/ mg TSP Precipitate; 6- 0.09 mg PEI/mg TSP Supernatant; 7-0.09 mg PEI/ mg TSP Precipitate; 8-0.18 mg PEI/ mg TSP Supernatant; 9-0.18 mg PEI/ mg TSP Precipitate; 10-0.36 mg PEI/ mg TSP Supernatant; 11-0.36 mg PEI/ mg TSP Precipitate B) Immunoglobulin remaining percentage and total soluble protein in supernatant. The immunoglobulin began to become affected by the higher dose and would get targeted by the polyelectrolyte.

## 4.3 Chromatography

### HIC

After polyelectrolyte precipitation was optimized to the point where applying the sample onto a column could be completed, the HIC step was optimized. A typical HIC elution profile is shown in Figure 6. The wash step removed impurities due most likely to their slightly hydrophobic nature. The remaining protein (including the majority of the immunoglobulin) eluted at low (<10 mS/cm) ionic strengths. Recovery percentages for this step were 71.9 ± 6.3. The resulting fractions for the HIC step are shown in Figure 6A. A typical chromatogram profile is given in Figure 6B. After concentration a fraction was taken for analysis, and the rest of the sample was directly applied to a MEP HyperCel™ resin.



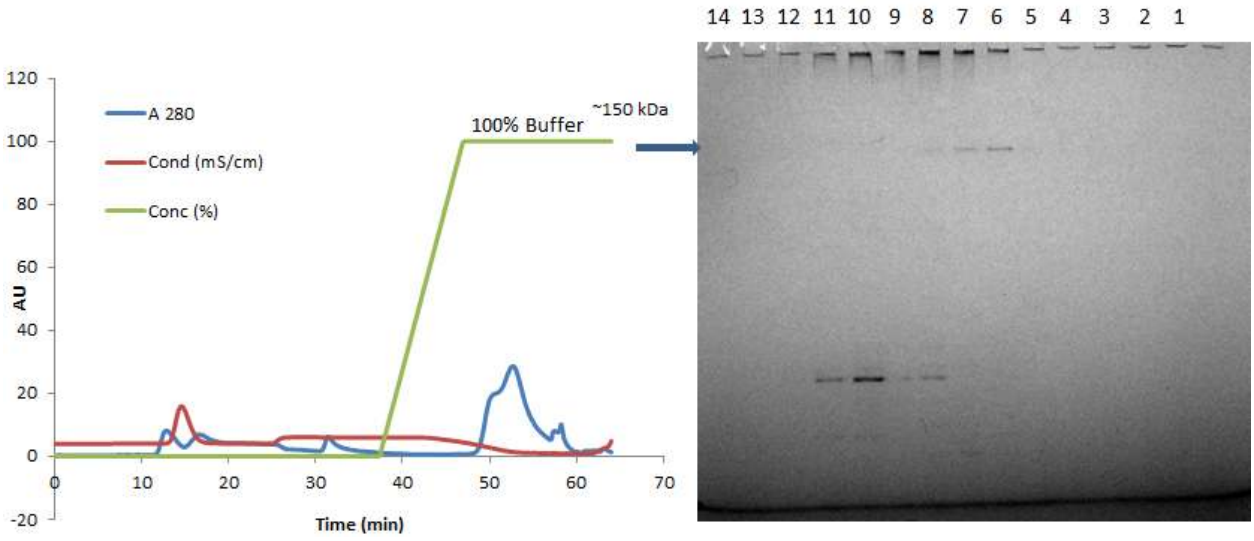

**A)**

**B)**

**Figure 7. HIC elution.** The antibody is marked by its approximate size of 150 KDa **A)** 1-Positive Control; 2- Initial extract; 3-Initial Dialysis; 4-PEI Result at 0.36 mg PEI/ mg TSP Dosage; 5-HIC Flow-through; 6-HIC Wash Step; 9-HIC Elution. The precipitation process clarifies the extract to the point where it can be successfully loaded onto a Phenyl Sepharose Fast Flow resin. The amount of impurities after this step is still high, most likely due to the hydrophobic nature of the remaining host cell proteins B) Typical Chromatogram for HIC step. The intermediate wash of 80 mS/cm is instrumental in preparing a somewhat more purified sample.

**HCIC**

Process optimization in this section was very important. Due to the large pH range given by the resin's manufacture, a broad pH array was chosen originally for the elution after the intermediate pH wash (in this case 100 mM sodium acetate at pH 5.6). The large range 5.6-3.6 was too broad and occurred too quickly but did show that the antibody was able to be separated from a large portion of the native impurities. Figure 7 shows this separation.




A) B)

**Figure 8. MEP gradient elution pH 5.6 to 3.6.** The antibody is marked by its approximate size of 150 KDa. A) Chromatogram for MEP HyperCel$^{TM}$ 1 mL Fractions were collected and neutralized. Going from right to left, the fractions were collected in order. The separation of antibody and a native impurity can be readily seen.

After the discovery that the majority of the antibody that was able to be eluted from the column at pH 5, a wash step of pH 5.6 was included, and then an elution step was applied at pH 5.0. A typical elution profile using this method is shown in Figure 9. The average percent recovery of immunoglobulin in this pure fraction was 35.5 ± 11.6. Some antibody remained bound to the column even after a subsequent wash of lower pH including pH 4.4.

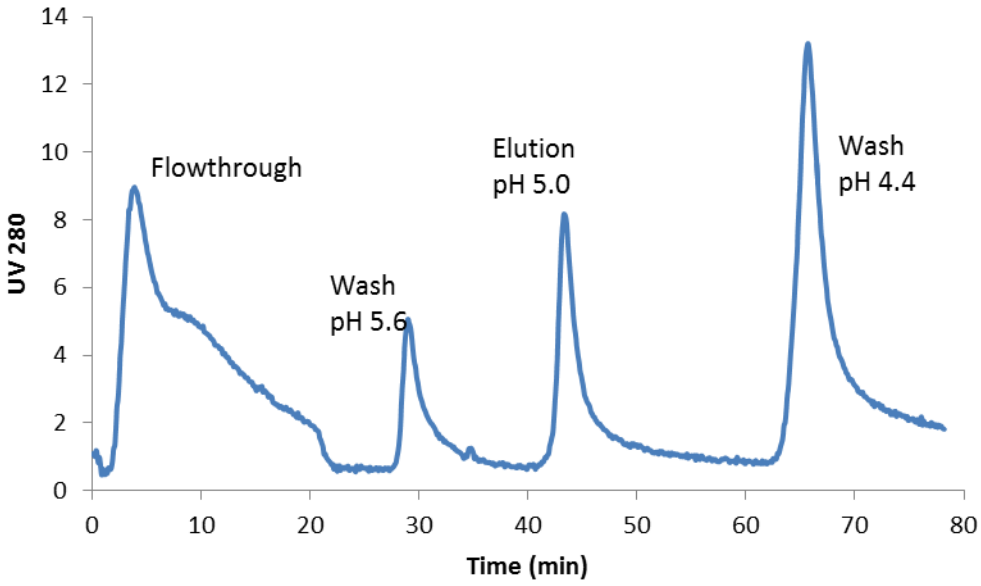



**Figure 9. Typical chromatogram for HCIC step**. The wash steps removed a large portion of the host cell proteins and mostly antibody eluted at pH 5.0

**Protein A Process**

After the sample was treated with PEI and solid-liquid phase separation was completed, the supernatant was directly applied to a Protein A column. The Protein A process included a flow-through step, a wash step at 5.0 with 0.5 M NaCl and 0.1% Tween-20 to attempt to remove host cell impurities, and an elution step at pH 2.5. A chromatogram for this process can be seen in Figure 10. The yield of the Protein A process as a single operation was around 85%.

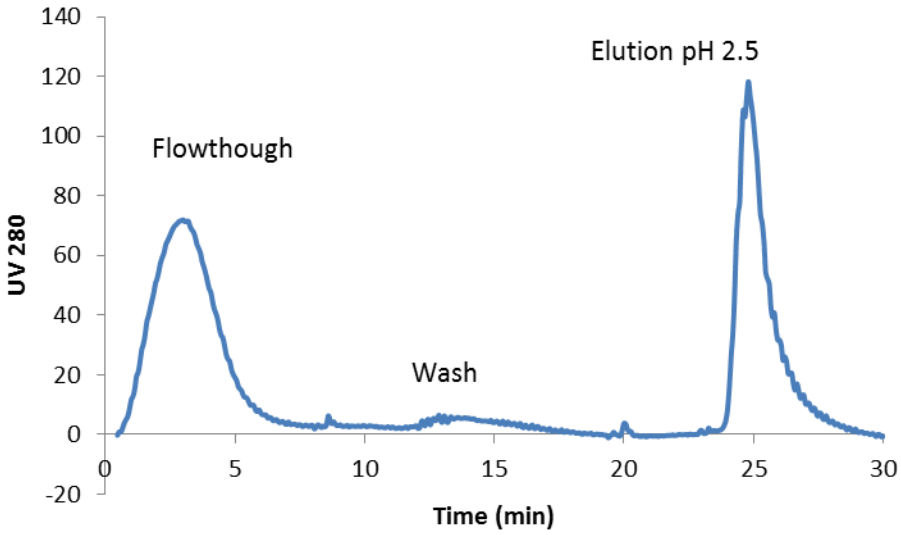



**Figure 10. Protein A purification of 6D8 mAb from native proteins.** The selectivity of Protein A is apparent; however, the wash step was ineffective in removing some of the native host impurities that non-specifically bound to the sorbent.

### 4.4 Process Summary and Comparison with Protein A

The ultimate goal of this research is to replace Protein A chromatography in the process of antibody purification from plants. In Table 2, the performance of the modified process is listed. Overall process recovery was around 19%. One issue in the protein loss during the process was the poor recovery during the MEP HyperCel™ step. The loss was theorized to have been for several reasons. The production of therapeutics from un-modified plants has been shown to produce heterogeneous proteins of different glycosylations and other PTMs (Cox et al., 2006). Variations between these molecules, in term of size, charge, etc. may be responsible for the poor elution recoveries. Different forms of the protein that were still properly assembled enough to where an ELISA would detect them may elute at different times on the HCIC step.

**Table 2. Overall process results.** The overall recovery was 19%. A large portion of the loss was due to the MEP HyperCel™ step, typical recovery for the ProA step was in the around 85%.

| Separation Process | Recovery % | Purification factor |
|---|---|---|
| Ultrafiltration (10K MWCO) | 95.80 | 1.16 |
| PEI Result (0.36 mg PEI/mg TSP) | 77.58 | 3.20 |
| HIC | 71.87 | 2.30 |
| MEP HyperCel™ | 35.53 | NA |

This process was compared to a Protein A resin. The entire modified process gel was shown is shown in Figure 11A. A major impurity was removed from the immunoglobulin in the HCIC wash step. The protein was estimated as pure by SDS-PAGE for Figure 11A. Although in order for this molecule to be administered to any living creature, significant analysis and polishing would be required. A process using Protein A after PEI precipitation is shown in Figure 11B. Typical recovery for the Protein A step was 85%; which is obviously much higher than the other process.

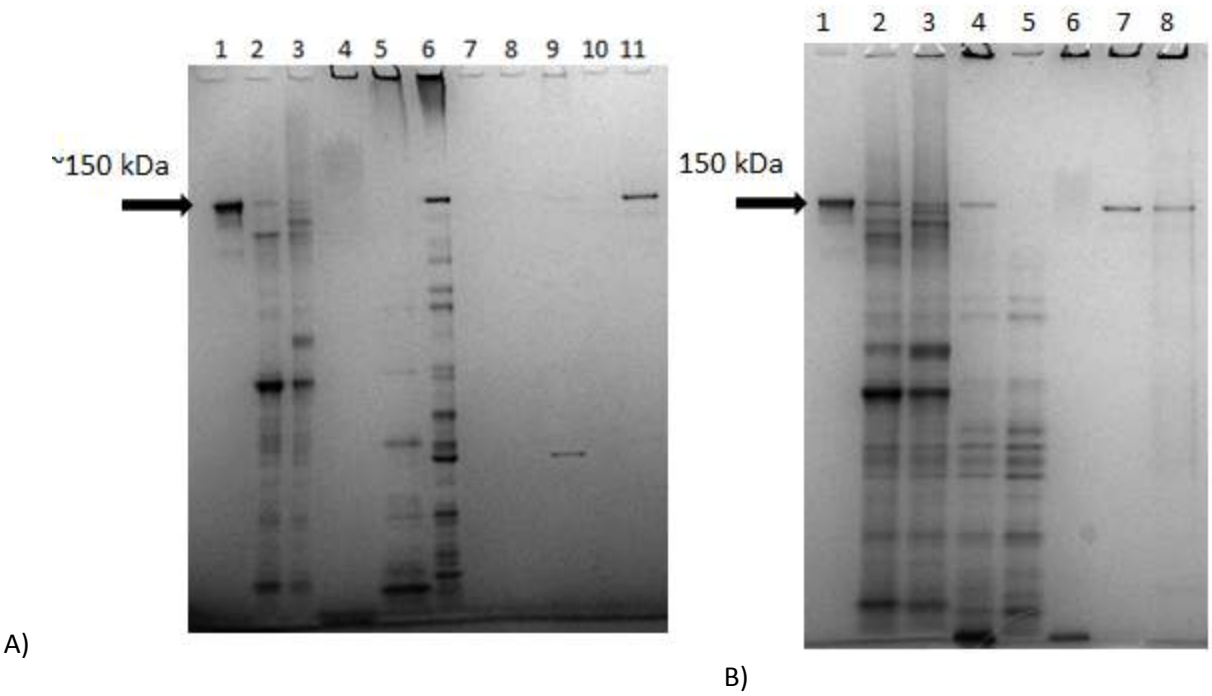



**Figure 11. Entire modified process gel (A) vs Protein A process (B)**. The antibody is marked by its approximate size of 150 KDa. A) Entire Process Gel 1-Positive Control; 2- Initial extract; 3-Initial Dialysis; 4-HIC Flo; 5-HIC Wash; 6-HIC Elution; 7-MEP Flo; 8-MEP Wash pH 5.6; 9-MEP Wash pH 4.4; 11-MEP Elution; 13-MEP Elution (reduced); 15- Postive control (reduced) B) 1-Positive Control; 2- Initial extract; 3-Initial Dialysis; 4-PEI Dosed Supernatant; 5-Pro A Flo; 6-Pro A Wash pH 5.0; 7- Pro A Wash 2 pH 4.0; 8-Pro A Elution; 11-Protein A Reduced

The proteins from both sources appear to be quite pure; however a Coomassie stain is not sensitive enough to declare the proteins as pure, so a silver stain of both processes appears below in Figure 12A. The non-specific binding of host cell impurities onto Protein A is apparent on this gel. Some impurities remain in the MEP HyperCel™ elution fraction, but western blots have indicated in the past that these impurities may be product related, not host cell related (data not shown). Also, some of the bands in the positive control are similar to those in the MEP HyperCel™ elution fraction, and those same bands do not appear in a gel where the modified process was applied to plants that were non-transgenic and did not express any antibody. The silver stained gel depicting the native proteins remaining after non-transgenic plants were subjected to the modified process is shown in Figure 12B.

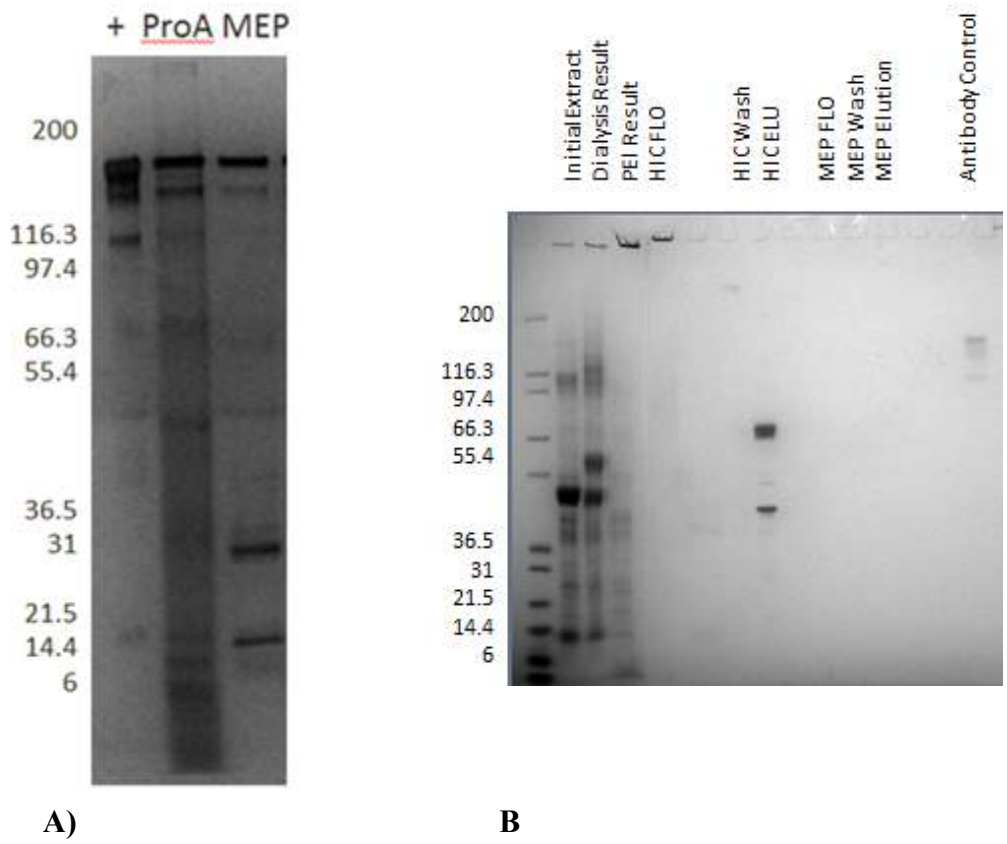



**Figure 12. Silver stain of Protein A elution fraction versus MEP HyperCel™ elution fraction.** A) Silver stained comparison of Protein A and MEP HyperCel™ elution fractions. The MEP fraction retains less impurities, and the remaining impurities may be product related due to their absence in a silver stained gel depicting the modified process using non-infected plants (B).

The Protein A sample was not clear and still contained some native, green plant material. In reality, another step would be required before and after Protein A to help protect the column, purify the antibody, and polish the product. The specificity of Protein A to the Fc region gives that particular resin unparalleled affinity towards immunoglobulins, which is why the technique has been so robust considering how long researchers have been attempting to replace it. It is not immune to non-specific interactions, and when dealing with this production platform, those interactions can negatively impact the column.

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# Chapter 5
# Conclusion

Monoclonal antibody expression in *N. benthamiana* represents a unique production system that may in the future have the ability to become a production host at the industrial level. The high titers achieved by some research groups increase its attractiveness. One of the major issues of this technology would be purification of a therapeutic. Protein A is used as a capture step when using mammalian cell culture to produce mAbs; however, directly applying a crude plant extract onto a Protein A resin results in significant fouling. There is a need to apply at least one clarification step prior to loading the sample onto a Protein A column. There are advantages to removing Protein A from the purification scheme entirely by replacing it with similarly performing resins due to some past issues involved with cost, ligand leaching into product, and non-robust CIP procedures.

Initial clarification steps must be used to remove the majority of host cell impurities to guarantee column robustness no matter what resin is used in the purification process. Polyelectrolyte precipitation combined with UF/DF shows an excellent ability to both clarify and purify crude plant extracts. Hydrophobic interaction chromatography followed by MEP HyperCel™ shows a good ability to purify an antibody against GP1 in Ebola virus to near homogeneous levels; however, the process yield was poor at 19%. Further optimization may allow for improved yield. Protein A, the industry standard, performed quite well with a recovery of 85%; however, the sample was not as clean as the other scheme yielded. Attempts to wash the impurities from Protein A with high salt and high detergents proved to be ineffective.

The modified process has several advantages over the Protein A process. The resins for both steps were use and re-used a multitude of times after the CIP steps proved to be extremely

effective. Also, the final product from the modified was more pure of native materials than the Protein A process. A single polishing step may be all that is required to fully purify the target protein.

In order to introduce this process at an industrial level there are some metaphorical mountains to climb. PEI at an industrial level may be unfeasible due to the limited size of centrifuge apparatuses; however, continuous centrifugation may be effective. An anion exchange step could be used in place of PEI to remove native impurities if the resin can be recycled, although the limited throughput could be an issue. The other resins used in this study were regenerated easily and proof of consistent performance could make this procedure applicable at the industrial level.

In conclusion, process development for purifying an immunoglobulin from a plant source is difficult. Every step will provide unique challenges that are not present in other production platforms. The need to develop high-throughput unit operations for the high amounts of biomass required is very important. Some processes that work with other organisms may not be as effective with plant tissue. Developing new purification strategies will be necessary to make the technology's wide-spread acceptance at the industrial level possible. In the realm of therapeutic protein production, a production platform has almost always met challenges before becoming accepted, and protein production in plants is no different. Reducing the cost wherever possible can only help to make the technology more attractive.

# Chapter 6
# Future Work

In order to use the antibody produced and purified in this study to actually be injected into an animal (and later to a human) a multitude of tasks must be accomplished. Polishing steps must be added to remove aggregates and remaining impurities. An UF/DF can be used to remove a majority of the remaining impurities. Using a higher MWCO in the very first UF/DF step (> 50 KDa but less than 100 kDa) could help to remove some of these materials. After this, further optimization of the process steps could yield better target protein recovery. Specifically optimizing the HCIC step by preparing a shallower gradient could help to yield better resolution from the native and product-related impurities. A HPLC analysis step must be used to determine if the purified antibody is indeed pure and demonstrates homogeneous structure. Then purified antibody must demonstrate *in vitro* ability to bind the antigen. Then toxic studies in animals may be conducted. Also, drug metabolism and pharmacology should be determined.