Purification of an acidic recombinant protein from transgenic tobacco

By

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Tobacco has been studied as a host for producing recombinant therapeutic proteins on a large-scale, commercial basis. However, the proteins expressed in tobacco usually need to be purified to high yield and purity from large amounts of biomass in order for their production to be commercially viable. The methods needed to purify proteins from tobacco are very challenging and not well studied. The objective of this research was to develop a process for the purification of the acidic model protein, recombinant β-glucuronidase (rGUS), from transgenic tobacco leaf tissue to high yield and purity.

Polyelectrolyte precipitation with polyethyleneimine (PEI) was identified as an initial purification step for purifying acidic recombinant proteins from tobacco. Polyethyleneimine precipitation allowed for high recovery and concentration of the target protein while removing large amounts of impurities from the initial extract. At dosages of 700 – 800 mg PEI/g total protein, nearly 100% of the rGUS activity was precipitated with generally more than 90% recovered from the pellet. In addition, more than 60% of the native tobacco proteins were removed in the process, resulting in a purification factor near 4.

Recombinant GUS was further purified by a step of hydrophobic interaction chromatography (HIC) followed by a step of hydroxyapatite chromatography (HAC). The HIC step served to remove PEI and other contaminants such as nucleic acids that were accumulated during the precipitation step, while the HAC step served to separate rGUS from the remaining native tobacco proteins, most notably ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). Nearly 40% of the initial rGUS activity was recovered as a near homogeneous fraction based on SDS-PAGE analysis after the three step process.

The main steps used in this process are anticipated to be scalable and do not rely on affinity separations, making the process potentially applicable to a wide variety of acidic recombinant proteins expressed in tobacco as well as other leafy crops.

Dedication

This thesis is dedicated to my wife, Emily Jarrett Holler. Thank you for all of your encouragement, support, and love during the pursuit of my academic studies. I am blessed to have by my side and forever yours.

Attribution

Christopher J. Holler is the major contributor and writer of the manuscripts in Chapter Three and Chapter Four of this thesis. Co-author Dr. Chenming Zhang, Ph.D., Chemical Engineering, Iowa State University, Iowa 1999, Committee Chair provided advice, supervision, funding, and laboratory support. Co-author Prof. David H. Vaughan, Ph.D., North Carolina State University, North Carolina, 1974 provided funding.

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Chapter One

Introduction and Objectives

Transgenic plants are being widely studied for the production of recombinant therapeutic proteins on a commercial scale. Plants offer many advantages over traditional microbial or transgenic animal systems and tobacco, in particular, shows great promise for producing biopharmaceuticals. While expression of valuable recombinant proteins in tobacco has been widely demonstrated, the purification processes needed to obtain large quantities of a highly pure product have not. In this work, the purification of an acidic model protein, recombinant β-glucuronidase (rGUS), from tobacco leaves was investigated to show the feasibility of producing acidic recombinant proteins in transgenic tobacco. All of the separation steps were chosen so that the overall process may be 1.) scalable for commercial production and 2.) applied to a wide variety of acidic recombinant proteins expressed in tobacco and other leafy crops.

The first objective was to investigate a non-chromatographic method as an initial step in the purification process. Fractionation and concentration of the target protein from large amounts of tobacco extract is desirable for reducing the amount of material to be further processed by chromatographic methods. Polyelectrolytes are charged polymers that bind to oppositely charged molecules in a solution. Polyethyleneimine (PEI) is a positively charged polymer at pH 7.0, and therefore was chosen to recover and partially purify rGUS from transgenic tobacco extracts. The specific questions to be answered are:

1. What does the precipitation profile look like for rGUS and native tobacco proteins with varying dosages of PEI?
2. Will the precipitated material be successfully recovered (redissolved / resuspended) after precipitation?
3. Will rGUS retain its activity after precipitation with PEI?
4. How much rGUS can be recovered and at what enrichment (purification)?
5. Will the PEI precipitation step be comparable or even advantageous to anion-exchange chromatography as a first step in the purification process?
6. Will the PEI precipitation step serve as an effective method in preparation for a subsequent chromatographic step in the purification process?

The second objective of this work was to investigate a general and potentially scalable process for the purification of rGUS from transgenic tobacco leaves to high yield and purity. The specific questions to be answered are:

1. Can rGUS be purified from transgenic tobacco using general (non-affinity) chromatographic methods following PEI precipitation?
2. What types of general chromatographic methods will be needed to purify rGUS and thus acidic proteins from transgenic tobacco?
3. Where in the process should the different protein separation techniques be incorporated?
4. What will be the final recovery and purity of rGUS after all purification steps?

Thesis Organization

This thesis contains five main chapters. Following the introduction in Chapter One, Chapter Two discusses the advantages and challenges of using plant systems, especially tobacco, for expressing recombinant proteins. This chapter discusses the general considerations for protein purification from tobacco and specific examples from the literature were briefly reviewed. The use of PEI precipitation and several chromatographic methods in purification studies was reviewed in detail as well. Finally, examples from the literature were reviewed pertaining to protein purification methods from plant systems using β-glucuronidase as the model protein.

Chapters Three and Four present the experimental work completed to address the objectives discussed above; and they are presented in journal format. The first paper (Chapter Three) focuses on PEI precipitation of rGUS and its feasibility for use as an initial step in the purification of acidic recombinant proteins from tobacco. The paper also compares the PEI precipitation step with anion-exchange chromatography in terms of rGUS recovery, purification, and preparation for hydrophobic interaction chromatography as the second step. The second paper (Chapter Four) builds on the results obtained from the first paper and presents a general purification scheme for purifying rGUS from transgenic tobacco to high yield and purity. Chapter Five presents the general conclusions for all experimental works.

Several appendices are included at the end of the thesis. Appendix A describes the activity assay used for the quantification of rGUS activity. Appendix B presents additional results on important experimental conditions encountered throughout the study. Appendix C and D present results obtained from size-exclusion chromatography and additional hydrophobic interaction chromatography experiments, respectively, which were attempted during the study. Appendix E presents additional data using hydroxyapatite chromatography in which tobacco extract was loaded directly to the column with no initial purification steps. Finally, Appendix F presents all of the methods created and used for the individual chromatography experiments completed during this study.

Chapter Two

Literature Review

2.1 Recombinant protein production in plants

Recombinant proteins have been expressed in plants for more than twenty years now. Initially, most studies focused on developing favorable agronomic traits such as insect resistance [1] and disease control [2, 3]. Within the past decade, however, plants have emerged as a serious candidate for expressing valuable therapeutic-based recombinant proteins on a commercial scale, vying for a share of the multi-billion dollar biotech industry. Several plant-derived diagnostic and research proteins have already reached the market [4, 5] and numerous other plant-derived biopharmaceuticals are in various stages of clinical trials [6, 7]. Antibodies, vaccines, growth regulators, human serum proteins, and hormones have all been efficiently expressed in a variety of plants [1, 8-11]. Crops such as cereals (maize, rice), legumes (soybean, pea), fruits and vegetables (potato, tomato, banana), and leafy plants (tobacco, alfalfa, lettuce) have all been among the target hosts for recombinant protein production. Each type of plant has its own unique advantages and disadvantages when it comes to the feasibility for production of recombinant proteins [6, 12].

In general, plants may be comparable or even advantageous to traditional systems, such as bacterial or mammalian cell cultures or transgenic animals, for the production of recombinant therapeutic proteins. Plant systems may prove to be safer, more economical, and more convenient than these traditional systems [13-16]. Plants do not harbor human pathogens; therefore, the threat of transmissible disease is eliminated. Furthermore, production of heterologous proteins in transgenic animals or mammalian cells raises safety and ethical concerns [12]. Whereas microbial systems cannot produce accurate post-translational modifications of eukaryotic proteins [9, 17], plants have the ability to produce functional proteins of eukaryotic origin, including human proteins [18, 19]. Production of recombinant proteins in traditional systems is still expensive and difficult to scale up; however, the framework for cultivating, harvesting, and processing large amounts of plant material is already established and well understood, which would help lower production cost [19]. Several studies have been done to assess the cost to produce recombinant proteins from plants compared with traditional

expression systems. It is estimated that production of recombinant proteins in plants may be 2-10% of the cost for the same protein production in microbial systems (i.e. *E. coli* fermentation) and 0.1% of the cost of mammalian cell cultures depending on the type of plant and expression level [9, 18].

While plants have many advantages for recombinant protein production, there are also limitations and concerns. Many of these concerns are associated with safety when producing biopharmaceuticals in plants. Contamination of a food or feed supply or release of recombinant protein products into the environment is a serious threat that must be closely regulated [1]. Furthermore, even though the protein synthesis pathways are largely conserved between plants and animals, some proteins may not be correctly processed [1]. Some leafy plant species, like alfalfa, can produce homogeneous glycan chains for glycoproteins; whereas tobacco produces heterogeneous glycan structures [12, 20]. Improper post-translational modifications of biopharmaceuticals can lead to immunogenic or toxic effects in humans [6, 9, 21]. Therefore, the expression and production of recombinant biopharmaceutical proteins in plants must be closely monitored by regulatory and safety standards, which are rapidly accumulating [6, 22, 23]

Another challenge for recombinant protein production in plants is low protein expression and accumulation, largely due to poor protein stability. Expression level and stability will ultimately dictate whether it is economically feasible to use a particular plant species for the production of a recombinant protein. Although recombinant protein expression levels vary considerably from plant to plant, typical expression levels of 0.01% to 0.1% total soluble protein (TSP) are commonly reported for biopharmaceutical proteins [17].

A final hurdle, and one of the most difficult to overcome, is the downstream processing (protein extraction and purification) needed to obtain large quantities of a highly pure protein. It is estimated that downstream processing may account for over 80% of the total cost associated with recombinant protein production in plants when a highly pure protein is needed [1, 24]. To circumvent this difficulty, many efforts have been devoted to the production of edible vaccines in corn, tomato, potato, banana and other food crops [9, 17]. Delivering the target protein orally avoids large expenses from downstream processing; however, this is not possible for many recombinant proteins or in some plant species, such as tobacco. Finally, there is a significant lack of published data regarding general purification processes that may have potential for commercial scale application and that do not rely on affinity steps. Therefore, new and

improved downstream processes must be investigated for the purification of recombinant proteins expressed in plant systems such as tobacco and other non-edible plants.

2.2 Tobacco as an expression host

Production and use of tobacco (*Nicotiana tabacum*), a major crop of the Southeastern United States, is on the decline due to the negative health issues associated with tobacco use as well as increasing federal regulations [25]. Therefore, alternative uses for this crop are being explored. Tobacco has long been used as a model system for the production of recombinant proteins and shows promise as a host for large-scale protein production [6, 8, 10, 20, 26]. Transformation procedures for the expression of foreign proteins in tobacco are well developed and accomplished by methods such as *Agrobacterium tumefaciens* mediated DNA-transfer [27] and plant viral vectors [28] such as tobacco mosaic virus (TMV). Tobacco is considered a relatively safe crop for recombinant protein production. Like all plants, tobacco does not harbor human pathogens, eliminating the threat of transmissible diseases. It is also a non-food and non-feed crop, thus the threat of contamination of a food or feed supply is minimized [1]. Recombinant protein expression in tobacco is usually directed to the leaf tissue, which translates to an abundance of transgenic biomass when plant production is increased. Standard farming practices for tobacco production are already well established and tobacco can be harvested several times a year [20]. Finally, recent advancements have allowed for targeted protein expression to the chloroplast, resulting in higher protein yields and stability [12, 29]. Numerous valuable recombinant therapeutic proteins have already been expressed in tobacco (Table 1) and many are undergoing clinical trials; however, none have been approved by the U.S. Food and Drug Administration [6]. Two biotech companies that are actively pursuing tobacco as a host system are Planet Biotechnology, Inc. and Meristem Therapeutics. As of 2004, these two companies are the only companies to have plant-derived pharmaceuticals in phase-II clinical trials [12].

While the expression of recombinant proteins in tobacco is well-studied, there are still many obstacles to overcome before tobacco can be utilized for large-scale production of therapeutic proteins. Protein expression in many crops, such as corn and canola, is often targeted to the seed which minimizes interactions with native plant components and also serves as a stable storage and transportation vessel [30-32]. However, tobacco seeds are extremely small

Table 1. Therapeutic recombinant proteins expressed in tobacco. Adapted from [9].

Protein	**Potential application/indication**	**Source**
Human protein C (serum protease)	Protein C pathway	[33]
Human hirudin variant 2	Indirect thrombin inhibitor	[34, 35]
Human granulocyte-macrophage colony-stimulating factor	Neutropenia	[36, 37]
Human erythropoietin	Anemia	[36, 37]
Human epidermal growth factor	Wound repair/control of cell proliferation	[36, 37]
Human serum albumin	Liver cirrhosis	[36-38]
Human hemoglobin	Blood substitute	[39]
Human homotrimeric collagen I	Collagen	[40]
Angiotensin-I-converting enzyme	Hypertension	[41]
Glucocerbrosidase	Gaucher's disease	[33, 42]
Guy's 13 (sIgA)	Dental caries; streptococcal antigen I or II	[43, 44]
T84.66 (IgG)	Cancer treatment; carcinoembyonic antigen	[45]
Recombinant HBsAG	Hepatitis B	[46, 47]
E. coli heat labile entertoxin LT-B	Cholera and E. coli diarrhea	[46, 48]
coat protein of Norwalk virus	Norwalk virus	[49]
Rabies virus glycoprotein	Rabies	[50]
HIV epitope (gp 120)	HIV	[51, 52]
Malarial B-cell epitope	Malaria	[46, 53]
Hemagglutinin	Influenza	[54]
c-Myc	Cancer	[54]

and would not be economical for the expression and production of recombinant proteins on a large scale. Therefore, protein expression in tobacco is targeted to the leaves due to the ability for production of large amounts of biomass and ease of scale-up [6].

Unfortunately, expressing recombinant proteins in vegetative tissue creates challenging issues when it comes to the recovery and purification of the target protein. Tobacco leaves contain an extremely high amount of native phenolic compounds, up to 30 mg/g dry weight, and toxic alkaloids, such as nicotine, that must be removed during the purification process [55, 56]. When the leaf material is processed by grinding or shearing, these compounds are released and can interfere with downstream processing by forming complexes with proteins in an aqueous extract [1, 57, 58] or by fouling resin during adsorption processes such as chromatography [6]. However, there are now some low-alkaloid cultivars that may be used for biopharmaceutical production [1]. Watery leaf tissue will also contain higher levels of proteolytic and microbial activity relative to seed tissue, making the environment much less stable during the initial harvest and extraction of the target protein [56]. Due to these components, fresh leaves should be processed immediately. The leaves can also be dried, freeze-dried, or frozen immediately after harvest, which may provide extended storage for some proteins [1, 59].

In addition to phenolics, alkaloids, and proteases, a tobacco extract contains numerous other plant compounds such as carbohydrates, nucleic acids, and native proteins that must be separated from the protein of interest. Tobacco proteins can be categorized into Fraction 1 or Fraction 2 proteins based on electrophoresis. Fraction 1 consists mainly of the photosynthetic chloroplast enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), which can account for up to 50% of the total soluble leaf protein (25% total leaf protein) and is found in nearly all leaf tissues [60]. Its polymeric molecular weight is approximately 560 kD and consists of eight large subunits and eight small subunits of 55 kD (pI 6.0) and 12.5 kD (pI 5.3), respectively [61]. Fraction 1 protein may be used as a nutritional protein similar to that of human or bovine milk proteins and have been shown to be superior to soybean proteins [62]. Fraction 2 is a mixture of soluble proteins and peptides extracted from both the chloroplast and cytoplasm, generally having molecular weights ranging from 3 kD to 100 kD, but which are not well-characterized [60]. It has been reported that tobacco proteins (F1 and F2) are overall acidic in nature and therefore extraction of a basic protein under acidic conditions would result in a lower purification burden [6, 63].

2.3 Downstream processing

2.3.1 General considerations

A major burden in the downstream processing of recombinant proteins from plants is the recovery and stability of the target protein in a plant extract. For tobacco, the leaves are usually ground up or sheared and the proteins are released, or extracted, into an aqueous buffer. Equipment for tissue disruption includes blenders, ball-bead mills, and homogenizers.

Factors such as pH, salt concentration, and temperature of the extraction buffer will affect the amount of protein extracted and also protein stability. Furthermore, steps should be taken to minimize the effects of phenolics, protein oxidation, and proteases during the extraction process. Free phenolics may bind to proteins in a solution and oxidative 'tanning' of proteins can cause structural modification of the protein, both often resulting in loss of activity or degradation. The addition of antioxidants (ascorbic acid), reducing agents (β-2-mercaptoethanol, BME; dithiothreitol, DTT), and phenolic-binding agents (polyvinylpolypyrrolidone, PVPP) to the extraction buffer may alleviate some of these interferences [64, 65]. To reduce the effects of proteases, the extraction buffer should be kept ice cold during the extraction process and protease inhibitors (phenylmethylsulphonyl fluoride, PMSF) may be added to the extraction buffer. Other additives such as detergents (Triton X-100, *N*-lauroylsarcosine) may be necessary to solubilize membrane proteins, and chelating agents (ethylenediaminetetraacetic acid, EDTA) may be included to reduce the effects of divalent cations such as Mn^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}, which can inhibit a protein's biological activity [66]. It is recommended; however, that a minimalist extraction buffer be selected as most additives will have marginal improvements on recoveries, but will increase processing costs significantly [16].

While the methods described above generally refer to the protein fractionation/recovery stage, the protein purification stage will usually include steps such as precipitation, filtration, and column chromatography among others. The physical characteristics of proteins can be used to select the appropriate separation step needed to recover a target protein. Size (molecular mass), shape (monomer, polymer), surface hydrophobicity, and net charge are commonly used protein characteristics exploited by separation steps. The overall charge of a protein depends on the protein's isoelectric point (pI), which is the pH where the protein will have a net charge of zero. At a system pH above the isoelectric point, the protein is negatively charged, and at a pH below the isoelectric point, the protein is positively charged. The system pH can be adjusted to change

the protein's overall net charge; however, most proteins have a small, defined pH range in which they will remain biologically active.

For plants expressing edible vaccines, extensive purification processes are not needed; however, this is not the case with tobacco [67, 68]. Many recombinant proteins, especially biopharmaceuticals, must be purified to a highly pure form (95-98% or greater) in order to be used safely in clinical trials and possible product formulation [69]. As downstream processing dictates the major cost for protein production, it is necessary to develop economical and scalable initial purification steps to remove large amounts of impurities before more costly steps, such as chromatography [6]. Generally, the components in a tobacco extract make it impractical to apply a crude extract directly to a column due to resin fouling and column plugging over repeated use [6]. It is also ideal to minimize the total number of purification steps used in an overall process as protein recovery generally decreases with increased number of steps.

There are two main concepts that are important to consider when reporting the efficiency of a purification process - recovery and enrichment. The recovery is defined as:

$$\text{Recovery} = \frac{protein\,(activity, mass, etc.)\ in\ analyzed\ fraction}{protein\,(activity, mass, etc.)\ in\ original\ fraction} \quad (1)$$

The enrichment ratio (E.R.), or purification factor, is defined as:

$$\text{Enrichment ratio} = \frac{[protein\,(activity, mass, etc.)/mg\ total\ protein]\ in\ analyzed\ fraction}{[protein\,(activity, mass, etc.)/mg\ total\ protein]\ in\ original\ fraction} \quad (2)$$

The enrichment ratio of any step is directly related to the initial purity of the target protein. For example, for a protein that is initially 1% pure, a maximum purification factor of 100 can be achieved. If a recombinant protein expressed in tobacco is on the order of 0.1% of the total soluble protein, the theoretical maximum enrichment will be 1000 resulting in a nearly 100% pure protein.

2.3.2 Polyelectrolyte precipitation

Precipitation methods are usually used as a method for processing large quantities of feed material, offering modest enrichment and high specificity at a low cost early in the purification process [58]. Incorporating a precipitation step in the purification process can significantly decrease the number of steps needed to purify a target protein by removing a large amount of impurities as well as providing concentration of the product feed [70]. Furthermore, precipitation processes can usually be scaled-up for commercial operations. Some common precipitants are acids or bases (isoelectric precipitation), salts ('salting out' with ammonium sulfate), organic solvents, polyelectrolytes, protein-binding dyes, and macroligands. "Selective" precipitation methods such as polyelectrolyte precipitation, protein-binding dyes, and macroligands tend to offer better purification in general than so-called "non-selective" methods such as isoelectric precipitation, ammonium sulfate precipitation, or organic solvents [70]. Selective precipitation processes are usually based largely on electrostatic interactions of the precipitant and protein which results in precipitation of the resulting complex.

Polyelectrolyte precipitation has been identified as a possible candidate for an initial, non-chromatographic purification step in this study. Polyelectrolytes are charged polymers at a specific pH that bind to oppositely charged molecules, including proteins, through electrostatic interactions when added to a mixture of components. The polymers are relatively inexpensive and usually small amounts are needed [58, 70]. Furthermore, the pellet can be redissolved in a higher salt buffer with no loss of the protein's biological activity [70]. The process can also result in significant sample concentration if the pellet is redissolved in a smaller volume, likely reducing subsequent processing costs.

The two main types of polyelectrolytes are cationic and anionic. Anionic polyelectrolytes such as poly(acrylic)acid (PAA), carboxymethyl cellulose (CMC), and Glass H (sodium hexametaphosphate) are negatively charged at neutral pH (pH ~ 7.0) and are used to fractionate basic proteins (pI $>$ 7). Cationic polyelectrolytes are positively charged at neutral pH and are used to fractionate acidic molecules including nucleic acids and proteins (pI $<$ 7). The most common type of cationic polyelectrolyte is polyethyleneimine (PEI). The linear form of PEI has the structure $H_2N(C_2H_4NH)_xC_2H_4NH_2$, and the pK_a value of its imino groups is 10-11 [71]. As the focus of this work deals with the purification of an acidic recombinant protein from tobacco, PEI precipitation was investigated as a non-chromatographic purification step.

The effects of polyelectrolyte precipitation are based on several factors such as polymer size and length, extract conditions including the amount of starting total protein and other impurities in the extract, and relative strength of interaction between polymers and molecules. PEI comes in various chain lengths and molecular weights including 0.8 kD, 25 kD, and 750 kD. Using a longer polymer will likely be able to bind larger amounts of acidic molecules. The nature of the starting extract plays an important role in polyelectrolyte precipitation efficiency. Native compounds such as phenolics, phytic acid, carbohydrates and nucleic acids may interfere with the ionic interactions needed to form protein-polyelectrolyte complexes by binding either to the polymer or protein. Furthermore, the ionic strength of the initial extract plays a critical role in the efficiency of polyelectrolyte binding. It has been shown that increasing the salt concentration in crude extracts of *E. coli* or wheat germ greatly decreases the percent of protein precipitated at any given PEI dose by disrupting electrostatic interactions [71]. The strength of interactions between PEI and negatively charged molecules is another important factor in polyelectrolyte precipitation. The surface characteristics of the molecule will dictate the relative electrostatic binding strength to the polymer, including the number and distribution of charge sites on a proteins surface [72]. A concern with PEI precipitation is the binding of the highly negatively charged nucleic acids found in a plant extract, which can interfere with protein binding. Furthermore, the polyelectrolyte-nucleic acid complex may strongly trap proteins as well [71]. By resuspending the precipitated pellet in a high salt buffer, the ionic interactions of the polyelectrolyte-protein-nucleic acid complex should be reduced or broken [71].

2.3.3 FPLC chromatography

One or more chromatography steps are almost always needed to obtain a high level of purity for recombinant protein purification. Chromatography processes are highly selective, and provide high resolving and separation power. However, these processes are more expensive than other methods and have several limitations [73]. One limitation is the slow binding and limited binding capacity due to diffusion limitations and steric hindrance when the molecules compete for interaction with the stationary phase. Second is the inability to handle crude or viscous extracts which contain impurities that can cause column plugging, column fouling, or lowered flow rates. A third limitation is the uncertainty of scale-up as bead deformation changes with higher pressure drops.

Fast performance liquid chromatography (FPLC) is generally used for purifying proteins as the columns can generally be scaled up for commercial use. There are many different types of chromatography used in FPLC, but the most commonly used are ion exchange, hydrophobic interaction, gel filtration, and affinity. Another lesser known type of chromatography that is becoming more popular is hydroxyapatite chromatography, which will be reviewed in more detail later in this chapter. Each method has a unique separation principle based on some physical characteristic of the target protein. Ion exchange chromatography separates proteins based on the overall net charge and charge distribution of the protein, and elution is accomplished by increasing the salt concentration in the buffer, which displaces the bound proteins from the functional ligands of the resin beads. Hydrophobic interaction chromatography separates proteins based on the strength of interaction between the hydrophobic patches located on the surface of the protein and the hydrophobic ligands of the stationary phase. Elution is accomplished by decreasing the chaotropic salt concentration in the buffer which disrupts the hydrophobic interactions between the bound protein and the stationary phase. Gel filtration, or size exclusion chromatography separates proteins based on their molecular weight, with larger molecules eluted prior to smaller molecules. Affinity chromatography separates proteins based on interactions with specially designed ligands specific to the target protein, such as antigen-antibody interactions. Out of all these methods, affinity chromatography is the most expensive due to the specificity of the designed stationary phase.

2.4 β-glucuronidase (GUS)

β-glucuronidase (GUS) is an enzyme that catalyzes the hydrolysis of β-glucuronides [66]. The *E. coli* gene encoding β-glucuronidase has been developed into one of the most widely used reporter genes in transgenic plants. The GUS reporter gene is used as a gene-fusion system to study and monitor gene expression and tissue specificity of promoter sequences in plants [74]. *E. coli* GUS is a homotetrameric protein, with four identical subunits of approximately 68.2 kD and a pI around pH 5.5 [75]. There are numerous reasons why GUS has been used as a "model" protein for recombinant protein expression and purification studies in transgenic plant systems. First, GUS is found endogenous in many animals and some bacterial species, but is almost entirely absent from higher plant species [66]. Therefore, any chance of background activity is eliminated. The enzyme is very stable under a wide range of detergents and ionic conditions,

and is largely resistant to protease degradation [66]. Furthermore, GUS is fairly resistant to thermal degradation with a half life time of two hours at 55°C and about 15 minutes at 60°C [66]. GUS in plant extracts may be stored at -70°C for prolonged periods of time and at 4°C for a few days without losing significant activity [76]. Lastly, many rapid and sensitive methods have been developed to quantify GUS activity, including histochemical, spectrophotometric, and fluorometric assays. GUS requires no cofactors, and can be assayed over a broad pH range, with the optimum between 5.0 and 7.8 for GUS activity [66].

2.5 Examples of purification studies

2.5.1 Protein purification from tobacco

Numerous recombinant proteins, including enzymes, antibodies, and vaccines have been expressed in tobacco. As a result, various methods have been studied to recover and purify these recombinant proteins, as well as some native tobacco proteins. Generally, the purification processes involve one or more affinity steps that offer high selectivity and specificity to the target protein. In one study, GUS with a calmodulin (CaM) affinity tag was purified on a phenothiazine affinity column to 20-fold purification and 85% yield after ammonium sulfate precipitation, dialysis, and concentration of the extract [77]. Similarly, histidine (His) tags have been used to purify recombinant proteins from tobacco using immobilized metal affinity chromatography (IMAC). Cationic peanut peroxidase (CPRX) with the addition of a His tag to the C-terminal was purified from tobacco using one step of IMAC [78]. Attachment of a His_6-tag to lactate dehydrogenase and purification on a Zn^{2+}-IMAC column increased yield from 7% to 55% and the purification factor from 21 to 82 [79]. Ricin, a plant protein toxin, was purified 288-fold on a lactose-agarose affinity column with 72% recovery [80]. Most of these studies involve molecular modification of the target protein and expensive affinity chromatography processes, which may not be feasible for large scale production of proteins as human therapeutics.

There have been a few reports on more generic processes used to purify proteins from tobacco leaves or cell suspensions, although most still rely on affinity steps or molecular modification. Recombinant trichosanthin, a basic ribosome-inactivating protein, was purified from tobacco extract by several steps of chromatography including gel filtration, anion-exchange, and cation-exchange; however purity and recovery data was not reported [81]. A

combination of anion exchange (DEAE) and size-exclusion chromatography (Sephacryl S-200) was used to purify a native tobacco protein, anionic peroxidase (MW 36 kD, pI 3.5), to a single band on SDS-PAGE with 80% recovery [82]. In this experiment, the extraction was carried out at pH 4.5, most likely significantly reducing the amount of native acidic proteins (i.e. Rubisco) co-extracted so that there was not a significant purification burden. Soluble native salicylic acid binding protein was purified by 250-fold from tobacco using anion-exchange chromatography (DEAE-Sephacel), size-exclusion chromatography (Sephacryl S-300), affinity chromatography (blue dextran-agarose), and another step of size-exclusion chromatography (Superose 6 HR 10/30) but with a recovery of only 16% [83]. Separation from the major interfering protein, Rubisco, occurred during the final step of size-exclusion chromatography. Human acetylcholinesterase-R (theoretical MW 67.8 kD, pI 5.96 estimated using ExPASy) was purified to homogeneity after extraction using procainamide affinity chromatography followed by ammonium sulfate precipitation and DEAE sepharose anion-exchange chromatography [84]. More than 400-fold purification was achieved but with a recovery of only 16%. Green fluorescence protein (GFP) fusion proteins were purified from tobacco cell cultures in three main steps [85]. Precipitation by ammonium sulfate at 30% (v/v) was first performed to remove particulate matter and aggregated material while maintaining the solubility of GFP. Hydrophobic interaction chromatography was performed next to remove background proteins and to elute GFP and fusions in a low salt buffer which could then be applied to anion-exchange chromatography. Secreted alkaline phosphatase (SEAP) and granulocyte-macrophage colony-stimulating factor (GMCSF), both as GFP fusions, as well as GFP itself were all recovered with yields greater than 70% and estimated purity over 80% with this process. Other purification processes studied on proteins expressed in tobacco are listed in Table 2.

2.5.2 PEI precipitation

Large scale purification of recombinant proteins from a crude tobacco extract by direct application to a chromatography column is impractical due to potential for resin fouling and clogging [6]. Therefore, non-chromatographic methods should be investigated early in the purification process to fractionate and concentrate large amounts of the extract. The effects of

Table 2. Downstream processing studies with tobacco. Adapted from [6, 65].

Protein	Purification	Notes	Source
"spiked" egg white lysozyme	Aqueous two-phase extraction	87% yield 4-fold purification 14-fold concentration	[63]
secretory IgA/G (Guy's 13)	Ultrafiltration Protein G affinity Sheep anti-mouse IgG1 sepharose column	Yield: 1 - 2.5 mg/kg fresh plant leaves > 95% purity from green tissue	[86]
anti-TMV IgG	Cross-flow filtration Protein A affinity Gel filtration	> 80% recovery High homogeneity on SDS-PAGE	[87, 88]
thermostable xylanase (chloroplast expression)	Heat precipitation (70°C) Anion-exchange	> 85% yield Major band on SDS-PAGE	[89]
GUS-polyhistidine-y-interferon fusion (chloroplast expression)	Anion exchange Immobilized Ni affinity Cation exchange	75% yield Purity not reported	[90]
antimicrobial peptide-CBD fusion	Size exclusion Chitin affinity	Yield and purity not reported	[91]
monoclonal antibody (IgG)	Expanded-bed protein A affinity	< 60% yield > 90% purity	[92]

polyethyleneimine (PEI) precipitation have been studied on a variety of biological systems. PEI precipitation has largely been used for the purification of nucleic acids; however, acidic proteins can be separated as well. A review of PEI precipitation including a detailed list of proteins and enzymes purified with a PEI fractionation step has been reported previously [71]. Some other examples of protein separation using PEI treatment include RNA polymerase from *Streptomyces aurefaciens* [93], acetyl Co-A carboxylase from parsley cell cultures and wheat germ [94], recombinant cysteine proteinase inhibitor stefin B from *E. coli* [95], and recombinant human tumor necrosis factor beta from *E. coli* cell extract [96].

PEI precipitation of β-glucuronidase (GUS) from several plant species has been previously attempted with varying results. Polyethyleneimine precipitation on canola (seed), soy (dried flakes), and corn (seed) extracts produced "spiked" GUS activity recoveries of 80%, 90%, and 81% and enrichment ratios of 18, 1.3, and 2.6 respectively [97]. In addition, nearly 100% of rGUSH6 (engineered to contain a six histidine fusion tail) was recovered from transgenic pea seed flour, but with only 1.5 enrichment, using PEI precipitation [98]. The dosage of PEI needed to precipitate proteins will vary depending on the type of plant extract. Generally, polymer is added based on a weight % of the total volume or as a ratio to the total protein present (mg PEI/g total protein (TP)). It has been shown that the amount of PEI needed to precipitate a percentage of proteins is dependent on the concentration of precipitable components in an extract [71]. In the papers that have studied GUS precipitation from plant extracts, usually low amounts of PEI were needed to fully precipitate GUS. These include 30 mg PEI/g TP, ~60 mg PEI/g TP, and 210 mg PEI/g TP, for canola, soy, and corn respectively [97]. In the study on purification of rGUSH6 from transgenic pea, approximately 80 mg PEI/g TP was needed for full rGUS precipitation [98]. However, there are currently no published reports of using PEI precipitation for the purification of recombinant proteins expressed in leafy plant tissue.

2.5.3 GUS purification

In addition to the studies reviewed above on GUS precipitation by PEI, there have been various studies on GUS stability and purification from other plant sources, using one or more chromatographic steps.

Several studies have been done to investigate the storage and stability of GUS in several plant species. The stability of rGUS for different time, temperature, and moisture content storage conditions was studied in corn (whole kernel and germ-rich) [31] and in canola as well [30, 99]. Likewise, the storage stability of rGUS in soy was studied [100]. Of most interest to studies using tobacco, the extraction and stability of rGUS was studied in two separate leafy crops. In one study, the stability of GUS was investigated in an alfalfa extract after protein extraction by maceration [101]. In this report, GUS degradation occurred even with the addition of several protease inhibitors. In a more recent paper, several types of extraction buffers were used to extract GUS from fresh lettuce leaves, and it was found that the reducing agent, DTT, was the most important component of the extraction buffer for minimizing GUS degradation [102]. In addition, it was reported that freeze-drying lettuce leaves extended the half-life of GUS considerably.

Several chromatographic schemes have been studied for the purification of GUS from *E. coli* as well as various plant sources. A strategy for purifying GUS from *E. coli* that included two stages of affinity chromatography (saccharolactone immobilized on cross-linked 4% beaded agarose) followed by one stage of weak anion exchange chromatography (DEAE-sepharose fast flow) with a final enzyme recovery near 50% has been reported [103]. Furthermore, in the same paper, "spiked" wild-type GUS was purified from canola with one step of anion exchange chromatography with a yield of 76.6% and an enrichment ratio of 55. Adding polyaspartate fusions increased the enrichment ratio from 55 to 191, but the recovery decreased to 53.4% when the fusion tail contained 15 aspartates. Recombinant GUS with 6 histidines added (GUSH6) was purified from transgenic pea with one step of anion exchange chromatography with a yield of 83%, but an enrichment ratio of only 3.1 [98]. In the same paper, GUSH6 was purified with two different metal-chelating resins on IMAC. The use of iminodiacetate (IDA) resin allowed for 97% GUS activity with an enrichment factor of 200, and the use of nitrilotriacetate (NTA) resin allowed for 100% GUS activity with an enrichment factor

of 260. In a previous study, GUSH6 was purified from canola extracts to near homogenous purity with one step of IMAC [104]. Recombinant GUS was purified from transgenic corn in four main steps consisting of adsorption with anion-exchange resin, hydrophobic interaction chromatography, anion-exchange chromatography, and size-exclusion chromatography [31]. Purity was more than 95% based on densitometric analysis, but with a recovery of only 10%. It was also noted here that the first ion-exchange chromatography step was the most important step as the sample was concentrated 6-fold and purified 8-fold. There have been virtually no examples of GUS purification from tobacco leaf tissue to date. An example of GUS-CaM affinity purification from tobacco was discussed above.

2.5.4 Hydroxyapatite chromatography

Chromatography types such as ionic exchange, hydrophobic interaction, and size-exclusion (gel filtration) are well reported and have been commonly used in the purification of recombinant proteins from various plant sources. A relatively new and lesser used method is hydroxyapatite chromatography (HAC). Hydroxyapatite has a structure of $(Ca_5(PO_4)_3OH)_2$ and is a form of calcium phosphate that can be used in the separation of biomolecules. The functional groups on the stationary phase are Ca^{2+}, PO_4^{3-}, and OH. Hydroxyapatite chromatography has some unique purification capabilities such as the separation of antibodies that differ in light chain composition, antibody fragments, supercoiled DNA from linear DNA, and single-stranded DNA from double-stranded DNA. Traditional hydroxyapatite is of crystalline form which offers excellent separation of biomolecules, but is not applicable to industrial scale processes.

However, a new form of hydroxyapatite, CHT ceramic hydroxyapatite, has been developed which offers many advantages over the crystalline form. Crystalline hydroxyapatite is sintered at high temperatures resulting in a modified ceramic form that increases both chemical and physical stability. The stationary phase is a spherical, macroporous form that can be used for repeated runs at high flow rates and high pressures in large columns. Two types of CHT ceramic hydroxyapatite are offered by Bio-Rad Laboratories (Hercules, CA, USA). Type I has a high protein binding capacity and is better suited for acidic proteins. Type II has a lower protein binding capacity and

is better suited for nucleic acid and immunoglobulin purification. Both types are offered in various particle sizes (20, 40, and 80 μm) so that scale-up can be readily accomplished.

Binding of components generally occurs under low salt conditions (< 10 mM sodium phosphate (NaPi)) in HAC and elution can be accomplished by simply increasing the NaPi concentration, similar to ion-exchange chromatography. Since both acidic and basic proteins can interact with stationary phase ions (Ca^{2+} and PO_4^{3-}), HAC is believed to exhibit a "mixed mode" ion exchange separation [105]. Although separation does not occur solely based on one physical property of a protein (i.e. charge density, molecular weight, or isoelectric point), proteins with lower pI's tend to elute before ones with higher pI's due to their affinity to the matrix. The optimum pH for the buffers used in HAC is 6.8, and the minimum pH tolerated is 5.5. At lower pH values, the addition of Ca^{2+} ions (calcium chloride) is needed in the mobile phase to reduce acidic dissolution of the CHT. Chelating agents such as EDTA cannot be used as they bind to and dissolve the support.

There has been little report of hydroxyapatite chromatography used for the separation of recombinant proteins from leafy crops. UDP-glucose (hydroxycoumarin 7-O-glucosyltransferase (CGTase)) was purified from a tobacco cell culture using a combination of anion-exchange chromatography, hydroxyapatite, gel filtration, a second step of anion-exchange chromatography, and affinity chromatography on UDP glucuronic acid agarose [106]. The protein was purified 1200-fold with 7% recovery. Similarly, hydroxylcinnamoyl-CoA was also purified to homogeneity and a 12% yield from a tobacco cell culture suspension using a four step process that included hydrophobic interaction (phenyl sepharose) and hydroxyapatite chromatography [107]. Gel filtration, ion exchange and hydroxyapatite chromatography were used in the purification of RNAi-associated ssRNA-specific ribonucleases from *Nicotiana benthiamiana*, a close relative of tobacco, although no purity or recovery yields were reported [108]. Finally, a 10-step process was reported for the purification of choline monooxygenase from spinach leaves which included polyethylene glycol precipitation, PEI precipitation, HIC, anion-exchange on choline-sepharose, DEAE sepharose, Mono-Q (NaCl elution), hydroxyapatite, Mono-Q (KAc elution), gel filtration, and SDS-PAGE [109]. Purification was over 600-fold, but with only 0.5% activity recovered.

2.6 Summary

Purification of recombinant proteins from transgenic tobacco is not well-studied by far. Downstream processing costs are likely to be the largest percentage of the overall production cost due to high recovery and purity demands, and the process is further complicated by the native components in tobacco. Scalability and economics should always be kept in mind when choosing the types and number of purification steps.

Due to the fact that little information exists on general purification procedures for acidic recombinant proteins from tobacco, there is a serious need to address this topic. Affinity protein purification methods might be necessary for specific or hard to isolate proteins expressed in tobacco; however, these procedures will be expensive, and may not be easily scalable for commercial production. Therefore, the goal of this study is to develop a general and potentially scalable purification process for the recovery and purification of an acidic recombinant protein from transgenic tobacco that may have implications for the purification of a wide variety of other acidic recombinant proteins expressed in tobacco and perhaps other leafy crops as well.

References

1. Twyman, R.M., E. Stoger, S. Schillberg, P. Christou, and R. Fischer. *Molecular farming in plants: host systems and expression technology.* Trends Biotechnol. 2003. 21(12): p. 570-8.
2. Broglie, K., I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C.J. Mauvais, and R. Broglie. *Transgenic Plants with Enhanced Resistance to the Fungal Pathogen Rhizoctonia-Solani.* Science. 1991. 254(5035): p. 1194-1197.
3. Nelson, R.S., S.M. Mccormick, X. Delannay, P. Dube, J. Layton, E.J. Anderson, M. Kaniewska, R.K. Proksch, R.B. Horsch, S.G. Rogers, R.T. Fraley, and R.N. Beachy. *Virus Tolerance, Plant-Growth, and Field Performance of Transgenic Tomato Plants Expressing Coat Protein from Tobacco Mosaic-Virus.* Bio-Technology. 1988. 6(4): p. 403-409.
4. Hood, E.E., D.R. Witcher, S. Maddock, T. Meyer, C. Baszczynski, M. Bailey, P. Flynn, J. Register, L. Marshall, D. Bond, E. Kulisek, A. Kusnadi, R. Evangelista, Z. Nikolov, C. Wooge, R.J. Mehigh, R. Hernan, W.K. Kappel, D. Ritland, C.P. Li, and J.A. Howard. *Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification.* Molecular Breeding. 1997. 3(4): p. 291-306.
5. Witcher, D.R., E.E. Hood, D. Peterson, M. Bailey, D. Bond, A. Kusnadi, R. Evangelista, Z. Nikolov, C. Wooge, R. Mehigh, W. Kappe, J. Register, and J.A. Howard. *Commercial production of beta-glucuronidase (GUS): a model system for the production of proteins in plants.* Molecular Breeding. 1998. 4(4): p. 301-312.
6. Menkhaus, T.J., Y. Bai, C. Zhang, Z.L. Nikolov, and C.E. Glatz. *Considerations for the recovery of recombinant proteins from plants.* Biotechnol Prog. 2004. 20(4): p. 1001-14.
7. Twyman, R.M., S. Schillberg, and R. Fischer. *Transgenic plants in the biopharmaceutical market.* Expert Opin Emerg Drugs. 2005. 10(1): p. 185-218.
8. Fischer, R. and N. Emans. *Molecular farming of pharmaceutical proteins.* Transgenic Res. 2000. 9(4-5): p. 279-99; discussion 277.

9. Giddings, G., G. Allison, D. Brooks, and A. Carter. *Transgenic plants as factories for biopharmaceuticals.* Nat Biotechnol. 2000. 18(11): p. 1151-5.
10. Stoger, E., M. Sack, Y. Perrin, C. Vaquero, E. Torres, R.M. Twyman, P. Christou, and R. Fischer. *Practical considerations for pharmaceutical antibody production in different crop systems.* Molecular Breeding. 2002. 9(3): p. 149-158.
11. Hood, E.E., S.L. Woodard, and M.E. Horn. *Monoclonal antibody manufacturing in transgenic plants--myths and realities.* Curr Opin Biotechnol. 2002. 13(6): p. 630-5.
12. Fischer, R., E. Stoger, S. Schillberg, P. Christou, and R.M. Twyman. *Plant-based production of biopharmaceuticals.* Curr Opin Plant Biol. 2004. 7(2): p. 152-8.
13. Fischer, R., J. Drossard, N. Emans, U. Commandeur, and S. Hellwig. *Towards molecular farming in the future: pichia pastoris-based production of single-chain antibody fragments.* Biotechnol Appl Biochem. 1999. 30 (Pt 2): p. 117-20.
14. Giddings, G. *Transgenic plants as protein factories.* Curr Opin Biotechnol. 2001. 12(5): p. 450-4.
15. Houdebine, L.M. *Antibody manufacture in transgenic animals and comparisons with other systems.* Curr Opin Biotechnol. 2002. 13(6): p. 625-9.
16. Larrick, J.W. and D.W. Thomas. *Producing proteins in transgenic plants and animals.* Curr Opin Biotechnol. 2001. 12(4): p. 411-8.
17. Daniell, H., S.J. Streatfield, and K. Wycoff. *Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants.* Trends Plant Sci. 2001. 6(5): p. 219-26.
18. Kusnadi, A.R., Z.L. Nikolov, and J.A. Howard. *Production of recombinant proteins in transgenic plants: Practical considerations.* Biotechnology and Bioengineering. 1997. 56(5): p. 473-484.
19. Whitelam, G.C., B. Cockburn, A.R. Gandecha, and M.R. Owen. *Heterologous protein production in transgenic plants.* Biotechnol Genet Eng Rev. 1993. 11: p. 1-29.
20. Schillberg, S., R.M. Twyman, and R. Fischer. *Opportunities for recombinant antigen and antibody expression in transgenic plants--technology assessment.* Vaccine. 2005. 23(15): p. 1764-9.

21. Hiatt, A., R. Cafferkey, and K. Bowdish. *Production of antibodies in transgenic plants.* Nature. 1989. 342(6245): p. 76-8.
22. Stein, K.E. and K.O. Webber. *The regulation of biologic products derived from bioengineered plants.* Curr Opin Biotechnol. 2001. 12(3): p. 308-11.
23. FDA. *Guidance for industry. Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals.* September 2002. URL: http://www.fda.gov/cber/gdlns/bioplant.pdf.
24. Kusnadi, A.R., E.E. Hood, D.R. Witcher, J.A. Howard, and Z.L. Nikolov. *Production and purification of two recombinant proteins from transgenic corn.* Biotechnol Prog. 1998. 14(1): p. 149-55.
25. USDA. *Tobacco*. Last updated January 16, 2007. URL: http://www.ers.usda.gov/Browse/Crops/Tobacco.htm.
26. Stoger, E., M. Sack, R. Fischer, and P. Christou. *Plantibodies: applications, advantages and bottlenecks.* Curr Opin Biotechnol. 2002. 13(2): p. 161-6.
27. Kapila, J., R. DeRycke, M. VanMontagu, and G. Angenon. *An Agrobacterium-mediated transient gene expression system for intact leaves.* Plant Science. 1997. 122(1): p. 101-108.
28. Verch, T., V. Yusibov, and H. Koprowski. *Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector.* Journal of Immunological Methods. 1998. 220(1-2): p. 69-75.
29. Daniell, H., S. Chebolu, S. Kumar, M. Singleton, and R. Falconer. *Chloroplast-derived vaccine antigens and other therapeutic proteins.* Vaccine. 2005. 23(15): p. 1779-1783.
30. Bai, Y. and Z.L. Nikolov. *Effect of processing on the recovery of recombinant beta-glucuronidase (rGUS) from transgenic canola.* Biotechnol Prog. 2001. 17(1): p. 168-74.
31. Kusnadi, A.R., R.L. Evangelista, E.E. Hood, J.A. Howard, and Z.L. Nikolov. *Processing of transgenic corn seed and its effect on the recovery of recombinant beta-glucuronidase.* Biotechnol Bioeng. 1998. 60(1): p. 44-52.
32. Stoger, E., C. Vaquero, E. Torres, M. Sack, L. Nicholson, J. Drossard, S. Williams, D. Keen, Y. Perrin, P. Christou, and R. Fischer. *Cereal crops as viable*

production and storage systems for pharmaceutical scFv antibodies. Plant Mol Biol. 2000. 42(4): p. 583-90.

33. Cramer, C.L., D.L. Weissenborn, K.K. Oishi, E.A. Grabau, S. Bennett, E. Ponce, G.A. Grabowski, and D.N. Radin. *Bioproduction of human enzymes in transgenic tobacco.* Ann N Y Acad Sci. 1996. 792: p. 62-71.
34. Parmenter, D.L., J.G. Boothe, G.J. van Rooijen, E.C. Yeung, and M.M. Moloney. *Production of biologically active hirudin in plant seeds using oleosin partitioning.* Plant Mol Biol. 1995. 29(6): p. 1167-80.
35. Boothe, J.G., J.A. Saponja, and D.L. Parmenter. *Molecular farming in plants: Oilseeds as vehicles for the production of pharmaceutical proteins.* Drug Development Research. 1997. 42(3-4): p. 172-181.
36. Ganz, P.R., R. Sardana, A.K. Dudani, E. Tackaberry, C. Sauder, and I. Altosaar, *Expression of human blood proteins in transgenic plants: the cytokine GM-CSF as a model protein.*, in *Transgenic plants: a production system for industrial and pharmaceutical proteins.*, M.R.L. Owen and J. Pen, Editors. 1996, John Wiley & Sons: London, UK. p. 281-287.
37. Goddijn, O.J.M. and J. Pen. *Plants as Bioreactors.* Trends in Biotechnology. 1995. 13(9): p. 379-387.
38. Sijmons, P.C., B.M. Dekker, B. Schrammeijer, T.C. Verwoerd, P.J. van den Elzen, and A. Hoekema. *Production of correctly processed human serum albumin in transgenic plants.* Biotechnology (N Y). 1990. 8(3): p. 217-21.
39. Dieryck, W., J. Pagnier, C. Poyart, M.C. Marden, V. Gruber, P. Bournat, S. Baudino, and B. Merot. *Human haemoglobin from transgenic tobacco.* Nature. 1997. 386(6620): p. 29-30.
40. Ruggiero, F., J.Y. Exposito, P. Bournat, V. Gruber, S. Perret, J. Comte, B. Olagnier, R. Garrone, and M. Theisen. *Triple helix assembly and processing of human collagen produced in transgenic tobacco plants.* Febs Letters. 2000. 469(1): p. 132-136.
41. Hamamoto, H., Y. Sugiyama, N. Nakagawa, E. Hashida, Y. Matsunaga, S. Takemoto, Y. Watanabe, and Y. Okada. *A new tobacco mosaic virus vector and*

its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. Biotechnology (N Y). 1993. 11(8): p. 930-2.

42. Cramer, C.L., J.G. Boothe, and K.K. Oishi. *Transgenic plants for therapeutic proteins: linking upstream and downstream strategies.* Curr Top Microbiol Immunol. 1999. 240: p. 95-118.
43. Ma, J.K.C., A. Hiatt, M. Hein, N.D. Vine, F. Wang, P. Stabila, C. Vandolleweerd, K. Mostov, and T. Lehner. *Generation and Assembly of Secretory Antibodies in Plants.* Science. 1995. 268(5211): p. 716-719.
44. Ma, J.K.C., B.Y. Hikmat, K. Wycoff, N.D. Vine, D. Chargelegue, L. Yu, M.B. Hein, and T. Lehner. *Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans.* Nature Medicine. 1998. 4(5): p. 601-606.
45. Vaquero, C., M. Sack, J. Chandler, J. Drossard, F. Schuster, M. Monecke, S. Schillberg, and R. Fischer. *Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves.* Proceedings of the National Academy of Sciences of the United States of America. 1999. 96(20): p. 11128-11133.
46. Tacket, C.O. and H.S. Mason. *A review of oral vaccination with transgenic vegetables.* Microbes Infect. 1999. 1(10): p. 777-83.
47. Ma, J.K. and A. Hiatt, *Expressing antibodies in plants for immunotherapy*, in *Transgenic plants: a production system for industrial and pharmaceutical proteins*, M.R.L. Owen and J. Pen, Editors. 1996, John Wiley & Sons: London, UK. p. 229-243.
48. Richter, L.J., Y. Thanavala, C.J. Arntzen, and H.S. Mason. *Production of hepatitis B surface antigen in transgenic plants for oral immunization.* Nature Biotechnology. 2000. 18(11): p. 1167-1171.
49. Dixon, R.A. and C.J. Arntzen. *Transgenic plant technology is entering the era of metabolic engineering.* Trends in Biotechnology. 1997. 15(11): p. 441-444.
50. McGarvey, P.B., J. Hammond, M.M. Dienelt, D.C. Hooper, Z.F. Fu, B. Dietzschold, H. Koprowski, and F.H. Michaels. *Expression of the rabies virus*

glycoprotein in transgenic tomatoes. Biotechnology (N Y). 1995. 13(13): p. 1484-7.

51. Doran, P.M. *Foreign protein production in plant tissue cultures.* Current Opinion in Biotechnology. 2000. 11(2): p. 199-204.
52. Mushegian, A.R. and R.J. Shepherd. *Genetic Elements of Plant-Viruses as Tools for Genetic-Engineering.* Microbiological Reviews. 1995. 59(4): p. 548-&.
53. Turpen, T.H., S.J. Reinl, Y. Charoenvit, S.L. Hoffman, V. Fallarme, and L.K. Grill. *Malarial Epitopes Expressed on the Surface of Recombinant Tobacco Mosaic-Virus.* Bio-Technology. 1995. 13(1): p. 53-57.
54. Beachy, R.N., J.H. Fitchen, and M.B. Hein. *Use of plant viruses for delivery of vaccine epitopes.* Engineering Plants for Commercial Products and Applications. 1996. 792: p. 43-49.
55. Davis, D.L. and M.T. Nielson, *Tobacco: Production, Chemistry and Technology*, ed. D.L. Davis and M.T. Nielson. 1999, Oxford: Blackwell Science. 3.
56. Moloney, M.M. *Molecular Farming in Plants - Achievements and Prospects.* Biotechnology & Biotechnological Equipment. 1995. 9(1): p. 3-9.
57. Cheryan, M. *Phytic acid interactions in food systems.* Crit Rev Food Sci Nutr. 1980. 13(4): p. 297-335.
58. Jervis, L. and W.S. Pierpoint. *Purification Technologies for Plant-Proteins.* Journal of Biotechnology. 1989. 11(2-3): p. 161-198.
59. Khoudi, H., S. Laberge, J.M. Ferullo, R. Bazin, A. Darveau, Y. Castonguay, G. Allard, R. Lemieux, and L.P. Vezina. *Production of a diagnostic monoclonal antibody in perennial alfalfa plants.* Biotechnol Bioeng. 1999. 64(2): p. 135-43.
60. Garger, S.J., R.B. Holtz, M.J. McCulloch, and T.H. Turpen. *Process for isolating and purifying viruses, soluble proteins, and peptides from plant sources.* U. S. Patent 6,033,895. 2000.
61. Kung, S.D. *Tobacco fraction 1 protein: a unique genetic marker.* Science. 1976. 191(4226): p. 429-434.
62. Akehurst, B.C., *Tobacco*. 2d ed. Tropical agriculture series. 1981, London ; New York: Longman. xiii, 764 p.

63. Balasubramaniam, D., C. Wilkinson, K. Van Cott, and C. Zhang. *Tobacco protein separation by aqueous two-phase extraction.* J Chromatogr A. 2003. 989(1): p. 119-29.

64. Loomis, W.D. *Removal of phenolic compounds during the isolation of plant enzymes.* Methods Enzymol. 1974. 31: p. 555-563.

65. Nikolov, Z.L. and S.L. Woodard. *Downstream processing of recombinant proteins from transgenic feedstock.* Curr Opin Biotechnol. 2004. 15(5): p. 479-86.

66. Jefferson, R.A. and K.J. Wilson, *The GUS gene fusion system*, in *Plant Molecular Biology Manual*, S.B. Gelvin, R.A. Schilperoort, and D.P.S. Verma, Editors. 1991, Kluwer Academic: Dordrecht, The Netherlands. p. 1-33.

67. Streatfield, S.J. and J.A. Howard. *Plant production systems for vaccines.* Expert Rev Vaccines. 2003. 2(6): p. 763-75.

68. Walmsley, A.M. and C.J. Arntzen. *Plant cell factories and mucosal vaccines.* Curr Opin Biotechnol. 2003. 14(2): p. 145-50.

69. Headon, D.R. and G. Walsh. *The industrial production of enzymes.* Biotechnol Adv. 1994. 12(4): p. 635-46.

70. Niederauer, M.Q. and C.E. Glatz, *Selective precipitation*, in *Advances in Biochemical Engineering/Biotechnology*, A. Fiechter, Editor. 1992, Springer Verlag: New York. p. 159-188.

71. Jendrisak, J. *The Use of Polyethyleneimine in Protein-Purification.* Journal of Cellular Biochemistry. 1987: p. 166-166.

72. Ledward, D.A., *Protein-polysaccharide interactions*, in *Polysaccharides in Food*, J.M.V. Blanshard and J.R. Mitchell, Editors. 1978, Butterworths: London. p. 205.

73. Senstad, C. and B. Mattiasson. *Precipitation of soluble affinity complexes by a second affinity interaction: a model study.* Biotechnol Appl Biochem. 1989. 11(1): p. 41-8.

74. Gallagher, S.R., *GUS protocols: using the GUS gene as a reporter of gene expression*. 1992, San Diego, CA: Academic Press, Inc.

75. Jefferson, R.A., S.M. Burgess, and D. Hirsh. *Beta-Glucuronidase from Escherichia coli as a gene-fusion marker.* Proc Natl Acad Sci U S A. 1986. 83(22): p. 8447-51.

76. Jefferson, R.A., M. Bevan, and T. Kavanagh. *The use of the Escherichia coli beta-glucuronidase as a gene fusion marker for studies of gene expression in higher plants.* Biochem Soc Trans. 1987. 15(1): p. 17-8.
77. Desai, U.A., G. Sur, S. Daunert, R. Babbitt, and Q. Li. *Expression and affinity purification of recombinant proteins from plants.* Protein Expr Purif. 2002. 25(1): p. 195-202.
78. Lige, B., S.W. Ma, D.L. Zhao, and R.B. van Huystee. *Cationic peanut peroxidase: Expression and characterization in transgenic tobacco and purification of the histidine-tagged protein.* Plant Science. 1998. 136(2): p. 159-168.
79. Mejare, M., G. Lilius, and L. Bulow. *Evaluation of genetically attached histidine affinity tails for purification of lactate dehydrogenase from transgenic tobacco.* Plant Science. 1998. 134(1): p. 103-114.
80. Sehnke, P.C. and R.J. Ferl. *Processing of preproricin in transgenic tobacco.* Protein Expr Purif. 1999. 15(2): p. 188-95.
81. Krishnan, R., K.A. McDonald, A.M. Dandekar, A.P. Jackman, and B. Falk. *Expression of recombinant trichosanthin, a ribosome-inactivating protein, in transgenic tobacco.* J Biotechnol. 2002. 97(1): p. 69-88.
82. Gazaryan, I.G. and L.M. Lagrimini. *Purification and unusual kinetic properties of a tobacco anionic peroxidase.* Phytochemistry. 1996. 41(4): p. 1029-34.
83. Chen, Z., J.W. Ricigliano, and D.F. Klessig. *Purification and characterization of a soluble salicylic acid-binding protein from tobacco.* Proc Natl Acad Sci U S A. 1993. 90(20): p. 9533-7.
84. Geyer, B.C., M. Muralidharan, I. Cherni, J. Doran, S.P. Fletcher, T. Evron, H. Soreq, and T.S. Mor. *Purification of transgenic plant-derived recombinant human acetylcholinesterase-R.* Chem Biol Interact. 2005. 157-158: p. 331-4.
85. Peckham, G.D., R.C. Bugos, and W.W. Su. *Purification of GFP fusion proteins from transgenic plant cell cultures.* Protein Expr Purif. 2006. 49(2): p. 183-9.
86. Chargelegue, D., N.D. Vine, C.J. van Dolleweerd, P.M.W. Drake, and J.K.C. Ma. *A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice.* Transgenic Research. 2000. 9(3): p. 187-194.

87. Fischer, R., K. Hoffmann, S. Schillberg, and N. Emans. *Antibody production by molecular farming in plants.* J Biol Regul Homeost Agents. 2000. 14(2): p. 83-92.

88. Fischer, R., Y.C. Liao, and J. Drossard. *Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture.* J Immunol Methods. 1999. 226(1-2): p. 1-10.

89. Leelavathi, S., N. Gupta, S. Maiti, A. Ghosh, and V.S. Reddy. *Overproduction of an alkali- and thermo-stable xylanase in tobacco chloroplasts and efficient recovery of the enzyme.* Molecular Breeding. 2003. 11(1): p. 59-67.

90. Leelavathi, S. and V.S. Reddy. *Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors.* Molecular Breeding. 2003. 11(1): p. 49-58.

91. Morassutti, C., F. De Amicis, B. Skerlavaj, M. Zanetti, and S. Marchetti. *Production of a recombinant antimicrobial peptide in transgenic plants using a modified VMA intein expression system.* Febs Letters. 2002. 519(1-3): p. 141-146.

92. Valdes, R., B. Reyes, T. Alvarez, J. Garcia, J.A. Montero, A. Figueroa, L. Gomez, S. Padilla, D. Geada, M.C. Abrahantes, L. Dorta, D. Fernandez, O. Mendoza, N. Ramirez, M. Rodriguez, M. Pujol, C. Borroto, and J. Brito. *Hepatitis B surface antigen immunopurification using a plant-derived specific antibody produced in large scale.* Biochem Biophys Res Commun. 2003. 310(3): p. 742-7.

93. Simuth, J., H. Sternbach, J. Zelinka, R.M. Chomutov, and A.A. Nedospasov. *DNA-dependent RNA polymerase from the chlorotetracycline producing strain of Streptomyces aureofaciens.* FEBS Lett. 1987. 218(1): p. 163-6.

94. Egin-Buhler, B., R. Loyal, and J. Ebel. *Comparison of acetyl-CoA carboxylases from parsley cell cultures and wheat germ.* Arch Biochem Biophys. 1980. 203(1): p. 90-100.

95. Jerala, R., L. Kroon-Zitko, and V. Turk. *Improved expression and evaluation of polyethyleneimine precipitation in isolation of recombinant cysteine proteinase inhibitor stefin B.* Protein Expr Purif. 1994. 5(1): p. 65-9.

96. Loh, K.C., Z.J. Yao, M.G. Yap, and M.C. Chung. *Role of polyethyleneimine in the purification of recombinant human tumour necrosis factor beta.* J Chromatogr A. 1997. 760(2): p. 165-71.

97. Menkhaus, T.J., S.U. Eriksson, P.B. Whitson, and C.E. Glatz. *Host selection as a downstream strategy: polyelectrolyte precipitation of beta-glucuronidase from plant extracts.* Biotechnol Bioeng. 2002. 77(2): p. 148-54.

98. Menkhaus, T.J., C. Pate, A. Krech, and C.E. Glatz. *Recombinant protein purification from pea.* Biotechnol Bioeng. 2004. 86(1): p. 108-14.

99. Bai, Y., Z.L. Nikolov, and C.E. Glatz. *Aqueous extraction of beta-glucuronidase from transgenic canola: kinetics and microstructure.* Biotechnol Prog. 2002. 18(6): p. 1301-5.

100. Holzmann, D. *Agracetus grows monoclonals in soy beans and corn plants.* Gen. Eng. News. 1994. 14(1): p. 34.

101. Austin, S., E.T. Bingham, R.G. Koegel, D.E. Mathews, M.N. Shahan, R.J. Straub, and R.R. Burgess. *An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa.* Ann N Y Acad Sci. 1994. 721: p. 234-44.

102. Joh, L.D., K.A. McDonald, and J.S. VanderGheynst. *Evaluating extraction and storage of a recombinant protein produced in agroinfiltrated lettuce.* Biotechnol Prog. 2006. 22(3): p. 723-30.

103. Zhang, C., R.T. Love, J.M. Jilka, and C.E. Glatz. *Genetic engineering strategies for purification of recombinant proteins from canola by anion exchange chromatography: an example of beta-glucuronidase.* Biotechnol Prog. 2001. 17(1): p. 161-7.

104. Zhang, C.M., S.A. Reslewic, and C.E. Glatz. *Suitability of immobilized metal affinity chromatography for protein purification from canola.* Biotechnol Bioeng. 2000. 68(1): p. 52-8.

105. Gorbunoff, M.J. *The interaction of proteins with hydroxyapatite. I. Role of protein charge and structure.* Anal Biochem. 1984. 136(2): p. 425-32.

106. Taguchi, G., H. Imura, Y. Maeda, R. Kodaira, N. Hayashida, M. Shimosaka, and M. Okazaki. *Purification and characterization of UDP-glucose: hydroxycoumarin 7-O-glucosyltransferase, with broad substrate specificity from tobacco cultured cells.* Plant Science. 2000. 157(1): p. 105-112.

107. Negrel, J. and F. Javelle. *Purification, characterization and partial amino acid sequencing of hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl)transferase from tobacco cell-suspension cultures.* Eur J Biochem. 1997. 247(3): p. 1127-35.

108. Omarov, R.T., J.J. Ciomperlik, and H.B. Scholthof. *RNAi-associated ssRNA-specific ribonucleases in Tombusvirus P19 mutant-infected plants and evidence for a discrete siRNA-containing effector complex.* Proc Natl Acad Sci U S A. 2007. 104(5): p. 1714-9.

109. Burnet, M., P.J. Lafontaine, and A.D. Hanson. *Assay, Purification, and Partial Characterization of Choline Monooxygenase from Spinach.* Plant Physiol. 1995. 108(2): p. 581-588.

Chapter Three

Polyethyleneimine precipitation versus anion exchange chromatography in fractionating recombinant β-glucuronidase from transgenic tobacco extract

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Abstract

Tobacco has been studied as a possible host for the production of recombinant proteins. In this report, recombinant β-glucuronidase (rGUS) was used as a model protein to study the feasibility of using polyethyleneimine (PEI) precipitation to fractionate acidic recombinant proteins from transgenic tobacco. Results showed that rGUS was preferentially precipitated when the PEI dosage was beyond 200 mg PEI/g total protein. At 700-800 mg PEI/g total protein, nearly 100% rGUS was precipitated with less than 40% native tobacco proteins co-precipitated. Approximately 85-90% of the rGUS activity could be recovered from the precipitation pellet for an enrichment ratio of 2.7 - 3.4. As a comparison, anion exchange chromatography (AEX) yielded similar results to PEI precipitation with 66-90% rGUS activity recovered and an enrichment ratio of 1.8 - 3.1. The rGUS was further purified by an additional hydrophobic interaction chromatographic (HIC) step after precipitation or AEX. Similar results in terms of rGUS activity recovered and enrichment were obtained. The major interfering protein at the end of all purification steps is most likely the Fraction 1 protein ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). The presence of this protein is likely the cause for the varying and somewhat low enrichment ratios, but it may be later removed via a size-exclusion chromatography step. PEI precipitation offers the advantage of allowing significant sample concentration prior to subsequent purification techniques such as chromatography and is much less expensive than chromatographic methods as well.

Through direct comparison, this study shows that PEI may be used as an initial fractionation step in replacement of AEX to facilitate the purification of acidic recombinant proteins from transgenic tobacco.

Keywords: Precipitation; Polyelectrolyte; Transgenic tobacco; Recombinant Protein; Glucuronidase; Chromatography

1. Introduction

Transgenic plants have been investigated as alternatives to microbial, mammalian cell, and transgenic animal systems for the production of recombinant proteins. Plant systems are cost-efficient in biomass production [1,2] and do not harbor human pathogens. Additionally, plants share similar protein synthesis pathways with animals and thus are capable of producing bioactive proteins [3]. There are many examples of fully functional mammalian proteins expressed in plants, ranging from human growth hormone [4] to multimeric antibodies [5,6].

Many plant systems have been used for the expression of recombinant proteins including corn [7], canola [8,9], rice [10-12], soy [13,14] and tobacco [15-17], each with its own unique advantages for practical applications [2]. Tobacco, as a non-food and non-feed crop, has emerged as a safer alternative for the production of therapeutic proteins. Other advantages are that tobacco is capable of producing large quantities of biomass through scale-up practices [18] and can be genetically modified with relative ease. Furthermore, many post-translational modifications similar to mammalian species can be performed [19]. However, one main challenge that faces the commercialization of tobacco as a host for the production of recombinant proteins is to economically recover and purify the recombinant protein from huge amounts of biomass. Tobacco extracts are a complex system containing plant nucleotides, phenolics, native plant proteins, and particulates, which complicate separation processes. Currently, chromatographic methods dominate most purification strategies due to their high selectivity and resolution. However, chromatographic steps are costly and are more suitable for the later stages of downstream processes. Therefore, for economic consideration, it may be beneficial to develop inexpensive, easily scalable, non-chromatographic techniques in the product

recovery stage to partially purify and concentrate the protein to prepare for the subsequent high-resolution chromatographic steps [20].

Polyelectrolyte precipitation is an inexpensive protein fractionation method that offers both concentration and partial purification of a target protein early in the separation process [21]. If developed properly, it may provide high selectivity as well. Polyelectrolytes are charged polymers that separate molecules of opposite charges at a certain pH based on electrostatic interactions [22,23]. Furthermore, the precipitate can be redissolved without affecting the bioactivity of the protein [22]. Two types of polymers, polyanionic and polycationic, have been used to recover proteins from various systems [23-29]. The most commonly used polycationic electrolyte (at neutral pH) is polyethyleneimine (PEI). The linear form of PEI has the structure of $H_2N(C_2H_4NH)_xC_2H_4NH_2$, and the p$K_a$ value of the imino groups is 10-11 [30]. PEI has been used in protein separation from various expression systems, such as RNA polymerase from *Streptomyces aurefaciens* [31], acetyl-CoA carboxylase from parsley cell cultures and wheat germ [32], and recombinant cysteine proteinase inhibitor stefin B from *E. coli* [33]. Protein precipitation by PEI is subjected to the effects of salt concentration and protein concentration. These effects have been reviewed in detail elsewhere [30].

A concern with PEI precipitation is the binding of highly negatively charged nucleic acids to the PEI, which can interfere with protein precipitation. In addition, the polyelectrolyte-nucleic acid complex may strongly trap proteins, resulting in lower recoveries of the target protein or loss of activity [30]. Other plant compounds such as phenolics and quinones (oxidized phenols) may also interfere with the protein-polyelectrolyte interactions by interacting with the target protein, causing conformational changes and loss of biological activity [21]. However, the inclusion of phenolic binding agents such as polyvinylpolypyrrolidone (PVPP) and reducing agents such as β-2-mercaptoethanol (BME) or dithiothreitol (DTT) in the plant extraction buffer may alleviate these interference [34].

Other non-chromatographic methods have been studied to separate proteins from tobacco extract. Anionic polyelectrolytes, poly(acrylic)acid (PAA), carboxymethyl cellulose (CMC), and Glass H (sodium hexametaphosphate), have been studied to

separate a basic model protein, lysozyme, from tobacco extract [29]. Also, aqueous two-phase extraction was studied as a means for separating spiked lysozyme from tobacco extract [35]. In this study, recombinant β-glucuronidase (rGUS) was used as an acidic model protein to test the effectiveness of PEI precipitation for rGUS recovery from transgenic tobacco extract. PEI precipitation, as an alternative to conventional chromatographic methods, was compared with a single stage of anion exchange chromatography by directly comparing the total rGUS recovery and enrichment. The impact of initial protein fractionation by PEI precipitation and anion exchange chromatography on the following separation was investigated by a subsequent hydrophobic interaction chromatography (HIC) step.

2. Materials and methods

2.1. Materials

Transgenic tobacco seeds containing the recombinant GUS gene regulated by Super P promoter were kindly provided by Dr. Luis Fabricio-Medina Bolivar (Arkansas State University) and were originally developed by Dr. Stanton B. Gelvin (Purdue University) [36]. PEI was purchased as a 50% (w/v) aqueous solution with an average molecular weight of 750 kDa from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl β-D-glucuronide (PNPG) and *p*-nitrophenol (PNP) were also purchased from Sigma. Bio-Rad Protein Assay and Bio-Safe Coomassie stain were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin was purchased from Pierce (Rockford, IL, USA). Dithiothreitol (DTT), kanamycin, and Murashige and Skoog basal salts and vitamins were obtained from Bioworld (Dublin, OH, USA). Greiner 96-well clear, flat bottom microplates were purchased from USA Scientific (Ocala, FL, USA). All SDS-PAGE products including 4-12% Bis-Tris Novex mini gels and 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), and antioxidant were purchased from Invitrogen (Carlsbad, CA, USA). Anion-exchange and hydrophobic interaction chromatography resins were purchased from GE Healthcare (Uppsala, Sweden). Microcon centrifugal filter devices were purchased from Millipore

(Bedford, MA, USA). All other standard laboratory grade chemicals and salts were either purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma.

2.2. Transgenic tobacco growth and protein extraction process

Transgenic tobacco seeds were prepared according to a published protocol [37]. Prepared seeds were placed aseptically in plastic boxes containing modified Murashige and Skoog (mMS) media [38] supplemented with 200 mg/L kanamycin (Kan_{200}) and allowed to germinate. Plants were grown in a temperature controlled (23 ºC) incubator and propagated periodically. After the first propagation, all plants were grown on Kan_{100} mMS media.

Fresh tobacco leaves were excised from the plant each time an extract was prepared. Leaf tissue was weighed out and placed in a 50 mL conical tube. Buffer was added to each tube at a ratio of 5 mL buffer:1 g leaf tissue. The extraction buffer consisted of 50 mM sodium phosphate (NaPi), pH 7 + 10 mM BME + 1mM EDTA. The samples were then homogenized until no large particulates remained (approximately one minute). Immediately after homogenization, 2% (w/v) pre-hydrated PVPP was added to the sample. The extract was then vortexed vigorously and allowed to set at room temperature for 15 minutes. After centrifugation at 4 ºC and 17,003 × g for 20 minutes, the supernatant was removed and filtered through a 0.22-µm syringe filter. It should be noted that the pH of the extract dropped to approximately pH 6.9 after extraction. The extracts were not titrated back to pH 7.

2.3. Analytical methods

Protein concentration was determined by Bio-Rad 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. Ten microliters of sample or standard was added to each empty well followed by 200 µL of prepared Bio-Rad reagent as given in the manufacturer's protocol. The samples were gently vortexed to mix and allowed to incubate at room temperature for 5 minutes. Absorbance measurements were read at 595 nm on a Bio-Tek Synergy microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

GUS is a homo-tetrameric enzyme and has an isoelectric point (p*I*) around pH 5.5. The molecular mass of each monomer is approximately 68.2 kDa [39,40]. The GUS activity assay used was a continuous spectrophotometric assay [41] based on the method developed by Jefferson and Wilson [42]. This assay utilizes the ability of GUS to hydrolyze *p*-nitrophenyl β-D-glucuronide (PNPG) to release chromophore *p*-nitrophenol (PNP), and GUS activity is expressed as unit/mL [42]. One unit of GUS activity was defined as the amount needed to liberate 1 nmol PNP/minute at room temperature and pH 7.0. A standard curve was created using various concentrations of PNP (solubilized in 50 mM NaPi, pH 7.0). All assays were carried out in 96-well microtiter plates and performed in triplicates. Four microliters of sample was added to each empty well, followed by 176 µL of 50 mM NaPi, pH 7.0 + 10 mM BME and 20 µL of 10 mM PNPG (solubilized in 50 mM NaPi, pH 7.0). The samples were gently vortexed to mix. Kinetic absorbance measurements were read at 405 nm every 50 s for a total of 8 min on a microplate reader. It is worth to point out that the results from a set of control experiments (data not shown) indicated that the GUS activity was not significantly affected (±10%) under different conditions encountered throughout this report.

SDS-PAGE samples were reduced and run on 4-12% Bis-Tris gels with MOPS as the running buffer. Thirteen microliters of sample was mixed with 5 µL LDS sample buffer and 2 µL of 500 m*M* DTT. The samples were vortexed and heated at 70 ºC for 10 min in a water bath. The gels were run for 50-60 minutes at 200 V. After running, the gels were washed with deionized (DI) water and stained with Bio-Safe Coomassie stain. The gels were scanned with a Bio-Rad ChemiDoc XRS imager and analyzed using Quantity One Software.

The enrichment ratio is used to assess the extent of purification of the target protein. The enrichment ratio (E.R.) is defined as follows:

$$E.R. = \frac{specific\ activity\ in\ the\ analyzed\ fraction\ (U/mg\ total\ protein)}{specific\ activity\ in\ the\ original\ extract\ (U/mg\ total\ protein)} \quad (1)$$

The maximum enrichment ratio is dependent on the initial purity of the target protein. A protein that is initially 1% pure will have a maximum enrichment ratio of 100. A protein that is initially 0.1% pure will have a maximum enrichment ratio of 1000.

2.4. Polyelectrolyte precipitation

Tobacco protein extract was obtained with 50 mM NaPi, pH 7, 10 mM BME, 1 mM EDTA. Total starting protein concentration was ca. 1.6 ± 0.33 mg/mL and the total starting rGUS activity was ca. 146 ± 24 U/mL after extraction. PEI was diluted to 10 mg/mL in DI water and adjusted to pH 7 with concentrated HCl. Each sample contained 0.5 mL of transgenic tobacco extract. The proper amounts of DI water and PEI stock were added as needed to bring the final volume up to 0.6 mL. Total dosage of PEI tested ranged from 0 to 800 mg PEI/g total protein. All precipitation samples were referenced for rGUS activity and total protein to a control sample which contained 0 mg PEI/g total protein and was otherwise treated in the same manner as all other samples containing PEI. Two replicates were run for all precipitation experiments.

After addition of the polyelectrolyte, the samples were vortexed vigorously for 10 s and then allowed to precipitate at room temperature for 30 min. Samples were then centrifuged (Marathon 16 KM, Fisher Scientific) for 20 minutes at 16,400 × g. The supernatant was removed by pipetting and saved for later analysis. The pellets were washed with 1 mL of DI water and then resuspended in 0.15 mL of extraction buffer containing 1.5 M NaCl. Since the pellets could not be resuspended by simply vortexing, the samples were sonicated (Fisher Sonic Dismembrator, Model 500) for 5 s and then re-centrifuged for 5 min at 16,400 × g to re-pellet the unwanted debris. The supernatant was removed from these samples and assayed as "pellet" data. Both the original supernatant and "pellet" samples were assayed for total protein and rGUS activity.

2.5. Chromatography

Chromatography experiments were performed using an AKTA Explorer 100 (GE Healthcare) fast-performance liquid chromatography (FPLC) system controlled by the Unicorn software. For all experiments, an HR 5/10 glass column, 10 cm × 5 cm (i.d.), (GE Healthcare) was packed to a bed height of approximately 5.1 cm (1 mL bed volume).

All flow rates used were 1 mL/min and fractions were collected in 1-mL aliquots. Fractions collected were assayed for rGUS activity and total protein.

Diethylaminoethyl (DEAE Sepharose Fast Flow) was used as the anion exchanger. A total of 2.5 mL of rGUS tobacco extract was loaded to the column. The equilibrating buffer was 50 mM NaPi, pH 7.0 + 10 mM BME (Buffer A1). After the sample was loaded and the flow-through collected, a linear gradient of Buffer B1 (Buffer A1 + 1.0 M NaCl) was applied from 0% to 75% over 40 min.

Phenyl Sepharose 6 Fast Flow (low substitution) was used for hydrophobic interaction chromatography. The equilibrating buffer was 50 mM NaPi, pH 7.0 + 1.5 M ammonium sulfate (AS) (Buffer A2). Proteins were eluted with a linear gradient of 50 mM NaPi, pH 7.0 (Buffer B2) from 0% to 100% B2 over 20 min.

3. Results and discussion

3.1. PEI precipitation

The precipitation profile of rGUS with PEI from transgenic tobacco extract is shown in Fig. 1. The amount of total protein (native tobacco protein) that is co-precipitated with rGUS is shown as well. A distinct precipitation profile can be seen for both total protein and rGUS activity. rGUS is preferentially precipitated before native tobacco proteins with increasing concentrations of PEI, starting around 200 mg PEI/g total protein. Based on GUS activity and protein assays, nearly 100% rGUS activity can be precipitated at high dosages of PEI (700-800 mg PEI/g total protein) with less than 40% total protein co-precipitated. In comparison with GUS precipitation from other plant species, GUS was completely precipitated from canola at 30 mg PEI/g total protein, from soy at 75 mg PEI/g total protein, and from corn at 210 mg PEI/g total protein in a "spiking" experiment [26]. In addition, 100% GUSH6 (GUS with 6 Histidine tails) was precipitated from transgenic pea extract at 100 mg PEI/g total protein, but with 70% of the native pea proteins co-precipitated [43]. The reason why tobacco requires much more PEI for complete precipitation of rGUS is that precipitation was carried out on extracts obtained from green leaf tissue, whereas precipitation from other plant sources was carried out on extracts obtained from ground seed tissue (corn, canola, pea) or dried flakes (soy). Tobacco contains very high amounts of polyphenolics [44] and the total

nucleic acid content is expected to be higher in green leaf tissue than in seed tissue. Furthermore, the total protein contents of the oil seed systems were significantly diluted prior to precipitation experiments. It was reported that upon carrying out precipitation on undiluted canola extract, four times more PEI was needed to precipitate out the same percentage of GUS [26].

Fig. 1 also shows the amount of rGUS activity and corresponding total protein recovered in the pellet for each sample. At 800 mg PEI/g total protein, almost 90% rGUS activity can be recovered, while only 33% total tobacco proteins are simultaneously recovered, leading to an enrichment ratio of 2.7. At 700 mg PEI/g total protein, nearly 85% rGUS activity can be recovered, with less than 30% total protein recovered, giving an enrichment ratio of 3.4. An enrichment ratio of 9.2 occurs at 400 mg PEI/g total protein, but only 53% rGUS activity can be recovered at this PEI dosage. PEI precipitation on canola, soy, and corn resulted in GUS activity recoveries of 80%, 90%, and 81% and enrichment ratios of 18, 1.3, and 2.6 respectively [26]. In addition, PEI precipitation of recombinant GUSH6 yielded near 100% recovery and an enrichment ratio of 1.5 [43]. These data indicate that the effectiveness of initial purification of GUS by PEI precipitation from various plant species is plant dependent.

As PEI concentration increases, there is a larger difference between the amount of rGUS precipitated and the amount of rGUS that can be recovered from the pellet (the gap between the two upper lines becomes wider in Fig. 1). This may be attributed to the complexes formed by PEI with nucleic acids and other native acidic proteins, and the complexes may strongly interact with or trap precipitated GUS. Consequently, pellets could not be resuspended in a high salt solution (0.5 - 2.5 M NaCl) by vortexing alone. Instead, physical disruption by sonication was needed to break up the precipitation pellet. Re-centrifugation was used to remove the smaller particulates not fully disrupted by sonication. This is in contrast to reports of GUS resuspension from other plant sources, including canola, corn, soy, and pea, which was accomplished by vortexing the pellets in a 2.5 M NaCl solution for 10 s [26,43]. Loss of rGUS activity is attributed to the incomplete disruption of the pellet or to conformational changes of the target protein through binding with interfering compounds. However, the inclusion of PVPP and BME in the extraction buffer plays a major role in minimizing phenolic interference and

oxidation, leading to higher activity recoveries. When precipitation experiments were attempted on tobacco extracts not containing PVPP or BME, 10 times less rGUS activity was initially extracted and less than 20% of the expected activity could be recovered from the pellet (data not shown).

The effects of PEI precipitation on rGUS tobacco extract can be seen by SDS-PAGE in Fig. 2. At 700 mg PEI/g total protein, a majority of native tobacco proteins remain in the supernatant. Several major bands can be seen in the recovered pellet fraction, including the rGUS band, which is completely absent from the supernatant fraction. The most notable bands occur at approximately 55 and 15 kDa, which make up a large percentage of native proteins seen in the crude rGUS extract as well. These proteins coincide with ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco), the major chloroplast storage protein found in most all vegetative tissues, and the most abundant protein in the world [45]. Rubisco is a hexadecameric protein with a total molecular weight of 560 kDa consisting of eight large subunits of approximately 50-60 kDa, pI 6.0 and eight small subunits of 12-20 kDa, pI 5.3 [2,45]. The precipitation and subsequent recovery of these proteins is the cause for low enrichment values seen for rGUS precipitation by PEI from tobacco. It is also the cause for large variations in GUS enrichment values, especially in the mid-dosage PEI range (300-600 mg PEI/g total protein) (data not shown). GUS enrichment ratios were more consistent at the low (<200 mg PEI/g total protein) and high (>600 mg PEI/g total protein) ranges where the presumed Rubisco was not precipitated or was largely precipitated, respectively. As a result, the amount of Rubisco extracted in any given experiment and precipitated at any given PEI dosage will directly dictate the precipitation profiles, rGUS enrichment, and to a lesser extent rGUS recovery.

Fig. 3 shows an SDS-PAGE gel with supernatant samples after precipitation with 200, 600, and 1000 mg PEI/g total protein, respectively. From the gel, it is clear that only a handful of native proteins are precipitated over this range and that Rubisco constitutes the mass majority, based on its decreasing band intensity. It has been reported previously that tobacco extract is overall acidic in nature [35]. However, the results reported here may provide new information on the characteristics of native tobacco proteins: (1) a large percentage of acidic tobacco proteins may be constituted by the Rubisco proteins, which

readily precipitates with PEI; or (2) precipitation of other less acidic tobacco proteins may be inhibited by the overwhelming competition of the Rubisco proteins binding to the PEI.

3.2. Anion-exchange chromatography

Anion exchange chromatography was carried out as a direct comparison to evaluate the effectiveness of PEI precipitation in recovering rGUS from transgenic tobacco extract. Fig. 4 shows that the majority of tobacco proteins that bind to the DEAE column are eluted in one major peak with a smaller initial shoulder peak during a simple linear salt gradient elution. A second, smaller, peak occurs afterwards, but contained very small amounts of protein and may likely be nucleic acids and other non-proteinaceous compounds. The elution profile of rGUS significantly overlaps the first major peak, which most likely consists of the Rubisco proteins and other negatively charged native tobacco proteins. Flow-through fractions were pooled and no rGUS activity was detected (data not shown). The accompanying SDS-PAGE gel for the AEX experiments is seen in Fig. 5. Individual fractions that displayed an enrichment factor greater than 1 for rGUS activity were pooled. A negative control AEX experiment was run on non-transformed tobacco extract (*N. tobacum* var. Xanthi) which showed the absence of the rGUS band in the crude extract and in the same pooled fractions, indicating there are no native tobacco proteins that overlap with rGUS, in terms of size. The presence of the Rubisco subunits is still very noticeable in the pooled fractions along with numerous other native tobacco proteins and rGUS. One contrast from the PEI precipitation experiments is that relatively less Rubisco proteins were recovered, but more native tobacco proteins were recovered, especially those of higher molecular weights. This may indicate that Rubisco interferes significantly with PEI precipitation but to a lesser extend with AEX.

Anion exchange chromatography has also been used to purify GUS from other plant sources. One step of anion exchange chromatography was used to purify "spiked" GUS from canola with a yield of 76.6% and an enrichment ratio of 55 [46]. Also, rGUSH6 was purified from transgenic pea with one step of anion exchange chromatography with a yield of 83% and an enrichment ratio of 3.1 [43]. The results

presented here closely follow those of transgenic pea as AEX experiments showed variable rGUS recoveries from 66% to 90% with enrichment ratios between 1.8 and 3.1 over three trials. This may be expected as both systems are overall acidic in nature, unlike canola which is largely basic. Overall, the single PEI precipitation step and anion-exchange step individually show very similar results in terms of overall rGUS recovery and enrichment.

3.3. Two-stage comparison using HIC

In order to investigate the impact of PEI precipitation on the subsequent purification steps, two stage experiments were carried out. A two-stage experiment was performed which consisted of a PEI precipitation step followed by a HIC step. This scheme was compared to a two-stage experiment which consisted of an anion-exchange chromatography step followed by a HIC step. Both experiments started with 2.5 mL of tobacco extract. Precipitation was carried out at 700 mg PEI/g total protein to ensure high rGUS recovery and some initial enrichment. The pellet was resuspended in 1.5 mL of 0.5 M NaCl, sonicated, and re-centrifuged as in previous precipitation experiments. It was found that nearly the same percentage of rGUS activity can be recovered from the pellet when resuspended in buffers with salt concentrations ranging from 0.5 - 2.5 M NaCl (results not shown). Anion-exchange chromatography was carried out as described above and a total of 6 fractions were collected, giving a total volume of 6 mL. Prior to loading to the HIC column, ammonium sulfate (AS) was added to all the samples to give a final concentration of 1.5 M AS. All samples were adjusted up to pH 7 with NaOH after the addition of AS. The HIC fractions were pooled for rGUS activity in the same manner as for the AEX experiments.

Fig. 6 and Fig. 7 show the HIC chromatograms after the precipitation and AEX steps respectively. Both show one main elution peak near the end of the elution gradient. Once again, the rGUS activity peak occurs near the end of the main peak and largely overlaps this peak. Fig. 8 shows the gel for both two-step schemes side-by-side. The final product after HIC looks similar, regardless of whether PEI precipitation or AEX was carried out as a first step. Final recoveries of rGUS for the precipitation-HIC scheme and AEX-HIC scheme were 53.5% and 51.2% respectively in the same final volumes (6 mL

pooled for each). In both cases, several native tobacco proteins with higher molecular weights than rGUS remain. However, at sizes smaller than rGUS, the only major proteins remaining are the two subunits that make up Rubisco. For a total of two trials, the final average enrichment ratios for precipitation-HIC and AEX-HIC schemes were 6.55 and 8.81 respectively. Apparently, the total enrichment of rGUS obtained is almost exclusively dependent on the amount of Rubisco recovered in the final pooled fraction after HIC, which will vary from experiment to experiment. Nevertheless, the presence of the Rubisco proteins may not be a major concern, because the proteins may conceivably be separated from rGUS by an additional size exclusion chromatography step.

4. Conclusion

The use of polyethyleneimine as a first step fractionation procedure is a viable option for the purification of acidic recombinant proteins from transgenic tobacco. PEI precipitation showed highly comparable results to that of AEX chromatography in terms of the range of rGUS recovery, 85-90% versus 66-90% respectively; and range of enrichment ratios, 2.7 - 3.4 versus 1.8 - 3.1 respectively. However, precipitation is much less expensive than chromatography and offers significant concentration of the sample, whereas AEX chromatography dilutes the initial sample more than 2-fold for a comparable recovery. Concentration of the sample by PEI precipitation prior to a chromatography step would greatly reduce overall purification costs.

The main interfering protein that remains after PEI precipitation, AEX chromatography, and hydrophobic chromatography is presumably Rubisco. Recovery of this protein in each step leads to low enrichment ratios of rGUS, even though rGUS recovery was still very good. Recovery of Rubisco also leads to variability in PEI precipitation and AEX data depending on how much of the protein is originally present in the extract and how much is recovered in the fractionation steps. Removal of Rubisco may likely be achieved by a final step of size-exclusion chromatography, although this will undoubtedly reduce total rGUS recovery as well. Nevertheless, this work has shown that incorporating a PEI precipitation step for the recovery of acidic recombinant proteins from tobacco is very realizable with many economic advantages over traditional chromatographic techniques.

Acknowledgement

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References

[1] L. Crosby, Biopharm-Appl. T. Bio. 16 (2003) 60.

[2] T.J. Menkhaus, Y. Bai, C. Zhang, Z.L. Nikolov, C.E. Glatz, Biotechnol. Prog. 20 (2004) 1001.

[3] G.C. Whitelam, J. Sci. Food Agric. 68 (1995) 1.

[4] A. Barta, K. Sommergruber, D. Thompson, K. Hartmuth, M.A. Matzke, A.J.M. Matzke, Plant Mol. Biol. 6 (1986) 347.

[5] L. Frigerio, N.D. Vine, E. Pedrazzini, M.B. Hein, F. Wang, J.K. Ma, A. Vitale, Plant Physiol. 123 (2000) 1483.

[6] H. Khoudi, S. Laberge, J.M. Ferullo, R. Bazin, A. Darveau, Y. Castonguay, G. Allard, R. Lemieux, L.P. Vezina, Biotechnol. Bioeng. 64 (1999) 135.

[7] E.E. Hood, D.R. Witcher, S. Maddock, T. Meyer, C. Baszczynski, M. Bailey, P. Flynn, J. Register, L. Marshall, D. Bond, E. Kulisek, A. Kusnadi, R. Evangelista, Z. Nikolov, C. Wooge, R.J. Mehigh, R. Hernan, W.K. Kappel, D. Ritland, C.P. Li, J.A. Howard, Mol. Breed. 3 (1997) 291.

[8] E. Krebbers, J. Vandekerckhove, Trends Biotechnol. 8 (1990) 1.

[9] J. Vandekerckhove, J. Vandamme, M. Vanlijsebettens, J. Botterman, M. Deblock, M. Vandewiele, A. Declercq, J. Leemans, M. Vanmontagu, E. Krebbers, Nat. Biotechnol. 7 (1989) 929.

[10] J.M. Huang, S. Nandi, L.Y. Wu, D. Yalda, G. Bartley, R. Rodriguez, B. Lonnerdal, N. Huang, Mol. Breed. 10 (2002) 83.

[11] S. Nandi, Y.A. Suzuki, J.M. Huang, D. Yalda, P. Pham, L.Y. Wu, G. Bartley, N. Huang, B. Lonnerdal, Plant Sci. 163 (2002) 713.

[12] E. Stoger, C. Vaquero, E. Torres, M. Sack, L. Nicholson, J. Drossard, S. Williams, D. Keen, Y. Perrin, P. Christou, R. Fischer, Plant Mol. Biol. 42 (2000) 583.

[13] D. Holzmann, Genet. Eng. News 14 (1994) 34.

[14] L. Zeitlin, S.S. Olmsted, T.R. Moench, M.S. Co, B.J. Martinell, V.M. Paradkar, D.R. Russell, C. Queen, R.A. Cone, K.J. Whaley, Nat. Biotechnol. 16 (1998) 1361.

[15] C.L. Cramer, D.L. Weissenborn, K.K. Oishi, E.A. Grabau, S. Bennett, E. Ponce, G.A. Grabowski, D.N. Radin, Ann. N. Y. Acad. Sci. (US) 792 (1996) 62.

[16] J. Pen, L. Molendijk, W.J. Quax, P.C. Sijmons, A.J. van Ooyen, P.J. van den Elzen, K. Rietveld, A. Hoekema, Nat. Biotechnol. (N Y) 10 (1992) 292.

[17] T.C. Verwoerd, P.A. van Paridon, A.J. van Ooyen, J.W. van Lent, A. Hoekema, J. Pen, Plant Physiol. 109 (1995) 1199.

[18] H. Daniell, S.J. Streatfield, K. Wycoff, Trends Plant. Sci. 6 (2001) 219.

[19] R. Fischer, E. Stoger, S. Schillberg, P. Christou, R.M. Twyman, Curr. Opin. Plant Biol. 7 (2004) 152.

[20] O.J.M. Goddijn, J. Pen, Trends Biotechnol. 13 (1995) 379.

[21] L. Jervis, W.S. Pierpoint, J. Biotechnol. 11 (1989) 161.

[22] M.Q. Niederauer, C.E. Glatz, in A. Fiechter (Editor), Advances in Biochemical Engineering/Biotechnology, Springer-Verlag, New York, NY 1992, p. 159.

[23] J.-Y. Shieh, MS Thesis. Department of Chemical Engineering, Iowa State University, Ames, Iowa, 1989.

[24] Y. Dharmadi, Q.L. Chang, C.E. Glatz, Enzyme Microb. Technol. 33 (2003) 596.

[25] P.M.T. Hansen, J. Hidalgo, I.A. Gould, J. Dairy Sci. 54 (1971) 830.

[26] T.J. Menkhaus, S.U. Eriksson, P.B. Whitson, C.E. Glatz, Biotechnol. Bioeng. 77 (2002) 148.

[27] M. Sternberg, D. Hershberger, Biochim. Biophys. Acta. 342 (1974) 195.

[28] F. Zaman, A.R. Kusnadi, C.E. Glatz, Biotechnol. Prog. 15 (1999) 488.

[29] C. Zhang, R. Lillie, J. Cotter, D. Vaughan, J. Chromatogr. A. 1069 (2005) 107.

[30] J. Jendrisak, J. Cell. Biochem. (1987) 166.

[31] J. Simuth, H. Sternbach, J. Zelinka, R.M. Chomutov, A.A. Nedospasov, FEBS Lett. 218 (1987) 163.

[32] B. Egin-Buhler, R. Loyal, J. Ebel, Arch. Biochem. Biophys. 203 (1980) 90.

[33] R. Jerala, L. Kroon-Zitko, V. Turk, Protein Expression Purif. 5 (1994) 65.

[34] W.D. Loomis, Methods Enzymol. 31 (1974) 555.

[35] D. Balasubramaniam, C. Wilkinson, K. Van Cott, C. Zhang, J. Chromatogr. A. 989 (2003) 119.

[36] M. Ni, D. Cui, J. Einstein, S. Narasimhulu, C.E. Vergara, S.B. Gelvin, Plant J. 7 (1995) 661.

[37] M.D. Neve, H.V. Houdt, H.-M. Bruyns, M.V. Montagu, A. Depicker, in C. Cunningham, A.J.R. Porter (Editors), Recombinant proteins from plants: production and isolation of clinically useful compounds, Humana Press, Totawa, NJ, 1998, p. 203.

[38] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473.

[39] S.R. Gallagher, in S.R. Gallagher (Editor), GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. Academic Press, San Diego, CA, 1992.

[40] R.A. Jefferson, S.M. Burgess, D. Hirsh, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 8447.

[41] S. Aich, L.T. Delbaere, R. Chen, BioTechniques 30 (2001) 846.

[42] R.A. Jefferson, K.J. Wilson, in S.B. Gelvin, R.A. Schilperoort, D.P.S. Verma (Editors), Plant Molecular Biology Manual, Kluwer Academic, Dordrecht, The Netherlands, 1991, p. 1.

[43] T.J. Menkhaus, C. Pate, A. Krech, C.E. Glatz, Biotechnol. Bioeng. 86 (2004) 108.

[44] D.L. Davis, M.T. Nielson (Editors), Tobacco: Production, Chemistry and Technology, Blackwell Science, Oxford, 1999, p. 3.

[45] P.R. Shewry, R.J. Fido, in S. Doonan (Editor), Methods in Molecular Biology. Humana Press, Totawa, NJ, 1996, p. 23.

[46] C. Zhang, R.T. Love, J.M. Jilka, C.E. Glatz, Biotechnol. Prog. 17 (2001) 161.

Figures

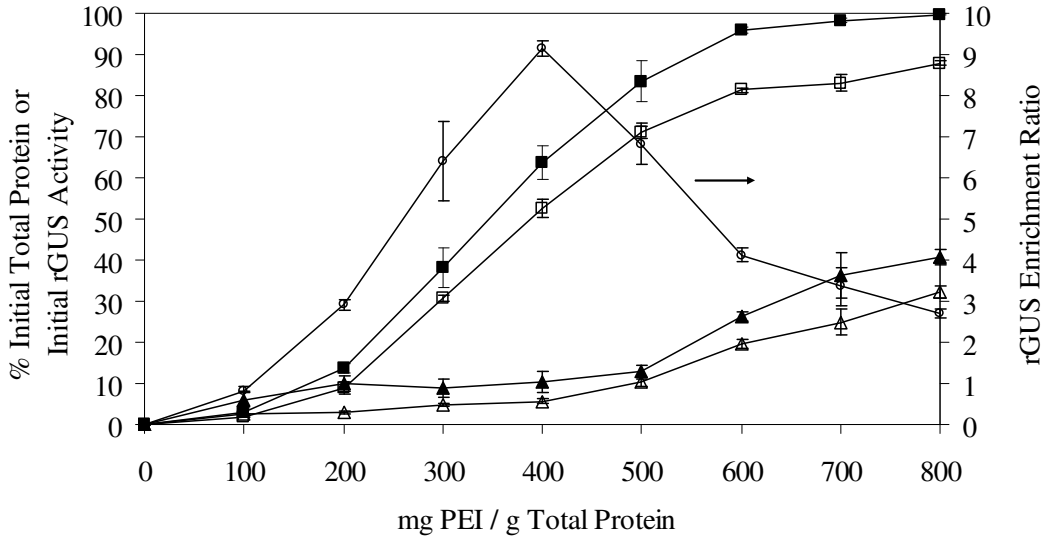

Fig. 1. Amount of recombinant GUS (rGUS) precipitated with PEI from tobacco extract (■) and rGUS activity recovered by dissolving the precipitate in 1.5 M NaCl (□); corresponding total protein precipitated from tobacco extract (▲) and recovered in pellet samples (Δ). The enrichment ratio (○) is shown at each dosage of PEI. Results are an average of two trials with error bars representing the range. Where error bars cannot be seen, the range was smaller than the data point symbol.

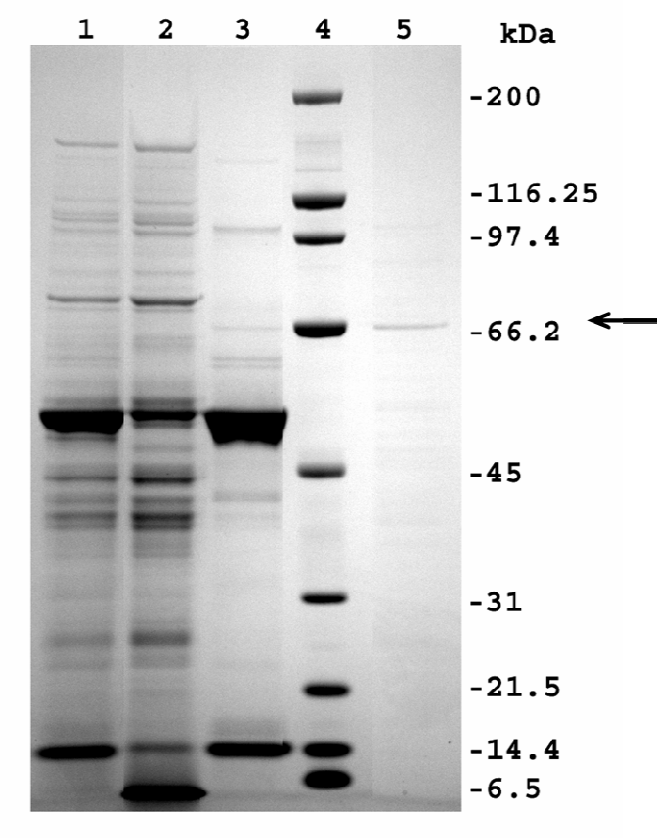

Fig. 2. SDS-PAGE analysis of precipitation of rGUS from tobacco using PEI. Gel was stained with Bio-Safe Coomassie stain. Lane 1: crude rGUS tobacco extract, diluted 5×; lane 2: supernatant fraction after precipitation with 700 mg PEI/g total protein, diluted 2×; lane 3: pellet fraction after precipitation with 700 mg PEI/g total protein, resuspended in 1.5M NaCl and diluted 5×; lane 4: broad-range molecular marker; lane 5: Sigma GUS standard. The arrow points to where the rGUS band is.

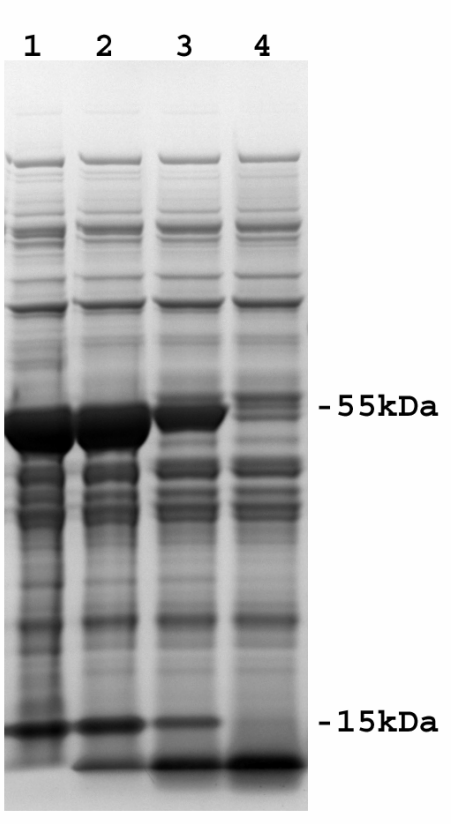

Fig. 3. SDS-PAGE showing Rubisco protein removal from tobacco extract with increasing PEI during precipitation experiments. Gel was stained with Bio-Safe Coomassie stain. Lanes 1–4: supernatant samples after precipitation with 0, 200, 600, and 1000 mg PEI/g total protein, respectively. All samples were diluted 2×. The relative sizes of the large and small subunits of Rubisco are marked.

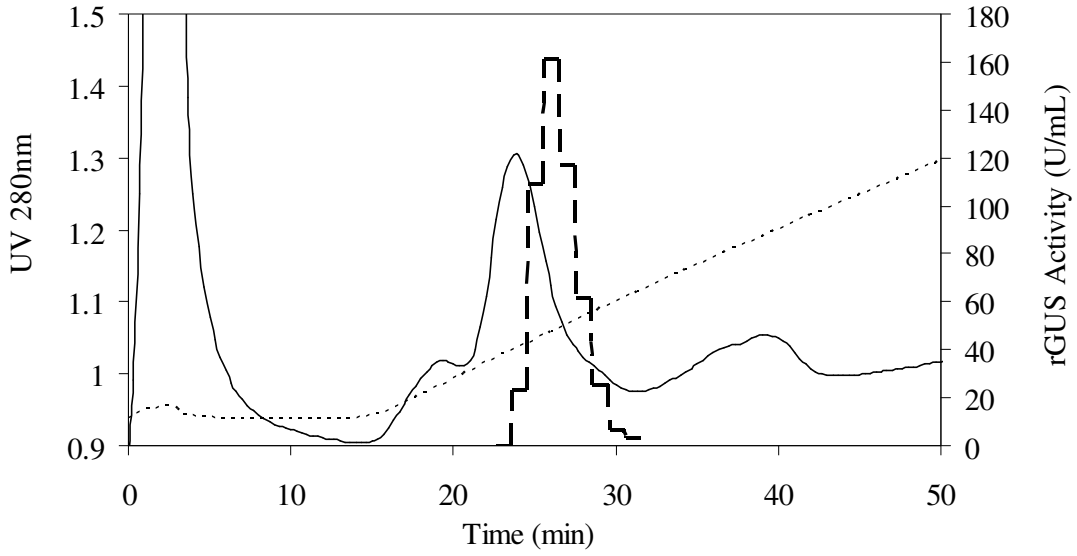

Fig. 4. DEAE anion-exchange chromatography of transgenic tobacco extract and corresponding fraction analysis of rGUS activity. Results were identical for two replicates. The thick dashed line represents the rGUS activity and the thin dotted line represents the elution gradient from 50mM NaPi + 10mM BME + 1mM EDTA, pH 7.0 (Buffer A1) to 75% Buffer B1 (A1 + 1.0 mM NaCl) over 40 min. Total extract volume loaded to the column was 2.5 mL.

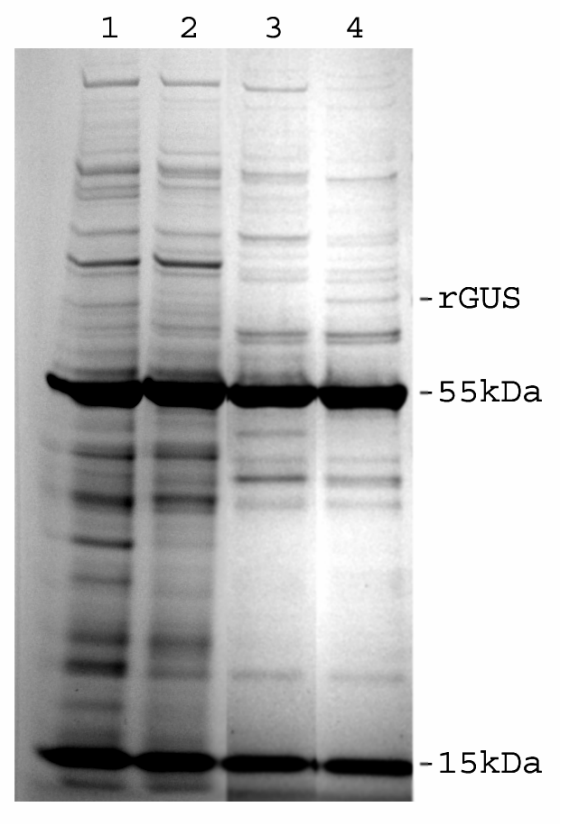

Fig. 5. SDS-PAGE of samples before and after anion exchange chromatography. Gel was stained with Bio-Safe Coomassie stain. Lane 1: non-transgenic crude Xanthi tobacco extract used as a negative control, diluted 5×; lane 2: crude rGUS extract, diluted 5×; lane 3: Xanthi (negative control) AEX pooled fraction comparable to pooled transgenic fractions; lane 4: rGUS AEX pooled fraction. The relative sizes of the large and small subunits of Rubisco are marked as well as the location of the rGUS band.

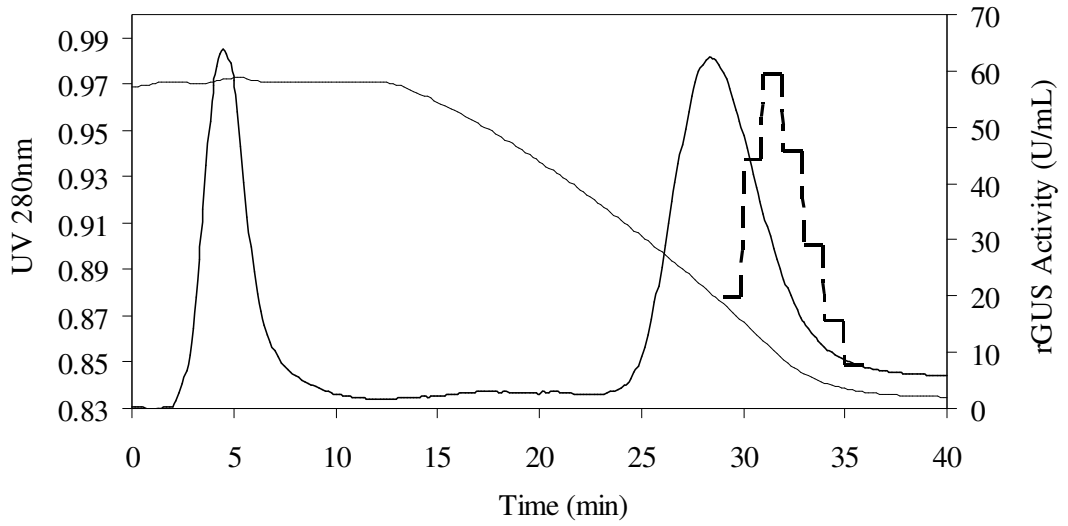

Fig. 6. Hydrophobic interaction chromatography of transgenic tobacco extract after precipitation with 700 mg PEI/g total protein. Approximately 1.5mL of sample was loaded to the column after PEI precipitation. The thick dashed line represents the rGUS activity and the thin dotted line represents the elution gradient from 50 mM NaPi + 1.5 M ammonium sulfate, pH 7.0 to 50 mM NaPi, pH 7.0.

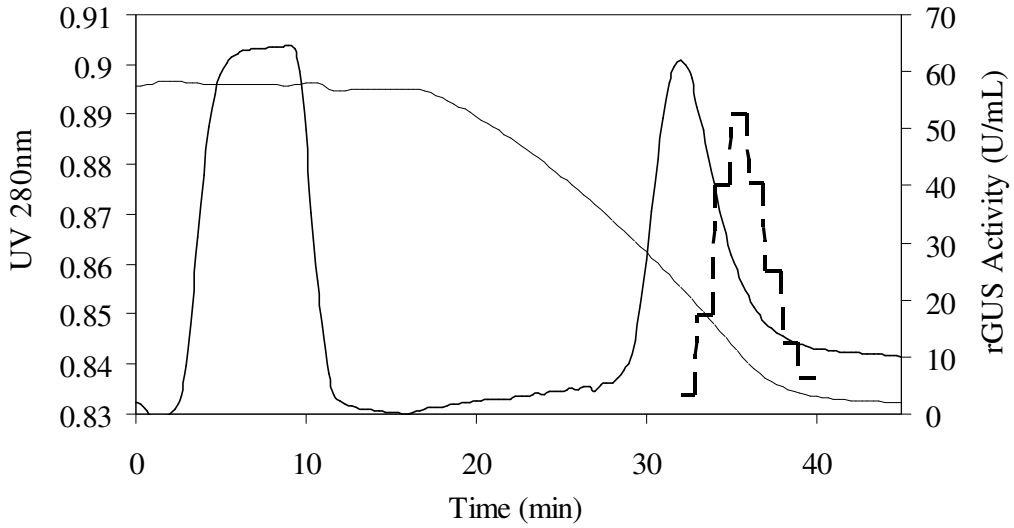

Fig. 7. Hydrophobic interaction chromatography of recombinant tobacco extract after anion exchange chromatography. Approximately 6mL of sample was loaded to the column after anion-exchange chromatography (DEAE). The thick dashed line represents the rGUS activity and the thin dotted line represents the elution gradient from 50mM NaPi + 1.5 M ammonium sulfate, pH 7.0 to 50 mM NaPi, pH 7.0.

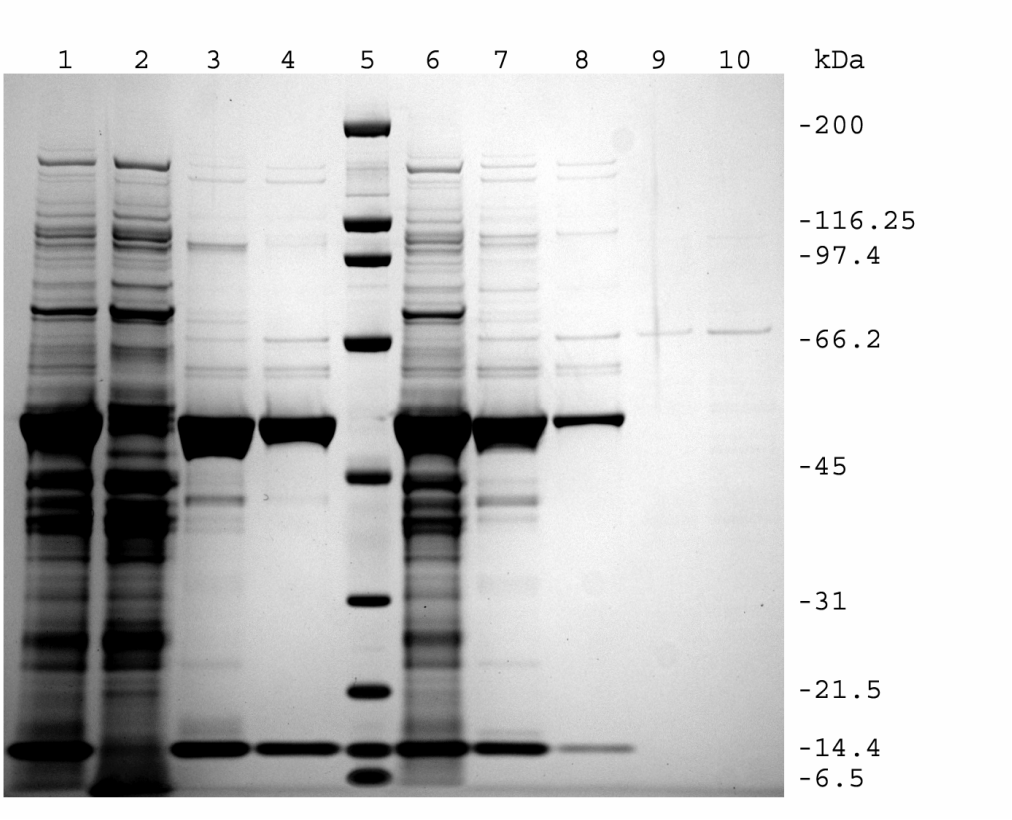

Fig. 8. SDS-PAGE of two-step purification schemes. Gel was stained with Bio-Safe Coomassie stain. Lanes 1,6: rGUS crude tobacco extract, diluted 2×; lane 2: supernatant fraction after precipitation with 700 mg PEI/g total protein; lane 3: pellet fraction after precipitation with 700 mg PEI/g total protein, resuspended in 0.5 M NaCl and diluted 2×; lane 4: final precipitation sample after HIC, concentrated 10×; lane 5: broad-range molecular marker; lane 7: AEX pooled fractions, concentrated 4×; lane 8: final AEX sample after HIC, concentrated 10×; lanes 9 and10: Sigma GUS standard. Concentration of samples was achieved with YM-10 Microcon centrifugal filter devices (MWCO 10,000).

Chapter Four

Development of a process for the purification of an acidic recombinant protein from transgenic tobacco*

Chris Holler, Chenming Zhang

A manuscript to be submitted to Biotechnology and Bioengineering

*work under patent consideration

Abstract

Tobacco has proven to be a promising alternative for the production of recombinant therapeutic proteins and offers numerous advantages over other plants as a host system. However, the recovery and purification steps needed to obtain a protein at high recovery and purity have not been well investigated. In this study, a process was developed to purify a model acidic protein, recombinant β-glucuronidase (rGUS), from transgenic tobacco leaf tissue, in three main steps after extraction: polyelectrolyte precipitation, hydrophobic interaction chromatography (HIC), and hydroxyapatite chromatography (HAC). Using this three-step process, up to 40% of the initial rGUS activity could be recovered to near homogeneous purity as judged by SDS-PAGE. This work demonstrates that acidic recombinant proteins expressed in tobacco may be purified to high yield with high purity in a minimal amount of steps that are suitable for scale-up. Furthermore, the general steps used in this process may suggest that a wide variety of acidic recombinant proteins may be purified in a similar manner from transgenic tobacco or other leafy crops.

Keywords: Transgenic tobacco; Recombinant protein; Downstream processing; Precipitation; Chromatography; β-glucuronidase

Introduction

Expression of recombinant therapeutic proteins in transgenic plants may have a tremendous impact on the biopharmaceutical industry and crop production around the world. Numerous recombinant proteins including antibodies, vaccines, hormones, and growth regulators have already been expressed in crops such as corn, rice, soybean, and tobacco, among many others. Tobacco, in particular, is an attractive host for the commercial production of recombinant therapeutic proteins (Stoger et al. 2002a; Stoger et al. 2002b). It is a non-food and non-feed crop so the threat of contamination of a food or feed supply is minimized. Furthermore, an abundance of biomass can be produced by simply planting more transgenic plants if the protein is expressed in the leaf tissue (Daniell et al. 2001). As with all plants, tobacco does not harbor human pathogens, which provides a safer alternative to mammalian or bacterial production systems.

Even if recombinant therapeutic proteins can be expressed efficiently in tobacco, there is still a great challenge in developing tobacco as an effective and economical host for producing these proteins on a commercial basis. Since therapeutic proteins expressed in tobacco can not be delivered orally, the protein must be purified to high purity for use in clinical trials. Downstream processing, which includes extraction and purification of the target protein, is estimated to account for 80% or more of the overall production cost (Kusnadi et al. 1998b). In addition, detailed protein purification methods are not-well studied in tobacco, or in other leafy crops (Nikolov and Woodard 2004).

An aqueous extract obtained from tobacco vegetative tissue is a complex mixture of native plant proteins, carbohydrates, nucleic acids, and other impurities such as phenolics and alkaloids that must be separated from the target protein. It has been shown that the overall nature of a tobacco extract is acidic in nature (Balasubramaniam et al. 2003). The overall acidity is largely attributed to the presence of the chloroplast storage protein, ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). Rubisco is found in most vegetative tissue and is the most abundant protein in the world. In tobacco, Rubisco may account for up to 50% of the total soluble protein (Garger et al. 2000; Shewry and Fido 1996). Rubisco is a hexadecameric protein with eight large subunits (average MW 50-60 kD, pI 6.0) and eight small subunits (average MW 12-20 kD, pI 5.3) (Menkhaus et al. 2004; Shewry and Fido 1996). Thus, purification of an acidic recombinant protein from tobacco may be more challenging than purification of a basic protein

(Balasubramaniam et al. 2003). Table I shows a sampling of acidic therapeutic proteins that have been expressed in tobacco which would greatly benefit from efficient purification processes.

In addition to native plant proteins, nucleic acids, and toxic alkaloids such as nicotine, the presence of phenolics in a tobacco extract could be problematic for protein purification. Phenolics may form complexes with proteins or interfere with adsorption processes such as column chromatography (Cheryan 1980; Jervis and Pierpoint 1989; Kusnadi et al. 1998a). The inclusion of a reducing agent such as β-2-mercaptoethanol (BME) or dithiothreitol (DTT) as well as a phenolic binding agent such as polyvinylpolypyrrolidone (PVPP) in an extraction buffer was needed to obtain stable and active recombinant β-glucuronidase (rGUS) extracted from transgenic tobacco leaves (Holler et al. 2007).

Incorporating a scalable non-chromatographic step early in a purification scheme may be advantageous for purifying recombinant proteins from large amounts of transgenic tobacco (Menkhaus et al. 2004). Application of a crude tobacco extract to a chromatography column is not feasible due to column fouling and plugging over extended use. Polyelectrolyte precipitation with polyethyleneimine (PEI) has been shown to be a promising method for removing large amounts of native tobacco impurities as well as providing high recovery and concentration of an acidic recombinant protein in preparation for chromatography steps (Holler et al. 2007).

Numerous methods for purifying recombinant therapeutic proteins from transgenic tobacco rely heavily on molecular modification of the protein or affinity steps (Desai et al. 2002; Lige et al. 1998; Mejare et al. 1998; Sehnke and Ferl 1999). However, these processes are expensive due to their high specificity and may not be feasible for large scale commercial production of therapeutic proteins. Therefore, it may be beneficial to develop purification schemes that utilize more general chromatography steps such as ion-exchange, hydrophobic interaction, and hydroxyapatite that can be used to purify a wide range of proteins.

In this study, a general and potentially scalable process has been developed for the purification of an acidic recombinant protein, β-glucuronidase (rGUS) from transgenic tobacco leaf tissue (Figure 1). The process outlines the extraction and recovery of rGUS

into a crude aqueous tobacco extract followed by a purification process that has three major steps including polyelectrolyte precipitation, hydrophobic interaction chromatography, and hydroxyapatite chromatography.

Materials and Methods

Transgenic tobacco growth and protein extraction

Transgenic tobacco plants were grown as described previously (Holler et al. 2007). Fresh tobacco leaves were excised from a plant each time an extract was prepared and the tissue was weighed out and placed in a 50 mL conical tube. The extraction buffer consisted of 50 mM sodium phosphate (NaPi), pH 7 + 10 mM BME + 1mM EDTA and all extractions were carried out at a ratio of 1:5 (w/v) at ice cold temperature. The sample was homogenized until no large particulates remained (approximately one minute). Immediately after homogenization, the sample was decanted to another tube containing a 2% (w/v) pre-hydrated PVPP solution. The extract was then vortexed vigorously and allowed to set at room temperature for 15 min. After centrifugation at 4 ºC and 17,003 × g for 20 minutes, the supernatant was removed and filtered through a 0.22-µm syringe filter.

Polyelectrolyte precipitation

Polyethyleneimine (PEI; long chain average MW 750 kD; 50% (w/v) aqueous solution from Sigma) was diluted to 10 mg/mL in deionized (DI) water and adjusted to pH 7 with concentrated HCl. This solution was added to 7 mL extract at a dosage of 800 mg PEI/g total protein. The necessary amounts of DI water and PEI stock were added to bring the final volume up to 8.4 mL.

After addition of the polyelectrolyte, the samples were vortexed vigorously for 10 s and then allowed to precipitate at room temperature for 30 min. Samples were then centrifuged for 20 min at 17,003 × g at room temperature. The supernatant was removed and saved for later analysis. The pellets were washed with 1 mL of DI water and then 1.5 mL of resuspension buffer (50 mM NaPi, pH 7.0 + 10 mM BME + 1 mM EDTA + 0.5 M NaCl) was added. Since the pellets could not be resuspended by simply vortexing, the samples were sonicated (Fisher Sonic Dismembrator, Model 500) for five seconds and

then re-centrifuged for 10 min at 17,003 × g to re-pellet the unwanted debris. The supernatant was removed from these samples and centrifuged again at 16,400 × g for 10 min (Marathon 16 KM, Fisher Scientific). The supernatant from this final step was collected and referred to as the "pellet" fraction.

FPLC chromatography

Chromatography experiments were performed using an ÄKTA Explorer 100 (GE Healthcare, Uppsala, Sweden) fast-performance liquid chromatography (FPLC) system controlled by the Unicorn software (version 3.10).

Phenyl Sepharose 6 Fast Flow (low substitution) was purchased from GE Healthcare and used for hydrophobic interaction chromatography. An HR 5/10 glass column (GE Healthcare), 10 cm × 5 cm (i.d.), was packed to a bed height of approximately 5.1 cm (1 mL bed volume). The equilibrating buffer was 50 mM NaPi, pH 7.0 + 1.5 M ammonium sulfate (AS) (Buffer A1). Proteins were eluted with a linear gradient of 50 mM NaPi, pH 7.0 (Buffer B1) from 0% to 100% B1 over 10 min. All flow rates used were 1 mL/min and fractions were collected in 2 mL aliquots.

CHT ceramic hydroxyapatite (Macro-prep Type I; 80 μm) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The resin was resuspended in 200 mM sodium phosphate buffer, pH 9 and packed as a slurry in a Tricorn column (GE Healthcare), 10 cm × 1 cm (i.d.), to a bed height of approximately 10.2 cm (8 mL bed volume). The equilibrating buffer was 10 mM NaPi, pH 6.8 (Buffer A2). Proteins were eluted over a linear gradient with 400 mM NaPi, pH 6.8 (Buffer B2) as the elution buffer. All flow rates used were 1 mL/min and fractions were collected in 1 mL aliquots.

Collected fractions were pooled and concentrated when needed in either Microcon (YM-10, MWCO 10,000) or Amicon Ultra (5,000 MWCO) centrifugal filter devices purchased from Millipore (Bedford, MA, USA).

Analytical methods

Protein concentration was determined by Bio-Rad assay (microtiter procedure) with bovine serum albumin (BSA) as the standard. All assays were carried out in Greiner 96-well, clear, flat bottom microtiter plates from USA Scientific (Ocala, FL, USA) and

performed in duplicates. Absorbance measurements were read at 595 nm on a Bio-Tek Synergy microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

GUS activity was measured as reported previously (Holler et al. 2007). This assay utilizes the ability of GUS to hydrolyze *p*-nitrophenyl β-D-glucuronide (PNPG) to release chromophore *p*-nitrophenol (PNP), and GUS activity is reported in units of activity (U)/mL (Jefferson and Wilson 1991). One unit of GUS activity was defined as the amount needed to liberate one nmol PNP/minute at room temperature and pH 7.0. Kinetic absorbance measurements were read at room temperature and 405 nm every 50 s for a total of eight minutes on a microplate reader.

SDS-PAGE samples were reduced with dithiothreitol (DTT) and run on 4-12% Novex Bis-Tris mini gels with MOPS as the running buffer obtained from Invitrogen (Carlsbad, CA, USA). After running, the gels were washed with DI water and bands were visualized using Bio-Safe Coomassie stain (Bio-Rad) or SilverQuest staining kit (Invitrogen). The gels were scanned with a Bio-Rad ChemiDoc XRS imager and analyzed using Quantity One Software.

Results and Discussion

Protein extraction from transgenic tobacco

The product recovery step is one of the most important steps in downstream processing as it will dictate how much initial protein is present for purification. The aim of the recovery step is to maximize target protein extraction into an aqueous medium while minimizing protein degradation. For many studies, an extract can be obtained by grinding frozen leaf tissue under liquid nitrogen followed by addition of an appropriate extraction buffer. However, this process is not feasible for large-scale extraction on large amounts of tobacco leaf biomass. Therefore, extraction was accomplished by homogenization which sheared the leaf tissue in an aqueous buffer. During this stage, there are several important factors to consider for minimizing protein degradation. First, 10 mM BME was added to the buffer prior to homogenization to keep the environment in a reduced state, preventing harmful oxidation that may alter the foreign protein's structure and render it inactive. Second, the buffer was kept at ice-cold temperature prior to homogenization to minimize proteolysis or protein denaturing due to increased

temperature during the homogenization process. Third, immediately after homogenization, the solution was added to pre-hydrated PVPP. The PVPP serves to bind free phenolics that may otherwise form complexes with proteins or foul chromatographic columns later used in the purification process. This is a critical step in tobacco protein extraction as tobacco can contain extremely high amounts of phenolics, up to 30 mg/g dry weight (Davis and Nielson 1999). Because PVPP is insoluble, it is easily removed in the pellet during the centrifugation step. After centrifugation, the remaining fine particulates are removed via filtration through a 0.22-μm syringe filter. Over nine independent experiments, the average starting total protein in a tobacco extract was 1.37 ± 0.54 mg/mL and the average initial rGUS activity was 128.2 ± 33.1 U/mL.

In the experiments conducted for this study, rGUS extraction was not optimized, but several different combinations of extraction buffers were investigated before a simple three component system was chosen. The combination of low salt (50 mM sodium phosphate), a reducing agent (10 mM BME), and an anti-chelating agent (1 mM EDTA) was found to consistently provide high amounts of initial rGUS activity. Additional components such as protease inhibitors (phenylmethylsulphonyl fluoride, PMSF) or detergents (Triton X-100, Tween 20, Sarcosyl) may be needed for some extraction procedures to minimize protease degradation or disrupt cellular membranes respectively, but these additives must be removed during the purification process.

PEI precipitation

After a transgenic tobacco extract was obtained, the first main step in the purification process was polyelectrolyte precipitation. Polyethyleneimine was added at a dosage of 800 mg PEI/g total protein to ensure near complete precipitation of rGUS and maximum recovery in the pellet fraction, as reported previously (Holler et al. 2007). While a dosage of 800 mg PEI/g total protein results in near complete precipitation of rGUS, a number of other native tobacco proteins co-precipitate, most notably the acidic chloroplast storage protein, ribulose 1,5-bisphosphate carboxylase-oxygenase (Rubisco). Increasing the PEI dosage will effectively increase the amount of Rubisco co-precipitated with rGUS, leading to modest enrichment values (Holler et al. 2007). We had previously reported that sonication and subsequent centrifugation of the pellet was necessary after

the addition of resuspension buffer to adequately recover rGUS from the precipitated pellet (Holler et al. 2007). An additional step of centrifugation was performed here in order to remove fine particulates before chromatography runs. This step did not significantly affect rGUS recovery or enrichment values of the precipitation step.

For the experiments reported here, the PEI precipitation data is presented in Table II. The PEI precipitation step was an important step in the purification process because it removed large amounts of impurities (approximately 65% native tobacco proteins) which should help improve column life and performance in later chromatography steps. Also, more than 95% of the target protein was recovered on average and the sample volume was greatly reduced (here 8.4 mL to 1.5 mL for a 5.6 × concentration) before chromatographic separation.

HIC optimization

Hydrophobic interaction chromatography (HIC) was carried out as the second main step in the purification scheme. After PEI precipitation, the sample (1.5 mL) was applied directly to the HIC column with no additional salt added. The sample was loaded to the column followed by an additional 2 mL of equilibration buffer (A1) on top of the sample, which ensured sufficient salt for binding. Our previous results suggest that a HIC step after PEI precipitation could not efficiently separate rGUS from many native tobacco proteins (i.e. Rubisco), leading to an enrichment ratio of approximately 6.55 with only 53.5% recovery (Holler et al. 2007). Octyl Sepharose FF and Butyl Sepharose FF resins were also investigated, but separation was not achieved and recoveries were lower than with Phenyl Sepharose FF (low substitution) (data not shown). Therefore, it was concluded that HIC chromatography would not be able to provide adequate separation to produce a high purity rGUS.

The HIC step was still found to serve a valuable purpose in the overall purification scheme. Several fractions collected during the flow-through were a light brownish color with extreme cloudiness observed upon diluting with low salt buffer. This suggests that both color-containing pigments (chlorophyll) and PEI are removed during this process. In addition, it is believed that a large amount of nucleic acids were also removed, either bound to PEI or in isolated form, as indicated by the large peak

observed at 215 nm on the chromatogram during the flow-through (data not shown). Other methods were attempted to remove PEI and nucleic acids including de-salting column and dialysis; however, in both cases precipitation of the proteins occurred and rGUS activity was not recorded. As a result, the HIC step also served as the only viable process for lowering the salt concentration in preparation for hydroxyapatite chromatography.

The HIC step in this process was thus optimized for maximum rGUS recovery with little regard for additional purification (enrichment) after PEI precipitation (Figure 2). The results of three identical HIC optimization trials are shown in Table III. Nearly 78% of the initial rGUS activity could be recovered after the HIC step (83.34% of the total activity loaded from the PEI step). The enrichment values were not calculated and the total volume collected from the HIC step was 10-12 mL (5-6 fractions).

Complete purification process

It was anticipated that size-exclusion chromatography could be used as a third step to completely separate rGUS from Rubisco based on the size of the polymeric forms of both proteins which are 270 and 560 kD, respectively (Holler et al. 2007). However, several runs using Sephacryl S-300 HR resin (globular protein fractionation range, MW 10 kD – 1500 kD packed in an XK 16/20 column, G.E. Healthcare) yielded little additional separation of Rubisco and rGUS after PEI precipitation and HIC, respectively (data not shown). Therefore, ceramic hydroxyapatite resin was investigated as an alternative chromatography step.

Hydroxyapatite (HA) can be used for the binding of acidic proteins and has the potential for scale-up. A low salt sample is required for the binding of proteins to the HA resin (10 mM NaPi). As up to 12 mL of sample was collected after the HIC step, it was desired to reduce the sample volume before loading to the HA column. Therefore, the samples were concentrated to less than 1 mL with Amicon Ultra-15 centrifugal filter devices and then subsequently diluted with DI water to ensure a NaPi concentration close to 10 mM and the estimated ammonium sulfate concentration to less than 100 mM. The total volume loaded to the HA column was approximately 3.5 mL. Preliminary experiments showed that rGUS may be separated from Rubisco and other native tobacco

proteins, at least partially, on a hydroxyapatite (HA) column after subsequent steps of PEI precipitation and HIC (Figure 3a). A simple linear elution gradient of 0% to 100% B2 over 80 minutes yielded between 40-50% of the initial rGUS activity during the elution. The pooling of 5 fractions resulted in a homogeneous band on SDS-PAGE which corresponded to nearly 25% of the initial rGUS activity (Figure 3b).

Recombinant GUS begins to elute just prior to the native tobacco proteins during hydroxyapatite chromatography separation (Figure 3a). Optimization by extended gradient over the salt concentration where proteins eluted (8% to 25% B over 60 min) in previous runs yielded greatly improved peak separation from the initial hydroxyapatite run (Figure 4a). For the optimized run, total rGUS activity recovered was still 50% for 28 fractions. Fractions 5-24 in Figure 4b show the overlap of rGUS and native tobacco proteins. Fractions 1-16 were pooled, which corresponded to nearly 40% of the initial rGUS activity, and run on SDS-PAGE (Figure 5a). Figure 5a shows the Coomassie stained image of the entire purification scheme and Figure 5b shows the silver stained (30× more sensitive than Coomassie for protein detection) lane of the purified rGUS fractions showing minor impurities. Therefore, this simple optimization procedure increased purified rGUS activity recovery significantly from 25% to 40%. Further optimization of this step may lend itself to recovering the maximum amount of rGUS as a pure fraction, up to 50% of the initial protein activity. The final enrichment ratio could not be calculated due to the fact that such a small amount of purified protein existed in the final sample. The results for the entire purification scheme are presented in Table IV. The results obtained from this study are quite remarkable when considering the fact that no affinity purification methods were used, keeping the overall scheme general for potential use with a wide range of acidic proteins expressed in tobacco or other leafy crops. In addition, it is anticipated that all of the steps incorporated in this process are scalable for large-scale commercial protein purification.

Conclusion

It has been shown here that an acidic recombinant protein (rGUS) can be efficiently purified from transgenic tobacco to high yield and purity in just three main steps after the initial extraction. The PEI precipitation step served as an effective non-

chromatographic step for initial fractionation and concentration of the target protein. The hydrophobic interaction chromatography step served the purpose of removing impurities such as PEI and nucleic acids obtained in the first step. The hydroxyapatite chromatography step served as the 'polishing' step where rGUS was effectively separated from the remaining native tobacco proteins, most notably Rubisco. Approximately 40% product yield (based on the initial rGUS activity) could be recovered after the final step, excluding the final concentration step needed for band visualization on SDS-PAGE. The rGUS was recovered to near homogeneous purity as judged by Coomassie and silver stained SDS-PAGE. These results suggest that acidic recombinant proteins may be efficiently purified from a transgenic tobacco extract in a minimum number of general steps. All of the steps incorporated in the suggested purification scheme have the potential to be scaled up for large-scale protein production.

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References

Balasubramaniam D, Wilkinson C, Van Cott K, Zhang C. 2003. Tobacco protein separation by aqueous two-phase extraction. J Chromatogr A 989(1):119-29.

Beachy RN, Fitchen JH, Hein MB. 1996. Use of plant viruses for delivery of vaccine epitopes. Engineering Plants for Commercial Products and Applications 792:43-49.

Cheryan M. 1980. Phytic acid interactions in food systems. Crit Rev Food Sci Nutr 13(4):297-335.

Cramer CL, Boothe JG, Oishi KK. 1999. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. Curr Top Microbiol Immunol 240:95-118.

Daniell H, Streatfield SJ, Wycoff K. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6(5):219-26.

Davis DL, Nielson MT. 1999. Tobacco: Production, Chemistry and Technology. Davis DL, Nielson MT, editors. Oxford: Blackwell Science. 3 p.

Desai UA, Sur G, Daunert S, Babbitt R, Li Q. 2002. Expression and affinity purification of recombinant proteins from plants. Protein Expr Purif 25(1):195-202.

Discipio RG, Davie EW. 1979. Characterization of Protein S, a Gamma-Carboxyglutamic Acid Containing Protein from Bovine and Human-Plasma. Biochemistry 18(5):899-904.

Fernandez-San Millan A, Mingo-Castel A, Miller M, Daniell H. 2003. A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. Plant Biotechnology Journal 1(2):71-79.

Ganz PR, Sardana R, Dudani AK, Tackaberry E, Sauder C, Altosaar I. 1996. Expression of human blood proteins in transgenic plants: the cytokine GM-CSF as a model protein. In: Owen MRL, Pen J, editors. Transgenic plants: a production system for industrial and pharmaceutical proteins. London, UK: John Wiley & Sons. p 281-287.

Garger SJ, Holtz RB, McCulloch MJ, Turpen TH. 2000. Process for isolating and purifying viruses, soluble proteins, and peptides from plant sources. U. S. Patent 6,033,895.

Goddijn OJM, Pen J. 1995. Plants as Bioreactors. Trends in Biotechnology 13(9):379-387.

Hamamoto H, Sugiyama Y, Nakagawa N, Hashida E, Matsunaga Y, Takemoto S, Watanabe Y, Okada Y. 1993. A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. Biotechnology (N Y) 11(8):930-2.

Haq TA, Mason HS, Clements JD, Arntzen CJ. 1995. Oral Immunization with a Recombinant Bacterial-Antigen Produced in Transgenic Plants. Science 268(5211):714-716.

Holler C, Vaughan D, Zhang C. 2007. Polyethyleneimine precipitation versus anion exchange chromatography in fractionating recombinant beta-glucuronidase from transgenic tobacco extract. J Chromatogr A 1142(1):98-105.

Jefferson RA, Wilson KJ. 1991. The GUS gene fusion system. In: Gelvin SB, Schilperoort RA, Verma DPS, editors. Plant Molecular Biology Manual. Dordrecht, The Netherlands: Kluwer Academic. p 1-33.

Jervis L, Pierpoint WS. 1989. Purification Technologies for Plant-Proteins. Journal of Biotechnology 11(2-3):161-198.

Kisiel W, Davie EW. 1981. Protein C. Methods Enzymol 80:320-332.

Kusnadi AR, Evangelista RL, Hood EE, Howard JA, Nikolov ZL. 1998a. Processing of transgenic corn seed and its effect on the recovery of recombinant beta-glucuronidase. Biotechnol Bioeng 60(1):44-52.

Kusnadi AR, Hood EE, Witcher DR, Howard JA, Nikolov ZL. 1998b. Production and purification of two recombinant proteins from transgenic corn. Biotechnol Prog 14(1):149-55.

Lige B, Ma SW, Zhao DL, van Huystee RB. 1998. Cationic peanut peroxidase: Expression and characterization in transgenic tobacco and purification of the histidine-tagged protein. Plant Science 136(2):159-168.

Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. 1996. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. Proceedings of the National Academy of Sciences of the United States of America 93(11):5335-5340.

Mejare M, Lilius G, Bulow L. 1998. Evaluation of genetically attached histidine affinity tails for purification of lactate dehydrogenase from transgenic tobacco. Plant Science 134(1):103-114.

Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE. 2004. Considerations for the recovery of recombinant proteins from plants. Biotechnol Prog 20(4):1001-14.

Nikolov ZL, Woodard SL. 2004. Downstream processing of recombinant proteins from transgenic feedstock. Curr Opin Biotechnol 15(5):479-86.

Sehnke PC, Ferl RJ. 1999. Processing of preproricin in transgenic tobacco. Protein Expr Purif 15(2):188-95.

Shewry PR, Fido RJ. 1996. Protein extraction from plant tissues. In: Doonan S, editor. Methods Mol Biol. Totawa, New Jersey: Humana Press. p 23-9.

Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ, Hoekema A. 1990. Production of correctly processed human serum albumin in transgenic plants. Biotechnology (N Y) 8(3):217-21.

Stoger E, Sack M, Fischer R, Christou P. 2002a. Plantibodies: applications, advantages and bottlenecks. Curr Opin Biotechnol 13(2):161-6.

Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R. 2002b. Practical considerations for pharmaceutical antibody production in different crop systems. Molecular Breeding 9(3):149-158.

Tables

Table I. Acidic therapeutic proteins expressed in transgenic tobacco.

Protein†	Potential application	MW (kD) subunit	pI	Expression Level‡	Source(s)
Human protein C (serum protease)	Protein C pathway	62[a]	4.4 - 4.8[b]	< 0.01% TSP[c]	(Kisiel and Davie 1981)[a] (Discipio and Davie 1979)[b] (Cramer et al. 1999)[c]
Norwalk capsid protein	Norwalk virus	58	5.25 - 5.5*	0.23% TSP	(Mason et al. 1996)
Angiotensin-I-converting enzyme inhibitor (coat protein-ACEI complex)	Hypertension	1.38*	6.00*	100 µg/g of fresh tissue	(Hamamoto et al. 1993)
Binding subunit of E. coli heat-labile enterotoxin (LT-B)	Cholera and E. coli diarrhea	11.6	5.05*	0.001% of TSP	(Haq et al. 1995)
Human serum albumin	Liver cirrhosis	67 - 69	5.85*	0.02% TSP (nuclear)[d], 11.1% (chloroplast)[e]	(Sijmons et al. 1990)[d] (Fernandez-San Millan et al. 2003)[e]
c-Myc	Cancer	49*	5.33*	Not reported	(Beachy et al. 1996)
Human granulocyte-macrophage colony-stimulating factor	Neutropenia	14.5*	5.21*	Not reported	(Ganz et al. 1996) (Goddijn and Pen 1995)

† For comparison: GUS MW 62.2 kD/subunit, ~270 kD total; pI 5.5.
‡ TSP, total soluble protein.
* Not reported in manuscript; theoretical values estimated using ExPASy calculator.

Table II. PEI precipitation data. Data is for a total of nine independent experiments.

	Total Protein (mg/mL)	**% total protein recovered in pellet**	**Enrichment**	**rGUS Recovery**
Average	1.37	36.05%	2.72	96.41%
Standard Deviation	0.54	5.64%	0.47	6.42%

Table III. HIC optimization data.

	Trial 1	Trial 2	Trial 3	Average	stdev
Starting total protein (mg/mL)	0.73	0.92	0.65	0.77	0.14
% rGUS activity recovered after PEI precipitation	92.84	97.12	94.48	94.81	2.16
Enrichment ratio after PEI precipitation	2.61	2.60	3.1	2.8	0.28
% rGUS activity recovered in flow-through (from original)	4.37	9.03	3.03	5.48	3.15
% rGUS activity recovered in pooled fractions (from original)	77.00	80.86	76.07	77.98	2.54
Total volume of pooled fractions after HIC (mL)	12	10	12		

Table IV. Data from the full purification scheme of rGUS from transgenic tobacco leaf tissue.

Step	Tot. Act. (U)	Final Vol. (mL)	E.R.	Recovery
Extraction	862.44	7	-	100.00%
PEI Precipitation	808.45	1.5	3.10	93.74%
HIC fractions (before concentration)	724.10	6	6.22	83.96%
HIC fractions (after concentration / dilution)	662.20	3.5	4.92	76.78%
HAC fractions (total rGUS)	438.42	28	-	50.84%
HAC fractions (purified rGUS)	349.66	16	-	40.54%

Figures

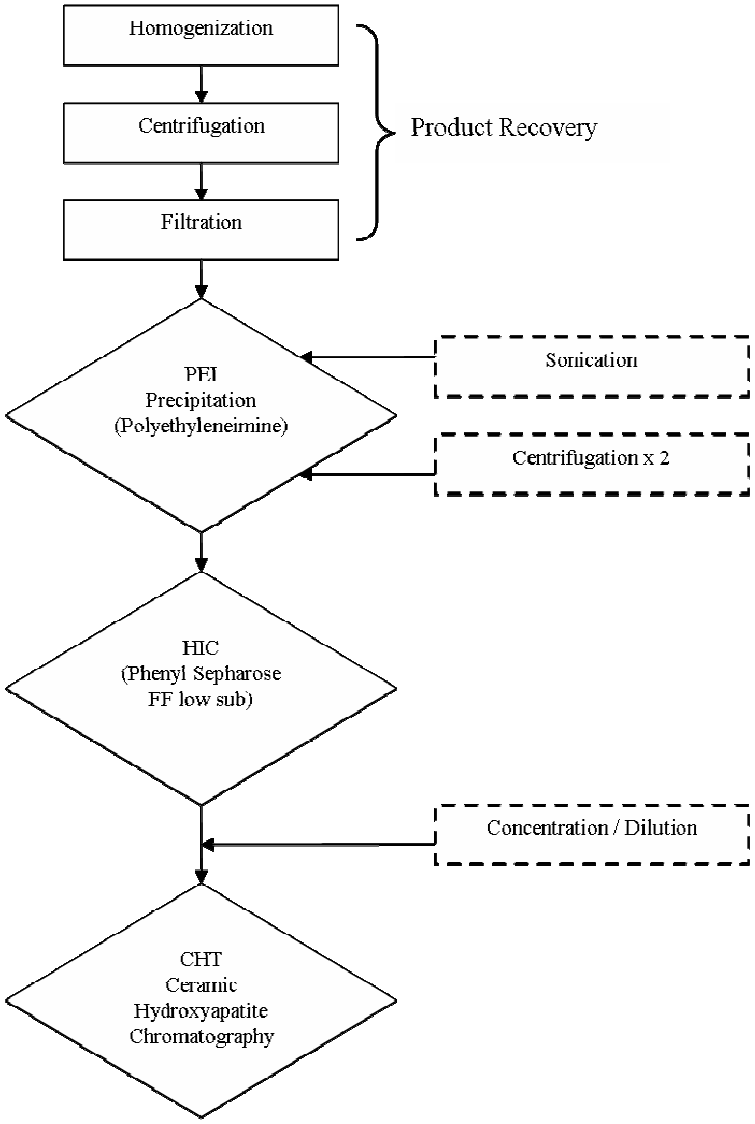

Figure 1. Diagram of proposed downstream processing scheme for the purification of an acidic recombinant protein from transgenic tobacco leaf tissue. The model protein studied was recombinant β-glucuronidase (pI 5.5) and the number of steps chosen was kept to the minimum needed to provide high recovery and purity of the target protein. In addition, the steps were kept general for the possible application to a wide range of acidic recombinant proteins expressed in tobacco or other leafy crops. An additional sample concentration step was used after hydroxyapatite chromatography to visualize the protein bands on SDS-PAGE but this step was not included as part of the overall purification scheme.

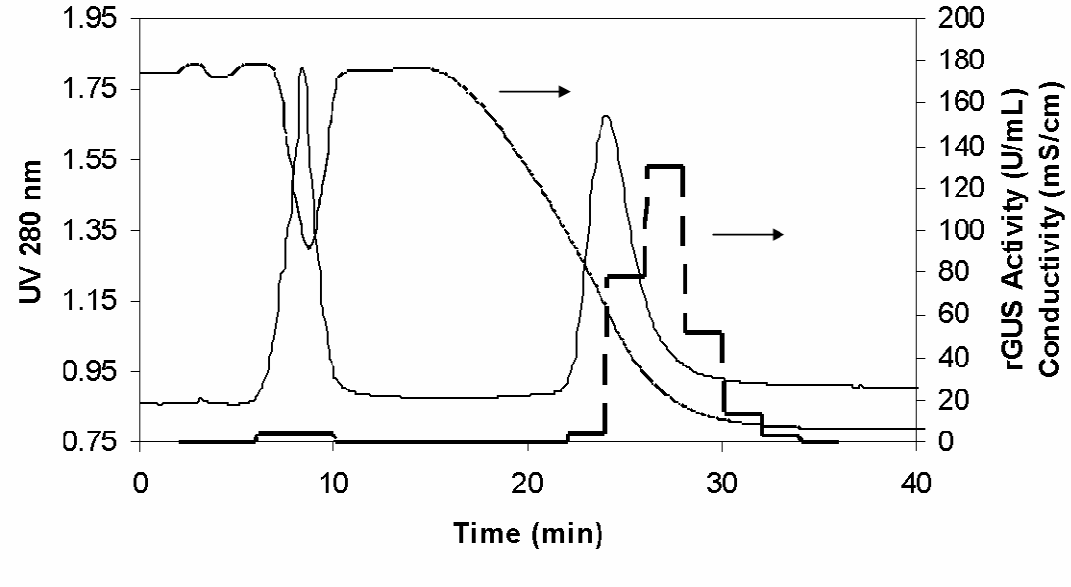

Figure 2. Representative chromatogram of the optimized hydrophobic interaction chromatography (HIC) step used as the second step in the purification of rGUS from transgenic tobacco. A total of 1.5 mL sample was loaded to the column after PEI precipitation at 800 mg PEI/g total protein. The thick dashed line represents the rGUS activity collected in each of the fractions and the thin dotted line represents the elution gradient from 50 mM NaPi, pH 7.0 + 1.5 M ammonium sulfate to 50 mM NaPi, pH 7.0.

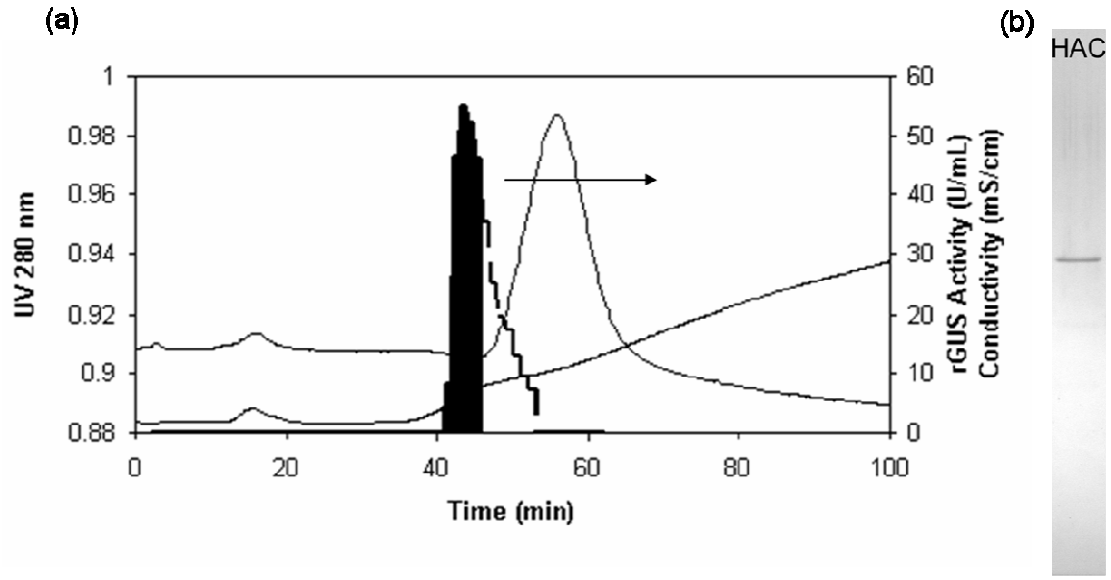

Figure 3. Initial hydroxyapatite chromatography (HAC) scheme used as the third step in the purification of rGUS from transgenic tobacco after PEI precipitation and HIC. The chromatogram shows the elution of rGUS (thick dashed line) prior to the elution of the main native tobacco proteins (a). The SDS-PAGE of the five pooled fractions (shaded in black) after concentration shows a homogeneous band at 68 kD corresponding to rGUS (b). The gel was silver stained for maximum detection of protein bands.

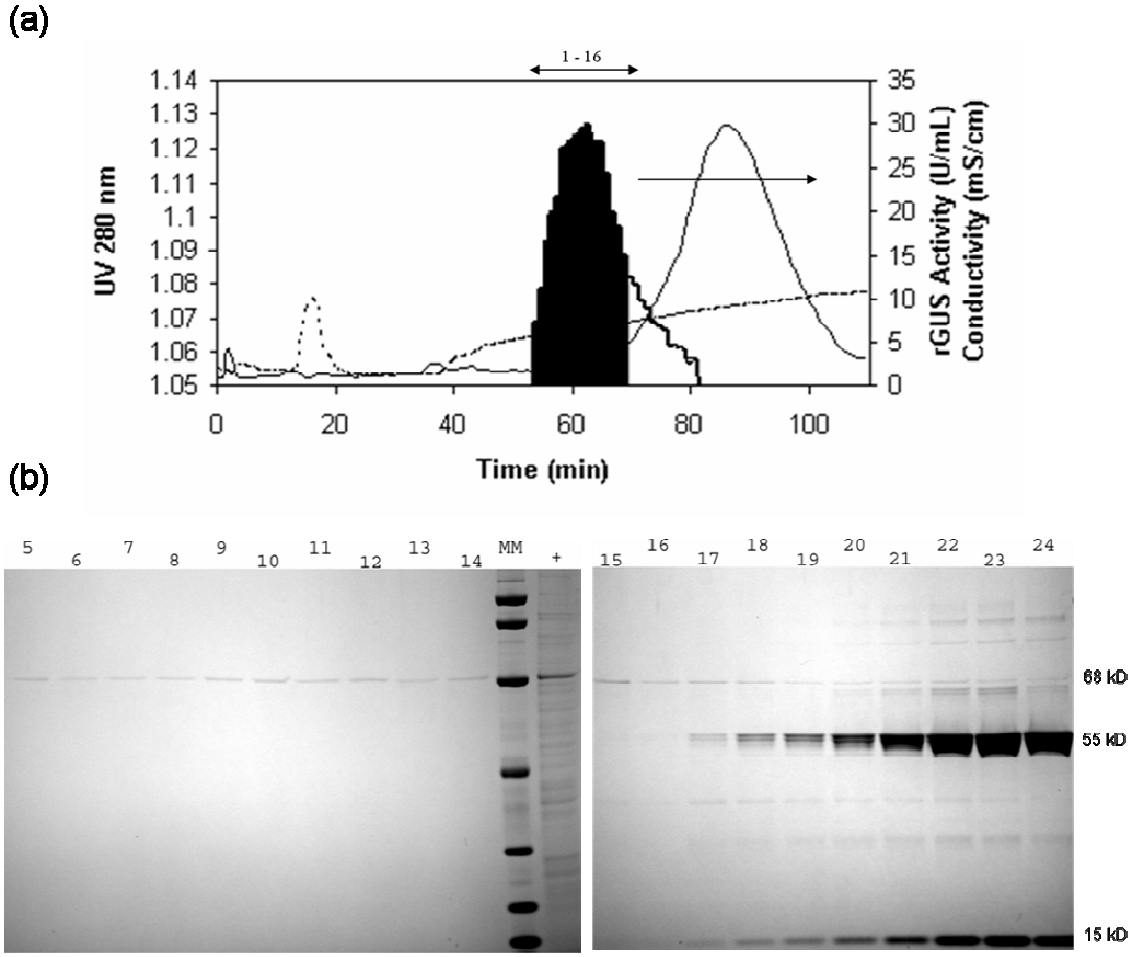

Figure 4. Optimized hydroxyapatite chromatography scheme. Chromatogram showing increased separation of rGUS from native tobacco proteins with the 16 pooled fractions shaded in black (a). SDS-PAGE of individual fractions (5-24) collected and concentrated using Microcon centrifugal concentration devices (b). The lane marked (+) is a Sigma GUS standard used as a positive control and the gels were stained with Bio-Safe Coomassie stain.

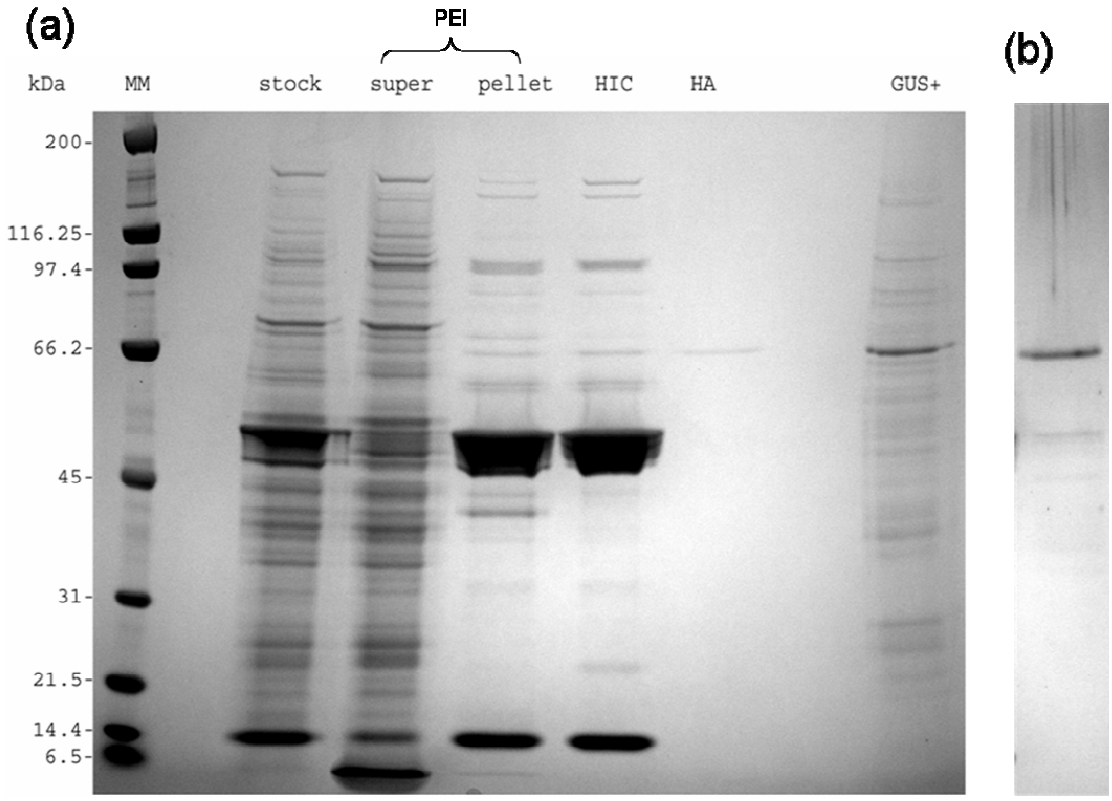

Figure 5. SDS-PAGE of the entire purification scheme. Stained with Bio-Safe Coomassie stain (a). The final product pooled from fractions 1-16 after HAC corresponds to approximately 40% of the initial rGUS activity and is nearly homogeneous in purity after staining with silver stain (b).

Chapter Five

Conclusions and Future Work

Conclusions

1. An extraction buffer consisting of low salt (50 mM sodium phosphate, NaPi), a reducing agent (10 mM β-2-mercaptoethanol, BME), and a chelating agent (1 mM EDTA) with the addition of a phenolic binding agent, polyvinylpolypyrrolidone (PVPP) after extraction, provided good extraction and stability of the recombinant protein, rGUS from transgenic tobacco leaves.

2. Polyelectrolyte precipitation using polyethyleneimine (PEI) was investigated as a first step in the purification of rGUS from transgenic tobacco. At dosages above 700 mg PEI/g total protein nearly 100% of the initial rGUS activity was precipitated with generally more than 90% recovered from the pellet after dissolving in a buffer containing a high concentration of NaCl.

3. PEI precipitation was compared with anion-exchange chromatography as a first step in the purification process. PEI precipitation yielded similar or better results than anion-exchange chromatography for recovery and enrichment of rGUS while also providing significant sample concentration. The two processes were further compared by adding a second step of hydrophobic interaction chromatography after the initial step. Once again similar results were obtained for both schemes, indicating that PEI precipitation may be a feasible alternative to an initial chromatography step.

4. The hydrophobic interaction chromatography step was kept as the second step in the purification process and optimized for maximum rGUS activity recovery. Hydrophobic interaction chromatography did not provide good separation of rGUS from native tobacco proteins, but the step was very important because impurities such as PEI and nucleic acids accumulated from the PEI precipitation

step were removed. In addition, the salt concentration was significantly reduced in preparation for hydroxyapatite chromatography. Total recovery from the optimized HIC step was nearly 80% of the initial rGUS activity.

5. The third and final step chosen in the purification scheme was hydroxyapatite chromatography. Hydroxyapatite chromatography provided good separation of rGUS and native tobacco proteins, and upon minimum optimization, yielded approximately 40% of the initial rGUS activity and a nearly homogeneous band on SDS-PAGE.

Future Work

1. All of the steps chosen for this process were selected to be potentially applicable for purifying a variety of acidic recombinant proteins expressed in tobacco and other leafy crops. However, the process should be carried out with one or more actual therapeutic acidic recombinant proteins expressed in tobacco or other leafy crops to verify the applicability of the overall processes. Additional optimization at each step would most likely be needed depending on the protein being purified.

2. It is anticipated that all steps of this process can be scaled up for large-scale purification. However, additional experiments would need to be performed on a pilot scale to determine the effectiveness of process scalability.

3. An economic evaluation of the overall process would be beneficial in determining the estimated production costs associated with the purification of acidic recombinant proteins from transgenic tobacco on a commercial scale.

4. In considering the overall purification process on a commercial scale, there must be methods developed to process the transgenic waste material collected from each step in the purification process.

Appendix A

GUS activity assay

The assay used throughout this study for determining the recombinant β-glucuronidase (rGUS) enzymatic activity is based on the ability of GUS to hydrolyze the substrate *p*-nitrophenyl β-D-glucuronide (PNPG) to form the chromophoric product *p*-nitrophenol (PNP). Accumulation of PNP was visualized by a yellow color change which was quantitatively measured at 405 nm. Determination of how much PNP is formed was a direct indicator of how much rGUS is present in a sample. In order to establish this relationship, PNP standards were created to obtain an equation that relates the absorbance at 405 nm to the amount of PNP present (nmol) in a sample. Two-hundred microliters of sample containing various concentrations of PNP were placed in each well of a 96-well microplate and read on a microplate reader (Bio-Tek Synergy) using KC4 software. Absorbance measurements were read using end-point determination at 405 nm. The absorbance values for three separate sets of PNP standards are presented in Table A1.

Table A1. Absorbance values at 405 nm for three sets of PNP standards.

Trial 1								
nmol PNP	**0**	**0.3125**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**
1	0.039	0.044	0.049	0.06	0.083	0.128	0.209	0.390
2	0.038	0.046	0.051	0.06	0.083	0.128	0.223	0.421
Average	0.039	0.045	0.050	0.060	0.083	0.128	0.216	0.406
Adjusted[1]	0.000	0.007	0.012	0.022	0.045	0.090	0.178	0.367
Slope	54.913							
Trial 2								
nmol PNP	**0**	**0.3125**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**
1	0.039	0.045	0.049	0.062	0.078	0.121	0.205	0.356
2	0.039	0.044	0.054	0.067	0.085	0.14	0.213	0.413
Average	0.039	0.045	0.052	0.065	0.082	0.131	0.209	0.385
Adjusted	0.000	0.006	0.013	0.026	0.043	0.092	0.170	0.346
Slope	57.853							
Trial 3								
nmol PNP	**0**	**0.3125**	**0.625**	**1.25**	**2.5**	**5**	**10**	**20**
1	0.040	0.045	0.046	0.057	0.076	0.113	0.19	0.326
2	0.039	0.044	0.049	0.061	0.08	0.126	0.205	0.376
Average	0.040	0.045	0.048	0.059	0.078	0.120	0.198	0.351
Adjusted	0.000	0.005	0.008	0.020	0.039	0.080	0.158	0.312
Slope	63.961							
Average Slope		**58.91**						

[1]Adjusted values are obtained by subtracting the average value of the '0' standard from the average values of the individual standards.

The standard curves obtained from the three sets of PNP standards are presented in Figure A1. The three slopes obtained from the individual curves were averaged to account for variation in creating the standards and the average slope obtained was 58.91 nmol PNP / abs_{405nm}. This is the slope that was used throughout all of the experiments when calculating the amount of rGUS activity in a sample.

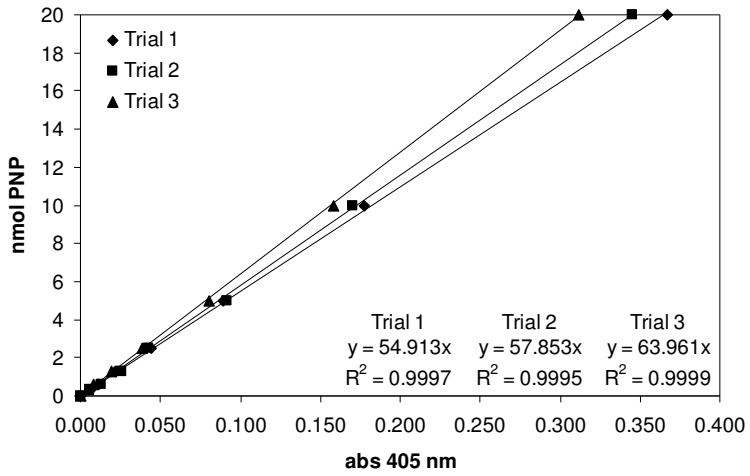

Figure A1. PNP standard curves.

During an activity assay, the absorbance measurements of the samples were recorded over a period of time. The accumulation of PNP increased linearly so that the slope of the resulting equation had the units abs_{405nm} / minute. The following calculations were then performed using the standard PNP slope to obtain the rGUS concentration in each sample. Note here that 1 unit (U) of rGUS activity is defined as the amount needed to liberate 1 nmol of PNP per minute at room temperature (RT ~ 25°C).

Sample rGUS activity calculation:

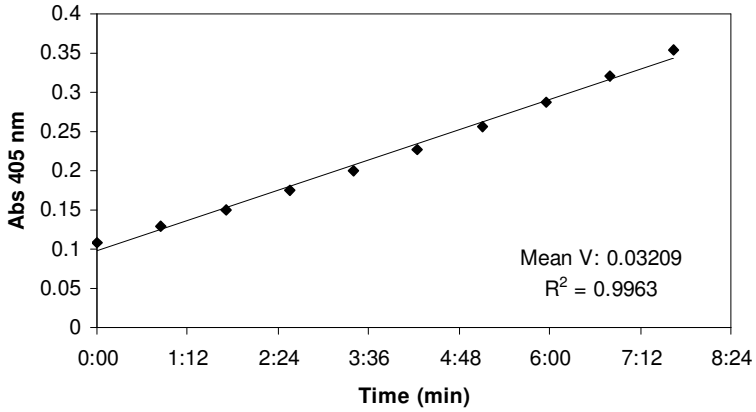

Figure A2. Sample readout from rGUS activity assay performed on a microplate reader using KC4 software.

Activity assay slope (Mean V): 0.03209 $\frac{abs_{405nm}}{\min}$ (from Figure A2)

Standard slope: $58.91\ \frac{nmol\ PNP}{abs_{405nm}}$ (From Table A1)

Total amount of rGUS activity units in the sample assayed:

$$0.03209\ \frac{abs_{405nm}}{\min} \times 58.91\ \frac{nmol\ PNP}{abs_{405nm}} = 1.89\ \frac{nmol\ PNP}{\min}\ ;$$

Therefore, since units (U) of GUS activity were expressed in nmol PNP produced per minute; there were 1.89 U of rGUS activity in the sample measured. For each activity assay performed, a total of 4 µL of sample was used. Therefore, to calculate the concentration of rGUS activity in the assayed sample:

Total rGUS concentration in sample assayed: $\frac{1.89\ U}{0.004\ mL} = 473\frac{U}{mL}$

Appendix B

Experimental conditions

Appendix B presents results on different experimental conditions which were important to the overall study.

The stability of components in a transgenic tobacco extract was investigated over a period of 20 days. Transgenic tobacco extract was obtained as first described in Chapter Three and then divided up into 2 mL aliquots. The aliquots were quick-frozen with liquid nitrogen and then stored in a -80°C freezer. The initial rGUS activity and total protein of the extract was recorded. Each time a data point was recorded, at least two extract samples were analyzed and their values were averaged with negligible variation.

A significant amount of total protein as well as rGUS activity was lost at -80°C storage over a period of 20 days as shown in Figure B1. This study indicated that fresh extracts should be used for each experiment.

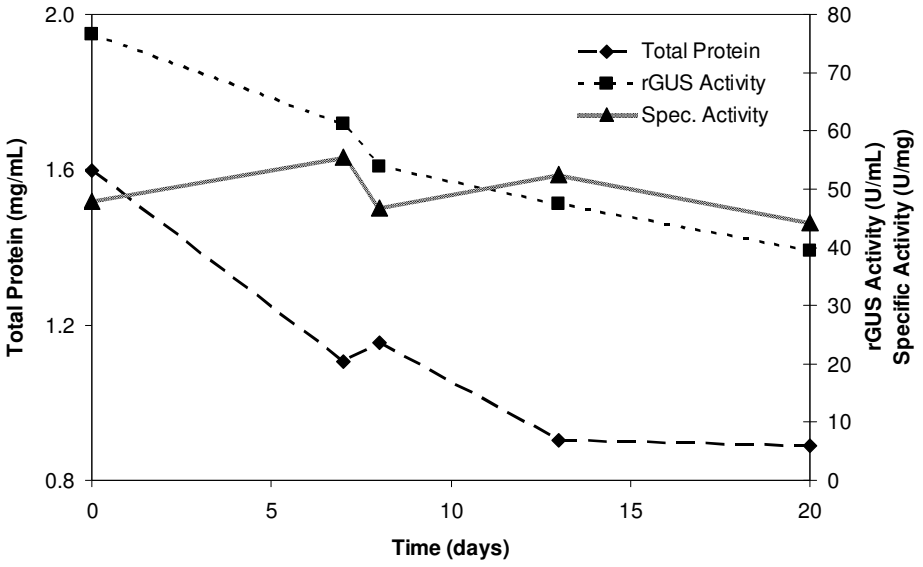

Figure B1. Recombinant GUS and total protein stability in transgenic tobacco extracts stored at -80°C.

The short term stability of rGUS in a transgenic tobacco extract over a 6 hour period at room temperature was also studied. Two extract samples were prepared as first described in Chapter Three and their initial activities after 6 hours at room temperature were recorded. The rGUS remained very stable in a tobacco extract over a 6 hour period as presented in Table B1.

Table B1. Short term rGUS stability over a 6 hour period at room temperature.

Samples	Replicate	Activity (U/mL)	Average (U/mL)
rGUS, 1 (RT, T = 0h)	1	55.60	50.45
	2	48.53	
	3	47.23	
rGUS, 2 (RT, T = 0h)	1	48.41	48.41
	2	49.10	
	3	47.78	
rGUS, 1 (RT, T = 6h)	1	45.20	46.55
	2	47.23	
	3	47.23	
rGUS, 2 (RT, T = 6h)	1	48.53	49.23
	2	48.73	
	3	50.44	

One of the experimental conditions tested was the effect that salt concentration had on rGUS enrichment and recovery after PEI precipitation. The rGUS activity and enrichment values for pellet samples after PEI precipitation at 700 mg PEI/g total protein are presented in Table B2. The pellet samples were dissolved in extraction buffer plus various concentrations of sodium chloride. The results indicated that the rGUS enrichment and activity recovery were not significantly affected by the concentration of NaCl (0.5 M – 2.5 M) in the buffers. However, at a concentration of 0.1 M NaCl, the pellet was not adequately redissolved and less than 15% of the initial rGUS activity was recovered.

Table B2. Recombinant GUS activity data for pellet samples dissolved in various concentrations of NaCl after PEI precipitation. Precipitations were carried out at 700 mg PEI/g total protein.

NaCl Conc.	Replicate	Activity (U/mL)	Average (U/mL)	Enrichment	Recovery
2.5 M	1	288.81	308.79	3.24	91.15%
	2	327.67			
	3	309.87			
2.0 M	1	283.80	300.88	3.80	88.82%
	2	304.12			
	3	314.73			
1.5 M	1	305.45	313.60	3.67	92.57%
	2	335.93			
	3	299.41			
1.0 M	1	316.35	342.12	4.09	100.99%
	2	352.43			
	3	357.58			
0.5 M	1	306.48	321.40	3.78	94.88%
	2	335.93			
	3	321.80			
0.1 M	1	36.10	44.52	3.46	13.14%
	2	47.03			
	3	50.44			

Another condition studied was the effect of the reducing agent (BME) concentration on protein extraction and rGUS activity. The data for four extracts containing various amounts of BME in the extraction buffer and the corresponding graph are presented in Table B3 and Figure B2, respectively. The results indicated that when BME is not present in the extraction buffer, very little rGUS activity was obtained. There are two possible conclusions that were drawn from these results. One is that without the reducing agent present, rapid oxidation of the rGUS occurred during homogenization, causing inactivation of the protein. Another is simply that rGUS needed to be in the presence of a reducing agent in order to be biologically active. It was also assumed that the effects of adding 2, 5, or 10 mM BME to the extraction buffer had virtually the same affect, and the variations seen were attributed to uncontrollable factors such as leaf selection and homogenization efficiency. In addition, even though rGUS activity was

affected by BME concentration, the amount of total protein extracted was not significantly affected.

Table B3. Extraction data with various concentrations of β-2-mercaptoethanol added to the extraction buffer. Extractions were carried out under the procedure first described in Chapter Three.

BME concentration (mM)	Total protein (mg/mL)	rGUS activity (U/mL)
0	0.75	20.75
2	1.03	119.46
5	1.15	226.71
10	0.90	141.99

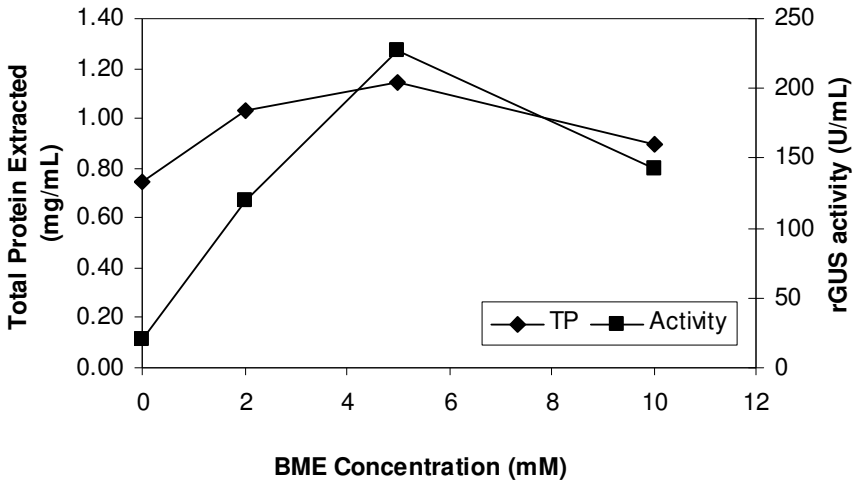

Figure B2. Total protein and rGUS activity extracted with various concentrations of β-2-mercaptoethanol in the extraction buffer.

Another brief study was conducted to determine the effect of adding various amounts of sodium chloride to the extraction samples before PEI precipitation. It was anticipated that adding small amounts of NaCl might inhibit large amounts of Rubisco precipitated while still allowing for large amounts of rGUS to precipitate. Instead, at NaCl concentrations above 0.1 M NaCl, native tobacco proteins and rGUS were not efficiently precipitated with PEI, as shown in Table B4, as the salt interferes with the electrostatic interactions needed to bind the molecules. Subsequently, the amount of

rGUS activity recovered from the pellet decreased significantly above 0.1 M NaCl as presented in Figure B3.

Table B4. Total protein and rGUS activity precipitated with PEI in the presence of various concentrations of NaCl.

NaCl Conc. (M)	% total protein precipitated	% rGUS activity precipitated
0	51.9%	100.0%
0.1	42.4%	96.6%
0.2	15.4%	33.9%
0.3	0.0%	1.0%
0.4	0.0%	0.0%
0.5	0.0%	0.0%

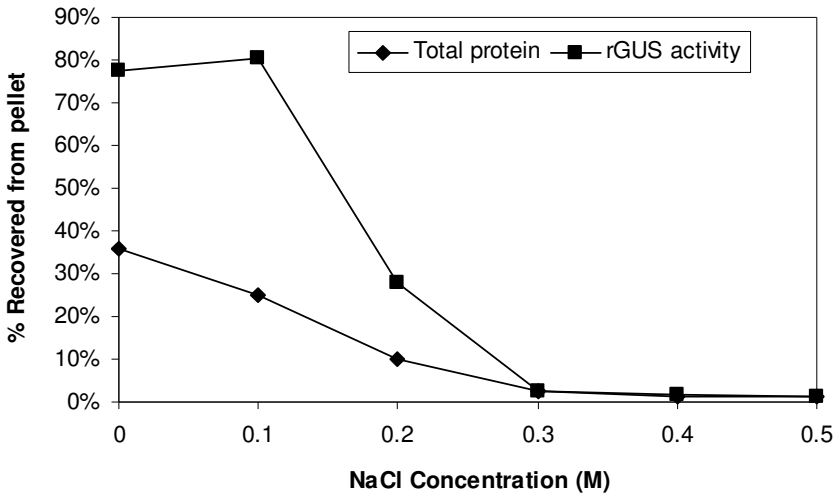

Figure B3. Total protein and rGUS activity recovered from the pellet after precipitation with PEI in the presence of various concentrations of NaCl.

It was also important to test various buffer conditions on the total protein assay (Bio-Rad) to ensure that there were no significant interferences with any of the components in a sample. Interference from sample components could result in inaccurate results for the total protein concentration of a sample. The absorbance readings of the BSA standards and the corresponding BSA standard curve are presented in Table B5 and Figure B4, respectively.

Table B5. BSA standards used for total protein concentration determination. Standards were diluted in 50 mM NaPi, pH 7.0.

Sample	BSA Conc (mg/mL)	Abs (595 nm)	Abs (595 nm)	Abs (Average)	Abs (adjusted avg)
A	0	0.273	0.286	0.280	0.000
B	0.075	0.397	0.408	0.403	0.123
C	0.15	0.510	0.506	0.508	0.229
D	0.3	0.670	0.653	0.662	0.382
E	0.45	0.754	0.793	0.774	0.494

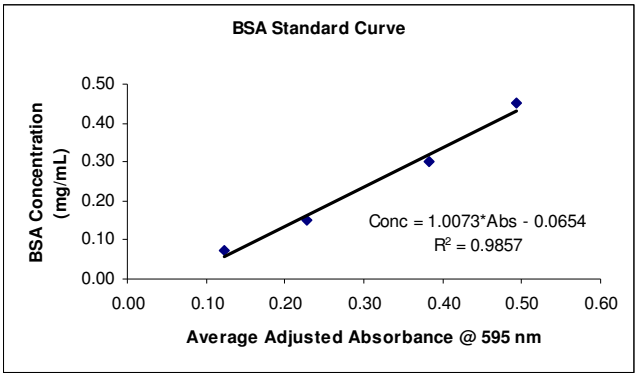

Figure B4. BSA standard curve. Standards were diluted in 50 mM NaPi, pH 7.0.

A standard of 0.3 mg/mL BSA was made up in four separate buffers commonly used in the experiments of this study. The absorbance readings for the standard protein sample in various buffers and at several dilutions are presented in Table B6. The results indicate that only extraction buffer (E.B.) + 1.5 M NaCl may cause significant interference with the Bio-Rad assay (error = greater than ±10% difference from the average). However, once the sample was diluted four times of its volume with 50 mM NaPi, pH 7.0, the values fell within ±10% of the average. It was assumed that the value for the sample 4 (4×) was in error due to the fact that the other samples containing ammonium sulfate were all within ±10% of the average. For samples containing sodium chloride, the samples were diluted with 50 mM NaPi, pH 7.0 until the salt concentration was less than 0.5 M NaCl to obtain more accurate results for the total protein concentration determination.

Table B6. Absorbance readings for total protein assay under various buffer conditions. The numbers correspond to the type of buffer and the numbers in parenthesis correspond to the dilution factor of each sample. Samples were diluted with 50 mM NaPi, pH 7.0.

Sample	Abs (595 nm)	Abs (595 nm)	Abs (average)	Adjusted average	Conc. (mg/mL)	Average and std deviation	% diff. from avg.
1 (0×)	0.672	0.680	0.676	0.397	0.334	0.312 ± 0.039	6.89%
2 (0×)	0.661	0.706	0.684	0.404	0.342		9.31%
3 (0×)	0.593	0.604	0.599	0.319	0.256		-18.09%
4 (0×)	0.658	0.663	0.661	0.381	0.318		1.89%
1 (2×)	0.494	0.497	0.496	0.216	0.152	0.151± 0.013	1.09%
2 (2×)	0.492	0.503	0.498	0.218	0.154		2.43%
3 (2×)	0.476	0.476	0.476	0.197	0.133		-11.96%
4 (2×)	0.502	0.511	0.507	0.227	0.163		8.45%
1 (4×)	0.402	0.409	0.406	0.126	0.062	0.061± 0.007	0.41%
2 (4×)	0.395	0.404	0.400	0.120	0.055		-9.45%
3 (4×)	0.397	0.404	0.401	0.121	0.056		-7.81%
4 (4×)	0.412	0.419	0.416	0.136	0.072		16.85%

	Buffers
1	50 mM NaPi, pH 7.0
2	Extraction Buffer (E.B.)[1]
3	E.B. + 1.5 M NaCl
4	E.B. + 1.5 M ammonium sulfate

[1]Extraction Buffer: 50 mM NaPi, pH 7.0 + 10 mM BME + 1 mM EDTA.

Appendix C

Size-exclusion chromatography

Size-exclusion chromatography (SEC)-FPLC was investigated as a method for separating rGUS from native tobacco proteins, most notably Rubisco, in a transgenic tobacco extract as hypothesized in Chapter Three and referred to in Chapter Four.

FPLC was attempted using Sephacryl S-300 HR resin (globular protein fractionation range, MW 10 kD – 1500 kD) from G.E. Healthcare. Approximately 20 mL of resin was packed in an XK 16/20 column (10 cm bed height) (G.E. Healthcare). A transgenic tobacco extract was obtained as first described in Chapter Three. Five milliliters of extract was precipitated at 800 mg PEI/g total protein. The pellet was collected and redissolved in 3 mL of E.B. + 1.5 M NaCl. The sample was then loaded directly onto a 1 mL HIC column (Phenyl Sepharose FF low substitution). Proteins were eluted over a linear gradient from 0% - 100% 50 mM NaPi, pH 7.0 over 20 minutes at 1 mL/min. The fractions displaying rGUS activity were collected (8 mL total) and concentrated in Amicon Ultra centrifugal filter devices (5,000 MWCO) down to approximately 0.75 mL. The sample was then loaded to the SEC column at a flow rate of 0.5 mL/min. After loading, buffer containing 50 mM NaPi, pH 7.0 + 0.15 M NaCl was pumped at a flow rate of 0.5 mL/min to elute proteins over a total of 60 minutes. A total of 8 mL of fractions displaying rGUS activity were collected and analyzed for rGUS activity.

As seen in Figure C1, rGUS could not be separated from Rubisco with the SEC column at the conditions tested. However, it was unclear as to why the proteins were not better separated. Theoretically, since rGUS has a polymeric molecular weight of 270 kD and Rubisco has a polymeric molecular weight of 560 kD, near baseline separation was anticipated due to the fact that these two proteins are more than a factor of two in size difference. It was speculated that rGUS may be binding to Rubisco in some way causing both proteins to elute simultaneously.

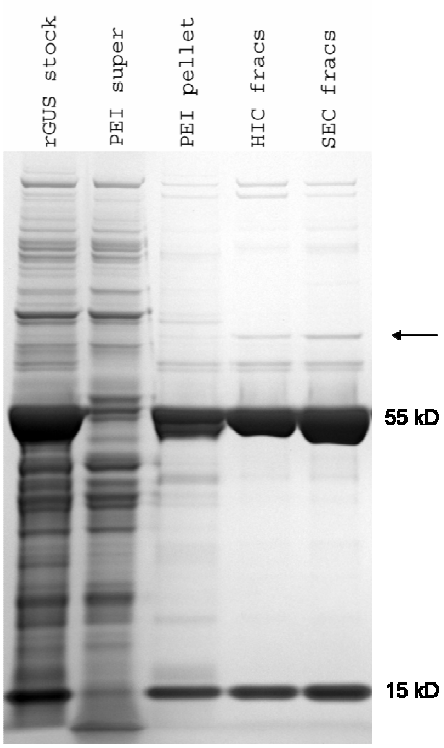

Figure C1. SDS-PAGE of SEC experiment. Gel was stained with Bio-Safe Coomassie stain. The arrow points to the rGUS band. Lanes 1 and 3 were diluted 2×, while lane 4 was concentrated 7× and lane 5 was concentrated approximately 30×.

As a control experiment, individual standards of a tobacco extract (enriched for Rubisco by PEI precipitation) and GUS (Sigma) were run on the SEC column to study each protein's elution profile. Therefore, a better conclusion was reached as to the separation behavior of the proteins on the SEC column. A total of 0.5 mL of each sample was loaded to the column in separate runs. The flow rate used was 0.5 mL/min throughout and the total elution time after sample loading was 60 minutes. The GUS fractions were collected and assayed for activity, whereas the tobacco fractions were collected and run on SDS-PAGE to confirm the presence of Rubisco. The overlaid chromatograms of the two individual runs are presented in Figure C2.

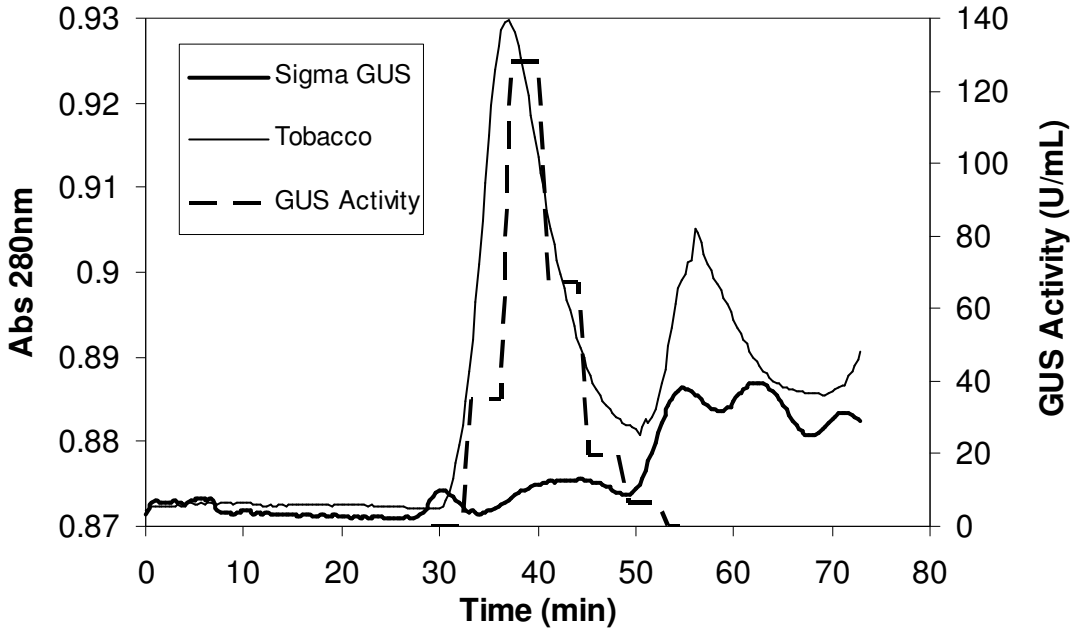

Figure C2. Overlaid chromatograms of tobacco and GUS standards on SEC.

The first large peak of the tobacco elution profile contains large amounts of Rubisco as confirmed by SDS-PAGE analysis (gel not shown). It appeared from the two independent runs that Rubisco and GUS eluted at nearly the same time, making separation of these two major proteins nearly impossible on the SEC column. It was thus concluded that the column used for these experiments was not adequate for use with SEC. Columns used for SEC generally should be very long and narrow to allow for proper separation of the molecules. The instructions manual for the resin used recommended packing in an XK 16/70 to a bed height of 30-60 cm. In contrast, the column used here was an XK 16/20 packed to a bed height of only 10 cm. Therefore, it was concluded that using a longer column may result in better separation; however, this was not investigated further.

Appendix D

Hydrophobic interaction chromatography scouting columns

During the investigation of hydrophobic interaction chromatography, two other resin types were studied along with Phenyl Sepharose FF (low substitution) – Butyl Sepharose FF and Octyl Sepharose FF. For these experiments, 1 mL HIC HiTrap scouting columns (G.E. Healthcare) were used, which were on hand.

For these experiments, 10 mL of transgenic extract was precipitated at 800 mg PEI/g total protein. The pellet was redissolved in 1 mL of extraction buffer containing 0.5 M NaCl and 1 mL of 2 M ammonium sulfate. Ammonium sulfate was added in these experiments as it was unclear at the time if additional salt was needed to ensure binding of the proteins to the column. Fractions were collected in 1 mL aliquots. The elution profile of proteins on the Butyl Sepharose FF HiTrap column is presented in Figure D1. The profile looked very similar to those obtained using Phenyl Sepharose FF (low sub) resin, with rGUS eluted towards the tail end of the main elution peak which was comprised of native tobacco proteins (largely Rubisco).

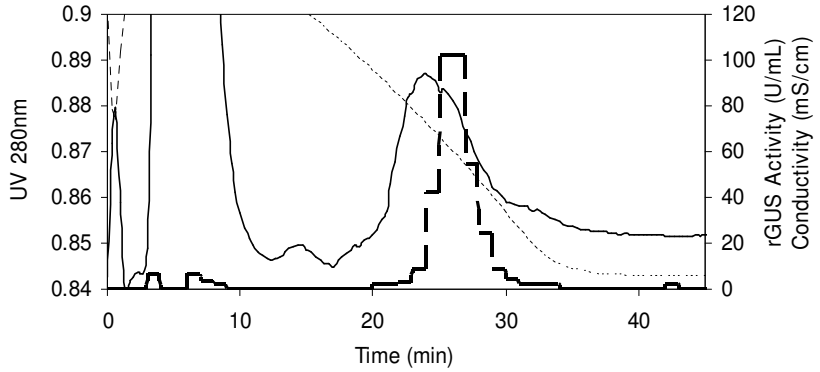

Figure D1. Protein elution profile on Butyl Sepharose FF. The thick dashed line represents the rGUS activity recorded in each fraction and the thin dotted line represents the conductivity of the elution buffer.

The elution profile of proteins on the Octyl Sepharose FF HiTrap column is presented in Figure D2. The elution peak of rGUS was very broad and coincided with

the elution of the main peak containing native tobacco proteins. The total rGUS activity recoveries for both resins are presented in Table D1.

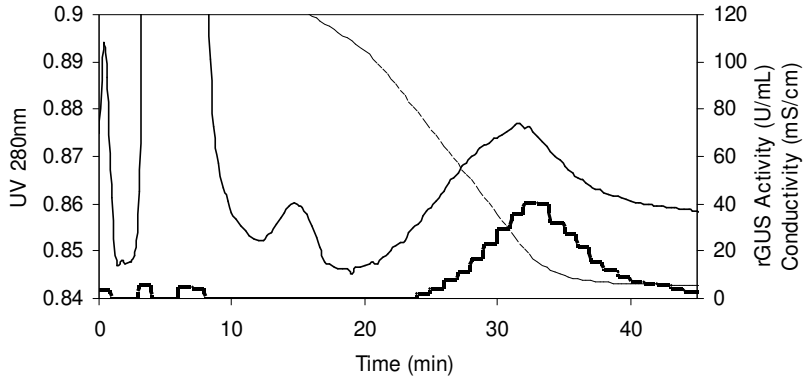

Figure D2. Protein elution profile on Octyl Sepharose FF. The thick dashed line represents the rGUS activity recorded in each fraction and the thin dotted line represents the conductivity of the elution buffer.

For both trials, the total rGUS activity recovered in the eluted fractions was greater than 50% of the initial activity, as shown in Table D1. However, since rGUS eluted at the same time of native tobacco proteins and the overall recoveries were no better than the results obtained using Phenyl Sepharose FF (low substitution), these resins were not investigated further.

Table D1. rGUS activity recoveries for Butyl Sepharose FF and Octyl Sepharose FF hydrophobic interaction chromatography runs. The rGUS recovery after the PEI precipitation step was near 100% for both runs.

Butyl Sepharose FF		rGUS Recovery	
Samples	Fractions	Total Activity (U)	% Initial
Flowthrough	6	19.94	3.28%
Fractions	14	361.20	59.48%

Octyl Sepharose FF		rGUS Recovery	
Samples	Fractions	Total Activity (U)	% Initial
Flow-through	5	14.25	2.10%
Fractions	24	359.54	52.94%

Appendix E

Direct load of extract on hydroxyapatite chromatography

Transgenic tobacco extract was applied directly to a hydroxyapatite chromatography column to determine the importance of initial purification steps. A total of 8 mL of transgenic tobacco extract was obtained as described in Chapter Four. Six milliliters of this extract was dialyzed in 10 mM NaPi, pH 6.8 buffer using Slide-A-Lyzer Dialysis cassettes (3500 MWCO) from Pierce (Rockford, IL, USA). Samples were dialyzed at 1:200 sample/buffer ratio for two hours at room temperature and then overnight at 4°C with new buffer. After dialysis, the samples were assayed for total protein and rGUS activity.

The hydroxyapatite column and elution procedure are described in Chapter Four (the extended gradient method was used – see Table F6). Approximately 7 mL dialyzed tobacco extract (14 mg total protein, 280 U rGUS activity) was loaded to the column and 1-mL fractions were collected, assayed for rGUS activity, and pooled as necessary. Figure E1 presents the chromatogram for the hydroxyapatite direct load experiment with the corresponding SDS-PAGE gel presented in Figure E2. There was not enough rGUS present in any individual fraction to obtain quantitative results using the activity assay. However, by visual inspection of the samples after two days at room temperature, yellow coloration was observed throughout all four of the pooled fractions, with the darkest coloration coming from Pool 1. Pool 1 corresponds to the conductivity range where purified rGUS was obtained during the three-step purification scheme described in Chapter Four. The results indicated that rGUS cannot be purified by a single step of hydroxyapatite chromatography and that initial purification steps are needed to remove many of the native tobacco proteins before separation on hydroxyapatite.

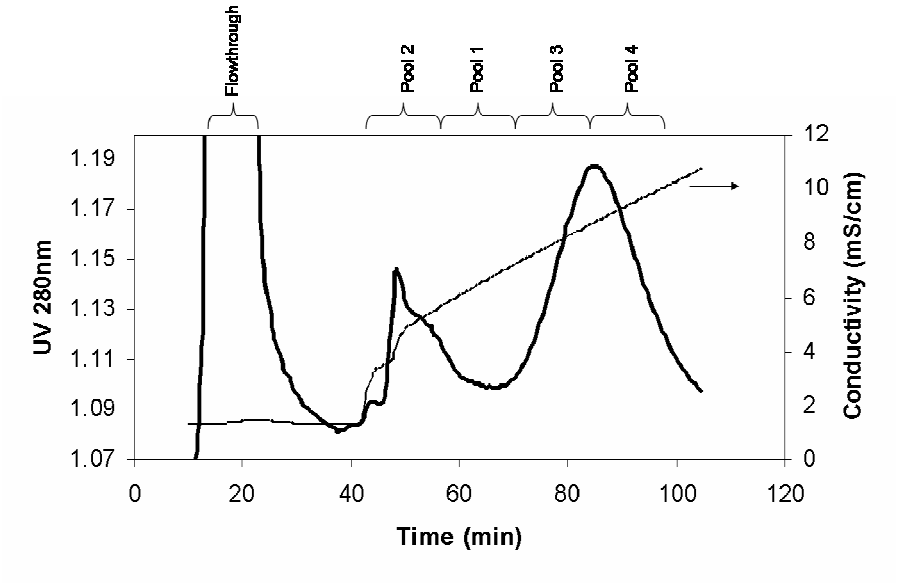

Figure E1. Chromatogram of hydroxyapatite chromatography with crude tobacco extract directly loaded onto the column.

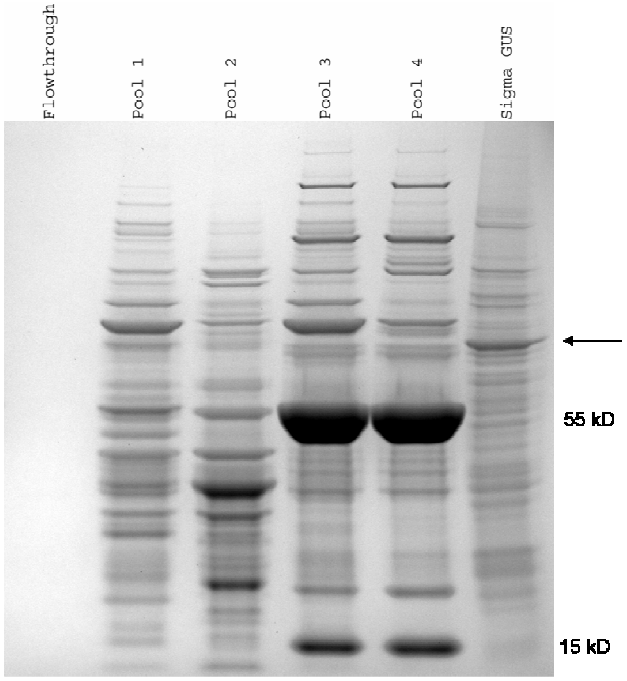

Figure E2. SDS-PAGE of direct load hydroxyapatite chromatography experiment. Gel was stained with Bio-Safe Coomassie stain. The arrow points to the GUS band of the Sigma stock. Lanes 2-5 were all concentrated approximately 20× in Amicon Ultra centrifugal filter devices (5000 MWCO).

Appendix F

FPLC chromatography methods

The chromatography methods presented in Appendix F were created using the Unicorn software (version 3.10) and carried out on an ÄKTA Explorer 100 FPLC chromatography system (G.E. Healthcare).

Table F1. FPLC method used for the anion-exchange chromatography experiments described in Chapter Three.

```
Method: C:\UNICORN\Local\fil\default\method\Chris\Chris AEX Run.m02
Main method:
  0.00  Base Time
  0.00  Flow 1.0 {ml/min}
  0.00  SampleValve S1
  0.00  ColumnPosition Position1Bypass
  1.5  SampleFlow 3.00 {ml/min}
  4.0  SampleFlow 0.00 {ml/min}
  5.0  SampleFlow 3.00 {ml/min}
  6.0  InjectionValve Inject
  8.00  SampleFlow 0.00 {ml/min}
  9.00  SampleFlow 1.00 {ml/min}
  11.00  ColumnPosition Position8
  11.00  Fractionation 18mm 1.000 {min} FirstTube Time
  13.50  SampleFlow .100 {ml/min}
  13.50  InjectionValve Load
  15.00  SampleFlow 0.00 {ml/min}
  23.00  Gradient 75.00 {%B} 40.00 {base}
  65.00  End_Method
```

Table F2. FPLC method used for the hydrophobic interaction chromatography step after anion-exchange chromatography in the two-stage experiment described in Chapter Three.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris HIC AEX.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 SampleValve S1
0.00 Flow 1.00 {ml/min}
1.50 SampleFlow 3 {ml/min}
4.00 SampleFlow 0 {ml/min}
5.00 SampleFlow 3 {ml/min}
6.00 InjectionValve Inject
8.00 SampleFlow 0.00 {ml/min}
9.00 SampleFlow 1.00 {ml/min}
9.00 Fractionation 18mm 1.000 {ml} FirstTube Volume
10.00 ColumnPosition Position7
20.00 SampleFlow 0.00 {ml/min}
20.00 InjectionValve Load
24.00 Gradient 100 {%B} 20.00 {base}
56.00 End_Method

Table F3. FPLC method used for the hydrophobic interaction chromatography step after PEI precipitation in the two-stage experiment described in Chapter Three.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris HIC Precip.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 SampleValve S1
0.00 Flow 1.00 {ml/min}
1.50 SampleFlow 3 {ml/min}
4.00 SampleFlow 0 {ml/min}
5.00 SampleFlow 3 {ml/min}
6.00 InjectionValve Inject
8.00 SampleFlow 0.00 {ml/min}
9.00 SampleFlow 1.00 {ml/min}
9.00 Fractionation 18mm 1.000 {ml} FirstTube Volume
10.00 ColumnPosition Position7
16.00 SampleFlow 0.00 {ml/min}
16.00 InjectionValve Load
20.00 Gradient 100 {%B} 20.00 {base}
52.00 End_Method

Table F4. FPLC method used for the hydrophobic interaction chromatography optimization experiment described in Chapter Four.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris HIC optimization 2.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 Flow 1.00 {ml/min}
5.00 SampleValve S1
5.00 SampleFlow 1.00 {ml/min}
5.00 InjectionValve Load
6.00 InjectionValve Inject
7.00 SampleFlow 0.00 {ml/min}
8.00 SampleFlow 1.00 {ml/min}
8.00 Fractionation 18mm 2.0 {ml} FirstTube Volume
8.50 ColumnPosition Position6
14.00 SampleFlow 0.00 {ml/min}
14.00 InjectionValve Load
18.00 Gradient 100 {%B} 10.00 {base}
47.00 ColumnPosition Position1Bypass
47.00 FractionationStop
49.00 End_Method

Table F5. FPLC method used for the for the initial hydroxyapatite chromatography experiment described in Chapter Four.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris Hydroxyapatite 2.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 Flow 1.00 {ml/min}
5.00 SampleValve S1
5.00 SampleFlow 1.00 {ml/min}
5.00 InjectionValve Load
6.00 InjectionValve Inject
7.00 SampleFlow 0.00 {ml/min}
8.00 SampleFlow 1.00 {ml/min}
8.00 Fractionation 18mm 1.00 {ml} FirstTube Volume
8.50 ColumnPosition Position7
15.00 SampleFlow 0.00 {ml/min}
15.00 InjectionValve Load
20.00 Gradient 100 {%B} 80 {base}
110.00 ColumnPosition Position1Bypass
110.00 FractionationStop
112.00 End_Method

Table F6. FPLC method used for the for the optimized hydroxyapatite chromatography experiment described in Chapter Four.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris Hydroxyapatite 2 Extended.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 Flow 1.00 {ml/min}
5.00 SampleValve S1
5.00 SampleFlow 1.00 {ml/min}
5.00 InjectionValve Load
6.00 InjectionValve Inject
7.00 SampleFlow 0.00 {ml/min}
8.00 SampleFlow 1.00 {ml/min}
8.00 Fractionation 18mm 2.00 {ml} FirstTube Volume
8.50 ColumnPosition Position7
20.00 SampleFlow 0.00 {ml/min}
20.00 InjectionValve Load
36.00 Gradient 8 {%B} 0 {base}
42.00 Fractionation 18mm 1.00 {ml} NextTube Volume
42.00 Gradient 25 {%B} 60.00 {base}
112.00 Fractionation 18mm 2.00 {ml} NextTube Volume
112.00 Gradient 100 {%B} 0.00 {base}
122.00 ColumnPosition Position1Bypass
127.00 End_Method

Table F7. FPLC method used for the size-exclusion chromatography experiments described in Appendix C.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris SEC2.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 Gradient 100 {%B} 0.00 {base}
0.00 SampleValve S1
0.00 Flow 1.00 {ml/min}
5.00 SampleFlow 3.00 {ml/min}
7.00 InjectionValve Inject
10.00 SampleFlow 0.00 {ml/min}
11.00 SampleFlow 0.5 {ml/min}
11.00 Fractionation 18mm 2.000 {ml} FirstTube Volume
13.00 ColumnPosition Position8
18.00 SampleFlow 0.00 {ml/min}
18.00 InjectionValve Load
18.00 Flow 0.5 {ml/min}
80.00 End_Method

Table F8. FPLC method used for the HiTrap hydrophobic interaction chromatography experiments described in Appendix D.

Method: C:\UNICORN\Local\fil\default\method\Chris\Chris HIC HiTrap 2 mL.m02
Main method:
0.00 Base Time
0.00 ColumnPosition Position1Bypass
0.00 Flow 1.00 {ml/min}
5.00 ColumnPosition Position5
10.00 ColumnPosition Position1Bypass
11.00 SampleValve S1
11.00 SampleFlow 1.00 {ml/min}
11.00 InjectionValve Load
12.00 InjectionValve Inject
13.00 SampleFlow 0.00 {ml/min}
14.00 SampleFlow 1.00 {ml/min}
14.00 Fractionation 18mm 1.0 {ml} FirstTube Volume
14.50 ColumnPosition Position5
19.00 SampleFlow 0.00 {ml/min}
19.00 InjectionValve Load
23.00 Gradient 100 {%B} 20.00 {base}
58.00 PumpBInlet B2
58.00 Gradient 100 {%B} 0.00 {base}
63.00 ColumnPosition Position1Bypass
65.00 End_Method

Vita

Christopher J. Holler was born April 30th, 1983 in Roanoke, Virginia. He graduated with a Bachelor of Science degree in Biological Systems Engineering with the bioprocess engineering option from Virginia Tech in May, 2005. Chris completed his Master of Science degree in Biological Systems Engineering in May 2007 under the guidance of Dr. Chenming Zhang. As a Master's student, he received first place in the Paul E. Torgersen Graduate Student Research Excellence Award competition in 2007. In the fall of 2007, Chris will begin attending the University of Kentucky's College of Medicine to obtain his doctoral degree in Biomedical Sciences.