DOWNSTREAM PROCESSING OF RECOMBINANT PROTEINS FROM TRANSGENIC PLANT SYSTEMS: PHENOLIC COMPOUNDS REMOVAL FROM MONOCLONAL ANTIBODY EXPRESSING *LEMNA MINOR* AND PURIFICATION OF RECOMBINANT BOVINE LYSOZYME FROM SUGARCANE

A Dissertation

by

GEORGIA OLIVEIRA FIGUEIREDO BARROS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Biological & Agricultural Engineering

Downstream Processing of Recombinant Proteins from Transgenic Plant Systems: Phenolic Compounds Removal from Monoclonal Antibody Expressing *Lemna minor* and Purification of Recombinant Bovine Lysozyme from Sugarcane

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ABSTRACT

Downstream Processing of Recombinant Proteins from Transgenic Plant Systems: Phenolic Compounds Removal from Monoclonal Antibody Expressing *Lemna minor* and Purification of Recombinant Bovine Lysozyme from Sugarcane. (May 2012)

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Transgenic plant systems have been proposed as bioreactors in the production of pharmaceutical and industrial proteins. The economic benefits of inexpensive plant production systems could be erased if the downstream processing ends up being expensive.

To avoid monoclonal antibody (mAb) modification or fouling of chromatography resins, removal of phenolics from plant extracts is desirable. Removal of major phenolics in *Lemna* extracts was evaluated by adsorption to PVPP, XAD-4, IRA-402 and Q-Sepharose resins. Analysis of phenolics adsorption to XAD-4, IRA-402 and Q-Sepharose showed superior dynamic binding capacities at pH 4.5 than at 7.5. The economic analysis using SuperPro Designer 7.0 indicated that addition of a phenolics adsorption step would increase mAb production cost only 20% by using IRA-402 compared to 35% for XAD-4 resin. The overall mAb processing cost can be reduced by implementing a phenolics removal step.

To understand phenolics-resin interactions, adsorption isotherms of phenolic compounds (chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside)

from different phenolic classes on three resins (IRA-402, PVPP, XAD-4) at pH 4.5 and 7.5 were determined. Differences in adsorption with the type of phenolics were observed, and PVPP was not efficient for phenolics removal.

Screening of sugarcane lines for bovine lysozyme (BvLz) accumulation indicated that expression levels are still inadequate for commercial development. To maximize BvLz extraction, pH and ionic strength were evaluated; five conditions resulted in equivalent BvLz/TSP ratio. Membrane filtration process using BvLz extracts attained partial removal of native proteins by the 100 kDa membrane step, but also BvLz loss (21-29%). Regardless of the extraction condition, at least 47% of the starting BvLz was lost during the membrane processing. None of the evaluated extraction conditions caused a substantial recovery of BvLz in the concentrate.

Alternative purification options for the IEX+HIC process, which achieved 95% BvLz purity, were tested. Direct loading of sugarcane extract concentrate on HIC and XAD-4 pretreatment of juice did not recover BvLz as effectively as the IEX chromatography. Pure BvLz was obtained by the XAD+HIC process, but higher purification fold and HIC yield were achieved by the IEX+HIC process, due to the complete separation of BvLz and 18-kDa protein.

To my parents, Otaviano and Denize

To my sister Giovana and my brother Breno

To my family, for their unconditional love and support

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1. INTRODUCTION

1.1. Transgenic Plants as Hosts for Protein Production

Advances in genetic engineering are resulting in the continuous development of new biological compounds and their manufacturing processes. Transgenic plant systems have been proposed as alternative bioreactors to mammalian cell culture for the production of a variety of proteins and other bioproducts (Goldstein and Thomas 2004; Menkhaus et al. 2004; Nikolov et al. 2009; Rybicki 2010). The majority of recombinant proteins are still being produced by microbial fermentation or mammalian cell culture, even though large-scale production is expensive and there is an added risk of animal virus contamination. Plant systems are being considered for the production of large molecules due to advantages such as the lower capital investment, simplicity of scale-up, and low risk of contamination by human pathogens.

The first plant-derived recombinant protein for commercial use was produced in corn (Hood et al. 1997; Woodard et al. 2003), but since then, a number of different food and non-food plants have been used for the production of recombinant proteins (Fischer et al. 2004; Howard et al. 2005). These plant systems include seed and leafy tissues as well as bioreactor-based plant systems, which may present particular advantages and disadvantages, depending on the bioproduct and its applications (Nikolov and Woodard 2004; Wilken and Nikolov 2012). The choice of plant host is determined by the cultivation methods and cost, the ease of molecular transformation, and processing considerations (Farinas et al. 2005a; Fischer et al. 2004; Howard et al. 2005). Recombinant protein

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This dissertation follows the style of Biotechnology and Bioengineering.

accumulation levels and the lack of efficient downstream processing are key drawbacks that need to be overcome for cost-effective recombinant protein production (Conley et al. 2011; Wilken and Nikolov 2012). With plants accepted as a viable alternative to fermentation systems, the demonstration of a cost-competitive downstream process will strengthen the lower cost provided by the upstream production in plants (Nikolov et al. 2009).

1.1.1. Pharmaceutical proteins

A variety of plant-derived products with diverse applications, ranging from baby formula additives (Kaiser 2008) to a component for HIV prevention (O'Keefe et al. 2009) or hepatitis B antibodies (Pujol et al. 2005; Valdés et al. 2003), demonstrate the feasibility of using transgenic plants for pharmaceutical manufacturing. High-value protein products such as interferon, human growth hormone (hGH), immunoglobulin G (IgG1), and secretory immunoglobulin A (sIgA) have been produced at significant levels in transgenic plants hosts (Fischer et al. 2009; Ma et al. 2003; Nikolov and Woodard 2004) and several products are already in or close to being in the market (Kaiser 2008).

Leafy and aquatic plants, such as tobacco and duckweed (*Lemna minor),* quickly generate biomass and, thus, are promising candidates for commercial production of pharmaceutical proteins (Cox et al. 2006; Twyman et al. 2003). *Lemna* doubles its biomass in 18-24 hours and has been engineered to express homogenously glycosylated monoclonal antibody (mAb) that closely mimics those produced in mammalian cell culture (Cox et al. 2006).

Because of production speed, transient plants are recently being considered as the most suitable system for vaccines production in case of pandemic outbreak (D' Aoust et al. 2010; Rybicki 2010), demonstrating the competitiveness of plant-made pharmaceutical

proteins with traditional technologies. Development of transient expression in plant systems enabled the low cost and ease of genetic manipulation, and could also provide high yield of proteins (Komarova et al. 2010), with the advantage of leaving no changes to the production plant (Pogue et al. 2010). New genes can be tested for expression and test quantities of the recombinant protein can be obtained in as little as 4–8 weeks after the initial molecular cloning event, rather than the months necessary for transgenic expression (Pogue et al. 2010; Rybicki 2010). Influenza virus-like particles (D' Aoust et al. 2010; D' Aoust et al. 2008) and human papillomavirus (Šmídková et al. 2010) are some of the antigens that have already been transiently expressed in plant tissue (Komarova et al. 2010; Rybicki 2010).

1.1.2. Industrial proteins

The total market for industrial enzymes reached $2.5 billion in 2009 (Demain and Vaishnav 2009). The leading protease, which is used as a detergent additive, accounts for 57% of the market. Other important industrial enzymes are amylase for starch hydrolysis, xylanase for pulp processing, xylose isomerase, lactase, lipase, and cellulase.

Over 80% of the commercial industrial enzymes are currently derived from microbial sources, either natural or recombinant (Demain and Vaishnav 2009; Howard et al. 2005), but transgenic plants have also been used to produce valuable industrial products, such as β-D-glucuronidase (GUS), avidin, laccase and trypsin (Hood 2002).

With the current state of technology for biomass conversion, the ongoing challenge is to meet the large mass requirement of cellulases. For example, 100,000 tons/year of the industrial enzyme xylose isomerase are required to isomerize D-glucose to D-fructose and amylase is used at an annual rate of 95,000 tons/year. To process the volumes of biomass

necessary to replace 30% of the gasoline consumed annually, 3.6 million tons of cellulase per year are needed (Howard et al. 2010).

In summary, protein production for industrial applications faces two main challenges: large volume requirement and low production cost. Because of the scale-up potential and relatively low-cost of plant systems, transgenic plants could potentially be a system of choice for large-scale production of industrial enzymes (Howard et al. 2005).

Corn has been the most used crop for the production of plant-made recombinant proteins. The reasons for that include the low commodity cost of corn, the current infrastructure for corn production, which can handle large acreages for industrial products, and the storage and transport of seeds, which are compatible with the high volume of protein production required for industrial enzymes (Howard et al. 2005). For the same reasons, plant-made proteins can be valuable co-products from plant biomass used for biofuel production. However, their full value would only be recognized after purification from the other plant components.

1.2. Downstream Processing of Plant-based Proteins

Along with the recombinant protein, transgenic plant extracts contain a mixture of native proteins and water-soluble non-protein impurities, such as nucleic acids, pigments, phenolic compounds, and polysaccharides (Hassan et al. 2008; Nikolov et al. 2009; Platis et al. 2008). Impurities in transgenic plant extracts present obstacles in the downstream processing, since they can interact with the target proteins or foul membranes and chromatographic resins during purification.

Based on final product requirements, the degree of removal of impurities varies. While volume and cost targets are indispensable for industrial enzymes, proteins for

agricultural and food industries offer the advantage of lower purity requirements compared to pharmaceutical proteins. For recombinant proteins intended for industrial uses, removal of all impurities is not necessary - only those that interfere with activity of the final product. On the other hand, complete removal (> 99%) of impurities is essential for pharmaceutical products.

The removal of plant components from extracts requires a varied degree of downstream processing which depends on the end application, that is, pharmaceutical, food or industrial non-food uses. Downstream processing can contribute up to 80% of the total manufacturing cost of pharmaceuticals and about 50% of industrial enzymes (Evangelista et al. 1998; Pogue et al. 2010). For plants to be accepted as a viable alternative to fermentation systems for industrial enzymes production, the demonstration of a cost-competitive downstream processing is important to complement the lower cost advantage of protein production in transgenic plants (Nikolov et al. 2009).

Because of superior resolution power and the variety of resins available, adsorption chromatography is the chief unit operation in the purification of recombinant proteins produced in plants (Nikolov and Woodard 2004; Wilken and Nikolov 2012). At the same time, the single largest cost for downstream processing purification is attributed to chromatography operations (Curling and Gottschalk 2007).

Alternative low-cost methods to substitute chromatography such as flocculation, crystallization, precipitation, microfiltration, or aqueous partitioning, can be used in downstream processing (Gottschalk 2011a; Gottschalk 2011b). Common strategies applied for pretreatment of plant extracts before chromatography have been recently reviewed by Wilken and Nikolov (2012). In order to remove plant impurities that affect

purification methods, precipitation, aqueous two-phase partitioning, adsorption, and membrane filtration are suggested as possible unit operations. Aqueous two-phase partitioning has been used to reduce the amount of phenolics, alkaloids, and pigments in leaf extracts (Hasmann et al. 2008; Platis et al. 2008). Acidification of plant extracts is another easy method which allows the partial removal of host protein (Cox et al. 2006; Nikolov et al. 2009). This dissertation will focus on membrane filtration and adsorption using inexpensive resins as pretreatment methods.

1.2.1. Membrane filtration

Membrane separations have historically been used as the first recovery step for many biologics produced by cell culture and microbial fermentation processes (Roush and Lu 2008). The fact that membrane processes operate at low temperature and pressures with no use of chemical additives minimizes undesirable effects in the labile bioproducts, making membrane separations suitable for processing biological molecules (Zeman and Zydney 1996). They also offer the advantages of low cost and ease of scale-up for commercial production of biotechnological products (Saxena et al. 2009). In the downstream processing of recombinant proteins, membrane filtration is used as a clarification step to remove impurities such as pigments, phenolic compounds, oil, and unwanted proteins (Nikolov et al. 2009), and to concentrate the final product. Examples of membrane separation applications in plant-based protein manufacturing processes include filtration processes for production of high-quality protein concentrates from alfalfa (D' Alvise et al. 2000), protein isolates from canola (Xu and Diosady 2002), and canola protein with reduced phenolics content (Berot et al. 2005). Several non-protein products have been obtained from plants extracts using membrane separations (Garger et al. 2000),

including concentration and fractionation of anthocyanins from grapes (Cardona et al. 2009; Kalbasi and Cisneros-Zevallos 2007).

Although a number of membrane processes are used for protein separations in the biotechnology industry, the greatest interest has been in the application of pressure-driven processes like microfiltration (MF) and ultrafiltration (UF). Microfiltration (MF) membranes are primarily used to retain the particulate impurities in the size range of 0.1-10.0 μm, while UF membranes used to retain impurities in the size range of 0.001-0.1 μm are designed to provide high retention of high molecular weight proteins and other macromolecules (Saxena et al. 2009). The choice of membrane is usually guided by its molecular weight cut-off (MWCO), which is typically defined by a 90% molecule/particle rejection at the MWCO.

Ultrafiltration (UF) membranes are cast from a variety of polymers, with polysulfone (PS), polyethersulfone (PES) and regenerated cellulose being of greatest interest for biopharmaceutical applications. Polyethersulfone (PES) is a widely used UF membrane material, because of its high rigidity, resistance, and good thermal and dimensional stabilities (Shi et al. 2007). Traditional PES membranes tend to adsorb protein as well as other biological components due to their hydrophobic nature (Shi et al. 2007; van Reis and Zydney 2007), leading to membrane fouling and flux reduction. Adsorption losses could be minimized by maintaining moderately high protein loading (>20 g/m^2), although significant yield losses due to adsorption are rare, and high protein concentration can promote protein aggregation (van Reis and Zydney 2007). Cellulose-type polymers offer a low protein binding as required by biotechnology applications, but cannot withstand harsh membrane cleaning procedures (Zeman and Zydney 1996). Regenerated cellulose

membranes are hydrophilic, which reduces protein adsorption compared to PES membranes and thus exhibit lower fouling and lower protein adsorption. Hence, these membranes are especially recommended for use when protein loading is low or the feed is highly fouling.

In tangential flow filtration (TFF), the feed stream flows tangentially along the membrane surface while an applied pressure forces part of the fluid through the membrane, which minimizes the accumulation of the retained components at the membrane surface.

1.2.2. Adsorption with inexpensive resins

Before transgenic plants became candidates for protein production, plant tissue has been used for years as a source of enzymes and proteins (Andersen and Sowers 1968; Loomis 1974), and phenolics were identified as an impediment in the purification of proteins. Phenolic compounds are a complex group of substances that have gained enormous attention in the last 10 years, due to their biological properties, natural anti-oxidant capacity, physiological effects, and other properties related to food quality (Manach et al. 2005; Williamson and Manach 2005). Depending on their structure, phenolic compounds can react with proteins and alter some of their physicochemical properties such as solubility, electrophoretic behavior, hydrophobicity, molecular weight, and secondary and tertiary structure (Rohn et al. 2002).

The significance of removing phenolic compounds from plant extracts before chromatography has being discussed (Miller et al. 2004; Platis and Labrou 2006; Woodard et al. 2009b), and different methods have been applied to perform this task. Amid the various approaches used to alleviate the problems caused by the presence of impurities in

plant extracts, inexpensive resins have been tested for phenolics removal before protein purification. Prehydrated polyvinylpirrolidone (PVPP) has been the most used resin for this purpose, in a variety of applications ranging from the fundamental isolation of plant enzymes (Loomis and Battaile 1966) to more recent recombinant protein purification from transgenic tobacco (Holler and Zhang 2008).

Adsorption of phenolics from spinach (Gray 1978), lettuce (D' Alvise et al. 2000), and apple juice (Saleh et al. 2008) using different polymeric resins shed light on the probable mechanisms of interaction that could explain phenolics adsorption. Based on these studies, it is known that phenolics adsorption to different resins may occur by hydrogen bonding, ionic (charge) interactions, and /or hydrophobic interactions.

Adsorption using inexpensive commercially available resins is commonly used to remove phenolic and other colored compounds from non-transgenic plants extracts (D' Alvise et al. 2000; Loomis 1974; Saleh et al. 2008; Xu and Diosady 2002), fermentation media (Fisher et al. 2008; van den Berg et al. 2008) and wastewater (Caetano et al. 2009; Carmona et al. 2006; Huang 2009; Li et al. 2001; Pissolatto et al. 1996). Relevant applications of adsorption using inexpensive resins for food processes, removal of phenolic compounds, detoxification of fermentation media, color removal, purification of sugar solutions and microbial metabolites were recently reviewed (Soto et al. 2011).

Polymeric resins have also been proven to be a desirable pretreatment option for processing extracts from plants that have been modified to produce recombinant proteins. It was previously demonstrated that *Lemna* phenolics could be removed by incubation of extracts with the anionic hydrophobic resin, Amberlite IRA-402 (Woodard et al. 2009b).

1.2.3. Chromatography

Process chromatography is the central downstream processing technology in all bio industries (Curling and Gottschalk 2007). Characteristics of chromatographic separation methods and the respective principles of gel filtration, ion exchange, hydrophobic interaction chromatography, reverse phase and affinity chromatography were described by Curling and Gottschalk (2007). Selection of the specific type of process chromatography is dependent on the properties of both recombinant protein and host impurity.

For example, downstream purification of mAb includes several chromatographic steps. Antibodies represent more than half of all biopharmaceutical products in development (Gottschalk 2011b), and although there are differences in properties and purification behavior between various mAbs, both cell culture and plant-based mAb purification processes have used Protein A affinity chromatography as the capture step in recent years (Kelley 2009; Shukla and Thömmes 2010). This mode of affinity chromatography is based on the specific binding affinity between the Fc region of mAbs and the Protein A ligand. Because of the specificity, host cell proteins, DNA, and other impurities from the cell culture process flow through the column while the product binds to the stationary phase, resulting in higher than 98% purity from a single step (Shukla and Thömmes 2010).

Chromatographic purification methods applied for plant-based recombinant proteins were summarized by Wilken and Nikolov (2012). It was observed that the initial capture of recombinant proteins from plant extracts is accomplished primarily by affinity and ion-exchange adsorption resins.

Ion-exchange resins are largely used in the biotechnology industry, and they are a common capture step for plant protein extracts (Harrison et al. 2003; Wilken and Nikolov 2012). Ion-exchange chromatography separates biomolecules on the basis of charge characteristics. Charged groups on the surface of a protein interact with oppositely charged groups immobilized on the ion-exchange medium. To develop the purification strategy, anion or cation exchange matrices, buffers and pH should be selected (Williams and Frasca 2001) depending on the recombinant protein properties. In many situations, manipulation of ionic strength and pH can improve protein binding capacity, concentration, and purification (D' Aoust et al. 2010; Pogue et al. 2010; Wilken and Nikolov 2012).

Hydrophobic type resins are usually positioned after ion-exchange steps in the purification train, since the recombinant proteins are eluted from the ion-exchange column with high salt concentrations. Hydrophobic interaction chromatography (HIC) has polar ligands substituted onto a neutral backbone and runs with an aqueous mobile phase (Curling and Gottschalk 2007). Because of relatively low recovery yields compared to affinity and ion-exchange resins, HIC is not usually considered as a capture step unless extract pretreatment requires the use of high salt concentrations (Wilken and Nikolov 2012).

As the most straightforward way to reduce downstream processing cost is to reduce reliance on high-cost chromatography steps (Wilken and Nikolov), lower-cost technologies are currently being investigated to substitute for chromatography in the biopharmaceutical industry. If the use of chromatography is still required for protein recovery, pretreatment of plant extracts prior to chromatography is one possible way to increase chromatographic

yield and consequently reduce the overall downstream processing cost. The removal of plant impurities before chromatography is desirable to increase the performance and lifetime of chromatography resins.

In the following sections of this dissertation, particular challenges in downstream processing of recombinant proteins from transgenic plant systems will be evaluated, using the examples of two different proteins expressed in two different plant systems. The focus will be on the impurities removal steps in the purification of a pharmaceutical protein (mAb) expressed in *Lemna minor* and in the purification of an industrial enzyme (bovine lysozyme) expressed in sugarcane. Section 2 reports the removal of phenolic impurities performed before the Protein A purification of mAb from *Lemna* extract and in Section 3 the adsorption isotherms of model phenolic solutions at pH 4.5 and 7.5 were determined, describing the interactions between five phenolic compounds commonly found in plant extracts and three types of inexpensive polymeric resins. In Section 4, conditions to minimize the native protein and maximize bovine lysozyme (BvLz) content in transgenic sugarcane extracts were investigated, as well as the recovery and purification of BvLz from sugarcane.

2. PHENOLICS REMOVAL FROM TRANSGENIC *LEMNA MINOR* EXTRACTS EXPRESSING MONOCLONAL ANTIBODY AND IMPACT ON MONOCLONAL ANTIBODY PRODUCTION COST*

2.1. Introduction

Transgenic plants have been used as hosts for a variety of protein products such as interferon, human growth hormone (hGH), immunoglobulin G (IgG1), and secretory immunoglobulin A (sIgA) antibodies at significant levels (Fischer et al. 2009; Ma et al. 2003; Nikolov and Woodard 2004). The use of plants as a recombinant expression platform for pharmaceutical proteins has been validated by products already in or close to being in the market (Kaiser 2008). Tobacco and a small aquatic organism known as *Lemna minor* (duckweed), are promising candidates for commercial production of pharmaceutical proteins. *Lemna minor* doubles its biomass in 18-24 hours and has been engineered to express homogenously glycosylated mAbs that closely mimic those produced in mammalian cell culture (Cox et al. 2006).

The extracts of *Lemna*, tobacco and other leafy plants can be complex and contain a number of water-soluble non-protein impurities including phenolics, amino acids, organic acids, carbohydrates, pectin, and nucleic acids (Hassan et al. 2008; Nikolov et al. 2009; Platis et al. 2008). Phenolic compounds are a potential concern in the production of protein therapeutics such as monoclonal antibodies (mAbs) for two reasons. First, they can interact with proteins through a number of mechanisms including hydrogen bonding, oxidative coupling, and ionic and/or hydrophobic interactions (Jervis and Pierpoint 1989; Loomis 1974). Second, the presence of phenolics in plant extracts (Woodard et al. 2009b)

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that could build up over time on a protein A-type affinity resin increases the probability of shortening resin lifetime. For plant-produced mAbs specifically, the cost of the protein A capture step is critical as the economic benefits of plant production systems could be erased if downstream processing ends up being more expensive than that of cell culture. Therefore, to avoid potential modification of the mAbs or fouling of expensive Protein A resins, a rapid and efficient removal of phenolics from plant extracts would be desirable. It was previously shown that the amount of IgG extracted from transgenic *Lemna* expressing a human mAb was similar regardless of whether pH 4.5 or pH 7.5 extraction was used (Woodard et al. 2009b). The pH 4.5 extract had 22% higher phenolics due to the presence of a ferulic acid form that was not found in the pH 7.5 extract. However, this difference would not be sufficient to eliminate their negative effect on the mAb purification train.

Although various methods for protein purification from plants allude to the need to remove phenolics from plant extracts, few papers on recombinant protein production from transgenic plants address this topic (Miller et al. 2004; Platis et al. 2008; Woodard et al. 2009b). Approaches for phenolics removal from plants include use of membrane filtration (D' Alvise et al. 2000; Xu and Diosady 2002), aqueous two-phase partitioning (Miller et al. 2004; Platis and Labrou 2006) and adsorption (Payne and Shuler 1988; Woodard et al. 2009b). The focus of this section has been on the latter since there are several inexpensive, commercially available resins which have been used in the past to remove phenolic and other colored compounds from non-transgenic plants (D' Alvise et al. 2000; Loomis 1974; Xu and Diosady 2002), fermentation media (Fisher et al. 2008; van den Berg et al. 2008) and wastewater (Carmona et al. 2006; Li et al. 2001; Pissolatto et al. 1996).

It was previously demonstrated that *Lemna* phenolics could be removed by incubation of extracts with the anionic hydrophobic resin, Amberlite IRA-402 (Woodard et

al. 2009b), but process relevant parameters were not examined in order to assess the cost impact of using a decolorization step in protein manufacturing. The objectives of this study were therefore 1) to identify major *Lemna* phenolics; 2) to evaluate their adsorption and elution from the commercially available resins XAD-4, IRA-402 and Q-Sepharose; and 3) to determine the cost of adding a phenolics removal step to a mAb purification train.

2.2. Materials

Transgenic *Lemna minor* was provided by Biolex Therapeutics (Pittsboro, NC) and stored at -80°C until use. Salts, acids and solvents were purchased from VWR (Radnor, PA). Ferulic acid and vitexin-2-O-rhamnoside standards of, respectively, 99 and 98% purity were purchased from Sigma-Aldrich (St. Louis, MO).

Rohm and Haas resins, IRA-402 and XAD-4, were purchased from Sigma-Aldrich, Q-Sepharose from GE Healthcare (Piscataway, NJ) and Polyclar® AT (PVPP) was purchased from VWR (Radnor, PA). XAD-4 is a hydrophobic polystyrene divinylbenzene resin (particle size 0.59 mm, surface area 725 m^2/g); IRA-402 is a strong anion exchange resin with a quaternary ammonium functional group added to polystyrene divinylbenzene matrix (particle size 0.45 mm, exchange capacity of 1.2 meq/mL wet volume); Q-Sepharose is an agarose based strong anion exchange resin with quaternary ammonium functional groups (particle size 0.105 mm, exchange capacity 0.2 meq/mL wet volume); and PVPP is an insoluble form of cross-linked polyvinylpyrrolidone. All the resins were conditioned per manufacturer's recommendations.

2.3. Methods

2.3.1. Extraction

Lemna extracts were homogenized in a blender with 100 mM acetate (pH 4.5) or phosphate buffer (pH 7.5) solutions by using 5:1 buffer-to-tissue ratio and two 30 s pulses at the highest speed. The two buffers contained 300 mM sodium chloride (NaCl) and 10 mM EDTA. After homogenization, the extracts were spun at 12,000 g for 30 min at 4˚C in a Beckman Allegra 25R centrifuge. The supernatants were filtered through a 0.45 μm filter and stored at 4°C for up to 5 days.

2.3.2. Phenolics profiling by RP-HPLC

Phenolics profiling was done using a system described previously (Woodard et al. 2009b). Extracts were run on a 5 μm 2.1 mm x 25 cm Supelco Discovery BIO Wide Pore C18 HPLC column connected to a Dionex Summit HPLC using a P680 pump and PDA-100 photodiode array detector to allow monitoring absorbance from 250 to 601 nm. The solvents consisted of (A) 0.05% formic acid in water and (B) 0.05% formic acid in acetonitrile. The gradient was from 0-50 min, 0 to 36% B; 50-55 min, 36-60% B; 55-60 min, 60-100% B at 0.25 mL/min.

2.3.3. **Lemna** *extract base hydrolysis*

Base hydrolysis of methanolic *Lemna* extract was done to convert O-glycosides to aglycones. Methanolic *Lemna* extract was treated with 2 M sodium hydroxide (NaOH), purged with nitrogen and incubated at room temperature with mixing for 2 hours protected from light and then neutralized using HCl.

2.3.4. Adsorption isotherms

Stock solutions (1 g/L) of ferulic acid and vitexin-2-O-rhamnoside were prepared by dissolving 5 mg of each in 0.25 mL of methanol and adding 4.75 mL of the appropriate

buffer. Sodium acetate and sodium phosphate (100 mM) buffer were used to make pH 4.5 and pH 7.5 solutions, respectively. Initial concentrations of 20, 50, 100, 150, and 200 mg/L were prepared by diluting stock solutions with the appropriate buffer. Before starting the equilibrium experiments the resins were pre-equilibrated in the respective buffer for 5 min. Equilibrium experiments were started by mixing 4 mL of each solution with 0.2 g of IRA-402, PVPP or XAD-4 resin. Solutions were gently agitated (20 rpm) by end-over-end rotation for 24 hours. All experiments were performed in duplicate. Supernatant absorbances were measured by a DU 640 Beckman spectrophotometer at the maximum wavelength for each compound (322 for ferulic acid, 342 nm for vitexin-2-O-rhamnoside) and converted to equilibrium concentrations using standard curves. The adsorbed amount at equilibrium was calculated from the difference between the initial and the equilibrium concentration of each phenolic compound in solution. Isotherms were generated by plotting the concentration of phenolics bound to the resin (Q) versus that in solution at equilibrium concentration (C*). The partition coefficient was estimated from initial slopes of the respective isotherms, that is, K_p = Q (mg/L)/C* (mg/L).

2.3.5. Breakthrough analysis of phenolics adsorption and resin regeneration

Each resin was packed into 1 cm x 10 cm Econo glass column (BioRad) to a 5-cm bed height, and equilibrated with 100 mM acetate (pH 4.5) or phosphate buffer (pH 7.5). *Lemna* extract (150 mL) was loaded at a superficial velocity of 38 cm/h onto the 4 mL bed volume column of IRA-402, Q-Sepharose, or XAD-4 resin. One column volume (CV) effluent fractions were collected during loading and their absorbances at 320 nm determined. The fractions were analyzed by HPLC and areas of the peaks with retention times between 20 and 50 min were summed. The resulting areas were then converted to concentrations as described before (Woodard et al. 2009b). Dynamic binding capacity (DBC) at a specified

breakthrough (%BT) was calculated from the difference between the amount of phenolic loaded and the amount that broke through the column. Loaded resins were washed with 5 CV of 100 mM acetate (pH 4.5) or phosphate (pH 7.5) buffer to remove interstitially retained phenolics and extract components. Adsorbed phenolics were eluted with 5 CV of 1 M NaCl in 100 mM of respective buffer followed by 5 CV of 95% aqueous ethyl alcohol (EtOH).

2.3.6. Process simulation and economics

A 100 kg/yr monoclonal antibody (mAb) purification process from transgenic *Lemna minor* extract was simulated with SuperPro Designer® 7.0 software (Intelligen, NJ). Using experimental data (Lisa Wilken, personal communication) and relevant information published elsewhere (Cox et al. 2006; Woodard et al. 2009b) the downstream processing train depicted in Figure 2.1 was assembled.

Adsorption with XAD-4 resin was operated for 10 cycles before replacement, assuming no loss in the dynamic binding capacity. Regeneration of XAD-4 resin between each cycle consisted of 5 CV buffer wash, 5 CV 95% EtOH and 5 CV buffer equilibration. The regeneration cycle was conducted at 800 cm/h. The second resin, IRA-402, was disposed after each cycle. To maintain process continuity two IRA-402 columns were operated in a staggered mode. Resin binding capacities of *Lemna* phenolics were experimentally determined as 0.89 g/L for XAD and 2.3 g/L for IRA-402. MAb yield from this step was experimentally determined as 89%. The resin cost of US$ 10/L (John Fisher, personal communication) was used; resin disposal cost was set at US$ 1/L. MAb purification by protein A (MAbSelect®, GE Healthcare) chromatography was simulated using the following inputs: dynamic binding capacity of 20 g/L; resin cost of US$ 8000/L and mAb yield of 90%. The number of usable cycles for MAbSelect® resin was used as

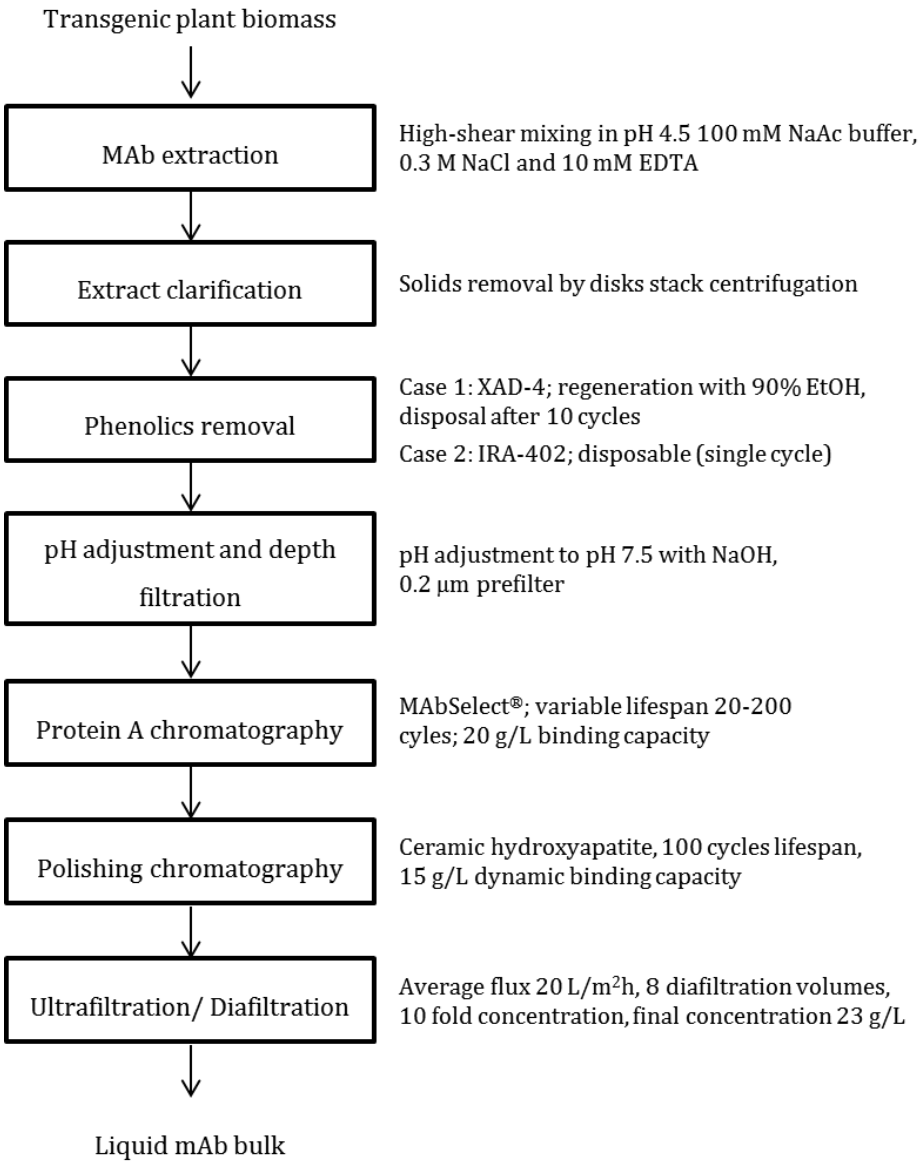


Figure 2.1: Downstream processing flow diagram of a monoclonal antibody (mAb) purification process from transgenic *Lemna minor* extract.

input for sensitivity analysis. For ceramic hydroxyapatite (Bio-Rad) chromatography, the binding capacity of resin was set at 15 g/L, resin cost at US$ 1000/L, resin lifespan at 100 cycles and mAb yield at 72%. Replacement cost of US$ 800/m^2 for UF membranes was

assumed.

Fixed capital investment was estimated from the equipment purchase cost using default SuperPro 7.0 factors (Lang factor of 6.7). The other fixed capital components (electrical, buildings, yard improvements, engineering, construction, etc.) were also estimated using SuperPro 7.0 default factors (Harrison et al. 2003). Operating labor was estimated by allocating operator hours required per equipment operation time. Labor-dependent cost items such as shift supervisors, QC/QA, and laboratory services were estimated as a percentage of operating labor: supervisors - 20%, lab services - 15%, QC/QA - 50% for chromatography steps and 25% for other unit operations. Direct production cost in this analysis included transgenic plant tissue, reagents, consumables, operating labor supervision, QC/QA and lab charges, operating supplies, maintenance, and utilities. Indirect cost included depreciation, taxes, insurance, and plant overhead cost (Peters et al. 2003). The cost of chemicals was obtained from the ICIS Chemical Business (www.icis.com) and industry collaborators.

2.4. Results and Discussion

2.4.1. Preliminary identification of phenolics in Lemna minor *extracts*

(Performed together with Dr. Susan Woodard)

In an earlier work (Woodard et al. 2009b), an RP-HPLC method was used to quantify the levels of phenolics extracted from transgenic *Lemna minor* at pH 4.5 or pH 7.5. Although this method was useful in assessing the relative levels of total phenolics present in these extracts, several of the major phenolic peaks were not completely resolved. To identify individual phenolic peaks, we decided to change the chromatographic method to one compatible with mass spectrometric identification. In HPLC-ESI-MS the mobile phase

modifier is typically formic acid at a concentration of 0.1%. This method caused the phenolic compounds to elute later and as a result some peaks were not well-resolved. By reducing the formic acid concentration to 0.05%, we were able to clearly delineate two peaks that were not resolved using the higher concentration of formic acid (Woodard, unpublished). Unfortunately, we were never able to identify any phenolics by mass spectrometry when ESI-MS detection was used with the HPLC method. Figure 2.2 shows RP-HPLC chromatographic profiles of methanolic *Lemna* extracts obtained with the 0.05% formic acid in water/0.05% formic acid in acetonitrile gradient. Although the number of phenolic peaks varies slightly with extraction and processing conditions, aqueous extracts (Figure 2.3) have a similar profile as the methanolic extract. At least 12 phenolics peaks were consistently detected by RP-HPLC in the methanolic extract (Table 2.1) and most of the peaks could also be seen in both aqueous extracts. All peaks eluting between 20 and 50 min look like phenolic compounds, based on their spectra.

A preliminary determination of the phenolic peaks (and the classes the majority of them belong to) was done by comparison to spectra of reference compounds or by comparison to published spectra (Mabry et al. 1970). For example, absorption maxima at 269 and 337 nm is characteristic of apigenin (5,7,4'-trihydroxyflavone) and C-glycosides of apigenin, such as vitexin (8-C-glucosylapigenin). Apigenin-7-glycosides have similar maxima but contain a more rounded trough compared to that of apigenin and vitexin. A double absorbance band at 255 and 270 nm with a stronger absorption band at about 350 nm is characteristic of luteolin (5,7,3',4'-tetrahydroxyflavone) and related glycosides. Although we were not able to definitively assign names to all of the different peaks in our chromatographic profile, the predominant peaks were categorized as apigenin-7-glycoside, ferulic acid-form, vitexin-form, depending on their UV-VIS spectra. At least two peaks of

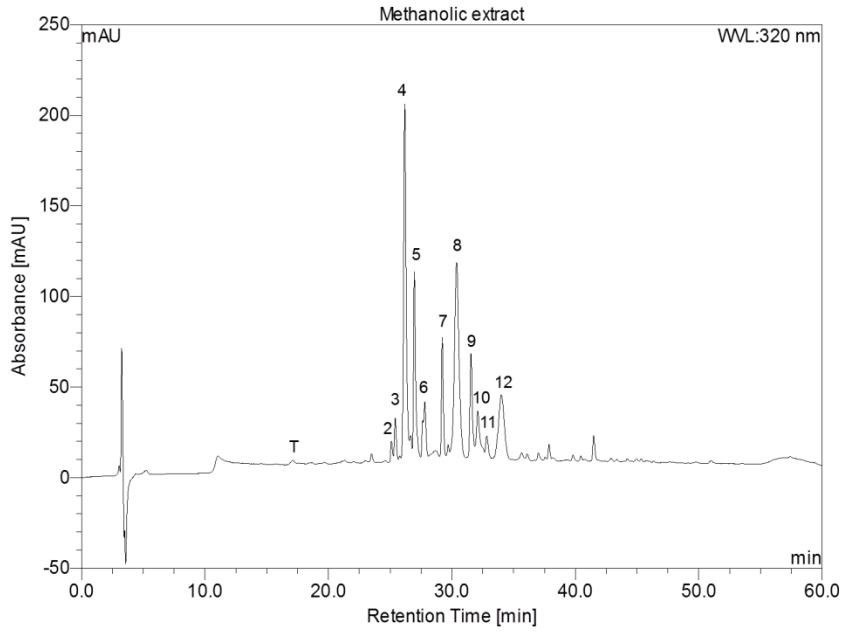


Figure 2.2: Phenolics profile by RP-HPLC of methanolic *Lemna* extract at 320 nm.

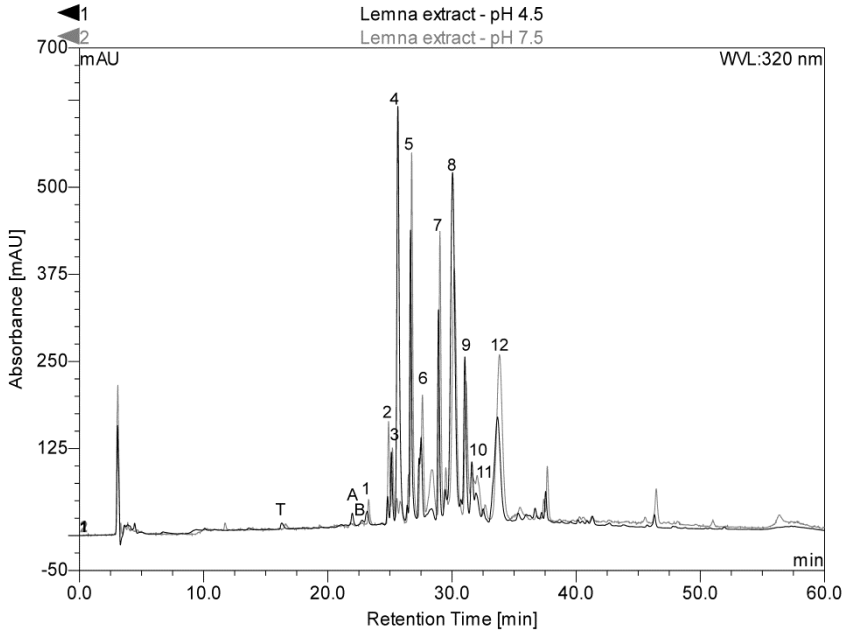


Figure 2.3: Phenolics profile by RP-HPLC of pH 4.5 and 7.5 aqueous *Lemna* extract at 320 nm.

Table 2.1: Peak assignments for phenolics profiled by RP-HPLC from *Lemna* extracts.

Peak	Peak retention time (min)	Tentative assignment
T	17.1	Tryptophan
A	21.1	Unknown
B	22.0	Unknown
C	23.2	Unknown
1	23.5	Luteolin-glycoside
2	25.1	Luteolin-glycoside
3	25.4	Apigenin-7-glycoside
4	26.2	Ferulic acid form
5	27.0	Apigenin-7-glycoside
6	27.8	Apigenin-7-glycoside
7	29.2	Apigenin-7-glycoside
8	30.4	Vitexin-form
9	31.6	Ferulic acid form
10	32.1	Mixed peak
11	32.8	Vitexin form
12	33.9	Vitexin form

each of these three compounds were observed in aqueous extracts. In addition, two minor peaks were designated as luteolin-glycosides.

The compounds eluting between 20 and 50 min (Figure 2.2 and 2.3) and having spectra consistent with one of the three categories (apigenin-7-glycoside, ferulic acid-form, or vitexin-form) of phenolic compounds comprised over 75% of the total peak area found in the pH 4.5 and 7.5 *Lemna* extracts. Peaks 4 and 9, with retention times of 26.2 and 31.6 min were classified as ferulic acid forms, peaks 3, 5, 6 and 7 eluting at 25.4, 26.9, 27.8 and

29.2 min as apigenin-7-glycoside forms, and those eluting at 30.4, 32.8 and 33.9 min (peaks 8, 11 and 12) as vitexin forms. There were several additional peaks that were not identified due to low abundance. Peaks 4 and 9 were further investigated because peak 4 (26.2 min) was present in pH 4.5 aqueous buffer extracts but not in the pH 7.5 phosphate buffer extract (Figure 2.3), whereas the presence of the spectrally similar peak 9 (RT 31.6 min) did not appear to be pH-dependent. Base hydrolysis of methanolic extract showed the 26.2 min peak shifted to 29.2 min where it overlapped with an apigenin peak (data not shown). The shift upon hydrolysis implies that the 26.2 min compound was glycosylated or some other base-labile form of a hydroxycinnamic acid. Since ferulic acid standard elutes at 29.1 min, we conclude that peak 4 is a hydrolysable form of ferulic acid.

The pH 4.5 extract (Figure 2.3) also has two small unidentified peaks (with phenolic acid signatures) which eluted before peak 1 (Peaks A and B; RT 21.1 and 22 min, respectively), while they are not seen in the pH 7.5 extract. Another unidentified peak in Table 2.1 with a phenolic acid signature (Peak C; RT 23.2 min) was present in very low concentrations in both methanolic and aqueous initial extracts, but was detected in the breakthrough fractions.

Based on retention times and spectral properties the majority of phenolic compounds in *Lemna* extracts are glycosylated flavones. Our conclusion that these flavones are glycosylated is based upon their having retention times that are much shorter than their aglycone counterparts. The presence of one or more glycosylation site on the molecule causes the compounds to be more polar and elute earlier. Based on the retention time patterns some of the flavones may be di- or even tri-glycosides. Unfortunately, none of the commercially available reference compounds had both the same exact retention time and spectral characteristics as the *Lemna* flavones.

2.4.2. Adsorption isotherms of ferulic acid and vitexin-2-O-rhamnoside

Adsorption isotherms of ferulic acid and vitexin-2-O-rhamnoside on PVPP, XAD-4, and IRA-402 resins at pH 4.5 and 7.5 are shown in Figure 2.4. The equilibrium data for the two phenolic compounds were generated from relatively low initial solution

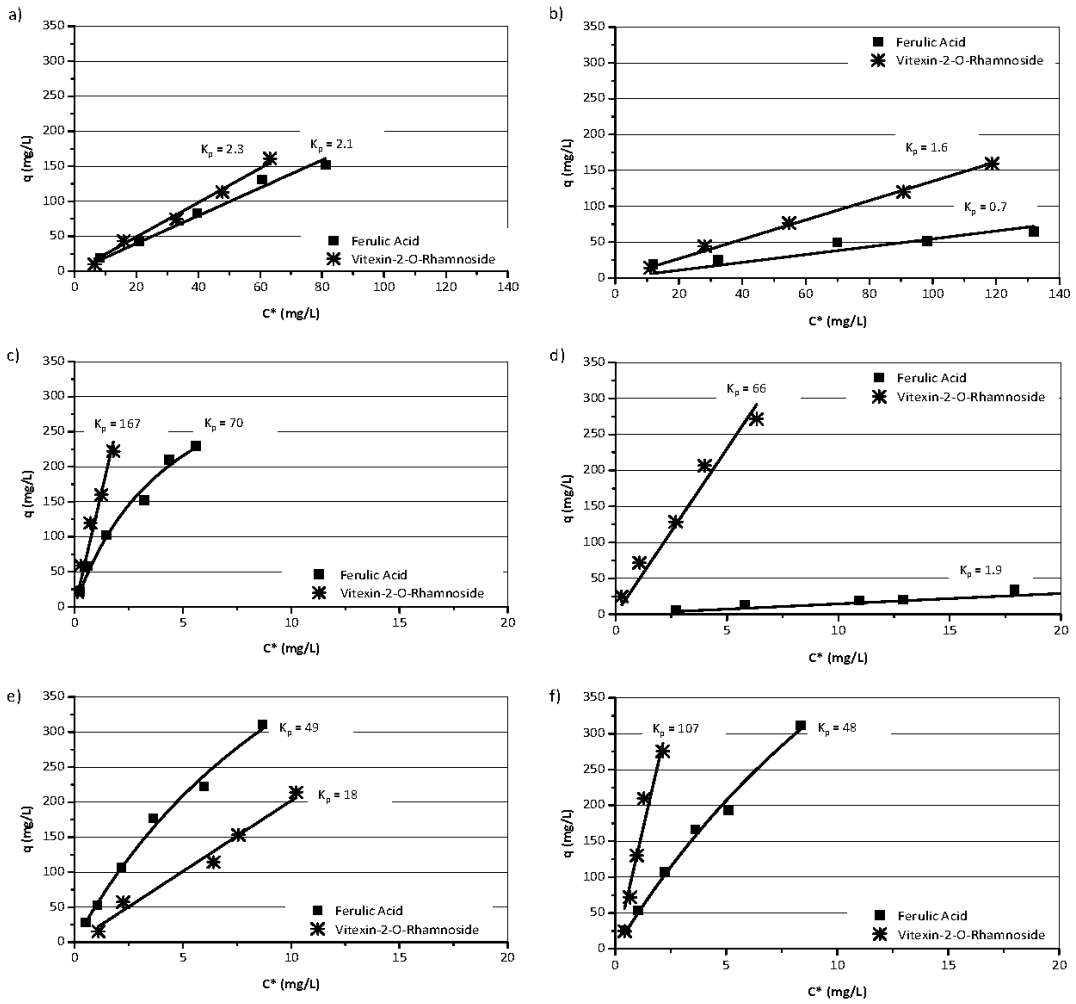


Figure 2.4: Equilibrium adsorption isotherms of ferulic acid and vitexin at room temperature. (a) PVPP at pH 4.5, (b) PVPP at pH 7.5, (c) XAD-4 at pH 4.5, (d) XAD-4 at pH 7.5, (e) IRA-402 at pH 4.5, (f) IRA-402 at pH 7.5.

concentrations (< 200 mg/L) due to their limited water solubility. In this concentration range, all vitexin isotherms were linear as well as those of ferulic acid on PVPP (Figures 2.4 a,b) and XAD-4 at pH 7.5 (Figure 2.4d). The other three isotherms of ferulic acid obtained with XAD-4 (Figure 2.4c) and IRA-402 (Figures 2.4 e,f) were best fitted to the Langmuir model. For both linear and Langmuir isotherms, the partition coefficient was estimated from the initial slopes of the respective linear portion of the isotherms. The partition coefficients obtained for either compound binding to PVPP were much lower than those for binding to XAD-4 or IRA-402. The partition coefficients estimates for ferulic acid and vitexin molecules with PVPP are in agreement with the conclusion of Andersen and Sowers (1968) that hydrogen bonding through aromatic hydroxyls governed the adsorption on PVPP. Furthermore, the same work demonstrated that the specificity of PVPP for the removal of phenolics from plant extracts was correlated to the number of free aromatic hydroxyl groups and was affected by the presence of undissociated carboxyl groups. Therefore, vitexin having three aromatic hydroxyls (pK_a = 6.7) (Rauha et al. 2001) and ferulic acid with one free aromatic hydroxyl (pK_a = 9.1) and one carboxyl group (pK_a = 4.4) would be expected to have similar partition coefficients at pH 4.5 (Figure 2.4a). The lower partition coefficient of vitexin (K_p = 1.6) and substantially reduced partition coefficient of ferulic acid (K_p = 0.7) at pH 7.5 compared to pH 4.5 could be explained by reduced hydrogen bonding because of partially dissociated vitexin hydroxyl groups and the fully dissociated ferulic acid carboxyl, respectively (Figure 2.4b).

The adsorption of the two phenolic compounds on XAD and IRA was somewhat more complex. The larger and more hydrophobic vitexin had a greater affinity for XAD-4 matrix than ferulic acid at pH 4.5 and 7.5 (Figures 2.4 c,d). At pH 7.5, the partition coefficients of both compounds were reduced, especially that of ferulic acid which dropped from a K_p of

70 to 1.9. The possible reason for this change is the prevalence of ferulate anion (pK_a = 4.4) in the aqueous phase at pH 7.5, which should have little affinity for the uncharged XAD resin.

Figures 2.4e and 2.4f compare the adsorption of the two molecules to IRA-402 at pH 4.5 and pH 7.5. There was very little difference in the adsorption of ferulic acid to IRA-402 at the two different pHs probably because of the dual binding mechanism inherent to this resin; hydrophobic interactions of undissociated ferulic acid dominate at pH 4.5 while charged interactions of ferulate anion prevail at pH 7.5. Vitexin-2-O-rhamnoside, on the other hand, interacted more favorably with IRA-402 at pH 7.5 than at pH 4.5. The cause for the greater partition coefficient of vitexin with IRA-402 at pH 7.5 is likely due to the fact that vitexin is able to bind both hydrophobically and ionically with the resin at pH 7.5 while at pH 4.5 only hydrophobic interactions occur. Regardless of the adsorption mechanism, hydrophobic resins XAD-4 and IRA-402 have greater affinity and equilibrium adsorption values than PVPP for the two representative phenolic compounds. Based on our results, IRA-402 and XAD-4 were selected for evaluating the removal of phenolics from *Lemna minor* extracts.

2.4.3. Breakthrough analysis of phenolics adsorption from pH 4.5 and 7.5 extracts

In addition to IRA-402 and XAD-4 resins, Q-Sepharose, a strong anion exchange resin with agarose (hydrophilic) matrix, in contrast to the hydrophobic IRA-402 matrix, was included in this experiment to assess the contribution of the anion ligand alone to phenolics retention. Extracts prepared at pH 4.5 and pH 7.5 were continuously loaded on each of the three resins to generate the breakthrough curves shown in Figure 2.5. Because it was not always possible to compare the adsorption capacity of the three adsorbents at

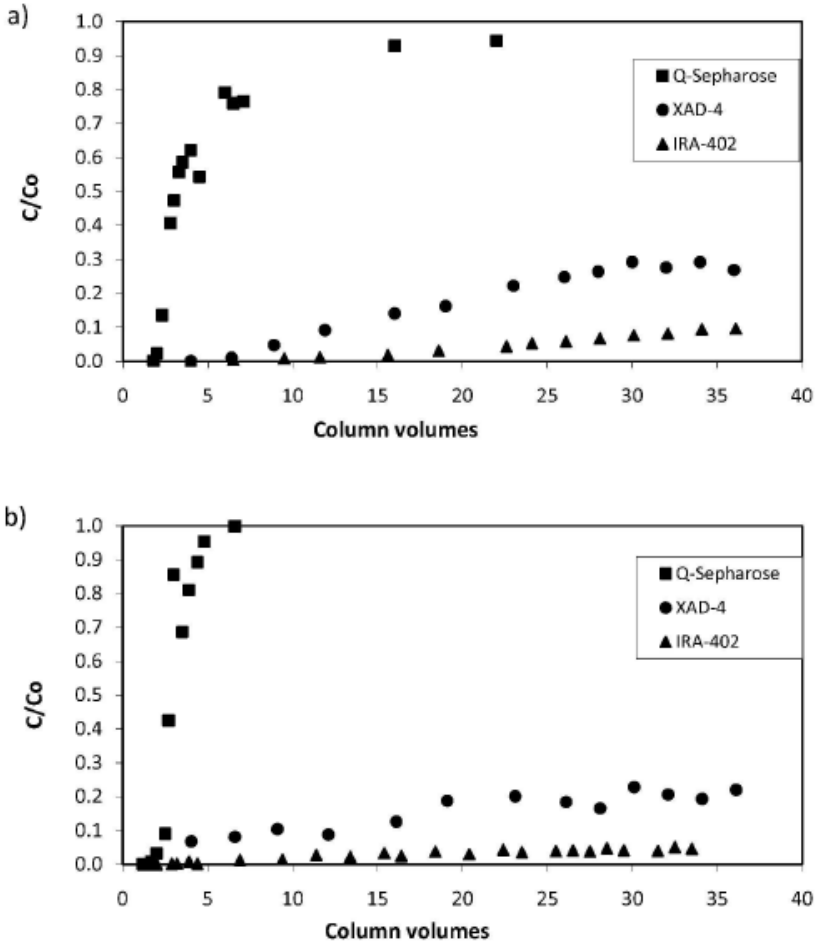


Figure 2.5: Breakthrough analysis of phenolics from (a) pH 4.5 and (b) pH 7.5 *Lemna* extract on different resins. Breakthrough curves were generated by plotting effluent phenolics concentration (C) as a fraction of the feed concentration (C_0) against the number of column volumes (CV) of extract loaded onto the column.

the same percentage breakthrough (%BT), Table 2.2 lists the estimated resin dynamic binding capacities (DBC) determined at the percent breakthrough closest to 5%.

The breakthrough curves in Figure 2.5 show that matrix hydrophobicity played an important role in adsorption of *Lemna* phenolics at either pH. Phenolics broke through the

Table 2.2: Dynamic binding capacities (DBC) of phenolics on adsorbent resins at pH 4.5 and pH 7.5.

pH	Resin	Percentage breakthrough (% BT)	Dinamic binding capacity (g/L resin)
4.5	Q-Sepharose	2	0.24
	XAD-4	5	0.89
	IRA-402	5	2.3
7.5	Q-Sepharose	3	0.15
	XAD-4	7	0.28
	IRA-402	5	1.8

Q-Sepharose column almost immediately at both pH 4.5 and 7.5 resulting in DBC of 0.24 and 0.15 g/L, respectively (Table 2.2). Dinamic binding capacity (DBC) of *Lemna* phenolics on XAD-4 was almost four-fold greater than that of Q-Sepharose at pH 4.5 and two-fold greater at pH 7.5. This trend was not surprising since hydrophobic interactions with polystyrene divinylbenzene matrix would be expected to drive phenolics adsorption. The IRA-402 resin had a considerably greater adsorption capacity compared to XAD-4 at both pHs indicating the added effect of ionic interactions to phenolics adsorption; the DBC of IRA-402 was 2.5 and 6 times higher than that of XAD-4 at pH 4.5 and 7.5, respectively. The ten-fold greater DBC of IRA-402 compared to Q-Sepharose is probably due to the combined effect of anionic ligand concentration (IRA = 1.2 meq/mL; Q-Sepharose = 0.2 meq/mL) and hydrophobic IRA matrix.

For all tested resins, DBCs were substantially greater at pH 4.5 than at pH 7.5 (Table 2.2). Due to the fact that extracts made at pH 7.5 had 3-fold higher protein and amino acids content than pH 4.5 extracts (Woodard et al. 2009b), a possible explanation for the lower

capacities observed at pH 7.5 is protein and amino acids binding to the resins. Interestingly, the resins with anionic groups, Q-Sepharose and IRA-402, were affected less by the adsorption pH than uncharged XAD-4 resin; XAD-4 had 300% reduction in DBC compared to 60 and 30% for Q-Sepharose and IRA-402.

Figure 2.6 shows the RP-HPLC profile analysis of fractions at breakthrough for each resin. The phenolics profiles of the breakthrough fractions indicate that their binding to the three adsorbents at pH 4.5 and 7.5 was different. At pH 4.5, primarily phenolic acids were breaking through on XAD-4 (peaks A, B, C and 4 in Figure 2.6a). On the anionic resins, phenolic acid peaks at pH 4.5 were retained by the resins, and the glycosylated flavones (peaks 3, 5, 6, 7, 8 and 12 in Figure 2.6a) appeared first in the breakthrough fractions. This is consistent with the measured ferulic acid and vitexin (flavone) partition coefficients on XAD-4 and IRA-402 at pH 4.5 (Figures 2.4 c,e). At pH 7.5, the anionic resins IRA-402 and Q-Sepharose were more effective than XAD-4 in retaining the majority of the predominant peaks (20-35 min). Only less-abundant, late eluting compounds (> 35 min) were detected in the breakthrough. Because IRA-402 showed higher overall adsorption than XAD-4, anionic resins seem the most appropriate to clean *Lemna* extracts before protein purification. The IRA-402 resin also has the advantage of exhibiting less variation in binding capacity than XAD-4.

2.4.4. Desorption of phenolics and resin regeneration

An important consideration for phenolics removal is the ease and extent of resin regeneration. The choice of solutions and elution sequence, 100 mM sodium acetate buffer, followed by 1 M NaCl and 95% EtOH, in addition to regeneration, was aimed at understanding the prevailing type of interactions with the absorbents (non-specific, ionic

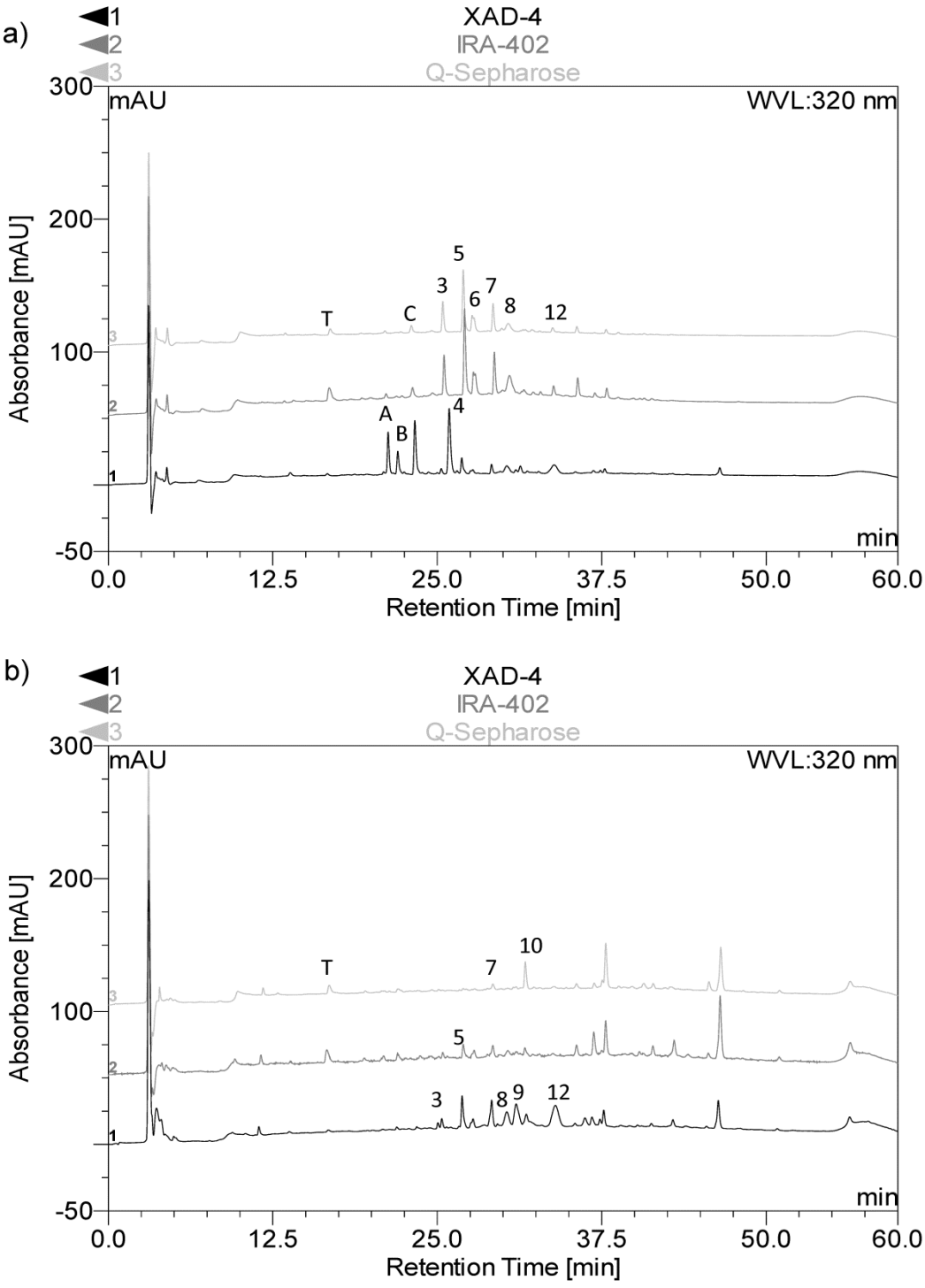


Figure 2.6: RP-HPLC profiling of phenolics from (a) pH 4.5 and (b) pH 7.5 *Lemna* extract at 5% breakthrough at 320 nm.

or hydrophobic). The effect of applied eluants on desorption of *Lemna* phenolics from the three tested resins is presented in Figure 2.7. Washing Q-Sepharose with three volumes of acetate buffer removed a significant fraction of phenolics retained in the interstitial column volume (Figure 2.7a). Buffered 1 M NaCl was effective in removing the remaining adsorbed phenolics. Specifically, 3rd and 4th CV application of the NaCl solution (fractions #8 and 9 in Figure 2.7a), which had the highest NaCl concentration (1 M), were the most effective in desorbing phenolics presumably bound through charge interactions. As expected for this matrix, no additional desorption of phenolics occurred when 95% EtOH was used as the eluant after NaCl, signifying a lack of hydrophobic interactions between *Lemna* phenolics and the agarose-based resin.

Figures 2.7b and 2.7c show desorption of phenolics from XAD-4 and IRA-402, respectively, using the same sequence of eluants. Desorption profiles with 100 mM acetate followed by 1 M NaCl were similar for both resins showing no specific desorption. Contrary to Q-Sepharose data, 1 M NaCl had little effect on phenolics desorption from IRA-402 suggesting that the strong hydrophobic interactions between the phenolics and the resin may have been enhanced by high salt concentration.

The elution of phenolics from XAD-4 and IRA-402 (Figures 2.7b and 2.7c) with 5 CV of 95% aqueous EtOH gave a different result. Five CV of 95% EtOH were apparently sufficient to remove a significant amount of phenolics from XAD-4 but not from IRA-402. The highest phenolics concentration was measured in the 2nd CV wash (fraction #12) for both resins but the concentration of desorbed phenolics in fraction #12 collected from XAD-4 elution (Figure 2.7b) was 6.5-fold higher than that from IRA-402 (Figure 2.7c). By comparing the amount of phenolics released to the amount loaded on these resins, we estimated that almost complete regeneration of Q-Sepharose could be achieved, as well as

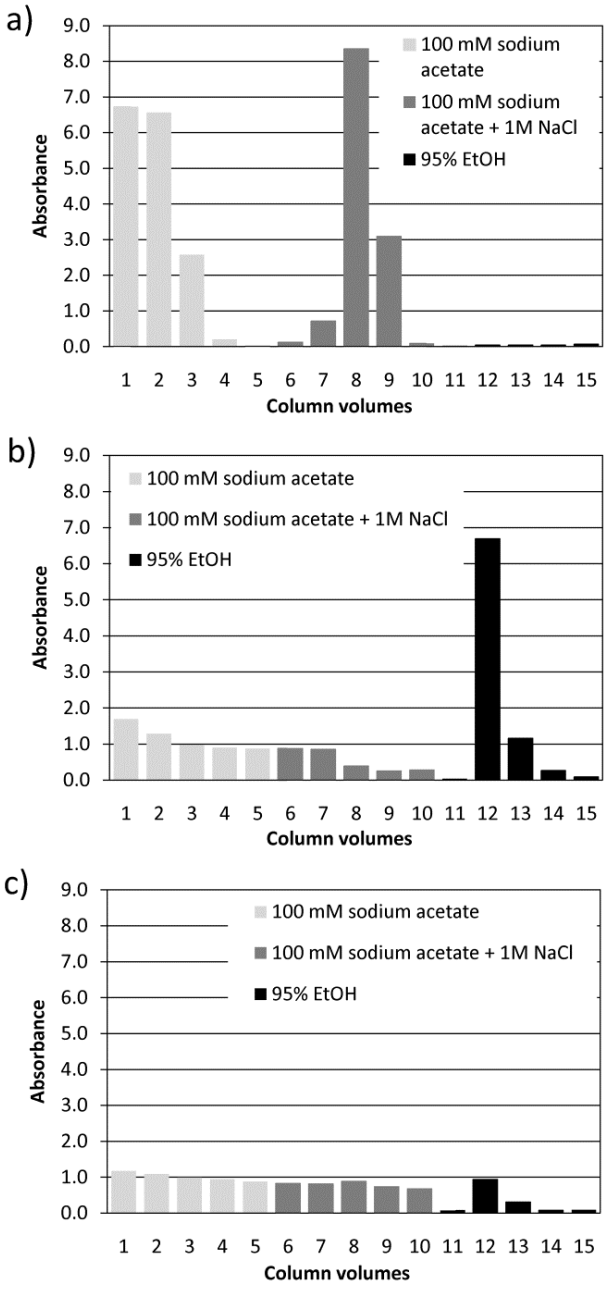


Figure 2.7: Desorption of pH 4.5 *Lemna* phenolics from (a) Q-Sepharose, (b) XAD-4, and (c) IRA-402 with 100 mM acetate buffer, 1 M NaCl and 95% EtOH.

40% regeneration of XAD-4, and only 10% of IRA-402. The low regeneration of IRA-402 indicates that the majority of the phenolics interacting with the resin are irreversibly bound through the formation of covalent bonds. The elution of *Lemna* phenolics adsorbed to the resins from pH 7.5 extracts showed the same trend as pH 4.5 extracts.

2.4.5. Production cost and sensitivity of a mAb purification train

Because the objective of making mAb in plants is to take advantage of low upstream production costs, determining the economic impact of adding a phenolics adsorption step to a mAb purification train was important. The mAb purification train evaluated in this study was designed to carry out phenolics adsorption from a pH 4.5 plant extract, because their adsorption to both IRA-402 and XAD-4 resins was superior at pH 4.5 compared to pH 7.5. Three process scenarios were simulated to determine the effect of phenolics removal on overall production cost. The protein A (ProA) scenario consisted of all the unit operations depicted in Figure 2.1 except for the phenolics removal step. Monoclonal antibody (mAb) eluted at pH 3 with 0.1 M citrate buffer was further purified on a ceramic hydroxyapatite column and then diafiltered and concentrated to yield a liquid bulk product. The other two cases (ProA+IRA and ProA+XAD) consisted of a phenolics adsorption step (IRA-402 or XAD-4 column) that was inserted before protein A chromatography (Figure 2.1). For additional process conditions refer to Figure 2.1 and Methods (Section 2.3.6.). To determine the cost benefit of increasing protein A lifetime, sensitivity analysis was performed by varying MabSelect® lifespan from 20 to 200 cycles. This range was selected as the most likely lower and upper end of protein A operational lifetime on a large scale (Kelley 2007; Sommerfeld and Strube 2005). The production costs (direct + indirect cost) from simulation analyses were normalized against the base case i.e. protein A (ProA) train with a lifespan of 20 cycles (Figure 2.8). The overall mAb yield for

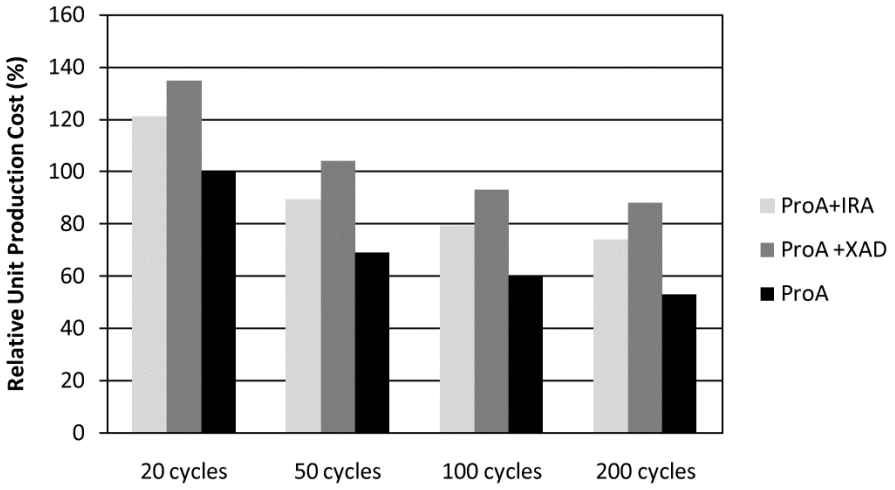


Figure 2.8: Effect of resin lifespan on unit production cost with and without phenolics removal step.

the ProA train was 60% while that for ProA+IRA or ProA+XAD train was 54%.

The data in Figure 2.8 demonstrate the significance of achievable resin cycles on the overall production cost. An increase of MabSelect® lifespan in the ProA train from 20 to 50 cycles would result in a 30% cost reduction and a further increase to 100 or 200 cycles would reduce cost by 40 and 47%, respectively. The greatest cost impact is achieved for the lifespan increase from 20-50 cycles, a trend also observed by Sommerfeld and Strube (2005). The introduction of a phenolics removal step to the ProA train adds additional cost of about 20% for IRA-402 resin and 35% for XAD-4. This increase stems primarily from the cost of consumables (resin) and reagents (ethanol for XAD-4 regeneration). The effect of increased lifespan of MabSelect® due to phenolics removal was similar to that observed with the ProA train. Compared to ProA base case, the increase of MabSelect® lifespan in ProA+IRA train from 20 to 50 cycles reduces production cost by 10% and from 20 to 100

or 200 cycles by 20 and 25%, respectively. Specifically, increasing the number of MAbSelect® cycles from 20 to 30 offsets direct material and labor cost resulting from the inclusion of an IRA-402 adsorption step. The ProA+XAD train, which is more expensive than the ProA+IRA train, would require an increase of MabSelect® lifespan to 60 cycles to offset additional XAD-related direct production costs. In all cases, inclusion of an XAD step was inferior to IRA, because of the lower dynamic binding capacity and high regeneration (solvent) cost. Single-use of IRA-402 resin also brings the typical advantage of disposables: no cleaning, regeneration and validation expenses.

The sensitivity analysis also shows that if the ProA train can be run for more than 50 cycles then there would be no cost reduction benefit from adding a phenolics adsorption step. In such cases, the driver for implementation of the phenolics removal step would be protection of the final product from undesirable protein-phenolics reactions. A decision for implementing phenolics removal step should be made on a case-by-case basis, by determining the reactivity of plant-derived phenolics and the ease of their removal from the final product during purification.

2.5. Conclusions

In this section, the majority of *Lemna* phenolics were identified to be glycosylated-flavones, primarily apigenin, vitexin, and some luteolin derivatives.

It was further demonstrated that the phenolic compounds present in *Lemna* extracts can effectively be removed using inexpensive adsorption resins prior to the affinity capture step. It was shown that hydrophobicity plays an important role in adsorption of *Lemna* phenolics; the DBCs of the resins with hydrophobic matrices XAD-4 and IRA-402 were, respectively, 3 and 10-fold greater than the DBC of the agarose-based resin Q-Sepharose.

By comparing the amount of phenolics released by the cleaning regime to the amount loaded on these resins, almost complete regeneration of Q-Sepharose could be achieved, 40% of XAD-4 could be regenerated, but only 10% of IRA-402. Because more than one mechanism is implicated in the binding of phenolics and IRA-402, the same reason that makes it a better resin for the phenolics removal also makes it harder to regenerate.

The economic analysis indicated that the addition of a phenolics adsorption step would increase the mAb production cost by only 20% for IRA-402 and 35% for XAD-4 resin. Furthermore, the cost of the added adsorption step could be offset by increasing the lifespan of protein A resin from 20 to 30 or more cycles, and an actual reduction of mAb production cost can be achieved by using disposable IRA-402. The use of an anionic resin has the added advantage of removing some negatively charged species such as acidic polysaccharide and nucleic acids.

Although no "one size fits all" approach is likely to work for all recombinant proteins from plant extracts, some of the findings reported in the study are universally applicable. The decision regarding the implementation of phenolics/pigments removal step and choice of available options has to be made on a case-by-case basis. The inclusion of a phenolics removal step may provide a more robust purification process that yields a product of uniform quality, a practice essential if the protein is to be used as a biotherapeutic.

3. REMOVAL OF PHENOLIC COMPOUNDS BY ADSORPTION ON POLYMERIC RESINS BEFORE PROTEIN PURIFICATION

3.1. Introduction

A variety of plant-derived pharmaceutical products, with diverse applications ranging from baby formula additives (Kaiser 2008) to a component for HIV prevention (O'Keefe et al. 2009) and hepatitis B antibodies (Valdés et al. 2003), demonstrate the feasibility of using transgenic plants as biofactories. Leafy and aquatic plants are frequently used to produce pharmaceutical proteins due to their quick biomass generation (Cox et al. 2006; Twyman et al. 2003). With plants accepted as a viable alternative to fermentation systems, the demonstration of a cost-competitive downstream process will strengthen the lower cost provided by the upstream production in plants (Nikolov et al. 2009). Together with the recombinant protein, there is a mixture of water-soluble impurities in the plant extracts, including nucleic acids, pigments, phenolic compounds and polysaccharides. The fact that every plant has a unique pool of impurities is still a disadvantage on the development of protein purification processes from plants.

Phenolic compounds are present in all plants, and because tobacco and *Lemna* are the current leading plant systems for production of therapeutics, the removal of phenolics is a critical issue to be addressed in order to reduce the downstream cost of plant-based proteins. Phenolics are a potential concern in the production of protein therapeutics such as monoclonal antibodies (mAb) for two reasons. First, they can interact with proteins through a number of mechanisms including hydrogen bonding, and ionic and/or hydrophobic interactions. (Jervis and Pierpoint 1989; Loomis 1974). Second, the presence of phenolics in plant extracts (Woodard et al. 2009b) that could accumulate over time on a

protein A-type affinity resin increases the probability of shortening resin lifetime. Thus, improvements in the downstream processing of recombinant proteins through phenolics removal will be the most objective way to reduce overall costs of biological processes for pharmaceutical proteins production.

The significance of removing phenolic compounds from plant extracts before chromatography steps was already pointed out (Miller et al. 2004; Platis and Labrou 2006; Woodard et al. 2009b), and different methods have been applied to perform this task. Adsorption is commonly used for removal of phenolics from wastewater (Caetano et al. 2009; Huang 2009), but not many applications for removal of phenolics from plant extracts are seen (D' Alvise et al. 2000; Saleh et al. 2008). Aqueous two-phase partitioning has also been used to reduce the amount of phenolics, alkaloids, and pigments in leaf extracts (Hasmann et al. 2008; Platis et al. 2008).

Before transgenic plants became candidates for protein production, plant tissue has been used for years as a source of enzymes and proteins (Andersen and Sowers 1968; Loomis 1974), and phenolics have been identified as a hurdle in the purification of proteins. Phenolic compounds are a complex group of substances that have gained enormous attention in the last 10 years, due to their biological properties, natural anti-oxidant capacity, physiological effects, and other properties related to food quality (Manach et al. 2005; Williamson and Manach 2005). Depending on their structure, phenolic compounds can react with proteins and alter some of their physicochemical properties such as solubility, electrophoretic behavior, hydrophobicity, molecular weight, and secondary and tertiary structure (Rohn et al. 2002).

Amid the various approaches used to alleviate the problems caused by phenolics present in plant extracts, several inexpensive resins have been tested for phenolics

removal before protein purification (D' Alvise et al. 2000). Prehydrated polyvinylpirrolidone (PVPP) was the most used resin for this purpose in a variety of applications ranging from the fundamental isolation of plant enzymes (Loomis and Battaile 1966) to more recent recombinant protein purification from transgenic tobacco (Holler and Zhang 2008). Gray (1978) compared PVPP to the polystyrene resins XAD-2 and Dowex-50 for adsorption of spinach polyphenols.

Adsorption of phenolics from spinach (Gray 1978), lettuce (D' Alvise et al. 2000) and apple juice (Saleh et al. 2008) using different polymeric resins shed light on the probable mechanisms of interaction that could explain phenolics adsorption. Based on these studies, it is known that phenolics adsorption to different resins may occur by hydrogen bonding, ionic (charge) interactions, and/ or hydrophobic interactions.

Because a great assortment of compounds is classified as phenolics and several polymeric adsorbents are available for removal of pigments and phenolics, understanding the type of interactions that governs phenolics adsorption would facilitate the choice of resin to be applied to a specific plant extract.

In order to understand the adsorption behavior of phenolic compounds from different plants, we selected five phenolic standards for this investigation: chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside. The chosen compounds belong to four classes of phenolic compounds (hydroxycinnamic acids, hydroxybenzoic acids, flanovols, and flavones) that are typically found in the plant extracts relevant to the work on protein purification from transgenic plants. Interactions of these compounds to three commercial types of polymeric resins (IRA-402, PVPP, XAD-4) were evaluated using equilibrium adsorption isotherms.

3.2. Materials

Acetic acid, phosphoric acid, sodium acetate, sodium phosphate dibasic and sodium phosphate monobasic were purchased from VWR (Radnor, PA).

Rohm and Haas resins, IRA-402 and XAD-4, were purchased from Sigma-Aldrich (St. Louis, MO), and Polyclar® AT (PVPP) was purchased from VWR. XAD-4 is a hydrophobic polystyrene divinylbenzene resin (particle size 0.59 mm, surface area 725 m^2/g); IRA-402 is a strong anion exchange resin with a quaternary ammonium functional group added to polystyrene divinylbenzene matrix (particle size 0.45 mm, exchange capacity of 1.2 meq/mL wet volume); and PVPP is an insoluble form of cross-linked polyvinylpyrrolidone (particle size 0.01-0.1 mm). All the resins were conditioned per manufacturer's recommendation.

Chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside standards of purity 98, 99, 95, 97 and 98%, respectively, were purchased from Sigma-Aldrich. Maximum absorption wavelengths are 275, 322, 326, 342 and 355 nm for syringic acid, ferulic acid, chlorogenic acid, vitexin, and rutin, respectively.

3.3. Methods

3.3.1. Calculation of pKa values

Values for pKa were calculated using the on-line software SPARC OnLine Calculator (Hilal et al. 1995) and ALOGPS 2.1 from the Virtual Computational Chemistry Laboratory (Tetko et al. 2005). As input for the software, the molecular structures of the compounds were drawn in a Java interface, which generated the pKa values.

3.3.2. Adsorption isotherms

Stock solutions (1 g/L) of the phenolic compounds chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside were prepared by dissolving 10 mg of each in 0.25 mL of methanol and adding 4.75 mL of the appropriate buffer. Sodium acetate and sodium phosphate buffer at 100 mM concentration were used to make pH 4.5 and pH 7.5 solutions, respectively. Initial concentrations of 20, 50, 100, 150, and 200 mg/L were prepared by diluting stock solutions with the appropriate buffer. Before starting the equilibrium experiments the resins were pre-equilibrated in the respective buffer for 5 minutes. After that, resins were vacuum filtered to eliminate intra-particle liquid, with the beads being supported by a 0.2 μm membrane.

Equilibrium experiments were started by mixing 4 mL of each solution with 0.2 g of IRA-402, PVPP or XAD-4 resin, making it a ratio of 5% (m/v). Solutions were gently agitated (20 rpm) by end-over-end rotation for 24 h at room temperature. Individual adsorption isotherms were generated at pH 4.5 and 7.5. Because rutin was not stable in the aqueous buffer at pH 4.5, the equilibration time for rutin at pH 4.5 was only 4 h. All experiments were performed in duplicate or in triplicate, in the cases that a substantial variation between the initial duplicates was seen.

Equilibrium solution absorbances were measured by a DU640 Beckham spectrophotometer and converted to equilibrium concentrations using standard curves. The adsorbed amount at equilibrium was calculated from the difference between the initial and equilibrium concentration of each phenolic compound in the solution. Isotherms were generated by plotting the concentration of phenolics bound to the resin (Q) versus that in solution at equilibrium concentration (C*). The partition coefficient was estimated from initial slopes of the respective isotherms, i.e., Kp = Q (mg/L)/ C* (mg/L).

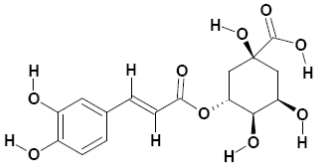
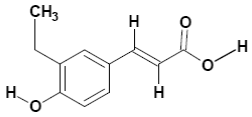
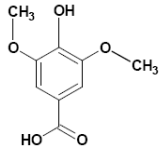
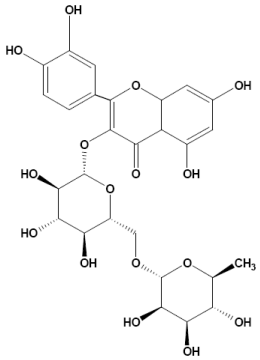
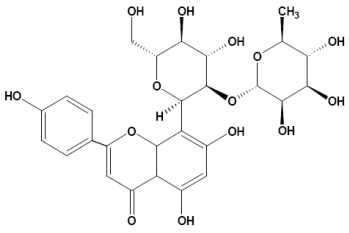
3.4. Results and Discussion

Structures, molecular weight (MW), classes and common sources of investigated phenolics are listed in Table 3.1. Ferulic acid is the most abundant phenolic compound in plants, being present in almost all types of plants and tissues. Chlorogenic acid was selected due to its large presence in tobacco, a plant commonly used for production of protein biotherapeutics. Syringic acid is found in sugarcane, a potential crop for industrial protein production, and other green tissues. Rutin and vitexin have been chosen as representatives of the flavonoid class. It should be noted that each plant contains a great variety of phenolic compounds and the selected sources of phenolics cited in Table 3.1 is not absolute. For example, sugarcane extracts contain also hydroxycinnamic acid compounds and flavones.

Adsorption isotherms of chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside on PVPP, XAD-4, and IRA-402 resins at pH 4.5 and 7.5 are shown in Figure 3.1. The equilibrium data were generated from relatively low initial phenolics concentrations (< 200 mg/L) as phenolics compounds are naturally present in low concentrations in plant aqueous extracts (Woodard et al. 2009b) due to their limited water solubility. In this concentration range, all chlorogenic acid, rutin and vitexin isotherms were linear. Ferulic acid and syringic acid isotherms generated with PVPP at pH 4.5 and 7.5 (Figures 3.1 a, b) and XAD-4 at pH 7.5 (Figure 3.1d) were also linear. The isotherms of ferulic and syringic acids obtained with XAD-4 at pH 4.5 (Figure 3.1c) and with IRA-402 at both pHs (Figures 3.1 e, f) were best fitted to the Langmuir model.

The estimated partition coefficients (Table 3.2) for most of the compounds, with the exception of syringic acid with PVPP at pH 7.5, were lower than those obtained with XAD-4 or IRA-402. In general, an increase in phenolics adsorption was observed with a decrease

Table 3.1: Classes, molecular weight, sources and structures of the five phenolic compounds investigated.

Phenolic compound	**MW (g/mol)**	**Structure**	**Class**	**Sources**
Chlorogenic acid	354.31		Hydroxycinnamic acid	*Lemna*, tobacco
Ferulic acid	194.18		Hydroxicinnamic acid	*Lemna*, rice
Syringic acid	198.17		Hydroxybenzoic acid	Sugarcane
Rutin	610.52		Flavonol	*Lemna*, tobacco
Vitexin-2-O-rhamnoside	578.52		Flavone	*Lemna*, sugarcane

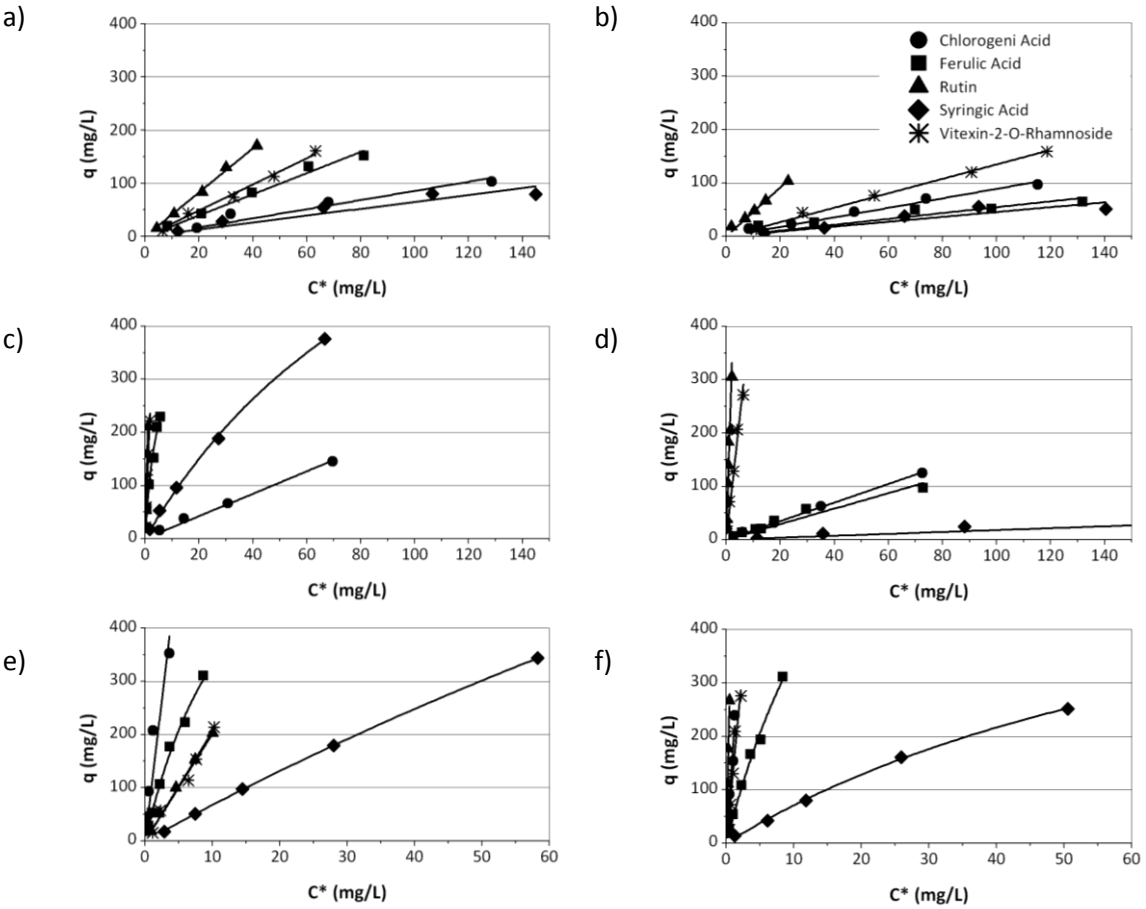


Figure 3.1: Equilibrium adsorption isotherms of chlorogenic acid, ferulic acid, rutin, syringic acid and vitexin-2-O-rhamnoside at room temperature. (a) PVPP at pH 4.5; (b) PVPP at pH 7.5; (c) XAD-4 at pH 4.5; (d) XAD-4 at pH 7.5; (e) IRA-402 at pH 4.5; (f) IRA-402 at pH 7.5.

of solute polarity.

The partition coefficient estimates for the interaction of phenolic molecules with PVPP are in agreement with previous data, which indicated that hydrogen bonding through aromatic hydroxyls governed the adsorption with PVPP (Andersen and Sowers 1968). For example, the more hydroxylated rutin (4 aromatic hydroxyl groups) had a larger partition

Table 3.2: Partition coefficients of phenolics on polymeric resins at pH 4.5 and pH 7.5 at room temperature.

pH	Resin	Partition coefficient (Kp)				
		Chlorogenic acid	**Ferulic acid**	**Syringic acid**	**Rutin**	**Vitexin-2-O-rhamnoside**
4.5	XAD-4	2.2	69.6	8.3	202	167
	IRA-402	167	48.8	6.8	21.9	17.8
	PVPP	1.3	2.1	0.8	3.9	2.3
7.5	XAD-4	1.8	1.9	0.3	195	66.4
	IRA-402	203	47.9	6.8	522	107
	PVPP	1.0	0.7	0.6	4.6	1.6

coefficient than vitexin (three aromatic hydroxyl groups) at both pHs. Also, both flavonoids interacted slightly stronger with PVPP matrix than the phenolic acids, which have one or two aromatic hydroxyl groups. Andersen and Sowers (1968) also demonstrated that the specificity of PVPP for the removal of phenolics from plant extracts was improved by the presence of undissociated carboxyl groups. This could possibly explain the differences between partition coefficients of ferulic, chlorogenic and syringic acids. At pH 4.5, 44% of ferulic acid carboxyl groups (pKa = 4.4) are undissociated, in contrast with 5% of chlorogenic acid carboxyl groups (pKa = 3.2) and 20% of syringic acid (pKa = 3.9). The larger fraction of dissociated carboxyls of chlorogenic acid at pH 4.5 could explain why chlorogenic acid with 2 hydroxyl groups has a lower partition coefficient than ferulic acid, which has only one hydroxyl group. At pH 4.5, ferulic acid also has a higher partition coefficient than syringic acid. Both acids have a single aromatic hydroxyl group, but ferulic acid has twice as much undissociated carboxyl (44%) compared to syringic acid

(20%). The influence of carboxyl groups on partitioning is not seen at pH 7.5, probably because the carboxyl groups of all phenolic acids are fully dissociated. As would be expected, chlorogenic acid has a slightly higher partition coefficient compared with ferulic and syringic acids. The lower partition coefficients of most phenolics at pH 7.5 (Figure 3.1b) compared to pH 4.5 (Figure 3.1a) could be explained by reduced hydrogen bonding, that is, the ionization of aromatic hydroxyl groups is higher at pH 7.5 than at pH 4.5.

The adsorption of the phenolic compounds on XAD-4 and IRA-402 involves interaction forces other than hydrogen bonding. The larger and more hydrophobic flavonoids had greater affinity for XAD-4 matrix than phenolic acids at pH 4.5 and 7.5 (Figures 3.1 c, d). At pH 7.5, the partition coefficients of ferulic acid, syringic acid and vitexin were lower in comparison to the coefficients at pH 4.5. The possible reason for this reduction is the prevalence of dissociated molecule forms in the liquid phase at pH 7.5, that is, dissociated forms are expected to have less affinity for the uncharged XAD resin (Bretag et al. 2009; Caetano et al. 2009; Kammerer et al. 2007). On the other hand, the partition coefficients of chlorogenic acid and rutin on XAD-4 were not affected by the pH change, which may be related to the fraction of dissociated forms already present at pH 4.5. At pH 4.5, 95% of the carboxyl groups of chlorogenic acid (pKa 3.2) are dissociated, and thus, only 5% would additionally dissociate at pH 7.5, resulting in a minor contribution to chlorogenic acid adsorption at pH 7.5. Similar behavior occurs with the hydroxyl groups of rutin. At pH 4.5, all rutin molecules were fully protonated, and the increase in pH to 7.5 causes only 7% of the hydroxyl groups to dissociate. Again, significant changes in adsorption could not be observed.

Figures 3.1e and 3.1f compare the adsorption of the five molecules to IRA-402 at pH 4.5 and pH 7.5. It was previously reported that anion exchange resins under alkaline

conditions had a maximum capacity for removal of phenols from aqueous solution, by combining hydrophobic and ion exchange interactions (Caetano et al. 2009; Carmona et al. 2006). It appears that the same mechanism applies for phenolics adsorption on IRA-402. The flavonoids rutin and vitexin-2-O-rhamnoside interacted more favorably with IRA-402 at pH 7.5 than at pH 4.5. Unlike XAD-4, which experienced the decreasing of hydrophobic interactions due to the prevalence of dissociated forms as the pH of the solution increased, both compounds have their interactions with the anionic resin enhanced by the dissociated forms that prevail at pH 7.5. A similar trend was observed with chlorogenic acid. Even though at pH 4.5 its carboxyl group was almost fully (95%) dissociated, 33% of the aromatic hydroxyl additionally dissociated at pH 7.5, resulting in a further increase of the partition coefficient from 167 at pH 4.5 to 203 at pH 7.5 (Table 3.2), suggesting that ionic interactions were added to the hydrophobic ones. In contrast to chlorogenic acid, ferulic and syringic acid binding to IRA-402 did not change with the change of solution pH. The fact that both acids have their carboxyl groups fully dissociated at pH 7.5 (compared to 80 and 56% at pH 4.5) suggests that their adsorption is probably affected by both charge and hydrophobic interactions.

3.5. Conclusions

Regardless of the adsorption mechanism, hydrophobic-matrix resins XAD-4 and IRA-402 exhibited a greater affinity than PVPP for the five phenolic compounds selected.

At pH 4.5, phenolic acids had ten-fold lower partition coefficients than flavonoids with XAD-4, indicating that XAD-4 would not be suitable for plant extracts containing large quantities of phenolic acids, but it would be adequate for capturing flavonoids such as rutin and vitexin. On the other hand, adsorption isotherms generated with ferulic and

chlorogenic acids on IRA-402 resulted in partition coefficient values higher than or closer to those of rutin and vitexin-2-O-rhamnoside. In the case of vitexin, it appears that XAD-4 would be a most adequate adsorbent at pH 4.5 and IRA-402 would be adequate at pH 7.5. The three phenolic acids had higher affinity for the charged resin than the non-charged ones, at pH 7.5.

4. RECOVERY AND PURIFICATION OF BOVINE LYSOZYME FROM SUGARCANE: EXTRACTION, MEMBRANE FILTRATION, AND PURIFICATION

4.1. Introduction

Lysozyme is a bacteriostatic enzyme whose ability to break bacterial cell walls gives this protein its anti-bacterial properties. Lysozyme is naturally found in many substances, but hen egg-white lysozyme (HEWLz) has become the major source of this enzyme for commercial production. Hen egg-white lysozyme is currently used as a preservative for fruits and vegetables, seafood, and meats, and lysozyme is also a pharmaceutical for the treatment of viral and bacterial infections as well as it is applied to improve the effectiveness of antibiotics. Likewise, the concentration of lysozyme on skin, as well as in tears and saliva, can be used as indicator of health (Proctor and Cunningham 1988). Recombinant human lysozyme (HuLz) purified from transgenic rice has been developed to be an additive for infant formula and electrolyte solution to reduce gastrointestinal infections (Huang et al. 2002). Also, human and bovine lysozyme genes have been cloned and transferred to enhance plant bacterial or fungal resistance (Gurupada and Subhash 2011).

Bovine lysozyme (BvLz) is considered more important industrially than other lysozymes because of its activity against gram-negative bacteria and fungi (Wilcox et al. 1997). In the case of plant pathogens, around 95% of them are gram-negative, including *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae*, and *Xanthomonas campestris*. Bovine lysozyme is, therefore, the most adequate lysozyme for agricultural applications, and since conventional use of heavy-metal containing sprays and antibiotics

for controlling bacterial plant pathogens are not environmentally acceptable, there is a potential market for BvLz use as an agricultural enzyme.

Bovine lysozyme is a 15 kDa-protein with a pI of 7.65, compared to 10.2 for HuLz and 10.7 for HEWLz. The optimum pH range for BvLz activity is between 4 and 6, whereas HEWLz's highest activity occurs between pH 5 and 7 (Dobson et al. 1984; Huang et al. 2002; Nonaka et al. 2009). Both HEWLz and BvLz contain 129 amino acids, HuLz contain 130 amino acids, and other variations in the structural parameters occur among lysozymes from diverse sources, such as the number of disulfide bonds or charged residues (Nonaka et al. 2009). Due to different properties, extraction and purification processes developed for HEWLz or HuLz may not be applicable to BvLz. At the same time, for transgenic plant processing considerations, BvLz resistance to proteases (Nonaka et al. 2009) gives an interesting advantage to the bovine form over human and hen egg-white lysozymes.

Human lysozyme has been expressed in several transgenic systems (Wilken and Nikolov 2006), and recombinant BvLz has been expressed in yeast (*Pichia pastoris*) (Digan et al. 1989), tobacco (Wilcox et al. 1997), and sugarcane (Mirkov and Irvine 2002).

Commercial sugarcane (*Saccharum ssp. hybridis*) is a fast-growing monocot which is the source of 70% of the sugar production worldwide (Lakshmanan et al. 2005). The growing interest in biofuels also makes sugarcane an important crop for renewable energy. Sugarcane bagasse is one of the major sources of lignocellulosic materials (Cardona et al. 2010), and because bioethanol from sugarcane is the only one competitive with gasoline (OECD-FAO 2008), sugarcane should remain one of the most important ethanol feedstocks over the coming decade (OECD-FAO 2011).

Increasing industrial use of sugarcane has led to considerable genetic improvements toward plant productivity. Developments in traditional sugarcane transformation were subsequently followed by pest resistant plants (Christy et al. 2009) and lines containing other high-value bioproducts. Currently, sugarcane is being considered as a biofactory for industrial compounds such as recombinant protein (Damaj et al. 2009; Harrison et al. 2011; Wang et al. 2005) and biopolymers (Anderson et al. 2011; Hustad 2009; McQualter et al. 2005; Petrasovits et al. 2007).

Recombinant protein expression levels are crucial to determine the feasibility of a plant system for production of bioproducts, as it dictates the economics of plants as hosts for recombinant proteins (Howard et al. 2005). In the case of BvLz use as an antibacterial, it is estimated that concentrations between 125 and 250 ppm are effective for its agricultural application (Mirkov and Fitzmaurice 1998).

For improvement of recombinant protein extraction from diverse plant tissues, the optimization of pH and ionic strength levels has been investigated (Azzoni et al. 2005; Farinas et al. 2005b; Wilken and Nikolov 2006; Zhang et al. 2005). During solid-liquid homogenization, the protein of interest is transferred to a buffered solution, so the choice of a given buffer solution is essential for adequate extraction levels, protein stability and further downstream processing and purification. In addition to the concentration of the desired protein, these studies also looked at the extracted impurities such as native proteins, phenolic compounds, oil and carbohydrates, which can negatively affect the subsequent downstream processing.

Sugar composes 80% of the sugarcane juice dry matter (Xande et al. 2010), but there are additional types of impurities that have been identified in sugarcane juice, including polysaccharides, glycoproteins, and free phenolics. Sugarcane, like other plants, has a

variety of phenolics in its composition, with distinct compounds present at different concentrations. Sugarcane juice phenolics (470 mg/L of juice) consist of chlorogenic acid, hydroxybenzoic acids, anthocyanins and 18 different flavonoids – forms of the flavones luteolin, apigenin and tricin (Paton 1992; Paton and Duong 1992). Phenolic compounds were found to be involved in the enzymatic browning of sugarcane, as they were substrates for polyphenol oxidase, which converts phenolics to polymeric colorants. Increase in color of sugarcane juice was proportional to a decrease in the concentration of chlorogenic acid and other flavonoids (Paton and Duong 1992). It is believed that colorants with molecular mass above 200 kDa are browning products of cell wall polysaccharides and comprise polysaccharides bonded to phenols and their oxidation products (Bento 2009).

Incomplete removal of impurities in conventional sugar processing has prompted many sugar experts to look at the possibility of pre-treating sugarcane juice by inserting a decolorization step prior to juice concentration by evaporation. Membrane filtration has been considered for enhancing the clarification of sugarcane juice (Ghosh et al. 2000) because it provided a better-quality sugar compared to the conventional chemical clarification process. The application of membrane technology may offer advantages in clarification and concentration of multicomponent solutions and suspensions like sugarcane juice, with the membrane-based processes combining technically efficient and energy saving processes, compared to the highly energy-consuming conventional concentration processing (Bhattacharya et al. 2001; Clarke 1997). The effect of different membranes and feed streams in the ultrafiltration (UF) of sugarcane juice has been evaluated and determined UF to be effective for removal of impurities (Balakrishnan et al. 2001). However, the application of membranes for sugarcane processing was limited due to significant flux reduction as a result of membrane fouling (Bhattacharya et al. 2001; Saha

et al. 2006). Membrane fouling and low flux continue to be the major hindrances in applying this technology on a large scale because of unfavorable economics (Sarode et al. 2001). Phenolics, polysaccharides and proteins are the sugarcane components believed to be responsible for fouling separation membranes (du Boil 1997; Godshall et al. 2001; Saha et al. 2007).

Decolorization of sugarcane juice could be accomplished by ultrafiltration followed by hydrophobic silica gel adsorption (Okuno and Tamaki 2002). Removal of colored compounds from sugarcane solutions have been performed using various types of adsorbents, from activated carbons and polymeric adsorbents, to polystyrene resins and anionic resins (Soto et al. 2011). Some sugar refineries use polystyrene or acrylic anion exchange resins, others use the two in series, and even a mixture of cation and anion exchange has been suggested (Bento 1998).

Adsorption with inexpensive commercially available resins is commonly used to remove phenolic and other colored compounds from non-transgenic plants extracts (D' Alvise et al. 2000; Loomis 1974; Saleh et al. 2008; Xu and Diosady 2002), fermentation media (Fisher et al. 2008; van den Berg et al. 2008) and wastewater (Caetano et al. 2009; Carmona et al. 2006; Huang 2009; Li et al. 2001; Pissolatto et al. 1996). Relevant applications of adsorption using inexpensive resins for food processes, removal of phenolic compounds, detoxification of fermentation media, color removal, and purification of sugar solutions and microbial metabolites were recently reviewed by Soto et al. (2011).

To date, many options for improvements in sugarcane processing have been explored, but downstream processing of recombinant proteins from transgenic sugarcane has not been reported.

To recover and purify recombinant BvLz produced in transgenic sugarcane, a downstream process consisting of sugarcane juice membrane concentration followed by two chromatography steps, cation exchange and hydrophobic interaction chromatography, was developed in the Bioseparations Lab (Appendix B). In order to progress with the downstream process development and envisioning the design of an economically-feasible process for purification of recombinant BvLz expressed in transgenic sugarcane, three portions of the downstream process where tackled: 1) recombinant BvLz extraction from transgenic sugarcane was maximized by evaluating the effects of nine buffer solutions, which combined different pH and salt levels, on BvLz recovery and native protein content in the extract; 2) membrane filtration performance of three selected extracts was assessed, determining the influence of extract pH and salt on the recovery of BvLz in the final concentrate; and 3) purification alternatives to using chromatography process, consisting of ion-exchange and hydrophobic interaction chromatography (IEX+HIC), were investigated, to eliminate the IEX step or substitute it for a less expensive unit operation that would achieve an effective impurity removal, and increase the HIC yield.

4.2. Materials

4.2.1. Transgenic sugarcane

Transgenic sugarcane plants were developed (Damaj et al. 2009) by our collaborator Dr. Erik Mirkov at Texas Agrilife Research and Extension Center at Weslaco, TX. Transgenic material was supplied to the Bioseparations Lab as shredded sugarcane stalks at -80°C. The shredded transgenic tissue was stored at -80°C until used for analysis and extraction studies.

Bench-scale extraction optimization and membrane filtration studies of recombinant BvLz were performed using the pSPU 32C line. The pSPU 32C line refers to the sugarcane line that is transgenic for the BvLz gene whose expression is driven by three constitutive promoters: a promoter for a sugarcane elongation factor 1α (pSEF1α), a promoter for the gene coding for a sugarcane proline rich protein (pPRP) (Yang et al. 2003) and maize ubiquitin 1 promoter (pUbi1) (Christensen and Quail 1996). The expression levels in the 32C tissue, determined by ELISA, varied between 2 and 3 mg/kg.

A number of pJSU sugarcane lines (131) containing BvLz constructs under the control of a combination of triple promoters were also shipped for expression analysis by activity and ELISA assays. The pJSU lines refer to the sugarcane lines that are transgenic for the BvLz gene whose expression is driven by one stem-regulated promoter, a promoter for a sugarcane jasmonate-inducible protein (pJAS), now called dirigent (Damaj et al. 2010); and two constitutive promoters: a promoter for a sugarcane elongation factor 1α (pSEFα) and maize ubiquitin 1 promoter (pUbi1).

4.2.2. Pilot-plant transgenic sugarcane concentrate

Pilot-plant pressed and concentrated juice for purification development was also supplied by Dr. Mirkov's group. Sugarcane juice concentrate preparation is described in Appendix B.1. The 3-kDa concentrate and samples from each step of the pilot-plant membrane filtration were received and analyzed for BvLz content. The concentrated juice containing 0.015% sodium azide (NaN_3) was stored at 4°C until further processing.

4.2.3. Reagents

Acetic acid, ammonium sulfate [$(NH_4)_2SO_4$], ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), 2-(*N*-morpholino)ethanesulfonic acid (MES), sodium chloride

(NaCl), sodium acetate, sodium hydroxide (NaOH), sodium phosphate monobasic and sodium phosphate dibasic, tris(hydroxymethyl)aminomethane (Tris) base, Tris hydrochloride as well as other reagents, if not otherwise mentioned, were purchased from VWR (Radnor, PA).

4.3. Methods

4.3.1. Analysis of BvLz accumulation in sugarcane lines

Extracts were prepared by homogenization of 100 g of sugarcane shredded stalks blended with pH 6 buffer solution (100 mM MES, 150 mM NaCl and 10 mM EDTA) at a 3:1 buffer-to-tissue ratio for 10 min in a blender with a 1 L stainless steel housing (Waring Laboratory & Science, Torrington, CT). Temperature was monitored during extraction and did not go over 35°C. The extracts were clarified by 12,000 g centrifugation for 30 min at 4°C in a Beckman Coulter Allegra 25R centrifuge followed by filtration of the supernatant using 0.45 μm surfactant-free cellulose acetate (SFCA) membrane Nalgene filter units (Thermo Fisher Scientific, Rochester, NY). The recovered solids were re-extracted using the same conditions of the first extraction. Bovine lysozyme concentrations in both extracts were analyzed by ELISA and/or activity assay.

4.3.2. Bench-scale extraction studies

Dry grinding of sugarcane stalks was performed using a KitchenAid® BCG100WH Blade Coffee Grinder, which was followed by an aqueous homogenization at 22°C using a Silverson® L4RT Laboratory Mixer. Ground stalks were homogenized twice using a 3:1 buffer-to-tissue ratio. The total of extraction time was 60 min (two-step extraction with 30 min each). The first 30 min of extraction was done by using the General Purpose Disintegrating working head at 4000 rpm followed by another 30 min extraction at 5000

rpm with the Square Hole High Shear. To determine the effect of pH and ionic strength, a full factorial experimental design (2^3) was implemented, with three or more repetitions for each experiment. The effect of pH on BvLz extraction yield was determined by using 50 mM sodium acetate (pH 4.5), 50 mM MES (pH6) and 50 mM Tris (pH 7.5). All extraction buffers contained 10 mM EDTA. The effect of ionic strength on BvLz extraction was evaluated at three different levels of NaCl concentration: 0, 50 and 150 mM (conductivities of approximately 3, 8, 17 mS/cm). To remove ground stalk solid residue, homogenized extracts were centrifuged (Beckman Coulter Allegra 25R, Brea, CA) for 30 minutes at 12,000 g and 4˚C. Crude extracts prepared at pH 6 and 7.5 were clarified by pH 4.5 precipitation followed by depth filtration (1 μm). After clarification, extract pHs were adjusted back to initial pH with 3 M NaOH, and filtered again through a 0.45 μm surfactant-free cellulose acetate membrane. Statistical analyses to compare the extracts were performed using the software SPSS 16.0. Interpretation of the results was structured using a significance level of 5%.

4.3.3. Bradford assay

Total soluble protein (TSP) content was determined by Bradford assay (Bradford 1976) in a microtiter format, using a Coomassie Plus Assay kit containing Coomassie Plus reagent and bovine serum albumin (BSA) as standard (Product # 23236, Thermo Scientific, Rochester, NY). To each well of the microtiter plate, 10 μL of sample or BSA standard and 300 μL of Coomassie Plus reagent were added. After 10 min of incubation, the absorbancies at 595 nm were measured by a Molecular Devices VersaMax® microplate reader using SoftMaxPro V4.6 software. Samples were analyzed in triplicate.

4.3.4. Enzymatic activity assay

BvLz concentrations were determined from the enzyme activity using *Micrococcus luteus* cell suspension (Sigma-Aldrich, St. Louis, MO) at pH 4 and 25°C. A *Micrococcus luteus* cell suspension of 0.0187% (w/v) was prepared using 50 mM sodium acetate buffer, pH 4, containing 60 mM NaCl; the same buffer was used for sample dilution. Bovine lysozyme activity was determined by measuring suspension absorbance ($A_{450\ nm}$) every 30 sec for 30 min using a Beckman Coulter DU640 spectrophotometer. Samples were analyzed in triplicate. Absorbance reduction over time was used to calculate units of enzyme activity. One unit of enzyme activity was defined as the amount of BvLz that produces absorbance change ($\Delta\ A_{450\ nm}$) of 0.001 per minute at pH 4 at 25°C. Enzyme concentration, in μg/mL, was calculated using specific activity of 6000 Units/mg BvLz.

4.3.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Bovine lysozyme concentrations were determined using a sandwich ELISA. Serum containing polyclonal antibodies against BvLz from tobacco (Wilcox et al. 1997) was produced in rabbits. Antibody purified from rabbit sera using Protein A-Sepharose (GE Healthcare, Piscataway, NJ) was used directly as a capture antibody (primary antibody). The purified antibody was also used to prepare the detection antibody (secondary antibody) by biotinylation using EZ-Link®Sulfo-NHS-LC-Biotinylation Kit (Product # 21435, Thermo Fisher Scientific, Rochester, NY). The standard curve was prepared using purified BvLz produced in *Pichia pastoris* (Digan et al. 1989) and quantified by protein sequencing. Microtiter plate wells coated with anti-BvLz (primary antibody) were blocked with 3% BSA. Appropriately diluted samples (100 μL) were added to each well and incubated at 37°C for 2 h. After washing the plate with 300 μL of PBST per well, 100 μL of 1:600 diluted biotinylated anti-BvLz (secondary antibody) were added and detected using

horseradish peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Rochester, NY) and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Product # T0440, Sigma-Aldrich, St. Louis, MO). After signal development the reaction was stopped by addition of 2 M HCl and the absorbances at 450 nm were measured by a Molecular Devices VersaMax® microplate reader using SoftMaxPro V4.6 software. Samples and standards were analyzed in triplicate.

4.3.6. Gel electrophoresis

Protein profiles of sugarcane extracts and process samples were evaluated by SDS-PAGE electrophoresis (Laemmli 1970) following manufacturer instructions (Invitrogen, Carlsbad, CA). Reduced protein samples were prepared mixing extracts to NuPAGE® LDS sample buffer and 2-mercaptoethanol (Sigma-Aldrich, St. Louis) and heated to 70°C for 10 min. Reduced protein samples and SeeBlue Plus2 standards (Cat. # LC5925) were loaded in NuPAGE® Novex® 4-12% Bis-Tris Mini Gels (1.5 mm x 10 wells), and the gel was run for 35 min at 200 V constant voltage. Protein was visualized by coomassie staining with SimplyBlue Safe Stain (Cat. # LC6065) or by silver staining with SilverQuest Silver Staining Kit™ (Cat. # LC6070). All the reagents and kits were purchased from Invitrogen (Carlsbad, CA), unless otherwise mentioned.

4.3.7. Western blot immunoassay

Presence of BvLz in sugarcane samples was detected by Western blot. After separation by gel electrophoresis in NuPAGE® Novex® 4-12% Bis-Tris Mini Gels (1.0 mm x 10 wells) from Invitrogen (Carlsbad, CA) as described in the previous section, the proteins were transferred to a PVDF membrane using a iBlot® Dry Blotting System (Invitrogen). The membrane was blocked with 1% BSA in TBST, and incubated sequentially with rabbit sera containing polyclonal antibodies against BvLz from tobacco (Wilcox et al. 1997)and

goat anti-rabbit IgG-AP (Sigma-Aldrich, St. Louis, MO). Bovine lysozyme bands were visualized by treating the membrane with the SigmaFast® BCIP/NBT substrate (Sigma-Aldrich) until the bands can be seen. After signal development, the membrane was immediately washed with water to stop the reaction.

4.3.8. UV-VIS scans

To determine the presence of phenolics and other pigments in the juice concentrate and processed samples the UV-Vis scans (200–800 nm) were performed using a Beckman Coulter DU640 spectrophotometer. Before scanning, samples from sugarcane juice concentrate before and after treatment with XAD-4 were diluted five-fold into the same pilot-plant diafiltration buffer (50 mM sodium acetate, pH 4.2).

4.3.9. Spiking of sugarcane with tobacco-derived BvLz

Purified and lyophilized tobacco-derived BvLz sample was provided by Dr. Erik Mirkov at Texas Agrilife Research and Extension Center at Weslaco, TX and stored at -20°C until use. Characterization of the received sample by Bradford, ELISA, activity assay, SDS-PAGE and western blot is described in Appendix C. For spiking of sugarcane juice and extracts, BvLz stock solutions were prepared using the estimated content of 0.12 g of BvLz per gram of lyophilized sample. A stock solution of 15 mg/mL tobacco-derived BvLz in extraction buffer (pH 6 100 mM MES, 150 mM NaCl, 10 mM EDTA) was prepared for membrane filtration experiments with spiked extracts. The addition of 7 mL stock solution to 500 mL of sugarcane extract resulted in spiked sugarcane extract with 22 μg BvLz/mL (22% of TSP). The stock solution prepared for HIC purification studies consisted of 8.5 mg/mL tobacco-derived BvLz in 50 mM sodium acetate buffer, pH 4.2. Sugarcane juice concentrate (41 mL) was spiked with 6.5 mL of the stock solution to obtain a BvLz content equivalent to 33% TSP.

4.3.10. Membrane filtration

Membrane processing of the extracts was performed using a Millipore® Labscale TFF system (Billerica, MA) at room temperature. The polyethersulfone (PES) flat-sheet membranes (Biomax 100 and Biomax 5, Millipore) had 50 cm^2 area and the molecular weight cut-offs (MWCO) of 100 kDa and 5 kDa, respectively. For 3 kDa concentration, a spiral wound module with regenerated cellulose membrane of 0.1 m^2 area attached to a Millipore® Prep/Scale holder was used. The extracts (~500 mL) were poured into the liquid reservoir and kept under constant agitation during filtration. Membrane filtration experiments consisted of membrane equilibration, 100 kDa filtration and 5 kDa or 3 kDa concentration steps.

Membrane equilibration was achieved by running the extract at a constant flow rate over the membrane surface for 5 min with the permeate valve completely closed in order to avoid flow through the membrane. After equilibration, the permeate valve was opened to allow the extract to flow through the membrane. Average permeate flow rate was determined using the time required to collect 100 mL of permeate. At the end of the concentration, retentate was diafiltered with two volumes of the same extraction buffer and then collected. To recover the remaining diafiltered retentate (holdup), 50 mL of clean buffer were recirculated through the system to wash out the residual extract. The holdup wash volume is referred to as “chase”.

After each filtration experiment, the membranes were rinsed with 50°C water and cleaned with 0.5 M NaOH, 0.5% Tergazyme detergent, and 250 ppm sodium hypochlorite. To assure proper cleaning of the membranes, water flow rates at different transmembrane pressures (TMP) before and after cleaning were compared. Bovine lysozyme and TSP

concentrations in permeate, retentate, and chase samples were analyzed using ELISA and Bradford methods, respectively.

4.3.11. Pretreatment of concentrated sugarcane juice with XAD-4

Amberlite XAD-4, a hydrophobic polystyrene divinylbenzene resin with particle size 0.59 mm and surface area 725 m^2/g (Supelco, St. Louis, MO), was soaked in methanol for at least 24 h before use (Payne and Shuler 1988). After methanol conditioning, the resin was rinsed several times with water and stored in 20% EtOH until use. The resin was packed into a 1 cm x 20 cm Tricorn column (GE Healthcare, Piscataway, NJ) to a 9.5-cm bed height (7.5 mL resin volume). Following the resin equilibration with pH 4.2 50 mM sodium acetate buffer, 35 mL of spiked transgenic sugarcane juice concentrate was loaded at 0.5 mL/min (38 cm/h superficial velocity) onto the XAD-4 column at room temperature. Wavelength scans, TSP and BvLz content of sugarcane juice concentrate before loading on XAD-4 column and collected flow through juice samples were determined using the previously described methods.

4.3.12. BvLz purification from sugarcane concentrate using HIC

Sugarcane juice or sugarcane extract concentrates spiked with tobacco–derived BvLz were purified by hydrophobic interaction chromatography (HIC). The effect of XAD pretreatment and ammonium sulfate concentration on the adsorption, as well as on purity and yield of BvLz was evaluated. Ammonium sulfate was added to sugarcane concentrates to a final concentration of 1.4 M or 2 M, and pH adjusted to 7. 5 with 3 M NaOH before loading on the HIC column. Sample load, equilibration buffer (25 mM Tris, 1.4 M or 2 M ammonium sulfate, pH 7. 5), and elution buffer (25 mM Tris, pH 7.5) were filtered using 0.2 μm SFCA Nalgene filter units (Thermo Fisher Scientific, Rochester, NY). Spiked sugarcane concentrate (32 mL) was loaded at a flow rate of 2 mL/min (255 cm/h superficial velocity)

into a 4.7 mL HiScreen Phenyl Sepharose FF column (GE Healthcare, Piscataway, NJ) with 10-cm bed height. After loading, the column was washed with 3 column volumes (CV) of the equilibration buffer, and bound BvLz was eluted with 20-CV linear ammonium sulfate gradient from 1.4 M or 2 M to 0 M ammonium sulfate in 25 mM Tris, pH 7.5.

Chromatographic experiments were performed at room temperatureusing an AKTA Purifier system (GE Healthcare, Piscataway, NJ) controlled by Unicorn 5.11 software; A280, A320 and conductivity were monitored throughout the chromatography run. Elution fractions of 10 mL were collected and analyzed by SDS-PAGE, ELISA and Bradford assays.

4.4. Results and Discussion

4.4.1. Screening of sugarcane lines for BvLz accumulation

More than 200 lines were generated by our collaborator, in order to test the effect of different promoter combination and promoter stacking, as well as plant age and fertilization dosages on BvLz accumulation in sugarcane stalks. Extracts from 131 transgenic sugarcane lines were analyzed using ELISA and activity assays to determine the plants expressing the highest amounts of BvLz.

For each line, the first extract was prepared by homogenization of the shredded stalks with the extraction buffer at a 1:3 buffer-to-tissue ratio. This ratio was implemented based on previous investigation by our group (Woodard et al. 2009a), which determined that this ratio guaranteed proper mixing and homogenization while keeping the dilution factor low.

When the expression level of the first extract measured by activity assay was above 1.3 mg/kg, the second extract was prepared by homogenization of the remaining solids from the first extraction in the same amount of buffer used in the first extraction. The sum

of BvLz in both extracts measured by ELISA was considered the expression level in the respective line. Total BvLz for each line was expressed in mg BvLz/kg of sugarcane stalks.

Only 16 lines had higher than 1.3 mg/kg BvLz content in the first extract (Table A.1 of Appendix A). However, to eliminate the possibility of underestimating BvLz content when using the activity assay, additional 70 lines were double extracted and the BvLz concentration was also measured by ELISA. The expression levels in the second extracts were typically 42 to 61% of the BvLz values measured in the respective first extracts. Based on this finding and on previous studies (Woodard et al. 2009a), a factor of 1.5 was used to estimate the total BvLz expression level of lines that only the first extracts were analyzed by activity, by multiplying the BvLz concentration in the first extract by this factor.

Expression levels of 18 lines were equal to or higher than 2 mg/kg; 7 lines had expression levels between 2 and 2.5 mg/kg and another 6 between 2.5 and 3.5 mg/kg. Five lines, pJSU 18, 19, 54, 298 and 299 exceeded 4.5 mg/kg i.e. 5.1, 4.6, 6.0, 5.4 and 5.1 mg BvLz/kg sugarcane, respectively. Results for all the lines analyzed are shown in Table A.1 of Appendix A.

4.4.2. Bench-scale extraction studies

(Performed together with Miguel Ballen)

Homogenization of the sugarcane stalk tissue at pH 6 was proven to release at least 50% more BvLz than direct stalk pressing (Woodard et al. 2009a). The second extraction performed in the two-step extraction bench scale experiments increased the BvLz recovery by approximately 30%. Because extract complexity depends on process conditions, BvLz extraction from transgenic sugarcane stalks was tested at constant temperature of 22°C and different buffer pH and ionic strengths. For each condition, concentrations of

extracted BvLz and TSP were quantified. The extraction conditions investigated included three pH levels (4.5, 6 and 7.5) and three salt concentrations (0, 50 and 150 mM NaCl). The selected range of pH between 4.5 and 7.5 includes pH for optimal activity of BvLz (pH 4.4) and pI of BvLz (pH 7.65). Previous data (Woodard, unpublished) demonstrated that salt concentrations higher than 150 mM NaCl did not increase the amount of extracted BvLz.

Removal of native protein by acidic pH precipitation was followed by gel electrophoresis (Figure 4.1) and quantified by Bradford (Figure 4.2). Total protein profiles in SDS-PAGE gels of extracts before and after precipitation are shown in Figure 4.1. Protein profiles (Figure 4.1) and TSP values (Figure 4.2) indicate that pH had an effect on the extraction of native proteins and salt concentration (ionic strength) did not result in an apparent change of extracted protein. Comparison of the crude extracts using the multiple comparison Tukey test confirmed that salt addition did not improve protein extractability, as there was no significant difference in the measured amount of TSP between the crude extracts prepared at the same pH.

The Tukey test classified the crude extracts into different homogeneous subsets based on the TSP concentrations (A, B or C in Figure 4.2), also confirming the positive pH effect on BvLz extraction. As pH increased, native sugarcane proteins became more soluble in the extraction buffer. This trend of increasing native protein solubility with increasing buffer pH has also been observed with other plant protein extractions (Azzoni et al. 2005; Wilken and Nikolov ; Wilken and Nikolov 2006; Wilken and Nikolov 2012; Woodard et al. 2009b).

For comparison of crude extracts and precipitated extracts, a t-paired test (Ott and Longnecker 2001) was used. Because this test is used for dependent samples, it compares the two groups (for example, extracts before and after precipitation) using the difference

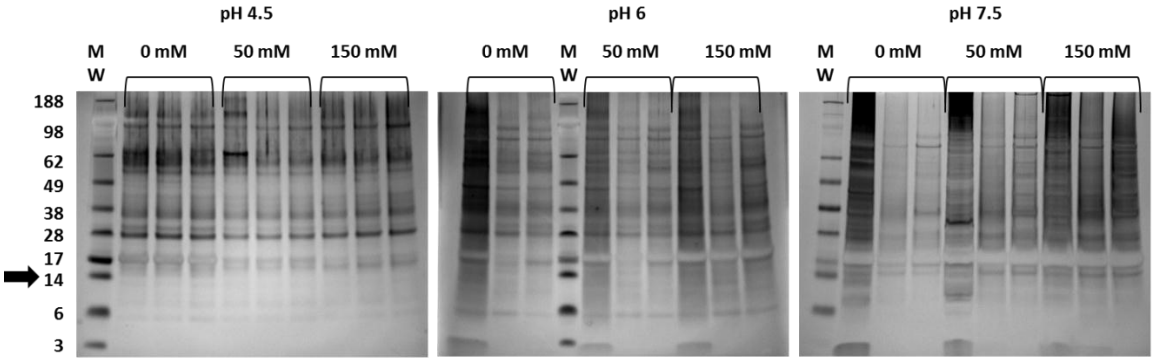


Figure 4.1: Protein profiles of sugarcane extracts at pH 4.5, 6 and 7.5, and salt concentration of 0-150 mM NaCl. The first lane of each salt concentration corresponds to the crude extract, the second to the extract after pH precipitation and 1 μm filtration, and the third lane to the extract after pH adjustment to the initial pH followed by 0.45 μm filtration. The arrow shows the position of BvLz.

between the pair of measurements for each sample. For the extracts generated at pH 6 and 7.5, there was a significant difference in the concentration of total soluble protein (TSP) before and after precipitation. Given that no pH change was necessary for pH 4.5 extract, comparison (before and after precipitation) was not performed for these extracts. .

Increase of salt concentration at pH 6 and 7.5 reduced the removal of the native proteins by acid precipitation, as the presence of NaCl prevented protein aggregation and, in consequence, protein precipitation. Based on TSP concentration, the precipitated extracts made from crude extracts at the same pH were classified in the same homogeneous subsets by the multiple comparison Tukey test (A', B', C' or D' in Figure 4.2). The amount of residual native protein (TSP) in Figure 4.2 showed an increasing trend that paralleled salt concentration.

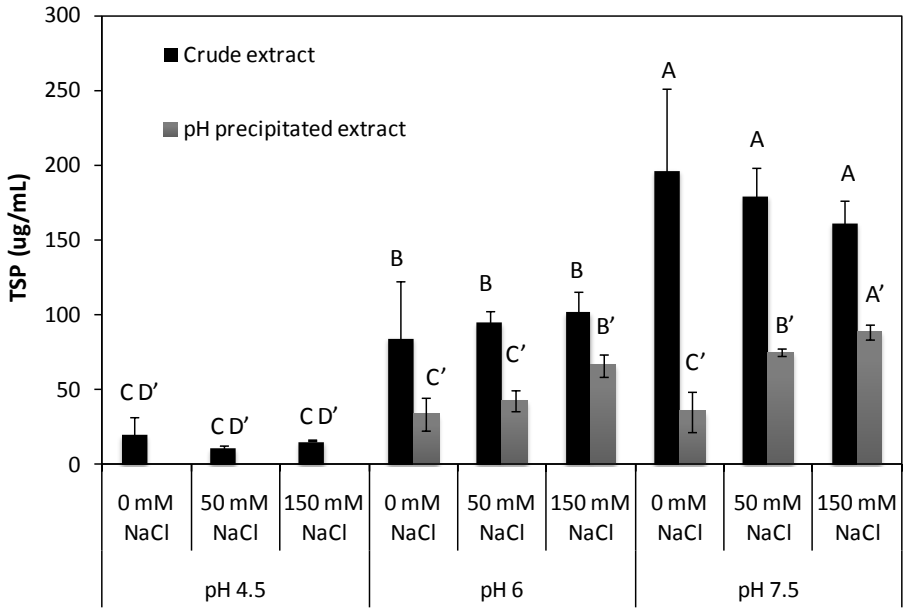


Figure 4.2: Total soluble protein (TSP) concentrations in crude and precipitated extracts measured by Bradford. Standard deviations between three replicates are represented by the error bars. Samples labeled with the same letter are classified as part of homogeneous subsets by the multiple comparison Tukey test.

Loss of BvLz during the acidic precipitation seemed to depend on the method used to measure BvLz concentration before and after precipitation. When the level of BvLz was compared using activity assay, BvLz concentration before and after precipitation was significantly different by the t-paired test. A difference between extracts before and after precipitation at each pH and salt concentration was not seen when the amount of BvLz in the extracts was measured by ELISA; the concentration of BvLz was statistically similar in

both extracts, indicating that no significant loss of lysozyme occurred as a result of the pH precipitation step.

After the pH precipitation and subsequent removal of interfering extract impurities, the activity of BvLz was higher than before the precipitation. This difference was probably caused by the removal of native protein from the extract by precipitation. It was hypothesized that the interaction between the *Micrococcus luteus* substrate and BvLz in the crude extract was reduced by the presence of unidentified extract impurities that caused underestimation of activity. The t-paired test comparing BvLz concentrations in precipitated extracts determined by activity assay and ELISA indicates that the two analytical methods give statistically similar ($p = 0.78$) BvLz estimates (Figure 4.3).

Analysis of variance between pH 4.5 extracts and precipitated pH 6 and pH 7.5 extracts indicated that there was an effect of pH and salt concentrations on the extraction of BvLz. The means were also compared by a multiple Tukey comparison test, which classifies the conditions in homogeneous subsets (higher means are labeled as A, followed successively by B and C).

Based on the results of BvLz extraction presented in this section, we can draw several conclusions. First, BvLz is less positively charged when the solution pH was closer to the pI (7.65), hence the amount of extracted BvLz was greater probably because of reduced interactions with negatively-charged components of the sugarcane solids. Second, the addition of salt to extraction buffers at pH 4.5 and 6 had the same positive effect on BvLz extraction due to reduced ionic interactions between BvLz and the solids. For example, amounts of BvLz extracted by pH 4.5 sodium acetate buffer with 50 and 150 mM NaCl were not different than the amount extracted by pH 6 buffer with no salt. Similarly, the amount of BvLz extracted by pH 6 buffer with 50 and 150 mM NaCl was equivalent

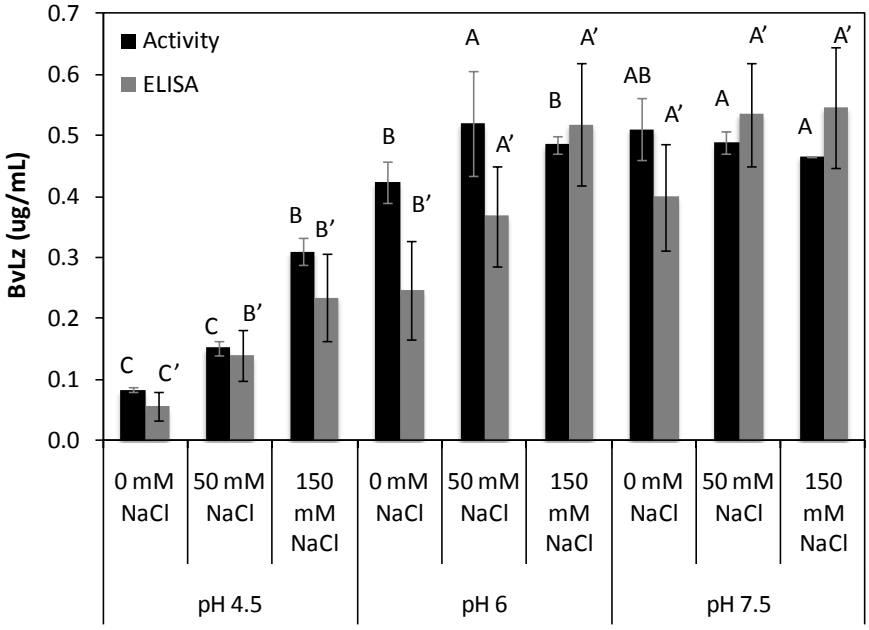


Figure 4.3: Bovine lysozyme (BvLz) concentration in pH precipitated extracts measured by activity assay and ELISA. The error bars represent standard deviations between three replicates. Samples labeled with the same letter are classified as part of homogeneous subsets by the multiple comparison Tukey test.

to the amount extracted at pH 7.5 without salt addition.

Finally, the lack of salt effect at pH 7.5 can be attributed to the close proximity of the extraction buffer pH to the pI of BvLz (7.65). At pH 7.5, with the net BvLz charge close to zero, there was no extra release of the target protein into the extract by addition of salt. Based on ELISA results, both pH 6 extraction with salt (50 or 150 mM NaCl) and pH 7.5 extraction independent of the salt concentration, maximize BvLz extraction.

Another important variable that could affect the efficiency of subsequent purification of the target protein (BvLz) is the TSP present in the extract. As discussed above (Figure 4.2), the

protein concentration in the extract is affected by pH and salt concentration, and extraction conditions could be manipulated to maximize BvLz and minimize TSP. The best extraction condition is often a compromise between the maximum extraction of the protein of interest and the minimum extraction of native protein impurities. One way to identify the optimal extraction conditions is to determine the amount of extracted target protein as a percentage of TSP at each different pH and salt concentration as shown in Figure 4.4. Extracts at pH 4.5 with 50 mM salt had the highest ration of BvLz in the total protein, but four other conditions (pH 4.5 with 150 mM NaCl, pH 6 with 0 mM NaCl, pH 6 with 50 mM

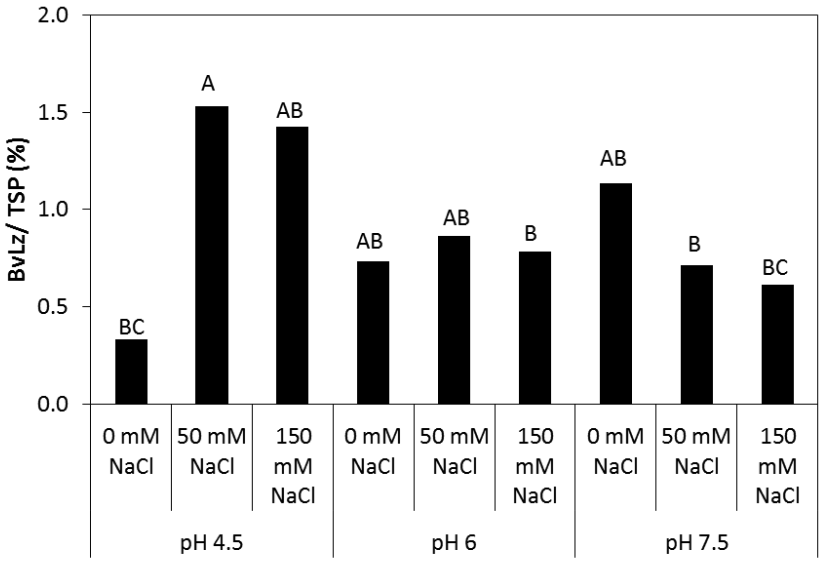


Figure 4.4: Bovine lysozyme (BvLz) expressed as percent of total soluble protein (TSP) in clarified extracts measured by activity assay and ELISA. Samples labeled with the same letter are classified as part of homogeneous subsets by the Tukey test.

NaCl and pH 7.5 with 0 mM NaCl) were considered statistically equivalent. The significant removal of native sugarcane protein that was achieved by precipitation of pH 6 and 7.5 extracts without NaCl was the reason for the observed similarity with pH 4.5 extracts.

4.4.3. Recovery and concentration of BvLz from transgenic sugarcane extracts by bench-scale cross-flow membrane filtration

The complete sugarcane bench-scale processing for BvLz recovery (Figure 4.5) consisted of extraction, centrifugation, extract pretreatment by pH precipitation, and a three-step membrane filtration (0.2 μm depth filtration followed by cross-flow filtrations 100 kDa for extract clarification and 5 kDa or 3 kDa for BvLz concentration).

To understand the influence of extraction conditions on BvLz membrane filtration, bench-scale membrane experiments were performed. From the previously determined conditions that maximize BvLz extraction, three types of extracts were selected for further development: a) Extracts prepared with pH 6 buffer containing 150 mM NaCl, that had the overall highest BvLz concentration, b) Extracts prepared with pH 4.5 buffer containing 150 mM NaCl, and c) Extracts prepared at pH 6 without NaCl, condition that are most suitable for scale-up. From downstream processing perspective, the absence of salt in the clarified extract allows the direct loading of the extract onto any ion exchange resin for further purification, besides the ease of preparation and lower reagent cost.

The performance of each membrane process was evaluated by ELISA (BvLz) and Bradford (TSP) analysis. Results are displayed in Tables 4.1 to 4.3.

Mass balance of BvLz for the 100 kDa step varied between 82 and 100% and that of TSP, 92 and 113% for the three extracts. For the concentration membrane (3 or 5 kDa), the mass balance for BvLz varied between 79 and 105%, while TSP mass balance was between

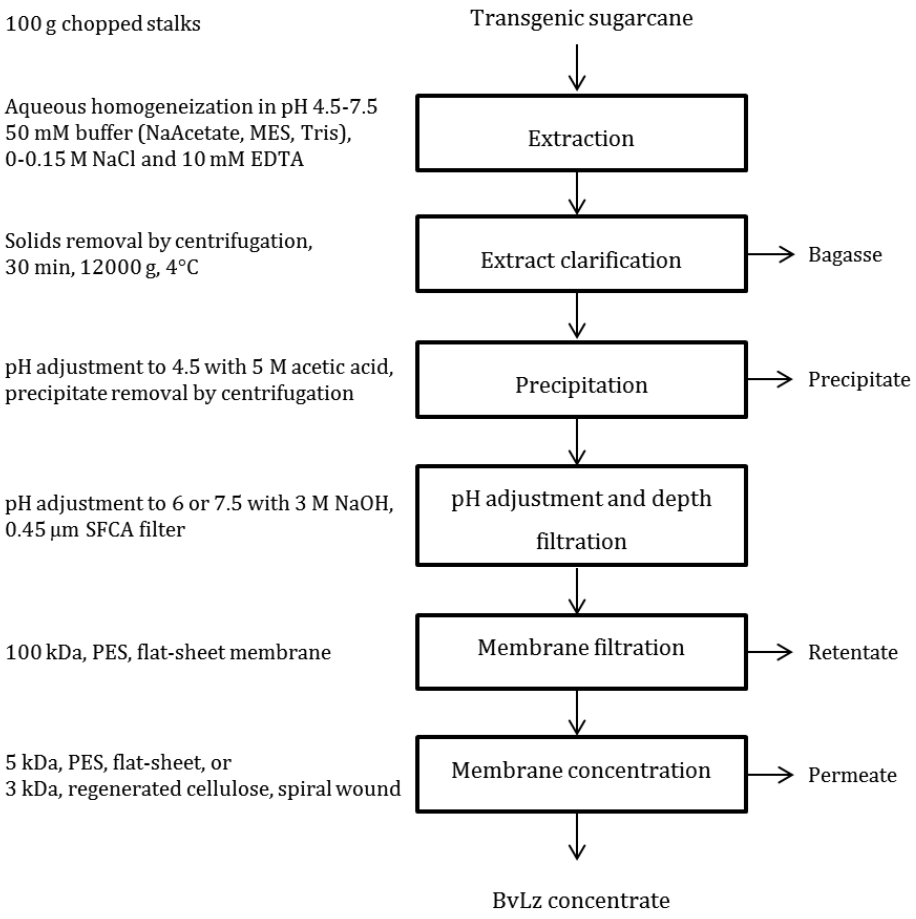


Figure 4.5: Sugarcane bench-scale processing for bovine lysozyme (BvLz) concentration using cross-flow membrane filtration.

74 and 126%. This variability is partly due to protein adsorption on the membrane as well as assay interfering compounds.

To compare the effect of extract condition on the performance of individual cross-flow filtration steps, data from Tables 4.1-4.3 are summarized and discussed separately.

Table 4.1: Bench-scale processing of sugarcane extracts at pH 6 and 150 mM NaCl. Bovine lysozyme (BvLz) mass and recovery determined by ELISA; total soluble protein (TSP) mass and recovery determined by Bradford.

	ELISA		Bradford	
Sample	**BvLz(μg)**	**Recovery (%)**	**TSP (mg)**	**Recovery (%)**
Crude extract	266	100	35	100
Precipitated (pH 4.5)	252	95	21	60
Filtered 0.2 μm	217	82	24	69
100 kDa retentate	25	9	15	43
100 kDa permeate	154	58	12	34
3 kDa concentrate	54	20	3	9
3 kDa chase	46	17		
3 kDa permeate	25	9	10	29

Table 4.2: Bench-scale processing of sugarcane extracts at pH 4.5 and 150 mM NaCl. Bovine lysozyme (BvLz) mass and recovery determined by ELISA; total soluble protein (TSP) mass and recovery determined by Bradford.

	ELISA		Bradford	
Sample	**BvLz (μg)**	**Recovery (%)**	**TSP (mg)**	**Recovery (%)**
Crude extract	141	100	15	100
Filtered 0.2μm	133	94	17	117
100 kDa retentate	28	20	3	23
100 kDa permeate	105	74	12	80
3 kDa concentrate	46	32	3	19
3 kDa chase	24	17	1	7
3 kDa permeate	13	9	11	84

Table 4.3: Bench-scale processing of sugarcane extracts at pH 6 and no NaCl. Bovine lysozyme (BvLz) mass and recovery determined by ELISA; total soluble protein (TSP) mass and recovery determined by Bradford.

	ELISA		Bradford	
Sample	**BvLz (μg)**	**Recovery (%)**	**TSP (mg)**	**Recovery (%)**
Crude extract	128	100	50	100
Precipitated pH 4.5	94	73	27	54
Filtered 0.2 μm	95	74	28	56
100 kDa retentate	12	9	8	16
100 kDa permeate	67	52	17	33
100 kDa chase	5	4	1	3
5 kDa concentrate	23	18	2	4
5 kDa chase	3	3	1	2
5 kDa permeate	43	34	10	20

4.4.3.1. Cross-flow filtration using 100 kDa membrane

The 100 kDa step was included in the membrane process for clarification of the extracts which was measured by the removal of TSP. Table 4.4 summarizes mass of BvLz and TSP before and after the bench-scale 100 kDa membrane filtration and the percentage recovery of BvLz and removal of native proteins. Results for extracts at pH 6 with and without NaCl were the average of two separate experiments.

In addition to different starting pH and NaCl concentration, each extract fed to the 100 kDa membrane had a different BvLz content. Nevertheless, comparable percentage recoveries (between 71 and 79%) were obtained, demonstrating that the 100 kDa membrane filtration was not affected by pH or ionic strength of extracts.

Table 4.4: Bovine lysozyme (BvLz) recovery and total soluble protein (TSP) removal from bench-scale 100 kDa membrane filtration of transgenic sugarcane extracts. BvLz (μg) was measured by ELISA and TSP (mg) was measured by Bradford.

	Stream	pH 6 150 mM NaCl	pH 4.5 150 mM NaCl	pH 6 0 mM NaCl
BvLz	100 kDa feed (μg)	217	133	95
	100 kDa permeate (μg)	154	105	67
	BvLz recovery (%)	71%	79%	71%
TSP	100 kDa feed (mg)	24	17	28
	100 kDa permeate (mg)	12	12	17
	TSP removal (%)	50%	29%	39%

The amount of TSP in the 100 kDa permeate indicates that a substantial fraction (29% to 50%) of larger than 100 kDa molecular weight proteins were removed from the extract. This was a rather unexpected outcome because the majority of native sugarcane proteins in the extracts determined by SDS-PAGE appeared to be smaller than 100 kDa (Figure 4.1). Therefore, one can conclude that larger than anticipated protein removal was due to fouling that had restricted the membrane pore openings and led to a greater protein rejection.

The reason for including the 100 kDa membrane filtration step was to improve the efficiency of the subsequent 3 or 5 kDa concentration step, since it was previously determined in the Bioseparations Lab that the additional clarification of sugarcane extracts by 100 kDa membrane filtration allowed achieving a higher permeate flux during and hen-egg white lysozyme concentration step (Ballen 2009). The potential increase of permeate

flow rates on these tight membranes would reduce operational time and the decrease in transmembrane pressure (TMP) would reduce the required pumping energy.

Partial removal of TSP (29-50%) from transgenic sugarcane extracts was indeed attained by the 100 kDa membrane step, but at expense of losing a portion of the BvLz (21-29%) that stayed in the retentate, likely bound to other large molecules. For that reason, the 100 kDa filtration step was eliminated for the purification experiments (Section 4.4.4) and also for the membrane filtration scale-up, as described in Appendix B.1.

4.4.3.2. Cross-flow concentration step using 3 or 5 kDa membrane

The concentration step for pH 6 extracts without NaCl was carried out using a flat-sheet 5 kDa polyethersulfone (PES) cartridge. Because substantial loss of BvLz occurred using this membrane (34% of the starting BvLz was detected in the 5 kDa permeate), the subsequent concentration steps at pH 4.5 and 6 with 150 mM NaCl were performed using a spiral wound 3 kDa regenerated cellulose membrane. The amount of BvLz in the feed and 3 kDa streams (permeate, concentrate and chase) of pH 4.5 and 6 extracts with 150 mM NaCl and the respective BvLz recoveries are given in Table 4.5.

Bovine lysozyme recovery in the concentrate from pH 4.5 extracts was slightly higher (67%) than that from pH 6 extracts (65%). Maximum transmission of proteins typically occurs at pH close to the isoelectric point (Mehta and Zydney 2006). Indeed, more BvLz was detected in the pH 6 permeates, presumably because BvLz (pI 7.65) has a lower net charge at pH 6 than at pH 4.5. However, the greater amount of BvLz permeating the membrane at pH 6 was compensated by the greater BvLz content in the feed, and the final recovery in percentage was not affected.

The overall performance of bench-scale cross-flow BvLz recovery and concentration

Table 4.5: Bovine lysozyme (BvLz) recovery from bench-scale 3 kDa membrane concentration of sugarcane extracts. BvLz values (μg) were measured by ELISA; data are average of two separate runs.

Stream	**pH 6 150 mM NaCl**	**pH 4.5 150 mM NaCl**
3 kDa feed (μg)	154	105
3 kDa permeate (μg)	25	13
3 kDa concentrate (μg)	54	46
3 kDa chase (μg)	46	24
Recovery (%)	65%	67%

process was examined by comparing the overall BvLz recovery in the 3 kDa or 5 kDa retentates and chases, the volume concentration factor (VCF = feed volume/ final retentate volume) and the concentration factor (CF = final BvLz concentration/ BvLz concentration in the feed). Volume concentration factor values of 4.5 for pH 4.5 extract and 4.6 for pH 6 extract, represent the volume reduction, which was similar for both extracts with 150 mM NaCl. In the same way, the concentration factor (CF) was also comparable for pH 4.5 and pH 6 extracts with 150 mM NaCl (2.2 and 1.7, respectively). The deviation of VCF from CF indicates BvLz losses during the cross-flow filtration process.

Overall BvLz recoveries (Tables 4.1-4.3) varied between 21% for pH 6 without NaCl extracts and 51% for pH 4.5 extracts containing 150 mM NaCl. Different sugarcane extraction preparation conditions produced extracts with different amounts of BvLz and TSP. The overall BvLz yield seem to be affected more by the pore size of the membrane used in the concentration step (5 kDa vs. 3 kDa MWCO) and membrane chemistry (PES vs. regenerated cellulose) than by extract pH and ionic strength. Extracts prepared at pH 4.5

with NaCl had a higher overall BvLz yield (51%) than pH 6 extracts (37%), possibly due to the lower amount of impurities in the pH 4.5 extract.

Regardless of the extraction condition and losses during pH precipitation, at least 47% of the BvLz initially present in the crude extract was lost during membrane processing by being retained by the 100 kDa membrane, permeated through the 3 kDa membrane or adsorbed on the membrane. The BvLz mass loss during membrane processing ranged between 63 and 117 μg (Tables 4.1-4.3), but the low BvLz concentration in the crude extracts probably magnified losses when they were expressed as percentage of initial BvLz concentration. For example, 22 μg of BvLz lost during the 3 kDa membrane concentration of pH 4.5 extract with 150 mM NaCl represents a loss of only 24 ng BvLz/cm^2 of membrane, although it translates in 16% of the initial BvLz lost.

Non-specific protein absorption on membrane surfaces is a function of membrane polymer chemistry. The PES membranes (100 kDa and 5 kDa used in this work) are classified by membrane manufacturer (Millipore) as low to moderate protein adsorbing and are recommended for solutions with more than 0.1 mg/mL protein. Regenerated cellulose membranes (spiral wound 3 kDa membranes) are considered ultra-low protein adsorbing materials and could be used with protein solutions containing less than 0.1 mg/mL protein. Crude BvLz extracts used in this study contained between 0.23 and 0.47 μg/mL BvLz and 0.025 and 0.062 mg/mL TSP, values that were at least 200-fold and 1.6-fold bellow the PES membrane threshold, respectively.

Because none of the three evaluated extraction conditions caused a substantial recovery of BvLz in the concentrate, extracts at pH 6 with 150 mM NaCl were selected for the subsequent experiments aimed at determining the cause of low BvLz recovery. A

purified BvLz solution (128 μg/mL tobacco-derived BvLz in pH 6 with 150 mM NaCl extraction buffer) was processed using the same downstream processing steps as the one shown in Figure 4.5. Characterization of tobacco-derived BvLz and analysis of samples collected during the bench-scale process are shown in Appendix C.

Membrane filtration of tobacco-derived BvLz solution resulted in 95% BvLz recovery in the 5 kDa concentrate. Bovine lysozyme degradation and aggregate impurities had no apparent effect on estimating the process yield using ELISA (only 5% of BvLz lost). On the other hand, the substantial difference between purified tobacco-derived BvLz recovery (95%) and that of transgenic sugarcane extracts (21-51%) can be attributed to interference from extract impurities. The higher percentage recovery achieved with the tobacco-derived BvLz was likely due to the absence of impurities in the standard solution. Even though the BvLz mass loss during processing of the tobacco-derived BvLz solution (3.4 mg) was at least 16-fold higher than the loss that occurred during processing of sugarcane extracts (0.2 mg), the enzyme loss expressed in percent of initial BvLz was minor (5%).

The strong effect of BvLz and impurities concentration in the starting extracts on the membrane filtration performance indicates that membrane processing alone would not be able to achieve a significant purification of BvLz from transgenic sugarcane extracts. Therefore, cross-flow filtration should be applied primarily for concentration and pretreatment of clarified sugarcane extracts before BvLz purification by chromatography.

4.4.4. Purification development: BvLz from sugarcane concentrates using hydrophobic interaction chromatography (HIC)

To scale-up the membrane filtration and develop required purification steps, the above-mentioned findings and additional discoveries were incorporated into the pilot-plant processing of transgenic sugarcane (Appendix B.1).

Another process modification in transitioning from bench to pilot scale was the preparation of BvLz extracts: in the pilot-plant, shredded sugarcane stalks were pressed to release juice containing BvLz, whereas on the bench, BvLz extracts were prepared by grinding chopped sugarcane stalks with aqueous buffer. To distinguish between these two methods, sugarcane juice will be used to describe pressed pilot-plant juice and sugarcane extract will refer to the Waring blender ground sugarcane in aqueous buffer.

Previous purification development work in the Bioseparations Lab using concentrated pilot-plant juice revealed that using a two-chromatography step purification consisting of ion exchange and hydrophobic interaction chromatography (IEX+HIC), 95% BvLz purity could be achieved (Appendix B.2). The use of a cation exchange resin as the first adsorption step had several benefits: a) removed part of colored impurities in the juice, b) reduced native protein amount in the HIC load, and c) concentrated BvLz. However, low BvLz recovery (20%) from the ion exchange step prompted the consideration of alternative purification options using less expensive unit operation that would achieve effective color and protein removal.

4.4.5. Concentration and purification of spiked extracts by HIC

To evaluate whether the decrease of color and protein level in the IEX step were indispensable benefits for satisfactory HIC performance, direct loading of sugarcane extract on HIC column was performed on bench scale. The reasons for testing direct loading of

extract on HIC were the lower TSP concentration of sugarcane extracts (52 μg/mL) than sugarcane juice (490 μg/mL) and less colored impurities (Figure 4.6).

Figure 4.6: Sugarcane juice (left) and sugarcane extract (right).

To be able to compare this process with the two-step (IEX+HIC) purification one, sugarcane extracts were spiked with tobacco-derived BvLz to a 20% TSP level. Spiked sugarcane extracts were concentrated using the same membrane filtration steps as the pilot-plant juice and then purified by hydrophobic interaction chromatography (Figure 4.7). The BvLz amount after each membrane step and respective yields are shown in Table 4.6.

The overall yield and the volume reduction (VCF) of 6.3 obtained by membrane concentration step of spiked sugarcane extract were comparable to the membrane

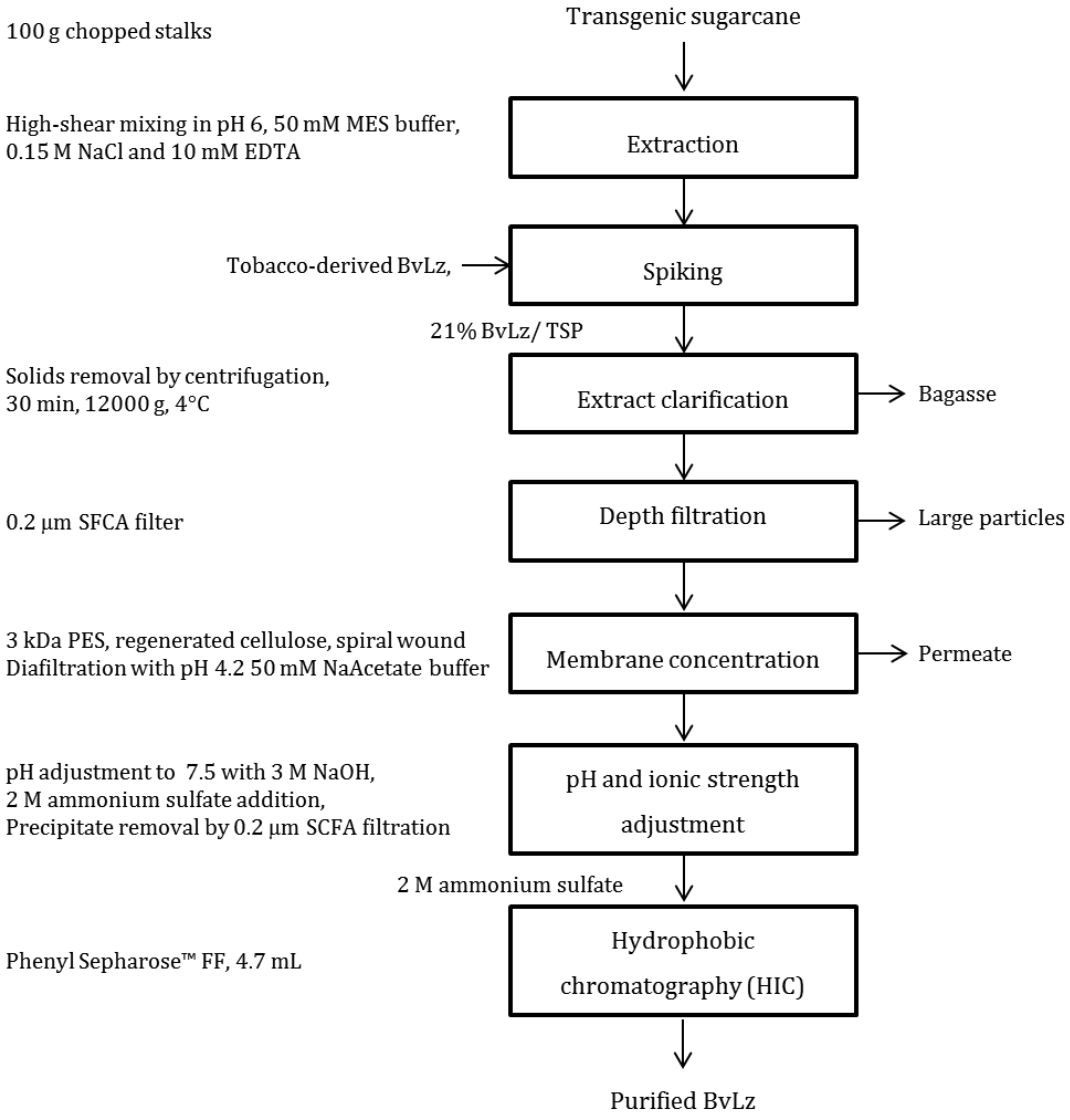


Figure 4.7: Bench-scale alternative processing for bovine lysozyme (BvLz) recovery and purification from spiked sugarcane extract.

Table 4.6: Bovine lysozyme (BvLz) recovery during membrane filtrations of spiked sugarcane extract (pH 6, 150 mM NaCl). BvLz values in mg were estimated by ELISA.

Sample	Volume (mL)	BvLz (mg)	Overall yield%
Spiked extract	507	11.2	100
Filtered 0.2 μm	506	10.3	92
3 kDa concentrate	80	7.1	65
Chase	115	1.8	17

concentration of transgenic extracts in Section 4.4.3.

Only 65% of the initial tobacco-derived BvLz in the spiked extract was recovered in the concentrate, compared to 95% of tobacco-derived BvLz that was recovered in the 5 kDa concentrate (Table C.1, Appendix C). This difference can be attributed to sugarcane impurities because more BvLz was lost during the processing of spiked extract (4.2 mg) than in the membrane filtration of tobacco-derived BvLz solution (3.4 mg).

Protein profiles of membrane-processed samples (Figure 4.8) show the presence of the major protein impurity in the crude extract, the 18 kDa dirigent protein (lanes 2-4). After spiking of the extract, the BvLz band size surpasses the 18 kDa sugarcane dirigent protein impurity (lane 5). Similarly to concentrated sugarcane juice (data not shown), the 18 kDa dirigent protein was concentrated together with BvLz, and it was present in substantial amounts in the 3 kDa concentrate of spiked sugarcane extract (lane 8).

The 3 kDa concentrated extract was loaded on the HIC column (Figure 4.9) and purified by separation from the 18 kDa sugarcane protein impurity. During the load, an increase in the

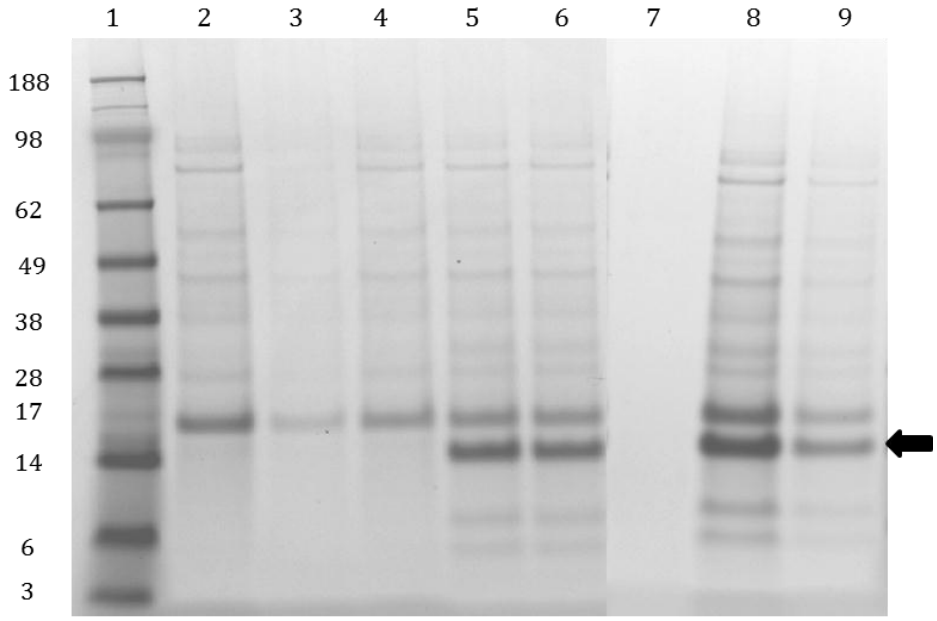


Figure 4.8: Protein profiles of bench-scale membrane recovery of sugarcane extract spiked with tobacco-derived bovine lysozyme (BvLz). Samples were loaded based on equal volumes. Lanes: (1) Molecular marker, (2) 1st sugarcane extract, (3) 2nd sugarcane extract, (4) Combined extracts, (5) Spiked BvLz sugarcane extract, (6) BvLz sugarcane extract after 0.2 μm depth filtration, (7) 3 kDa permeate, (8) 3 kDa concentrate, (9) Chase (membrane wash). The arrow shows the position of BvLz.

280 nm absorbance of the solution flowing through the column was observed. However, protein analysis by Bradford and gel electrophoresis (Figure 4.10) showed that flow through fractions did not contain detectable protein. Because phenolic compounds absorb at both 280 and 320 nm, the apparent absorbance increase at 280 nm was likely caused by phenolic compounds, because the 320 nm signal increased proportionally with the 280 nm absorbance.

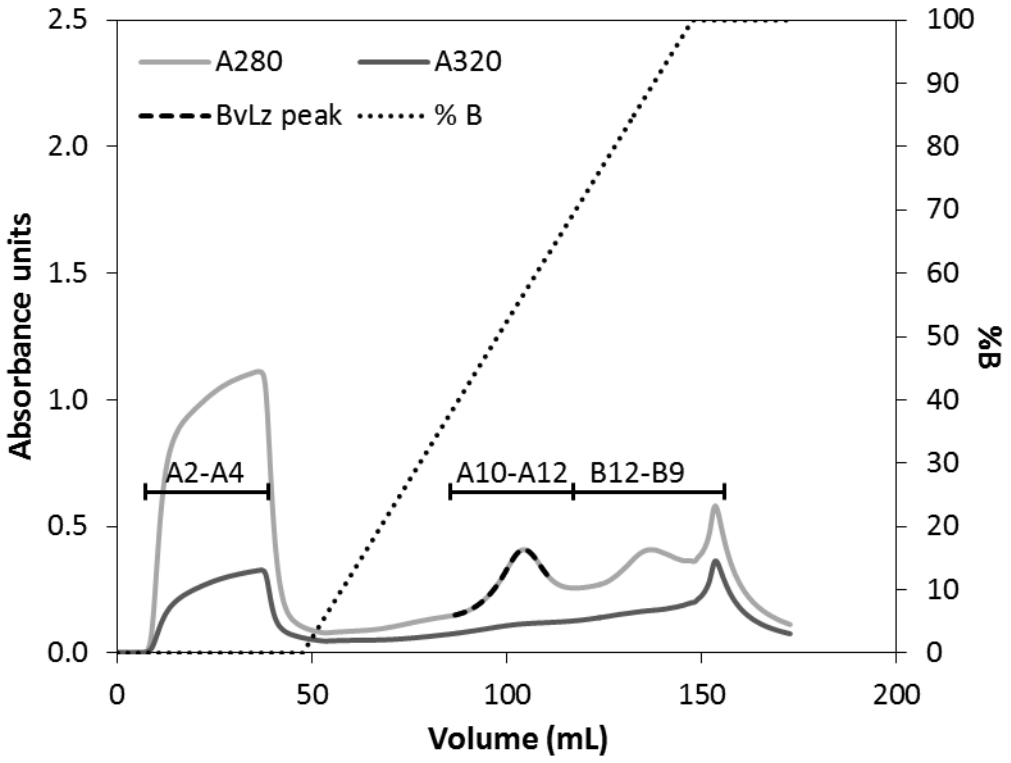


Figure 4.9: Purification profile of spiked 3 kDa concentrate on a hydrophobic interaction chromatography (HIC). Percentage of elution buffer B, A280 and 320 nm monitored using AKTApurifier™. The dashed line shows the position of BvLz.

The protein bands of the eluted fractions show that the BvLz elution peak (A10-A12) does not contain the 18 kDa dirigent (Figure 4.10). The BvLz elution peak in Figure 4.9 did not reach the baseline, and the lack of complete resolution was evidenced by the protein profiles of the subsequent fractions (B12-B9), where BvLz eluted together with the sugarcane impurity.

Analysis of the elution fractions (Table 4.7) confirmed the incomplete separation of BvLz from the native sugarcane proteins. Although a large part of BvLz in the load (88%) was recovered in the elution, fractions containing the majority of BvLz elution peak (A10-

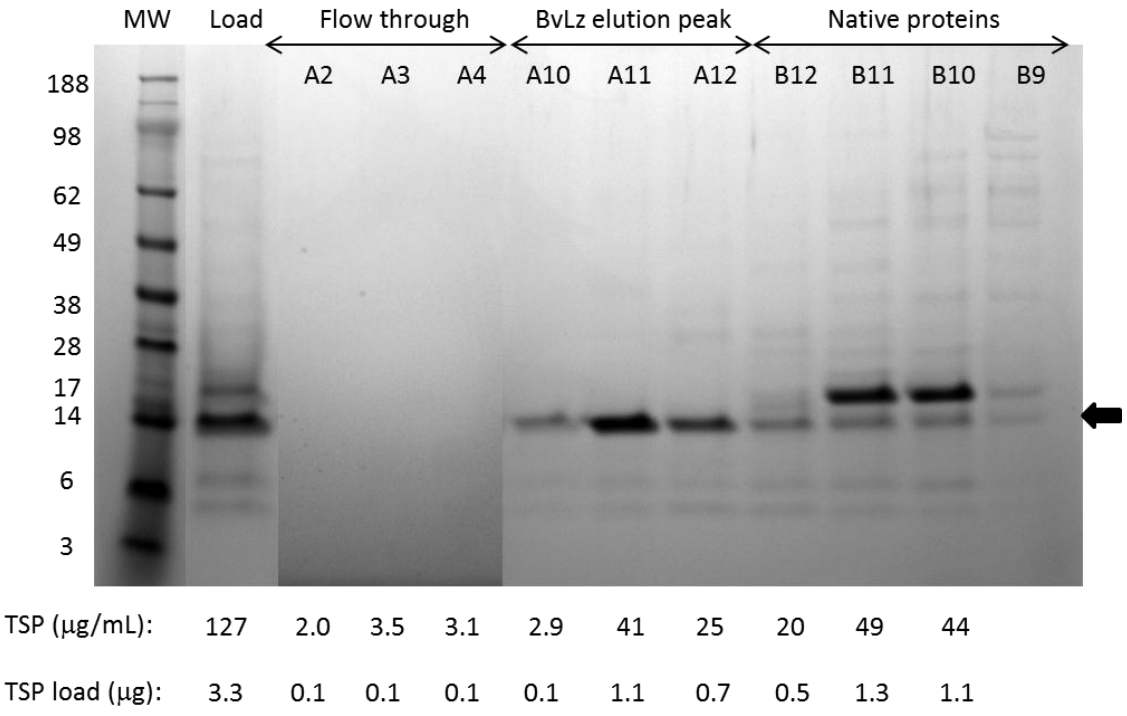


Figure 4.10: Protein profiles for molecular weight marker, hydrophobic interaction chromatography (HIC) load, and BvLz and native protein fractions from HIC of 3 kDa concentrated sugarcane extract. The arrow shows the position of BvLz.

A12) represented 69% of loaded BvLz and were only 50% pure. Protein load per mL resin can be ruled out as the cause of low selectivity (performance) of the hydrophobic chromatography step because the TSP concentration that was loaded on the HIC column (510 µg/mL) for IEX+HIC process was greater than the native protein concentration in the spiked sugarcane extract (127 µg/mL). Thus, it was hypothesized that interference from non-protein impurities was likely the cause for the reduced yield and BvLz purity. The comparison of HIC loads in the IEX+HIC process (Figure B.5, lane 2) and this one (Figure 4.10, lane 2) shows a slight difference in the protein profile. The most prominent

Table 4.7: Bovine lysozyme (BvLz) mass in elution peak fractions and respective purities (BvLz/ TSP). BvLz values in μg were measured by ELISA.

Sample	BvLz (μg)	Purity (%)
Load	898	19
A10	66	44
A11	404	62
A12	150	35
B12	77	24
B11	47	7
B10	30	5
B9	16	7

difference between the two elution profiles in Figures 4.9 and B.4 (Appendix B) is the amount of phenolics and other colored impurities (A320 line) that co-elute with the BvLz peak.

In summary, the assumed advantages of sugarcane extract, lower content of TSP and colored impurities, did not result in a comparable better purification assembly than the IEX+HIC process.

4.4.6. Concentration and purification of spiked and pretreated sugarcane juice by HIC

As observed previously, one of the benefits of IEX step in the IEX+HIC purification process was the adsorption and removal of colored impurities. Assuming that partial protein purification by cation exchange is not required before the HIC step, the removal of colored impurities from the juice should allow bypassing the IEX chromatography step. To remove non-protein and colored impurities from the sugarcane juice, uncharged polystyrene-based resin (XAD-4) was used. To simulate the concentration effect of IEX

step before HIC in the two-chromatography purification process (IEX+HIC), sugarcane juice concentrate was spiked with tobacco-derived BvLz to approximately the same concentration achieved after the IEX column. The XAD+HIC purification process was performed as described in Figure 4.11.

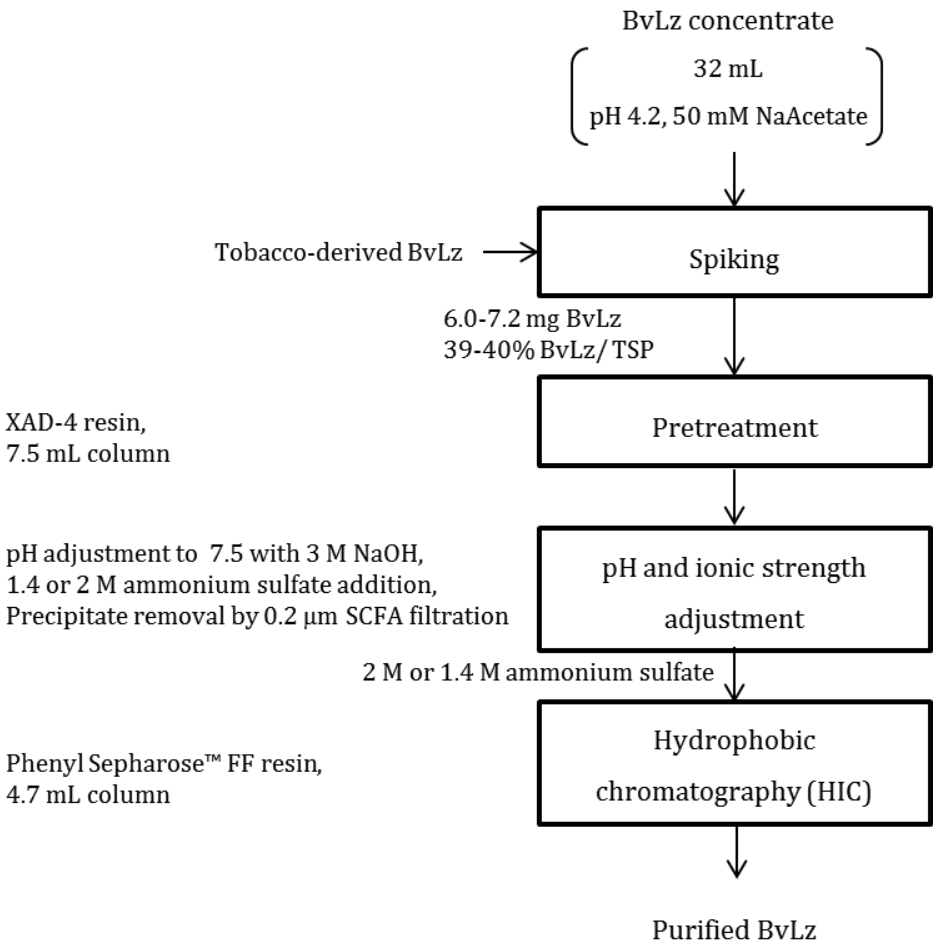


Figure 4.11: Sugarcane bench-scale process for bovine lysozyme (BvLz) purification using XAD-4 pretreatment and hydrophobic interaction chromatography (XAD+HIC).

Removal of impurities by XAD-4 adsorption was performed at pH 4.2. Low pH was the most efficient for XAD-4 removal of phenolics from *Lemna minor* (Section 2), and the sugarcane juice concentrate leaving the membrane filtration was diafiltered into 50 mM sodium acetate at pH 4.2.

Samples of sugarcane juice concentrate, spiked and XAD-4 treated were analyzed by spectrophotometry and gel electrophoresis, and their wavelength spectra and protein profiles compared (Figure 4.12). The wavelength spectrum of the sugarcane juice concentrate did not change after spiking with tobacco-derived BvLz. In contrast, a change

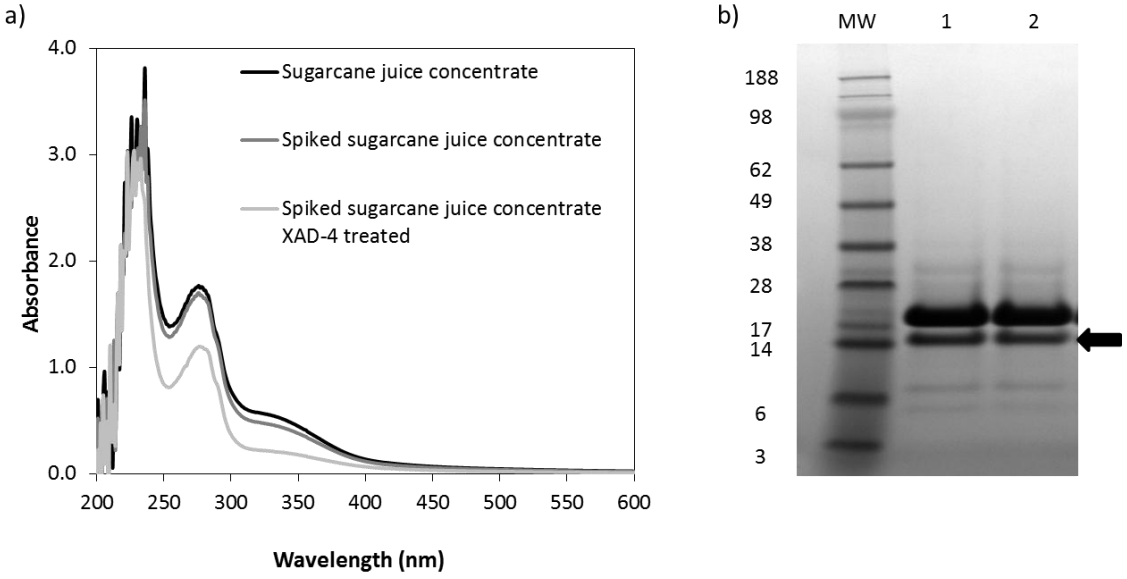


Figure 4.12: Sugarcane juice concentrates before and after XAD-4 pretreatment: (a) wavelength spectra and (b) protein profiles. Samples in the gel electrophoresis were loaded based on equal volumes: (MW) Molecular marker, (1) spiked sugarcane juice concentrate, and (2) XAD-4 treated spiked sugarcane juice concentrate.

of wavelength spectra of sugarcane juice concentrates before and after XAD-4 treatment was observed. Absorbance decreased between 250 and 400 nm, suggesting that the removed compounds absorb this range. No substantial change in the protein profiles was observed and 2.6% reduction in TSP was measured by Bradford. Only 8.3% of initial BvLz was lost in XAD-4 treatment.

In preparation for the HIC step, ammonium sulfate was added to the spiked sugarcane juice concentrate. Two HIC runs were performed, using different concentrations of ammonium sulfate in the load. The addition of 2 M ammonium sulfate (49% saturation) resulted in partial precipitation of BvLz (24%). No BvLz precipitated with the addition of 1.4 M $(NH_4)_2SO_4$ (34% saturation). In spite of BvLz losses due to precipitation, 2 M ammonium sulfate concentration in the load was necessary to assure complete binding of BvLz on the hydrophobic column. At 1.4 M ammonium sulfate, BvLz was detected in the flow through fractions (Figure C.4, Appendix C). Spiked and XAD-treated juice concentrate were loaded at 2 M or 1.4 M ammonium sulfate onto the hydrophobic column, and the results compared (Figure 4.13).

Although the protein bands of the HIC loads after XAD-4 treatment (Figure 4.14, lane 2) and after IEX (Figure B.5, lane 1) looked similar, the HIC elution profiles were different between the two runs (Figure 4.13 and Figure B.4, Appendix B). The second peak in the elution profiles consists primarily of the 18-kDa dirigent protein (fractions A7-A12 in Figure 4.14a and fractions A7-B12 in Figure 4.14b).

Since the presence of ammonium sulfate strengthens hydrophobic interactions, greater BvLz adsorption to HIC resin was observed for the feed containing 2 M ammonium sulfate, than 1.4 M salt. In the same way, BvLz eluted earlier (lower %B) when less ammonium

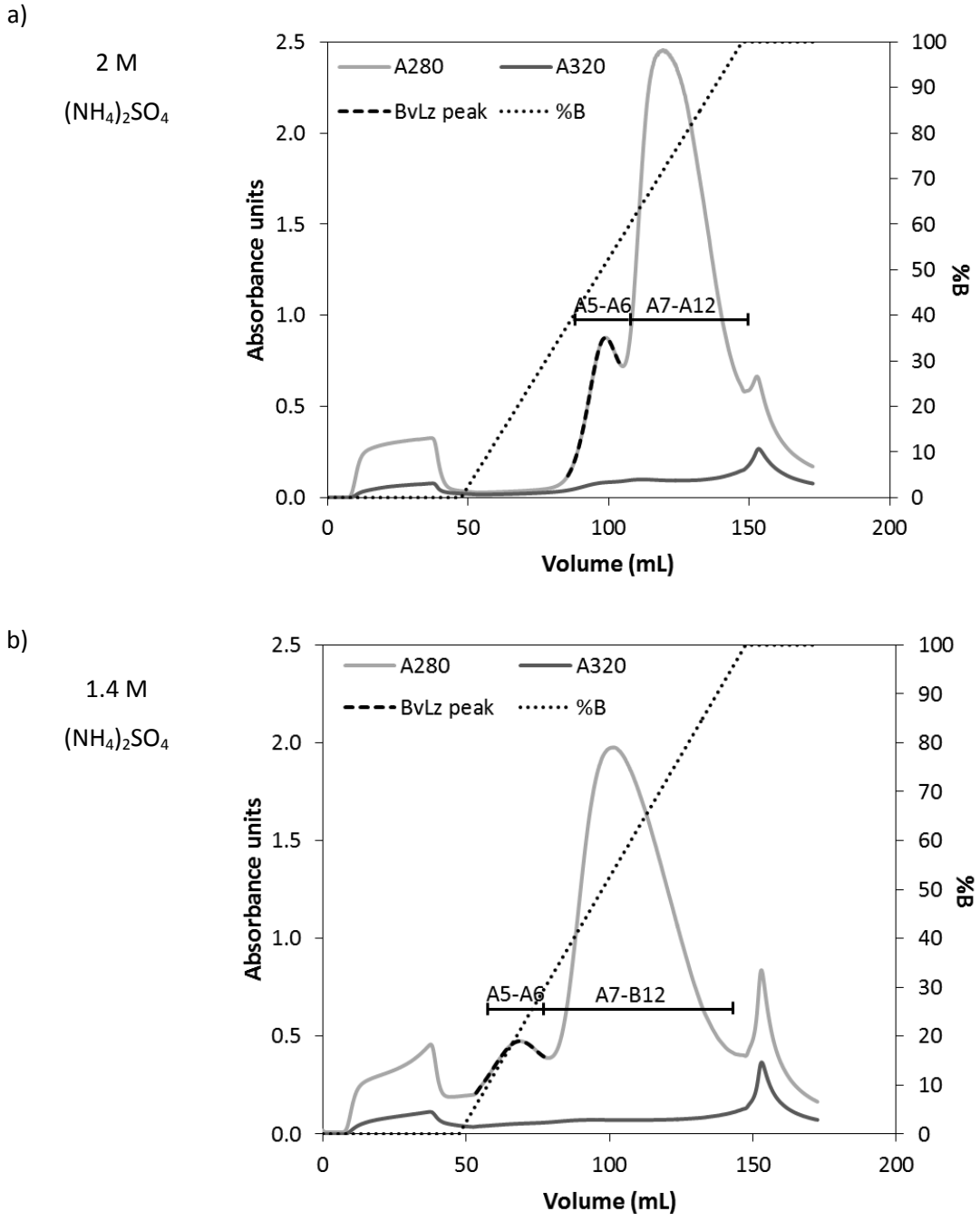


Figure 4.13: Purification profiles of sugarcane juice treated with XAD-4 and loaded on a hydrophobic interaction chromatography (HIC) with (a) 2 M or (b) 1.4 M ammonium sulfate in the load. Percentage of elution buffer B, A280 and 320 nm were monitored using AKTApurifier™. The dashed line indicated BvLz.

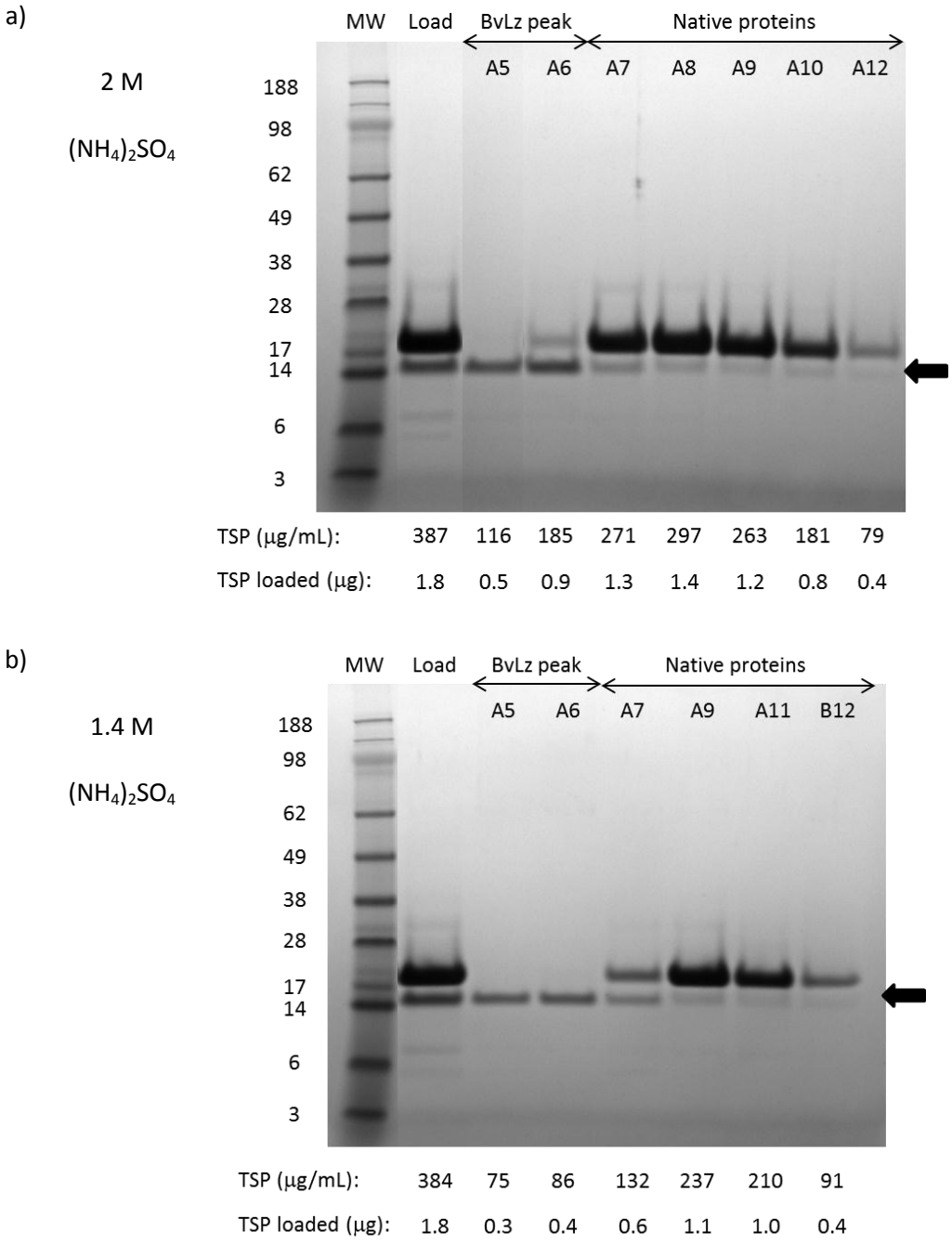


Figure 4.14: Protein profiles of molecular marker, HIC load, and elution fractions from hydrophobic interaction chromatography (HIC) of spiked sugarcane juice concentrate XAD-4 treated. Ammonium sulfate concentration in the load was (a) 2 M or (b) 1.4 M. The arrows shows the position of BvLz.

sulfate was present in the load (Figure 4.13b). Actually, for both cases, BvLz started eluting when the ammonium sulfate in the mobile phase reached almost the same concentration: 1.25 M versus 1.22 M. However, the difference of 0.03 M ammonium sulfate in the mobile phase was sufficient to initiate an earlier elution of BvLz from the column when the adsorption was performed with 1.4 M ammonium sulfate. In the stronger BvLz adsorption to the hydrophobic column performed with 2 M ammonium sulfate, BvLz started eluting later (1.25 M ammonium sulfate) and, for that reason, the second fraction (A6) of the BvLz elution peak was contaminated with the 18-kDa dirigent protein (Figure 4.14a). A better separation between BvLz and the 18-kDa impurity was seen when BvLz was adsorbed with 1.4 M ammonium sulfate.

Quantitative analysis of the elution fractions show that pure BvLz could be recovered in 20 mL (4.3 CV), independent of ammonium sulfate concentrations in the load. Lower yield of pure BvLz in the 1.4 M salt run (Table 4.8) was probably a result of the lower overall recovery of BvLz in the elution fractions. Almost 90% of loaded BvLz was recovered in the eluted fractions (A5-A10) when the load had 2 M salt, but only 63% of the loaded BvLz was in the eluted fractions when adsorption was performed with 1.4 M ammonium sulfate (data not shown). When adsorption was performed with 2 M ammonium sulfate, 66% of the loaded BvLz was recovered as pure BvLz in 20 mL. Recovery of 43% of loaded BvLz as pure BvLz was obtained when the adsorption was performed with 1.4 M ammonium sulfate.

Besides the difference in the HIC yields, the overall purification yields for the XAD+HIC process (Table 4.8) were similar for either ammonium sulfate concentration. BvLz loss due to precipitation after the addition of 2 M ammonium sulfate in preparation for the HIC load was compensated by the lower HIC recovery (43%) achieved with 1.4 M

Table 4.8: Purification table of the XAD+HIC process for bovine lysozyme (BvLz) purification from sugarcane juice concentrate. BvLz values in mg were measured by ELISA.

		2 M ammonium sulfate			1.4 M ammonium sulfate		
Sample	**Volume (mL)**	**BvLz (mg)**	**Purification Yield (%)**	**HIC Yield (%)**	**BvLz (mg)**	**Purification Yield (%)**	**HIC Yield (%)**
XAD load	32	7.3	100		4.9	100	
After XAD	32	6.7	92		4.5	92	
HIC load	32	5.0	69	100	4.8	97	100
HIC pool	20	3.3	45	66	2.0	41	43

ammonium sulfate.

4.4.7. Comparison between purification processes

Even though ion exchange resins are heavily used in protein separations, chromatography is considered an expensive operation, which is usually applied for high-value proteins and not for industrial enzymes. To resolve BvLz from a major sugarcane impurity, the 18-kDa native protein, HIC was found to be indispensable in the BvLz purification process. In the two-chromatography (IEX+HIC) process developed for BvLz purification from sugarcane juice concentrate (Appendix B.2), the IEX column was important as a preparation step for the HIC, providing concentration of BvLz and removal of impurities.

Comparison of parameters and results for the three HIC purification processes are summarized in Table 4.9.

Because sugarcane extracts were cleaner than juice, less TSP was present in the sugarcane concentrate, and consequently, lower percentage of the HIC column binding

Table 4.9: Purification parameters and results of bovine lysozyme (BvLz) purification by hydrophobic interation chromatography (HIC).

	IEX+HIC	**HIC**	**XAD+HIC**	
Starting purification	Juice	Extract	Juice	
$[NH_4]_2SO_4$ in the load (M)	1.4	2	2	1.4
Load volume (CV)	6.8	6.8	6.8	6.8
Column binding capacity used (%)	5.3	1.4	7.8	7.4
BvLz mass in the load (mg)	7.0	0.9	7.3	4.9
BvLz/TSP in the load (%)	21	19	40	39
BvLz elution volume (CV)	5.3	4.3	4.3	4.3
Start of BvLz elution (M $[NH_4]_2SO_4$)	1.25	1.22	1.22	1.25
BvLz purity (%)	95	50	108	126
Purification fold	4.5	2.6	2.7	3.2
HIC yield (%)	200	--	66	43
Overall yield (%)	41	--	45	41

capacity was used. BvLz content in the load was lower for the sugarcane extract than the load for the purifications from sugarcane juice, resulting in a smaller BvLz peak (Figure 4.9). Concentrated extract can be directly loaded on the hydrophobic column without previous XAD-4 pretreatment or IEX purification, but only 50% purity could be achieved.

The experiments including the XAD-4 pretreatment step in the purification train (XAD+HIC) instead of IEX were conducted with sugarcane juice concentrate. The XAD-4 pretreatment did not remove native protein or concentrated BvLz as effectively as the IEX chromatography. Pure BvLz was also obtained by the XAD+HIC process, although the HIC yield (66%) was not as high as in the IEX+HIC process (200%).

When comparing XAD+HIC to IEX+HIC, we were comparing recombinant BvLz from transgenic sugarcane to tobacco-derived BvLz spiked in sugarcane juice. The protein profile of the HIC load after IEX (Figure B.5, Appendix B) shows the 15-kDa BvLz monomer and small amounts of additional BvLz forms. The same was true for the protein profile of the HIC after XAD (Figure 4.12).

Different BvLz forms interacted differently with the HIC column; the smaller degraded BvLz form eluted earlier, and the dimer after the other forms (Figure C.4, Appendix C). Thus, the distribution of the three forms within the BvLz sample may have also influenced the resolution of BvLz peaks and contributed to the observed difference in the HIC purification profiles from the (Figures 4.13 and Figure B.4 in Appendix B).

The two-chromatography IEX+HIC process has a clear advantage over the other two alternative processes. Higher purification fold and yield were achieved for the HIC after IEX, due to the complete separation of BvLz and the 18-kDa protein.

4.5. Conclusions

In this section, the screening of 131 transgenic sugarcane lines did not find satisfactory accumulation of recombinant BvLz on the sugarcane stalks, with only five lines exceeding the expression level of 4.5 mg/kg,

To maximize the extraction of recombinant BvLz from transgenic sugarcane, the investigation of aqueous buffers with different pH and ionic strength levels indicated that the protein concentration in the sugarcane extracts is affected by pH and salt concentration. Protein precipitation of pH 6 and 7.5 extracts at acidic pH achieved a significant removal of native sugarcane protein. Extracts at pH 4.5 with 50 mM salt had the highest of BvLz to TSP ratio, but other four conditions (pH 4.5 with 150 mM NaCl, pH 6

with 0 mM NaCl, pH 6 with 50 mM NaCl and pH 7.5 with 0 mM NaCl) were considered statistically equivalent. Concentrations of BvLz from 0.31 to 0.52 μg/mL corresponded to 0.31-0.52 ppm, at least 240-fold lower than the target values of 125-250 ppm required for BvLz anti-bacterial treatment of seeds (Mirkov and Fitzmaurice 1998).

Using three extract conditions (pH 4.5 with 150 mM NaCl, pH 6 without and with 150 mM NaCl) that maximized BvLz extraction, a three-step membrane filtration (0.2 μm depth filtration followed by cross-flow filtrations 100 kDa for extract clarification and 5 kDa or 3 kDa for BvLz concentration) was performed for concentration of BvLz. Partial removal of TSP (29-50%) from transgenic sugarcane extracts was attained by the 100 kDa membrane step, but a portion of the BvLz (21-29%) was lost. Because of that the 100 kDa filtration step was eliminated for the purification experiments and also for the membrane filtration scale-up. Regardless of the extraction condition, at least 47% of the BvLz initially present in the crude extract was lost during membrane processing. Higher BvLz recovery was obtained by using the 3 kDa regenerated cellulose membrane rather than 5 kDa PES membrane, but none of the three evaluated extraction conditions caused a substantial recovery of BvLz in the concentrate. Because membrane processing alone would not be able to achieve a significant purification of BvLz from transgenic sugarcane extracts, cross-flow filtration was applied primarily for concentration and pretreatment of sugarcane extracts, and a subsequent BvLz purification by chromatography was needed.

Alternative purification options were evaluated to substitute the two-chromatography process (IEX+HIC), which achieved 95% BvLz purity and total recovery of the BvLz loaded into the HIC step. Direct loading of spiked sugarcane extract concentrate on the HIC step, although containing less colored impurities and native proteins than the sugarcane juice, recovered 69% of loaded BvLz at only 50% purity. In the XAD+HIC

process, BvLz loss during XAD-4 step was only 8.3%, but XAD-4 pretreatment did not remove native protein or concentrate BvLz as effectively as the IEX chromatography. Even though pure BvLz was obtained by the XAD+HIC process, the HIC yield was not as high as in the IEX-HIC process.

The IEX column was important as a preparation step for the HIC, providing concentration of BvLz and removal of impurities. After the cation exchange chromatography, higher purification fold and yield were achieved by the HIC step, due to the complete separation of BvLz and 18-kDa protein.

5. SUMMARY AND CONCLUSIONS

Transgenic plant systems have been proposed as alternative bioreactors to microbial and mammalian cell culture for the production of proteins and other bioproducts due to advantages such as the lower capital investment, simplicity of scale-up, and low risk of contamination by human pathogens.

Seed and leafy tissue systems offer particular advantages and disadvantages that depend on the bioproduct and its applications. Recombinant protein accumulation levels and the lack of efficient downstream processing are key drawbacks that have to be overcome to achieve cost-effective recombinant protein production.

Leafy and aquatic plants are promising candidates for commercial production of pharmaceutical proteins, due to their quick generation of biomass. In addition, they are amenable for transient expression and are considered the most suitable system for rapid vaccine production in case of pandemic outbreak. The easy scale-up and relatively low-cost of plant systems could potentially meet the two main challenges in the large-scale production of industrial enzymes: large volume requirement and low production cost.

Along with the recombinant protein, transgenic plant extracts contain a mixture of native proteins and water-soluble non-protein impurities, such as pigments and phenolic compounds. Impurities in transgenic plant extracts present obstacles in the downstream processing because they can interact with the target proteins or foul membranes and chromatographic resins during purification. The removal of plant components from extracts requires a varied degree of downstream processing, which depends on the end application, that is, pharmaceutical or industrial uses. Downstream processing can contribute up to 80% of the total manufacturing cost of pharmaceuticals and about 50% of

industrial enzymes. For plants to be accepted as a viable alternative to fermentation systems for industrial enzymes production, the demonstration of a cost-competitive downstream processing will complement the lower cost advantage of protein production in transgenic plants.

Because of superior resolution power and the variety of resins available, adsorption chromatography is at the same time the chief unit operation in the purification of recombinant proteins produced in plants and the single largest cost for downstream processing purification. Thus, the most straightforward way to reduce downstream processing cost is to reduce reliance on high-cost chromatography steps.

This dissertation examines the applicability of membrane filtration and adsorption as pretreatment methods for removal of plant impurities that interfered with purification development. Particular challenges that were encountered in downstream processing of recombinant proteins include the impurities removal during purification of a pharmaceutical protein (monoclonal antibody) expressed in *Lemna minor* and of an industrial enzyme (bovine lysozyme) expressed in sugarcane.

Section 2 reported the removal of phenolic impurities from monoclonal antibody (mAb)-expressing *Lemna* extract*s* before Protein A affinity chromatography. Identified *Lemna* phenolics were glycosylated-flavones, apigenin, vitexin, and luteolin derivatives. Their adsorption and elution from the commercially available resins XAD-4, IRA-402 and Q-Sepharose were investigated. It was demonstrated that the phenolic compounds present in *Lemna* extracts can effectively be removed using inexpensive adsorption resins prior to the affinity step. Hydrophobicity of studied adsorbents played an important role in adsorption of *Lemna* phenolics; the dynamic binding capacities (DBCs) of the resins with hydrophobic matrices (XAD-4 and IRA-402) were 3 and 10-fold greater than the DBC of

agarose-based resin (Q-Sepharose). The cost of adding a phenolics removal step to a mAb purification train was determined, and the economic analysis indicated that the addition of a phenolics adsorption step would increase mAb production cost by only 20% for IRA-402 and 35% for XAD-4 resin. The cost of the added adsorption step could be offset by increasing the lifespan of protein A resin from 20 to 30 or more cycles, and an actual reduction of mAb production cost can be achieved by using disposable IRA-402. In addition of increasing the lifespan of protein A resin without increasing the production cost, the inclusion of a phenolics removal step has the potential to generate a product of uniform quality, an important factor for a pharmaceutical protein. Although the outcomes were obtained for *Lemna* extracts, the approach for deciding which would be the most suitable styrene-based resins for the extract pretreatment can be extended for other green tissues, whose phenolic compounds can be an obstacle in the purification of plant-made proteins.

In Section 3, the equilibrium adsorption isotherms of model phenolic solutions at pH 4.5 and 7.5 were determined, describing the interactions between five phenolic compounds commonly found in plant extracts (chlorogenic acid, ferulic acid, rutin, syringic acid, vitexin-2-O-rhamnoside) and three commercial types of polymeric resins (IRA-402, PVPP, XAD-4). The chosen compounds belong to four classes of phenolic compounds (hydroxycinnamic acids, hydroxybenzoic acids, flanovols, and flavones) that are typically found in the plant extracts relevant to our work on recombinant protein purification from transgenic plants. Regardless of the adsorption mechanism, hydrophobic-matrix resins XAD-4 and IRA-402 exhibited a greater affinity than PVPP for the five representative phenolic compounds. At pH 4.5, phenolic acids had ten-fold lower partition coefficients than flavonoids with XAD-4, indicating that XAD-4 would not be suitable if plant extracts

contain large quantities of phenolic acids, but it would be adequate for capturing flavonoids such as rutin and vitexin. Adsorption isotherms generated with ferulic and chlorogenic acids on IRA-402 resulted in partition coefficient values higher than or closer to those of rutin and vitexin-2-O-rhamnoside. In the case of vitexin, it appears that XAD-4 would be a better adsorbent at pH 4.5 and IRA-402 at pH 7.5. At pH 7.5, phenolic acids had higher affinity for the charged resin than the non-charged one. The decision regarding the implementation of phenolics/pigments removal step and choice of available options has to be made on a case-by-case basis, considering the type of phenolics present in the plant extract.

In Section 4, advances made in the development of a downstream process to recover and purify bovine lysozyme from sugarcane were reported. As the screening of 131 transgenic sugarcane lines did not result in a sufficient accumulation of recombinant BvLz in sugarcane stalks, conditions to minimize native protein impurities and maximize BvLz extraction from transgenic sugarcane extracts were investigated. Combination of different pH and salt levels in the extraction buffers indicated that BvLz and total soluble protein (TSP) concentrations in the sugarcane extracts were affected by pH and salt concentration. Five extraction conditions that resulted in the highest BvLz to TSP ratio were considered statistically equivalent. Concentrations of BvLz in extracts ranged from 0.31 to 0.52 ppm and were at least 240-fold lower than the required values (125-250 ppm) for BvLz anti-bacterial effect in the treatment of seeds. Due to the low expression levels, it was concluded that preparation of a sugarcane concentrate would be necessary for BvLz concentration.

Membrane filtration performance of three extracts was also assessed in Section 4. The effect of extract pH and ion strength on the recovery of bovine lysozyme using a three-

step membrane filtration (0.2 μm depth filtration followed by cross-flow filtrations 100 kDa and 5 kDa or 3 kDa) was determined. Regardless of the extraction condition, more than 47% of the BvLz initially present in the crude extract was lost during membrane processing. Partial removal of soluble protein (29-50%) from transgenic sugarcane extracts was achieved by 100 kDa membrane step, but 21 to 29% BvLz was lost. Higher BvLz recovery was obtained by using a 3 kDa regenerated cellulose membrane rather than 5 kDa PES membrane. None of the three evaluated extraction conditions resulted in a substantial recovery of BvLz in the concentrate. Because membrane processing alone did not result in a significant purification of BvLz from transgenic sugarcane extracts, it was concluded that cross-flow filtration would be suitable only for concentration and pretreatment of clarified sugarcane extracts, and a subsequent BvLz purification by chromatography would be required.

Two process alternatives were investigated to develop a less expensive option than the two chromatography process consisting of ion exchange and hydrophobic interaction chromatography (IEX+HIC), which achieved 95% bovine lysozyme purity. The goal of considered alternatives was to eliminate the ion exchange (IEX) chromatography step or substitute it for a less expensive unit operation that would achieve effective color and protein removal before the hydrophobic interaction chromatography (HIC). Although sugarcane extracts contained less colored impurities and native proteins than the sugarcane juice, direct loading of spiked sugarcane extract concentrate on the HIC step recovered only 69% of loaded BvLz with 50% purity. When the IEX column was substituted for XAD-4 treatment (XAD+HIC) process, the BvLz loss during XAD-4 step was lower (8.3% vs 80%), but XAD-4 pretreatment did not remove native protein or concentrated BvLz as effectively as the IEX chromatography. Even though, pure BvLz was

obtained by the XAD+HIC process, the HIC yield was not as high as in the IEX+HIC process. The IEX column was found to be an important preparation step for the HIC, providing concentration of BvLz and removal of impurities. After the cation exchange chromatography, higher purification fold and process yield were achieved by the HIC step, due to the complete separation of BvLz and 18-kDa protein.

The pretreatment of plant extracts using styrene-based resins was effective for the removal of *Lemna* phenolics, succeeding in the development of a less expensive downstream process for purification of monoclonal antibody expressed in *Lemna minor*. Nonetheless, the pretreatment of sugarcane juice with the same resins did not result in a satisfactory improvement of bovine lysozyme purification; combination of ionic exchange and hydrophobic interaction chromatography was still the most efficient process.

If the use of chromatography is required for protein recovery, pretreatment of plant extracts prior to chromatography is one possible way to increase chromatographic yield and consequently reduce the overall downstream processing cost. However, the fact that each plant has a unique pool of impurities is still a challenge for the development of protein purification processes from plants, and the purification scheme should be determined case-by-case, depending on the recombinant protein and plant host impurities properties, and also considering the bioproduct application.

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APPENDIX A

A.1. Analysis of Sugarcane Stalks for Bovine Lysozyme (BvLz) Accumulation

More than 200 lines were generated by our collaborator, in order to test the effect of different promoter combination and promoter stacking, as well as plant age and fertilization dosages on bovine lysozyme (BvLz) accumulation in sugarcane stalks. Extracts from 131 transgenic sugarcane lines were analyzed using ELISA and activity assays to determine the plants expressing the highest amounts of BvLz.

Expression levels of 18 lines were equal to or higher than 2 mg/kg; 7 lines had expression levels between 2 and 2.5 mg/kg and another 6 between 2.5 and 3.5 mg/kg. Five lines, pJSU 18, 19, 54, 298 and 299 exceeded 4.5 mg/kg i.e. 5.1, 4.6, 6.0, 5.4 and 5.1 mg BvLz/kg sugarcane, respectively. Results for all the lines analyzed are shown on Table A.1.

Protein expression level is a critical variable in assessing the feasibility of a plant system for production of industrial enzymes or pharmaceutical proteins because it dictates the economics of plants as hosts for recombinant proteins . In the case of BvLz intended for use as an anti-bacterial agent, it was estimated that enzyme concentrations between 125 and 250 ppm would be effective for agricultural application of BvLz as an antibacterial agent . For example, greenhouse tomato plants exposed to the gram-negative bacteria *Pseudomonas syringae* and then treated with 250 ppm of BvLz exhibited fewer lesions than untreated plants.

Table A.1: Bovine lysozyme accumulation in sugarcane stalks of triple promoter plants.

Sample	**By activity assay (mg/kg)[a]**		**By ELISA (mg/kg)**	
	1st extract BvLz[b]	***Estimated total BvLz[c]***	***1st extract BvLz***	***Total BvLz measured[d]***
DT 57C	0.5	0.8		
pJSU #4			0.5	0.7
pJSU #18			3.2	5.1
pJSU #19			3.2	4.6
pJSU #20			1.8	2.8
pJSU #42			0.9	1.3
pJSU #44			2.0	2.9
pJSU #54			4.1	6.0
pJSU #73			1.8	3.1
pJSU #76	1.1	1.7		
pJSU #88			0.8	1.1
pJSU #89			1.4	1.9
pJSU #90			0.7	1.0
pJSU #91			0.9	1.3
pJSU #92			1.8	2.6
pJSU #95			1.4	1.8
pJSU #98			0.7	1.1
pJSU #99			0.6	0.9
pJSU #102			1.3	1.9
pJSU #120			0.9	1.2
pJSU #122	0.9	1.4		
pJSU #123			0.7	1.0
pJSU #126			0.5	0.8
pJSU #127			1.7	2.3
pJSU #128	0.5	0.8		
pJSU #131			0.8	1.2
pJSU #140			0.6	0.9
pJSU #142	0.9	1.4		
pJSU #145	1.0	1.5		

Table A.1 continued

Sample	**By activity assay (mg/kg)[a]**		**By ELISA (mg/kg)**	
	1st extract BvLz[b]	***Estimated total BvLz[c]***	***1st extract BvLz***	***Total BvLz measured[d]***
pJSU #160			0.6	0.9
pJSU #163	1.1	1.7		
pJSU #170	1.0	1.5		
pJSU #171	1.0	1.5		
pJSU #174	0.9	1.4		
pJSU #175	1.0	1.5		
pJSU #176			0.9	1.4
pJSU #177	0.9	1.4		
pJSU #180			1.1	1.5
pJSU #181	1.2	1.8		
pJSU #182			0.7	1.1
pJSU #183			0.3	0.5
pJSU #186			0.6	1.0
pJSU #187			0.8	1.1
pJSU #190			0.4	0.6
pJSU #191	0.9	1.4		
pJSU #192			1.0	1.5
pJSU #193			0.9	1.4
pJSU #194			0.5	0.7
pJSU #195			0.5	0.8
pJSU #197	1.5	2.3	1.5	2.3
pJSU #199	0.5	0.8		
pJSU #202			0.9	1.7
pJSU #207	0.9	1.4		
pJSU #213			0.6	0.9
pJSU #215	0.9	1.4		
pJSU #220				2.1
pJSU #225	1.1	1.7		
pJSU #226	1.3	2.0	0.9	1.3

Table A.1 continued

Sample	**By activity assay (mg/kg)[a]**		**By ELISA (mg/kg)**	
	1st extract BvLz[b]	***Estimated total BvLz[c]***	***1st extract BvLz***	***Total BvLz measured[d]***
pJSU #227	1.1	1.7		
pJSU #230	1.3	2.0		
pJSU #236	1.0	1.5	0.8	1.2
pJSU #237			1.2	1.8
pJSU #238			1.4	2.0
pJSU #242	0.8	1.2		
pJSU #243	1.1	1.6		
pJSU #247	0.8	1.2		
pJSU #248	0.9	1.4		1.1
pJSU #249	0.6	0.9		
pJSU #250	0.7	1.1		
pJSU #258	1.0	1.5		1.4
pJSU #259	0.9	1.4		1.0
pJSU #266			2.2	3.3
pJSU #267	0.8	1.2		
pJSU #268	1.0	1.5		
pJSU#269	1.2	1.8	1.5	2.3
pJSU #271	1.1	1.7		
pJSU #275	0.8	1.2		
pJSU #296	1.4	1.9	2.3	3.5
pJSU #297	1.1	1.7		
pJSU #298	1.3	2.0	3.6	5.4
pJSU #299	1.3	2.0	3.0	5.1
pJSU #300	0.6	0.9		
pJSU #302	1.0	1.5		
pJSU #303	1.0	1.5		
pJSU #304	1.1	1.7		

Table A.1 continued

	By activity assay (mg/kg)[a]		By ELISA (mg/kg)	
Sample	*1st extract BvLz[b]*	*Estimated total BvLz[c]*	*1st extract BvLz*	*Total BvLz measured[d]*
pJSU #314	0.7	1.1		
pJSU #315	0.8	1.2		
pJSU #316	0.7	1.1		
pJSU #317	0.7	1.1		
pJSU #322	0.5	0.8		
pJSU #341	1.0	1.5		
pJSU #342	0.9	1.4		
pJSU #343	0.8	1.2		
pJSU #344	1.0	1.5	1.4	2.2
pJSU #345	0.8	1.2		
pJSU #347	0.9	1.4		
pJSU #348	0.7	1.1		
pJSU 18 – HF	1.3	2.0		
pJSU 18 – HF1	0.5	0.8	0.3	0.5
pJSU 18 - HF2	0.7	1.1		
pJSU 18 – LF	1.7	2.6		1.3
pJSU 18 – LF1			0.3	0.5
pJSU 18 - LF2	0.6	0.9		
pJSU 18 - LF3	1.7	2.6		1.3
pJSU 19 – HF	1.5	2.3		1.1
pJSU 19 – HF1	0.6	0.9		
pJSU 19 - HF2	0.6	0.9		
pJSU 19 – LF1	0.2	0.3	0.2	0.3
pJSU 19 - LF2	0.6	0.9		
pJSU 19 - MF	1.4	2.1		1.0

Table A.1 continued

	By activity assay (mg/kg)[a]		By ELISA (mg/kg)	
Sample	***1st extract BvLz[b]***	***Estimated total BvLz[c]***	***1st extract BvLz***	***Total BvLz measured[d]***
pJSU 44 - HF	1.5	2.3		
pJSU 44 - HF1	1.0	1.5		
pJSU 44 - LF	1.4	2.1		2.1
pJSU 44 - LF1	1.1	1.7		
pJSU 54 - HF	1.7	2.6		1.4
pJSU 54 – HF1			0.4	0.6
pJSU 54 - HF2	0.8	1.2		
pJSU 54 – LF1	0.5	0.8	0.3	0.6
pJSU 54 - LF2	0.9	1.4		
pJSU 54 - MF	1.9	2.9		1.6
pSPU 32C - HF1	0.6	0.9	0.4	0.7
pSPU 32C - HF2	0.4	0.6		
pSPU 32C - HF1	0.6	0.9	0.4	0.7
pSPU 32C - HF2	0.4	0.6		
pSPU 32C - HF3	0.8	1.2		
pSPU 32C - LF	1.5	2.3		1.2
pSPU 32C - LF1	0.6	0.9	0.4	0.7
pSPU 32C - LF2			0.3	0.5
pSPU 32C - LF3	0.5	0.8		
pSPU 32C - MF	1.3	2.0		1.3

[a] Calculated using specific activity of 6000 Units/mg.

[b] Single extracts were made for screening lines by activity.

[c] BvLz concentration in the 2nd extract was estimated by extrapolation.

[d] ELISA of 1st and 2nd extracts was performed to determine total BvLz.

APPENDIX B

B.1. Preparation of Pilot-plant Sugarcane Juice

Pilot-plant pressed and concentrated juice for purification development was also supplied by our collaborator Dr. Erik Mirkov at Texas Agrilife Research and Extension Center at Weslaco, TX. To determine the potential benefit of a 100 kDa membrane clarification/ separation step before the juice concentration on a 3-kDa membrane, bench scale studies have been conducted using sugarcane extracts spiked with 100 μg/mL hen egg-white lysozyme . The additional clarification of sugarcane extracts by 100 kDa membrane filtration allowed achieving a higher permeate flux during hen-egg white lysozyme concentration step. However, the 100 kDa filtration with BvLz extracts resulted in approximately 25% BvLz loss. Loss of recombinant BvLz (21-29%) was also observed during the 100 kDa filtration of transgenic sugarcane extracts (Section 4.3). In addition, higher BvLz recovery obtained by using the 3 kDa membrane rather than 5 kDa PES membrane indicated that 3 kDa cross-flow filtration would be a more robust step and higher yielding step for BvLz concentration. Based on the above information, the 100 kDa membrane filtration step was not used and the 5-kDa hollow fiber membrane module (3 m^2) was replaced with a 5 m^2 of 3 kDa PES membrane in the pilot-plant process.

The block diagram of the pilot-plant process for preparing bovine lysozyme (BvLz) concentrate is given in Figure B.1. The sugarcane stalks from a mixture of pJSU lines were first shredded by a hammer mill and then pressed using a pilot-size tandem mill. The crude juice was clarified through a 5 μm vibratory screen to remove large particles

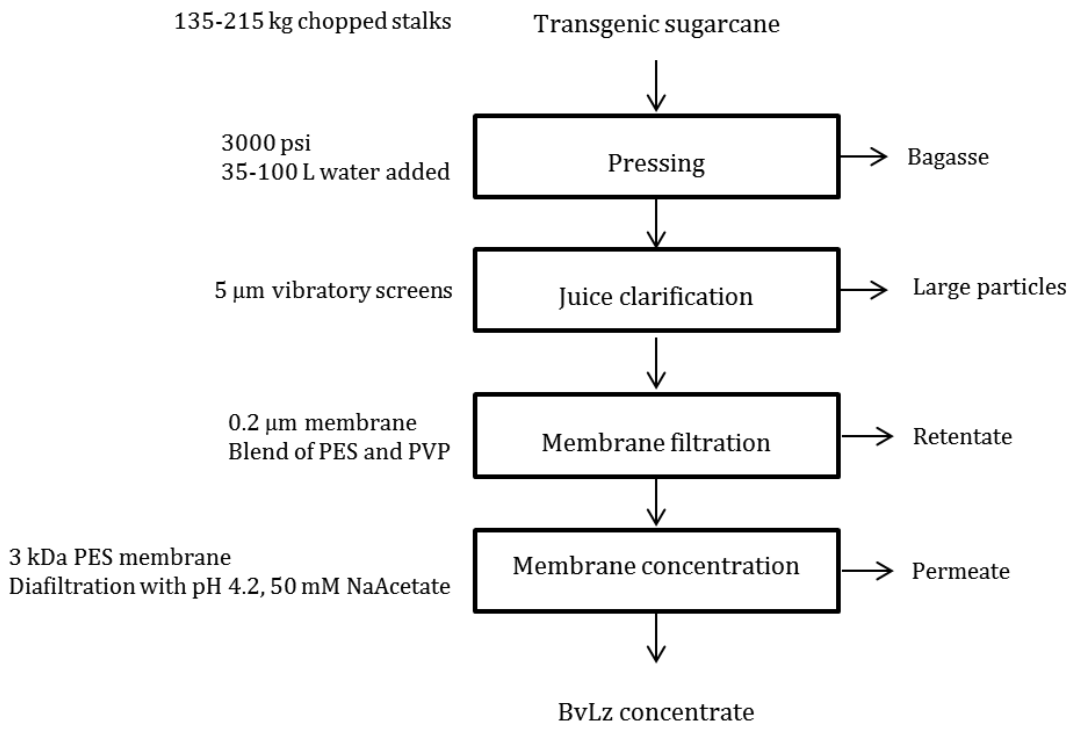

Figure B.1: Pilot-plant process for 3 kDa sugarcane juice concentrate preparation for bovine lysozyme (BvLz) recovery from transgenic sugarcane.

followed by a two-step membrane filtration consisting of 0.2 µm and 3 kDa hollow fiber modules . The permeate from the 0.2 µm membrane filtration step was concentrated by a 3-kDa polyethersulfone (PES) membrane.

B.2. Two-chromatography Process for Purification of Bovine Lysozyme (BvLz) from Sugarcane Juice Concentrate

To increase bovine lysozyme (BvLz) purity, a purification method using concentrated pilot-plant juice was developed in the Bioseparations Lab (Figure B.2). The downstream process consisting of two chromatography steps (IEX+HIC), cation exchange and

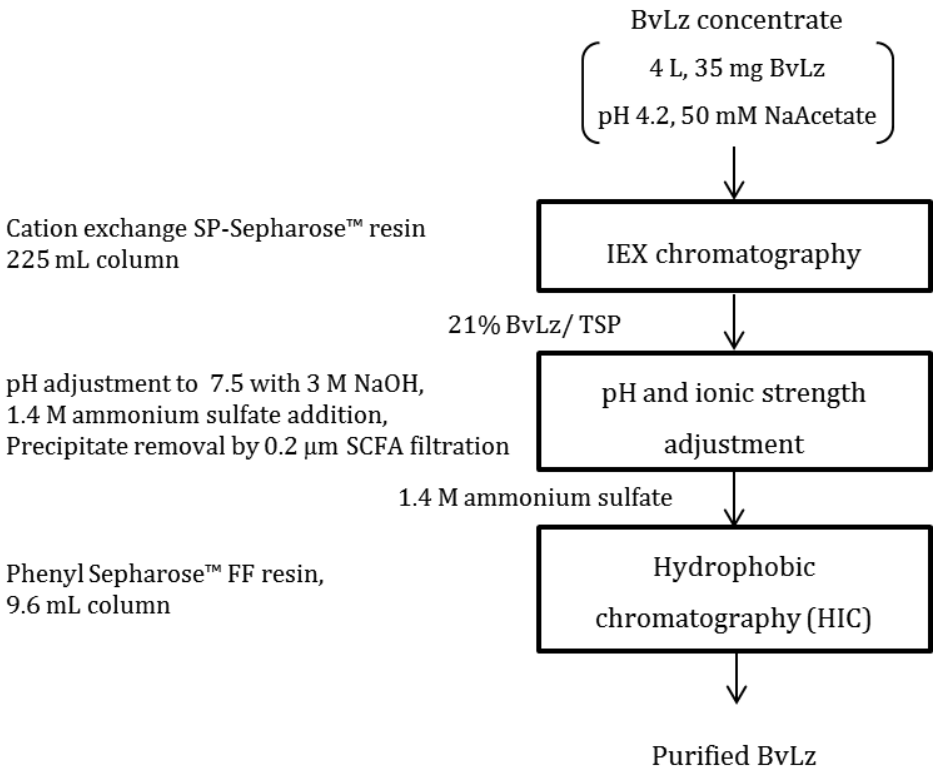

Figure B.2: Pilot-plant two-chromatography (IEX+HIC) process for bovine lysozyme (BvLz) recovery and purification from transgenic sugarcane.

hydrophobic interaction chromatography, achieved 95% pure BvLz , and was used as a reference process to compare with alternative BvLz purification options.

The IEX resin in the IEX-HIC process adsorbed a significant amount of colored compounds during the loading the sugarcane juice concentrate (Figure B.3a). This step also reduced the amount of sugarcane native proteins present in the feed in almost three-fold, from 1.48 g to 0.51 g. Although the majority of colored impurities were removed during the IEX capture step, residual color still accumulated on the top of the HIC column (Figure B.3b).

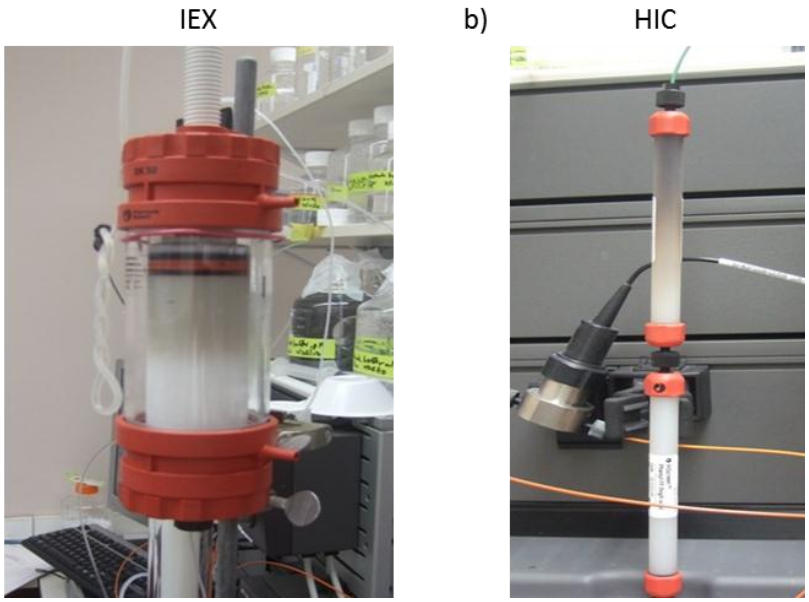

Figure B.3: (a) Ion exchange chromatographic (IEX) column after loading of 3 kDa sugarcane juice concentrate and (b) hydrophobic interaction chromatography (HIC) column after loading of IEX pool.

The IEX chromatography increased BvLz concentration in 13-fold, but recovery was only 20%. Together with the BvLz capture and concentration on the IEX resin, this step also concentrated an 18-kDa protein impurity (Figure B.3, lane 1). The 18-kDa protein was identified as a dirigent protein (MW 17.7 kDa, pI 5.6-6.3) which has similar properties to BvLz (MW 14.4 kDa, pI 6.5) and could not be separated from the BvLz by IEX chromatography. Because of the dirigent protein in the IEX pool, the BvLz purity after the capture column was also low - only 21%. Although the BvLz yield from the capture chromatography was low, it was an important step for the subsequent purification of BvLz on the HIC column.

The main purification challenge of separating the 18-kDa protein from BvLz was solved by using HIC, which resulted in complete separation of BvLz from the dirigent and other protein impurities. A baseline separation of BvLz peak from the 18-kDa dirigent was achieved as indicate in Figure B.4. The efficient purification of BvLz was confirmed by the gel electrophoresis (Figure B.5). Collected BvLz peak fractions had a single BvLz band and no 18-kDa protein or other protein impurity. Purity of fractions from the BvLz peak is shown on Table B.1.

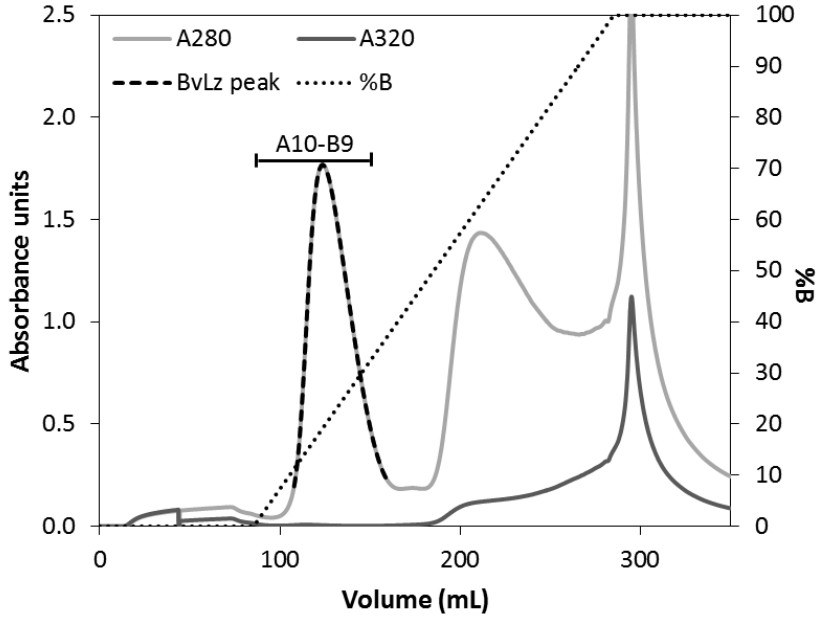

Figure B.4: Protein (A280) and phenolics (A320) elution profiles from hydrophobic interaction chromatography (HIC) of sugarcane juice concentrate pooled after ion exchange chromatography (IEX). Percentage of elution buffer B, A280 and 320 nm monitored using AKTApurifier™. The dashed line indicates the position of BvLz.

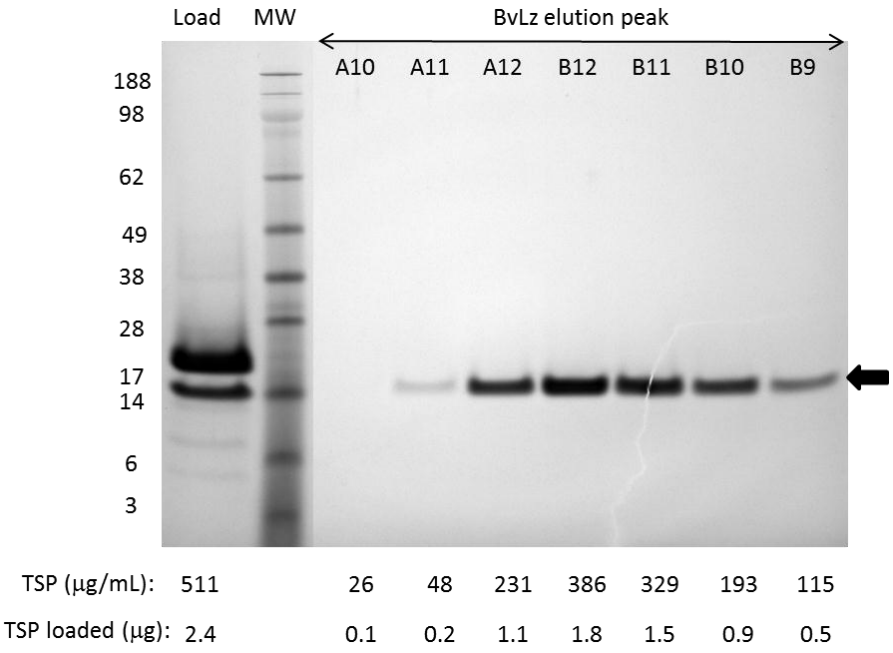

Figure B.5: Protein profiles for hydrophobic interaction chromatography (HIC) load, molecular marker and bovine lysozyme (BvLz) elution peak fractions from HIC of sugarcane juice concentrate pooled after ion exchange chromatography (IEX). The arrow shows the position of BvLz.

The purification table with volumes and BvLz content (Table B.2) summarizes the IEX-HIC process for BvLz purification from sugarcane juice. BvLz was completely recovered in the HIC eluate with an estimated purity of 95%. The recovery of 172% was attributed to sugarcane juice interference with the ELISA assay as discussed before (Section 4.4.2).

Table B.1: Bovine lysozyme (BvLz) in elution peak fractions and respective purities (BvLz/TSP). BvLz values in μg were measured by ELISA.

Sample	BvLz (μg)	Purity (%)
Load	6960	21%
A12	2839	123%
B12	3469	90%
B11	3506	106%
B10	1633	85%
B9	518	45%

Table B.2: Purification table of the two-chromatography process (IEX+HIC) for bovine lysozyme (BvLz) purification from pilot-plant sugarcane juice concentrate: ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC). BvLz values were measured by ELISA (HIC pool after dialysis sample measured by A280 nm, extinction coefficient = 2.84).

Sample	Volume (L)	BvLz (mg)	Purification yield (%)	HIC yield (%)
IEX load	4.0	35	100	
HIC load	0.06	7	20	100
HIC pool	0.05	12	35	171
HIC pool after dialysis	0.08	14	41	200

APPENDIX C

C.1. Characterization of Tobacco-derived Bovine Lysozyme (BvLz) Used in Spiking Experiments

Purified and lyophilized, tobacco-derived BvLz sample was provided by Dr. Erik Mirkov at Texas Agrilife Research and Extension Center at Weslaco, TX and stored at -20°C until use. Partially purified BvLz from tobacco was characterized by gel electrophoresis and western blot and results are shown in Figure C.1.

The four bands detected by gel electrophoresis (Figure C.1a) were also detected by western blotting (Figure C.1b). A 30-kDa-dimer and two small molecular weight forms of BvLz (~ 6 kDa) were also detected. The estimated concentration of the BvLz monomer (MW 15 kDa) was 45% of the BvLz bands seen on the gel (Figure C.2).

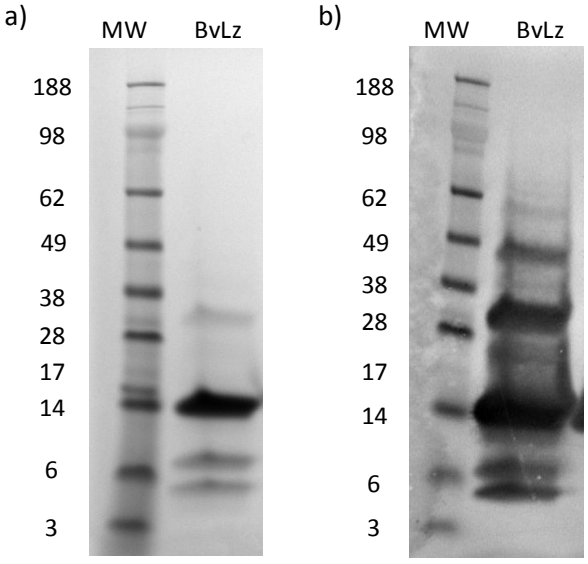

Figure C.1: (a) Gel electrophoresis and (b) western blotting of tobacco-derived bovine lysozyme (BvLz) solution in 25 mM Tris buffer.

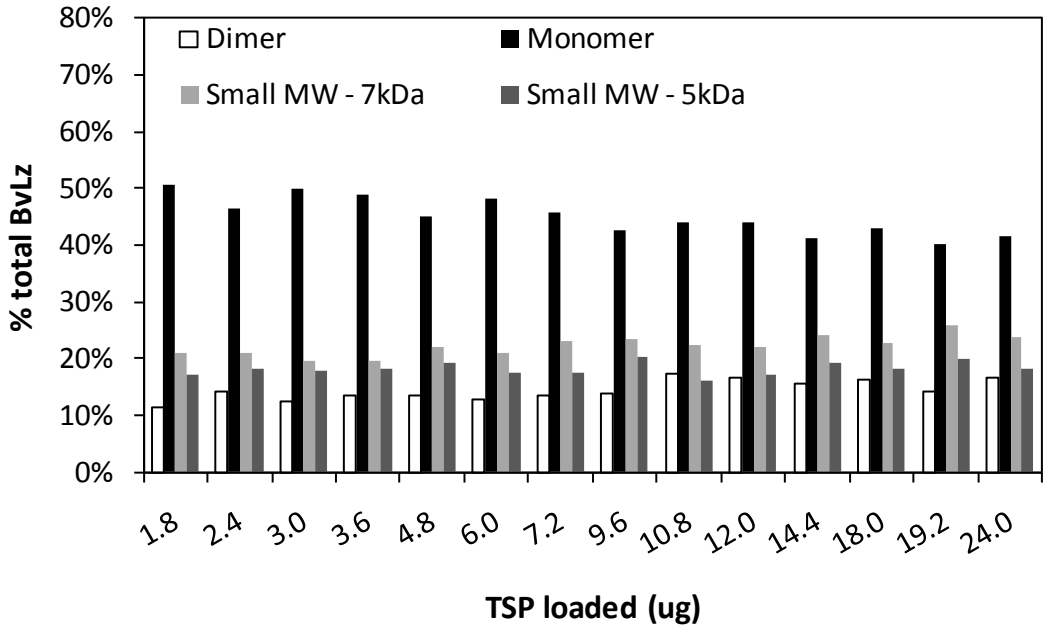

Figure C.2: Percentage of bovine lysozyme (BvLz) bands detected in protein profiles of tobacco-derived BvLz by gel electrophoresis. Percentages were estimated using band areas and intensities values provided by the software Kodak 1D 3.6.

The protein content of lyophilized BvLz sample was 0.24 g TSP/g powder as determined by Bradford and 0.12 g BvLz/g (50% of TSP) based on ELISA and activity assays. The ionic strength of 1 g/L tobacco-derived BvLz sample in PBS buffer was determined by conductivity, and estimated to be equivalent to 189 mg NaCl/g lyophilized sample.

C.2. Cross-flow Filtration of Standard Tobacco-derived Bovine Lysozyme (BvLz)

A purified standard bovine lysozyme (BvLz) solution (128 μg/mL tobacco-derived BvLz in pH 6 and 150 mM NaCl extraction buffer) was processed using the same

downstream processing steps as the sugarcane extract (Figure 4.5). To achieve the recommended protein concentration (> 0.1 mg/mL) for optimal performance of polyethersulfone (PES) flat-sheet membrane filtration, tobacco BvLz solution was made to contain approximately 200-fold greater BvLz concentration than original transgenic sugarcane extracts.

Purified tobacco-derived BvLz in pH 6 buffer with 150 mM NaCl (Figure C.3, lane 2), in addition to monomeric BvLz (MW 15 kDa), contained BvLz degradation products (~5 and 7 kDa) and ~30 kDa aggregate.

The mass of BvLz (mg) after each step and the respective yields are shown on Table C.1. Membrane filtration of tobacco-derived BvLz solution resulted in 95% BvLz recovery in the 5 kDa concentrate. BvLz degradation and aggregate impurities had no apparent effect on estimating the process yield using ELISA (only 5% of BvLz lost). The substantial difference between purified BvLz recovery (95%) and that of transgenic sugarcane extracts (65% in Table 4.6) can be attributed to interference from extract impurities. Even though the BvLz mass loss (3.4 mg) during processing of the tobacco-derived BvLz solution was at least 16-fold higher than that loss occurred during processing of sugarcane extracts (0.2 mg), the enzyme loss expressed in percent of initial BvLz was minor (5%).

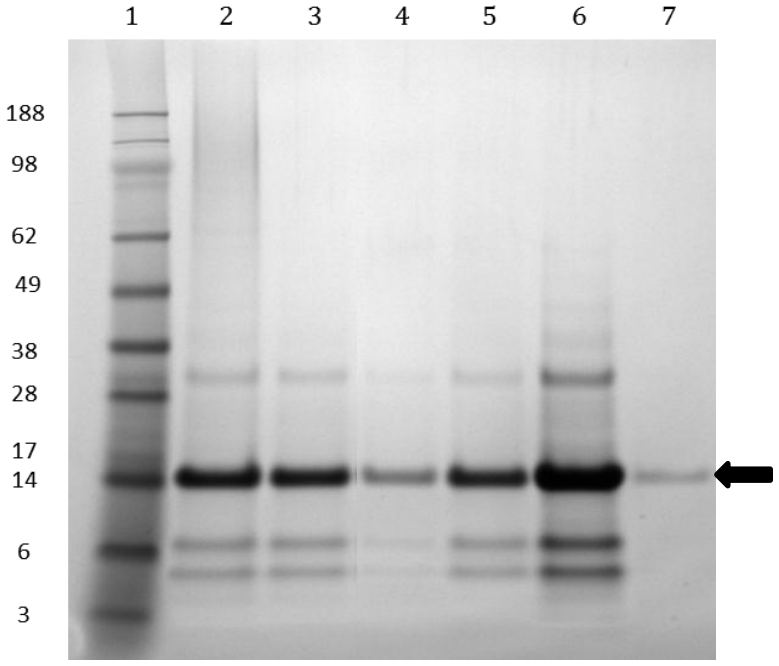

Figure C.3: Protein profiles of bench-scale membrane processing of tobacco-derived bovine lysozyme (BvLz) solution. Samples were loaded based on equal volumes. Lanes: (1) Molecular marker, (2) Tobacco-derived BvLz solution in pH 6 with 150 mM NaCl buffer, (3) Tobacco-derived BvLz solution after pH 4.5 precipitation, (4) 100 kDa retentate, (5) 100 kDa permeate, (6) 5 kDa concentrate, (7) 5 kDa permeate. The arrow shows the position of BvLz monomer.

Table C.1: Bovine lysozyme (BvLz) overall yields for bench-scale membrane processing of tobacco-derived BvLz in pH 6 with 150 mM NaCl. BvLz values (mg) were measured by ELISA.

Stream	BvLz (mg)	Overall yield (%)
Feed solution	64.3	100
Filtered 0.2 μm	60.6	94
100 kDa permeate	56.5	88
5 kDa concentrate	60.9	95

C.3. Tobacco-derived Bovine Lysozyme (BvLz) Adsorption and Elution

Before spiking of sugarcane extract and membrane concentrated sugarcane juice with tobacco-derived BvLz, the effect of tobacco impurities, and the interaction of tobacco-derived BvLz with the hydrophobic resin was evaluated. A standard solution of 100 μg/mL tobacco-derived BvLz prepared in 25 mM Tris with 1.4 M $(NH_4)_2SO_4$ was loaded on the hydrophobic interaction chromatography (HIC) step. Protein profiles of the samples collected from the HIC purification are shown in gels (Figure C.4).

Protein profiles of the flowthrough fractions demonstrated that tobacco-derived BvLz was not completely adsorbed to the hydrophobic resin. The different BvLz forms present in the tobacco-derived sample apparently interacted in different ways with the chromatography resin; the 15-kDa monomer being the only BvLz form in the earlier flow through fractions (A3-A4), probably because the monomer BvLz required higher ammonium sulfate concentration for adsorption. The low-molecular weight degradation products started coming off the column after approximately 50 mL (10.6 CV) were loaded,

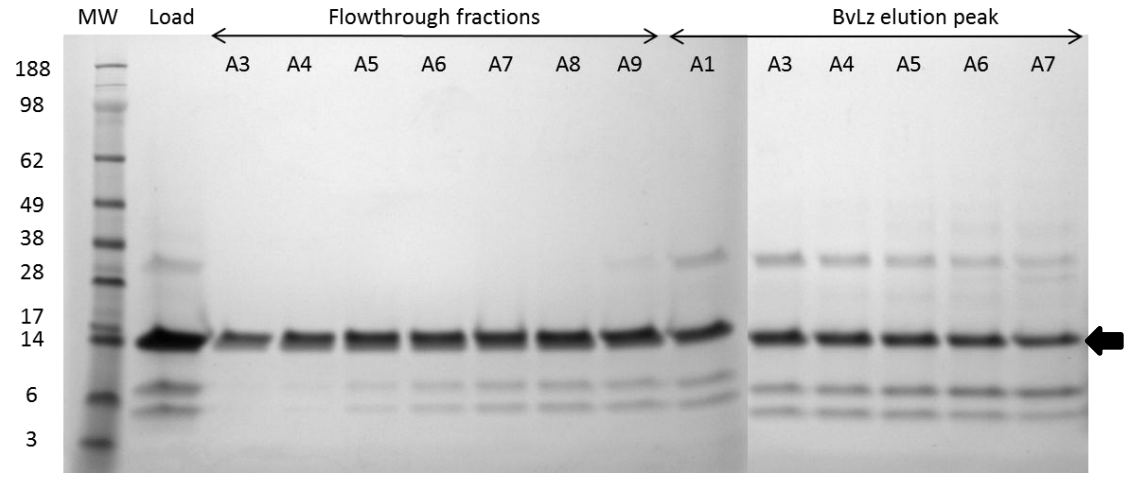

Figure C.4: Protein profiles of the hydrophobic interaction chromatography (HIC) load, flow through fractions and tobacco-derived BvLz peak fractions from HIC. The arrow shows the position of BvLz.

while the aggregate BvLz was strongly bound to the column, only leaving the column in the elution fractions. Because of the presence of extra forms of BvLz (low-molecular weight and aggregate BvLz) in the tobacco-derived sample, binding capacity of the tobacco-derived 15-kDa monomer appeared to be lower than the sugarcane one. To avoid losing BvLz in the flow through fractions, the salt concentration was increased to 2 M ammonium sulfate in the load. With higher ammonium sulfate concentration, no BvLz was detected in the flow through fractions (data not shown) and the dynamic binding capacity of tobacco-derived BvLz in the hydrophobic column was estimated as 13.7 mg/ mL resin.

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