

Bioprocess development: Extraction and purification of human serum albumin from transgenic rice

by

Kseniya Alekseyevna Sheshukova

B.S., Kansas State University, 2019

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Biological and Agricultural Engineering  
College of Engineering

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2019

Approved by:

Major Professor  
Dr. Lisa R. Wilken

# **Copyright**

© Kseniya Alekseyevna Sheshukova 2019.

## Abstract

Transgenic plant systems have successfully been used to express recombinant proteins, including rice seed-expressed recombinant human serum albumin (rHSA). The development of an efficient and integrated rHSA extraction and purification process would allow rHSA to be used for various medical applications such as replacing lost fluid and restoring blood volume, modulating inflammatory reactions, stabilizing vaccines, as well as treatment for cirrhosis, ascites, acute respiratory distress syndrome, etc., without risk of contamination of human pathogens and viruses. Developing an efficient extraction process is critical as the step determines recombinant protein concentration and purity, quantity of impurities, and process volume. Therefore, the effect of pH and time on the extraction and stability of rHSA was evaluated. The amount of rHSA in clarified extracts increased with pH (from 0.9 mg/g at pH 3.5 to 9.6 mg/g at pH 6.0) but not over time and the total soluble protein in extracts also increased with pH (from 3.9 mg/g to 19.7 mg/g). Extraction conditions that maximized rHSA purity were not optimal for rHSA stability as pH 3.5 extraction resulted in high purity (78%) but degraded over time by 56%. Similar purities were observed in pH 4.0 extracts yet rHSA remained stable. rHSA degradation was not observed in pH 4.5 and 6.0 extracts but higher native protein concentrations decreased purity which would increase purification requirements. Strategies such as pH and temperature adjustment were effective for reducing rHSA degradation in pH 3.5 rice extracts. Low temperature, pH 3.5 extraction retained high purity (97%) and stability but required higher energy inputs to maintain the low temperature.

The effect of extraction conditions on subsequent purification using ion exchange chromatography (IEX) was evaluated using batch adsorption and dynamic binding capacity

studies. Batch adsorption was performed to identify binding conditions that maximize rHSA adsorption and to evaluate the impact of extraction conditions on adsorption kinetics using Q Sepharose™ FastFlow® resin. A residence time of 5 min was determined to be sufficient for rHSA adsorption. Acetate and citrate (pH adjusted with TRIS), and phosphate buffers were tested for rHSA binding studies at pH 8.0. Acetate buffer was selected to maintain low conductivities and implemented during extraction to minimize processing operations and cost. The rHSA saturation binding capacities in pH 4.0, 4.5, and 6.0 extracts adjusted to pH 8.0 varied from 69 mg rHSA/mL resin (pH 4.5 extract adjusted to 8.0) to 79 mg rHSA/mL resin (pH 4.0 extracted adjusted to 8.0). Purification studies were then completed at 10% binding capacity to evaluate resultant rHSA purities and yields by using Blue Sepharose™ High Performance affinity resin. Purity and yield also varied with extraction pH with pH 4.5 extract providing an IEX fraction with the most desirable results (>95% purity and 76% yield).

# Table of Contents

List of Figures .....	viii
List of Tables .....	x
Acknowledgements .....	xi
Dedication .....	xii
1. Introduction.....	1
Genetic Manipulation of Organisms .....	1
Protein Expression Systems .....	2
Downstream Processing of Protein Expression Systems .....	5
Plant-Based Expression Systems .....	7
Leafy Expression Systems .....	10
Bioreactor Expression Systems.....	12
Seed Expression Systems.....	13
Transient vs Stable Expression .....	14
Rice Seed as a Host System.....	15
Rice Grain Physiology and Composition.....	15
Benefits of Rice Seed as an Expression System .....	17
Recombinant Protein Expression in Rice Seed.....	18
Extraction of Recombinant Proteins from Rice Seed .....	21
Capture and Purification by Anion Exchange Chromatography .....	22
Human Serum Albumin .....	23
Structure and Properties .....	24
Medical Applications .....	25
Human Serum Albumin Expression in Other Systems .....	26
Objectives .....	28
Analysis of Recombinant Human Serum Albumin Extraction and Degradation in Transgenic Rice Extracts .....	29
Purification of Recombinant Human Serum Albumin from Transgenic Rice Through Adsorption.....	29

2. Analysis of Recombinant Human Serum Albumin Extraction and Degradation in Transgenic	
Rice Extracts .....	30
Abstract.....	30
Introduction.....	31
Materials and Methods.....	36
Materials .....	36
Analytical Methods.....	36
Total Protein Quantification .....	36
Protein Analysis by SDS-PAGE®.....	37
rHSA Quantification .....	37
Statistical Analysis.....	37
Experimental Methods .....	38
Extraction Kinetics.....	38
Effect of pH, Temperature, and rHSA Stability .....	38
Effect of Storage pH on Stability of rHSA in Extracts .....	38
Stability of Purified rHSA in Buffer.....	39
Stability of rHSA and BSA Spiking into Transgenic Rice Extracts.....	39
Effect of Temperature on rHSA in Extracts .....	40
Stability of rHSA at Different Temperatures in pH 3.5 Buffer .....	41
Results and Discussion .....	41
Extraction Kinetics.....	41
Effect of pH and Time on Extraction of TSP .....	41
Effect of pH and Time on Extraction of rHSA .....	43
Effect of pH on rHSA stability .....	48
Effect of Storage pH on Stability of rHSA in Extracts.....	48
Stability of Purified rHSA in Buffer.....	50
Stability of rHSA and BSA Spiked into Transgenic Rice Extracts .....	51
Effect of Temperature on rHSA in Extracts .....	53
Stability of rHSA at Lower Temperatures in pH 3.5 Buffer.....	56
Conclusions.....	57

3. Purification of Recombinant Human Serum Albumin from Transgenic Rice Extracts Through Adsorption.....	60
Abstract.....	60
Introduction.....	61
Materials & Methods .....	66
Materials .....	66
Analytical Methods.....	66
Total Protein Quantification .....	66
Protein Analysis by NU-PAGE® .....	66
Experimental Methods .....	67
Buffer Selection through Consecutive Batch Binding.....	67
Conductivity and Protein Load Effects on rHSA Adsorption .....	69
Adsorption Kinetics of rHSA in Transgenic Rice Extracts .....	69
Static Binding Capacity of rHSA in Transgenic Rice Extracts .....	70
Dynamic Binding Capacity of rHSA in Transgenic Rice Extracts Using ÄKTA Pure....	71
rHSA Purity Quantification Using Affinity Chromatography.....	72
Results & Discussion .....	73
Identification of Compatible Purification Conditions.....	73
Buffer Selection Through Consecutive Batch Binding .....	74
Conductivity and Protein Load Effects on rHSA Adsorption .....	75
Adsorption Kinetics of rHSA in Transgenic Rice Extracts .....	76
Consecutive Batch Binding of rHSA in Transgenic Rice Extracts.....	81
Breakthrough of rHSA in Transgenic Rice Extracts Using Anion Exchange Chromatography with ÄKTA Pure.....	82
rHSA Purity Quantification using Affinity Chromatography with Anion Exchange Chromatography Elution Fractions Using ÄKTA Pure.....	87
Conclusions.....	91
4. Conclusions.....	94
Summary of Results.....	94
Future Work .....	95
5. References.....	97

## List of Figures

Figure 1-1. Downstream processing diagram with unit operation for products from bacteria, yeast, mammalian cells, and plants .....	7
Figure 2-1. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from (a) pH 3.5 extracts (wells 2–8), (b) pH 4.0 extracts (wells 2–8), (c) pH 4.5 extracts (wells 2–8), and (d) pH 6.0 extracts (wells 2–8).....	46
Figure 2-2. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from extraction maintained at pH 3.5 (wells 2–5) and adjusted to pH 4.5 (wells 6–10).....	49
Figure 2-3. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from extraction maintained at pH 6.0 (well 2) and adjusted to pH 4.5 (well 3) and pH 3.5 (well 4) at 120 min .....	50
Figure 2-4. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from pure rHSA in pH 3.5 buffer (wells 2–6) .....	51
Figure 2-5. Protein profiles of extracts by NU-PAGE® electrophoresis gel with (a) samples from the spiking of pure HSA in pH 3.5 extracts (wells 2–8) and (b) samples from the spiking of pure BSA in pH 3.5 extracts (wells 2–8) .....	52
Figure 2-6. Average TSP (mg TSP/g flour) extracted at pH 3.5 low temperature (LT) and pH 3.5 room temperature (RT) throughout extraction (0 to 60 min).....	54
Figure 2-7. Protein profiles of samples by NU-PAGE® electrophoresis gel with samples from room temperature extraction at 23°C (wells 2–5) and samples from pure rHSA in pH 3.5 buffer at 5°C (wells 7–10).....	56
Figure 2-8. Protein profiles of extracts by Western blot with samples from room temperature extraction at 23°C (wells 2–5) and samples from pure rHSA in pH 3.5 buffer at 5°C (wells 7–10) .....	57
Figure 3-1. Process diagram of consecutive batch binding to simulate dynamic binding of pure rHSA in citrate, acetate, and phosphate buffer .....	68
Figure 3-2. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from (a) pH 4.0 adjusted to 8.0 extract (wells 2-8), (b) pH 4.5 adjusted to 8.0 extract (wells 2-8), and (c) pH 6 adjusted to 8.0 extract (wells 2-8) .....	80



Figure 3-3. Consecutive batch binding breakthrough curves of rHSA in pH-adjusted extracts...	82
Figure 3-4. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 4.0 adjusted to 8.0 extract .....	83
Figure 3-5. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 4.5 adjusted to 8.0 extract .....	84
Figure 3-6. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 6.0 adjusted to 8.0 extract .....	85
Figure 3-7. Dynamic binding elution chromatograms of loading to 10% breakthrough with pH 4.0 adjusted to 8.0 extract (solid line), pH 4.5 adjusted to 8.0 extract (dashed line), and pH 6.0 adjusted to 8.0 extract (dotted line).....	86
Figure 3-8. NuPAGE <sup>®</sup> gel electrophoresis of pH 4.0 and 4.5 adjusted to 8.0 extract and elution fractions loaded to the same rHSA concentration of 0.25 mg/mL .....	87
Figure 3-9. NuPAGE <sup>®</sup> gel electrophoresis of pH 4.0 adjusted to 8.0 anion exchange chromatography (AEX) samples and affinity chromatography results after loading combined AEX elution samples.....	88

## List of Tables

Table 1-1. Comparison of bacteria, yeast, mammalian cells, and plants for recombinant protein expression .....	4
Table 1-2. Summary of various downstream processing of recombinant proteins in transgenic rice seed endosperms .....	20
Table 2-1. Average TSP (mg TSP/g flour) extracted at pH 3.5, pH 4.0, pH 4.5, and pH 6.0 throughout extraction (0 to 60 min) .....	42
Table 2-2. Average rHSA concentration (mg/g flour) extracted at pH 3.5, pH 4.0, pH 4.5, and pH 6.0 throughout extraction (0 to 60 min) .....	44
Table 2-3. Effect of pH and extraction time on rHSA protein purity (%) .....	47
Table 2-4. Effect of pH and extraction time on the rHSA to native rice protein concentration ratio .....	48
Table 2-5. Effect of temperature and extraction time on rHSA protein purity (%) .....	55
Table 2-6. Effect of temperature and extraction time on the rHSA to native rice protein concentration ratio .....	55
Table 3-1. Conductivity and concentration effect on purified rHSA adsorption .....	76
Table 3-2. Percentage of rHSA bound and eluted in pH 4.0, 4.5, and 6.0 extracts using Q Sepharose™ FastFlow® in adsorption kinetics studies .....	81
Table 3-3. Purities and yields of anion exchange chromatography elution extracts for each extraction pH condition .....	89
Table 3-4. The amount of total soluble protein (TSP) and rHSA, and purities and yields of anion exchange chromatography elution extracts for each extraction pH condition .....	90

## **Acknowledgements**

I would like to thank my major professor, Dr. Wilken, who has been incredibly patient and has provided an unyielding amount of support throughout the years. Without such an incredible mentor, I would not have had as many opportunities and experiences that have professionally and personally influenced me to be the individual I am today. I also thank my committee members, Dr. Donghai Wang and Dr. Susan Sun for their support and input in my research project. I also thank all of the laboratory members who have supported me since my first year in the Bioseparations and Bioprocessing Lab and all of my colleagues and faculty members in the Department of Biological and Agricultural Engineering at Kansas State University.

I would like to thank the department for providing funding as an undergraduate student and as a graduate teaching assistant. I thank the College of Engineering for providing funding through the Raj and Diana Nathan Undergraduate Research Experience Award and the Office of Undergraduate Research & Creative Inquiry for funding through Travel Grants. I also thank Ventria Bioscience for providing the necessary supplies. This project would not be possible without the transgenic rice and purified protein

Finally, thank you to my parents, brother, and closest friends for your continuous motivation and support.

## **Dedication**

To my advisor and all of my professors who have guided me throughout my academic career. To my family and close friends who have supported me during the hardest times from the beginning. To Zack, I am so grateful to have had your love and support. Your influence on my life is still felt every day. Thank you everyone.

# 1. Introduction

## Genetic Manipulation of Organisms

The genetic manipulation of organisms is the modification of genetic material through engineering techniques like transformation or transduction with the intent to improve the organism or produce a non-native product. This was first accomplished in 1973 when a gene for frog ribosomal RNA was transferred into *Escherichia coli* (*E.coli*) through a plasmid and successfully expressed the foreign gene in multiple bacterial colonies (Cohen et al., 1973). This was followed by the integration of retrovirus DNA (Moloney leukemia virus) into early mouse embryos that developed leukemia to prove that inserted transgenes can be passed on to future generations (Jaenisch, 1976). Shortly after, the commercial production of products through transgenic organisms began to rise with the development of the hormone, somatostatin in 1977 (Houghes, 2011), human insulin in 1978 (Crea et al., 1978), and human growth hormone in 1979 (Ayyar, 2011), all expressed in *E. coli* by the first biotechnology company, Genentech. With these biotechnological advancements, select yeast, bacteria, and mammalian organisms were commonly chosen to continue the development of genetic engineering. These traditional expression systems have been used to produce many recombinant protein products which have contributed to the large market size of \$1.6 billion in 2017 which is expected to exceed \$2850 million by 2022 (MarketandMarkets™, 2017). While many recombinant proteins are expressed for food, chemical, cosmetic, and other industries, most of the market size is comprised of products for biotechnological industries. Recently, the production of recombinant proteins from traditional expression systems has expanded focus to plant systems with the first Food and Drug Administration (FDA) approved recombinant protein from a transgenic plant in 2012. This product was ELELYSO™ (taliglucerase alfa) from carrot root, a plant cell suspension for the

treatment of type 1 Gaucher's disease by Protalix BioTherapeutics of Carmiel, Israel (Fox, 2012), which was a landmark in the plant biotechnology field.

## **Protein Expression Systems**

Different expression systems have been successfully used to produce various recombinant proteins, however, 70% of approved recombinant proteins are currently produced in Chinese hamster ovary (CHO) cells (Butler and Spearman, 2014). While CHO cells are widely used, they are expensive to culture and difficult to scale-up compared to other systems (Table 1-1). Yeast, such as *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*), are the second most commonly used systems to produce recombinant proteins (Butler and Spearman, 2014) because high yields can be achieved through fermentation and post-translational modifications of proteins is similar to eukaryotic glycosylation (Vieira Gomes et al., 2018). Bacteria, like *E. coli*, is another expression platform used as it is a highly researched and established as a cell factory with many molecular tools available (Rosano and Ceccarelli, 2014) to achieve expression levels up to 30% of total cellular protein (Baeshen et al., 2015). However, bacteria are not capable of performing protein glycosylation, which can be necessary for proteins with biological activity. Therefore, expressed proteins accumulate within inclusion bodies, which require additional processing steps to solubilize inclusion bodies followed by protein refolding (Singh et al., 2015), which can drastically reduce protein yields. Downstream processing costs can be minimized through the secretion of recombinant proteins into a medium (Darah et al., 2011), while upstream processing additives can improve yields and do not increase production costs as raising production titers is mainly limited to the cell line, media optimization, expression levels, *etc.* (Gronemeyer et al., 2014). The cost of recombinant protein production using bacteria is significantly lower than CHO cells and comparable to plant platforms. Protein expression

levels in plant systems have increased throughout the past decade to be comparable or greater than the expression levels in bacteria and yeast systems while also being able to produce complex proteins and retain their bioactivity through post-translational modifications (Streatfield, 2007). Plant systems are classified as a leafy, seed, and bioreactor system, each with advantages and disadvantages. Overall, plant systems are able to efficiently express recombinant proteins and are easy to scale from a pilot- to large-scale production process (He et al., 2011).

**Table 1-1. Comparison of bacteria, yeast, mammalian cells, and plants for recombinant protein expression.**

	<b>Bacteria</b>	<b>Yeast</b>	<b>Mammalian cells</b>	<b>Plants</b>
<b>Common cell host</b>	<i>Escherichia coli</i>	<i>Saccharomyces cerevisiae</i> , <i>Pichia pastoris</i>	Chinese hamster ovary, chicken eggs	<i>Nicotiana tabacum</i> , <i>Nicotiana benthamiana</i> , <i>Oryza sativa</i> , <i>Zea mays</i>
<b>Select expression levels</b>	1-1.5 mg MazF/mL ( <i>E. Coli</i> ); 7.5 mg human interleukin-6/mL ( <i>E. coli</i> ); 12.5 mg human I-glutamate decarboxylase 65/mL ( <i>E. coli</i> )	300 mg recombinant human chitinase/L/day ( <i>P. pastoris</i> ); 1.5 g insulin precursor/L ( <i>P. pastoris</i> ); 10 mg norovirus capsid/mL ( <i>P. pastoris</i> )	1.5 mg Avidin/egg; 10 g recombinant antibody and Fc-fusion protein/ L CHO	114.3 µg human glutamic acid decarboxylase/g fresh leaf weight and 2 mg human antibody/g fresh leaf weight, ≥0.37 g Griffithsin/kg fresh leaf weight ( <i>N. benthamiana</i> ); 1.6% heat labile enterotoxin B secreted of TSP ( <i>N. benthamiana</i> culture); 0.366 g human α-1-antitrypsin/kg brown rice ( <i>O. sativa</i> )
<b>Glycosylation</b>	No	Most	Yes	Yes
<b>Advantages</b>	Rapid expression time, low cost, easy to scale-up,	Moderate expression time	Short expression time (transient expression)	Low cost, easy to scale-up, fast expression time (transient expression)
<b>Disadvantages</b>	Protein misfolding possible, no protein secretion, inclusion body formation, and protein inactivity	N-linked glycan structures differ from mammalian proteins, and fermentation required to improve yields,	High cost, low protein yields, difficult to scale-up, and long expression time (stable expression)	Long expression time (stable expression), glycosylation patterns differ from eukaryotes
<b>References</b>	Davis et al., 2000; Kim et al., 2008; Rosano and Ceccarelli, 2014; Suzuki et al., 2006	Andersen and Krummen, 2002; Vieira Gomes et al., 2018; Xia et al., 2007	Hood and Howard, 2014; Huang et al., 2010; Wurm, 2008	Alam et al., 2018; Avesani et al., 2014; Larsen and Curtis, 2012; Zhang et al., 2013; Zischewski et al., 2016



## **Downstream Processing of Protein Expression Systems**

The recovery of biological products from the various expression systems has been utilized in the production of pharmaceutical, nutraceutical, and industrial products. The predicted sales forecast of processed biological products is estimated to be over \$300 billion by 2050 (DiMasi et al., 2003), therefore upstream and downstream process development is important. Upstream processing of biological products is necessary to establish high expression levels through genetic engineering and desired cell accumulation through optimized growth conditions using robust and scalable continuous or batch operations (Clincke et al., 2013; Li et al., 2010). Parameters such as pH, conductivity, nutrients, and temperatures are used to formulate optimal media conditions for cell growth before transfer to production bioreactors (Gronemeyer et al., 2014). In plant-based systems, transgenic plants are grown in monitored environmental conditions that cause high tissue yields with the desired product. Lastly, cells are harvested and lysed (if product is intracellular) before downstream processing. Upstream processing costs typically do not increase with scale up while downstream processing costs increase linearly (Gronemeyer et al., 2014) and comprise 80% to 90% of pharmaceutical manufacturing cost (Sabalza et al., 2014). Therefore, the development of an efficient and cost-effective downstream processing is critical.

Downstream processing consists of four main stages:

- Solid-liquid separation/clarification- removal of cells, cell debris, or particulates
- Product isolation- removal of impurities with different properties from desired product
- Purification- removal of impurities with similar biochemical properties to desired product

- Polishing- removal or deactivation of remaining contaminants and product packaging in a stable liquid or solid form

The selection of unit operations in each stage is dependent on the expression system used and biochemical properties of the target product. The initial stage varies if the product is produced inside the cell or excreted, as cell disruption using homogenization, grinding, or milling (Hopkins, 1991) would be required for an intracellular product but not for an extracellular product. Traditional systems can produce intracellular or extracellular products, however, plant systems such as leafy and seed can only produce intracellular products while a bioreactor system can produce either. Afterwards, solid-liquid separation can occur through filtration, centrifugation, sedimentation, flocculation, and precipitation (Svarovsky, 2000). Liquid-liquid or solid-liquid extraction with water, solvent, or buffer to extract the product into a soluble form is also commonly used. Product isolation can occur using adsorption, membrane filtration, or precipitation (Harrison et al., 2015) to prepare the product for purification. Since the impurities in the purification stage typically resemble the product physically and chemically, this stage can contribute to majority of the downstream processing costs. Therefore, efficient planning is required to determine if more than one unit operation is required and whether retaining end product purity and yield is important. This stage can be accomplished through crystallization, fractional precipitation, and different chromatography methods like ion-exchange, size exclusion, affinity, reversed phase, and hydrophobic interactions chromatography (Harrison et al., 2015). Once the product has been isolated and purified, unnecessary liquids are removed, and the solid product is converted to a crystalline form through drying and crystallization. Figure 1-1 highlights the possible unit operations for an intracellular product (from bacteria, yeast, mammalian, leafy, seed, and plant bioreactor systems) or extracellular product (from bacteria,

yeast, mammalian, and plant bioreactor systems). The following section highlights plant-based expression systems, specifically leafy, bioreactor, and seed systems.

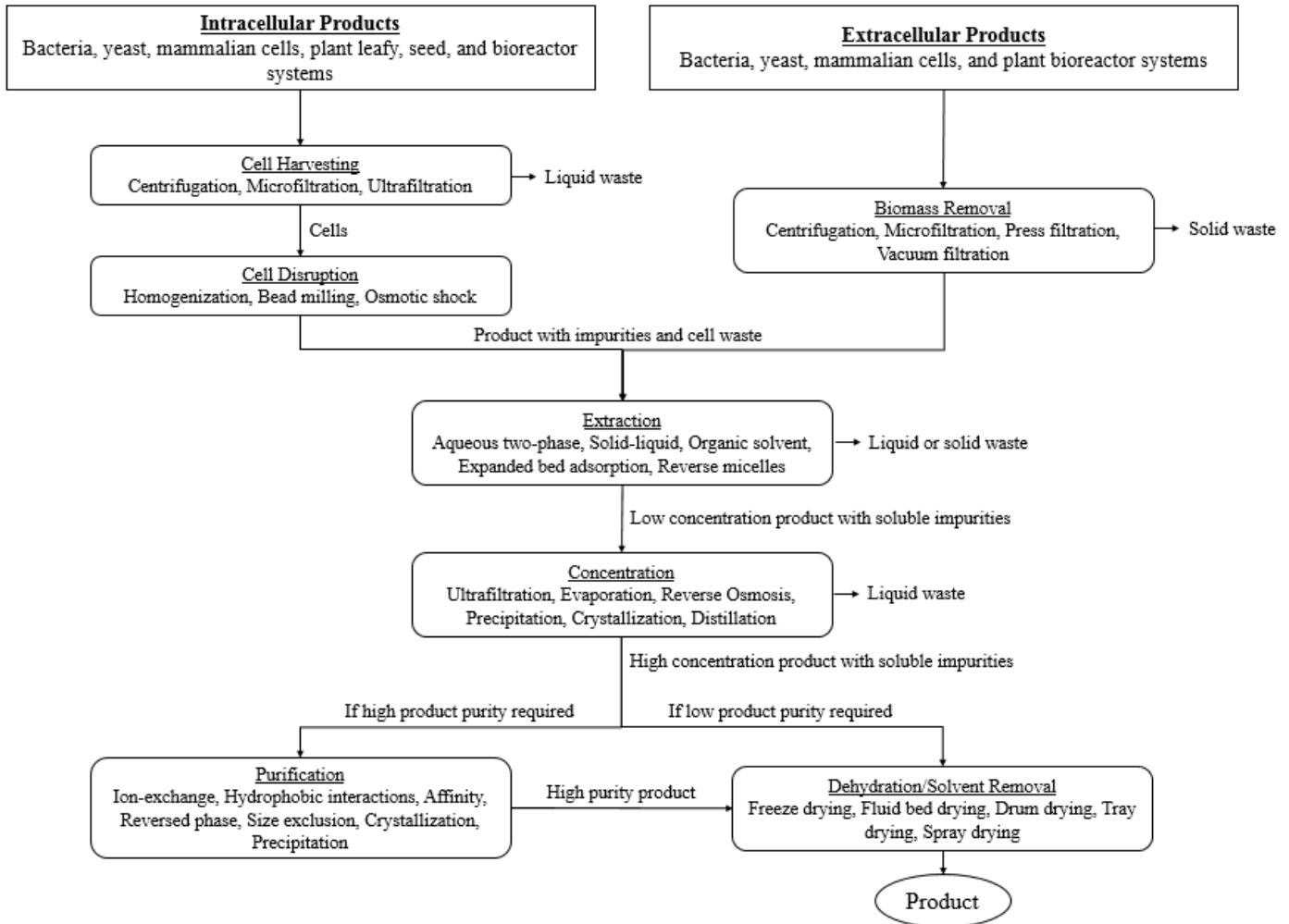


Figure 1-1. Downstream processing diagram with unit operation for products from bacteria, yeast, mammalian cells, and plants (adapted from Petrides et al., 1989).

### Plant-Based Expression Systems

While plants have been selectively bred for over 11,000 years, the use of genetically manipulating plant DNA has become a controversial topic in agriculture and medicine. However, over the past 20 years the number of commercially available products from transgenic plant

systems and modified crops has increased drastically with thirty genetically modified plant types approved by the International Service for Acquisition of Agri-biotech Applications (ISAAA) as of October 2018. These transgenic plants encompass over 12% of global cropland (189.8 million hectares), a 110-fold increase from the initial 1.7 million hectares in 1996 (Brookes and Barfoot, 2018). Initially, the main purposes for use of genetically modified crops were to improve crop yields, reduce farm costs, increase profit, improve environmental conditions, and increase the nutritional value (ISAAA, 2017). These were accomplished through genetic alterations for herbicide resistance, insect resistance, virus resistance, drought tolerance, nutrition, and longevity (National Academies of Sciences, 2016). The first crop to be genetically altered was the tobacco plant for antibiotic resistance in 1983 (Bawa and Anilakumar, 2013) and the first product produced in a genetically modified plant was recombinant human growth hormone from tobacco and sunflower in 1986 (Barta et al., 1986). However, transgenic food products were not approved by the FDA for commercial production until 1994 when FLAVR SAVR™, a genetically modified tomato using an antisense polygalacturonase gene to regulate fruit ripening (Kramer and Redenbaugh, 1994) was available for purchase. Since then, the use of transgenic plants for the production of molecules such as therapeutics has been explored.

Transgenic plant systems are used for the expression of different products including recombinant proteins due to multiple advantages in comparison to prokaryote and other eukaryote platforms. The cost of processing using plant systems has the potential to be less than traditional expression platforms as upstream processing costs can be as low as \$1.00-\$2.00 per kg of protein (Tusé et al., 2014) and downstream processing costs are either comparable or less (Yao et al., 2015). For example, Tusé et al., 2014 compared costs for tobacco-expressed cellulase and demonstrated more than 30% reduction in unit production costs and an 85% reduction in

initial capital investments compared to a fungal fermentation-based system. Scaling up the processes also requires less funding as existing agricultural bases are already established with large crop acreage available (Lau and Sun, 2009). The need for altering or increasing manufacturing facilities and equipment for processing and storage is not as expensive in comparison to using non-plant systems and contains the highest safety levels, even though transgenic plants have a high public perception of risk (Goldstein and Thomas, 2004). Traditional expression systems for recombinant proteins such as CHO cells require \$14 to \$22 million per 250,000 L batch in media costs (Buyel et al., 2017) while plant-based systems growth requirements cost around \$4.5 million per batch of the same size (Buyel and Fischer, 2012). Plant systems have become more economically feasible than CHO cells at higher expression levels such as 2.0 g monoclonal antibody per kg leaf fresh weight (Zischewski et al., 2016). They are also not limited to gene size and can glycosylate recombinant proteins with a high protein folding accuracy, yield, and homogeneity due to their protein synthesis similarities with mammalian cells (Goldstein and Thomas, 2004). Plant and mammalian *N*-glycan biosynthesis in the initial endoplasmic reticulum-associated phase is highly similar (Bosch and Schots, 2010) but differs in the final Golgi apparatus-associated phase to produce sugar (xylose and/or fucose) residue attached to the core glycan (Lerouge et al., 1998). Plant and mammalian *O*-glycans can be structurally different depending on the species and could elicit an immune response in humans but plant systems remain a strong alternative for expression of therapeutic glycoprotein with glycosylation optimization (Gomord et al., 2010). A majority of biotherapeutic products are glycoproteins (Seeberger and Cummings, 2015), therefore, proper glycosylation of proteins is important as it influences protein folding, solubility, stability, biological activity, and proteolysis resistance (Gomord et al., 2010). Plant cells do not express pathogenic or viral agents like

mammalian cells (Kuo et al., 2013) or endotoxins like bacterial cells (Wang and Quinn, 2010), which eliminates risk of contamination and benefits processing costs as those impurities can be difficult to remove (Goldstein and Thomas, 2004).

While many biopharmaceuticals have the potential to be produced in transgenic plants, the use of plant-based systems has been slow in comparison to yeast, bacteria, and mammalian cell culture systems due to commercial regulations and negative public perception (Yuan et al., 2011). Therefore, many biotechnology companies have focused on recombinant protein production for non-clinical applications like process reagents, enzymes, and diagnostics (Hood and Requesens, 2012). However, there are companies that have commercially produced recombinant proteins using novel expression technologies in seed, leafy, and bioreactor expression systems such as MagnICON<sup>®</sup> Transient Expression for leaves (Pogue et al., 2010), ProCellEx<sup>®</sup> Stable Expression for cell cultures (Fox, 2012), and ExpressTec Stable Expression for seed (Alfano et al., 2014), systems.

### ***Leafy Expression Systems***

Leafy systems are commonly used for recombinant protein expression due to the high protein yields (Twyman et al., 2003) and rapid growth cycles that produces large quantities of biomass for harvest (Daniell et al., 2001). A commonly used leafy expression system is tobacco due to the established transformation methods and high biomass yields (Abiri et al., 2016). Once harvested, recombinant proteins have been known to undergo proteolytic degradation and require immediate treatment of freezing or desiccation to maintain bioactivity (Yao et al., 2015). This is due to the high physiological activity of the tissue, therefore, it is critical to limit processing time to maintain protein integrity (Ma et al., 2003). Leafy tissues also have the presence of

phenolics/pigments and native proteases that can interfere with processing steps by reducing binding capacity and/or altering recombinant protein bioactivity (Dixon et al., 2018). Conditions can be modified to improve recombinant protein solubilization during extraction by using stabilizing additives such as protease inhibitors (Leibly et al., 2012) or minimize proteolytic degradation by reducing the extraction temperature (Ryan, 2011) which would improve subsequent purification. Some cases require high ionic strength buffers to improve recombinant protein solubility during extraction but would impede purification by reducing the binding capacity and ability to precipitate proteins (Dixon et al., 2018).

Some commercially available biopharmaceuticals produced using leafy tissues include ZMapp™ from tobacco leaves for Ebola treatment (Qiu et al., 2014), CaroRx® from tobacco leaves to protect against tooth decay (Wycoff, 2005), and a vaccine for non-Hodgkin's Lymphoma that is expressed in *Nicotiana benthamiana* (*N. benthamiana*) leaves (Lee and Ko, 2017). Another product expressed in leafy systems include human somatotropin, which has been successfully produced in transgenic tobacco chloroplasts with protein levels over 100-fold higher compared to nuclear transgenic systems. The tobacco chloroplast were able to produce somatotropin and a *Bacillus thuringiensis* insecticide protein at 7% and 45% of the total plant protein respectively (Chargelegue et al., 2001).

The Canadian biotechnology company, Medicago, has developed an influenza vaccine with virus-like particles produced in *N. benthamiana* (Pillet et al., 2016) using their Proficia® transient expression technology (Espinosa-Leal et al., 2018). Once clinical trials have been completed and approved by the FDA, the *N. benthamiana* influenza vaccine is expected to be released for commercial use (Loh et al., 2017). The use of a plant leafy tissue expression system to produce influenza vaccines is novel as common systems include cell-cultures (Soema et al.,

2015) and embryonated chicken eggs which can illicit an allergic response (Turner et al., 2015). The potential for other protein-based products expressed in *N. benthamiana* using Proficia<sup>®</sup> is possible due to the versatility of the expression system and rapid plant growth of 5 to 6 weeks with the vaccine hemagglutinin being produced in 19 days (D'Aoust et al., 2010) which result in high tissue yields annually. However, batches may not be consistent due to the variability of growth conditions unless controlled like in a bioreactor system.

### ***Bioreactor Expression Systems***

Bioreactor systems are used to cultivate intracellular and extracellular recombinant proteins which dictate the necessary unit operations before extraction as previously visible in Figure 1-1. Bioreactor systems are most similar to traditional expression systems and use culture media that typically contains components that do not interfere with downstream processing (Dixon et al., 2018). The need for cell lysis or protein solubilization may also not be necessary as most recombinant proteins are secreted into the culture media, but commonly at low concentrations (Dixon et al., 2018). However, intracellular recombinant proteins are directed to specific cellular compartments such as the cytosol, vacuole, endoplasmic reticulum, or chloroplast (Tekoah et al., 2015) and, thus, would require cell lysis before extraction. Production batches tend to be consistent due to the controlled growth conditions which is not always possible with seed or leafy systems (Xu et al., 2012) as plant growth is dependent on its environment and nutrients. Bioreactor systems can also possess impurities such as native proteins and polysaccharides, which can cause antigenic effects and increase cell suspension viscosity that affects purification steps (Farrell et al., 2015). Native protein solubility can be minimized by extracting at the isoelectric point of the native proteins followed by membrane filtration to further reduce the amount of native proteins present (Dixon et al., 2018). However,



polysaccharides can decrease membrane filtration flux due to their high viscosity (Hellwig et al., 2004) and cause cells to accumulate on surfaces, requiring additional processing steps to remove polysaccharides. Plant bioreactors pose as an alternative to traditional expression systems such as CHO cells as they produce high expression levels of 5 g to 10 g per L in optimal conditions (Kelley, 2007) but require a high financial investment due to cell media costs of \$55 to \$90 per L (Buyel et al., 2017).

Popular cells used in bioreactor systems include tobacco, specifically *Nicotiana tabacum* (*N. tabacum*), and carrot (Hellwig et al., 2004), however other cell suspensions like rice (Huang and McDonald, 2012) have become more frequently researched. Commercial products produced in a bioreactor expression system include ELELYSO™ enzyme replacement expressed in carrot and tobacco cell culture (Fox, 2012) and Protalix (glucocerebrosidase), a treatment for Gaucher's disease expressed in carrot cell culture (Fox, 2012). Other recombinant proteins include the USDA-approved Newcastle virus vaccine (Miller et al., 2009) and human interleukin-2 and -4 (Hellwig et al., 2004), both expressed in *N. tabacum* cell suspension.

### ***Seed Expression Systems***

While cell cultures and leafy tissue are used frequently, plant seeds have become of interest for biopharmaceutical production due to their advantages of high expression, stability, and mass production. Seeds are able to accumulate a high amount of recombinant protein while minimizing impurities like phenolics (Yao et al., 2015) that can lower purification requirements. Recombinant proteins from transgenic seeds have been known to be biologically active and stable for many years at room temperature (Dockal et al., 2000; Oakes et al., 2009; Ramírez et al., 2001), due to the presence of endogenous protease inhibitors (Ryan, 1990) and are able to

withstand changes in conditions like pH and temperature, which can reduce storage costs. Extracting at a lower pH can be beneficial as it reduces the solubility of native proteins, however, seeds can contain phytic acid which is soluble at a low pH (Dixon et al., 2018). Phytic acid can impact purification as it can shield the protein charge on a recombinant protein and prevents adsorption to cation exchange resin, reducing the binding capacity of anion exchange resin or forming phytate-cation precipitate in columns (Wilken and Nikolov, 2012b).

Commonly used seed-based platforms for recombinant protein expression include corn and *Oryza sativa* (*O. sativa*) rice. For example, ProdiGene used corn to express TrypZean<sup>®</sup> (recombinant bovine trypsin) for use in animal cell culture (Krishnan and Woodard, 2014) and rice seed was used to express VEN 150 for the treatment of human immunodeficiency virus (HIV)-associated chronic inflammation (Yao et al., 2015). Other recombinant proteins expressed in seed-based systems include human  $\alpha$ -1-antitrypsin expressed in rice seed for inflammation treatment (Zhang et al., 2013) and human coagulation factor IX expressed in soybean seed vacuoles for the treatment of factor IX hemophilia (Cunha et al., 2011).

### **Transient vs Stable Expression**

The method for expressing foreign products varies with seed, leafy, and bioreactor plant systems as transient or stable expression can be used. Transient expression is when transfected cells express a transgene but it is not integrated into the genome as the plasmid DNA is maintained in the nucleus causing the transgene to not be replicated and expressed for a finite time (Chen et al., 2016). Stable expression uses transfected cells with integrated transgenes in the genome, thus the gene is replicated and a stably transfected cell line is created (Stepanenko and Heng, 2017). Transient expression systems are able to produce consistent high protein yields

unlike stable expression systems due to the absence of the “position effect” (Komarova et al., 2010). Stable expression is also more complex to perform and can cause a non-target region of the plasmid to be integrated into the genome and expressed. As such, stable expression is more compatible with linear DNA while transient expression is more compatible with supercoiled DNA (Chen et al., 2016). Typically, stable expression is used throughout bacteria, yeast, mammalian cells, and plant systems, but transient expression is commonly used for mammalian and plants cells.

### **Rice Seed as a Host System**

The interest in large-scale rice biopharmaceuticals has steadily increased over the past decades (San Juan, 2018), as such, seed systems have successfully produced multiple recombinant proteins. These products include human lysozyme (Huang et al., 2002), human lactoferrin (Nandi et al., 2002), human serum albumin (US8158857B2, 2010), human transferrin (Zhang et al., 2010), human  $\alpha$ -1-antitrypsin (Zhang et al., 2013), vaccine antigens (Daniell et al., 2005), cholera toxin B vaccine (Kashima et al., 2016), mouse interleukin-4 and -6 (Fujiwara et al., 2016), griffithsin (Vamvaka, et al., 2016a), BP178 peptide (Montesinos et al., 2017), and 2G12 antibody (Vamvaka, et al., 2016b). This is due to the many advantages rice plant seeds have to offer including high expression levels of recombinant proteins.

### **Rice Grain Physiology and Composition**

Rice is a monocot plant that requires 30 to 45 days after pollination for seed maturation (Ou et al., 2014) and undergoes a vegetative and reproductive phase. The grain development of the reproductive phase dictates potential seed yield (Sehgal et al., 2018) which can be maximized

in optimal environmental conditions (Nawaz and Farooq, 2017). *Oryza sativa* is the most commonly used species of rice for genetic manipulation and recombinant protein production through its seed endosperm. It is classified as a cereal seed and is comprised of the grain hull which protects the brown rice. The brown rice contains the embryo, pericarp, seed coat, nucellus, aleurone layer, and endosperm (Juliano and Tuaño, 2019). The endosperm makes up 80% of the total seed weight and aids in maintaining seed stability throughout different environmental conditions (Takaiwa, 2013). The embryo contains the relevant genetic information and is made of the scutellum, plumule, radicle, and epiblast (Champagne et al., 2004). The brown rice is enclosed in the lemma and palea to create the grain hull, which is removed by dehusking, milling, and grinding for processing.

The removal of the grain hull to expose the endosperm and embryo is beneficial for processing to eliminate large impurities that could interfere with operations. For example, the removal of the aleurone layer through fractionation reduced the phytic acid content by 50% to 80% (Coulibaly et al., 2011) to improve purification as phytic acid is negatively charged above a pH of 1 (Costello et al., 1976). Protein-phytic acid interactions can form which shifts the isoelectric point and solubility of proteins to interfere with protein extraction (Cheryan and Rackis, 1980). This was demonstrated in human lysozyme expressed in rice extracts where phytic acid caused precipitation to occur and clogged the cation exchange chromatography column, hindering purification methods (Wilken and Nikolov, 2010). For this reason, removal of the aleurone layer is beneficial and has been implemented by Ventria Bioscience as current transgenic rice flour extracts do not precipitate in the column during anion exchange chromatography (Sheshukova and Wilken, 2018).

Majority of the proteins in rice seed are found in the sub-aleurone layer, the aleurone layer and embryo contain majority of the lipid content, and starch granules are mainly found in the endosperm (Azhakanandam et al., 2000). The embryo contains the highest amount of protease activity involving proteases such as peptidase and proteinase (Horiguchi and Kitagishi, 1976). *Oryza sativa* seed generally contain 8% total protein with 480 soluble native proteins, out of which 302 have been identified (Yang et al., 2013). Most of the protein functions are varied or unknown but 25% of the proteins are used for metabolic processes and 17% are used for immune defense (Yang et al., 2013). The majority of the proteins have isoelectric points between 5 and 7 with molecular masses between 15 kDa to 95 kDa (Yang et al., 2013). Therefore, impurities such as native proteins, phytic acid, and proteases are important processing considerations as additional steps may be necessary to remove or inactivate the impurity like aleurone layer removal, use of protease inhibitors, or supplementary purification steps.

### **Benefits of Rice Seed as an Expression System**

Rice seed endosperm is an excellent recombinant protein expression system due to its reduced risk of contamination with other non-transgenic species as rice is self-pollinated and possesses a cross-pollination frequency of <1% (Rong et al., 2007). The maximum distance rice pollen grains can travel from the plant is less than 110 meters and the pollen grain has a short lifespan of five min for possible pollination (Song et al., 2004), further improving the biosafety of rice as a transgenic platform. Rice is generally regarded as safe (GRAS) for human consumption with rice products considered to be hypoallergenic for direct oral administration (Takaiwa et al., 2015) which can reduce future purification costs of therapeutics. As a host system, rice seed is commonly reported to be advantageous due to recombinant protein stability (Ryan, 1990) as it contains less impurities such as phenolic compounds and complex protein

mixtures (Prabhu and Jayadeep, 2015) than leafy tissues and bioreactor systems. Rice seed is also able to express high amounts of different recombinant proteins (Takaiwa et al., 2007) which is beneficial for downstream processing costs. Expressed proteins have been shown to possess a high insensitivity to changes in pH and temperature conditions which are extremely beneficial in storage and transportation (Takaiwa et al., 2007), as well as be biologically active and properly folded, establishing the endosperm cell capacity of expressing foreign proteins (Ou et al., 2014). For example, human serum albumin (HSA) expressed in *O. sativa* seed was comparable to plasma derived HSA (pHSA) as it retained its tertiary structure, 17 disulfide bonds, and biological activity (He et al., 2011).

### **Recombinant Protein Expression in Rice Seed**

The development of rice specific expression systems has aided in expressing recombinant proteins at high levels. Healthgen Biotechnology Corp. in Wuhuan, China has successfully developed an expression system called OryzExpress that has produced recombinant human serum albumin, antitrypsin, protease inhibitors, and IGF-1 (Satya and Sarkar, 2018). Another system called ExpressTec was developed by Ventria Bioscience and has successfully produced human leukemia inhibitory factor (Alfano et al., 2014), proinsulin-transferrin fusion protein (Chen et al., 2018), human lactoferrin (Nandi et al., 2002), human lysozyme (Hennegan et al., 2005), and HSA (Frahm et al., 2014) for commercial sale.

Small-, pilot-, and large-scale downstream processing examples in Table 1-2, include many different recombinant proteins expressed in rice seed endosperm. Human interleukin-10 was extracted and purified from *O. sativa* using extraction and precipitation followed by different chromatography methods that increased purity but decreased the recovery with each

step (Fujiwara et al., 2010). As a result, only 4.3% of human interleukin-10 was recovered at 100% purity after the last chromatography step from the initial 96.3% recovery and 65.2% purity after the first step (Fujiwara et al., 2010). A pilot-scale downstream processing of recombinant human  $\alpha$ -1-antitrypsin from rice seed recovered 0.366 g/kg brown rice (18.9%) with a purity of 97% (Zhang et al., 2013). *Oryza sativa* recombinant human serum albumin (OsrHSA) expression was the highest of multiple recombinant proteins from rice seed at 8 g/kg brown rice and had a high yield (2.75 g/kg brown rice) after three chromatography steps (He et al., 2011). Other recombinant proteins such as recombinant human granulocyte-macrophage colony stimulating factor from rice endosperm had high expression levels of 7.5 g/kg brown rice (Ning et al., 2008), but purification was not performed and thus this example was not used for comparison.

When comparing multiple recombinant proteins from transgenic rice seed as seen in Table 1-2 **Error! Reference source not found.**, the results varied with expression levels ranging from 0.2 g/kg up to 10 g/kg brown rice and yields ranging from 0.0083 g/kg to 2.75 g/kg brown rice. The purities of the final product after purification for recombinant human basic fibroblast growth factor, lactoferrin, and transferrin resulted in >95% purity. Purification of OsrHSA,  $\alpha$ -1-antitrypsin, and human interleukin-10 resulted in >99.45%, >97%, and 100% purities respectively. Increasing the number of chromatography steps improved product purities but decreased yields of  $\alpha$ -1-antitrypsin and OsrHSA like human interleukin-10.

**Table 1-2. Summary of various downstream processing of recombinant proteins in transgenic rice seed endosperms.**

<b>Recombinant Protein</b>	<b>Scale</b>	<b>Expression level (g/kg brown rice)</b>	<b>Extraction tissue to buffer ratio</b>	<b>Buffer used in solid-liquid extraction</b>	<b>Purification steps</b>	<b>Yield (g/kg brown rice)</b>	<b>Purity (%)</b>	<b>Recovery (%)</b>	<b>References</b>
<b>Human interleukin-10</b>	Lab	1.2	1:20	pH 7.4 50 mM Tris-HCl and 0.5 M NaCl with 1% CTAB and 10 mM $\beta$ -mercaptoethanol	1. Immobilized metal ion affinity chromatography 2. Immobilized metal ion affinity chromatography 3. Anion exchange chromatography 4. Cation exchange chromatography 5. Size-exclusion chromatography	0.05	100	4.3	Fujiwara et al., 2010
<b><math>\alpha</math>-1-antitrypsin</b>	Pilot	2.2	1:10	pH 7 45 mM phosphate with 1 mM $\beta$ -mercaptoethanol	1. Anion exchange chromatography 2. Ceramic hydroxyapatite chromatography 3. Multimodal anion exchange chromatography	0.366	>97	18.89	Zhang et al., 2013
<b>Human basic fibroblast growth factor</b>	Large	0.19	1:5	pH 7.5 50 mM phosphate and 0.25 M NaCl with 1 mM EDTA and reduced L-Glutathione	1. Affinity chromatography	0.0083 3	>95	4.49	An et al., 2013
<b>Human lactoferrin</b>	Pilot	5	1:10	pH 7 20 mM sodium phosphate and 0.3 M NaCl	1. Anion exchange chromatography	NR	>95	68	Nandi et al., 2002
<b><i>Oryza sativa</i>-expressed human serum albumin</b>	Large	8	1:10	pH 7.5 25 mM phosphate and 50 mM NaCl	1. Multimodal cation exchange chromatography 2. Anion exchange chromatography 3. Hydrophobic interactions chromatography	2.75	>99.45	55.8	He et al., 2011
<b>Transferrin</b>	Lab	10	1:10	pH 7.5 25 mM Tris-HCl	1. Anion exchange chromatography	NR	>95	60	Zhang et al., 2010

NR- not reported



## **Extraction of Recombinant Proteins from Rice Seed**

Extraction is a critical operation in downstream processing that can reduce subsequent purification requirements and costs by using conditions favorable for high product yields and purity. This is accomplished by maximizing the amount of recombinant protein expressed and minimizing the amount of native proteins and impurities. Therefore, extraction conditions like pH, ionic strength, extraction time, and temperature all influence the solubility of proteins present in transgenic plants. pH has a strong effect on protein extractability from transgenic seed systems as the amount of total soluble protein (TSP) increases with extraction pH, from a pH of 4.5 (5-6 mg/g flour) to 10 (15-16 mg/g flour), as demonstrated by Wilken and Nikolov (2006). The effect of pH on extractability is also a function the isoelectric point (pI) of the recombinant protein as solubility typically decreases when the extraction pH is close to the pI of the target protein (Malhotra and Coupland, 2004). However, a combination of pH and electrostatic effects can alter this behavior. For, example, extraction of human lysozyme from transgenic rice was lower at pH 6.5 than at pH 10 despite the protein having a pI of 10. The negative pH effect on protein extractability was attributed to ionic interactions between negatively charged rice constituents and positively charged human lysozyme (Wilken and Nikolov, 2006; Wilken and Nikolov, 2010). The lower extractability of human lysozyme at pH 6.5 was overcome by increasing the ionic strength (through addition of sodium chloride), which disrupts the ionic interactions between the target protein and oppositely-charged plant impurities (Wilken and Nikolov, 2006). At pHs where ionic interactions are not dominate, increasing sodium chloride did not improve recombinant protein extractability. Extraction temperature can also effect solubility and stability of recombinant proteins as increasing temperature improved solubility in transgenic corn (Shukla et al., 2000). However, increasing the temperature can cause the target

protein to inactivate and degrade (Shukla et al., 2000). With careful optimization of extraction conditions, the purity and yield of recombinant proteins after extraction can be improved which could decrease clarification and purification requirements and significantly reduce processing costs.

### **Capture and Purification by Anion Exchange Chromatography**

For a purified product to meet pharmaceutical regulations, most if not all of the impurities must be removed for commercialization. Typical purities of reagents and diagnostic chemicals is  $\geq 95\%$  and  $\geq 99\%$  for pharmaceutical grade drugs (Krishnan and Woodard, 2014). Minimizing the amount of impurities before purification is important as purification is the most expensive unit operation of downstream processing (Gronemeyer et al., 2014). In addition, reducing the number of purification steps is critical to overall process cost and yield. A process yield of  $>50\%$  is a common target for a commercial purification process (Krishnan and Woodard, 2014). A common method of purification of recombinant proteins is using ion exchange chromatography due to its robustness, scalability, and high binding capacities (Lenhoff, 2016). There is also predictability of molecule elution patterns as the adsorption of protein to resin is dependent on charge. Selecting whether to use cation or anion exchange is dependent on the pI of the selected protein. Anion exchange is used if the buffer pH conditions are above the protein pI during purification and vice versa for cation exchange.

Anion exchange chromatography is most compatible with acidic proteins like HSA for purification, therefore, Q Sepharose™ FastFlow® resin (binding capacity of 120 mg HSA/mL) was selected for this study. This strong anion exchanger contains a quaternary ammonium that is fully charged from pH 4 to 10 and is widely used in industrial downstream processes due to its

ease of scale-up (GE Healthcare, 2016). This positively charged ligand binds to the negatively charged groups on the protein's surface through reversible electrostatic interactions. A solution containing the protein is loaded onto a column containing anion exchange resin to allow selective protein binding. During this process, all proteins with the opposite charge flow through the column allowing for the removal of impurities. An elution buffer with counter-ions is then gradually applied to compete with and displace the captured protein, releasing the protein from the resin's charged ligand.

Other resins like DEAE Sepharose™ FastFlow® provide broad selectivity and scale-up ease like Q Sepharose™ FastFlow® resin with a similar binding capacity of 110 mg HSA/mL resin. High resolution chromatography resin includes Q Sepharose™ High Performance which can provide better molecule separation than DEAE and Q Sepharose™ resin but has a lower binding capacity of 50 mg HSA/mL resin.

## **Human Serum Albumin**

Studies for novel medical therapies have recently focused on recombinant proteins instead of traditional small molecule drugs. These therapeutic proteins can be highly complex and exceed 100 kDa (Lagassé et al., 2017), requiring significantly more processing steps to manufacture and produce than small molecule drugs like ibuprofen (Schellekens, 2009). Since 2011, more than 62 recombinant proteins have been approved for various diseases and conditions, with almost half of the commercial products being monoclonal antibodies (Lagassé et al., 2017). The other products were coagulation factors, enzymes, fusion or hormones proteins, growth factors, and plasma proteins (Lagassé et al., 2017). Of the plasma proteins, recombinant HSA (rHSA) has not been approved for therapeutic use even though there is a large demand

worldwide (Yang et al., 2018) and HSA constitutes majority of proteins found in human plasma (Otagiri and Chuang, 2016). Therefore, studies on rHSA are necessary to meet demands and improve the therapeutic protein market.

## **Structure and Properties**

Human serum albumin is an acidic protein with a molecular weight of 66.5 kDa, pI of 4.7, and is the most abundant protein in human blood (around 60% of blood plasma). It is known to be structurally stable due to its large quantity of disulfide bonds and has a 76% similarity with bovine serum albumin in its sequence identity, a commonly used stable diagnostic protein (Michnik et. al., 2006).

Human serum albumin (P02768) is an unglycosylated single peptide chain of 585 amino acids, three equal domains, and 17 disulfide bounds. The first 24 amino acids are the single peptide and propeptide which are cleaved post translationally to form the precursor protein of 585 residues. These disulfide bonds are located at residue 77 to 86, 99 to 115, 114 to 193, 192 to 201, 224 to 270, 269 to 277, 289 to 303, 302, to 313, 340 to 385, 384 to 393, 416 to 462, 461 to 472, 485 to 501, 500 to 511, 538 to 583, and 582 to 591 (Saber et al., 1977). It is also composed of 35 helices and 14 beta strands that form the tertiary structure. The three domains are located at residues 19 to 210, 211 to 403, and 404 to 601 with six subdomains identified as IA, IB, IIA, IIB, IIIA, and IIIB (Carter et al., 1989). Human serum albumin also has a high affinity for metal ions, specifically plasma zinc with binding sites at residue 91, 123, 271, and 273 (Lu et al., 2008), all located in the first two domains. The third domain does not participate in any binding (Dockal et al., 2000). Human serum albumin also has a high affinity for other ligands such as fatty acids, cations, and water. Human serum albumin is synthesized in the liver and enables transportation

of numerous endogenous and exogenous compounds such as amino acids, fatty acids, bile acids, hormones, toxic metabolites, metals, and drugs (Otagiri and Chuang, 2009). However, it is also susceptible to conformational changes (Dockal et al., 2000) in its domains in low pH such as 3.5. The protein unfolds and a helix to beta sheet and helix to coil transition occurs. By pH 2.5, the protein is expanded to its full extent while maintaining its disulfide bonds. In alkaline solutions (pH 6 to 9) residues 1 to 387 display a conformational transition which affects the N-terminus and impacts ligand binding. When comparing rice expressed rHSA and pHSA, they are structurally equivalent using X-Ray crystallography as all 17 disulfide bonds are visible to suggest rHSA would be biologically active (He et al., 2011).

## **Medical Applications**

Human serum albumin has a half-life of 19 days and is synthesized in the liver by hepatocytes and secreted into the blood circulation to make up 60-65% of proteins in blood that helps maintain 80% of the colloid osmotic pressure of plasma (Otagiri and Chuang, 2009). There have been theories of a high HSA concentration in blood plasma improving overall health (Peters, 1996) however, a direct correlation has not been proven. A healthy adult can synthesize 13.8 g daily but requires 360 g total, with only one third present in the bloodstream for blood donation, causing limitations (Otagiri and Chuang, 2009). Human serum albumin has many medical applications for the treatment of burns, liver cirrhosis, hypoalbuminemia, cardiopulmonary bypass, hemorrhagic shock, nephritic syndrome, and hypoproteinemia (He et al., 2011; Mendez et al., 2005). Human serum albumin has also been evaluated for use as a carrier of oxygen (Tsuchida et al., 2009), stabilizing agent (Kratz, 2008), peptide fusion platform (Subramanian et al., 2007), animal-free cell culture supplement and process reagent (Wilken and Nikolov, 2012a), and as a pharmaceutical excipient to protect the product from aggregation,

degradation, and adsorption of a pharmaceutical to the storage container (Yamasaki and Anraku, 2016). HSA is also used to bind to warfarin, naproxin, and ibuprofen (Watanabe et al., 2001). Current commercially available products include nanoscale drug delivery systems, Abraxane and Albuferon. Abraxane is a 130 nm nanoparticle that attached to Paclitaxel for treatment of breast cancer (Petrelli et al., 2010) and Albuferon is an interferon  $\alpha$ -2b/albumin fusion protein (Kratz, 2008).

### **Human Serum Albumin Expression in Other Systems**

There is a large annual demand of more than of 600 tons of HSA worldwide with China requiring 420 tons in 2015 (Yang et al., 2018). However, China was only able to produce 75% of the needed HSA market due to the increasing therapeutic applications of HSA. Limitations arise because the traditional technique of obtaining HSA through blood donation and plasma fractionation provides high risk of contamination by human pathogens so additional treatment steps like heating at 60°C for 10 h in sodium octanoate and N-acetyl-L-tryptophanate is required (Anraku et al., 2007). Additional screening is necessary to determine if all impurities have been eliminated, which adds costs to processing. Therefore, alternatives to produce rHSA instead of pHSA have been explored using different expression systems.

Currently, rHSA has been expressed in multiple bacteria, yeast, and plant systems. *Escherichia coli* was the first platform to express rHSA due to its well established molecular and industrial base but resulted in insoluble rHSA due to protein aggregation and improper protein folding (Latta et al., 1987). However, this was remedied with a multistep process that optimized cell growth parameters to enhance rHSA solubility and improve protein folding through the use of chaperone-assisted folding (Sharma and Chaudhuri, 2017). Transgenic *Bacillus subtilis*

expressed soluble rHSA but resulted in incorrect protein folding at the N-terminus (Saunders et al., 1987).

Many yeast strains have been used to express rHSA with varying yields depending on the transformation technique. *Saccharomyces cerevisiae* produced between 0.02 g to 0.334 g rHSA/L media (Baizhi Li et al., 2011; Saunders et al., 1987) and *P. pastoris* produced 8 g to 12 g rHSA/L media using a repeated fed-batch culture (Belew et al., 2008) and 1.34 g rHSA/L media with a cyclic fed-batch culture (Bushell et al., 2003). However, proteolytic degradation due to acidic proteases can occur when rHSA is secreted into the culture broth with *P. pastoris* and *S. cerevisiae* (Kang et al., 2000; Kobayashi et al., 2000). Therefore new expression systems like PichiaPink™ have been created to achieve high expression levels (12 g/L) while retaining stability (Baizhi Li et al., 2011).

Leafy and bioreactor plant systems have also been used to express rHSA at varying levels with stability. rHSA expressed in tobacco leaf only accounted for 0.02% of TSP due to proteolytic degradation within tissue (Sijmons et al., 1990) while rHSA accounted for 0.2% of TSP in potato tubers (Farran et al., 2002). Higher amounts of rHSA were achieved (11.1% of TSP) with tobacco leaf chloroplasts but the protein suffered proteolytic degradation and required the formation of inclusion bodies (Fernández-San Millán et al., 2003).

More success has been achieved using seeds instead of leafy tissue or cell culture with OsrHSA reaching 10.6% of TSP in *O. sativa* endosperm cells (He et al., 2011). High expression of OsrHSA was achieved (8 g/kg brown rice) and after three chromatography steps, recovery of 55.8% and purity of 99.5% was reported. However, rHSA expressed in *O. sativa* was biologically active but showed variability in glycation after processing when rHSA from

different suppliers was tested (Frahm et al., 2014). *Oryza sativa*-expressed rHSA was also more thermally stable in comparison to *P. pastoris*-expressed rHSA (Frahm et al., 2012). Current products on the market of rHSA include Cellastim™, expressed in transgenic rice seed (Smith et al., 2015) which has been available since 2006 for cell culture and diagnostic applications, rather than for pharmaceutical use.

Current methods of downstream processing of rHSA from plant seeds vary but may include grinding, extraction, clarification, cation exchange chromatography, hydrophobic interactions chromatography, anion exchange chromatography, followed by ultrafiltration (Chen et al., 2013). These processes typically include many unit operations to achieve a purity satisfactory for commercial use. A dose of HSA typically includes 10 g or more per vial with near 100% purity for clinical use. If impurities are present, a systematic reaction to the impurities could occur in the patient; therefore, extra procedures are necessary when using pHSA and rHSA. For a system to express rHSA from rice seed, an expression level of 2.75 g HSA/kg brown rice is sufficient as a purity of >99% OsrHSA had immunogenicity similarly to pHSA since liver cirrhosis was successfully treated in rats (He et al., 2011). Therefore, a cost-effective process that can produce over >99% purity and  $\geq 2.75$  g/kg brown rice of rHSA is needed.

## **Objectives**

The overall goal of the thesis is to develop an integrated and cost-efficient downstream process of recombinant human serum albumin from transgenic *O. sativa* seeds. This is accomplished by analyzing different conditions and rHSA stability during extraction followed by subsequent purification of select extraction parameters which are compatible with anion exchange chromatography.



## **Analysis of Recombinant Human Serum Albumin Extraction and Degradation in Transgenic Rice Extracts**

To determine extraction conditions favorable to maximize rHSA recovery and minimize native protein recovery while maintaining rHSA stability, 1) the effect of pH and time on the solubility of rHSA and native protein was determined by performing extraction kinetics at pH 3.5, 4.0, 4.5, and 6.0 for 60 min, 2) the effect of storage pH on rHSA in extracts was evaluated through pH adjustment, 3) the stability of rHSA in pH 3.5 extract was evaluated by the addition of purified rHSA and adjusting extraction conditions such as temperature to minimize degradation, and 4) rHSA purity and the ratio of rHSA to native protein for subsequent purification was evaluated using densitometry.

## **Purification of Recombinant Human Serum Albumin from Transgenic Rice Through Adsorption**

To develop an integrated downstream process of rHSA extraction and purification, the purification was evaluated by: 1) determining rHSA adsorption parameters such as buffer, residence time, and conductivity compatible with extraction conditions, 2) determining the binding capacity of rHSA in extract with anion exchange resin through consecutive batch binding to simulate dynamic binding, 3) evaluating the dynamic binding capacity of rHSA in extract at saturation and rHSA purity and yield at 10% breakthrough, and 4) quantifying anion exchange chromatography results with affinity chromatography.

## **2. Analysis of Recombinant Human Serum Albumin Extraction and Degradation in Transgenic Rice Extracts**

### **Abstract**

Transgenic plant systems have successfully been used to express recombinant proteins, including rice seed-expressed recombinant human serum albumin (rHSA), without the risk of contamination of human pathogens. Developing an efficient extraction process is critical as the step determines recombinant protein concentration and purity, quantity of impurities, and process volume. This article evaluates the effect of pH and time on the extraction and stability of rHSA. The amount of rHSA in clarified extract after 60 min of solubilization increased with pH from 0.9 mg/g (pH 3.5) to 9.6 mg/g (pH 6.0), but not over time as 10 min was sufficient for solubilization. Total soluble protein in extracts also increased with pH from 3.9 mg/g (pH 3.5) to 19.7 mg/g (pH 6.0) in clarified extract. Extraction conditions that maximized rHSA purity were not optimal for rHSA stability and yield. Extraction at pH 3.5 resulted in high purity (78%), however, rHSA degraded over time. Similar purities (78%) were observed in pH 4.0 extracts yet rHSA remained stable. rHSA degradation was not observed in pH 4.5 and 6.0 extracts but higher native protein concentrations decreased purity. Strategies such as pH and temperature adjustment were effective for reducing rHSA degradation in pH 3.5 rice extracts. Low temperature pH 3.5 extraction retained high purity (97%) and rHSA stability. While seed-expressed recombinant proteins are known to be stable for up to 3 years, the degradation of rHSA was notably extensive (56% within 60 min) when extracted at low pH.

---

\*Reprinted with permission from “Analysis of recombinant human serum albumin extraction and degradation in transgenic rice extracts” by Kseniya A. Sheshukova and Lisa R. Wilken, 2018. *Biotechnol Prog* 34(3):681-691. © 2018 by American Chemical Society.

## Introduction

Human serum albumin (HSA) is an acidic protein with a molecular weight of 66.5 kDa (He and Carter 1992), an isoelectric point (pI) of 4.7 (He and Carter 1992), and the most abundant protein in human blood (around 60% of blood plasma) (Huang et al. 2010). HSA is an unglycosylated single peptide chain of 585 amino acids (He and Carter 1992), three equal domains, and 17 disulfide bonds (He et al. 2011). HSA is synthesized in the liver and enables transportation of numerous endogenous and exogenous compounds such as amino acids, fatty acids, bile acids, hormones, toxic metabolites, metals, and drugs (Otagiri and Chuang, 2009). HSA has many medical applications including the treatment of liver cirrhosis, serious burns, hemorrhagic shock, nephritic syndrome, and hypoproteinemia (He et al. 2011). HSA has been used as an excipient for vaccines and therapeutic protein drugs (Huang et al. 2010; Marth and Kleinhappl 2001), and more recently, has been evaluated for use as a carrier of oxygen (Tsuchida et al., 2009), for nanodelivery of drugs (Cai et al., 2006), as a peptide fusion platform (Subramanian et al., 2007), and as an animal-free cell culture supplement and process reagent (Wilken and Nikolov 2012a).

With a high demand of 500 tons of HSA per year worldwide and a limited supply of human plasma-derived human serum albumin (pHSA) (Fernández-San Millán et al., 2003), other attempts at HSA production through various expression systems have been executed. Production of recombinant human serum albumin (rHSA) is more desired, compared to pHSA, as it eliminates the risk of transmitting blood-derived infectious pathogens such as, HIV and hepatitis (Chamberland et al., 2001), while being structurally equivalent (by X-ray crystallography) and possessing similar biological activity (He et al. 2011). Some of the expression systems for rHSA

include *Escherichia coli* (Latta et al., 1987), *Saccharomyces cerevisiae* (Sleep et al., 1990), *Kluyveromyces lactis* (Fleer et al., 1991), *Pichia pastoris* (Kobayashi et al., 2000), transgenic animals (Barash et al., 1993), and transgenic plants (Fernández-San Millán et al. 2003; Sijmons et al. 1990; Farran et al. 2002; Huang et al. 2005), Reported expression levels of rHSA include 11.1% of total soluble protein (TSP) by tobacco leaf chloroplasts (Fernández-San Millán et al., 2003) and 11.5% of TSP by rice cell culture using a sugar starvation-induced promoter (Huang et al. 2005). Using rice seed as a production platform for rHSA resulted in commercially competitive expression levels with rHSA constituting at least 10.0% of the TSP in the transgenic rice harvested seeds (Huang et al. 2010). Previously high expression levels (greater than 0.5% of total dry weight) have been reported for other recombinant proteins expressed in transgenic rice seeds (Huang et al. 2002).

Transgenic plants have become an alternative for microbial and mammalian cell culture systems for recombinant protein production because they do not carry or spread mammalian diseases and viruses (Goldstein and Thomas, 2004). Transgenic plants can also possess a high expression level of recombinant proteins, which can reduce subsequent purification requirements and create a cost-effective production method (Twyman et al., 2003). Seed-expressed recombinant proteins are considered to be less susceptible to degradation due to the high protein content (10% to 40% in comparison with leafy tissues <5% of wet weight) and low protease content (Boothe et al., 2010), and the presence of endogenous protease inhibitors that prevents degradation of the recombinant protein throughout processing (Menkhaus et al., 2004; C. A. Ryan, 1990; Takaiwa et al., 2007). Recombinant proteins expressed in seeds have been known to be stable at room temperature for years including anti-hepatitis B surface antigen single-chain Fv antibody fragment expressed in tobacco seed, which was stable and retained full biological

activity for 1.5 years at room temperature (Dockal et al., 2000). Recombinant hirudin expressed in canola seed, was stable for 3 years (Ramírez et al., 2001) and recombinant cholera toxin B subunit maintained immunogenicity and stability for more than 1.5 years at room temperature in *Oryza sativa* seed (Nochi et al., 2007). In addition to the seed-specific advantages, rice has a GRAS (generally regarded as safe) status (Nandi et al., 2002), established production infrastructure, relatively high protein content, low risk of contamination by human pathogens, and high expression levels and relatively fast production scale up (Stoger et al., 2005), which are important considerations for selecting a transgenic host. To utilize these benefits of transgenic plants, efficient downstream processes for extraction and purification of recombinant proteins must be developed.

Downstream processing of transgenic plant tissue includes primary recovery and purification steps. Primary recovery includes fractionation and/or milling, product extraction/release, and solid–liquid separations. Extraction is a critical recovery step because it determines the recombinant protein concentration and purity, the type and quantity of impurities that have to be removed during purification, and volume of extract that must be processed (Wilken and Nikolov 2012b). Optimization of extraction conditions can improve product stability throughout processing and reduce the number and cost of purification steps. Variables typically evaluated for extraction optimization include extraction pH, ionic strength, temperature, and time.

Several studies have shown that pH has a strong effect on protein extractability from transgenic seed flour (Azzoni, Farinas, and Miranda 2005; Farina et al. 2002; Wilken and Nikolov 2006). In general, the amount of TSP extracted increases with extraction pH. For example, the amount of TSP in transgenic rice extracts (with recombinant human lysozyme)

increased from 5–6 mg/g flour at pH 4.5 to 7 mg/g at pH 6.5, 8 mg/g at pH 7.5, and 15–16 mg/g at pH 10 (Wilken and Nikolov 2006). The amount of recombinant protein extracted also varies greatly with extraction pH and is also a function of the pI of the recombinant protein. For example, the amount of native protein extracted from transgenic flour increased from 2–3 mg/g flour at pH 4.5 to 5–6 mg/g flour at pH 6.5, 7–8 mg/g flour at pH 7.5, and 12–13 mg/g flour at pH 10 (Wilken and Nikolov 2006). However, the amount of human lysozyme extracted decreased from 3 to 4 mg/g flour at pH 4.5 to 1–2 mg/g flour at pH 6.5 and 0–1 mg/g flour at pH 7.5 (Wilken and Nikolov 2006). However, an increase in solubility of human lysozyme occurred at pH 10 to 2–3 mg/g flour, despite the pI (10.5) being close to the pH (Huang, Rodriguez, and Hagie 2003). Extracts prepared from the high-expressing rice flour had recombinant protein concentrations up to 70% of TSP by careful selection of extraction conditions (Huang, Rodriguez, and Hagie 2003). The effect of temperature on extraction is another parameter to consider as recombinant human proinsulin extraction from transgenic corn showed a positive effect with temperature increase. The protein concentrations achieved for extraction performed at 25°C ( $18.87 \pm 0.48$  mg/L) were 10% less than at a higher temperature of 40°C ( $20.72 \pm 1.06$  mg/L) (Farinas et al., 2007). Other protein extraction studies with transgenic corn show increased solubility with an increase of temperature. Protein solubility was highest at 50°C (evaluated temperatures of 20°C to 60°C) and extraction above 60°C showed protein denaturation as well as an increase in cost to maintain the high temperature (Shukla et al., 2000). Other studies have shown a correlation between protein extraction and ionic strength, although this relationship is not as significant as the effect of pH on extractability.

The inherent stability of the recombinant protein is also an important consideration for selection of processing conditions. HSA is known to be a relatively stable protein due to the high

disulfide bond frequency (17 disulfide bonds (He et al. 2011b)) and subdomains which provide structural stabilization (Banach et al., 2016) and the wide temperature range at which HSA maintains biological activity (Shukla et al., 2000). Thus, HSA has been used as a pharmaceutical excipient to protect against product degradation and loss by adsorption (Yamasaki and Anraku, 2016). However, HSA is susceptible to changes in its molecular structure by pH and temperature (Fernández-San Millán et al., 2003). One study performed on HSA displayed thermal denaturation of 12% at 65°C and 45% at 75°C (Wetzel et al., 1980). While protein unfolding is significant at 55°C, the process is reversible at temperatures up to 68°C (Wetzel et al., 1980). The impact of temperature on HSA provides flexibility in temperature selection during extraction as HSA is structurally stable up to 50°C (Wetzel et al., 1980). With respect to pH, HSA is susceptible to conformational changes (Dockal et al., 2000) in its domains at low pH such as 3.5. The protein unfolds and structural elements are disrupted. As the pH decreases to pH 2.5, the protein expands and more conformational transitions occur but disulfide bonds are preserved and, thus, the full length structure remains intact (Dockal et al., 2000). In alkaline solutions (pH 7.0 to 9.0), amino acid residues 1 to 387 display a conformational transition which affects the N-terminus and impacts ligand binding (Dockal et al., 2000). Therefore, conditions such as temperature and pH can impact both recombinant protein stability and extractability.

To take advantage of the numerous applications that HSA provides without the risks of pHSA, an alternative expression system, such as rice seed, is needed. The use of transgenic rice for production of rHSA is very promising, but extraction conditions must be investigated for the development of a cost-effective processing method. We discuss the effect of pH on the amount of rHSA and TSP extracted from transgenic rice flour and the effect of extract and storage pH on rHSA stability.

## Materials and Methods

### Materials

Transgenic *Oryza sativa* flour (40% TSP) was provided by InVitria, a division of Ventria Bioscience (Junction City, KS). The rice was transformed using ExpressTec (Ventria Bioscience) with a chimeric gene comprising a globulin promoter (SEQ ID NO: 6) with upregulated activity during rice seed maturation and DNA sequence encoding human serum albumin (Huang et al. 2010). The rice flour was stored at 4°C throughout the research. Human serum albumin (>96% purity, rice-derived) and bovine serum albumin (>98% purity) were purchased from Sigma-Aldrich (St. Louis, MO) and used as analytical standards and for stability experiments. All other reagents and supplies were purchased from VWR Scientific.

### Analytical Methods

#### *Total Protein Quantification*

Total soluble protein 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 (Thermo Fisher Scientific, Waltham, MA). Absorbances at 595 nm were measured by a SpectraMax Pro 384 microplate reader using SoftMaxPro V4.6 software. Samples were analyzed in triplicate. TSP concentrations in extracts were expressed as milligrams protein per gram flour.



### ***Protein Analysis by SDS-PAGE®***

The protein profiles of extracts were evaluated by electrophoresis as described by Laemmli (Laemmli 1970). Samples were loaded on 4%–12% *bis*-tris gels under reducing conditions and ran according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Extract samples were prepared for gel electrophoresis immediately after clarification by centrifugation.

### ***rHSA Quantification***

rHSA concentration was determined using ImageJ software (Rasband, 1997) to analyze relative concentrations on NU-PAGE® gels (Tan et al., 2009). rHSA concentrations of extract samples were determined by densitometry using purified reference rHSA and a blank. Concentrations of pure rHSA samples were verified by measuring the absorbance of each sample at 280 nm (extinction coefficient of 0.549 mL/mg cm). Two concentrations of rice-derived HSA (>96% purity) were used as standards for comparison of staining intensities to that of rHSA bands at 66.5 kDa in extract samples. Standard concentrations were within range of rHSA concentrations observed in extracts.

### ***Statistical Analysis***

Statistical analysis of rHSA purity over time was performed on each time sample in comparison with the initial time point (10 min) using a two-tailed *t*-test ( $p < 0.05$ ) for each pH. If the resulting *p* value was below 0.05, the purity at that time point had significantly decreased in comparison to the 10 min purity.

## **Experimental Methods**

### ***Extraction Kinetics***

The effect of pH was determined by investigating the amount of rHSA and total protein extracted at various pHs. Experiments were performed at pH 3.5, pH 4.0, pH 4.5, and pH 6.0 using 50 mM sodium chloride in 50 mM sodium citrate buffer for pH 3.5, pH 4.0, and pH 4.5 and 50 mM sodium chloride in 50 mM sodium phosphate buffer for pH 6.0. Low pH reduces the amount of native protein extracted and extracting at least one pH unit below/above the pI is recommended as extraction at the pI limits protein extractability (S Ozturk and Hu, 2005). The conductivities of buffers were measured by EC Meter Model 2052 (VWR Scientific) and reported in mS. Experiments at the four pHs were conducted with transgenic rice flour in triplicate. For each experiment, 20 g of transgenic rice flour was added to 100 mL of extraction buffer and mixed continuously for 1 hour on a magnetic stir plate. An extraction sample (1 mL) was taken every 10 min for 60 min total. The kinetic samples were centrifuged (VWR Micro 2416 microcentrifuge) at 9,100×g for 3 min to separate the suspended solids. After the final 60 min extract sample was taken, the remaining mixture was centrifuged (Thermo Scientific Sorvall RC 6 Plus Centrifuge) at 10,000×g for 15 min to separate the insoluble solids and then filtered with a 0.45 µm SFCA filter (Thermo Fisher Scientific, Waltham, MA). Gel samples were immediately prepared after centrifugation (Tan et al., 2009). Extract supernatants were analyzed for total protein and rHSA content. To determine the amount of protein in the clarified extracts, TSP was determined by Bradford assay. The concentration of rHSA in clarified extracts was determined by analyzing protein profiles of extracts using NU-PAGE® gel electrophoresis.

### ***Effect of pH, Temperature, and rHSA Stability***

#### ***Effect of Storage pH on Stability of rHSA in Extracts***

Stability was tested by evaluating rHSA concentration over time throughout storage of extracts (as a function of pH). Based on extraction kinetics, an extraction time of 20 min was selected for further analysis of rHSA stability in extracts. pH 3.5 and pH 6.0 extracts were prepared as described above and stored at the extraction pH or pH adjusted to evaluate rHSA stability over time. The pH 3.5 extract was either kept at pH 3.5 for storage or adjusted to pH 4.5 with 3 M NaOH and then stored for 120 min. The pH 6.0 extract was maintained at pH 6.0 or adjusted to pH 3.5 or pH 4.5 with 0.1 M HCl and then stored for 120 min. All extracts were stored at room temperature (23°C). pH 3.5 stored extract samples were taken every 15 min for 45 min while adjusted pH 4.5 samples were taken every 30 min for 120 min. pH 6.0 stored extracts, pH 4.5 adjusted extract, and pH 3.5 adjusted extract samples were taken at 120 min. All samples were prepared immediately for gel analysis.

#### ***Stability of Purified rHSA in Buffer***

To ensure the rHSA degradation at low pH was not due to pH instability, the stability of purified rHSA in buffer was evaluated. rHSA stability was tested by measuring the rHSA concentration of purified rHSA in buffer over time by NU-PAGE<sup>®</sup> gel electrophoresis. A 1 mg/mL solution of purified rHSA was prepared and mixed in pH 3.5 50 mM sodium citrate with 50 mM sodium chloride buffer (11.87 mS) and time samples were taken every 30 min over 120 min at room temperature of 23°C. All samples were prepared immediately for gel analysis.

#### ***Stability of rHSA and BSA Spiking into Transgenic Rice Extracts***

rHSA stability was tested by spiking purified rHSA or BSA in clarified extract and measuring the rHSA or BSA concentration over time. Clarified pH 3.5 extract was stored at room temperature (23°C) until all expressed rHSA was degraded and then spiked with 6.2 mg of pure solid rHSA and 6.2 mg of pure solid BSA (0.62 mg/mL). Spiked solutions were mixed until

solids had solubilized. The amount of rHSA and BSA spiked into extract was consistent with rHSA concentrations measured in transgenic extracts. Spiked extracts were analyzed for 90 min with a sample taken every 15 min and immediately prepared for gel analysis. Samples were analyzed by Bradford TSP microplate assay and NU-PAGE<sup>®</sup> gel electrophoresis.

### ***Effect of Temperature on rHSA in Extracts***

The effect of temperature was tested by evaluating the results of pH 3.5 extracts at decreased temperature. Extraction kinetics were performed with 20 g of transgenic rice flour mixed in 100 mL of pH 3.5 50 mM sodium citrate with 50 mM sodium chloride (12.46 mS). The temperature was reduced by dry ice surrounding the centrifuge bottle, keeping an average constant temperature of 5°C. Time samples were taken every 10 min over 60 min. After the 60 min extract was taken, the remaining mixture was centrifuged (Thermo Scientific Sorvall RC 6 Plus Centrifuge) and filtered with a 0.45 µm SFCA filter (Thermo Fisher Scientific, Waltham, MA). Samples were immediately prepared for gel analysis and analyzed by Bradford TSP microplate assay and NU-PAGE<sup>®</sup> gel electrophoresis. A Western blot was performed with gel sample preparation of 1:60 ratio of sample in 1× tris-buffered saline. Gel to membrane transfer occurred using the iBlot<sup>®</sup> Gel Transfer Device and Mini iBlot<sup>®</sup> Nitrocellulose Gel Transfer Stacks (Thermo Fisher Scientific, Waltham, MA) for 7 min. Membrane was carefully transferred for blocking in 5% milk (SACO<sup>®</sup> Real Skim Milk) in 1× Tris-Buffered Saline with TWEEN<sup>®</sup> 20 (TBST) overnight. The blot was then washed with TBST three times for 5 min before incubation in the primary antibody (rabbit anti-human albumin antibody) using a 1:10,000 dilution for 90 min. The blot was then washed with TBST three times for 5 min before incubation in the conjugated secondary antibody (goat anti-rabbit IgG–alkaline phosphatase) using a 1:15,000 dilution for 90 min. To develop the blot, the membrane was incubated in a solution of

SIGMAFAST™ BCIP®/NBT until bands were visible. Distilled water was used to stop development.

### ***Stability of rHSA at Different Temperatures in pH 3.5 Buffer***

The stability of rHSA in pH 3.5 buffer was tested at two different temperature conditions to evaluate stability of rHSA. Extraction kinetics were performed with 6.2 mg pure solid rHSA in 10 mL of pH 3.5 50 mM sodium citrate buffer with 50 mM sodium chloride (13.13 mS) in two 15 mL conicals. One conical was kept at room temperature (23°C) and the remaining conical was stored at a constant temperature of 5°C. Samples were taken immediately after solubilization and then every 30 min for 90 min. Samples were immediately prepared for gel analysis and analyzed by NU-PAGE® gel electrophoresis and Western blot.

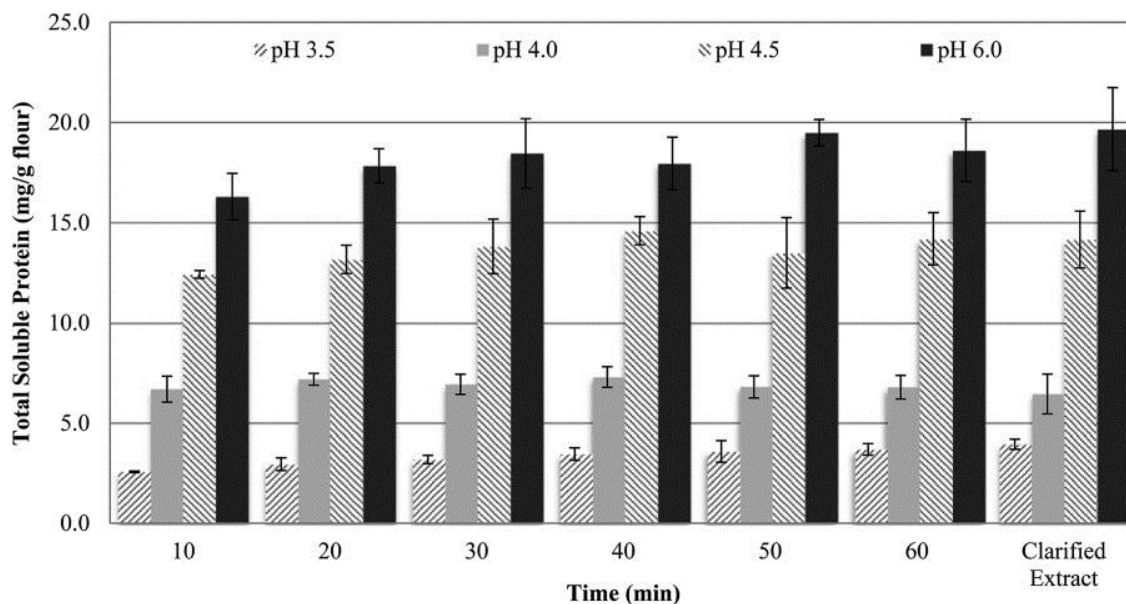
## **Results and Discussion**

### **Extraction Kinetics**

#### ***Effect of pH and Time on Extraction of TSP***

The average amount of TSP extracted from rice flour is shown in Table 2-1. pH 3.5 extraction resulted in the lowest concentrations of total protein with 2.3 ( $\pm 0.1$ ) mg TSP/g flour present in 10 min extract to 3.9 ( $\pm 0.3$ ) mg TSP/g flour in the final clarified extract. pH 4.0 extracts had 6.7 ( $\pm 0.6$ ) mg TSP/g flour at 10 min and 6.4 ( $\pm 1.0$ ) mg TSP/g flour in the clarified extract. While pH 4.5 had 12.4 ( $\pm 0.2$ ) mg TSP/g flour at 10 min and 14.2 ( $\pm 1.4$ ) mg TSP/g flour in the clarified extract. pH 6.0 extraction resulted in the highest amount of extracted TSP with 16.3 ( $\pm 1.2$ ) mg/g flour at the first extraction sample to 19.7 ( $\pm 2.1$ ) mg/g flour in the clarified extract. TSP in pH 6.0 extracts were six- to eight-fold greater than that in pH 3.5 extracts. The

increase in TSP with pH is consistent with trends observed for other transgenic rice-derived proteins (Wilken and Nikolov 2012b; Wilken and Nikolov 2006). The drastic increase in the amount of TSP extracted at pH 4.5 compared to pH 4.0 was initially surprising for such a small increase in pH. This increase in TSP and native protein was unexpected as native rice proteins have isoelectric points in the selected pH which can reduce solubility. The amount of native protein extracted (at 30 min) increased from 2.3 mg/g to 6.8 mg/g with an increase of extraction pH from 4.0 to 4.5. Later analysis also showed a significant increase in rHSA solubility with the same extraction pH increase.



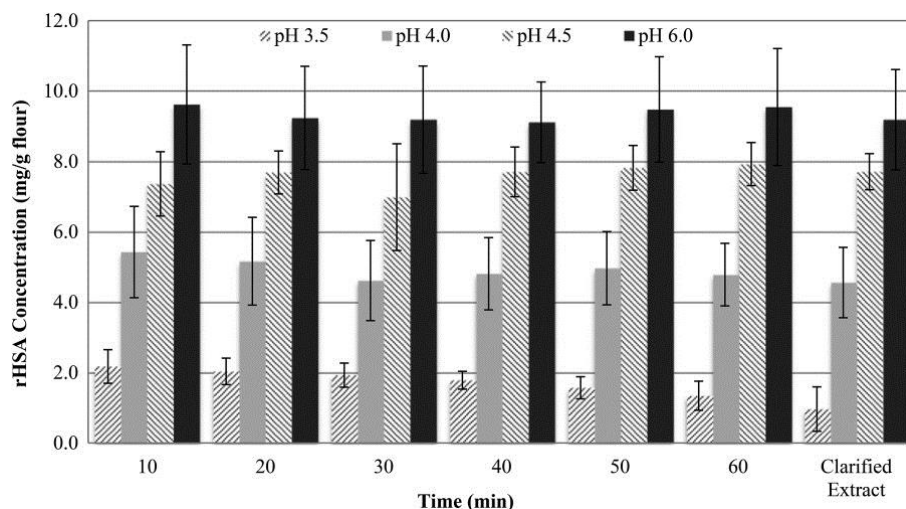
**Table 2-1. Average TSP (mg TSP/g flour) extracted at pH 3.5, pH 4.0, pH 4.5, and pH 6.0 throughout extraction (0 to 60 min). The final filtrate (Clarified Extract) represents the 60 min extract after the final centrifugation and dead-end filtration. Error bars indicate standard deviation of triplicate extractions.**

A study involving rice expressed human lysozyme showed a significantly lower amount of extracted TSP at pH 4.5 when compared to rHSA rice extracts at the same conditions (Wilken and Nikolov 2006). At 30 min, the amount of TSP in rHSA extracts was 13.3 mg/g flour, while the amount of TSP in human lysozyme extracts was 5.7 mg/g flour. The amount of soluble rice

protein in rHSA extracts (6.8 mg/g) was almost threefold greater than in human lysozyme extracts (2.4 mg/g). However, the observation of substantially lower amounts of extracted rice protein from transgenic flour expressing human lysozyme was due to the reported reduction of salt-soluble globulins in high-lysozyme-expressing rice seed (Huang, Rodriguez, and Hagie 2003). The same study also showed a fairly steady increase of around 1 to 2 mg TSP/g flour, unlike the drastic increase in rHSA extracts of approximately 5 mg TSP/g flour per 1 pH unit increase.

### ***Effect of pH and Time on Extraction of rHSA***

The average amount of rHSA extracted from rice flour (in mg rHSA/g flour) is shown in Table 2-2. pH 3.5 resulted in the lowest concentration of rHSA, which varied from 2.2 ( $\pm 0.5$ ) mg/g flour for 10 min extracts to 0.9 ( $\pm 0.6$ ) mg/g flour for final clarified extracts. The concentration of rHSA in pH 4.0, pH 4.5, and pH 6.0 extracts remained stable throughout extraction and resulted in rHSA concentrations of 5.4 ( $\pm 1.3$ ) mg/g flour, 7.4 ( $\pm 0.9$ ) mg/g flour, and 9.6 ( $\pm 1.7$ ) mg/g flour, for each respective pH, at 10 min. These results were higher or comparable to the levels of other recombinant proteins extracted from transgenic rice seed flours. For example, reported extraction yields of other recombinant proteins expressed in transgenic rice were 4.4 mg/g flour for human lysosome (Wilken and Nikolov 2012b), 6 mg/g flour for human lactoferrin (Nandi et al., 2002), and 10 mg/g flour transferrin (D. Zhang et al., 2010). Table 2-1 also shows that the amount of rHSA extracted did not increase after 20 to 30 min for all pHs evaluated. Thus, increasing the extraction time beyond 20 to 30 min would add to processing costs without a direct benefit.



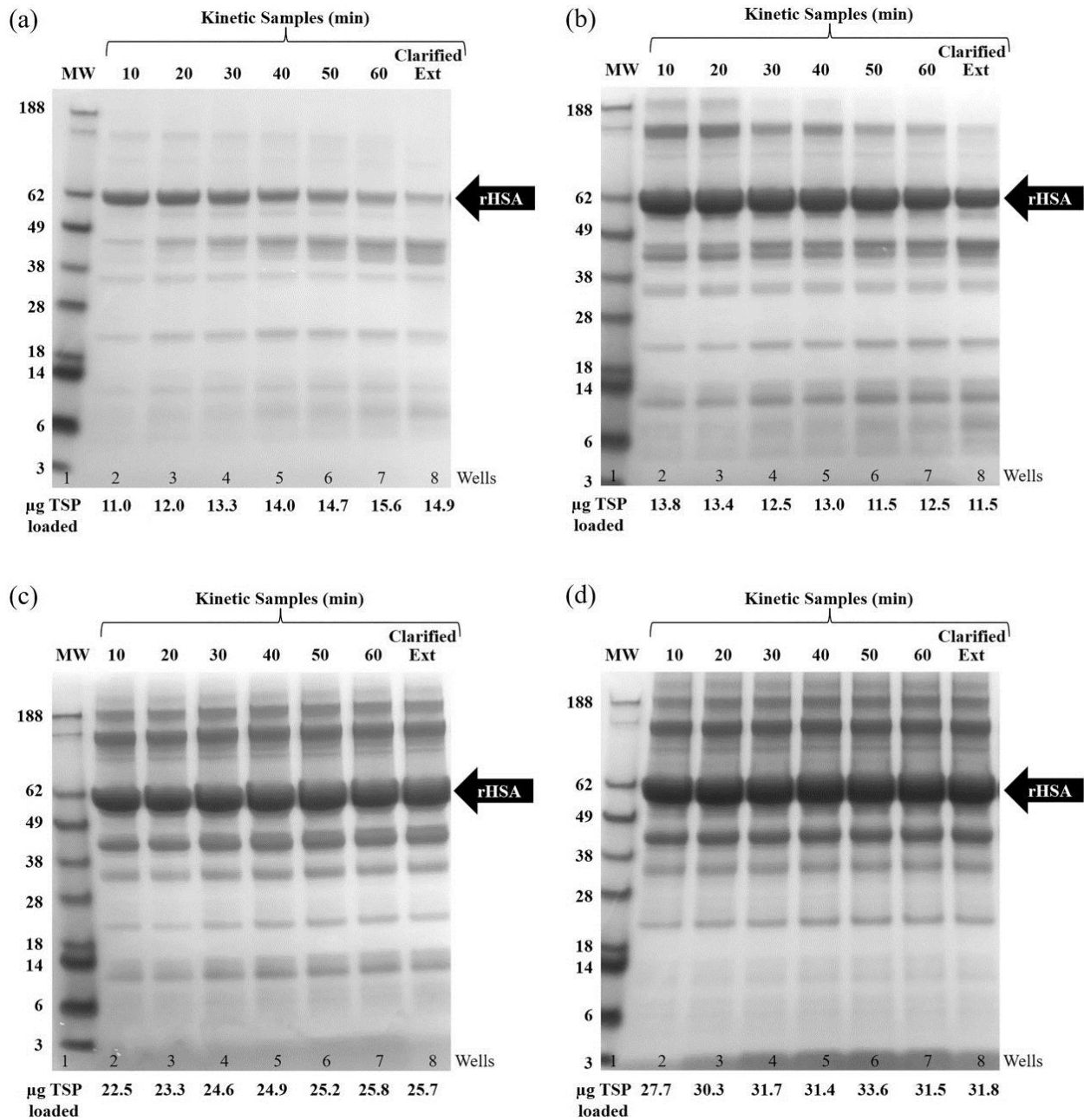
**Table 2-2. Average rHSA concentration (mg/g flour) extracted at pH 3.5, pH 4.0, pH 4.5, and pH 6.0 throughout extraction (0 to 60 min). The final filtrate (Clarified Extract) represents the 60 min extract after the final centrifugation and dead-end filtration. Error bars indicate standard deviation of triplicate extractions.**

The concentration of rHSA decreased as the extraction time increased at pH 3.5, which indicated that rHSA was being degraded during extraction. A decrease in rHSA concentration was not observed at pH 4.0, 4.5, and 6.0. The degradation of rHSA during pH 3.5 extraction was unexpected as recombinant proteins extracted from seeds are typically stable due to the presence of native protease inhibitors and low metabolic activity in seeds (Wilken and Nikolov 2012b), thus, significant degradation of rice seed-expressed recombinant proteins has yet to be reported. However, HSA has been reported to be highly susceptible to proteolytic degradation in other transgenic systems (Fernández-San Millán et al., 2003; Kang et al., 2000) and conformational changes are known to occur at pH 3.5 and below (Del Giudice et al., 2017; Dockal et al., 2000). Thus, further exploration of rHSA stability was warranted. In addition, there was significant variability in rHSA concentrations calculated using densitometry.

Gel electrophoresis was used to evaluate the protein profiles of the extracts over time. Degradation of rHSA over time at pH 3.5 is apparent in the gel (Figure 2-1a) and confirmed by



Western blot (as discussed later). The intensity of the 66.5 kDa molecular weight band (full length rHSA) decreased over time while the intensity of lower molecular weight bands increased with time and are likely degradation products. For pH 4.0 extract samples, gel analysis showed a slight decrease in the intensity and size of molecular weight bands at 66.5 kDa (Figure 2-1b). However, the calculated TSP (Table 2-1) and rHSA concentrations (Table 2-2) were not significantly reduced over time (within range of standard deviation). pH 4.5 and pH 6.0 gels show consistent rHSA band intensity over time at 66.5 kDa and a larger amount of native rice proteins as demonstrated in Figure 2-1a and Figure 2-1d, respectively. This analysis correlates with the increase of TSP in pH 4.5 and pH 6.0 (12.4 mg/g and 16.3 mg/g, respectively) from pH 4.0 (6.7 mg/g) at 10 min.



**Figure 2-1. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from (a) pH 3.5 extracts (wells 2–8), (b) pH 4.0 extracts (wells 2–8), (c) pH 4.5 extracts (wells 2–8), and (d) pH 6.0 extracts (wells 2–8), showing protein molecular weight profiles. The final extract (clarified extract) was clarified by centrifugation and dead-end filtration after the 60 min time period. The arrows show molecular weight placement of rHSA.**

The extraction conditions that maximize recombinant human serum albumin concentration and minimize the amount of soluble rice protein in the extracts were identified by calculating the rHSA protein purity (percentage of rHSA in total protein) and ratio of rHSA to

solubilized native rice protein. Selecting extraction conditions that maximizes these parameters will reduce purification requirements. rHSA purities (Table 2-3) significantly decreased over time in pH 3.5 extracts from  $79\% \pm 17.2\%$  (10 min) to  $26\% \pm 15.6\%$  (clarified extract) but stayed fairly consistent on average throughout pH 4.0 (71%) and pH 4.5 (53%) extracts. The decrease in rHSA purity with time in pH 3.5 extracts was due to the decrease in rHSA concentration and not from a significant increase in TSP with time. This is also apparent in the corresponding gels (Table 2-1) and rHSA concentration data (Table 2-1). The significant decrease in rHSA purity with time for pH 6.0 extraction from 65.3% to 51.3% (Table 2-3) can be attributed to the increase in native protein.

**Table 2-3. Effect of pH and extraction time on rHSA protein purity (%).**

pH	10 min	20 min	30 min	40 min	50 min	60 min	Clarified Ext.
3.5	78.9 ± 17.2	64.9 ± 12.1	58.2 ± 10.5	51.4 ± 6.3	44.2* ± 5.2	38.4* ± 8.6	26.0* ± 15.6
4.0	78.8 ± 12.0	71.7 ± 16.4	67.1 ± 18.2	66.2 ± 15.2	73.8 ± 18.2	70.1 ± 9.2	70.0 ± 4.4
4.5	56.9 ± 8.8	55.9 ± 6.0	47.3 ± 15.3	50.3 ± 5.9	56.2 ± 9.7	53.2 ± 3.9	52.5 ± 3.1
6.0	65.3 ± 1.5	56.7* ± 2.4	54.9* ± 0.2	54.8* ± 0.5	52.8* ± 3.9	56.1* ± 2.0	51.3* ± 1.1

Errors represent the standard deviations of triplicate experiments. An asterisk (\*) designates that the purity is significantly different within the pH group in comparison with each 10 min time sample ( $p < 0.05$ ).

Extraction conditions that maximize rHSA concentration and minimize the amount of soluble rice protein in the extracts were identified by calculating the ratio of extracted rHSA to solubilized native rice protein (Table 2-3). This rHSA to native protein ratio, along with rHSA extractability should be considered when identifying optimal extraction conditions for high-expressing plants. A high ratio signifies a high purity which is beneficial for reducing cost in subsequent downstream processing, however, the amount of rHSA extracted should be considered. The ratio was calculated by dividing the rHSA concentration by the native rice protein concentration, which was found by subtracting the rHSA concentration from the total soluble protein concentration. The highest ratios were observed in 10 min extracts at pH 3.5

(ratio of 5.6) and pH 4.0 (ratio of 4.3). The ratio was much lower for the 10 min extract at pH 4.5 (1.5) and 6.0 (1.4) which was likely due to the drastic increase in TSP at pH 4.5 and pH 6.0 compared to pH 4.0 with less than proportional increase in rHSA. pH 4.0 extract contained the highest ratio over time, ending with 2.4 at the clarified extract compared to pH 3.5 (0.3), pH 4.5 (1.2), and pH 6.0 (0.9). The rHSA to native rice protein concentration ratio for the final clarified extracts decreased over time for all pH levels, but most significantly for pH 3.5 extraction (from 5.6 to 0.3). Due to the unique degradation behavior observed at pH 3.5, we further explored the impact of pH on rHSA stability in buffer and in extract to ensure conformational changes or bottle adsorption was not the cause.

**Table 2-4. Effect of pH and extraction time on the rHSA to native rice protein concentration ratio.**

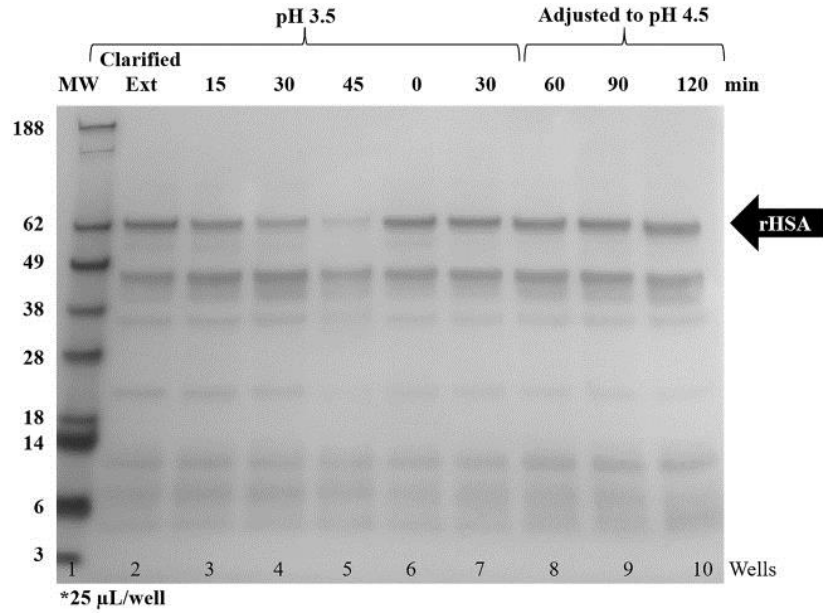
pH	10 min	20 min	30 min	40 min	50 min	60 min	Clarified Ext.
3.5	5.6	2.2	1.5	1.1	0.8	0.6	0.3
4.0	4.3	2.6	2.0	1.9	2.7	2.4	2.4
4.5	1.5	1.4	1.0	1.1	1.4	1.3	1.2
6.0	1.4	1.1	1.0	1.0	0.9	0.6	0.9

## **Effect of pH on rHSA stability**

### *Effect of Storage pH on Stability of rHSA in Extracts*

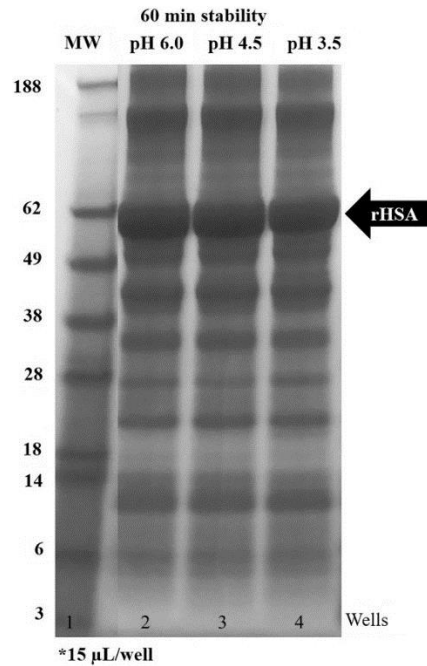
Two experiments were run at pH 3.5 and pH 6.0 to evaluate the effect of extract and storage pH on rHSA stability. The pH 3.5 extract was either stored at the extraction pH or adjusted to pH 4.5. The pH 6.0 extract was either stored at the extraction pH or adjusted to pH 4.5 or 3.5. All extracts were maintained at room temperature (23°C) for 120 min. As shown in Figure 2-2, rHSA extracted and stored in the transgenic rice extract at pH 3.5 was degraded over time (as indicated by the decrease in rHSA band intensity over time and the increase of band intensities of lower molecular weight degradation products). However, rHSA in pH 3.5 extract

adjusted to pH 4.5 was stable as indicated by a consistent band intensity at 66.5 kDa throughout storage.



**Figure 2-2. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from extraction maintained at pH 3.5 (wells 2–5) and adjusted to pH 4.5 (wells 6–10). The arrow shows molecular weight placement of rHSA at 66.5 kDa.**

The results of either maintaining the extract at pH 6.0, adjusting it to pH 4.5, or to pH 3.5 are shown in Figure 2-3. For these cases, rHSA was stable as indicated by consistent band intensity at 66.5 kDa (full-length rHSA) throughout storage at all investigated pHs. Thus, degradation of rHSA cannot be attributed to low pH only.



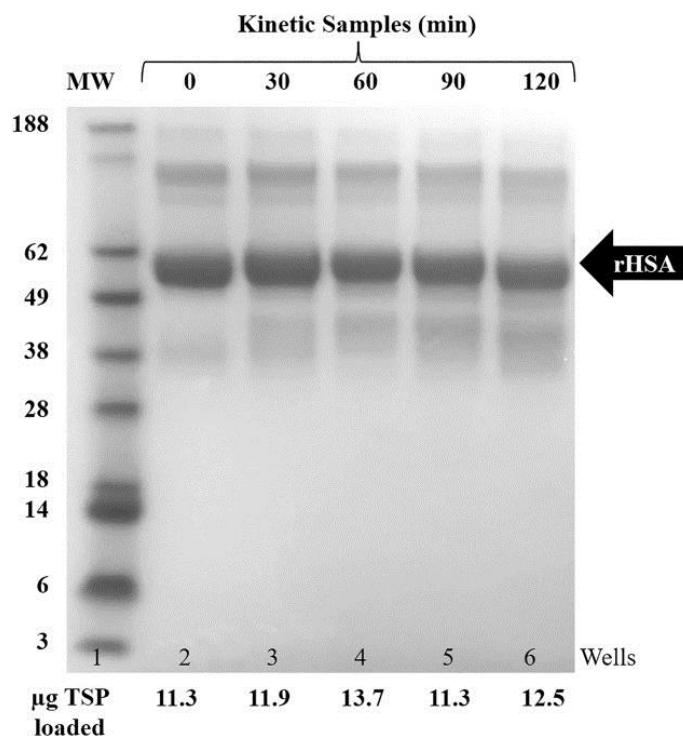
**Figure 2-3. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from extraction maintained at pH 6.0 (well 2) and adjusted to pH 4.5 (well 3) and pH 3.5 (well 4) at 120 min. The arrow shows molecular weight placement of rHSA at 66.5 kDa.**

Both experiments demonstrated that rHSA was stable in transgenic rice flour extract if pH 3.5 extract was adjusted to pH 4.5 or 6.0 for storage or if pH 6.0 extract was maintained at pH 6.0 or adjusted to a pH of 3.5 to 4.5. For these cases, TSP and rHSA concentrations remained constant during storage, which indicates that the cause of degradation is not due to pH instability alone. These experiments seem to suggest a difference in extracted components (such as a protease) or activity of the component (if protease) between pH 3.5 and pH 6.0, which causes the degradation in rHSA at low pH.

#### ***Stability of Purified rHSA in Buffer***

To further confirm that the rice extract was responsible for rHSA degradation and not pH instability of rHSA or adsorption to polyethylene and polypropylene copolymer bottles used for extraction, the stability of purified rHSA was evaluated in buffer of the same pH and ionic

strength (pH 3.5 50 mM sodium citrate buffer with 50 mM sodium chloride) as the pH 3.5 extraction buffer. This experiment showed that rHSA in only pH 3.5 buffer was not degraded or adsorbed as shown by the stable band intensity over time in Figure 2-4 and consistent TSP (about 12  $\mu\text{g}$  per well) and rHSA concentrations over 120 min at room temperature (23°C). This suggests rHSA instability in pH 3.5 extracts was not due to pH sensitivity of rHSA or adsorption to polyethylene and polypropylene copolymer bottles but rather caused by a rice impurity co-extracted with rHSA.

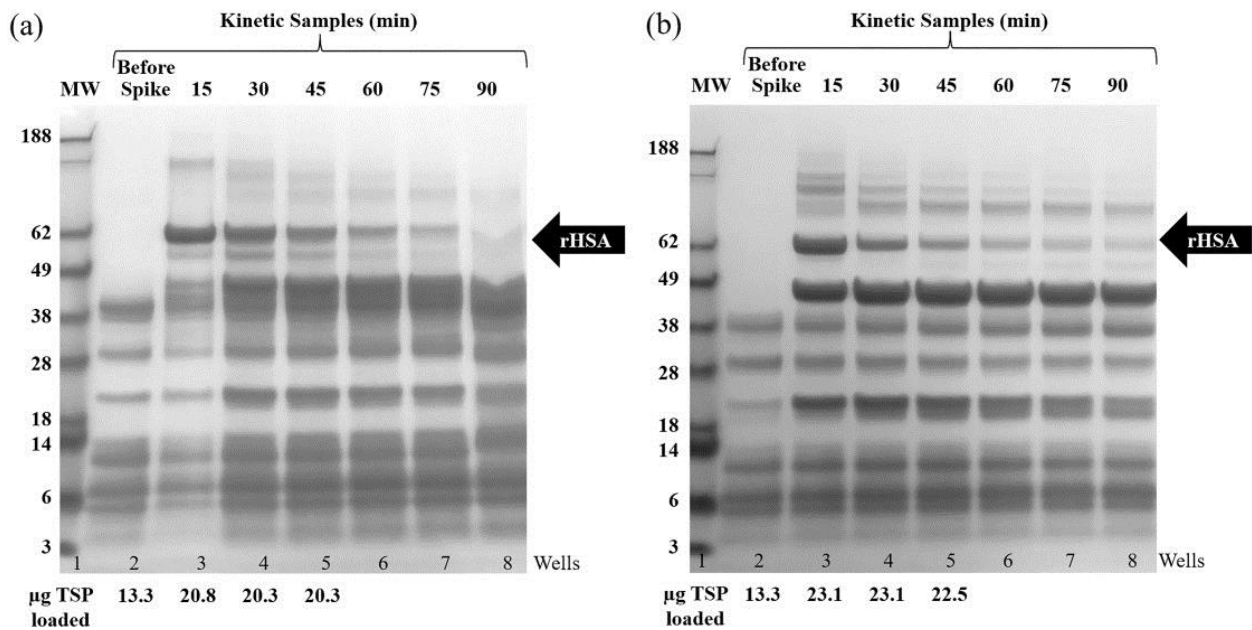


**Figure 2-4. Protein profiles of extracts by NU-PAGE® electrophoresis gel with samples from pure rHSA in pH 3.5 buffer (wells 2–6). The arrow shows molecular weight placement of rHSA at 66.5 kDa. Gels were loaded on a volume basis.**

### *Stability of rHSA and BSA Spiked into Transgenic Rice Extracts*

Spiking of purified rHSA and BSA (commonly used as a standard for its known stability (Bradford, 1976)), which share 76% amino acid sequence homology (Huang, Kim, and Dass

2004), in room temperature pH 3.5 extracts demonstrated continuous degradation of rHSA and BSA, which was similar to behavior observed in pH 3.5 extracts. When rHSA was spiked into pH 3.5 extracts, the TSP per well was 20.8  $\mu\text{g}$  at 15 min to 20.3  $\mu\text{g}$  at 45 min. The amount of full-length rHSA detected in the gel was reduced over time. Gel electrophoresis showed further analysis of TSP was unnecessary, as degradation was visible from decreasing band intensity at 66.5 kDa in both gels (Figure 2-5). This clarifies the assumption of degradation related to pH 3.5 extracts alone as rHSA and BSA continued to degrade in pH 3.5 extracts after spiking and was not due to pH sensitivity of rHSA, as proven by Figure 2-4 earlier. To improve rHSA storage stability in pH 3.5 extract, we evaluated the impact of decreasing temperature during extraction.



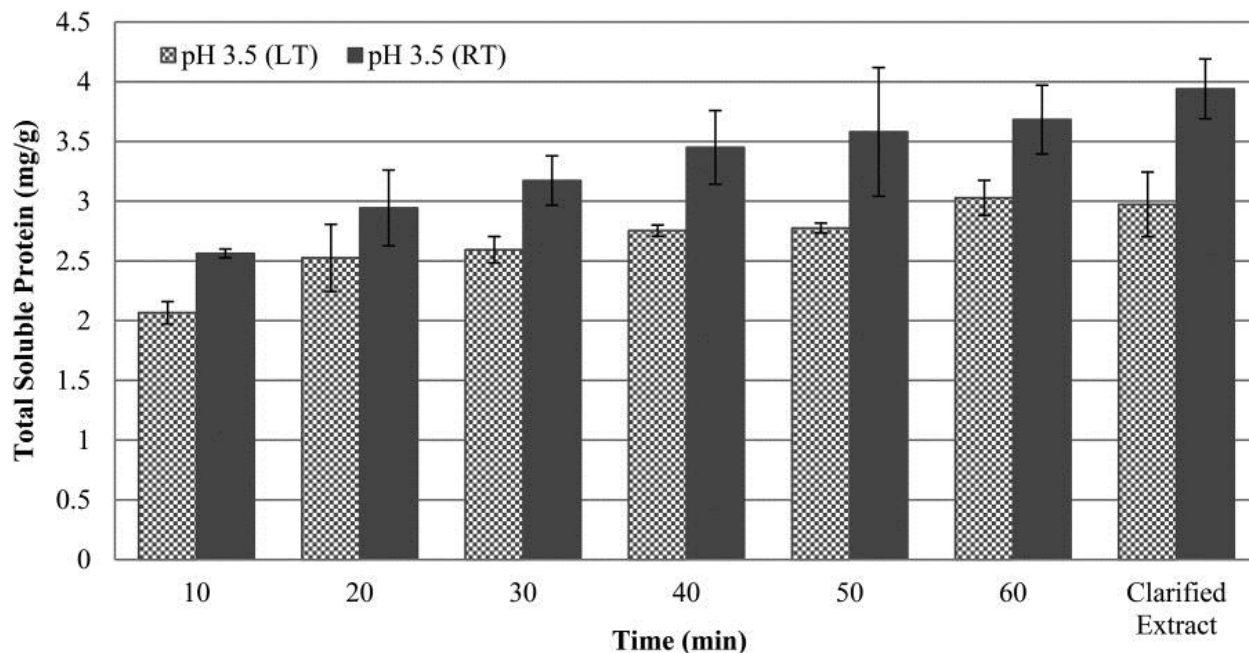
**Figure 2-5. Protein profiles of extracts by NU-PAGE® electrophoresis gel with (a) samples from the spiking of pure HSA in pH 3.5 extracts (wells 2–8) and (b) samples from the spiking of pure BSA in pH 3.5 extracts (wells 2–8). The arrows show molecular weight placement of rHSA and BSA.**



### ***Effect of Temperature on rHSA in Extracts***

To evaluate the feasibility of extracting rHSA at a lower temperature (5°C), the amount of full-length rHSA and TSP extracted over time at pH 3.5 was measured. Maintaining a low temperature of 5°C throughout extraction at pH 3.5 resulted in stability of full length rHSA, as indicated by the bands at 66.5 kDa in a Western blot (results not shown). The resulting rHSA concentrations at 10 min (2.0 mg/g flour) and in the clarified extract (2.1 mg/g flour) are similar to the rHSA concentration of pH 3.5 at room temperature at 10 min (2.2 mg/g flour). Because lower temperature typically reduces protein extractability (Farinas et al., 2007; Shukla et al., 2000), the amount of TSP and rHSA was evaluated and compared to that at room temperature.

TSP in the pH 3.5 low temperature extracts was higher by 0.5 mg/g (10 min) to 1.0 mg/g (clarified extract) than TSP in pH 3.5 extracts at room temperature. The total soluble protein concentration was 2.1 ( $\pm 0.1$ ) mg/g flour at 10 min, 2.6 ( $\pm 0.1$ ) mg/g flour at 30 min, and 3.0 ( $\pm 0.3$ ) mg/g flour in the clarified extract compared to the room temperature concentrations of 2.6 ( $\pm 0.04$ ) mg/g flour at 10 min, 3.2 ( $\pm 0.2$ ) mg/g flour at 30 min, and 3.9 ( $\pm 0.3$ ) mg/g flour in the clarified extract (Figure 2-6).



**Figure 2-6. Average TSP (mg TSP/g flour) extracted at pH 3.5 low temperature (LT) and pH 3.5 room temperature (RT) throughout extraction (0 to 60 min). The final filtrate (Clarified Extract) represents the 60 min extract after the final centrifugation and dead-end filtration. The error bars indicate standard deviation of triplicate extractions.**

The rHSA concentration in pH 3.5 extracts at a low temperature throughout extraction was very similar to the initial concentration of pH 3.5 extract at room temperature. At room temperature, rHSA concentration decreased over time while the low temperature extract maintained a stable rHSA concentration. Thus, decreasing the temperature during extraction resulted in increased stability of rHSA which may be attributed to inactive protease(s) or reduction in protease solubility or activity at the lower temperature. Further studies using room temperature extraction and then adjusting the extract to 5°C would provide additional insight into the cause of rHSA degradation in pH 3.5 extracts.

Purity of rHSA at a temperature of 5°C decreased over time due to the increase in native protein; however, the initial and final purities (96.1% to 71.5%) of the extracts (Table 1) were the highest compared to the other conditions (Table 2-5). The decrease in purity of the low

temperature pH 3.5 extracts can be attributed to the increase in TSP as the rHSA concentration remained constant.

**Table 2-5. Effect of temperature and extraction time on rHSA protein purity (%).**

pH 3.5	10 min	20 min	30 min	40 min	50 min	60 min	Clarified Ext.
<b>LT (5°C)</b>	96.1 ± 18.2	83.1 ± 14.0	83.8 ± 14.4	80.9 ± 14.2	75.0 ± 12.3	67.3 ± 17.1	71.5 ± 13.7
<b>RT (23°C)</b>	78.9 ± 17.2	64.9 ± 12.1	58.2 ± 10.5	51.4 ± 6.3	44.2 ± 5.2	38.4 ± 8.6	26.0 ± 15.6

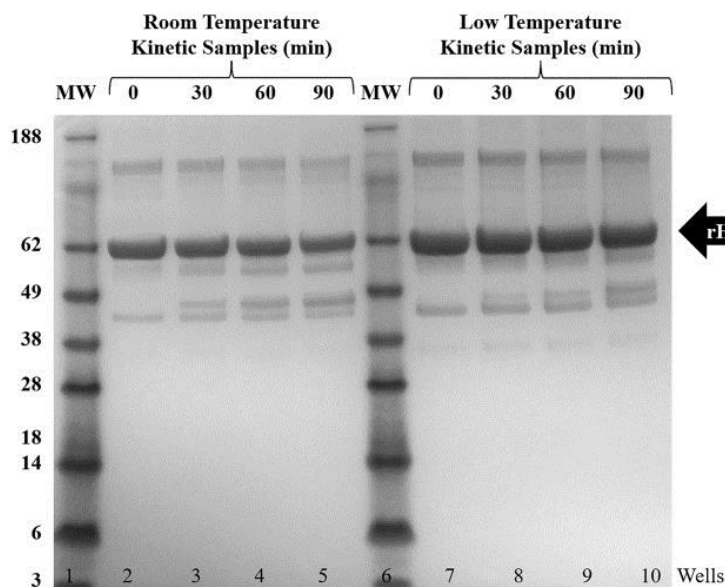
The ratio of rHSA to native protein (Table 1) was used to compare extraction conditions and to identify favorable conditions that may reduce further processing and purification costs. The ratio of the pH 3.5 low temperature extract at 10 min was fivefold higher (24.4) compared to the other conditions due to the high initial purity as well as the small amount of native protein (0.08 mg/g flour) compared to rHSA (1.98 mg/g flour). Although a decrease in the ratio is evident, this is due to the increase in native protein (0.08 mg/g flour to 0.43 mg/g flour) as the rHSA concentration remained constant, similarly to the purity. The final ratio of the clarified extract (2.5) is comparable to the pH 4.0 ratio of 2.4 which suggests extraction conditions at pH 3.5 low temperature and pH 4.0 at room temperature (Table 2-6) are more desirable than extracting at pH 3.5, 4.5, or 6.0 at room temperature. Ten min extraction at pH 4.0 is more suitable for downstream processing due to the stability of rHSA at room temperature (would not require temperature control), high initial purity, and higher rHSA concentration than low temperature pH 3.5 extraction.

**Table 2-6. Effect of temperature and extraction time on the rHSA to native rice protein concentration ratio.**

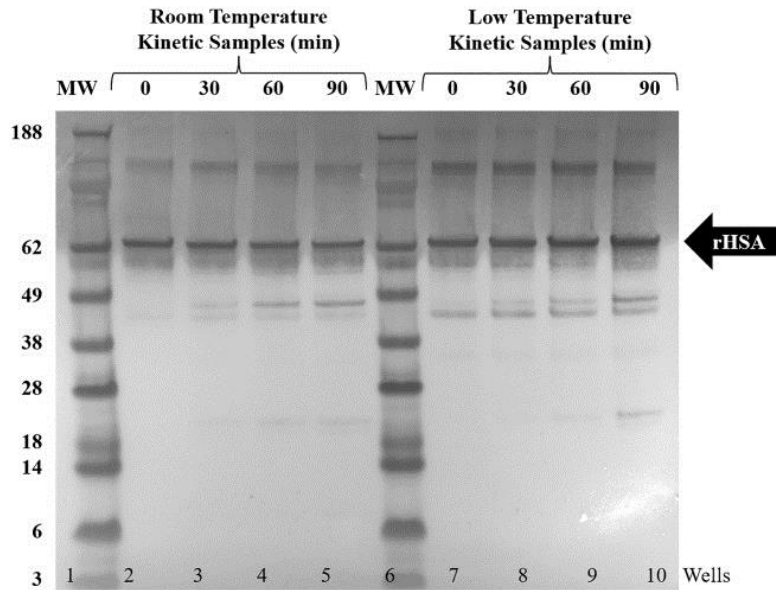
pH 3.5	10 min	20 min	30 min	40 min	50 min	60 min	Clarified Ext.
<b>LT (5°C)</b>	24.4	4.9	5.2	4.2	3.0	2.1	2.5
<b>RT (23°C)</b>	5.6	2.2	1.5	1.1	0.8	0.6	0.3

### *Stability of rHSA at Lower Temperatures in pH 3.5 Buffer*

Pure rHSA stability was tested in pH 3.5 buffer at a low temperature (5°C) to verify that decreasing the temperature did not affect rHSA. rHSA in the pH 3.5 buffer was determined to be stable based off the consistent band intensities at 66.5 kDa over time in the NU-PAGE® electrophoresis gel (Figure 2-7). The Western blot also demonstrated stability in the bands over time at 66.5 kDa (Figure 2-8).



**Figure 2-7. Protein profiles of samples by NU-PAGE® electrophoresis gel with samples from room temperature extraction at 23°C (wells 2–5) and samples from pure rHSA in pH 3.5 buffer at 5°C (wells 7–10). The arrow shows molecular weight placement of rHSA at 66.5 kD**



**Figure 2-8. Protein profiles of extracts by Western blot with samples from room temperature extraction at 23°C (wells 2–5) and samples from pure rHSA in pH 3.5 buffer at 5°C (wells 7–10). The arrow shows molecular weight placement of rHSA at 66.5 kDa.**

Both NU-PAGE<sup>®</sup> electrophoresis gel and Western blot showed pure rHSA stability at reduced temperatures in pH 3.5 buffer. These results further indicate that the instability of rHSA could be due to protease activity and the reduced temperature may have decreased activities of the proteases.

## Conclusions

Extracting at pH 4.0 50 mM sodium citrate with 50 mM sodium chloride for 10 min at room temperature was most favorable for maximizing rHSA concentration and achieving the highest ratio of rHSA to native protein. This study demonstrates the degradation of rHSA in pH 3.5 extract but not higher pH extracts. Results indicate this is not due to the effect of pH and time on stability or rHSA stability alone since pure rHSA is stable in pH 3.5 buffer (stable concentration and presence of rHSA band) but degrades if pure rHSA or BSA is spiked into pH 3.5 extract. While 60% of the rHSA extracted at 10 min degraded after the 60 min extraction and

clarification, rHSA did not degrade during pH 4.0, 4.5, or 6.0 extractions. However, degradation did not occur in pH 3.5 extracts if the extraction temperature was reduced to 5°C. In addition, rHSA purity levels were initially as high as 79% for pH 3.5 and pH 4.0 at room temperature.

The expression levels demonstrated by this study are very high compared to the reported 11.1% from transgenic chloroplasts (Daniell et al., 2005; Fernández-San Millán et al., 2003). rHSA constituted up to 10.0% of total protein (Huang et al. 2010), but by modifying the extraction conditions, we were able to extract rHSA as 79% of TSP at room temperature and 96% of TSP at a reduced temperature. Thus, examining extraction conditions is necessary. Purity levels this high could facilitate cost effective purification for numerous applications. However, the amount of extracted rHSA combined with purity and stability should be considered for purification. An extraction pH of 4.5 or 6.0 provides more rHSA for purification although the lower purity can increase the number of purification steps. Less rHSA is extracted at lower pH conditions (pH 3.5 at low temperature and pH 4.0) but the high purity can reduce the costs by requiring less purification steps. Downstream processing with pH 3.5 room temperature extract is not desired due to the instability of rHSA. The results observed in this study contradicts experimental observations in transgenic rice (Nochi et al., 2007), corn (Menkhaus et al., 2004), canola (Menkhaus et al., 2004) seeds as well as current literature which indicates that seed-based expression is primarily desirable due to the inherent stability of a recombinant protein. Despite this observation, the expression level is one of the highest reported and the degradation of rHSA was controlled by simple pH or temperature adjustment.

Expressing a recombinant protein to such levels could potentially impact expression of proteases or make recombinant proteins more susceptible but further research would be required. Future work should include analyzing protease activity in extracts and the biological activity of

rHSA. The conformational stability of rHSA in extracts at pH 3.5 should also be analyzed using fluorescence spectroscopy and/or CD-spectroscopy and further characterization of the clarified extract using mass spectrometry is necessary. To our knowledge, degradation of a recombinant protein in rice seed extract has not been reported and thus warrants further investigation.

### **3. Purification of Recombinant Human Serum Albumin from Transgenic Rice Extracts Through Adsorption**

#### **Abstract**

Transgenic plant systems have successfully been used to express a variety of recombinant proteins, including rice seed-expressed recombinant human serum albumin (rHSA). The development of an efficient and integrated rHSA extraction and purification process would allow rHSA to be used for various medical applications such as a stabilizer in vaccinations without the risk of contamination of pathogens and viruses. Extraction is a critical step that determines recombinant protein concentration and purity and the type and quantity of impurities that must be removed during purification. Previous studies indicated that pH 3.5 extraction resulted in high purity (79%) but rHSA was unstable and degraded over time. Extraction at pH 4.5 and 6.0 provided stable rHSA yields but higher native rice protein concentrations, which would increase purification requirements. To develop an integrated process, the effects of extraction conditions on subsequent purification using ion exchange chromatography must be evaluated. The impact of extraction conditions on purification efficiency was evaluated through adsorption kinetics with pure rHSA. Once suitable conditions for rHSA binding were identified, adsorption of rHSA from transgenic rice extracts was evaluated. Anion exchange chromatography with Q Sepharose™ FastFlow® resin was selected because rHSA has an isoelectric point of 4.7. Acetate and citrate buffers (pH adjusted with TRIS) and phosphate buffers were tested for rHSA binding studies at pH 8.0. Acetate buffer resulted in the lowest conductivity after pH adjustment, which increased the binding capacity of rHSA and was selected to minimize processing operations and cost. The rHSA saturation binding capacities in pH 4.0, 4.5, and 6.0 extracts adjusted to pH 8.0 varied from 69 mg rHSA/mL resin (pH 4.5 extract adjusted to 8.0) to 79 mg rHSA/mL resin (pH 4.0



extracted adjusted to 8.0). Purification studies were then completed at 10% binding capacity to evaluate resultant rHSA purities and yields. Purity and yield after anion exchange chromatography varied with extraction pH with pH 4.5 extract (adjusted to pH 8.0) resulting in the most desirable purity and yield (>95% purity and 76% yield). Purification results of rHSA were analyzed for the development of an integrated, cost-efficient downstream process.

## **Introduction**

Genetically modified organisms (GMO's) have become a major success in the agricultural and biological fields, specifically plants which offer the utilization of a host without risk of spreading or carrying human diseases (Goldstein and Thomas, 2004). Many GMO's have been used in the production of compounds for pharmaceutical purposes as well as production of food for human consumption. Transgenic plants have become an alternative to traditional systems for recombinant protein expression due to the exclusion of possible mammalian pathogen and virus contamination as well as the high expression level of recombinant proteins which reduce purification requirements and create a more cost-effective method (Twyman et al., 2003). Rice is a relatively popular choice for a seed-based transgenic plant host due to its generally regarded as safe status (Nandi et al., 2002), established production infrastructure, relatively high protein content, low risk of contamination by human pathogens, low phenolic concentrations, and the presence of endogenous protease inhibitors that prevent degradation of recombinant proteins throughout processing (Wilken and Nikolov, 2006).

Multiple recombinant proteins have been expressed in transgenic rice seed such as recombinant human serum albumin (rHSA), a 66.5 kDa acidic protein comprised of 585 amino acids. With an isoelectric point (pI) of 4.7, this protein comprises majority of human blood

plasma and is synthesized in the liver. Human serum albumin (HSA) enables the transportation of endogenous and exogenous compounds such as hormones, fatty acids, amino acids, and metals (Otagiri and Chuang, 2009). With the many functions of HSA, the protein has been used in medical applications as an excipient for vaccines and therapeutic drugs (Marth and Kleinhappl, 2001), blood volume expander, and treatment for liver cirrhosis, hemorrhagic shock, burns, and hypoproteinemia (He et al., 2011), carrier of oxygen (Tsuchida et al., 2009), and nanodelivery of drugs (Cai et al., 2006).

To utilize the benefits of recombinant proteins such as HSA from transgenic plants, an efficient downstream process that includes primary recovery and purification steps must be developed. Extraction is a critical step of primary recovery that can reduce the number and cost of subsequent purification steps. This is done by determining the recombinant protein concentration and purity, type and quantity of impurities, and volume of extract to be processed (Wilken and Nikolov, 2012a). Our previous extraction studies evaluated the effect of pH and time on rHSA extraction and stability of rHSA in transgenic rice extracts. These extraction studies showed that the amount of rHSA extracted was highest at pH 6.0 (compared to pH 3.5, 4.0, and 4.5) but co-extraction of native rice protein resulted in a lower HSA purity in clarified extract than at pH 3.5 and 4.0. Due to high expression levels of rHSA, 2.2 ( $\pm 0.5$ ) mg rHSA per g flour was extracted at pH 3.5 with 78% purity, 5.4 ( $\pm 1.3$ ) mg/g at pH 4.0 with 78% purity, 7.4 ( $\pm 0.9$ ) mg/g at pH 4.5 and 57% purity, and 9.6 ( $\pm 1.7$ ) mg/g at pH 6 with 65% purity (all in 50 mM buffer with 50 mM sodium chloride). pH 3.5 extracts were unstable due to rHSA degradation over time and thus, resulted in pH 3.5 being unsuitable for further ion exchange adsorption studies even though this condition maximized rHSA purity and minimized native protein concentrations more effectively than at other pHs (Sheshukova and Wilken, 2018).

Stability studies showed rHSA degradation was not due to pH instability but possible protease activity at low pH. Decreasing the extraction temperature improved stability and prevented degradation of pH 3.5 extracts. However, reducing the temperature adds an additional cost to processing and is not feasible. pH 4.5 and 6.0 resulted in rHSA stability, but did not minimize native rice protein as total soluble protein (TSP) concentrations increased with pH. (Sheshukova and Wilken, 2018). Although higher pH conditions resulted in lower rHSA purities than at pH 3.5, extraction at pH 4.0, 4.5, or 6.0 are potential suitable conditions for generating an integrated process for extraction and purification.

There are many chromatographic methods for protein purification such as hydrophobic interaction, ion-exchange, size exclusion, and affinity chromatography. Ion-exchange chromatography is used to concentrate, separate, and purify substances (Jungbauer and Hahn, 2009) through electrostatic attraction between the solute and charged groups of the resin. Ion-exchange resin is selected based on its wide availability, low cost, and durability (Wilken and Nikolov, 2012a) and the physiochemical properties (isoelectric point, hydrophobicity, molecular weight, etc.) of the target protein. This method can give high resolution of macromolecules and is a less expensive alternative to other methods previously used for rHSA purification such as affinity chromatography and hydrophobic interaction chromatography. Recombinant HSA was purified to >95% purity with a 48% recovery from transgenic tobacco suspension culture using Capto™ Blue as an affinity medium (Sun et al., 2011). Another study resulted in a 99% rHSA purity from *Pichia pastoris* broths after hydrophobic interaction chromatography (Dong et al., 2012). The salt requirement for hydrophobic interaction chromatography (HIC) would increase purification costs as an additional buffer exchange for salt removal following purification would

be required. For these reasons, an alternative chromatography method such as ion-exchange is desired.

Ion-exchange allows for pH adjustment of solution for adsorption instead and elutes solutes by changing pH or ionic strength through use of a salt gradient (Doran, 2013). Purification of plant-derived recombinant proteins is primarily done through adsorption chromatography and acidic proteins such as rHSA can be captured by anion-exchange chromatography to achieve high purities and concentrations which is common in the biotechnology industry (Wilken and Nikolov, 2012a). Binding conditions affect protein charge including pH, ionic strength, and presence of oppositely charged molecules, which can impact the binding capacity of any protein including rHSA. The determination of protein binding capacity is critical as it dictates the amount of resin required for adsorption. A low binding capacity would require a larger column volume of resin and increase process cost. Selection of buffer is also dependent on the isoelectric point of the protein as it is recommended that buffer pH must be at least one pH unit above or below the pI to have sufficient charge difference between the protein and resin. In general, a protein will carry a net negative charge when the pH is greater than the pI and thus, will be suited for anion exchange chromatography. A net positive charge results when the pH is below the pI, allowing the protein to bind to cation exchange chromatography resin. Simple anions should be used for buffers in anion exchange chromatography such as chloride or acetate and simple cations for cation exchange chromatography such as sodium or potassium (Harrison et al., 2015). Buffers should also be selected with a pKa value close to the selected pH for adsorption which is dependent on the protein pI (Jungbauer and Hahn, 2009). Increasing the ionic strength can cause proteins to aggregate, undergo a conformational change, and cause non-

specific binding. However, it can also improve protein solubility by reducing the zeta potential and reduce binding of proteins with a low affinity to the resin, ultimately enhancing separation.

Selection of extraction conditions most compatible with subsequent adsorption to ion exchange resin is critical in reducing the number of steps and process costs. Studies recommend a buffer with low ionic strength to increase protein-to-resin binding by minimizing interference of buffer ions. Extraction buffers often include sodium chloride which can enhance extraction of the recombinant protein, but can also reduce protein binding capacity. Concentrations between 10 mM to 100 mM of buffer (conductivity of 1 mS/cm to 5 mS/cm) are chosen to improve binding during the flow through phase of chromatography (Jungbauer and Hahn, 2009) which are commonly used in charge based adsorption. Maintaining a constant pH throughout binding is critical as a pH shift can reduce the binding affinity and impact the adsorption behavior.

Once extraction and purification conditions are optimized, investigation for the development of a cost-effective processing method must be conducted. With a high demand of 500 tons of HSA per year worldwide and a limited supply of HSA primarily from collected human plasma (Fernández-San Millán et al., 2003). An alternative expression system, such as rice seed, can take advantage of minimized risks of plasma-derived HSA that include human immunodeficiency virus and hepatitis (Chamberland et al., 2001). The objectives of this study were to determine suitable conditions for anion exchange adsorption and purification of rHSA which are compatible with extraction by: 1) evaluating adsorption kinetics to determine an appropriate binding buffer and ionic strength, 2) continually challenging the resin with extract through consecutive batch binding to simulate dynamic binding for determining residence time and binding capacity, and 3) evaluating the dynamic binding capacity of rHSA at saturation and then evaluating purification parameters such as yield and purity at 10% breakthrough.

## **Materials & Methods**

### **Materials**

Transgenic rice flour and pure rHSA were provided by Ventria Bioscience (Junction City, KS). The rice flour was stored at 4°C throughout the research. Human serum albumin (>96% purity, rice-derived) and bovine serum albumin (>98% purity) were purchased from Sigma-Aldrich (St. Louis, MO) and used as analytical standards. All other reagents and supplies were purchased from VWR Scientific and GE Healthcare Life Sciences.

### **Analytical Methods**

#### ***Total Protein Quantification***

Total soluble protein (TSP) 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 (Thermo Scientific, Rochester, NY). Absorbances at 595 nm were measured by a SpectraMax Pro 384 microplate reader using SoftMaxPro V4.6 software. Samples were analyzed in triplicate and diluted as necessary to be within the linear range of the standard curve.

#### ***Protein Analysis by NU-PAGE®***

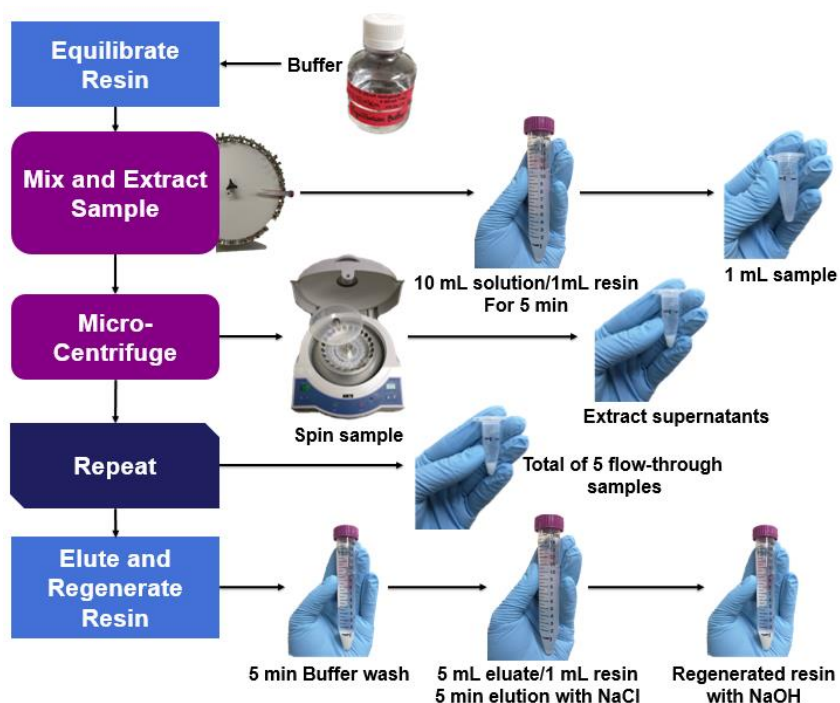
The protein profiles of extracts and chromatography fractions were evaluated by electrophoresis as described by Laemmli (1970). Samples were loaded on 4-12% bis-tris gels under reducing conditions and ran according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Clarified extracts pH adjusted for adsorption, supernatants after adsorption (as a function of time), and elution samples were prepared for gel electrophoresis immediately after clarification by centrifugation.

## Experimental Methods

### *Buffer Selection through Consecutive Batch Binding*

Selection of buffer conditions to maximize adsorption of rHSA on the strong ion exchange resin, Q Sepharose™ FastFlow® was determined through completion of resin adsorption kinetics. Buffers (25 mM sodium citrate or 25 mM sodium acetate with 25 mM sodium chloride) were adjusted from 4.5 to pH 8.0 using 25 mM tris(hydroxymethyl)aminomethane (TRIS) for an integrated process compatible with rHSA extraction conditions. 25 mM sodium phosphate was created at the same ionic strength and pH adjusted to 8.0 with sodium hydroxide for buffer comparison and to maintain pH 6.0 sodium phosphate extraction conditions. The conductivities of buffers were measured by EC Meter Model 2052 (VWR Scientific) and reported in mS/cm. Each buffer was used to equilibrate 1 mL of Q Sepharose™ FastFlow® resin (GE Healthcare Life Sciences, United Kingdom) by mixing 10 mL of buffer with the resin on a rotator as visible in Figure 3-1. After mixing, the solution was centrifuged at 5,000 x g for 5 min and then buffer removed by transfer pipette. New buffer was added and the process was repeated four times to ensure adequate equilibration of the resin. Ten mL of purified rHSA (0.6 mg/mL) was pH adjusted to 8.0 with 25 mM TRIS (pH 4.5 citrate and acetate buffer) and sodium hydroxide (pH 6.0 phosphate buffer) before added to the equilibrated resin and mixed continuously for 5 min. The selected. The selected rHSA concentration was based on the approximate concentration of rHSA in transgenic rice extracts and the binding time was determined through previous adsorption kinetics where a sample was taken every 2 min for a total of 10 min of contact with the ion exchange resin. The selected times were based on potential residence times for rHSA adsorption in a packed chromatography column. A 5 min residence time resulted in adequate rHSA adsorption to resin. After 5 min, the

sample was centrifuged (VWR® Micro 2416 microcentrifuge) at 9,100 x g for 1 min to separate the resin from binding buffer and a sample of the supernatant was taken. This process was repeated a total of five times to simulate dynamic binding of pure rHSA to a chromatography column after which the resin was washed with 10 mL of equilibration buffer and eluted with 5 mL of 0.5 M sodium chloride and 5 mL 1 M sodium chloride.



**Figure 3-1. Process diagram of consecutive batch binding to simulate dynamic binding of pure rHSA in citrate, acetate, and phosphate buffer.**

TSP of the supernatant samples was determined by Bradford assay (Bradford, 1976) in a microtiter format, using a Coomassie Plus Assay kit containing Coomassie Plus reagent and BSA as a standard (Thermo Scientific, Rochester, NY). The amount of rHSA bound was analyzed through Bradford microplate assay by subtracting the unbound analyzed mass (TSP concentration multiplied by loaded volume) from the initial loaded mass (TSP concentration



multiplied by loaded volume) and reporting in mg rHSA per mL resin. The percentage of rHSA bound was calculated by dividing the mass of bound protein by the initial loaded mass.

### ***Conductivity and Protein Load Effects on rHSA Adsorption***

For further ion exchange adsorption studies, pH adjusted acetate buffer was selected based on initial buffer screening results. To evaluate if dilution of the purified rHSA solution would increase adsorption, rHSA load solution was diluted with water (to evaluate conductivity effect) or binding buffer (to evaluate rHSA concentration effect). Resins were equilibrated as indicated above using the corresponding buffers (prepared same as the load solution with rHSA). Ten mL of purified rHSA (6 mg at 2.2 mS/cm), purified rHSA diluted two-fold with 25 mM sodium acetate and 25 mM sodium chloride buffer adjusted to pH 8.0 (3 mg at 4.2 mS/cm), and purified rHSA diluted two-fold with water (6 mg at 4.2 mS/cm) were used with equilibrated resin for adsorption kinetics. Samples were taken at 2 min and 8 min (minimum and maximum times) and resin and solution was separated by centrifugation at 9,100 x g for 1 min, and the supernatant was removed for analysis. The resin was then washed with 10 mL of equilibrium buffer for 5 min to evaluate rHSA concentration in the interstitial space. Bound rHSA was eluted by adding 1 mL of 1 M sodium chloride in pH 8.0 buffer and mixing for 10 min. Eluant was obtained through solid-liquid separation by centrifuge. Process was repeated with 1 mL of 1.5 M sodium chloride in pH 8.0 buffer. Samples were analyzed by Bradford TSP microplate assay.

### ***Adsorption Kinetics of rHSA in Transgenic Rice Extracts***

Once suitable conditions for adsorption of purified rHSA were identified, adsorption of rHSA from transgenic rice extracts was evaluated to determine the impact of rice flour impurities on binding to ion exchange resin. Previous extraction studies determined that an extraction time of 20 min was sufficient for rHSA solubilization from transgenic rice flour (Sheshukova and

Wilken, 2018). To prepare rHSA-expressed transgenic rice extracts, 20 g of rice flour was mixed with extraction buffer for 20 min and maintained at extraction pH with 1 M acetic acid. The extraction buffers used were pH 4.0 and 4.5 in 25 mM sodium acetate with 25 mM sodium chloride and pH 6.0 in 25 mM sodium phosphate with 25 mM sodium chloride. After 20 min, the mixture was centrifuged (Thermo Scientific Sorvall RC 6 Plus Centrifuge) at 10,000 x g for 15 min to separate the insoluble solids and then filtered with a 0.45 µm SFCA filter (Thermo Fisher Scientific, Waltham, MA). Prior to adsorption, pH 4.0 and pH 4.5 filtered extract was adjusted to pH 8 with 1 M TRIS to improve buffering capacity at the adjusted pH. pH 6.0 filtered extract was adjusted to pH 8 with 1 M sodium hydroxide. Each pH-adjusted extract was filtered with a 0.45 µm SFCA filter (Thermo Fisher Scientific, Waltham, MA). For the adsorption studies, 10 mL of pH-adjusted extract was mixed with 1 mL of Q Sepharose™ FastFlow® resin continuously on a rotator as seen in Figure 3-1. Kinetic samples at 1, 2, 4, 6, 8, and 10 min were taken, centrifuged to remove suspended resin, and the supernatant was collected for analysis. The resin was then washed with 10 mL of equilibrium buffer for 5 min to evaluate rHSA concentration in the interstitial space. Bound rHSA was eluted by adding 1 mL of 1 M sodium chloride in pH 8.0 buffer and mixing for 10 min. Eluant was obtained through solid-liquid separation by centrifuge. Process was repeated with 1 mL of 1.5 M sodium chloride in pH 8.0 buffer. Samples were analyzed by Bradford TSP microplate assay and immediately prepared (Laemmli, 1970) for NU-PAGE® gel electrophoresis.

### ***Static Binding Capacity of rHSA in Transgenic Rice Extracts***

Consecutive adsorption of the extracts was evaluated to determine the static binding capacity of rHSA using a 5 min residence time. This was performed by adding 10 mL of pH-adjusted and filtered extract to 1 mL of equilibrated Q Sepharose™ FastFlow® resin as

previously described in the section above. The extract and resin slurry was mixed on a rotator for 5 min before a sample was taken and centrifuged for 1 min at 9,100 x g. The remaining solution was centrifuged at 10,000 x g for 15 min and remaining supernatant was removed before another 10 mL of extract was added to the resin. The process was then repeated for a total of seven times before the resin was washed with 10 mL of equilibrium buffer for 5 min. The resin slurry was centrifuged again and the equilibrium buffer was carefully disposed of before elution. 5 mL of pH-adjusted 25 mM sodium acetate with 0.5 M sodium chloride buffer was mixed with the resin for 5 min. The resin slurry was centrifuged and elution buffer disposed of before the process was repeated with pH-adjusted 25 mM sodium acetate with 1 M sodium chloride. Samples were analyzed by Bradford TSP microplate assay and immediately prepared (Laemmli, 1970) for NU-PAGE<sup>®</sup> gel electrophoresis.

#### ***Dynamic Binding Capacity of rHSA in Transgenic Rice Extracts Using ÄKTA Pure***

Lab-scale anion exchange chromatography was performed using the ÄKTA Pure System interfaced with UNICORN<sup>™</sup> 7 control software and a 1 mL HiTrap Q Sepharose<sup>™</sup> FastFlow<sup>®</sup> column (7 mm x 25 mm) (GE Healthcare Life Sciences, United Kingdom). Extract was prepared according to method above using pH 4.0 and 4.5 25 mM sodium acetate with 25 mM sodium chloride and pH adjusted to 8.0 with 25 mM TRIS and pH 6.0 25 mM sodium acetate with 25 mM sodium chloride and 25 mM TRIS pH adjusted with 1 M sodium hydroxide prior to loading on column. The system was equilibrated for 20 column volumes (CV) with pH-adjusted to 8.0 25 mM sodium acetate with 25 mM sodium chloride and 25 mM TRIS. 50 mL of pH 4.0 or 4.5 adjusted to 8.0 rice extract and 55 mL of pH 6.0 extract adjusted to 8.0 was loaded on the column at 0.2 mL/min (30 cm/h) to fully saturate the resin. Afterwards, the column was washed with equilibrium buffer for 5 CV to remove unbound materials and liquid within the interstitial space.

A linear salt gradient was applied to the column with 1 M sodium chloride for 20 CV before a final column wash for 5 CV. Fractions (5 mL) were collected during sample load and wash and 2 mL fractions were collected during elution. Load volumes were decreased to 26 mL of pH 4.0 and 4.5 extracts pH adjusted to 8.0 and 23 mL of pH 6.0 extract pH adjusted to 8.0 for 10% rHSA breakthrough. Samples were analyzed by Bradford TSP microplate assay and immediately prepared (Laemmli, 1970) for NU-PAGE<sup>®</sup> gel electrophoresis.

### ***rHSA Purity Quantification Using Affinity Chromatography***

Lab-scale affinity chromatography for HSA quantification in anion exchange elution fractions was performed using the ÄKTA Pure System interfaced with UNICORN<sup>™</sup> 7 control software with and a 1 mL HiTrap Blue Sepharose<sup>™</sup> High Performance column (GE Healthcare Life Sciences, United Kingdom), (7 mm x 25 mm). To prepare the anion exchange elution fractions for analysis, fractions were pH adjusted to 7.0 and diluted to a concentration of <1 mg/mL and a conductivity between 5 to 5.5 mS/cm. The system was equilibrated for 10 CV with pH 7.0 25 mM sodium phosphate (5.3 mS/cm) before 2 mL of sample was loaded at 1 mL/min (155 cm/h). After loading, the column was washed with 5 CV equilibrium buffer and a step elution with 10 CV of 2 M sodium chloride (130 mS/cm) was applied. 1 mL fractions were collected and analyzed by Bradford microplate assay. Elution peaks were also analyzed by integrating the absorbance vs volume curve in UNICORN<sup>™</sup> 7 using Equation 3-1. Absorbances were converted to concentrations using an extinction coefficient of 0.549 mL/mg·cm (Sancataldo et al., 2014). The concentration of rHSA ( $C$ ) in the elution peaks was analyzed by the Beer-Lambert law using an HSA extinction coefficient ( $\epsilon$ ) of 0.549 mL/mg·cm by measuring the absorbance ( $A$ ) and the flow cell path length ( $l$ ).

$$C_{rHSA} = \frac{A}{\varepsilon \times l} = \frac{A}{0.549 \frac{mL}{mg \cdot cm} \times 0.2 \text{ cm}}$$

**Equation 3-1. Beer-Lambert law used to calculate rHSA concentration.**

## **Results & Discussion**

### **Identification of Compatible Purification Conditions**

Our overall research objective is to develop an integrated process for rHSA extraction and purification and thus, the impact of the potential extraction buffer systems for rHSA binding must be considered. For each buffer system, the pH was adjusted to 8.0 for adsorption for rHSA to carry a sufficient negative charge for binding to the positively charged anion exchange resin. The three pHs selected were from previous extraction studies that resulted in rHSA stability at pH 4.0, 4.5, and 6.0 (Sheshukova and Wilken, 2018). Adsorption studies using kinetics can be beneficial in determining the residence time, or the length of time optimal for rHSA adsorption to the resin which can be used to determine the appropriate linear velocity of solution flowing through the column in dynamic binding. Determining the binding capacity of rHSA by performing consecutive batch binding to simulate dynamic binding is useful to calculate the necessary column volume of resin required to bind a specific amount of rHSA. This can aid in determining the extract load volume required to saturate the resin, or occupy all binding sites. Results can provide insight on rHSA adsorption behavior by plotting the percent protein breakthrough over protein load and evaluating the slope of each curve. Breakthrough curve shape can indicate high or low product loss if shallow or sharp respectively and the flexibility of loading until a certain percent breakthrough is reached. Using the results of adsorption and consecutive batch binding studies to determine appropriate conditions for dynamic binding, the amount of protein bound and eluted can be calculated and elution purities and yields analyzed.

The determination of rHSA purity after purification is important as it must be >95% pure for reagents and chemicals and >99% for therapeutic drugs (Krishnan and Woodard, 2014).

### ***Buffer Selection Through Consecutive Batch Binding***

Selection of optimal ion-exchange adsorption conditions such as pH and ionic strength is critical in the development of an efficient purification process for recombinant proteins such as rHSA by improving binding capacity which minimizes resin volume requirements and potential process costs. Evaluation of purified rHSA at conditions favorable for rHSA adsorption must be conducted prior to evaluation of rHSA binding in transgenic rice extracts. Binding conditions were tested with purified rHSA in acetate, citrate, and phosphate buffers. Results of the percentage of purified rHSA bound to the Q Sepharose™ FastFlow® resin were as a function of buffer system.

Buffer selection for adsorption must be compatible with extraction conditions and the results demonstrate that citrate possessed poor binding in comparison to acetate and phosphate as less than 7% of purified rHSA bound. Although citrate was used as an extraction buffer in previous studies, adsorption studies suggest it is not suitable for anion exchange. Phosphate and acetate buffer resulted in higher adsorption of rHSA with 42% and 85% of pure rHSA binding to resin respectively. However, all buffer systems were successful in eluting the majority of bound rHSA from the resin as 20%, 67%, and 78% of protein eluted using citrate, phosphate, and acetate buffer systems respectively. Results also showed a binding capacity of 49 mg pure rHSA/mL resin with acetate buffer compared to 23 mg pure rHSA/mL resin for phosphate buffer and 6 mg pure rHSA/mL resin for citrate buffer. Ultimately, acetate buffer was chosen for further adsorption studies due to its higher binding results and financially being more cost-effective compared to phosphate as monobasic and dibasic phosphate is require for buffers. Low

binding in citrate and phosphate buffer could be due to the high conductivities (>10 mS/cm) as acetate buffer and pure rHSA in acetate buffer possessed conductivities below 6 mS/cm.

Phosphate has also been known to interact with positively charged anion exchangers as it absorbs to the stationary phase at a higher affinity as half of the phosphate ions carry two charges in sodium phosphate buffer (Vajda et al., 2016).

### ***Conductivity and Protein Load Effects on rHSA Adsorption***

The effect of conductivity and rHSA load volume was observed to determine the effect of ionic strength and concentration on rHSA binding to resin. The conductivity of solution during charged-based adsorption is important as it can affect the binding capacity and purification costs. Increasing the conductivity can decrease the electrostatic repulsion encountered by impurities with the recombinant protein bound at the resin surface (Hardin et al., 2009), thus reducing protein-resin interactions. The amount of target protein loaded on the column is also important as loading until saturation results in product loss in the flow through.

Conductivity and protein load effects did not impact the amount of rHSA bound to resin as 100% of rHSA was bound for all time samples at all conditions (Table 3-1). Unexpectedly, the amount of rHSA eluted was quite low (33% - 52%) given that elution from ion exchange resins is typically performed using up to 1 M sodium chloride or binding buffer sodium chloride concentrations between 20 mM to 50 mM. Poor elution could be due to the volume of eluate (1 mL or 1 CV), hydrophobic effects of organic compounds (Rahmani and Mohseni, 2017) in the extract, or possible precipitation of protein in solution. Further studies conducted were maintained at a conductivity closer to the binding buffer (4-7 mS) to improve protein and resin interaction as suggested by Jungbauer and Hahn (2009). Protein binding improvement from initial buffer testing (improved from 50% to 100%) may have been caused by pH instability as

acetate buffer used for extraction was pH adjusted for anion exchange adsorption and the required pH was beyond its buffering capacity. This may have created localized pH shift which impacted adsorption efficiency.

**Table 3-1. Conductivity and concentration effect on purified rHSA adsorption.**

Sample	Conductivity (mS)	Load (mg)	% rHSA Bound	% Eluted
No dilution	4.2	6.18	100	39.1
2-fold dilution with buffer	4.2	2.98	100	32.8
2-fold dilution with water	2.2	2.92	100	51.9

### *Adsorption Kinetics of rHSA in Transgenic Rice Extracts*

To select preliminary anion exchange adsorption conditions (buffer selection, pH, etc.), our previous extraction conditions and results were considered. Extraction in pH 3.5 and 4.0 buffer resulted in high initial purities (78%) but rHSA concentrations were low and rHSA degraded over time in pH 3.5 buffer. However, pH 3.5 rHSA extracts were stabilized by reducing the extraction temperature below room temperature but this is an additional processing cost, which makes this condition unsuitable for an integrated extraction and purification process. rHSA concentration in pH 4.0, 4.5, and 6.0 extracts were 2.5, 3.4, and 4.4 -fold higher than in pH 3.5 extract at 10 min and remained stable over time. Therefore, the three conditions in pH 4.0, 4.5, and 6.0 buffer were selected for further purification studies.

Adsorption of rHSA from pH 4.0, 4.5, and 6.0 was then evaluated by adjusting extracts to binding pH 8 to evaluate the impact of native rice extract impurities on HSA adsorption. Extracts were pH adjusted to increase the difference between the binding pH and pI of rHSA (4.7) and increase the charge on HSA and improve adsorption efficiency. Acetate buffers were pH adjusted with TRIS as pH 8.0 is beyond the buffering capacity of acetate. Addition of TRIS allowed for improved buffering capacity at the adsorption pH. Typical buffers used for anion

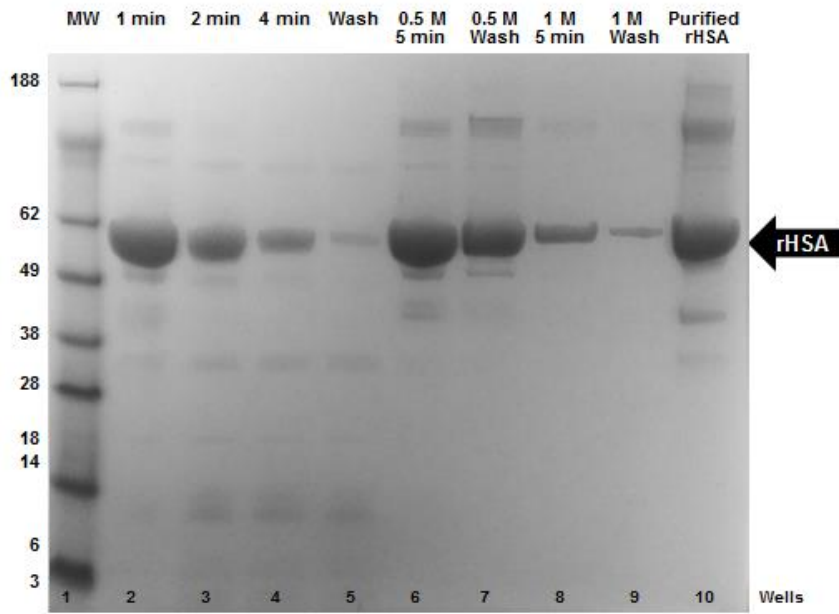


exchange chromatography include TRIS-HCl (Jiang et al., 2015), TRIS, L-histidine, ethanolamine (dos Santos et al., 2017) *etc.*, while acetate, citrate, and phosphate buffers are commonly used with cation exchange resin (Pabst and Carta, 2007) due to the low buffering ranges of each buffer varying from 3 mS/cm to 8 mS/cm. However, these buffers were selected because extraction is optimal in the acidic pH range and these ions have buffering capacity at the specified extraction pH. As anion exchange is performed at pHs above the pI, higher buffering capacities are required, thus the pH 4.0 and 4.5 acetate buffers were pH adjusted above the pI of rHSA (4.7) with TRIS to improve the buffering capacity at pH 8.0.

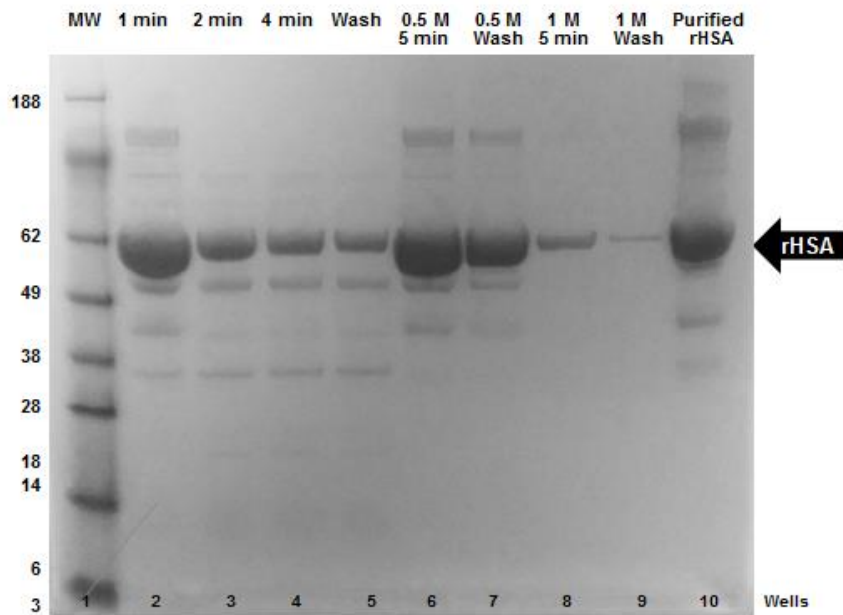
Gel electrophoresis was used to evaluate molecular weight profiles in the initial pH adjusted extract (load) and flow through (Figure 3-2) to establish the amount of bound rHSA over time. As rHSA binds to the resin ligand surface, the resin saturates and prevents anymore binding of rHSA. Observing the rHSA band can help determine the length of time necessary for rHSA to bind to the resin ligand surface. This binding time is beneficial in determining the appropriate residence time or the period of time necessary for extract to flow through the column for full rHSA binding, which allows for calculation of the corresponding linear velocity and load flow rate. The efficiency of binding was evaluated by the presence or absence of rHSA in the resin binding supernatants. The extent of rHSA adsorption can be assessed by the lack of or presence of rHSA in the resin supernatant. Adsorption of rHSA to the resin is reflected in a depletion, reduction, or removal of rHSA from the extract feed as measured by the amount of rHSA remaining in the liquid solution (supernatant). Relative concentrations of rHSA remaining were determined by observing band intensities at the 66.5 kDa molecular weight. Purified rHSA was used at a 1 mg/mL concentration to compare to other samples. Figure 3-2a shows the molecular weight profiles of pH 4.0 extract adjusted to 8 (load), adsorption supernatant samples

at 2 and 4 min and the subsequent wash. The gel also shows the elution sample protein profile using 0.5 M sodium chloride buffer and 1 M sodium chloride buffer. When comparing wells, there is a noticeable decrease in only the rHSA band intensity (other rice protein band intensities did not change). For example, the 30 kDa protein remained in solution as rHSA was bound to the resin (as observed in wells 3 and 4). The elution wells indicate rHSA is being eluted, however, rHSA purity analysis is required to determine if purification was achieved by comparing to the initial extract purity. Figure 3-2b and c show similar results except with pH 4.5 and pH 6.0 extracts adjusted to pH 8.0, respectively. All gels show depletion of rHSA band intensity from the resin supernatant samples, which is desired for optimal purification. TSP increase is visible in non-rHSA bands in supernatants after incubation of pH 4.0 extract (adjusted to 8.0) and pH 6.0 extract (adjusted to 8.0) with resin. This is due to the increase in native protein extracted with pH, causing the presence of more impurities. More bands at higher intensities are visible in Figure 3-2c compared to Figure 3-2a because of the higher native protein concentration at pH 6.0 than at pH 4.0. The presence of other proteins during elution (protein around 49 kDa and 98 kDa) implies rHSA was not fully purified as some native proteins were also bound. This is visible in Figure 3-2 as the bands visible in elution are not present in the flow through (well 2-4) and the wash. Four min was sufficient to oversaturate the resin for all tested conditions.

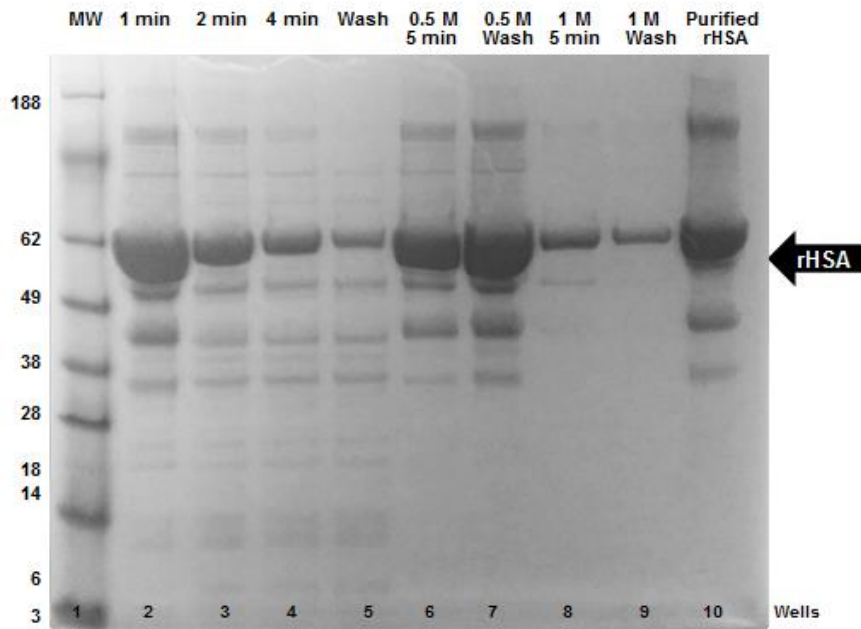
(a)



(b)



(c)



**Figure 3-2. Protein profiles of extracts by NU-PAGE<sup>®</sup> electrophoresis gel with samples from (a) pH 4.0 adjusted to 8.0 extract (wells 2-8), (b) pH 4.5 adjusted to 8.0 extract (wells 2-8), and (c) pH 6 adjusted to 8.0 extract (wells 2-8), showing protein molecular weight profiles. Well 2 includes initial load of pH adjusted extract. Wells 3-5 include flow through and wells 6-9 include eluted proteins. Well 10 contains purified rHSA as standard. The arrow indicates the molecular weight placement of rHSA at 66.5 kDa.**

Results showed a larger percent of bound rHSA eluting with 0.5 M sodium chloride buffer compared to 1 M sodium chloride buffer. The cause could be caused by hydrophobic interactions during the high salt concentration elution which could increase non-specific binding of rHSA to polymeric ion-exchange resin. 0.5 M sodium chloride elution band intensities (wells 6-7) seem larger than the extract due to the initial load being 10 mL of solution compared to the much smaller 1 mL elution, resulting in a more concentrated appearance. Also, only 39% of bound protein eluted at pH 4.0 while 84% of protein was bound to the resin (Table 3-2). 33% of bound protein eluted at pH 4.5 and only 26% of bound protein eluted at pH 6.0. While only TSP was measured in the supernatants, due to the decreasing band intensity of rHSA and constant band intensities of native proteins, it can be assumed mainly rHSA was bound to the resin.

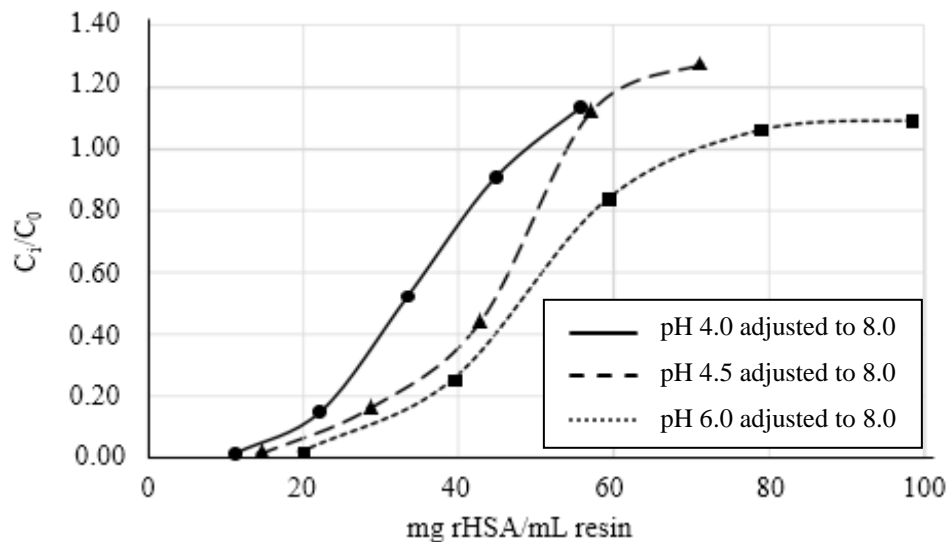
**Table 3-2. Percentage of rHSA bound and eluted in pH 4.0, 4.5, and 6.0 extracts using Q Sepharose™ FastFlow® in adsorption kinetics studies.**

Sample	Conductivity (mS)	Initial TSP Conc. (mg/mL)	% Protein Bound	% Protein Eluted
pH 4.0 Extract	6.1	2.10	84.4	32.6
pH 4.5 Extract	5.5	2.51	86.1	32.9
pH 6.0 Extract	3.5	3.02	91.3	26.4

***Consecutive Batch Binding of rHSA in Transgenic Rice Extracts***

Simulated dynamic binding capacities of the three conditions (pH 4.0, pH 4.5, and pH 6.0 extracts adjusted to 8.0) were evaluated by consecutively challenging the resin to simulate a breakthrough curves under static conditions as seen in Figure 3-3. Consecutive binding experiments allows for the determination of a dynamic binding capacity at a lab bench scale and short experimentation time. To determine the total amount of rHSA that can bind to the resin. This is important as it determines how much sample to load and the amount of resin required for purification. This was done by analyzing the amount of rHSA present in the flow through samples (unbound rHSA) and plotting the amount of rHSA challenging the resin and the flow through rHSA concentration over the initial rHSA load ( $C_i/C_0$ ). The  $C_i/C_0$  is above 100% for some samples due to error in rHSA quantification using ImageJ Analysis. This data demonstrates the efficiency of the chosen purification conditions due to 100% of rHSA bound to the resin over time for all conditions. Static binding studies resulted in minimal protein loss as 83.3%, 81.8%, and 92.6% of total protein loaded was recovered throughout binding and elution of pH 4.0, 4.5, and 6.0 extracts adjusted to 8.0 for adsorption, respectively. The breakthrough curves also show how an increase in extraction pH increases the amount of total rHSA that can bind to the resin (Figure 3-3). The static binding capacities of the three conditions at 10% breakthrough ( $C_i/C_0$  at 0.1) resulted in 18.22 mg rHSA/mL resin, 22.56 mg rHSA/mL resin, and 26.38 mg rHSA/mL

resin for pH 4.0, 4.5, and 6.0, adjusted to 8.0, respectively. This is expected due to the increase of rHSA and native protein with increasing pH (Sheshukova and Wilken, 2018).



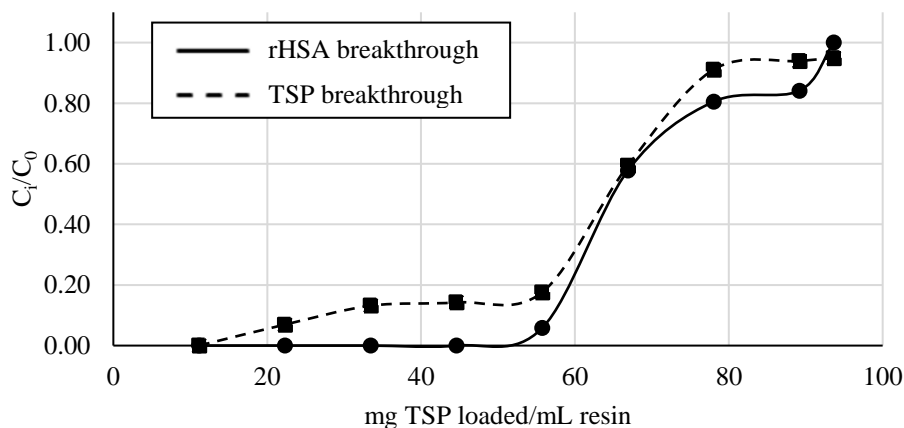
**Figure 3-3. Consecutive batch binding breakthrough curves of rHSA in pH-adjusted extracts. pH 4.0 adjusted to 8.0 extract is represented by the solid line, pH 4.5 adjusted to 8.0 extract is represented by the dashed line, and pH 6.0 adjusted to 8.0 extract is represented by the dotted line. Where  $C_i/C_0$  is the concentration of the unbound rHSA over the load concentration.**

Using the rHSA binding capacities at saturation, the volume of extract for each pH condition to achieve 100% breakthrough was calculated. The load volumes were determined to be 50 mL for pH adjusted pH 4.0 and 4.5 extracts and 55 mL for pH adjusted pH 6.0 extract. These volumes of extract were then loaded on a prepacked Q Sepharose™ Fast Flow® column using to determine the dynamic binding capacity and 5% and 10% breakthrough.

### **Breakthrough of rHSA in Transgenic Rice Extracts Using Anion Exchange Chromatography with ÄKTA Pure**

Using the load volumes determined previously, 50 mL of pH 4.0 adjusted to 8.0 extract was loaded on the ÄKTA Pure to reach rHSA saturation. The breakthrough curves where the column reaches full saturation is shown in Figure 3-4. The breakthrough of native protein during

load was also plotted to determine if binding of proteins with similar properties to rHSA bound to the column. Results indicate most native proteins are not bound and thus, flowed through the column as shown in the small increase in slope on the dashed line in Figure 3-4. This is again evident in the secondary increase in slope as the binding sites of the column are filled with rHSA and remaining protein (rHSA and native protein) flows through.

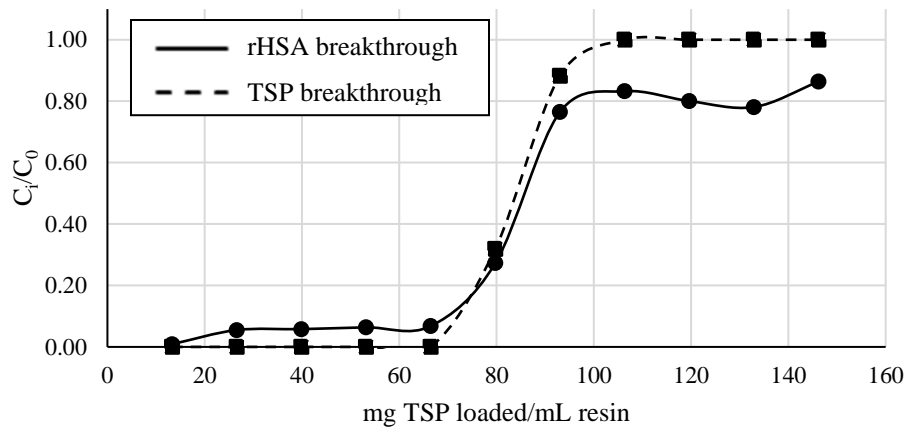


**Figure 3-4. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 4.0 adjusted to 8.0 extract. Where  $C_i/C_0$  is the concentration of the unbound protein (TSP or rHSA) over the load concentration for each respective line.**

Using 5% and 10% breakthrough for binding evaluation and comparison is standard in industry (Grushka and Grinberg, 2009). When comparing 5% and 10% breakthrough, certain parameters such as breakthrough curve slope are analyzed, such as by calculating the area under the curve (amount of unbound protein). A shallow slope will designate more target protein loss to reach each respective breakthrough capacity but will be less sensitive to load variability. A steep slope indicates more protein is bound, however, it will be sensitive to the amount of sample loaded. Therefore, the dynamic binding capacity at 5% and 10% for pH 4.0 adjusted to 8.0 extract was analyzed using the breakthrough curve in Figure 3-4. At 5%, the amount of rHSA bound was 67.4 mg/mL resin (0% of rHSA lost) and at 10%, the amount of rHSA bound increased to 68.9 mg/mL resin (2% of rHSA lost). This is expected as more protein is loaded, the

column begins to saturate, resulting in a breakthrough curve. The binding capacity at 5% and 10% is high and indicates over 67 mg of rHSA can be bound in 1 mL of resin which is beneficial in processing. The current parameters such as residence time can be scaled-up with a larger column which would allow for more rHSA binding if needed.

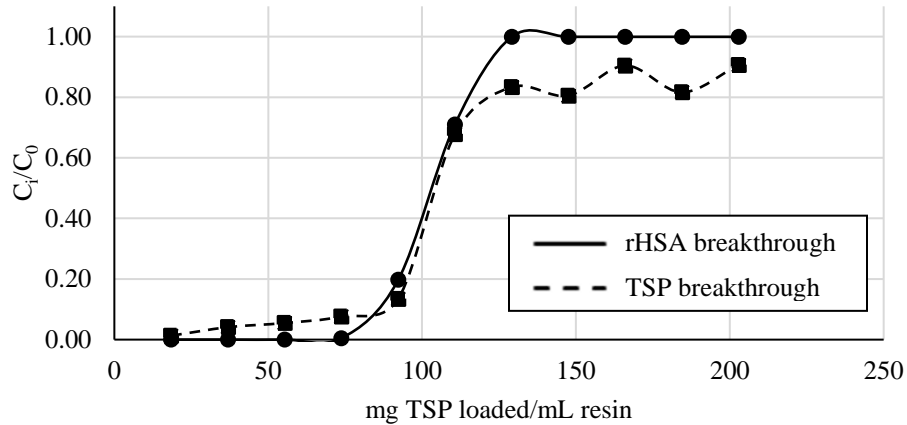
Due to the increase of bound rHSA from 5% to 10%, loading until 10% is acceptable as more total protein can be recovered during elution with minimal rHSA loss as >98% of loaded rHSA was bound. At saturation, 79.2 mg rHSA/mL resin was bound (68%) and 116.3 mg rHSA loaded.



**Figure 3-5. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 4.5 adjusted to 8.0 extract. Where  $C_i/C_0$  is the concentration of the unbound protein (TSP or rHSA) over the load concentration for each respective line.**

The dynamic binding capacity of pH 4.5 adjusted to 8.0 extract at 5% and 10% is visible in Figure 3-5. The amount of bound and loaded rHSA did not increase significantly from 5% to 10% breakthrough (loaded 61.1 mg rHSA, bound 60.5 mg rHSA and loaded 63.0 mg rHSA, bound 61.8 mg rHSA, respectively). The amount of bound rHSA decreased from 99% to 98%, therefore, loading to 10% breakthrough for pH 4.5 adjusted to 8.0 extract is acceptable. At saturation, only 68.7 mg rHSA was bound (53%) when 130.3 mg rHSA was loaded.

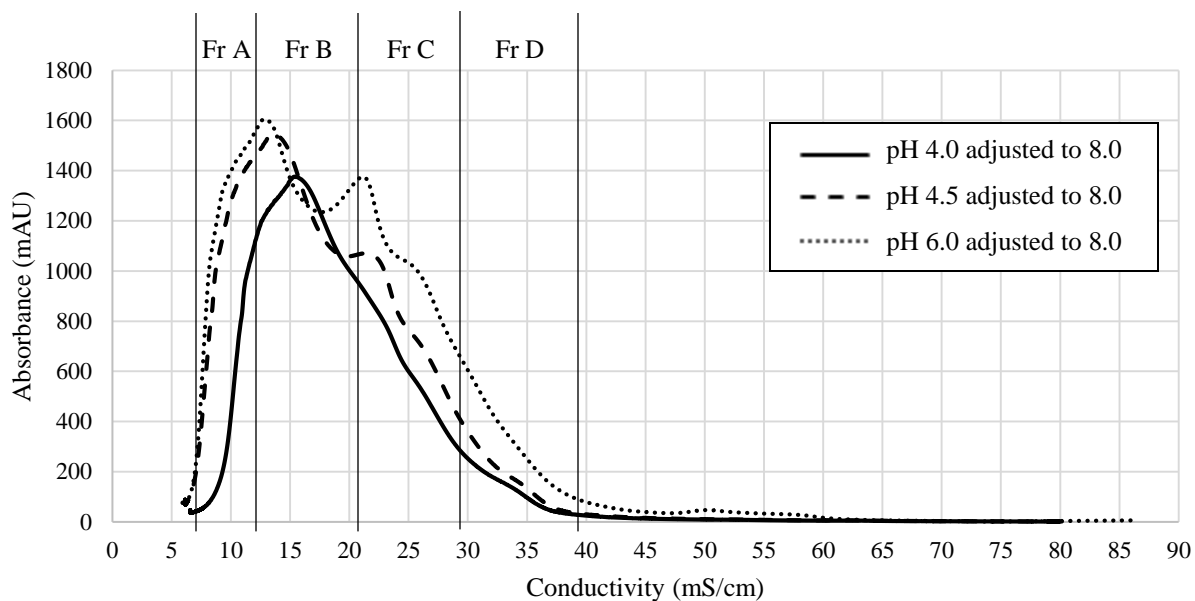




**Figure 3-6. Dynamic binding breakthrough curves of rHSA (solid line) and total soluble protein (dashed line) in pH 6.0 adjusted to 8.0 extract. Where  $C_i/C_0$  is the concentration of the unbound protein (TSP or rHSA) over the load concentration for each respective line.**

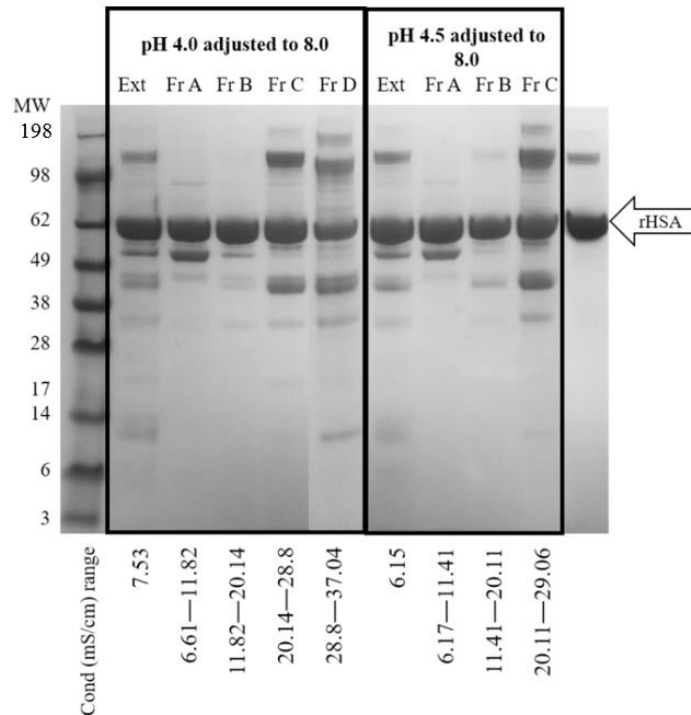
The dynamic binding capacity of pH 6.0 adjusted to 8.0 extract increased from pH 4.0 and 4.5 adjusted to 8.0 extracts (Figure 3-6). At 5% breakthrough, 62.8 mg rHSA was bound (99%) and at 10%, 65.9 mg rHSA bound to the resin (98%). At saturation, 76.4 mg rHSA bound (46%) with 165 mg rHSA loaded on the column.

The amount of bound rHSA in extract at saturation varied with pH but was greater than 69 mg/mL resin for all conditions. The binding capacity of rHSA could be improved with further optimization of operating conditions such as pH, ionic strength, residence time, linear velocity, *etc.* Jungbauer and Hahn, (2009) suggest increasing the residence time (by decreasing the flow rate) or increasing the bed height to further improve binding capacity of proteins.



**Figure 3-7. Dynamic binding elution chromatograms of loading to 10% breakthrough with pH 4.0 adjusted to 8.0 extract (solid line), pH 4.5 adjusted to 8.0 extract (dashed line), and pH 6.0 adjusted to 8.0 extract (dotted line).**

Once binding capacities of rHSA at 10% were identified, the required volumes were calculated to be 26 mL for pH 4.0 and 4.5 adjusted to 8.0 extracts and 23 mL for pH 6.0 adjusted to 8.0 extract. Extract was loaded to 10% breakthrough, a common industry standard (Grushka and Grinberg, 2009). Once loaded, the elution chromatogram was observed to visually begin to separate between 15 mS/cm and 20 mS/cm with increasing extraction pH as viewed in Figure 3-7. This can be attributed to an increased amount of lower molecular weight native proteins extracted with pH (Figure 3-8). Most of the extracted native proteins would possess a negative charge at the binding pH of 8.0 as rice native proteins between 15 kDa to 95 kDa have a pI between 5 and 7 (Yang et al., 2013). Therefore, when binding at a higher extraction pH such as pH 6.0, the presence of more native proteins will result in more proteins with a higher affinity for the resin, which causes the formation of separated peaks on the chromatogram.

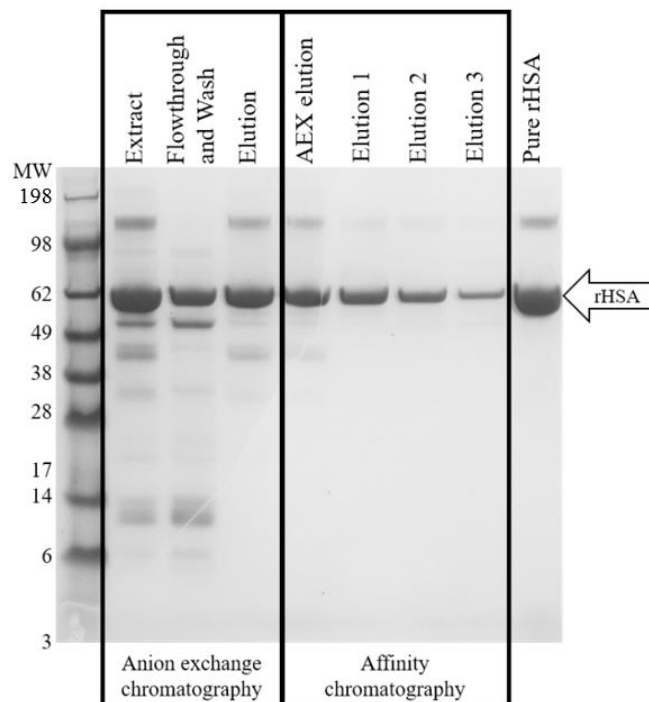


**Figure 3-8. NuPAGE<sup>®</sup> gel electrophoresis of pH 4.0 and 4.5 adjusted to 8.0 extract and elution fractions loaded to the same rHSA concentration of 0.25 mg/mL. The main rHSA band is indicated by the arrow pointing toward the pure rHSA sample. Elution fraction conductivity ranges are included below the gel in mS/cm.**

The amount of native protein visually increases with conductivity in both pH 4.0 and 4.5 adjusted to 8.0 elutions and the protein band between 98 and 198 kDa becomes more intense. This band is similar to the higher molecular weight band seen in the pure rHSA sample that is visible in Figure 3-8 and could possibly be a dimer as the band was also present in HSA specific affinity chromatography elution fractions of extract (Figure 3-9). Fr A through D for all pH conditions (Figure 3-7) were also loaded on the HSA specific affinity column to identify purities and yields to determine which extraction pH condition provides the best results of purified rHSA in downstream processing.

***rHSA Purity Quantification using Affinity Chromatography with Anion Exchange Chromatography Elution Fractions Using ÄKTA Pure***

The anion exchange chromatography elution fractions selected encompassed the full chromatogram peak. The peak was comprised of four, 2 mL fractions with the initial fraction eluting between 6 mS/cm and 12 mS/cm and each fraction after, increasing by 10 mS/cm. Blue Sepharose™ Fast Flow was selected as the affinity resin as the blue dye binds blood proteins such as HSA, interferon, lipoproteins, and blood coagulation factors specifically (GE Healthcare, 2018). The affinity ligand would only bind rHSA and not native rice protein impurities in extract as the transgenic rice flour only expresses one human blood protein. Therefore, the affinity chromatography elution peak was assumed to contain majority or only rHSA as visible in Figure 3-9 with the corresponding bands in pure rHSA samples matching affinity chromatography elution fraction bands.



**Figure 3-9. NuPAGE® gel electrophoresis of pH 4.0 adjusted to 8.0 anion exchange chromatography (AEX) samples and affinity chromatography results after loading combined AEX elution samples.**

Each AEX elution fraction was loaded on the affinity column and all chromatogram samples were taken to analyze using mass balance through TSP results. Results indicated that

90% or more of all protein loaded was accounted for in the affinity chromatography mass balances so the elution peak was confidently analyzed to determine the amount of rHSA present. The concentration of rHSA in the elution peaks was analyzed by the Beer-Lambert law using an extinction coefficient of 0.549 mL/mg·cm. Resultant rHSA concentration was multiplied by the elution peak volume to obtain the amount of rHSA (mg) and compared to the sample load. Results of purity and yield for each pH condition is listed in Table 3-3. Purity and yield varied throughout the elution peak visible in Figure 3-7 for each pH condition, with pH 4.5 adjusted to 8.0 extract resulting in the most pure elution peak (60%) which contained >99% of rHSA content present in the loading sample. Fr A was more pure (81%) in pH 4.0 adjusted to 8.0 extract but containing the smallest yield (12%). For pH 4.5 adjusted to 8.0 extract, elution Fr B was the most pure (>99%) and contained the highest rHSA yield (76%), while pH 6.0 adjusted to 8.0 extract contained the highest yield in Fr B and the highest purity in Fr C.

**Table 3-3. Purities and yields of anion exchange chromatography elution extracts for each extraction pH condition. Fr D was not included as it contained no detectible amounts of rHSA in pH 4.0 and 4.5 adjusted to 8.0 extracts.**

	pH 4.0				pH 4.5				pH 6.0			
	<i>Fr A</i>	<i>Fr B</i>	<i>Fr C</i>	<i>Fr A-D</i>	<i>Fr A</i>	<i>Fr B</i>	<i>Fr C</i>	<i>Fr A-D</i>	<i>Fr A</i>	<i>Fr B</i>	<i>Fr C</i>	<i>Fr A-D</i>
<b>Purity (%)</b>	80.6	78.4	71.2	58.1	69.4	>99.0	51.9	59.9	72.5	56.3	79.0	51.9
<b>Yield (%)</b>	12.4	57.0	17.2	87.1	25.1	75.7	17.3	>99.0	20.1	29.1	26.4	75.6

Elution fractions can also be combined to improve purity and yields as shown in Table 3-4. In pH 4.5 adjusted to 8.0 extract, combining the first two and first three elution fractions resulted in >99% purity and yield (35 mg and 41 mg rHSA respectively). Combining the full peak of pH 6.0 adjusted to 8.0 elution fractions instead of three improved the yield by around 10% but did not change the purity.

**Table 3-4. The amount of total soluble protein (TSP) and rHSA, and purities and yields of anion exchange chromatography elution extracts for each extraction pH condition. Fr D was not included as it contained no detectible amounts of rHSA in pH 4.0 and 4.5 adjusted to 8.0 extracts.**

	pH 4.0		pH 4.5		pH 6.0	
	<i>Fr A, B</i>	<i>Fr A, B, C</i>	<i>Fr A, B</i>	<i>Fr A, B, C</i>	<i>Fr A, B</i>	<i>Fr A, B, C</i>
<b>TSP (mg)</b>	29.2	37.2	34.4	35.7	39.1	55.6
<b>rHSA (mg)</b>	24.1	29.7	35.0	40.6	18.4	31.5
<b>Purity (%)</b>	82.6	79.9	>99.0	>99.0	47.1	56.7
<b>Yield (%)</b>	73.0	90.1	>99.0	>99.0	42.6	64.3

However, after comparing the elution fraction purities to the extract load purities, only pH 4.5 and 6.0 adjusted to 8.0 extracts have improved purities. pH 4.0 adjusted to 8.0 extract has an initial purity of 78% (Sheshukova and Wilken, 2018) and after anion exchange chromatography the highest purity of any of the elution fractions was 81% (Table 3-3). Initial purity calculations in extract could have been over-estimated and rHSA purity in anion exchange elution could be improved by isolating the rHSA elution conductivity range or possibly adjusting adsorption pH. For rHSA to be comparable to industry standards, it must be >95% pure for reagents and chemicals and >99% for therapeutic drugs (Krishnan and Woodard, 2014). Typically, purities higher than 95% are achieved through more than one chromatography step. Therefore, Fr B from the purification of pH 4.5 extract adjusted to 8.0 would satisfy these purity conditions (rHSA purity >99%), as visible in Table 3-3.

The testing efficiency of rHSA purification can be analyzed with other methods of purification such as membrane filtration, hydrophobic interactions chromatography, or size exclusion as an additive or alternative step to produce rHSA with comparable purity to market rHSA.

## Conclusions

The development of an efficient and integrated extraction and purification process for recombinant proteins is critical for furthering research in biotechnology and pharmacology. Adsorption kinetics was utilized to determine the most suitable conditions to maximize the amount of rHSA bound to resin and maximize the amount of bound rHSA eluted. Acetate buffer was chosen as a more cost-effective reagent over phosphate and citrate to maintain consistent and lower conductivities (around 6 mS/cm) throughout binding as binding capacity decreased in pH adjusted phosphate and citrate buffer (>10 mS/cm). Once buffer conditions were identified, conductivity and concentration effect on purified rHSA was analyzed by comparing pure solution to diluted pure solution with buffer (maintain constant conductivity) or with water (maintain constant concentration). Results indicated all conditions bound almost 100% of the purified rHSA, with an increase in the percentage of rHSA eluted from 33% to 52% in two-fold with buffer and two-fold with water respectively. Concentration change did not indicate improvement in binding or elution. Adsorption kinetics performed on previously determined stable extraction conditions of pH 4.0, 4.5, and 6.0 (Sheshukova and Wilken, 2018) showed saturation of the resin after 4 min. All extracts were pH adjusted to the binding pH of 8.0 either with TRIS (pH 4.0 and 4.5) or with 1 M sodium hydroxide (pH 6.0 extraction buffer already included TRIS). Using acetate buffer with an anion exchange resin is typically not utilized as anion exchange pH ranges are outside the buffering capacity of acetate, thus the addition of TRIS improves the buffering capacity to allow stable pH throughout purification.

Adsorption kinetics resulted in the largest amount of bound protein at pH 6.0 at 91%, although only 26% of the bound protein was eluted. The highest amount of bound protein eluted

was at pH 4.5 with 33%. Further studies to determine breakthrough of rHSA at different conditions analyzed the static binding capacity at 10% after full binding of rHSA to the resin occurred. Load volumes of extract (50 mL for pH 4.0 and 4.5 extracts, and 55 mL for pH 6.0 extracts) was determined before applying to a purification system with a 1 mL column with anion exchange chromatography resin to create a dynamic binding breakthrough curve. The calculated dynamic binding capacity at 5% and 10% was 4-fold greater than what was determined in consecutive batch binding for pH 4.0 adjusted to 8.0 extract and over 2-fold greater for pH 4.5 and 6.0 adjusted to 8.0 extracts. The amount of rHSA bound at 5% and 10% was close to 98%, thus, indicating loading to 10% for all pH conditions was more beneficial due to more rHSA in total being loaded on the column. Once the anion exchange column was loaded to 10% breakthrough capacity, elution samples were taken and then chromatogram was analyzed. Gel electrophoresis results showed an increase of native protein with conductivity during the elution which would decrease fraction purities. Elution fractions were then analyzed using affinity chromatography to determine the amount rHSA present in each fraction. Purities and yields varied with extraction pH and fractions were then selectively combined to provide options of improving purity or yield. While pH 4.0 extraction was selected as a desired condition due to the high initial purity (79%) and stability (Sheshukova and Wilken, 2018), sample purities did not improve after anion exchange chromatography which indicates this pH conditions is not the most optimal. pH 4.5 and 6.0 adjusted to 8.0 extracts demonstrated an improvement in purity after anion exchange and even full yield and 100% purity was achieved when combining pH 4.5 adjusted to 8.0 Fr A and B (6 to 20 mS/cm).

While anion exchange chromatography was successful in purifying rHSA from pH 4.5 and 6.0 adjusted to 8.0 extracts, additional or different purification methods may need to be



considered to increase rHSA purity. HIC would be compatible following anion exchange as elution fractions are concentrated and in high salt buffer which is the loading requirements for HIC. Affinity chromatography using Blue Sepharose™ resin was successful in selectively binding rHSA in AEX elution fractions and in extract with minimal native protein binding. Examining purification conditions is a necessary part of creating an efficient and optimized downstream process that could be applied to the production of rHSA to be utilized in many possible medical applications.

## 4. Conclusions

### Summary of Results

To develop an integrated and cost-efficient downstream processing system of recombinant human serum albumin (rHSA) from transgenic *Oryza sativa* seeds, different extraction conditions were analyzed and those deemed acceptable were explored further in subsequent purification studies. Extraction conditions that maximized rHSA purity were at the lower pHs evaluated (3.5 and 4.0) in pH 50 mM sodium citrate with 50 mM sodium chloride. Low pH extraction resulted in a very high initial rHSA purity of 78% but for pH 3.5 extracts, the rHSA degraded 60% by 60 min while pH 4.0 extracts remained stable. Instability of pH 3.5 extracts was not due to pH as pure rHSA remained stable at pH 3.5 while additional protein (bovine serum albumin) spiked into pH 3.5 extract also degraded along with the recombinant rHSA. Decreasing the extraction temperature improved stability in pH 3.5 extracts but would increase processing costs. rHSA extract at pH 4.5 and 6.0 in 50 mM sodium citrate and 50 mM sodium phosphate buffer respectively, with 50 mM sodium chloride remained stable throughout the 60 min extraction but resulted in lower rHSA purities of <60%. rHSA yields increased with extraction pH as did native proteins. With these results, extraction conditions favorable for purification studies were pH 4.0, 4.5, and 6.0 as rHSA remained stable throughout extraction and provided various purities and yields to analyze.

Once the extraction conditions were selected, purification conditions such as buffer type, ionic strength, residence time, and binding capacity were determined using adsorption kinetics, consecutive batch binding, and dynamic binding. The buffer most compatible for binding pH 4.0 and 4.5 extracts was acetate using TRIS for pH adjustment to 8.0, which is above the isoelectric

point (pI) of rHSA (4.7). TRIS was used to provide buffering capacity (pH 7.5-8.0) as needed for adsorption at pH 8.0. Acetate has a buffering range of pH 4.8-5.2 so the addition of TRIS assists with pH adjustment and provided sufficient buffering to maintain pH through adsorption. This also resulted in lower overall conductivity for loading, which helped maintain the binding capacity. Phosphate buffer provided sufficient buffering capacity but resulted in higher overall conductivity and reduced rHSA binding capacity. Purification of rHSA extracts using anion exchange resulted in high binding capacities at saturation and at 10% breakthrough. rHSA binding capacities varied with pH. The binding capacity at saturation ranged from 68.7 mg rHSA/mL resin to 79.2 mg rHSA/mL resin and the binding capacity at 10% ranged from 61.8 mg rHSA/mL resin to 68.9 mg rHSA/mL resin. Afterwards, pH adjusted extracts were loaded to 10% breakthrough to evaluate purification potential for each condition. While pH 4.5 adjusted to 8.0 extract resulted in the lowest binding capacity, the highest rHSA purity (>99%) was achieved along with the highest yield (76%). These results indicate that rHSA in extract was purified 1.7-fold and a high yield of rHSA was obtained as >50% yield is a common target of commercial processes (Krishnan and Woodard, 2014)

## **Future Work**

While acceptable purities and yields were achieved with pH 4.5 adjusted to 8.0 elution fractions, chromatography methods such as hydrophobic interactions chromatography (HIC) are suitable for purification to further improve rHSA purities and yields. This is due to anion exchange elution fractions containing high salt concentrations which is necessary for HIC as high salt exposes more hydrophobic regions on the protein that bind to the resin (Cummins and O'Connor, 2011). This eliminates the need for buffer exchange which can increase costs.

Another method of improving purities and yields is by modifying the current anion exchange chromatography gradient elution to a step elution between 6-20 mS/cm where majority of rHSA elutes. This can isolate rHSA and prevent any bound native proteins from eluting above 20 mS/cm.

Initial studies were conducted to evaluate various buffer systems and combinations that were suitable for both extraction and purification. However, further testing of other buffer systems used in anion exchange chromatography such as TRIS-HCl should be tested and compared to determine if acetate is the most suitable binding buffer. Testing of other binding pHs such as 7.0 or 7.5 should also be explored to determine if reducing the adsorption pH will minimize native protein binding. Rice proteins tend to have isoelectric points between 5 and 7 (Yang et al., 2013) so loading extract closer to the isoelectric point of native proteins may improve purification-fold, although there may be a decrease in the binding capacity of rHSA. However, loading at a pH closer to the extraction pH could reduce processing costs from extract pH adjustment.

The biological activity of rHSA was not tested. To determine if current extraction and purification methods are feasible for commercial production an activity assay, fluorescence spectroscopy, X-ray crystallography, nuclear magnetic resonance spectroscopy, and/or mass spectrometry would be required to compare rHSA biochemical activity, structure, and function to plasma derived HSA. Lastly, an economic evaluation of the proposed processing system using extraction and purification results is necessary. A program such as SuperPro Designer would help establish which conditions are the most economical and could identify the major economic and processing bottlenecks.

## 5. References

- Abiri, R., Valdiani, A., Maziah, M., Shaharuddin, N. A., Sahebi, M., Yusof, Z. N. B., ... Talei, D. (2016). A Critical Review of the Concept of Transgenic Plants: Insights into Pharmaceutical Biotechnology and Molecular Farming. *Current Issues in Molecular Biology*, 18, 21–42. <https://doi.org/10.21775/cimb.018.021>
- Alam, A., Jiang, L., Kittleson, G. A., Steadman, K. D., Nandi, S., Fuqua, J. L., ... McDonald, K. A. (2018). Technoeconomic Modeling of Plant-Based Griffithsin Manufacturing. *Frontiers in Bioengineering and Biotechnology*, 6, 102. <https://doi.org/10.3389/fbioe.2018.00102>
- Alfano, R., Youngblood, B. A., Zhang, D., Huang, N., MacDonald, C. C. (2014). Human leukemia inhibitory factor produced by the ExpressTec method from rice (*Oryza sativa* L.) is active in human neural stem cells and mouse induced pluripotent stem cells. *Bioengineered*, 5(3), 180–5. <https://doi.org/10.4161/bioe.28996>
- An, N., Ou, J., Jiang, D., Zhang, L., Liu, J., Fu, K., ... Yang, D. (2013). Expression of a functional recombinant human basic fibroblast growth factor from transgenic rice seeds. *International Journal of Molecular Sciences*, 14(2), 3556–67. <https://doi.org/10.3390/ijms14023556>
- Andersen, D. C., Krummen, L. (2002). Recombinant protein expression for therapeutic applications. *Current Opinion in Biotechnology*, 13(2), 117–123. [https://doi.org/10.1016/S0958-1669\(02\)00300-2](https://doi.org/10.1016/S0958-1669(02)00300-2)
- Anraku, M., Kouno, Y., Kai, T., Tsurusaki, Y., Yamasaki, K., Otagiri, M. (2007). The role of N-acetyl-methioninate as a new stabilizer for albumin products. *International Journal of Pharmaceutics*, 329(1–2), 19–24. <https://doi.org/10.1016/J.IJPHARM.2006.08.014>
- Avesani, L., Merlin, M., Gecchele, E., Capaldi, S., Brozzetti, A., Falorni, A., Pezzotti, M. (2014). Comparative analysis of different biofactories for the production of a major diabetes autoantigen. *Transgenic Research*, 23(2), 281–291. <https://doi.org/10.1007/s11248-013-9749-9>
- Ayyar, V. S. (2011). History of growth hormone therapy. *Indian Journal of Endocrinology and Metabolism*, 15 Suppl 3(Suppl3), S162-5. <https://doi.org/10.4103/2230-8210.84852>
- Azhakanandam, K., Power, J. B., Lowe, K. C., Cocking, E. C., Tongdang, T., Jumel, K., ... Davey, M. R. (2000). Qualitative Assessment of Aromatic Indica Rice (*Oryza sativa* L.): Proteins, Lipids and Starch in Grain from Somatic Embryo- and Seed-Derived Plants.

- Journal of Plant Physiology*, 156(5–6), 783–789. [https://doi.org/10.1016/S0176-1617\(00\)80248-5](https://doi.org/10.1016/S0176-1617(00)80248-5)
- Azzoni, A. R., Farinas, C. S., Miranda, E. A. (2005). Transgenic corn seed for recombinant protein production: relevant aspects on the aqueous extraction of native components. *Journal of the Science of Food and Agriculture*, 85(4), 609–614. <https://doi.org/10.1002/jsfa.2018>
- Baeshen, M. N., Al-Hejin, A. M., Bora, R. S., Ahmed, M. M. M., Ramadan, H. A. I., Saini, K. S., ... Redwan, E. M. (2015). Production of Biopharmaceuticals in *E. coli*: Current Scenario and Future Perspectives. *Journal of Microbiology and Biotechnology*, 25(7), 953–962. <https://doi.org/10.4014/jmb.1412.12079>
- Baizhi Li, B., Cao, Y., Zhou, L., Liang, C., Sun, F. (2011). A novel protein expression system- *PichiaPink*<sup>TM</sup>- and a protocol for fast and efficient recombinant protein expression. *African Journal of Biotechnology*, 10(83), 19464–19472. <https://doi.org/10.5897/AJB11.1513>
- Banach, M., Kalinowska, B., Konieczny, L., Roterman, I. (2016). Role of Disulfide Bonds in Stabilizing the Conformation of Selected Enzymes—An Approach Based on Divergence Entropy Applied to the Structure of Hydrophobic Core in Proteins. *Entropy*, 18(3), 67. <https://doi.org/10.3390/e18030067>
- Barash, I., Faerman, A., Baruch, A., Nathan, M., Hurwitz, D. R., Shani, M. (1993). Synthesis and secretion of human serum albumin by mammary gland explants of virgin and lactating transgenic mice. *Transgenic Research*, 2(5), 266–276. <https://doi.org/10.1007/BF01968839>
- Barta, A., Sommergruber, K., Thompson, D., Hartmuth, K., Matzke, M. A., Matzke, A. J. M. (1986). The expression of a nopaline synthase human growth hormone chimaeric gene in transformed tobacco and sunflower callus tissue. *Plant Molecular Biology*, 6(5), 347–357. <https://doi.org/10.1007/BF00034942>
- Bawa, A. S., Anilakumar, K. R. (2013). Genetically modified foods: safety, risks and public concerns-a review. *Journal of Food Science and Technology*, 50(6), 1035–46. <https://doi.org/10.1007/s13197-012-0899-1>
- Belew, M., Li, Yan, M., Zhang, Wei, Caldwell, K. (2008). Purification of Recombinant Human Serum Albumin (rHSA) Produced by Genetically Modified *Pichia Pastoris*. *Separation Science and Technology*, 43(11–12), 3134–3153. <https://doi.org/10.1080/01496390802221857>

- Boothe, J., Nykiforuk, C., Shen, Y., Zaplachinski, S., Szarka, S., Kuhlman, P., ... Moloney, M. M. (2010). Seed-based expression systems for plant molecular farming. *Plant Biotechnology Journal*, 8(5), 588–606. <https://doi.org/10.1111/j.1467-7652.2010.00511.x>
- Bosch, D., Schots, A. (2010). Plant glycans: friend or foe in vaccine development? *Expert Review of Vaccines*, 9(8), 835–842. <https://doi.org/10.1586/erv.10.83>
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- Brookes, G., Barfoot, P. (2018). Farm income and production impacts of using GM crop technology 1996–2016. *GM Crops & Food*, 9(2), 59–89. <https://doi.org/10.1080/21645698.2018.1464866>
- Bushell, M. E., Rowe, M., Avignone-Rossa, C. A., Wardell, J. N. (2003). Cyclic fed-batch culture for production of human serum albumin in *Pichia pastoris*. *Biotechnology and Bioengineering*, 82(6), 678–683. <https://doi.org/10.1002/bit.10616>
- Butler, M., Spearman, M. (2014). The choice of mammalian cell host and possibilities for glycosylation engineering. *Current Opinion in Biotechnology*, 30, 107–112. <https://doi.org/10.1016/J.COPBIO.2014.06.010>
- Buyel, J. F., Fischer, R. (2012). Predictive models for transient protein expression in tobacco (*Nicotiana tabacum* L.) can optimize process time, yield, and downstream costs. *Biotechnology and Bioengineering*, 109(10), 2575–2588. <https://doi.org/10.1002/bit.24523>
- Buyel, J. F., Twyman, R. M., Fischer, R. (2017). Very-large-scale production of antibodies in plants: The biologization of manufacturing. *Biotechnology Advances*, 35(4), 458–465. <https://doi.org/10.1016/J.BIOTECHADV.2017.03.011>
- Cai, C., Zhou, K., Wu, Y., Wu, L. (2006). Enhanced liver targeting of 5-fluorouracil using galactosylated human serum albumin as a carrier molecule. *Journal of Drug Targeting*, 14(2), 55–61. <https://doi.org/10.1080/10611860600613324>
- Carter, D. C., He, X. M., Munson, S. H., Twigg, P. D., Gernert, K. M., Broom, M. B., Miller, T. Y. (1989). Three-dimensional structure of human serum albumin. *Science (New York, N.Y.)*, 244(4909), 1195–8. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2727704>
- Chamberland, M. E., Alter, H. J., Busch, M. P., Nemo, G., Ricketts, M. (2001). Emerging infectious disease issues in blood safety. *Emerging Infectious Diseases*, 7(3 Suppl), 552–3.

- <https://doi.org/10.3201/eid0707.010731>
- Champagne, E. T., Wood, D. F., Juliano, B. O., Bechtel, D. B. (2004). *Chapter 4: the Rice Grain and Its Gross Composition. RICE: Chemistry and Technology.*  
<https://doi.org/10.1094/1891127349.004>
- Chargelegue, D., Obregon, P., Drake, P. M. (2001). Transgenic plants for vaccine production: expectations and limitations. *Trends in Plant Science*, 6(11), 495–6.  
[https://doi.org/10.1016/S1360-1385\(01\)02123-9](https://doi.org/10.1016/S1360-1385(01)02123-9)
- Chen, Q., Dent, M., Hurtado, J., Stahnke, J., McNulty, A., Leuzinger, K., Lai, H. (2016). Transient Protein Expression by Agroinfiltration in Lettuce. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 1385, pp. 55–67). [https://doi.org/10.1007/978-1-4939-3289-4\\_4](https://doi.org/10.1007/978-1-4939-3289-4_4)
- Chen, Y.-S., Zaro, J., Zhang, D., Huang, N., Simon, A., Shen, W.-C., ... Shen, W.-C. (2018). Characterization and Oral Delivery of Proinsulin-Transferrin Fusion Protein Expressed Using ExpressTec. *International Journal of Molecular Sciences*, 19(2), 378.  
<https://doi.org/10.3390/ijms19020378>
- Chen, Z., He, Y., Shi, B., Yang, D. (2013). Human serum albumin from recombinant DNA technology: Challenges and strategies. *Biochimica et Biophysica Acta - General Subjects*, 1830(12), 5515–5525. <https://doi.org/10.1016/j.bbagen.2013.04.037>
- Cheryan, M., Rackis, J. J. (1980). Phytic acid interactions in food systems. *C R C Critical Reviews in Food Science and Nutrition*, 13(4), 297–335.  
<https://doi.org/10.1080/10408398009527293>
- Clincke, M.-F., Mölleryd, C., Zhang, Y., Lindskog, E., Walsh, K., Chotteau, V. (2013). Very high density of CHO cells in perfusion by ATF or TFF in WAVE bioreactor™. Part I. Effect of the cell density on the process. *Biotechnology Progress*, 29(3), 754–67.  
<https://doi.org/10.1002/btpr.1704>
- Cohen, S. N., Chang, A. C., Boyer, H. W., Helling, R. B. (1973). Construction of biologically functional bacterial plasmids in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 70(11), 3240–4. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/4594039>
- Costello, A. J. R., Glonek, T., Myers, T. C. (1976). <sup>31</sup>P Nuclear magnetic resonance  $\square$  pH titrations of myo-inositol hexaphosphate. *Carbohydrate Research*, 46(2), 159–171.  
[https://doi.org/10.1016/S0008-6215\(00\)84287-1](https://doi.org/10.1016/S0008-6215(00)84287-1)



- Coulibaly, A., Kouakou, B., Chen, J. (2011). Phytic Acid in Cereal Grains: Structure, Healthy or Harmful Ways to Reduce Phytic Acid in Cereal Grains and Their Effects on Nutritional Quality. *American Journal of Plant Nutrition and Fertilization Technology*, 1(1), 1–22. <https://doi.org/10.3923/ajpnft.2011.1.22>
- Crea, R., Kraszewski, A., Hirose, T., Itakura, K. (1978). Chemical synthesis of genes for human insulin. *Proceedings of the National Academy of Sciences of the United States of America*, 75(12), 5765–9. <https://doi.org/10.1073/PNAS.75.12.5765>
- Cunha, N. B., Murad, A. M., Ramos, G. L., Maranhão, A. Q., Brígido, M. M., Araújo, A. C. G., ... Rech, E. L. (2011). Accumulation of functional recombinant human coagulation factor IX in transgenic soybean seeds. *Transgenic Research*, 20(4), 841–855. <https://doi.org/10.1007/s11248-010-9461-y>
- D'Aoust, M.-A., Couture, M. M.-J., Charland, N., Trépanier, S., Landry, N., Ors, F., Vézina, L.-P. (2010). The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza. *Plant Biotechnology Journal*, 8(5), 607–619. <https://doi.org/10.1111/j.1467-7652.2009.00496.x>
- Daniell, H., Chebolu, S., Kumar, S., Singleton, M., Falconer, R. (2005). Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine*, 23(15), 1779–1783. <https://doi.org/10.1016/J.VACCINE.2004.11.004>
- Daniell, H., Streatfield, S. J., Wycoff, K. (2001). Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends in Plant Science*, 6(5), 219–226. [https://doi.org/10.1016/S1360-1385\(01\)01922-7](https://doi.org/10.1016/S1360-1385(01)01922-7)
- Darah, I., Sumathi, G., Jain, K., Lim, S. H. (2011). Tannase enzyme production by entrapped cells of *Aspergillus niger* FETL FT3 in submerged culture system. *Bioprocess and Biosystems Engineering*, 34(7), 795–801. <https://doi.org/10.1007/s00449-011-0529-8>
- Davis, K. M., Foos, T., Bates, C. S., Tucker, E., Hsu, C.-C., Chen, W., ... Wu, J.-Y. (2000). A Novel Method for Expression and Large-Scale Production of Human Brain -Glutamate Decarboxylase. *Biochemical and Biophysical Research Communications*, 267(3), 777–782. <https://doi.org/10.1006/bbrc.1999.2038>
- Del Giudice, A., Dicko, C., Galantini, L., Pavel, N. V. (2017). Time-Dependent pH Scanning of the Acid-Induced Unfolding of Human Serum Albumin Reveals Stabilization of the Native Form by Palmitic Acid Binding. *The Journal of Physical Chemistry B*, 121(17), 4388–4399.

<https://doi.org/10.1021/acs.jpcc.7b01342>

DiMasi, J. A., Hansen, R. W., Grabowski, H. G. (2003). The price of innovation: new estimates of drug development costs. *Journal of Health Economics*, 22, 151–185.

[https://doi.org/10.1016/S0167-6296\(02\)00126-1](https://doi.org/10.1016/S0167-6296(02)00126-1)

Dixon, C., Wilken, L. R., Woodard, S. L., Barros, G. O. F. (2018). The Impact of Six Critical Impurities on Recombinant Protein Recovery and Purification from Plant Hosts. In *Molecular Pharming* (pp. 137–180). Hoboken, NJ, USA: John Wiley & Sons, Inc.

<https://doi.org/10.1002/9781118801512.ch7>

Dockal, M., Carter, D. C., Rüker, F. (2000). Conformational transitions of the three recombinant domains of human serum albumin depending on pH. *The Journal of Biological Chemistry*, 275(5), 3042–50. <https://doi.org/10.1074/JBC.275.5.3042>

Dong, Y., Zhang, F., Wang, Z., Du, L., Hao, A., Jiang, B., ... Xiu, Z. (2012). Extraction and purification of recombinant human serum albumin from *Pichia pastoris* broths using aqueous two-phase system combined with hydrophobic interaction chromatography. *Journal of Chromatography A*, 1245, 143–149.

<https://doi.org/10.1016/J.CHROMA.2012.05.041>

Doran, P. M. (2013). *Bioprocess engineering principles*. Elsevier/Academic Press. Retrieved from <https://www.sciencedirect.com/book/9780122208515/bioprocess-engineering-principles>

dos Santos, R., Carvalho, A. L., Roque, A. C. A. (2017). Renaissance of protein crystallization and precipitation in biopharmaceuticals purification. *Biotechnology Advances*, 35(1), 41–50. <https://doi.org/10.1016/J.BIOTECHADV.2016.11.005>

Espinosa-Leal, C. A., Puente-Garza, C. A., García-Lara, S. (2018). In vitro plant tissue culture: means for production of biological active compounds. *Planta*, 248(1), 1–18.

<https://doi.org/10.1007/s00425-018-2910-1>

Farina, C., Vargas, V., Heydari, N., Kümpfel, T., Meinel, E., Hohlfeld, R. (2002). Treatment with glatiramer acetate induces specific IgG4 antibodies in multiple sclerosis patients. *Journal of Neuroimmunology*, 123(1–2), 188–92. [https://doi.org/10.1016/S0165-5728\(01\)00490-8](https://doi.org/10.1016/S0165-5728(01)00490-8)

Farinas, C. S., Leite, A., Miranda, E. A. (2007). Recombinant human proinsulin from transgenic corn endosperm: solvent screening and extraction studies. *Brazilian Journal of Chemical Engineering*, 24(3), 315–323. <https://doi.org/10.1590/S0104-66322007000300002>

- Farran, I., Sánchez-Serrano, J. J., Medina, J. F., Prieto, J., Mingo-Castel, A. M. (2002). Targeted Expression of Human Serum Albumin to Potato Tubers. *Transgenic Research*, 11(4), 337–346. <https://doi.org/10.1023/A:1016356510770>
- Farrell, A., Mittermayr, S., Morrissey, B., Mc Loughlin, N., Navas Iglesias, N., Marison, I. W., Bones, J. (2015). Quantitative Host Cell Protein Analysis Using Two Dimensional Data Independent LC–MS<sup>E</sup>. *Analytical Chemistry*, 87(18), 9186–9193. <https://doi.org/10.1021/acs.analchem.5b01377>
- Fernández-San Millán, A., Mingo-Castel, A., Miller, M., Daniell, H. (2003). A chloroplast transgenic approach to hyper-express and purify Human Serum Albumin, a protein highly susceptible to proteolytic degradation. *Plant Biotechnology Journal*, 1(2), 71–79. <https://doi.org/10.1046/j.1467-7652.2003.00008.x>
- Fleer, R., Yeh, P., Amellal, N., Maury, I., Fournier, A., Bacchetta, F., ... Mayaux, J. F. (1991). Stable Multicopy Vectors for High–Level Secretion of Recombinant Human Serum Albumin by *Kluyveromyces* Yeasts. *Bio/Technology*, 9(10), 968–975. <https://doi.org/10.1038/nbt1091-968>
- Fox, J. L. (2012). First plant-made biologic approved. *Nature Biotechnology*, 30(6), 472–472. <https://doi.org/10.1038/nbt0612-472>
- Frahm, G. E., Cyr, T. D., Smith, D. G. S., Walrond, L. D., Johnston, M. J. W. (2012). Investigation of the Differences in Thermal Stability of Two Recombinant Human Serum Albumins with 1,2-Dipalmitoyl- *sn* -glycero-3-phosphocholine Liposomes by UV Circular Dichroism Spectropolarimetry. *The Journal of Physical Chemistry B*, 116(15), 4661–4670. <https://doi.org/10.1021/jp2125729>
- Frahm, G. E., Smith, D. G. S., Kane, A., Lorbetskie, B., Cyr, T. D., Girard, M., Johnston, M. J. W. (2014). Determination of Supplier-to-Supplier and Lot-to-Lot Variability in Glycation of Recombinant Human Serum Albumin Expressed in *Oryza sativa*. *PLoS ONE*, 9(10), e109893. <https://doi.org/10.1371/journal.pone.0109893>
- Fujiwara, Y., Aiki, Y., Yang, L., Takaiwa, F., Kosaka, A., Tsuji, N. M., ... Sekikawa, K. (2010). Extraction and purification of human interleukin-10 from transgenic rice seeds. *Protein Expression and Purification*, 72(1), 125–130. <https://doi.org/10.1016/J.PEP.2010.02.008>
- Fujiwara, Y., Yang, L., Takaiwa, F., Sekikawa, K. (2016). Expression and Purification of Recombinant Mouse Interleukin-4 and -6 from Transgenic Rice Seeds. *Molecular*

- Biotechnology*, 58(4), 223–231. <https://doi.org/10.1007/s12033-016-9920-7>
- GE Healthcare. (2016). Ion Exchange Chromatography Principles and Methods. Chicago: GE Healthcare. Retrieved from [www.gelifesciences.com/handbooks](http://www.gelifesciences.com/handbooks)
- Goldstein, D. A., Thomas, J. A. (2004). Biopharmaceuticals derived from genetically modified plants. *QJM*, 97(11), 705–716. <https://doi.org/10.1093/qjmed/hch121>
- Gomord, V., Fitchette, A.-C., Menu-Bouaouiche, L., Saint-Jore-Dupas, C., Plasson, C., Michaud, D., Faye, L. (2010). Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotechnology Journal*, 8(5), 564–587. <https://doi.org/10.1111/j.1467-7652.2009.00497.x>
- Gronemeyer, P., Ditz, R., Strube, J. (2014). Trends in Upstream and Downstream Process Development for Antibody Manufacturing. *Bioengineering*, 1(4), 188–212. <https://doi.org/10.3390/bioengineering1040188>
- Grushka, E., Grinberg, N. (2009). *Advances in Chromatography. Volume 47*. CRC Press.
- Hardin, A. M., Harinarayan, C., Malmquist, G., Axén, A., van Reis, R. (2009). Ion exchange chromatography of monoclonal antibodies: effect of resin ligand density on dynamic binding capacity. *Journal of Chromatography. A*, 1216(20), 4366–71. <https://doi.org/10.1016/j.chroma.2008.08.047>
- Harrison, R. G., Todd, P., Rudge, S. R., Petrides, D. P. (2015). *Bioseparations science and engineering*. (K. E. Gubbins, M. A. Barteau, D. A. Lauffenburger, M. Morari, W. H. Ray, & W. B. Russel, Eds.) (2nd ed.). New York: Oxford University Press.
- He, X. M., Carter, D. C. (1992). Atomic structure and chemistry of human serum albumin. *Nature*, 358(6383), 209–215. <https://doi.org/10.1038/358209a0>
- He, Y., Ning, T., Xie, T., Qiu, Q., Zhang, L., Sun, Y., ... Yang, D. (2011). Large-scale production of functional human serum albumin from transgenic rice seeds. *Proceedings of the National Academy of Sciences*, 108(47), 19078–19083. <https://doi.org/10.1073/pnas.1109736108>
- Hellwig, S., Drossard, J., Twyman, R. M., Fischer, R. (2004). Plant cell cultures for the production of recombinant proteins. *Nature Biotechnology*, 22(11), 1415–1422. <https://doi.org/10.1038/nbt1027>
- Hennegan, K., Yang, D., Nguyen, D., Wu, L., Goding, J., Huang, J., ... Watkins, S. C. (2005). Improvement of Human lysozyme Expression in Transgenic Rice Grain by Combining

- Wheat (*Triticum aestivum*) puroindoline b and Rice (*Oryza sativa*) Gt1 Promoters and Signal Peptides. *Transgenic Research*, 14(5), 583–592. <https://doi.org/10.1007/s11248-004-6702-y>
- Hood, E. E., Howard, J. A. (2014). Commercial Plant-Produced Recombinant Avidin (pp. 15–25). [https://doi.org/10.1007/978-3-662-43836-7\\_2](https://doi.org/10.1007/978-3-662-43836-7_2)
- Hood, E. E., Requesens, D. V. (2012). Production of Industrial Proteins in Plants. In *Molecular Farming in Plants: Recent Advances and Future Prospects* (pp. 161–181). Dordrecht: Springer Netherlands. [https://doi.org/10.1007/978-94-007-2217-0\\_8](https://doi.org/10.1007/978-94-007-2217-0_8)
- Hopkins, T. R. (1991). Physical and chemical cell disruption for the recovery of intracellular proteins. *Bioprocess Technology*, 12, 57–83. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1367090>
- Horiguchi, T., Kitagishi, K. (1976). Studies on rice seed protease. *Soil Science and Plant Nutrition*, 22(1), 73–80. <https://doi.org/10.1080/00380768.1976.10432968>
- Houghes, S. S. (2011). *Genentech: The Beginnings of Biotech - Sally Smith Hughes - Google Books*. Chicago: The University of Chicago Press.
- Huang, B. X., Kim, H.-Y., Dass, C. (2004). Probing three-dimensional structure of bovine serum albumin by chemical cross-linking and mass spectrometry. *Journal of the American Society for Mass Spectrometry*, 15(8), 1237–1247. <https://doi.org/10.1016/j.jasms.2004.05.004>
- Huang, J., Nandi, S., Wu, L., Yalda, D., Bartley, G., Rodriguez, R., ... Huang, N. (2002). Expression of natural antimicrobial human lysozyme in rice grains. *Molecular Breeding*, 10(1/2), 83–94. <https://doi.org/10.1023/A:1020355511981>
- Huang, L.-F., Liu, Y.-K., Lu, C.-A., Hsieh, S.-L., Yu, S.-M. (2005). Production of human serum albumin by sugar starvation induced promoter and rice cell culture. *Transgenic Research*, 14(5), 569–581. <https://doi.org/10.1007/s11248-004-6481-5>
- Huang, N., Rodriguez, R., Hagie, F. (2003, August 12). *US20040111766A1*. Retrieved from <https://patents.google.com/patent/US20040111766>
- Huang, N., Rodriguez, R. L., Hagie, F. E., Stalker, D. M. (2010, March 31). *US8158857B2*. Retrieved from <https://patents.google.com/patent/US8158857>
- Huang, T.-K., McDonald, K. A. (2012). Bioreactor systems for in vitro production of foreign proteins using plant cell cultures. *Biotechnology Advances*, 30(2), 398–409. <https://doi.org/10.1016/j.biotechadv.2011.07.016>

- Huang, Y.-M., Hu, W., Rustandi, E., Chang, K., Yusuf-Makagiansar, H., Ryll, T. (2010). Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment. *Biotechnology Progress*, 26(5), 1400–1410. <https://doi.org/10.1002/btpr.436>
- ISAAA. (2017). *Global Status of Commercialized Biotech/GM Crops in 2017: Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 Years*. Ithaca, NY. <https://doi.org/978-1-892456-67-2>
- Jaenisch, R. (1976). Germ line integration and Mendelian transmission of the exogenous Moloney leukemia virus. *Proceedings of the National Academy of Sciences of the United States of America*, 73(4), 1260–4. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1063407>
- Jiang, L., Jin, Y., Marcus, R. K. (2015). Polyethylenimine modified poly(ethylene terephthalate) capillary channeled-polymer fibers for anion exchange chromatography of proteins. *Journal of Chromatography A*, 1410, 200–209. <https://doi.org/10.1016/J.CHROMA.2015.07.102>
- Juliano, B. O., Tuaño, A. P. P. (2019). Gross structure and composition of the rice grain. *Rice*, 31–53. <https://doi.org/10.1016/B978-0-12-811508-4.00002-2>
- Jungbauer, A., Hahn, R. (2009). Chapter 22 Ion-Exchange Chromatography. *Methods in Enzymology*, 463, 349–371. [https://doi.org/10.1016/S0076-6879\(09\)63022-6](https://doi.org/10.1016/S0076-6879(09)63022-6)
- Kang, H. A., Choi, E.-S., Hong, W.-K., Kim, J.-Y., Ko, S.-M., Sohn, J.-H., Rhee, S. K. (2000). Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, 53(5), 575–582. <https://doi.org/10.1007/s002530051659>
- Kashima, K., Yuki, Y., Mejima, M., Kurokawa, S., Suzuki, Y., Minakawa, S., ... Kiyono, H. (2016). Good manufacturing practices production of a purification-free oral cholera vaccine expressed in transgenic rice plants. *Plant Cell Reports*, 35(3), 667–679. <https://doi.org/10.1007/s00299-015-1911-9>
- Kelley, B. (2007). Very Large Scale Monoclonal Antibody Purification: The Case for Conventional Unit Operations. *Biotechnology Progress*, 0(0), 0–0. <https://doi.org/10.1021/bp070117s>
- Kim, T. W., Chung, B. H., Chang, Y. K. (2008). Production of Soluble Human Interleukin-6 in Cytoplasm by Fed-Batch Culture of Recombinant *E. coli*. *Biotechnology Progress*, 21(2),

- 524–531. <https://doi.org/10.1021/bp049645j>
- Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Ohi, H., ... Ohmura, T. (2000). High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. *Journal of Bioscience and Bioengineering*, 89(1), 55–61. [https://doi.org/10.1016/S1389-1723\(00\)88050-0](https://doi.org/10.1016/S1389-1723(00)88050-0)
- Komarova, T. V., Baschieri, S., Donini, M., Marusic, C., Benvenuto, E., Dorokhov, Y. L. (2010). Transient expression systems for plant-derived biopharmaceuticals. *Expert Review of Vaccines*, 9(8), 859–876. <https://doi.org/10.1586/erv.10.85>
- Kramer, M. G., Redenbaugh, K. (1994). Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR tomato story. *Euphytica*, 79(3), 293–297. <https://doi.org/10.1007/BF00022530>
- Kratz, F. (2008). Albumin as a drug carrier: Design of prodrugs, drug conjugates and nanoparticles. *Journal of Controlled Release*, 132(3), 171–183. <https://doi.org/10.1016/J.JCONREL.2008.05.010>
- Krishnan, A., Woodard, S. L. (2014). TrypZean™: An Animal-Free Alternative to Bovine Trypsin (pp. 43–63). Springer, Berlin, Heidelberg. [https://doi.org/10.1007/978-3-662-43836-7\\_4](https://doi.org/10.1007/978-3-662-43836-7_4)
- Kuo, Y. C., Tan, C. C., Ku, J. T., Hsu, W. C., Su, S. C., Lu, C. A., Huang, L. F. (2013). Improving pharmaceutical protein production in *Oryza sativa*. *International Journal of Molecular Sciences*, 14(5), 8719–8739. <https://doi.org/10.3390/ijms14058719>
- Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227(5259), 680–685. <https://doi.org/10.1038/227680a0>
- Lagassé, H. A. D., Alexaki, A., Simhadri, V. L., Katagiri, N. H., Jankowski, W., Sauna, Z. E., Kimchi-Sarfaty, C. (2017). Recent advances in (therapeutic protein) drug development. *F1000Research*, 6, 113. <https://doi.org/10.12688/f1000research.9970.1>
- Larsen, J. S., Curtis, W. R. (2012). RNA viral vectors for improved *Agrobacterium*-mediated transient expression of heterologous proteins in *Nicotiana benthamiana* cell suspensions and hairy roots. *BMC Biotechnology*, 12(1), 21. <https://doi.org/10.1186/1472-6750-12-21>
- Latta, M., Knapp, M., Sarmientos, P., Bréfort, G., Becquart, J., Guerrier, L., ... Mayaux, J.-F. (1987). Synthesis and Purification of Mature Human Serum Albumin from *E. Coli*. *Nature Biotechnology*, 5(12), 1309–1314. <https://doi.org/10.1038/nbt1287-1309>

- Lau, O. S., Sun, S. S. M. (2009). Plant seeds as bioreactors for recombinant protein production. *Biotechnology Advances*, 27(6), 1015–1022.  
<https://doi.org/10.1016/j.biotechadv.2009.05.005>
- Lee, J. H., Ko, K. (2017). Production of Recombinant Anti-Cancer Vaccines in Plants. *Biomolecules & Therapeutics*, 25(4), 345–353. <https://doi.org/10.4062/biomolther.2016.126>
- Leibly, D. J., Nguyen, T. N., Kao, L. T., Hewitt, S. N., Barrett, L. K., Van Voorhis, W. C. (2012). Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins. *PloS One*, 7(12), e52482. <https://doi.org/10.1371/journal.pone.0052482>
- Lenhoff, A. M. (2016). Ion-exchange chromatography of proteins: the inside story. *Materials Today: Proceedings*, 3(10), 3559–3567. <https://doi.org/10.1016/J.MATPR.2016.10.038>
- Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fischette-Lainé, A.-C., Gomord, V., Faye, L. (1998). N-Glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Molecular Biology*, 38(1/2), 31–48. <https://doi.org/10.1023/A:1006012005654>
- Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., Amanullah, A. (2010). Cell culture processes for monoclonal antibody production. *MAbs*, 2(5), 466–79.  
<https://doi.org/10.4161/MABS.2.5.12720>
- Loh, H.-S., Green, B. J., Yusibov, V. (2017). Using transgenic plants and modified plant viruses for the development of treatments for human diseases. *Current Opinion in Virology*, 26, 81–89. <https://doi.org/10.1016/J.COVIRO.2017.07.019>
- Lu, J., Stewart, A. J., Sadler, P. J., Pinheiro, T. J. T., Blindauer, C. A. (2008). Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. *Biochemical Society Transactions*, 36(6), 1317–1321. <https://doi.org/10.1042/BST0361317>
- Ma, J. K.-C., Drake, P. M. W., Christou, P. (2003). The production of recombinant pharmaceutical proteins in plants. *Nature Reviews Genetics*, 4(10), 794–805.  
<https://doi.org/10.1038/nrg1177>
- Malhotra, A., Coupland, J. N. (2004). The effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates. *Food Hydrocolloids*, 18(1), 101–108.  
[https://doi.org/10.1016/S0268-005X\(03\)00047-X](https://doi.org/10.1016/S0268-005X(03)00047-X)
- MarketandMarkets. (2017). *Protein Expression Market by Type & Application - Global Forecast 2022* | MarketsandMarkets. Pune, India. Retrieved from <https://www.marketsandmarkets.com/Market-Reports/protein-expression-market->



180323924.html

- Marth, E., Kleinhapl, B. (2001). Albumin is a necessary stabilizer of TBE-vaccine to avoid fever in children after vaccination. *Vaccine*, 20(3–4), 532–7. [https://doi.org/10.1016/S0264-410X\(01\)00329-2](https://doi.org/10.1016/S0264-410X(01)00329-2)
- Mendez, C. M., McClain, C. J., Marsano, L. S. (2005). Albumin Therapy in Clinical Practice. *Nutrition in Clinical Practice*, 20(3), 314–320. <https://doi.org/10.1177/0115426505020003314>
- Menkhaus, T. J., Bai, Y., Zhang, C., Nikolov, Z. L., Glatz, C. E. (2004). Considerations for the recovery of recombinant proteins from plants. *Biotechnology Progress*, 20(4), 1001–1014. <https://doi.org/10.1021/bp040011m>
- Miller, P. J., Estevez, C., Yu, Q., Suarez, D. L., King, D. J. (2009). Comparison of Viral Shedding Following Vaccination With Inactivated and Live Newcastle Disease Vaccines Formulated With Wild-Type and Recombinant Viruses. *Avian Diseases*, 53(1), 39–49. <https://doi.org/10.1637/8407-071208-Reg.1>
- Montesinos, L., Bundó, M., Badosa, E., San Segundo, B., Coca, M., Montesinos, E. (2017). Production of BP178, a derivative of the synthetic antibacterial peptide BP100, in the rice seed endosperm. *BMC Plant Biology*, 17(1), 63. <https://doi.org/10.1186/s12870-017-1011-9>
- Nandi, S., Suzuki, Y. A., Huang, J., Yalda, D., Pham, P., Wu, L., ... Lönnardal, B. (2002). Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Science*, 163(4), 713–722. [https://doi.org/10.1016/S0168-9452\(02\)00165-6](https://doi.org/10.1016/S0168-9452(02)00165-6)
- National Academies of Sciences, E. and M. (2016). *Genetically Engineered Crops*. Washington, D.C.: National Academies Press. <https://doi.org/10.17226/23395>
- Nawaz, A., Farooq, M. (2017). Rice Physiology. In *Rice Production Worldwide* (pp. 455–485). Cham: Springer International Publishing. [https://doi.org/10.1007/978-3-319-47516-5\\_17](https://doi.org/10.1007/978-3-319-47516-5_17)
- Ning, T., Xie, T., Qiu, Q., Yang, W., Zhou, S., Zhou, L., ... Yang, D. (2008). Oral administration of recombinant human granulocyte-macrophage colony stimulating factor expressed in rice endosperm can increase leukocytes in mice. *Biotechnology Letters*, 30(9), 1679–1686. <https://doi.org/10.1007/s10529-008-9717-2>
- Nochi, T., Takagi, H., Yuki, Y., Yang, L., Masumura, T., Mejima, M., ... Kiyono, H. (2007). Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proceedings of the National Academy of Sciences*, 104(26), 10986–10991.

<https://doi.org/10.1073/PNAS.0703766104>

Oakes, J. L., Bost, K. L., Piller, K. J. (2009). Stability of a soybean seed-derived vaccine antigen following long-term storage, processing and transport in the absence of a cold chain.

*Journal of the Science of Food and Agriculture*, 89(13), 2191–2199.

<https://doi.org/10.1002/jsfa.3705>

Otagiri, M., Chuang, V. T. G. (2009). Pharmaceutically important pre- and posttranslational modifications on human serum albumin. *Biological & Pharmaceutical Bulletin*, 32(4), 527–34. <https://doi.org/https://doi.org/10.1248/bpb.32.527>

Otagiri, M., Giam Chuang, V. T. (2016). *Albumin in Medicine*. (M. Otagiri & V. T. G. Chuang, Eds.), *Albumin in Medicine: Pathological and Clinical Applications*. Singapore: Springer Singapore. <https://doi.org/10.1007/978-981-10-2116-9>

Ou, J., Guo, Z., Shi, J., Wang, X., Liu, J., Shi, B., ... Yang, D. (2014). Transgenic rice endosperm as a bioreactor for molecular pharming. *Plant Cell Reports*, 33(4), 585–594.

<https://doi.org/10.1007/s00299-013-1559-2>

Ozturk, S., Hu, W. (2005). *Cell culture technology for pharmaceutical and cell-based therapies*. (S. Ozturk & W.-S. Hu, Eds.). New York: Taylor & Francis.

Pabst, T. M., Carta, G. (2007). pH transitions in cation exchange chromatographic columns containing weak acid groups. *Journal of Chromatography A*, 1142(1), 19–31.

<https://doi.org/10.1016/J.CHROMA.2006.08.066>

Peters, T. (1996). *All about albumin : biochemistry, genetics, and medical applications*. Academic Press.

Petrelli, F., Borgonovo, K., Barni, S. (2010). Targeted delivery for breast cancer therapy: the history of nanoparticle-albumin-bound paclitaxel. *Expert Opinion on Pharmacotherapy*,

11(8), 1413–1432. <https://doi.org/10.1517/14656561003796562>

Petrides, D., Cooney, C., Evans, L. (1989). An introduction to biochemical process design. In M. Winkler (Ed.), *Chemical Engineering Problems in Biotechnology* (1st ed., p. 351). New York: American Institute of Chemical Engineering.

Pillet, S., Aubin, É., Trépanier, S., Bussière, D., Dargis, M., Poulin, J.-F., ... Landry, N. (2016). A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults. *Clinical Immunology*, 168, 72–87.

<https://doi.org/10.1016/J.CLIM.2016.03.008>

- Pogue, G. P., Vojdani, F., Palmer, K. E., Hiatt, E., Hume, S., Phelps, J., ... Bratcher, B. (2010). Production of pharmaceutical-grade recombinant aprotinin and a monoclonal antibody product using plant-based transient expression systems. *Plant Biotechnology Journal*, 8(5), 638–654. <https://doi.org/10.1111/j.1467-7652.2009.00495.x>
- Prabhu, A. A., Jayadeep, A. (2015). Enzymatic processing of pigmented and non pigmented rice bran on changes in oryzanol, polyphenols and antioxidant activity. *Journal of Food Science and Technology*, 52(10), 6538–6546. <https://doi.org/10.1007/s13197-015-1761-z>
- Qiu, X., Wong, G., Audet, J., Bello, A., Fernando, L., Alimonti, J. B., ... Kobinger, G. P. (2014). Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*, 514(7520), 47–53. <https://doi.org/10.1038/nature13777>
- Rahmani, S., Mohseni, M. (2017). The role of hydrophobic properties in ion exchange removal of organic compounds from water. *The Canadian Journal of Chemical Engineering*, 95(8), 1449–1455. <https://doi.org/10.1002/cjce.22823>
- Ramírez, N., Oramas, P., Ayala, M., Rodríguez, M., Pérez, M., Gavilondo, J. (2001). Expression and long-term stability of a recombinant single-chain Fv antibody fragment in transgenic *Nicotiana tabacum* seeds. *Biotechnology Letters*, 23(1), 47–49. <https://doi.org/10.1023/A:1026728721966>
- Rasband, W. (1997). ImageJ. Bethesda, MD: US National Institutes of Health.
- Rong, J., Lu, B.-R., Song, Z., Su, J., Snow, A. A., Zhang, X., ... Wang, F. (2007). Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields. *New Phytologist*, 173(2), 346–353. <https://doi.org/10.1111/j.1469-8137.2006.01906.x>
- Rosano, G. L., Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in Microbiology*, 5, 172. <https://doi.org/10.3389/fmicb.2014.00172>
- Ryan, B. J. (2011). Avoiding Proteolysis During Protein Chromatography. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 681, pp. 61–71). [https://doi.org/10.1007/978-1-60761-913-0\\_4](https://doi.org/10.1007/978-1-60761-913-0_4)
- Ryan, C. A. (1990). Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens. *Annual Review of Phytopathology*, 28(1), 425–449. <https://doi.org/10.1146/annurev.py.28.090190.002233>
- Sabalza, M., Christou, P., Capell, T. (2014). Recombinant plant-derived pharmaceutical proteins:

- current technical and economic bottlenecks. *Biotechnology Letters*, 36(12), 2367–2379.  
<https://doi.org/10.1007/s10529-014-1621-3>
- Saber, M. A., Stöckbauer, P., Morávek, L., Meloun, B. (1977). Disulfide bonds in human serum albumin. *Collection of Czechoslovak Chemical Communications*, 42(2), 564–579.  
<https://doi.org/10.1135/cccc19770564>
- San Juan, A. A. (2018). Review of Biopharmaceuticals and Nutraceuticals from Rice Grain: Exploiting the Endosperm, Germ and Bran for High-value Innovation Rice By-products. *Philippine Journal of Science*, 147(3), 431–439.
- Sancataldo, G., Vetri, V., Foderà, V., Di Cara, G., Militello, V., Leone, M. (2014). Oxidation Enhances Human Serum Albumin Thermal Stability and Changes the Routes of Amyloid Fibril Formation. *PLoS ONE*, 9(1), e84552. <https://doi.org/10.1371/journal.pone.0084552>
- Satya, P., Sarkar, D. (2018). Plant Biotechnology and Crop Improvement. *Biotechnology for Sustainable Agriculture*, 93–140. <https://doi.org/10.1016/B978-0-12-812160-3.00004-0>
- Saunders, C. W., Schmidt, B. J., Mallonee, R. L., Guyer, M. S. (1987). Secretion of human serum albumin from *Bacillus subtilis*. *Journal of Bacteriology*, 169(7), 2917–25.  
<https://doi.org/10.1128/JB.169.7.2917-2925.1987>
- Schellekens, H. (2009). Biosimilar therapeutics-what do we need to consider? *NDT Plus*, 2(Suppl\_1), i27–i36. <https://doi.org/10.1093/ndtplus/sfn177>
- Seeberger, P. H., Cummings, R. D. (2015). *Glycans in Biotechnology and the Pharmaceutical Industry. Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press.  
<https://doi.org/10.1101/GLYCOBIOLOGY.3E.057>
- Sehgal, A., Sita, K., Siddique, K. H. M., Kumar, R., Bhogireddy, S., Varshney, R. K., ... Nayyar, H. (2018). Drought or/and Heat-Stress Effects on Seed Filling in Food Crops: Impacts on Functional Biochemistry, Seed Yields, and Nutritional Quality. *Frontiers in Plant Science*, 9, 1705. <https://doi.org/10.3389/fpls.2018.01705>
- Sharma, A., Chaudhuri, T. K. (2017). Revisiting *Escherichia coli* as microbial factory for enhanced production of human serum albumin. *Microbial Cell Factories*, 16(1), 173.  
<https://doi.org/10.1186/s12934-017-0784-8>
- Sheshukova, K. A., Wilken, L. R. (2018). Analysis of Recombinant Human Serum Albumin Extraction and Degradation in Transgenic Rice Extracts. *Biotechnology Progress*, 1–11.  
<https://doi.org/10.1002/btpr.2609>

- Shukla, R., Cheryan, M., DeVor, R. E. (2000). Solvent Extraction of Zein from Dry-Milled Corn. *Cereal Chemistry Journal*, 77(6), 724–730.  
<https://doi.org/10.1094/CCHEM.2000.77.6.724>
- Sijmons, P. C., Dekker, B. M. M., Schrammeijer, B., Verwoerd, T. C., van den Elzen, P. J. M., Hoekema, A. (1990). Production of Correctly Processed Human Serum Albumin in Transgenic Plants. *Nature Biotechnology*, 8(3), 217–221. <https://doi.org/10.1038/nbt0390-217>
- Singh, A., Upadhyay, V., Upadhyay, A. K., Singh, S. M., Panda, A. K. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial Cell Factories*, 14, 41. <https://doi.org/10.1186/s12934-015-0222-8>
- Sleep, D., Belfield, G. P., Goodey, A. R. (1990). The Secretion of Human Serum Albumin from the Yeast Saccharomyces cerevisiae Using Five Different Leader Sequences. *Nature Biotechnology*, 8(1), 42–46. <https://doi.org/10.1038/nbt0190-42>
- Smith, D. G. S., Frahm, G. E., Kane, A., Lorbetskie, B., Girard, M., Johnston, M. J. W., Cyr, T. D. (2015). Data set for mass spectrometric analysis of recombinant human serum albumin from various expression systems. *Data in Brief*, 4, 583–586.  
<https://doi.org/10.1016/J.DIB.2015.07.024>
- Soema, P. C., Kompier, R., Amorij, J.-P., Kersten, G. F. A. (2015). Current and next generation influenza vaccines: Formulation and production strategies. *European Journal of Pharmaceutics and Biopharmaceutics*, 94, 251–263.  
<https://doi.org/10.1016/J.EJPB.2015.05.023>
- Song, Z., Lu, B.-R., Chen, J. (2004). Pollen flow of cultivated rice measured under experimental conditions. *Biodiversity and Conservation*, 13(3), 579–590.  
<https://doi.org/10.1023/B:BIOC.0000009491.24573.1d>
- Stepanenko, A. A., Heng, H. H. (2017). Transient and stable vector transfection: Pitfalls, off-target effects, artifacts. *Mutation Research/Reviews in Mutation Research*, 773, 91–103.  
<https://doi.org/10.1016/j.mrrev.2017.05.002>
- Stoger, E., Ma, J. K.-C., Fischer, R., Christou, P. (2005). Sowing the seeds of success: pharmaceutical proteins from plants. *Current Opinion in Biotechnology*, 16(2), 167–173.  
<https://doi.org/10.1016/j.copbio.2005.01.005>
- Streatfield, S. J. (2007). Approaches to achieve high-level heterologous protein production in

- plants. *Plant Biotechnology Journal*, 5(1), 2–15. <https://doi.org/10.1111/j.1467-7652.2006.00216.x>
- Subramanian, G. M., Fiscella, M., Lamousé-Smith, A., Zeuzem, S., McHutchison, J. G. (2007). Albinterferon  $\alpha$ -2b: a genetic fusion protein for the treatment of chronic hepatitis C. *Nature Biotechnology*, 25(12), 1411–1419. <https://doi.org/10.1038/nbt1364>
- Sun, Q.-Y., Ding, L.-W., Lomonossoff, G. P., Sun, Y.-B., Luo, M., Li, C.-Q., ... Xu, Z.-F. (2011). Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture. *Journal of Biotechnology*, 155(2), 164–172. <https://doi.org/10.1016/J.JBIOTECH.2011.06.033>
- Suzuki, M., Roy, R., Zheng, H., Woychik, N., Inouye, M. (2006). Bacterial bioreactors for high yield production of recombinant protein. *The Journal of Biological Chemistry*, 281(49), 37559–65. <https://doi.org/10.1074/jbc.M608806200>
- Svarovsky, L. (2000). *Solid-liquid separation*. Butterworth-Heinemann.
- Takaiwa, F. (2013). Increasing the production yield of recombinant protein in transgenic seeds by expanding the deposition space within the intracellular compartment. *Bioengineered*, 4(3), 136–139. <https://doi.org/10.4161/bioe.24187>
- Takaiwa, F., Takagi, H., Hirose, S., Wakasa, Y. (2007). Endosperm tissue is good production platform for artificial recombinant proteins in transgenic rice. *Plant Biotechnology Journal*, 5(1), 84–92. <https://doi.org/10.1111/j.1467-7652.2006.00220.x>
- Takaiwa, F., Wakasa, Y., Takagi, H., Hiroi, T. (2015). Rice seed for delivery of vaccines to gut mucosal immune tissues. *Plant Biotechnology Journal*, 13(8), 1041–1055. <https://doi.org/10.1111/pbi.12423>
- Tan, H. Y., Ng, T. W., Liew, O. W. (2009). Adapted liquid crystal display backlighting unit for densitometry of stained polyacrylamide electrophoresis gels. *ELECTROPHORESIS*, 30(6), 987–990. <https://doi.org/10.1002/elps.200800363>
- Tekoah, Y., Shulman, A., Kizhner, T., Ruderfer, I., Fux, L., Nataf, Y., ... Shaaltiel, Y. (2015). Large-scale production of pharmaceutical proteins in plant cell culture-the protalix experience. *Plant Biotechnology Journal*, 13(8), 1199–1208. <https://doi.org/10.1111/pbi.12428>
- Tsuchida, E., Sou, K., Nakagawa, A., Sakai, H., Komatsu, T., Kobayashi, K. (2009). Artificial Oxygen Carriers, Hemoglobin Vesicles and Albumin–Hemes, Based on Bioconjugate

- Chemistry. *Bioconjugate Chemistry*, 20(8), 1419–1440. <https://doi.org/10.1021/bc800431d>
- Turner, P. J., Southern, J., Andrews, N. J., Miller, E., Erlewyn-Lajeunesse, M., Doyle, C., ...  
Turner, P. J. (2015). Safety of live attenuated influenza vaccine in atopic children with egg allergy. *Journal of Allergy and Clinical Immunology*, 136(2), 376–381.  
<https://doi.org/10.1016/J.JACI.2014.12.1925>
- Tusé, D., Tu, T., McDonald, K. A. (2014). Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *BioMed Research International*, 2014, 256135. <https://doi.org/10.1155/2014/256135>
- Twyman, R. M., Stoger, E., Schillberg, S., Christou, P., Fischer, R. (2003). Molecular farming in plants: host systems and expression technology. *Trends in Biotechnology*, 21(12), 570–578. <https://doi.org/10.1016/J.TIBTECH.2003.10.002>
- Vajda, J., Weber, D., Stefaniak, S., Hundt, B., Rathfelder, T., Müller, E. (2016). Mono- and polyprotic buffer systems in anion exchange chromatography of influenza virus particles. *Journal of Chromatography A*, 1448, 73–80.  
<https://doi.org/https://doi.org/10.1016/j.chroma.2016.04.047>
- Vamvaka, E., Arcalis, E., Ramessar, K., Evans, A., O’Keefe, B. R., Shattock, R. J., ... Capell, T. (2016). Rice endosperm is cost-effective for the production of recombinant griffithsin with potent activity against HIV. *Plant Biotechnology Journal*, 14(6), 1427–1437.  
<https://doi.org/10.1111/pbi.12507>
- Vamvaka, E., Twyman, R. M., Murad, A. M., Melnik, S., Teh, A. Y.-H., Arcalis, E., ... Capell, T. (2016). Rice endosperm produces an underglycosylated and potent form of the HIV-neutralizing monoclonal antibody 2G12. *Plant Biotechnology Journal*, 14(1), 97–108.  
<https://doi.org/10.1111/pbi.12360>
- Vieira Gomes, A. M., Souza Carmo, T., Silva Carvalho, L., Mendonça Bahia, F., Parachin, N. S. (2018). Comparison of Yeasts as Hosts for Recombinant Protein Production. *Microorganisms*, 6(2). <https://doi.org/10.3390/microorganisms6020038>
- Wang, X., Quinn, P. J. (2010). *Endotoxins : Structure, function and recognition*. Springer Verlag.
- Watanabe, H., Kragh-Hansen, U., Tanase, S., Nakajou, K., Mitarai, M., Iwao, Y., ... Otagiri, M. (2001). Conformational stability and warfarin-binding properties of human serum albumin studied by recombinant mutants. *The Biochemical Journal*, 357(Pt 1), 269–74.

<https://doi.org/10.1042/BJ3570269>

- Wetzel, R., Becker, M., Behlke, J., Billwitz, H., Böhm, S., Ebert, B., ... Lassmann, G. (1980). Temperature behaviour of human serum albumin. *European Journal of Biochemistry*, *104*(2), 469–78. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/6244951>
- Wilken, L., Nikolov, Z. (2006). Factors Influencing Recombinant Human Lysozyme Extraction and Cation Exchange Adsorption. *Biotechnology Progress*, *22*(3), 745–752. <https://doi.org/10.1021/bp0600536>
- Wilken, L. R., Nikolov, Z. L. (2010). Evaluation of alternatives for human lysozyme purification from transgenic rice: Impact of phytic acid and buffer. *Biotechnology Progress*, *26*(5), 1303–1311. <https://doi.org/10.1002/btpr.434>
- Wilken, L. R., Nikolov, Z. L. (2012a). Downstream Processing of Transgenic Plant Systems: Protein Recovery and Purification Strategies. In *Molecular Farming in Plants: Recent Advances and Future Prospects* (pp. 217–257). Dordrecht: Springer Netherlands. [https://doi.org/10.1007/978-94-007-2217-0\\_11](https://doi.org/10.1007/978-94-007-2217-0_11)
- Wilken, L. R., Nikolov, Z. L. (2012b). Recovery and purification of plant-made recombinant proteins. *Biotechnology Advances*, *30*(2), 419–433. <https://doi.org/10.1016/j.biotechadv.2011.07.020>
- Wurm, F. M. (2008). Manufacture of Recombinant Biopharmaceutical Proteins by Cultivated Mammalian Cells in Bioreactors. In *Modern Biopharmaceuticals* (pp. 723–759). Weinheim, Germany: Wiley-VCH Verlag GmbH. <https://doi.org/10.1002/9783527620982.ch31>
- Wycoff, K. (2005). Secretory IgA Antibodies from Plants. *Current Pharmaceutical Design*, *11*(19), 2429–2437. <https://doi.org/10.2174/1381612054367508>
- Xia, M., Farkas, T., Jiang, X. (2007). Norovirus capsid protein expressed in yeast forms virus-like particles and stimulates systemic and mucosal immunity in mice following an oral administration of raw yeast extracts. *Journal of Medical Virology*, *79*(1), 74–83. <https://doi.org/10.1002/jmv.20762>
- Xu, J., Dolan, M. C., Medrano, G., Cramer, C. L., Weathers, P. J. (2012). Green factory: Plants as bioproduction platforms for recombinant proteins. *Biotechnology Advances*, *30*(5), 1171–1184. <https://doi.org/10.1016/J.BIOTECHADV.2011.08.020>
- Yamasaki, K., Anraku, M. (2016). Stability of Albumin and Stabilization of Albumin Preparations. In *Albumin in Medicine* (pp. 25–49). Singapore: Springer Singapore.



[https://doi.org/10.1007/978-981-10-2116-9\\_2](https://doi.org/10.1007/978-981-10-2116-9_2)

- Yang, D., Ou, J., Shi, J., Guo, Z., Shi, B., Abiri, N. (2018). Transgenic Rice for the Production of Recombinant Pharmaceutical Proteins. In *Molecular Pharming* (pp. 275–307). Hoboken, NJ, USA: John Wiley & Sons, Inc. <https://doi.org/10.1002/9781118801512.ch11>
- Yang, Y., Dai, L., Xia, H., Zhu, K., Liu, H., Chen, K. (2013). Protein profile of rice (*Oryza sativa*) seeds. *Genetics and Molecular Biology*, *36*(1), 87–92. <https://doi.org/10.1590/S1415-47572013000100012>
- Yao, J., Weng, Y., Dickey, A., Wang, K. Y. (2015). Plants as factories for human pharmaceuticals: Applications and challenges. *International Journal of Molecular Sciences*, *16*(12), 28549–28565. <https://doi.org/10.3390/ijms161226122>
- Yuan, D., Bassie, L., Sabalza, M., Miralpeix, B., Dashevskaya, S., Farre, G., ... Christou, P. (2011). The potential impact of plant biotechnology on the Millennium Development Goals. *Plant Cell Reports*, *30*(3), 249–265. <https://doi.org/10.1007/s00299-010-0987-5>
- Zhang, D., Nandi, S., Bryan, P., Pettit, S., Nguyen, D., Santos, M. A., Huang, N. (2010). Expression, purification, and characterization of recombinant human transferrin from rice (*Oryza sativa* L.). *Protein Expression and Purification*, *74*(1), 69–79. <https://doi.org/10.1016/J.PEP.2010.04.019>
- Zhang, L., Shi, J., Jiang, D., Stupak, J., Ou, J., Qiu, Q., ... Yang, D. (2013). Expression and characterization of recombinant human alpha-antitrypsin in transgenic rice seed. *Journal of Biotechnology*, *164*(2), 300–308. <https://doi.org/10.1016/j.jbiotec.2013.01.008>
- Zischewski, J., Sack, M., Fischer, R. (2016). Overcoming low yields of plant-made antibodies by a protein engineering approach. *Biotechnology Journal*, *11*(1), 107–116. <https://doi.org/10.1002/biot.201500255>