RECOVERY OF RECOMBINANT AND NATIVE PROTEINS FROM RICE AND CORN SEED

A Dissertation

by

LISA RACHELLE WILKEN

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

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Biological & Agricultural Engineering

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Approved by:

Chair of Committee,	Zivko L. Nikolov
Committee Members,	M. Elena Castell-Perez
	Cady R. Engler
	Martin B. Dickman
Head of Department,	Gary Riskowski

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ABSTRACT

Recovery of Recombinant and Native Proteins from Rice and Corn Seed. (August 2009)

Lisa Rachelle Wilken, B.S., Kansas State University

Chair of Advisory Committee: Dr. Zivko L. Nikolov

Plants are potential sources of valuable recombinant and native proteins that can be purified for pharmaceutical, nutraceutical, and food applications. Transgenic rice and corn germ were evaluated for the production of novel protein products. This dissertation addresses: 1) the extraction and purification of the recombinant protein, human lysozyme (HuLZ), from transgenic rice and 2) the processing of dry-milled corn germ for the production of high protein germ and corn protein concentrate (CPC).

The factors affecting the extraction and purification of HuLZ from rice were evaluated. Ionic strength and pH was used to optimize HuLZ extraction and cation exchange purification. The selected conditions, pH 4.5 with 50 mM NaCl, were a compromise between HuLZ extractability and binding capacity, resulting in 90% purity. Process simulation was used to assess the HuLZ purification efficiency and showed that the processing costs were comparable to native lysozyme purification from egg-white, the current predominant lysozyme source.

Higher purity HuLZ (95%) could be achieved using pH 4.5 extraction followed by pH 6 adsorption, but the binding capacity was unexpectedly reduced by 80%. The rice impurity, phytic acid, was identified as the potential cause of the unacceptably low

capacity. Enzymatic (phytase) treatment prior to adsorption improved purification, implicating phytic acid as the primary culprit. Two processing methods were proposed to reduce this interference: 1) pH 10 extraction followed by pH 4.5 precipitation and pH 6 adsorption and 2) pH 4.5 extraction and pH 6 adsorption in the presence of TRIS counter-ions. Both methods improved the binding capacity from 8.6 mg/mL to >25 mg/mL and maintained HuLZ purity.

Processing of dry-milled corn germ to increase protein and oil content was evaluated using germ wet milling. In this novel method, dry-milled germ is soaked and wet processed to produce higher value protein products. Lab-scale and pilot-scale experiments identified soaking conditions that reduced germ starch content, enhanced protein and oil content, and maintained germ PDI (protein dispersibility index). Soaking at neutral pH and 25°C maintained germ PDI and improved CPC yield from defatted germ flour. CPC with >75% protein purity was produced using protein precipitation or membrane filtration.

DEDICATION

To all the exceptional teachers and mentors I have had throughout my education and to my family who waited a long time for the end but cheered for me from the start.

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I thank my committee chair, Dr. Nikolov, for inspiring me to pursue a Ph.D., guiding my professional development, and for providing invaluable research and teaching opportunities. I am sincerely grateful for all the time he invested in my education and for allowing and encouraging me to pursue new challenges. I feel very fortunate to have such a wonderful role model and mentor. I also thank my committee members: Dr. Elena Castell, Dr. Cady Engler, and Dr. Marty Dickman for supporting this endeavor. I thank Susan Woodard and Steve White whose experience and guidance in the Bioseparations Lab have been very valuable to me. Thanks also to all the other past and present members of the Bioseparations Lab.

I am also thankful for my friends and colleagues in the Department of Biological & Agricultural Engineering at Texas A&M University. Thanks also to the department for providing funding as a teaching assistant; I enjoyed my teaching experiences and I learned a lot from both the students and professors. I am especially thankful for the continual support and advice of Dr. Phil Barnes from Kansas State University.

Finally, thanks to my parents, brothers, sisters, nieces, and nephews for their patience and understanding.

NOMENCLATURE

CM	Carboxymethyl
CPC	Corn protein concentrate
CV	Column volumes
db	Dry base
DBC	Dynamic binding capacity
DCG	Defatted corn germ
DFC	Direct fixed cost
DDGS	Distillers dried grains with solubles
EXT	Extract
HCl	Hydrochloric acid
HewLZ	Hen egg-white lysozyme
HuLZ	Human lysozyme
kDa	Kilodalton
MW	Molecular weight
mS	milliSiemens
NaAcetate	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PA	Phytic acid
PDI	Protein dispersibility index

pI	Isoelectric point
Ppt	Precipitate
PO_4	Phosphate
RO	Reverse osmosis
RT	Room temperature (25°C)
SBC	Saturation binding capacity
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	Sulfopropyl
TCI	Total capital investment
TSP	Total soluble protein
UF/DF	Ultrafiltration/diafiltration

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

FIGURE Page

LIST OF TABLES

TABLE Page

CHAPTER I

INTRODUCTION

BACKGROUND INTRODUCTION

Plants have been used as a source of natural products throughout history, dating back thousands of years. Plant-based products have numerous, diverse industrial applications that continue to expand with the development of new bioprocess and genetic engineering technologies. One such technology was the development of plant transformation which led to the use of plant systems for the production of recombinant proteins for pharmaceutical, nutraceutical, and industrial applications.

A significant amount of effort has been dedicated to various crops, including rice, as a platform for recombinant protein production. Several recombinant proteins have successfully been expressed in rice, including human lysozyme, which has recently reached commercial levels and the field release of transgenic rice seed was approved by the USDA in 2007. Although InVitria (a rice biotechnology company) is producing small amounts of human lysozyme as a cell culture reagent, comparative process analysis of the existing hen egg-white lysozyme product currently on the market has not been done.

Each plant system presents different processing challenges because each has a unique composition based on the recombinant protein properties and the host system composition. Extraction conditions (solid-liquid ratio, pH, ionic strength, temperature)

This dissertation follows the style of Biotechnology and Bioengineering.

dictate extract composition and may need to be optimized to reduce impurities that interfere with subsequent purification steps. Specific impurities may dictate the sequence of unit operations and operating conditions for extraction and purification. Once the critical extract impurities have been identified, the extraction conditions can be manipulated to minimize their impact on selected recovery and purification steps. Therefore, further understanding of the rice system components and their interactions would facilitate process development. The first objective of this dissertation was to develop a process for the extraction and purification of recombinant human lysozyme from rice using a single chromatography step and to evaluate rice extract impurities that may interfere with purification. Cation exchange chromatography was used for purification based on the highly basic nature of lysozyme and also compared to hen egg-white lysozyme purification.

The use of plants to produce biofuels also provides new and exciting opportunities for traditional food crops that have expanded significantly in the past decade. Ethanol plants are seeking novel fractionation methods for pre-processing due to increasing pressure to make ethanol from corn, the common feedstock in the United States, more cost-competitive while also addressing concerns about using a food crop for fuel.

Corn germ is a non-fermentable corn kernel fraction that is a promising source of higher value food protein based on its protein composition, amino acid distribution, and functional properties. To capture the potential value of germ protein, carefully designed fractionation and extraction processes are required to preserve the protein

quality for food applications. The effect of processing conditions on the germ protein quality has not been addressed and few studies for producing protein-rich concentrates from corn germ are available. Typical methods used for germ processing may need to be modified to prevent protein loss and denaturation throughout fractionation, defatting, grinding, and protein extraction. The second objective of this dissertation was to determine the effect of processing conditions on germ protein quality and to develop a process for producing high protein products from dry-milled corn germ.

LITERATURE REVIEW

Plants as Recombinant Protein Production Systems. Since the first successful transformation of a higher plant in 1983 (Fraley et al., 1983), numerous heterologous proteins have been produced in a wide variety of plant systems. Seed crops, green-tissue plants, and aquatic plants have been used for the expression of human biopharmaceuticals (Giddings et al., 2000), nutraceuticals (Adkins and Lönnerdal, 2004), antibodies (Nikolov et al., 2009; Stöger et al., 2002), industrial enzymes (Ponstein et al., 1996), and vaccine antigens (Sala et al., 2003). Plants have a low cost of production and a relatively simple transition to large-scale production by increasing acreage instead of adding expensive, specialized manufacturing facilities. The cost of producing recombinant proteins in plants has been estimated to be 2-10% of the cost using traditional microbial fermentation systems and 0.01% of the cost of using mammalian cell culture (Hood et al., 2002). Plants are also advantageous because they are efficient protein producers, free from human pathogens, capable of post-translational modifications, and many have natural protein storage organelles. Disadvantages include

the long development time for transformation, regeneration, and production of transgenic plants (Twyman, 2004), regulatory uncertainty (Doran, 2000), and recovery and purification challenges.

Rice as a Host System for Recombinant Proteins. Several recombinant proteins have been successful expressed in rice seed including α-interferon (Zhu et al., 1994), human lactoferrin (Nandi et al., 2002), human serum albumin (Huang et al., 2005), human lysozyme (Huang et al., 2002a), antibodies (Stöger et al., 2000), vaccine antigens (Nochi et al., 2007; Wu et al., 2007), and allergens (Takaiwa et al., 2007; Yang et al., 2008a). Recently, the USDA (2007) approved field release of transgenic rice expressing human lysozyme, lactoferrin, and serum albumin in Kansas and clinical trials using rice-dervied human lysozyme and lactoferrin in oral rehydration solutions have been successfully completed (Zavaleta et al., 2007).

As a seed crop, rice offers many advantages over microbial fermentations, mammalian cell cultures, and transgenic animals as a host system including an extensive knowledge base regarding its cultivation, harvesting, storage, and processing (Kusnadi et al., 1997). Rice is also advantageous because seeds allow for long-term storage and have low concentrations of phenolic compounds which pose problems in downstream processing of green tissues (Ma et al., 2003; Twyman, 2004). Rice is a self-pollinating crop so there is a low rate of gene transfer (Huang, 2004). Rice is also generally regarded as safe (GRAS) and rice-based foods are considered hypoallergenic so products in rice also have the option of oral delivery without extensive purification (Nandi et al., 2002; Yang et al., 2008b). The presence of endogenous protease inhibitors should

ensure the stability of the recombinant protein throughout extraction and purification (Menkhaus et al., 2004a). The use of rice as a platform for recombinant protein production is promising, but high expression levels and efficient downstream processing are needed.

Recombinant Human Lysozyme Expression in Rice. The highest expression of recombinant human lysozyme, 0.5-0.9% dry weight, has been achieved in rice (*Oryza sativa*) seed (Hennegan et al., 2005; Huang, 2004). The tissue-specific rice storage protein promoter, glutelin-1, was used to maximize the expression level and to direct human lysozyme accumulation into the endosperm in type II protein bodies (Hennegan et al., 2005; Hwang et al., 2002). Recombinant human lysozyme expressed in rice has identical physical and biological characteristics as the native form (Huang et al., 2006b). This includes the isoelectric point (pI) , overall charge, N-terminal sequence, specific activities, and surface structure (Huang et al., 2002b).

Properties of Human Lysozyme. Human lysozyme (EC 3.2.1.17), N-acetylmuramoyl-hydrolase, found in tears, saliva, nasal secretions, and breast milk, consists of 130 amino acids with a molecular weight of 15 kDa and isoelectric point (pI) of 10.2 Lysozyme has the ability to hydrolyze the β-(1-4) linkages between N-acetylglucosamine and N-acetylmuramic acid of peptidoglycan (Jollès, 1996), giving the enzyme its antibacterial properties. Human lysozyme also has antiviral, antifungal, and antiinflammatory properties and numerous potential therapeutic applications to a broad spectrum of human diseases.

The unglycosylated protein is present in human milk at concentrations of 50-400 μg/mL but its use is restricted by the limited supply and risk of viral and microbial contamination. To increase the supply of human lysozyme, various production systems have been explored. Human lysozyme has been expressed in mouse milk (Maga et al., 1995) as well as tobacco (Nakajima et al., 1997). However, recombinant human lysozyme produced by these systems was not economically viable due to low expression levels and processing constraints. With the high expression levels of human lysozyme in rice, there is an opportunity to make lysozyme production viable for a variety of applications. To a large degree, market penetration for pharmaceutical, nutraceutical, and food applications hinges on the final product cost of which downstream processing contributes a significant share.

Downstream Processing of Transgenic Seed Crops. Depending on product type and application, downstream processing can account for greater than 80% of the total operating costs (Evangelista et al., 1998) and thus, the full potential of transgenic crops can only be realized with the development of efficient and selective extraction and purification processes. As the transgenic plant platform matures, research and development interest will shift from upstream to downstream processing to improve overall productivity, which has been the case for more established biotechnology (Gottschalk, 2008). Thus, the development of efficient downstream processes as early as possible is essential for favorable economics (Basaran and Rodriguez-Cerezo, 2008).

The general downstream processing strategy for seed crops is given in Figure 1.1. Grinding or fractionation is typically the first step for seed processing to reduce the

particle size for extraction. The extraction (solid-liquid separation) step uses water or buffer to extract water soluble components from the flour containing the recombinant protein. After extraction, the crude extract is clarified by separating the liquid (clarified extract) from the spent solids. This is typically done using centrifugation and in some cases includes an additional dead-end filtration step (Nikolov and Woodard, 2004). The recombinant protein is then purified from the clarified extract by one or more steps (capture and purification) using methods such as chromatography, membrane filtration, and precipitation. The capture step is used to concentrate the product and partially remove extract components and the purification is the final polishing step. In this study, the goal was to develop a single chromatography step to replace the capture and purification steps.

The processing of plant extracts is challenging because each transgenic plant extract is a complex mixture that has unique characteristics based on the extract composition (ions, proteins, sugars, phenolics) and recombinant protein properties (charge, size, hydrophobicity). Therefore, further understanding of the plant system components and their interactions is critical to designing an integrated, optimized purification process.

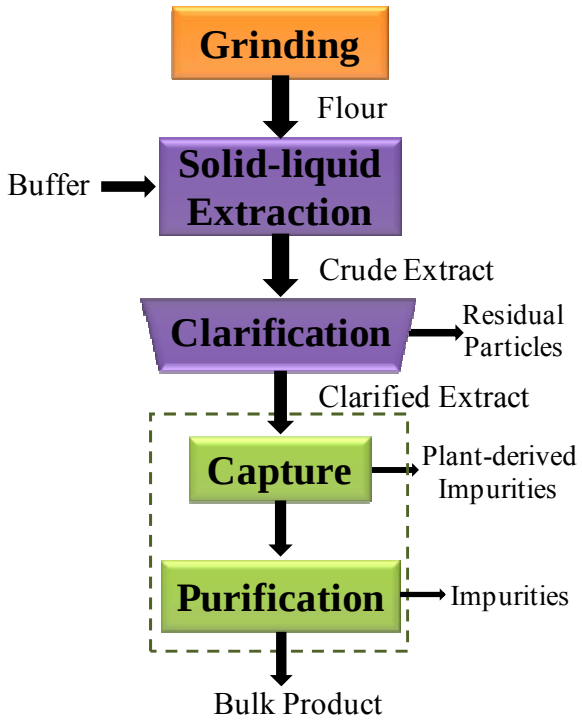

Figure 1.1. General processing strategy for protein purification from seed crops. The focus of Chapters II, III, and IV was to capture and purify lysozyme with a single chromatography step as indicated be the dashed box. Figure adapted from Nikolov and Woodard (2004).

Extraction of Recombinant Proteins from Seed Crops. To reduce purification requirements, extraction conditions that maximize the concentration of recombinant protein and minimize the amount of soluble native protein in the extracts are desirable. Selecting the appropriate extraction conditions is an important factor for reducing the purification costs of recombinant proteins (Azzoni et al., 2002; Menkhaus et al., 2004a; Nikolov and Woodard, 2004). Several studies have specifically addressed the effect of particle size, pH, and ionic strength on the extractability of recombinant and/or native transgenic plant proteins (Azzoni et al., 2005; Bai and Nikolov, 2001; Bai et al., 2002; Farinas et al., 2005; Menkhaus et al., 2004b; Zhang et al., 2005), but only a few in the

context of process integration, optimization, or cost analysis (Azzoni et al., 2002; Kusnadi et al., 1998; Menkhaus et al., 2002; Menkhaus et al., 2004b; Nandi et al., 2005). In general, pH and ionic strength had the greatest effect on protein extractability (Azzoni et al., 2005; Balasubramaniam et al., 2003; Farinas et al., 2005). Extraction conditions determine the extract volume and composition and can be optimized to improve the efficacy of subsequent purification steps.

Capture and Purification by Cation Exchange. Regardless of the selected platform, host system cell components and product- and process-related contaminates must be removed to produce a purified product that meets product specifications (Drossard, 2004). Ion-exchange chromatography is the most widely used purification method in biotechnology due to its resolving power, versatility, high protein binding capacity, robustness, reproducibility, and ease of scale-up (Jacob and Frech, 2007). Ion-exchange is also a common method employed to capture and purify recombinant proteins from plants (Huang et al., 2002a; Menkhaus et al., 2004a; Nandi et al., 2002; Woodard et al., 2003). Cation exchange chromatography has been the preferred method for lysozyme purification and other basic proteins. The strong cation exchange resin, SP-Sepharose FF, was selected for this study since the sulfopropyl (SP) ligand is fully charged from pH 4 to 8 and is commercially available and commonly used at a large scale (Jacob and Frech, 2007).

Adsorption of a protein to a cation exchange resin occurs primarily through Coulombic (electrostatic) interactions between the negatively charged ligand on the resin (stationary phase) and positively charged groups on the protein surface. The solution

containing the protein of interest is loaded onto packed-bed (column) of cation exchange resin, the column is washed to remove unbound material, and the bound protein is eluted with a step gradient or linear gradient of increasing ionic strength. Step gradient is typically used for capture operations and linear gradient is used for high-resolution separations. Linear gradient was used in this study since the goal was to achieve highly pure lysozyme with a single chromatography step.

The two most important resin attributes for preparative linear gradient separation are resin selectivity and dynamic binding capacity (Jacob and Frech, 2007). Proper selectivity leads to higher final purity of the product. The binding capacity influences the operating costs of chromatography because a resin with a higher capacity results in a smaller column volume (less resin), increased productivity, and reduced buffer volume (Strube et al., 2007). These two attributes, binding capacity and selectivity, were considered for lysozyme purification.

Protein molecules are amphoteric so the actual protein charge will depend on pH, ionic strength, and buffer type (Winzor, 2004). Factors that can contribute to or interfere with electrostatic interactions between the resin, recombinant protein, or rice impurities can alter the protein adsorption behavior. Therefore, identifying plant impurities and understanding their interactions are critical to designing an integrated, optimized purification process.

Rice Impurites: Proteins and Phytic Acid. Aqueous extracts of transgenic rice contain rice protein and may also contain a mixture of starch, ash (phosphorus, potassium, magnesium, chlorine, calcium), fiber, and lipids impurities which must be

removed during purification (Juliano, 1980). One of the objectives of this work was to examine the effect of rice proteins and phytic acid on human lysozyme purification by cation exchange chromatography so a brief introduction to each of these components is provided.

Rice Proteins. The properties of rice proteins should be considered when selecting extraction conditions and purification processes. The processing conditions have to be selected based on the type and quantity of native proteins present in rice and properties such as pI and molecular weight.

The protein content of brown rice is generally 8%, composed of about 80% glutelin, 10% globulin, 5% albumin, and 5% prolamin. The primary protein groups that would potentially be extracted using typical aqueous buffers would be the water-soluble albumin and salt-soluble globulin. The major albumin fraction is a 60 kDa monomer with an isoelectric point (pI) of 6.5 (Mawal et al., 1987). The reported pIs of globulin range from pH 5 to 7 (Ellepola and Ma, 2006). Rice proteins are reportedly similar to corn proteins based on pI and molecular weights, implying that purification studies for corn may also be applicable to rice (Menkhaus et al., 2004a).

Phytic Acid. Phytic acid is the primary storage form of phosphate found in all eurokaryotic cells, particularly cereal grains and legumes. Phytic acid is the phosphoric ester of inositol (hexahydroxycyclohexane) with 12 ionizable protons with pKa values from 1 to 12 (Turner et al., 2002). Nine dissociation sites are acidic making phytic acid negatively charged when the pH is above 1 (Costello et al., 1976).

Brown rice contains between 0.84-0.99% phytic acid in the form of potassium, magnesium, and calcium phytate salts that are concentrated in the germ and aluerone layer (Reddy, 2002). The binding of magnesium and calcium to phytic acid and solubility of phytate salts is highly dependent on pH, ionic strength, and the molar ratio of the cation to phytic acid (Crea et al., 2008; Crea et al., 2006) so the behavior of phytic acid in a complex extract is difficult to predict and will depend on the selected extraction conditions.

Phytic acid can also form binary and tertiary complexes with proteins in the extract. The type of complex and extent of interaction depends on pH, cation concentration and charge, and protein properties (Cheryan, 1980). Generally, binary complexes are formed at acidic pH and ternary complexes at neutral pH (Selle et al., 2000). Basic proteins like human lysozyme could form binary complexes over a wide pH range. Previous studies noted the formation of phytic acid and lysozyme complexes between pH 6 and 9.5 (Okafo et al., 1994; Okafo et al., 1995; Rodriguez and Li, 1999). Protein-phytic acid interactions have been shown to interfere with protein extraction (Hussain and Bushuk, 1992) by shifting the isoelectric point and the solubility profile of proteins (Wolf and Sathe, 1998).

Processing of Corn for Food, Feed, and Ethanol. Although corn processing methods like wet milling, dry milling, and dry grinding have been well established, the conversion from petroleum based fuels to corn-based biofuels resulted in the need for expanding plant processing expertise. Novel processing strategies are needed to maintain a competitive processing cost for ethanol by producing higher value co-

products. Recently proposed strategies include removal of non-fermentable seed parts (germ and fiber) to increase fermentation efficiency (Ponnampalam et al., 2004; Wahjudi et al., 2000) and the development of higher value co-products from fiber and germ. Germ wet milling is one new processing strategy developed to produce protein products from dry-milled germ, a co-product from ethanol plants (Lohrmann et al., 2008). This strategy uses fundamental concepts of both dry milling and wet milling so an overview of each process is provided.

Corn Wet Milling. Corn ethanol facilities using wet mills are capital-intensive and operated primarily by large agri-business companies (Erickson and Carr, 2009). Although this was the traditional fractionation method, only one new wet mill ethanol facility has been constructed since 1997 (Erickson and Carr, 2009). As shown in Figure 1.2, wet milling was designed to fractionate corn into individual components (oil, fiber, protein, and starch) using chemical and physical methods. The key for this highly effective separation method is the steeping conditions. Corn wet milling provides good fractionation of germ as measured by yield and oil content but usually at the expense of protein quality. The corn kernels are soaked with sulfur dioxide (a reducing agent) at a high temperature (45-50°C) and low pH for 24-36 h to increase starch and protein separation efficiency. These conditions cause proteolysis and protein aggregation (Parris et al., 2006) and a significant amount of albumin, globulin, and some glutelin proteins are lost in soluble fractions (Wilson, 1994).

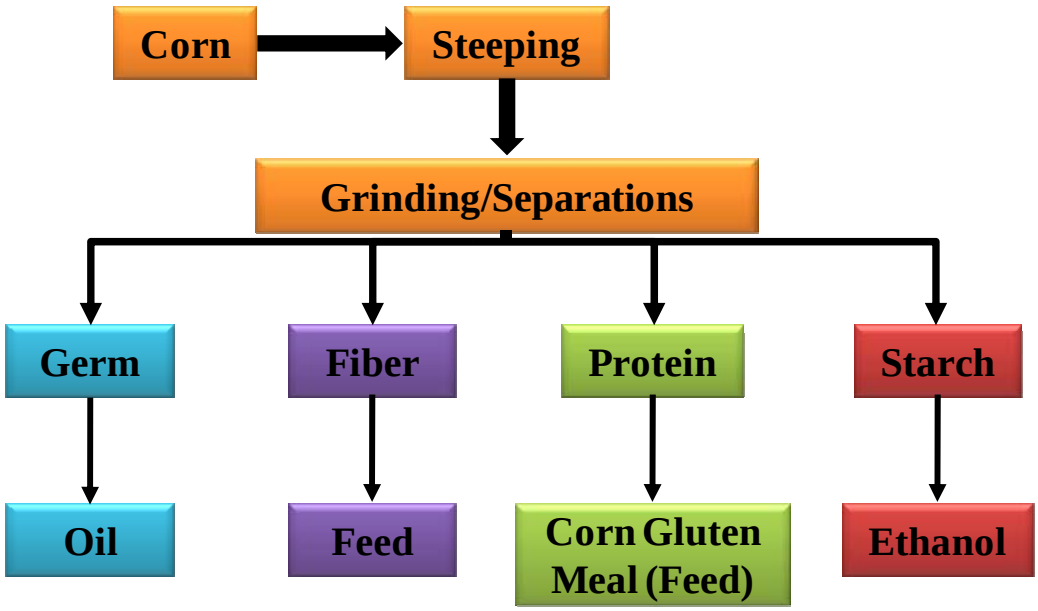

Figure 1.2. Overview of corn wet milling, a technique that uses mechanical and chemical methods to separate corn giving germ and starch as the main products.

Corn Dry Milling. Unlike wet milling, dry milling separates corn into fractions such as germ, pericarp, and endosperm using mechanical and physical methods (Figure 1.3). In corn dry milling, the kernels are tempered to increase moisture content to 20-25% and then degerminated. Corn is tempered at room temperature for 2 h or less and thus, preserves important germ protein properties. Dry milling yields relatively low purity germ (≤ 23% oil and 15% protein) with significant residual endosperm starch attached to the germ. The market for dry milled germ has been limited to corn grits, meal, and flour applications to brewing and cereal food (Duensing et al., 2003).

In the past, the dry milling industry was much smaller than the wet milling industry (Wright, 1994) but corn-to-ethanol plants are transitioning to dry milling processing as a pre-processing method for ethanol production to make corn a more cost

effective feedstock. This transition will significantly expand the dry milled germ supply. There is an opportunity to further increase the germ co-product value by producing higher protein products for food applications.

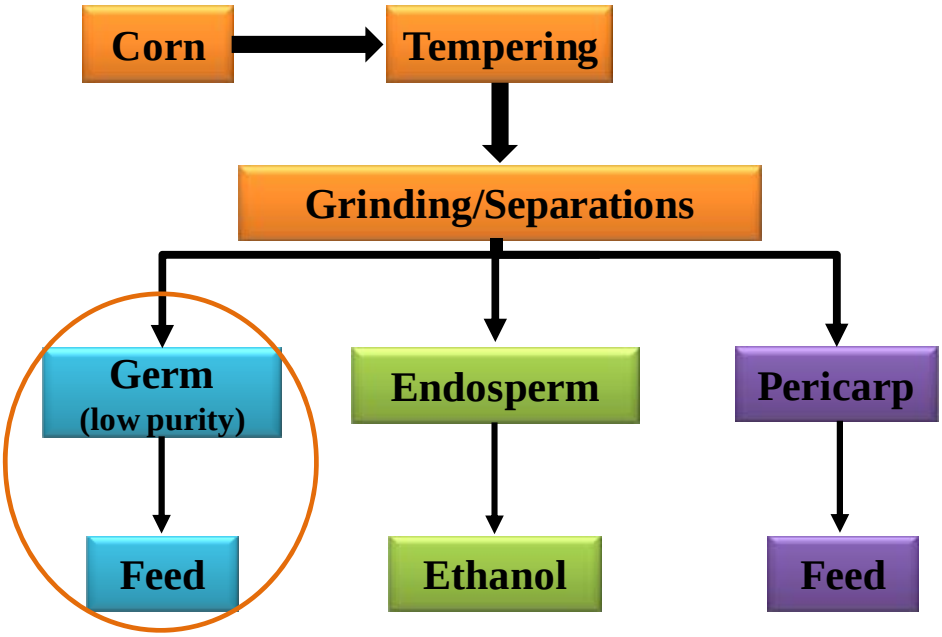

Figure 1.3. Overview of corn dry milling, a technique that uses mechanical and physical methods to separate corn into three main fractions. The section circled is the focus of Chapter V, improving dry milled germ quality and protein recovery.

Germ Wet Milling. Germ wet milling was recently developed to address the processing limitations, starch and oil loss, for producing cost effective protein co-products from dry-milled corn (Lohrmann et al., 2008). The method given in Figure 1.4 consists of three steps: soaking, grinding, and separation to produce clean germ with enhanced protein content and reduced starch content (Lohrmann et al., 2008). The purpose of the soaking step is primarily to remove the attached endosperm from dry-

milled germ. After soaking, the water with starch can then be used for ethanol fermentation. The soaked germ is then processed by grinding to remove attached endosperm solids. The clean germ having higher protein and oil content and reduced starch content than dry-milled germ is then recovered by density separation. The clean germ is recovered as the light fraction and the endosperm solids as the heavy fraction. Determining the effect of soaking conditions such as time, temperature, and pH on germ composition is an important consideration since the germ will be used to prepare corn protein concentrate.

Protein Concentrates. Currently, soy protein concentrates and isolates dominate the food market so literature discussing extraction and purification of germ protein is limited. Conventional methods for extraction of corn protein for food applications include alcohol and/or alkali extraction. Protein precipitation by pH adjustment or addition of alcohol has been used to recover and concentrate proteins from extracts. Membrane filtration has been implemented instead of protein precipitation, primarily for soy protein enrichment (Muralidhara et al., 2003; Thomas et al., 2001) but also for protein recovery from whole corn extracts (Kampen, 1995; Lawhorn, 1986). Rao et al. (2002) showed that the composition, protein structure, and functionality of membrane concentrated soy protein differed from precipitated protein concentrate. Membrane filtration has not been considered before as a method to prepare corn protein concentrate or isolate from high protein alkali germ extracts.

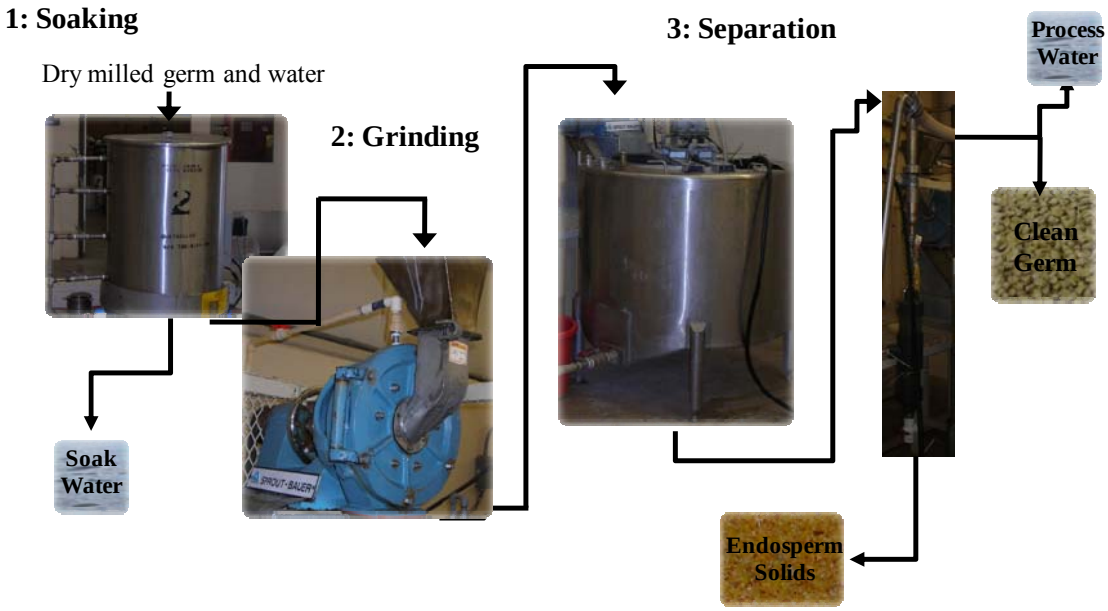

Figure 1.4 Overview of germ wet milling. The method consists of 3 steps: soaking, grinding, and separation to produce clean germ. The objective is to produce germ with a higher protein and oil content and lower starch content than dry milled germ.

OBJECTIVES

Process Analysis of Human Lysozyme Purification from Transgenic Rice. To take advantage of the high-expressing human lysozyme source and to make lysozyme production viable for a variety of applications, several objectives for downstream processing were addressed as listed below:

1. To determine the effect of pH and ionic strength on human lysozyme extraction and cation exchange adsorption.
2. To study the effect of rice extract impurities (e.g. proteins and phytic acid) on the cation exchange adsorption of human lysozyme.
3. To compare the adsorption of purified human lysozyme to hen egg-white lysozyme and the competitive binding of host proteins (rice or egg-white).
4. To compare the downstream processing costs for the proposed human lysozyme and hen egg-white lysozyme purification methods.
5. To identify rice impurities that interfere with purification, based on lysozyme binding capacity and purity, and propose and evaluate alternative processing trains that minimize interference.

Development of Protein Products from Dry-Milled Corn Germ. To produce higher value protein products (high-protein germ and corn protein concentrate) from dry milled corn germ, the following specific objectives were addressed:

1. To quantify the amount of protein and phytic acid leached during soaking of dry milled corn germ based on soak time, temperature, and pH.

2. To determine the effect of the soaking conditions (pH, temperature, time) on the composition of clean corn germ (soaked and separated dry milled germ).
3. To compare lab-scale and pilot-scale germ soaking studies.
4. To evaluate the effect of processing conditions on the protein dispersibility index of corn germ.
5. To develop a method to produce corn protein concentrate from clean germ.

DISSERTATION ORGANIZATION

This dissertation is composed of six chapters and covers two separate processing projects: 1) Process analysis of human lysozyme purification from transgenic rice and 2) Development of protein products from dry-milled corn germ. Chapter I provides a general overview of the use of plants for recovery of protein products, including the use transgenic rice as a platform for recombinant human lysozyme production and a brief introduction to corn processing.

Chapters II-V are written in journal article format (Biotechnology and Bioengineering) and report the experimental work for both projects. The objectives for the first study, process analysis of human lysozyme purification, are addressed in Chapters II-IV. The first paper (Chapter II) examines the factors influencing the extraction and cation exchange adsorption of human lysozyme. The effect of pH and ionic strength on lysozyme and total soluble protein extraction were investigated to identify conditions that would potentially reduce the purification burden in the subsequent adsorption step. The saturation binding capacity of human lysozyme was evaluated as a function of pH and ionic strength to determine whether the optimal

extraction conditions would be compatible with cation exchange adsorption. The second paper (Chapter III) expands the adsorption studies to include hen egg-white lysozyme and egg-white as a benchmark to assess human lysozyme purification efficiency from rice extract. The third paper (Chapter IV) studies the interference of rice extract (as measured by binding capacity and lysozyme purity) on human lysozyme purification and the methods evaluated to identify the interfering component. Alternative purification schemes were proposed and tested to alleviate the interference.

The fourth paper (Chapter V) presents the experimental work for the second study, which was the development of protein products (high protein germ and corn protein concentrate) from dry-milled corn germ. The effects of processing conditions (pH, temperature, time, and process-scale) on corn germ quality were addressed and a method was developed to produce corn protein concentrate.

The general conclusions are given in Chapter VI along with future research needs for both projects.

CHAPTER II

FACTORS INFLUENCING RECOMBINANT HUMAN LYSOZYME EXTRACTION AND CATION EXCHANGE ADSORPTION*

OVERVIEW

Human lysozyme has numerous potential therapeutic applications to a broad spectrum of human diseases. This glycosidic enzyme is present in tears, saliva, nasal secretions, and milk - sources not amendable for commercial development. Recently however, a high expression level of recombinant human lysozyme (0.5% dry weight) was achieved in transgenic rice seed. This paper evaluates the effects of pH and ionic strength on rice protein and lysozyme extractability, as well as their interactions with the strong cation exchange resin, SP-Sepharose™ FF. The extraction conditions that maximized lysozyme yield and the ratio of extracted human lysozyme to native rice protein were not optimal for lysozyme adsorption. The conditions that gave the highest extracted lysozyme-to-native protein ratio were pH 4.5 and 100 mM NaCl in 50 mM sodium acetate buffer. At pH 4.5, salt concentrations above 100 mM NaCl reduced the lysozyme to protein ratio. The best conditions for lysozyme adsorption were pH 4.5 and 50 mM sodium acetate buffer. Lysozyme extraction and subsequent adsorption at pH 4.5 and 50 mM NaCl was an acceptable compromise between lysozyme extractability, adsorption, and purity. The primary recovery of human lysozyme from pH 6 extracts,

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irrespective of ionic strength, was inferior to that using pH 4.5 with unacceptably low saturation capacities and lysozyme purity. High purity was achieved with a single chromatography step by adjusting the pH 4.5 extract to pH 6 before adsorption. The disadvantage of this approach was the drastically lower saturation capacity compared to adsorption at pH 4.5.

INTRODUCTION

Human lysozyme, a 15 kDa glycosidic enzyme with a pI of 10.2, is found in tears, saliva, nasal secretions, and milk. Human lysozyme has antibacterial, antiviral, antiinflammatory, and antifungal properties with numerous potential therapeutic applications to a broad spectrum of human diseases (Huang et al., 2006a; Huang et al., 2006b; Jollès, 1996; Lee-Huang et al., 2005; Zavaleta et al., 2007).

Breast milk contains 50-400 μg/mL lysozyme and is the primary source of human lysozyme, but its use is restricted by the limited supply and potential risk of viral and microbial contamination (Huang et al., 2006b). Recombinant human lysozyme has been expressed in transgenic mouse milk (Maga et al., 1995), *Aspergillus oryzae* (Tsuchiya et al., 1992), tobacco leaves (Nakajima et al., 1997), carrot (Takaichi and Oeda, 2000), rice seed (Huang et al., 2002a; Yang et al., 2003), rice cell culture (Huang et al., 2002b), and yeasts (Maullu et al., 1999; Oka et al., 1999; Yoshimura et al., 1988). The highest expression level of recombinant lysozyme of 0.5% dry weight was achieved in transgenic rice seed (Huang et al., 2002a).

Transgenic plants have recently become a viable alternative to microbial and mammalian cell culture systems for large-scale recombinant protein production.

Transgenic plants are particularly attractive as bioreactors because they do not carry or propagate human diseases and mammalian viral vectors (Goldstein and Thomas, 2004). In addition, the expression of recombinant proteins in edible plant tissues could reduce protein purification requirements for some applications (Twyman et al., 2003). Corn, potato, canola, sunflower, soybean, tobacco, and rice are some of the crops that have been used for the expression of human biopharmaceuticals, antibodies, and vaccines (Menkhaus et al., 2004a). Cereal crops, such as rice, are particularly well-suited for such applications because their seeds allow for long-term storage and have low concentrations of phenolic compounds, which cause problems in the downstream processing of tobacco and other leaf tissue-derived recombinant proteins (Ma et al., 2003; Nikolov and Woodard, 2004). Rice has a GRAS (generally regarding as safe) status and rice-based foods are considered hypoallergenic (Nandi et al., 2002). Therefore, human lysozyme produced from transgenic rice could be used in a partially purified form as a baby formula additive or oral rehydration solution to reduce gastrointestinal tract infections (Huang et al., 2002b; Zavaleta et al., 2007).

Several previous studies specifically investigated the extractability of recombinant and/or native transgenic plant proteins as a function of particle size, pH, and ionic strength (Azzoni et al., 2005; Bai and Nikolov, 2001; Bai et al., 2002; Farinas et al., 2005; Menkhaus et al., 2004b; Zhang et al., 2005), but only a few in the context of integrated recovery (Azzoni et al., 2002; Kusnadi et al., 1998; Menkhaus et al., 2004b). Generally, these studies found that ionic strength and pH had a strong effect on extractability, with pH having a greater impact than ionic strength (Azzoni et al., 2005;

Balasubramaniam et al., 2003; Farinas et al., 2005). The lowest soluble protein concentrations measured were typically at or below pH 4 and highest at or above pH 7 (Azzoni et al., 2005; Azzoni et al., 2002; Balasubramaniam et al., 2003; Farinas et al., 2005). Azzoni et al. (2002) found that selecting the appropriate extraction conditions was crucial in reducing the purification costs of aprotinin from corn seed. Therefore, extraction conditions such as solid-liquid ratio, pH, and ionic strength determine the extract volume and composition and can be optimized to improve the efficacy of subsequent purification steps.

Ion-exchange chromatography is the biotechnology industry standard for capturing proteins (Ladiwala et al., 2003) and can be used to concentrate and purify recombinant proteins derived from plants (Menkhaus et al., 2004a). However, determining the optimal chromatography conditions for complex mixtures such as plant extracts remains a challenge because each transgenic extract has unique characteristics based on the host system's composition (ions, lipids, proteins, sugars, phenolics) and recombinant protein properties (charge, size, hydrophobicity).

Various strategies for human lysozyme purification have been reported (Boesman-Finkelstein and Finkelstein, 1982; Huang et al., 2002a; Jollès and Jollès, 1967; Parry et al., 1969; Wang and Kloer, 1984). Most reported processes used weak cation exchangers combined with ammonium sulfate precipitation and diafiltration to produce purified human lysozyme at a relatively low process yield (Jollès and Jollès, 1967; Parry et al., 1969). Human lysozyme has also been purified from milk by affinity chromatography followed by size exclusion chromatography (Boesman-Finkelstein and

Finkelstein, 1982; Wang and Kloer, 1984). Since the affinity resin uses an animal-derived ligand and is more expensive than ion exchange chromatography resin, this process may not be suitable for low-cost product applications. Huang et al. (2002a) used strong cation exchange adsorption followed by a size exclusion chromatography step to purify human lysozyme from rice flour. Little purification was achieved by the cation exchange step as gel analysis showed similar protein profiles for the crude extract and eluate. Furthermore, lysozyme was present in the cation exchange flowthrough, which reduced the overall yield to 60%. The reason for this inefficient purification was not investigated. A preliminary process simulation and cost analysis of the purification process reported by Huang et al. (2002a) using SuperPro Designer (Intelligen, Inc.) identified the chromatography steps and extraction step as major downstream cost-contributing factors (unpublished data).

To take advantage of the high-expressing human lysozyme source and to make lysozyme production viable for a variety of applications, we believe that a further investigation of the factors that affect the extraction and chromatography steps is warranted. In this chapter, we evaluate the effects of pH and ionic strength on rice protein and lysozyme extractability and their interactions with a strong cation exchange resin. We discuss the process conditions necessary to achieve optimal recovery and purification of human lysozyme from transgenic rice flour as well.

MATERIALS & METHODS

Materials. Transgenic and control rice flour was provided by Ventria Bioscience (Sacramento, CA). The rice flour (20-100 mesh size) was stored at 4ºC throughout the

research. The control flour was of the same genetic background as the transgenic flour (cultivar Taipei 309). Recombinant human lysozyme (>93% purity) was also provided by Ventria Bioscience and used as an analytical standard.

Analytical Methods. ***Total Protein and Lysozyme Quantification.*** Total soluble protein (TSP) was determined using the Bradford *(35)* method with bovine serum albumin as a standard. Lysozyme concentration was determined by enzymatic assay, which measures the decrease in turbidity of a *Micrococcus luteus* cell suspension (Shugar, 1952). A modified microtiter plate method was used with a 0.03% (w/v) cell suspension and 0-15 μg/mL purified human lysozyme standards. Control flour extract was used in the preparation of standards to account for the interference of extract components. Cell suspension (250 μL) was added to each sample (50 μL) and the activity was monitored at 450 nm for 4 min. Lysozyme concentration in the extracts was expressed as milligrams pure lysozyme per gram flour.

Protein Analysis by SDS-PAGE. The protein profiles of extracts and chromatography fractions were evaluated by electrophoresis as described by Laemmli (1970). Samples were loaded on 8-16% tris-glycine gels under non-reducing conditions.

Experimental Methods. ***Effect of pH and Ionic Strength on Protein Extraction.*** The effect of buffer ionic strength was determined by investigating the amount of lysozyme and total protein extracted at various sodium chloride concentrations. Five sets of experiments were performed at pH 4.5 and pH 6.5 using different sodium chloride concentrations (0, 50, 100, 150, and 300 mM) in 50 mM sodium acetate and sodium phosphate buffers, respectively. The conductivities of

buffers and extracts were measured by EC Meter Model 2052 (VWR Scientific) and reported in mS. The effect of buffer pH on protein extraction was determined with 50 mM NaCl at pH 4.5, 6.5, and 7.5. Sodium phosphate buffer (50 mM) with 50 mM NaCl was used for pH 7.5 extractions. At each pH and sodium chloride concentration, experiments were conducted with lysozyme flour in triplicate. For each experiment, 20 g of flour was added to 100 mL of extraction buffer and mixed continuously for 1 hr. The extraction time was selected on the basis of kinetics experiments conducted at pH 4.5 and 6.5 over 120 min (data not shown). The time studies showed that the maximum lysozyme concentration in the supernatant was achieved by 30 min. The samples were centrifuged (Beckman Coulter Allegra 25R) at 12,000 x g for 15 min to separate the suspended solids. Extract supernatants were analyzed for total protein and lysozyme content. To determine the amount of lysozyme and protein in the interstitial volume of the centrifuged solids, 50 mL of the same extraction buffer was added to the wet solids, mixed for 2 min, and centrifuged at 12,000 x g for 15 min. The 50 mL wash volume (2 times centrifuged solids volume) and short contact time were selected to mimic large scale washing of the filter cake.

Effect of pH and Ionic Strength on Dynamic Saturation Binding Capacity (SBC) of Human Lysozyme. Adsorption and elution experiments were conducted with a strong cation exchange resin (SP-Sepharose^{TM} FF) using a Bio-Rad (Hercules, CA) BioLogic LP chromatography system equipped with UV and conductivity detectors and interfaced with LP DataView Software. SP-Sepharose^{TM} FF resin (GE Healthcare, Piscataway, NJ) was packed to a height of 2.2 cm in a 1 x 10 cm Bio-Rad Econo column

(1.73 mL bed volume). Lysozyme solutions (0.5 mg/mL) were prepared with purified recombinant human lysozyme (Ventria Bioscience) and binding buffer. Binding buffers of various ionic strengths (0, 50, 100, and 150 mM NaCl) were prepared in 50 mM sodium acetate for pH 4.5 experiments and 50 mM sodium phosphate for pH 6. At pH 7.5, the binding capacity was evaluated at a single sodium chloride concentration (50 mM) in 50 mM sodium phosphate buffer. For each experiment, the column was equilibrated with binding buffer and the lysozyme solution was loaded at a flow rate of 2 mL/min (150 cm/h) until saturation. The bound lysozyme was eluted by applying 1 M NaCl in 50 mM buffer of the same pH as the binding buffer. Elution fractions (3 mL) were collected, pooled, and analyzed for lysozyme content by absorption at 280 nm (Beckman DU 600) using an extinction coefficient of 2.64 mL/mg-cm. The SBC was estimated from the breakthrough curve and compared to the SBC calculated from eluted lysozyme. The SBC values reported are averages and are expressed as milligrams lysozyme bound per milliliter resin.

Binding and Elution of Transgenic Rice Extracts. The effect of rice extract on the binding capacity, purity, and yield of lysozyme was investigated at pH 4.5 and 6. Each lysozyme rice flour extract was prepared as described previously with 50 mM NaCl in either 50 mM sodium acetate, pH 4.5, (buffer A) or 50 mM sodium phosphate, pH 6 (buffer B). In a separate experiment, a pH 4.5 extract was adjusted to pH 6 using 1 M sodium phosphate, pH 7, before loading it onto the ion exchange column. Extracts were filtered through filter paper (Micro Filtration Systems No. 5C) followed by a 0.45 μm cellulose acetate filter (Corning) to produce a clarified extract. Clarified extracts

were loaded at a flow rate of 1 mL/min (300 cm/h) onto a 0.5 x 10 cm SP-Sepharose™ FF column (Tricorn, GE Healthcare) until lysozyme saturation. The column was washed with 5 column volumes (CV) of the respective binding buffer, and the bound protein was eluted by linear gradient from 0 to 1 M NaCl in buffer A (pH 4.5) or buffer B (pH 6) over 20 CV. Fractions (3 mL) were collected throughout loading and elution. The fractions collected during loading were analyzed for lysozyme concentration, and lysozyme breakthrough curves were constructed to estimate SBC. The elution peak fractions were analyzed for lysozyme and total protein content and by SDS-PAGE. Lysozyme yields were calculated as a percent of total bound lysozyme. Lysozyme purity of elution peak fractions was calculated by lysozyme activity and total protein.

RESULTS & DISCUSSION

Effect of pH and Ionic Strength on Protein Extractability. The amount of lysozyme extracted from rice flour as a function of buffer pH and ionic strength is shown in Figure 2.1. The conductivities of the various extracts were measured and reported to ensure that similar ionic strength effects were compared at all pHs. At pH 4.5, the amount of extracted lysozyme varied from 2.7 (±0.2) to 4.4 (±0.5) mg/g flour as the NaCl concentration varied from 0 to 300 mM. No substantial difference in extracted lysozyme was observed above 100 mM NaCl. At pH 6.5, the amount of lysozyme extracted varied from 0.4 (±0.01) to 4.0 (±0.03) mg/g flour and lysozyme extractability increased almost linearly with ionic strength. The amount of extracted lysozyme was lower at pH 6.5 than pH 4.5 at all ionic strengths, with the largest differences measured at NaCl concentrations below 150 mM. To determine whether lysozyme extractability

would continue to decrease with increasing pH, an additional extraction experiment was carried out at pH 7.5 and 50 mM NaCl. Under these conditions, only 0.4 mg/g of lysozyme was extracted at pH 7.5 compared to 1.1 mg/g at pH 6.5 and 3.3 mg/g at pH 4.5.

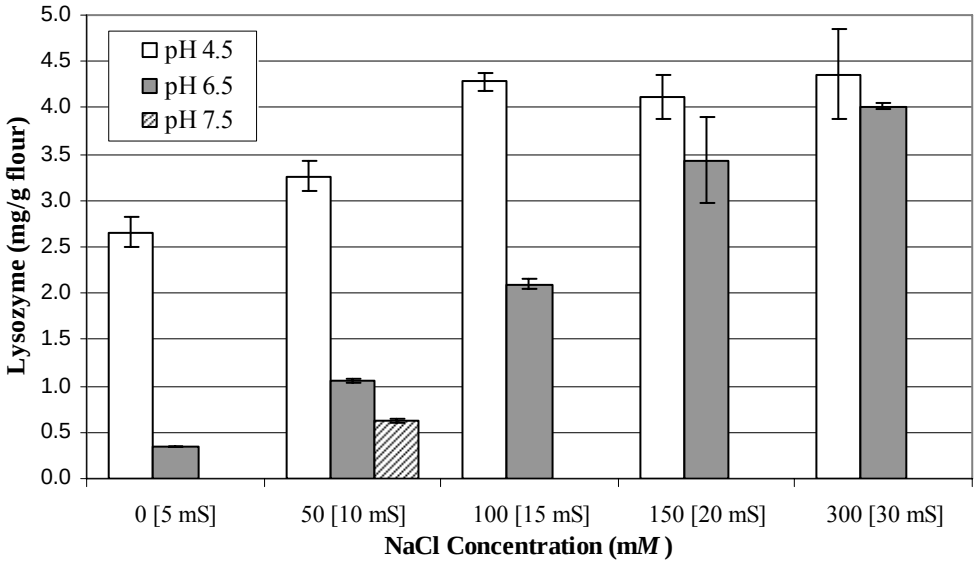


Figure 2.1. Effect of pH and ionic strength on the extraction of human lysozyme (mg lysozyme/g flour) from transgenic rice. Conductivity is given in brackets. Error bars indicate standard deviation of triplicate extractions.

Residual lysozyme entrapped in centrifuged solids was removed with the respective extraction buffer. For pH 4.5, the amount of lysozyme recovered in the buffer wash varied from 0.23 mg/g to 0.51 mg/g. On average, lysozyme content in the wash accounted for 10% of the total extracted lysozyme. At pH 6.5, the amount of lysozyme recovered in the wash step varied from 0 (below detectable limit) to 0.56 mg/g as NaCl concentration varied from 0 to 300 mM. Nearly 20% of total extracted lysozyme was

recovered from the centrifuged solids for the pH 6.5 extracts containing 50-300 mM NaCl.

Figure 2.2 shows the effect of pH and ionic strength on total soluble protein (TSP). TSP increased as both pH and ionic strength increased. TSP varied from 4.3 (±0.16) to 8.0 (±0.79) mg/g at pH 4.5 and from 5.7 (±0.5) to 9.9 (±0.6) mg/g at pH 6.5 as NaCl concentration varied from 0 to 300 mM. The effect of three pHs on TSP extraction at 50 mM NaCl was also included for comparison. Total soluble protein increased from 5.7 mg/g at pH 4.5 to 8.2 mg/g at pH 7.5, even though the amount of extracted lysozyme was five times lower at pH 7.5 than at pH 4.5. At 50 mM NaCl, extracted lysozyme was nearly 60% of the TSP at pH 4.5, 15% at pH 6.5, and 10% at pH 7.5.

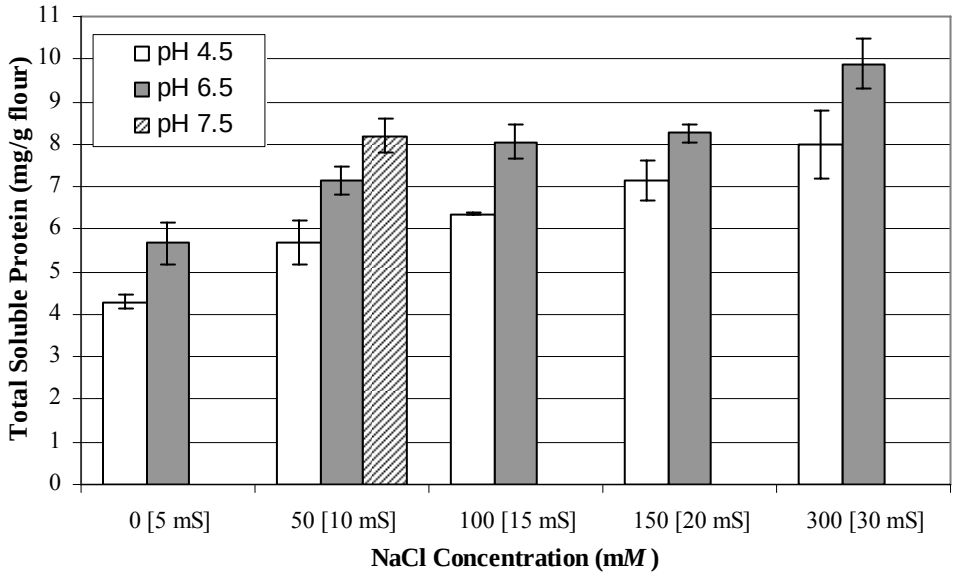


Figure 2.2. Effect of pH and ionic strength on the extraction of total soluble protein (TSP) from transgenic rice. TSP reported as mg extracted protein/g flour. Conductivity is given in brackets. Error bars indicate standard deviation of triplicate extractions.

Since lysozyme was a substantial fraction of TSP that varied broadly with extraction conditions, soluble rice protein content in transgenic extracts was calculated by subtracting lysozyme concentration from TSP (Figure 2.3). The extracted soluble rice protein at pH 4.5 varied from 1.6 to 3.6 mg/g and was significantly lower than that at pH 6.5, which ranged from 5.3 to 6.1 mg/g (Figure 2.3). The largest difference in soluble rice protein between pH 4.5 and 6.5 was measured at the lowest ionic strength (3.3-fold) and smallest at 300 mM NaCl (1.6-fold). The same trend in the solubilization of rice proteins with NaCl concentration was observed with control (nontransgenic) flour (Figure 2.3). Interestingly, the concentration of soluble rice protein was greater in the control extracts than in the transgenic extracts for all ionic strengths at both pHs. Soluble rice protein was 6% to 42% lower in transgenic rice extracts than control rice extracts. The largest difference (mg/g basis) between the two rice flours was measured at pH 6.5 and 300 mM NaCl, where the amount of extracted rice protein was 8.3 mg/g for the control flour and 5.9 mg/g for transgenic flour. This experimental observation of substantially lower amounts of extracted rice protein from transgenic flour than control flour supports the reported reduction of salt-soluble globulins in high-lysozyme-expressing rice seed (Huang et al., 2006b).

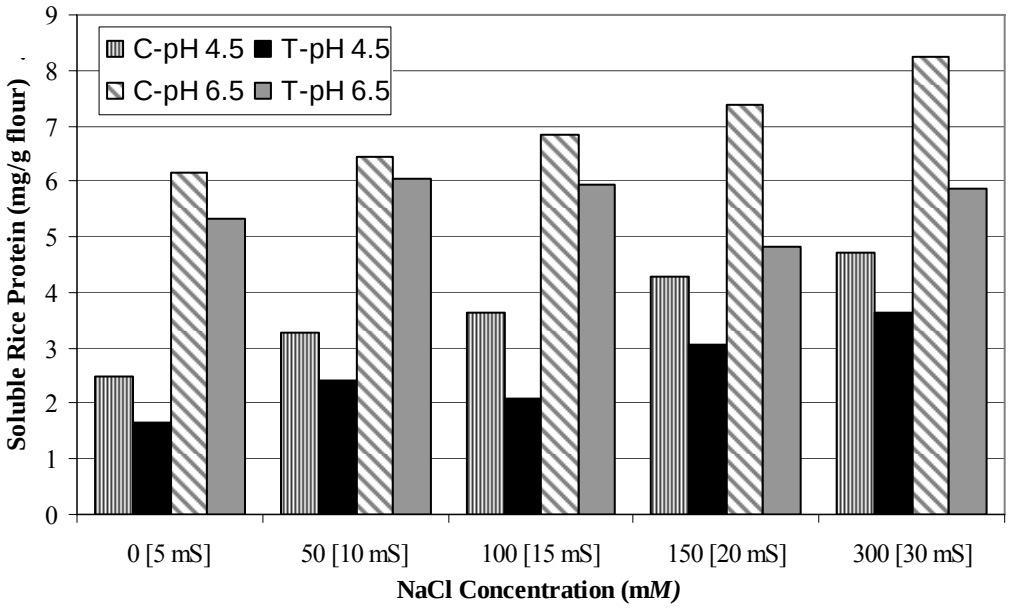


Figure 2.3. Comparison of soluble rice protein in control extracts (C) and transgenic extracts (T) as a function of ionic strength and pH. For transgenic flour extract, lysozyme was subtracted from total soluble protein extracted to determine the amount of rice protein extracted. Soluble rice protein reported as mg extracted rice protein/g flour. Conductivity is given in brackets.

To reduce purification requirements, extraction conditions that maximize lysozyme concentration and minimize the amount of soluble rice protein in the extracts were identified by plotting the ratio of extracted lysozyme to solubilized rice protein (Figure 2.4). This ratio provides a better measure of optimal extraction conditions for high-expressing plants because it is based upon extracted rice protein instead of TSP, which includes lysozyme.

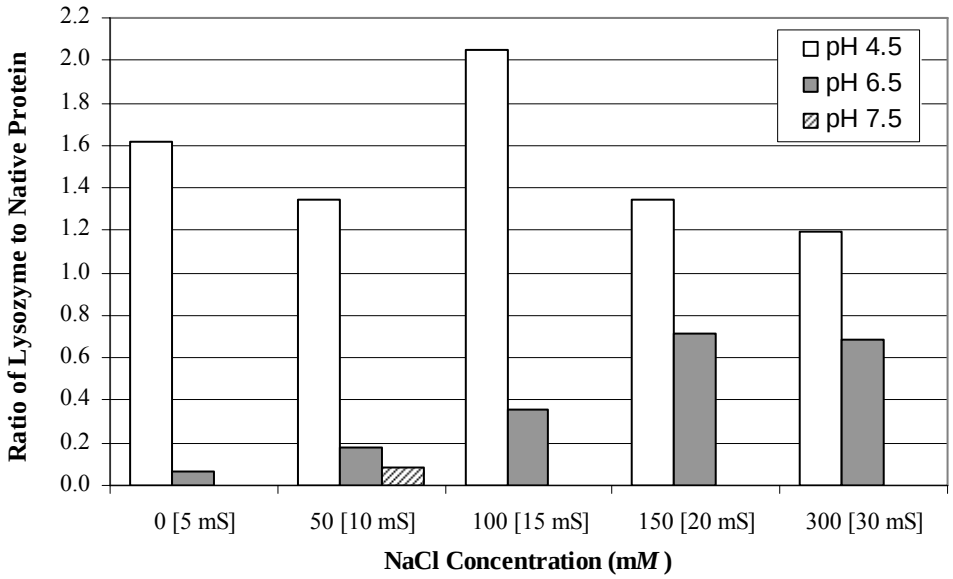


Figure 2.4. Effect of pH and ionic strength on protein composition of extracts. Protein composition expressed as ratio of lysozyme to native rice protein in extract. Conductivity is given in brackets.

As Figure 2.4 shows, this ratio indicates that pH 4.5 was more favorable lwilknethan pH 6.5 and 7.5 for all ionic strengths investigated. The extraction conditions that gave the most favorable protein composition were at pH 4.5 with 100 mM NaCl (15 mS) followed by 0 mM NaCl (5 mS). However, the latter condition was significantly inferior to 100 mM NaCl because the extracted lysozyme drops from 4.5 mg/g to 2.6 mg/g (Figure 2.1). The consistently lower rice protein concentration at pH 4.5 compared with those at 6.5 and 7.5 indicates that the majority of extractable rice proteins are in the acidic pI range. The need to add sodium hydroxide to maintain pH during the pH 6.5 and 7.5 extraction experiments supports this conclusion. The properties of rice proteins given in the Rice Proteome Database (http://gene64.dna.affrc.go.jp/RPD/main_en.html) further support this observation. The

reduced extractability of lysozyme at pH 6.5 and 7.5 (Figure 2.1) was unexpected since lysozyme is highly water-soluble and positively charged (pI~10) in this pH range. The negative pH effect on lysozyme extractability from pH 4.5 to 7.5 could be a consequence of ionic interactions between acidic proteins and negatively charged phytic acid with positively charged human lysozyme. The difference in lysozyme content between pH 4.5 and 6.5 extracts decreased as ionic strength increased (Figure 2.1), which also supports this ionic interaction hypothesis.

Effect of pH and Ionic Strength on Dynamic Saturation Binding Capacity (SBC) of Human Lysozyme. To assess the effect of extract pH and ionic strength on the adsorption of lysozyme to a cation exchange resin, the SBC of purified lysozyme as a function of pH and salt concentration was measured. For this investigation, pH 6 solutions were used instead of pH 6.5 because preliminary adsorption data with purified human lysozyme indicated a stronger than expected negative pH effect on the SBC.

The saturation capacities of human lysozyme on SP-Sepharose^{TM} FF are shown in Figure 2.5. The SBC of human lysozyme decreased proportionally as the pH and conductivity increased. At pH 4.5, the saturation capacity increased from 25 to 77 mg/mL resin as the conductivity decreased from 20 to 5 mS. Similarly, the capacity at pH 6 varied from 6 to 63 mg/mL. The effect of pH on the SBC was more pronounced at the higher sodium chloride concentrations. The saturation capacities at pH 4.5 and 6 differed by only 20% at the lowest ionic strength (5 mS), but the difference increased to 80% at the highest ionic strength (20 mS). The saturation capacities at pH 4.5, 6, and

7.5 were compared at 10 mS (50 mM NaCl) ionic strength. The capacity decreased 40% as pH increased from 4.5 to 6 and an additional 40% from 6 to 7.5.

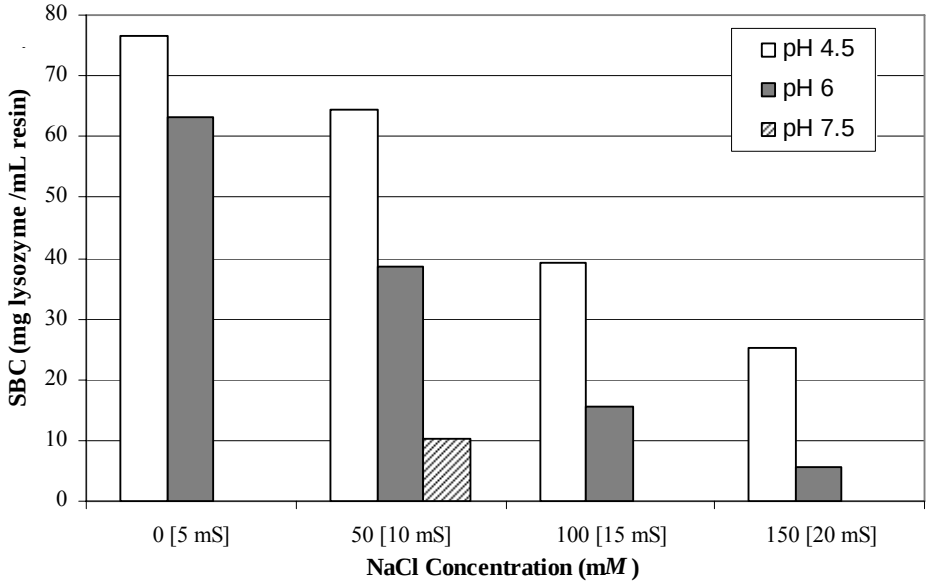


Figure 2.5. Effect of pH and ionic strength on the saturation binding capacity (SBC) of purified human lysozyme solution (0.5 mg/mL) on SP-Sepharose™ FF. Values are averages of the SBC calculated from the breakthrough curve and elution lysozyme and reported as mg lysozyme bound per mL resin. Conductivity is given in brackets.

The highest SBC at pH 4.5 and 6 were measured in 50 mM buffer (5 mS). The addition of 50 mM NaCl to the buffer (10 mS) reduced saturation capacity by 16% at pH 4.5 and 40% at pH 6. Thus, the highest capacities of lysozyme were achieved at the lowest ionic strength (5 mS) but the greatest lysozyme to rice protein extraction ratios (see Figure 2.4) were obtained at 100 mM NaCl (15 mS) for pH 4.5 and greater than 100 mM NaCl (15 mS) for pH 6.5. On the basis of these results, ionic strength and pH of the transgenic rice extract and the amount of rice-derived impurities appear to be critical parameters for optimal lysozyme recovery and purification.

Binding and Elution of Transgenic Rice Extracts. To investigate the effect of rice proteins and other extract impurities on the SBC of human lysozyme, extracts were prepared with 50 mM NaCl (10 mS). The selected ionic strength was a compromise between the conditions needed to achieve the highest binding capacity and the conditions that maximized the lysozyme to rice protein extraction ratio.

pH 4.5 Extract. The elution chromatogram and corresponding gel analysis of the clarified pH 4.5 extract that was loaded onto the SP-Sepharose™ cation exchange are presented in Figure 2.6. Lysozyme accounted for 67 mg of the 96 mg of total protein eluted from the SP-Sepharose™ column. The estimated purity of lysozyme in the entire protein pool was 70%, which was slightly higher than that of the loaded extract. Fractions 1 through 5 of the elution peak contained predominately lysozyme with rice proteins coeluting in an increasing amount starting with fraction 2. The purity and yield of lysozyme in the first five pooled fractions (1 through 5) were 89% and 93%, respectively. A greater recovery yield could be achieved by pooling the first six fractions (98%) at the expense of purity (85%). Lysozyme eluted at 28 mS (measured at peak), which corresponds to the conductivity required for elution of purified human lysozyme (data not shown). Rice proteins that coeluted at lower than 30 mS conductivity were primarily low molecular weight. The higher molecular weight protein impurities eluted at higher ionic strengths (fractions 4 to 6). The required purity depends on the application and determines how the fractions should be combined. Further improvement in lysozyme purity under these loading conditions is unlikely because control rice proteins (also extracted at pH 4.5) eluted in the same 28-32 mS conductivity

range. The SBC of the crude lysozyme was 43 mg/mL, a 30% capacity reduction compared to purified human lysozyme (Figure 2.5). Reducing the loading linear flow rate to 100 cm/h would only potentially increase lysozyme binding capacity but would not improve its purity.

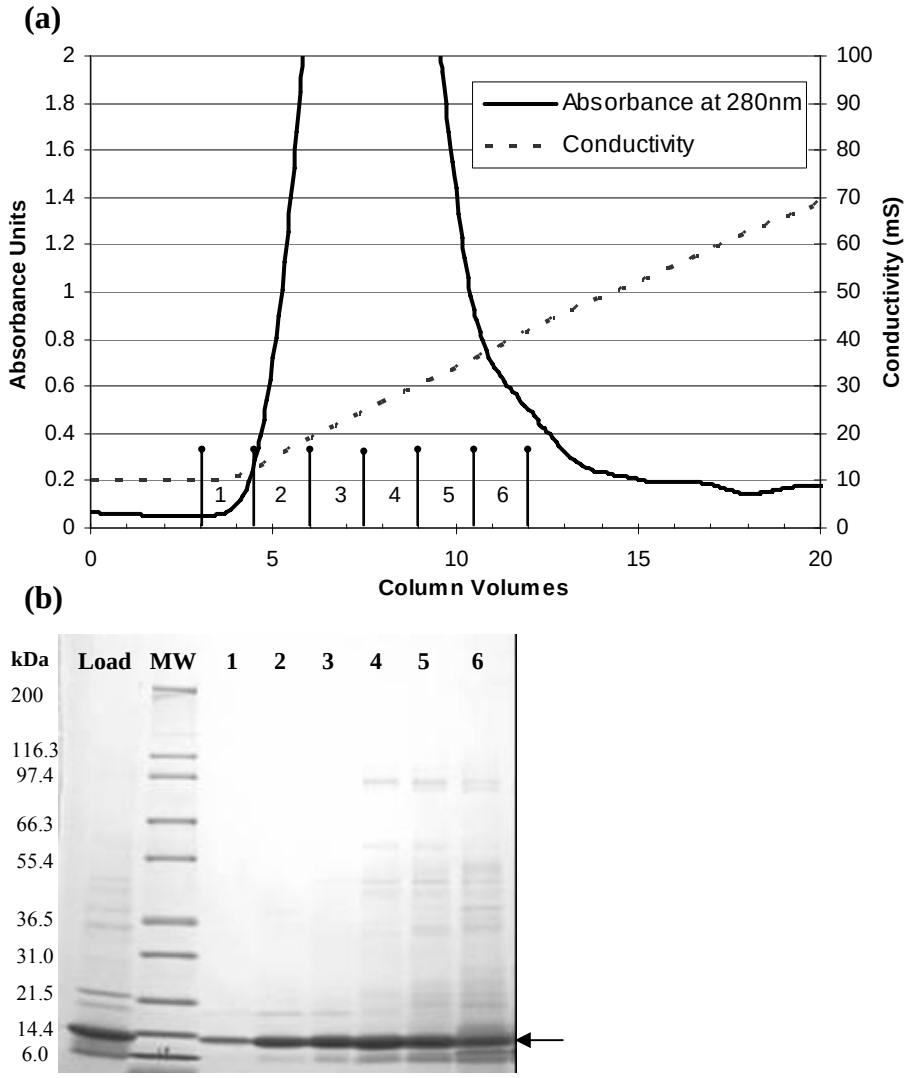


Figure 2.6. Chromatogram with pH 4.5 elution profile (a) showing peak fractions analyzed by SDS-PAGE (b). Load solution was pH 4.5 clarified lysozyme flour extract. Protein profile for load, molecular weight markers (MW), and elution peak fractions (1-6). Numbered lanes correspond to fraction number in elution peak chromatogram. The arrow shows the position of human lysozyme.

pH 6 Extract. Figure 2.7 shows the elution chromatogram and SDS-PAGE analysis of eluted fractions for pH 6 extract. The loaded pH 6 extract (initial purity of 25%) contained less lysozyme and more soluble rice protein than the pH 4.5 extract. Most of the rice proteins coeluted with lysozyme in fractions 2 through 4, with observed enrichment in both lysozyme and rice protein at 55 kDa. The low molecular weight proteins (ca. 6 kDa) that were present in both pH 6 and 4.5 extracts eluted in similar conductivity ranges (30-50 mS). These low molecular weight rice proteins were soluble at both pHs and are probably more basic than human lysozyme, which eluted at a lower conductivity (12-40 mS).

Fraction 1 had the highest lysozyme purity (85%) but contained only 1.5% of the total bound lysozyme. Subsequent fractions (2-4) also contained lysozyme, but had low purity (40-50%) since several native rice proteins coeluted with lysozyme. The purity and yield of the lysozyme pool (fractions 1 through 4) were 47% and 99%, respectively. This evidence suggests that pH 6 extract would require an additional purification step to achieve 90% purity. The SBC of the crude lysozyme was 24 mg/mL, which was 30% lower than the SBC measured with the purified human lysozyme at pH 6 and 50 mM NaCl (Figure 2.5).

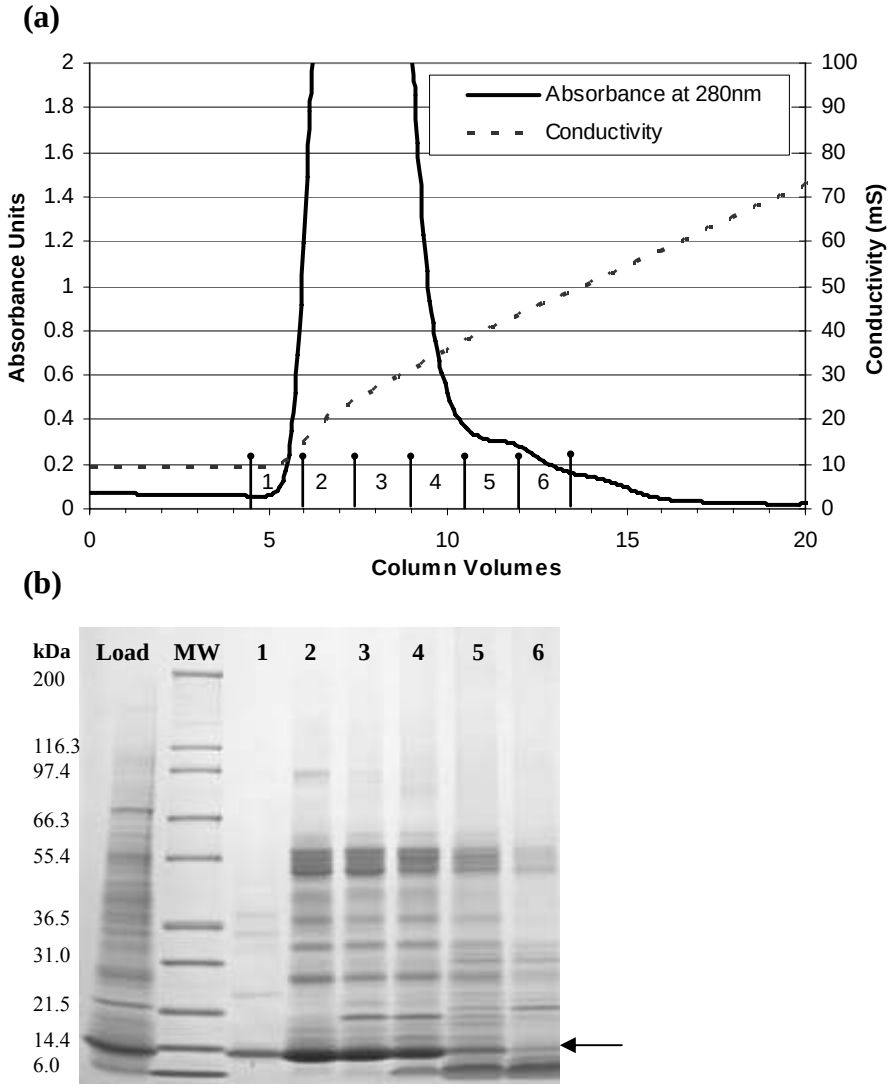


Figure 2.7. Chromatogram with pH 6 elution profile (a) showing peak fractions analyzed by SDS-PAGE (b). Load solution was pH 6 clarified lysozyme flour extract. Protein profile for pH 6 extract load, molecular weight markers (MW), and elution peak fractions (1-6). Numbered lanes correspond to fraction number in elution peak chromatogram. The arrow shows the position of human lysozyme.

pH 4.5 Extract Adjusted to pH 6 for Binding. The analysis of eluted rice proteins at pH 4.5 suggested that by combining extraction at pH 4.5 with extract loading at pH 6, a higher lysozyme yield and purity could potentially be achieved. The working hypothesis was that the slightly acidic and neutral proteins (pI 6-7) extracted at pH 4.5 do not bind to SP-Sepharose^{TM} resin at pH 6 and 10 mS conductivity.

The elution profiles of pH 4.5 control extract and pH 4.5 control extract adjusted to pH 6 showed a significant reduction of bound rice proteins (Figure 2.8). Figure 2.9 contains the elution chromatogram and SDS-PAGE analysis of the flowthrough and eluted fractions. Most of the rice proteins in the clarified extract did not bind to the cation exchange resin as the protein profiles of the flowthroughs (FT 1 and FT 2) were nearly identical to those of the load. The 11 mg of total protein that was eluted from the SP-Sepharose^{TM} resin consisted primarily of lysozyme (10 mg) and low molecular weight rice proteins (6-15 kDa). Lysozyme eluted at 29 mS, preceding the rice proteins that appeared in fractions 4-5. The lysozyme pool formed from fractions 1 to 4 accounted for 92% of the total eluted protein. The purity and yield of the lysozyme pool collected in fractions 1 to 3 (10-35 mS) were 95% and 90%, respectively. The yield of bound lysozyme increased to 100% with the addition of fraction 4 to the pool with a slight decrease in purity (from 95% to 92%). This process confirmed our hypothesis that combining pH 4.5 extraction with pH 6 adsorption would give a high yield and purity. However, the binding capability of lysozyme was drastically reduced.

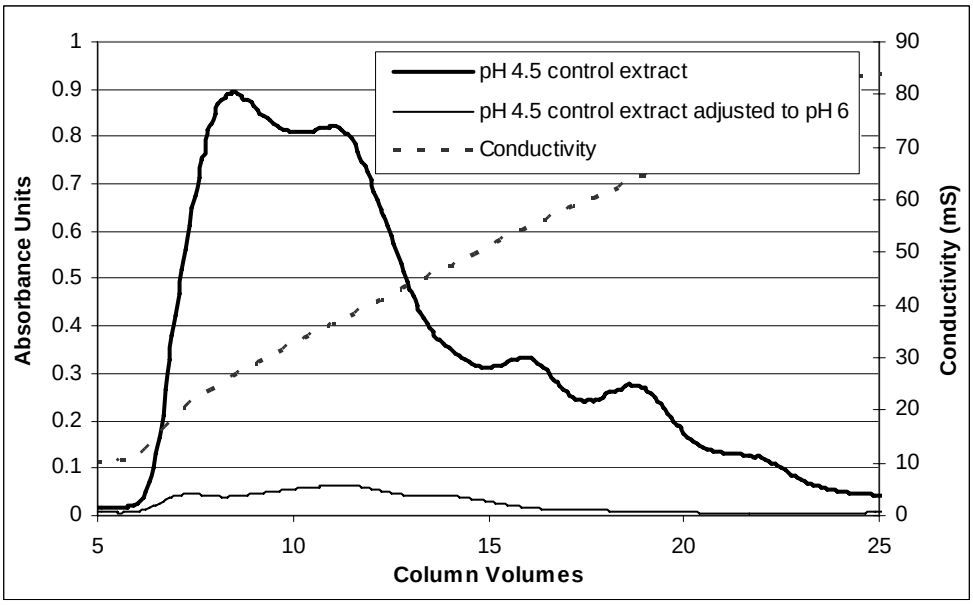


Figure 2.8. Elution profile comparison for pH 4.5 control extract and pH 4.5 control extract adjusted to pH 6 showing the reduction of bound protein at pH 6.

The binding capacity estimated from the lysozyme breakthrough curve was 8.6 mg/mL, almost 80% lower than the SBC of purified human lysozyme at pH 6 and 50 mM NaCl. We speculate that ferulic acid and/or phytic acid in the rice extract may have interfered with lysozyme binding causing an early lysozyme breakthrough. A small amount of ferulic acid, measured by RP-HPLC, eluted with sodium hydroxide during the resin regeneration step. The total amount of soluble ferulic acid in brown rice is only 0.02 mg/g (Adom and Liu, 2002), and probably too small to cause an 80% reduction in lysozyme binding. Phytic acid, which is present at 1 mg/g in brown rice (Juliano and Bechtel, 1985), is known to form binary and tertiary complexes with positively charged proteins and cations (Cheryan, 1980). At pH 4.5 and 6, phytic acid would be negatively charged and could form complexes with positively charged lysozyme. Since a drastic

reduction in binding capability was only observed in this last adsorption case, no attempt was made to prove that phytic acid caused the apparent SBC reduction. The mechanism of interference from phytic acid and/or other extract impurities would require a separate investigation.

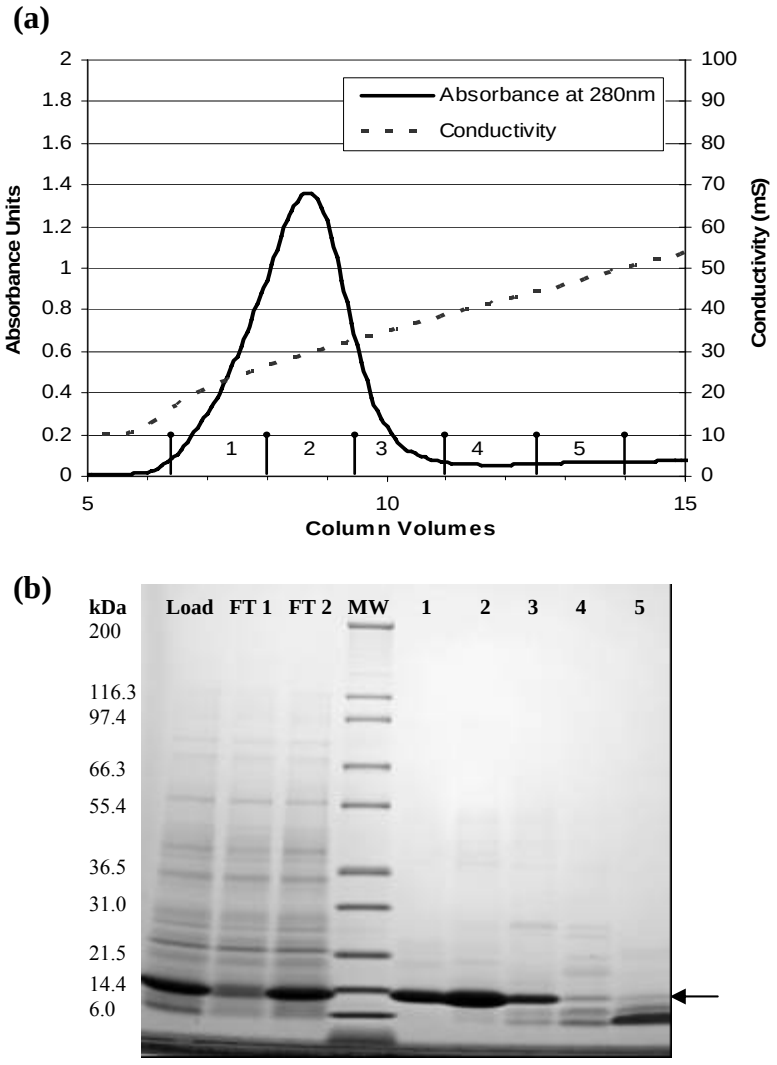


Figure 2.9. Elution peak profile (a) showing fractions analyzed by SDS-PAGE (b). Protein profile for load (pH 4.5 extract adjusted to pH 6), flowthrough (FT 1, FT 2), molecular weight markers (MW), and elution peak fractions (1-5). Numbered lanes correspond to fraction number in elution peak chromatogram. FT 1 collected at the beginning of lysozyme breakthrough (8.4 mg lysozyme loaded), FT 2 collected at lysozyme saturation (12.4 mg lysozyme loaded). The arrow shows the position of human lysozyme.

SUMMARY

This study demonstrated that the best conditions for extraction are not necessarily optimal for lysozyme adsorption. The most favorable extraction conditions for maximizing lysozyme concentration and achieving the highest ratio of lysozyme to native protein were pH 4.5 and 100 mM NaCl (15 mS). In terms of lysozyme adsorption, pH 4.5 extract with low ionic strength (5 mS) had the highest binding capacity. Data indicate that direct application of pH 4.5 extract containing 100 mM NaCl (15 mS) would not be as effective on a large scale for capturing and purifying human lysozyme because the SBC of 40 mg/mL was rather modest compared with the 75 mg/mL SBC at 5 mS. The 15 mS extract could be diluted with water to 10 mS or 5 mS to improve binding capacity, but dilution would increase the extract volume 2- to 3-fold, resulting in a longer loading time on the cation exchanger. Removal of NaCl by diafiltration is an alternative but at a cost. For applications where 90% purity of lysozyme is satisfactory, pH 4.5 and 10 mS extraction and adsorption appears to be an acceptable compromise. For greater than 95% purity, the adjustment of pH 4.5 extract to pH 6 before ion exchange adsorption offers an exciting opportunity providing the binding capacity is increased from 8 to 30-40 mg/mL. The identification of interfering extract impurities would be critical for the design of an optimal downstream process with a single adsorption chromatography step.

CHAPTER III

EVALUATION AND ECONOMIC ANALYSIS OF RECOMBINANT HUMAN LYSOZYME AND HEN EGG-WHITE LYSOZYME PURIFICATION

OVERVIEW

Human lysozyme and hen egg-white lysozyme have antibacterial, antiviral, and antifungal properties with numerous potential commercial applications. Currently, hen egg-white lysozyme dominates low cost applications but the recent high-level expression of human lysozyme in rice could provide an economical source of lysozyme if purification costs were comparable. This study evaluates human lysozyme and hen egg-white lysozyme adsorption to the cation exchange resin, SP-Sepharose™ FF, and the effect of rice extract on lysozyme purification. The dynamic binding capacities of human lysozyme were lower, except in one case, than those of hen egg-white at pH 4.5, 6, and 7.5 with ionic strengths from 0 to 100 mM (5-20 mS). Ionic strength and pH had similar effects on the capacities, but human lysozyme was more sensitive to these factors than hen egg-white lysozyme. At pH 4.5 and in the presence of rice extract, the dynamic binding capacities of human and hen egg-white lysozymes were reduced by 20-30%. Similarly, in the presence of rice extract the capacities were reduced 32-39% at pH 6. Hen egg-white lysozyme was purified from egg-white using two major steps (protein precipitation and ion-exchange chromatography). The hen egg-white purification data was then used as a benchmark to assess the effectiveness of human lysozyme recovery and purification from transgenic rice. Process simulation for human lysozyme

purification from rice and hen lysozyme purification from egg-white demonstrated that production costs would be comparable on a manufacturing scale.

INTRODUCTION

Lysozyme (EC 3.2.1.17) is a glycosidic enzyme and bioactive protein found in hen egg-white and human tears and milk (Jollès, 1996). All lysozymes have the same enzyme specificity but can have different physico-chemical properties (isoelectric point, molecular weight), amino acid composition, and specific activity. For example, there is only a 60% homology between hen egg-white lysozyme (HewLZ) and human lysozyme (HuLZ) which results in slightly different isoelectric points (pI) and substantially different specific activities against a cell wall suspension of *Micrococcus luteus* (Huang et al., 2002b). In addition, antibodies against HewLZ and HuLZ do not cross-react (Faure and Jollès, 1970) signifying important structural differences (Huang et al., 2006b). Human lysozyme, found in tears, saliva, nasal secretions, and breast milk, consists of 130 amino acids with a molecular weight of 15 kDa and an isoelectric point of 10.2 (Huang et al., 2002a). HewLZ contains 129 amino acids with a molecular weight of 14 kDa, similar pI (≈11), but 3-4 fold lower N-acetylmuramoyl specific activity than human lysozyme.

Depending on the application and purity level, hen egg-white lysozyme can be produced at a relatively low cost of $150/kg (Cook, 2004) and dominates lower cost applications. HewLZ is used in the U.S. to control bacterial growth in food/wine production and as a feed antibacterial agent (Proctor and Cunningham, 1988). In 1998, hen egg-white lysozyme was given GRAS (generally recognized as safe) status by the

FDA. In Europe, hen egg-white lysozyme has been approved under the EU Additive Directive 95/2/EC and is used extensively as a chemical-free preservative in the cheese making industry instead of nitrate and as a sulfate substitute in wine making. Lysozyme is also used for pharmaceutical applications (Sava, 1996) and is present in many over the counter medications in Asia and Europe.

Depending on the application, rice-derived human lysozyme may be advantageous in situations where non-animal source protein and product hypoallergenicity are required (Huang et al., 2002a). Potential applications of HuLZ include an anti-infective agent for oral infections (Huang et al., 2006a), as a baby formula additive to reduce gastrointestinal tract infections (Huang et al., 2002b), and as a cell lysing agent (InVitria™, Fort Collins, CO). The recent high-level expression of HuLZ in transgenic rice combined with inherently higher specific activity provides an inexpensive source of human lysozyme for applications similar to those of HewLZ (i.e. where animal source and hypoallergenicity have no marketing/application advantage). If one can demonstrate that the downstream processing cost for HuLZ was comparable to HewLZ then pricing and market penetration of HuLZ would primarily be driven by the unique market and product requirements such as specific activity, hypoallergenicity and non-animal source protein.

Various purification strategies have been employed for purification of both lysozymes with cation-exchange chromatography being one of the prevailing methods (Guérin-Dubiard et al., 2005; Li-Chan et al., 1986). HewLZ is commercially purified either by direct salt precipitation (Alderton and Fevold, 1946; Alderton et al., 1945) or

by cation exchange adsorption (Guérin-Dubiard et al., 2005; Li-Chan et al., 1986) and can be used as a benchmark to assess the effectiveness of cation exchange-based purification processes of HuLZ. Although the adsorption behavior of purified hen egg-white lysozyme has been thoroughly investigated (Carta et al., 2005; Chang and Lenhoff, 1998; Dismer and Hubbuch, 2007; Dziennik et al., 2005; Ladiwala et al., 2005; Nash and Chase, 1998; Skidmore et al., 1990; Whitley et al., 1989), the conditions presented by rice extract components and human lysozyme are unique and require additional evaluation. In Chapter II, we established the effect of pH and ionic strength on adsorption capacity of HuLZ to the cation exchanger, SP-Sepharose™ FF, and identified potential process bottlenecks that required further investigation (Wilken and Nikolov, 2006b). For example, the saturation binding capacity data showed a stronger than expected negative pH and salt effect, even though binding studies were conducted at pHs well below the reported pI of human lysozyme. In this paper we further examine the effect of rice extract on human lysozyme and hen egg white purification and compare manufacturing costs of simulated downstream processing trains for HuLZ and native HewLZ.

MATERIALS & METHODS

Materials. Recombinant human lysozyme (>93% purity), human lysozyme (HuLZ) expressing rice flour (transgenic flour), and control rice flour were provided by Ventria Bioscience (Sacramento, CA). The transgenic and control flour were from the same genetic background. Hen egg-white lysozyme (HewLZ) was obtained from

Calbiochem (LaJolla, CA). Grade A large eggs were purchased from a local grocery store.

Analytical Methods. ***Total Protein and Lysozyme Quantification.*** Total soluble protein (TSP) was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Lysozyme concentrations were determined by enzymatic assay for extracts and by UV absorbance at 280 nm for highly pure lysozyme solutions. The enzymatic assay measures the decrease in turbidity of a *M. luteus* cell suspension (Shugar, 1952). To measure lysozyme content, a modified microtiter plate method was used with a 0.03% (w/v) cell suspension and either 0-15 μg/mL HuLZ standards or 0-50 μg/mL HewLZ standards. The range of lysozyme standards differed because HewLZ has a 3-fold lower specific activity than HuLZ. Control flour extract was used in the preparation of standards to account for the interference of extract components. Cell suspension (250 μL) was added to each 50 μL sample and activity was monitored at 450 nm for 4 min with a Versamax plate reader (Molecular Devices). When lysozyme was the only UV-absorbing solution component, lysozyme concentration was determined by absorption at 280 nm (Beckman DU 600) using an extinction coefficient of 2.46 mL/mg-cm for human lysozyme and 2.64 mL/mg-cm for hen egg-white lysozyme.

Protein Analysis by SDS-PAGE. The protein molecular weight profiles of extracts and chromatography fractions were evaluated by electrophoresis as described by Laemmli (1970). Samples were loaded on 8-16% tris-glycine gels under non-reducing conditions with Mark12^{TM} protein standards (Invitrogen).

Experimental Methods. ***Effect of pH and Ionic Strength on Dynamic Binding Capacity (DBC) of Human Lysozyme and Hen Egg-white Lysozyme.*** The breakthrough capacities of HuLZ and HewLZ on a strong cation exchange resin (SP-Sepharose™ FF) were determined using a Bio-Rad (Hercules, CA) BioLogic LP chromatography system interfaced with LP DataView Software. SP-Sepharose™ FF resin (GE Healthcare, Piscataway, NJ) was packed to a height of 2.2 cm in a 1 cm x 10 cm column (Bio-Rad). HuLZ solutions (0.5 mg/mL) and HewLZ solutions (0.5 mg/mL) were prepared with each respective binding buffer. Binding buffers of various ionic strengths (0, 50, 100, and 150 mM NaCl) were prepared in 50 mM sodium acetate (pH 4.5 experiments) or 50 mM sodium phosphate (pH 6 experiments). At pH 7.5, the breakthrough capacity was evaluated at a single sodium chloride concentration (50 mM) using 50 mM sodium phosphate buffer.

For each experiment, the column was equilibrated with binding buffer and the lysozyme solution was loaded at a flow rate of 2 mL/min (150 cm/h) until saturation. Absorbance at 280 nm was monitored throughout loading. The absorbances were converted to lysozyme concentrations using a path length of 2 mm and an extinction coefficient of 2.64 mg/mL-cm for hen egg-white lysozyme or 2.46 mg/mL-cm for human lysozyme. The 5% breakthrough capacity was calculated by determining the amount of lysozyme bound to the column once the outlet concentration of lysozyme was 5% of the load concentration. The DBC values reported are expressed as milligrams lysozyme bound per milliliter resin.

Preparation of Control and Lysozyme Rice Flour Extracts. To prepare extracts, 20 g of control or transgenic rice flour was added to 100 mL extraction buffer and mixed continuously for 1 h. The extraction buffers used for extraction were 50 mM sodium acetate, 50 mM NaCl (pH 4.5) or 50 mM sodium phosphate, 100 mM NaCl (pH 6). Extracts were centrifuged at 12,000 x g for 15 min and then filtered through No. 5C filter paper (Micro Filtration Systems) followed by a 0.45 μm cellulose acetate filter (Corning) to produce a clarified extract. For HewLZ spiked extract, HewLZ was added to clarified control extract and mixed. The concentration of HewLZ spiked into control extract was comparable to the HuLZ concentration in transgenic extract at pH 4.5 (10 mS) or pH 6 (15 mS).

Effect of Rice Extract on the Binding and Elution of Human Lysozyme and Spiked Hen Egg-white Lysozyme. The binding capacities of HuLZ and HewLZ in pH 4.5 and pH 6 rice extracts were determined to understand how background impurities would impact the binding capacity and purification of both lysozymes.

pH 4.5 Binding and Elution. HuLZ rice flour and HewLZ spiked control rice flour extracts were prepared as described previously. The column was equilibrated with pH 4.5 50 mM sodium acetate with 50 mM NaCl and the clarified extract was loaded onto a 2 mL (0.5 cm x 10 cm) SP-Sepharose™ FF column at a flow rate of 1 mL/min (300 cm/h). The column was washed with 10 column volumes (CV) of equilibration buffer and bound protein was eluted by a linear gradient from 0.05 to 1 M NaCl in pH 4.5 50 mM sodium acetate over 20 CV. Lysozyme breakthrough curves (HuLZ and HewLZ) were generated by collecting 3 mL fractions throughout column loading and

measuring the lysozyme activity in each fraction. The DBC was determined by calculating the amount of lysozyme loaded when the column outlet concentration was 5% of the load concentration. To determine if the eluted HewLZ would have greater purity than the eluted HuLZ, the column was loaded to 80% of the saturation capacity and elution peak fractions were analyzed for lysozyme and total protein content.

pH 6 Binding and Elution. The binding capacity and eluted lysozyme purities were determined under saturation conditions at pH 6 to further evaluate and compare the interactions of HuLZ and HewLZ with SP-Sepharose™ FF in rice extracts. The saturation binding capacities (SBC) were determined instead of DBCs at 5% breakthrough because very little protein was bound from pH 6 HuLZ rice extract at 15 mS in a preliminary experiment.

The column was equilibrated with pH 6 50 mM sodium phosphate with 50 mM NaCl and the clarified extract was loaded onto a 2 mL (0.5 cm x 10 cm) SP-Sepharose™ FF column at a flow rate of 1 mL/min (300 cm/h). The column was washed with 10 column volumes (CV) of equilibration buffer and bound protein was eluted over 20 CV with a linear gradient from 0.05 to 1 M NaCl in pH 6 50 mM sodium phosphate. Elution peak fractions (3 mL) were collected, analyzed for lysozyme and total soluble protein, and pooled based on purity. The SBCs were determined by summing the lysozyme content (activity assay) in all the elution peak fractions.

Binding and Elution of Control Extract. Control rice flour extract was prepared as described previously with 50 mM NaCl in 50 mM sodium acetate, pH 4.5. Clarified extract was loaded onto a 2 mL (0.5 cm x 10 cm) SP-Sepharose™ FF column at a flow

rate of 1 mL/min (300 cm/h). The volume of control extract loaded corresponded to the volume of either transgenic or spiked control rice extract needed to reach lysozyme saturation. The column was washed with 5 column volumes (CV) of the respective binding buffer and the bound protein was eluted by linear gradient from 0 to 1 M NaCl in 50 mM sodium acetate with 50 mM NaCl over 20 CV. Fractions (3 mL) were collected throughout elution and analyzed for total soluble protein and by SDS-PAGE.

Effect of Egg-white Protein on Hen Egg-white Lysozyme Purification. Egg-white was diluted 1:3 with RO water, adjusted to pH 6 with 5 M phosphoric acid, and mixed for 1 h at room temperature to precipitate ovomucin (Nakamura et al., 1961). After precipitation, the solution was centrifuged for 30 min at 12,000 x g and 25°C. The liquid was recovered, sodium phosphate was added to get 50 mM buffer with 0, 50, or 100 mM NaCl, and the solution was filtered with a 0.2 μm surfactant-free cellulose acetate filter system (Nalgene).

Three sodium chloride concentrations were evaluated to determine the effect of conductivity on the binding capacity and lysozyme purity using SP-Sepharose™ FF. The capacity of hen egg-white lysozyme on the weak cation exchanger, CM-Sepharose™ FF (GE Healthcare), was evaluated at a single ionic strength (50 mM sodium phosphate and 50 mM NaCl). For each experiment, the column was equilibrated with 50 mM sodium phosphate buffer (pH 6) with 0, 50, or 100 mM NaCl (buffer A) over 10 CV. The clarified egg-white solution was loaded at 1 mL/min (300 cm/h) onto a 2 mL SP-Sepharose™ FF column (0.5 cm x 10 cm) until saturation. The column was washed with 10 CV of buffer A and the bound protein was eluted by linear

gradient from 0 to 1 M NaCl in buffer A. Fractions (3 mL) were collected throughout elution and analyzed for lysozyme, total soluble protein, and by SDS-PAGE. The lysozyme saturation capacity was calculated by measuring the activity in the elution peak fractions.

Process Simulation & Economics. To estimate manufacturing costs, SuperPro Designer 7.0 software (Intelligen Inc.) was used to simulate HuLZ extraction and purification from transgenic rice and HewLZ purification from egg-white. The simulated processes were: 1) pH 4.5 extraction and adsorption of HuLZ and 2) pH 6 adsorption of HewLZ. Using our experimental data and other published information, the HuLZ process was assembled in six sections: 1) grinding to remove rice hull, 2) pH 4.5 extraction, 3) solid-liquid separation by decanter centrifuge, 4) ion-exchange capture at pH 4.5, 5) ultrafiltration/diafiltration (UF/DF) buffer exchange and product concentration, and 6) spray drying. HewLZ purification consisted of five sections: 1) precipitation of ovomucin at pH 6, 2) solid-liquid separation by disk-stack centrifuge, 3) ion-exchange capture at pH 6, 4) UF/DF, and 5) spray drying. The processes were designed to produce 1000 kg lysozyme per year in 330 batches.

The equipment size was estimated by the simulation software using component mass balance, operating time, and the required throughput. Equipment cost was estimated using either SuperPro built-in models or user-define models based on vendor price quotes. The total capital investment included equipment cost, direct fixed capital (DFC), working capital, and start-up cost. The DFC was calculated from the total

equipment cost using a Lang factor of 3. Working capital and start-up costs were assumed to be 3% and 5% of DFC (Peters and Timmerhaus, 1991).

Lysozyme purification by SP-Sepharose^{TM} FF (HuLZ) and CM-Sepharose^{TM} FF (HewLZ) was simulated using experimentally determined capacities and yields and assumed values for the resin cost of $500/L and lifespan of 500 cycles. Other consumables used for each process include UF membranes ($1000/m^2 with 150 cycle lifespan) and dead-end filtration cartridge ($100/m^2, 100 cycle lifespan). Operation data such as buffer composition, column volumes, and linear velocities for the column wash, elution, regeneration, and equilibration were consistent with experimental procedures listed previously. The cost of chemicals was obtained from the ICIS Chemical Business (www.icis.com) and SuperPro database.

The operating labor for each process was estimated by summing the allocated operator hours required for each procedure per equipment operation time. Lab services with QC/QA were assumed to be 15% of the operating labor (Harrison et al., 2003). The total labor includes the operating and the labor-dependent costs.

The total operating cost for each downstream processing train was determined from the direct process costs (reagents and consumables, labor and operating supplies, and utilities and waste disposal) and indirect process costs (maintenance and depreciation).

The lysozyme production cost on a mass basis was converted to a per unit activity basis using specific activities of 150,000 units/mg for HuLZ and 50,000 units/mg for HewLZ. One unit activity is defined as the amount of lysozyme that

produces a change of 0.001 in the absorbance at 450 nm per minute at the standard lysozyme assay conditions.

RESULTS & DISCUSSION

Effect of pH and Ionic Strength on Dynamic Binding Capacity (DBC) of Human Lysozyme and Hen Egg-white Lysozyme. The capacities were evaluated and compared to understand how the differences between HuLZ and HewLZ properties (pI, activities) and amino acid sequence (homology and charge distribution) translate into different adsorption behavior to the cation exchange resin. The dynamic binding capacities at 5% breakthrough of human and hen egg-white lysozyme on SP-Sepharose™ FF are reported in Figures 3.1-3.3. The binding capacities typically decreased as the pH and conductivity increased with HewLZ having greater capacities than HuLZ. At pH 4.5 (Figure 3.1), the dynamic capacity decreased from 69 to 20 mg/mL for HuLZ and from 65 to 42 mg/mL for HewLZ as the conductivity increased from 5 to 20 mS. The negative effect of ionic strength on both enzymes was more pronounced at pH 6 (Figure 3.2). The dynamic capacity at pH 6 dropped from 62 to 4 mg/ml for HuLZ and from 73 to 6 mg/ml for HewLZ as the conductivity increased from 5 to 20 mS (Figure 3.2).

The two enzymes responded differently in the presence of acetate buffer or phosphate buffer with no salt addition. The binding capacities of both lysozymes at pH 4.5 (acetate buffer) were similar, whereas at pH 6 (phosphate buffer) they differed by 10 mg/ml. This difference was caused by the slight increase of HewLZ binding with the concomitant decrease of HuLZ binding. At pH 4.5, the addition of 50 mM NaCl to

acetate buffer slightly increased DBC of HewLZ. This positive salt effect could possibly be caused by reduced repulsion between free and adsorbed lysozyme and enhanced protein transport within the resin particle (Chang and Lenhoff, 1998; Zydney et al., 2009).

At pH 4.5, HuLZ susceptibility to ionic strength change was greater than that of HewLZ, and at pH 6, both enzymes were equally affected (Figure 3.1 and 3.2). At pH 4.5 and the highest ionic strength (15 mS), the HewLZ capacity (42 mg/mL) was double the HuLZ capacity (20 mg/mL). The capacities at pH 6 and 15 mS were only 6 mg/mL for HewLZ and 4 mg/mL for HuLZ. To determine whether the DBC difference would continue to increase with increasing pH, an additional DBC experiment was carried out at pH 7.5 and 50 mM NaCl (10 mS). The DBC of HuLZ was 12%, 37%, and 74% lower than HewLZ at pH 4.5, 6, and 7.5, respectively (Figure 3.3). The stronger effect of ionic strength and pH on HuLZ than HewLZ suggests different surface charge distribution and/or different positive net charge. Examination of protein models using Protein Explorer (Martz, 2002) revealed two clusters of basic residues, primarily lysine, on HewLZ but a more even distribution of basic charges (arginine) on the surface HuLZ which may explain the ionic strength effect.

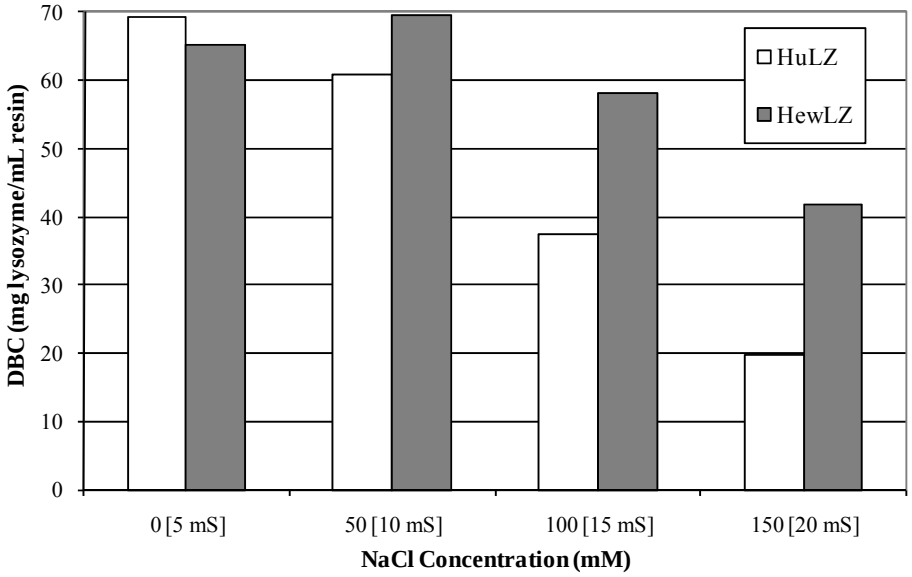


Figure 3.1. Effect of ionic strength on the binding capacities of purified human lysozyme (HuLZ) and hen egg-white lysozyme (HewLZ) solutions (0.5 mg/mL) on SP-Sepharose^{TM} FF at pH 4.5. Dynamic binding (DBC) were measured at 5% breakthrough with a linear velocity of 150 cm/h and reported as mg lysozyme bound/mL resin.

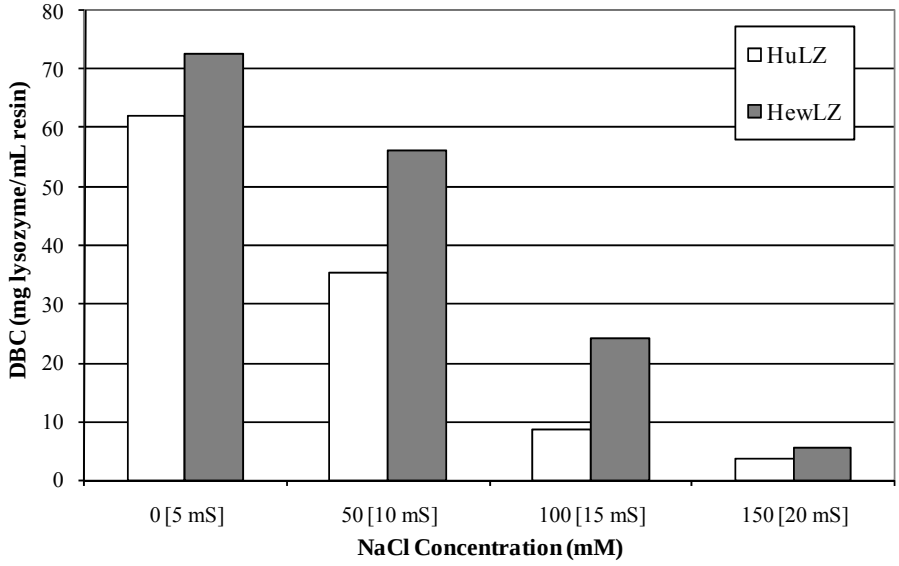


Figure 3.2. Effect of ionic strength on the binding capacities of purified human lysozyme (HuLZ) and hen egg-white lysozyme (HewLZ) solutions (0.5 mg/mL) on SP-Sepharose^{TM} FF at pH 6. Dynamic binding capacities (DBC) were measured at 5% breakthrough with a linear velocity of 150 cm/h and reported as mg lysozyme bound/mL resin.

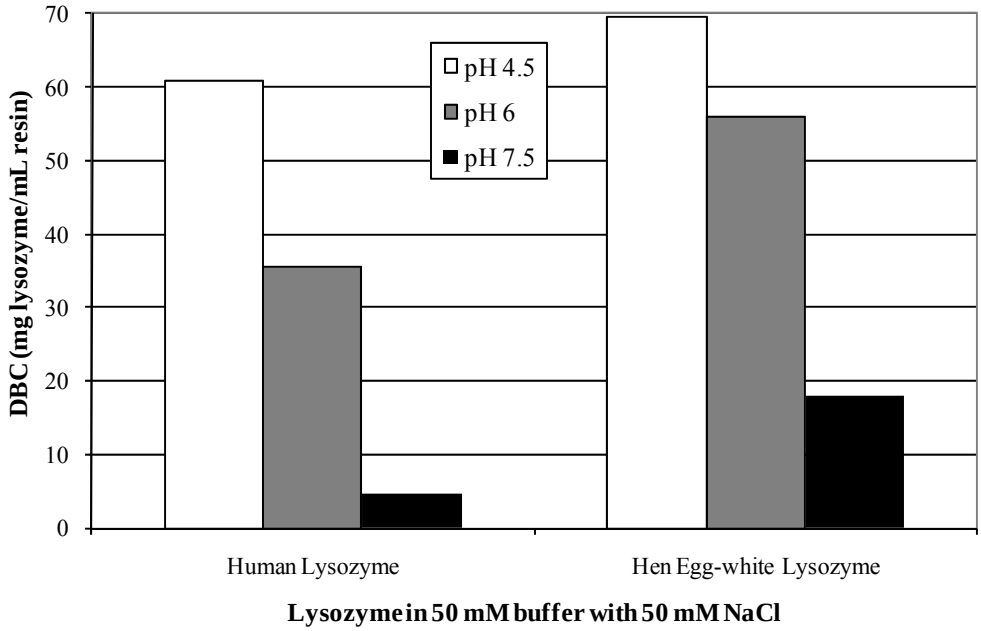


Figure 3.3. Effect of pH on the binding capacities of purified human lysozyme and hen egg-white lysozyme (0.5 mg/mL) on SP-Sepharose™ at an ionic strength of 50 mM NaCl (10 mS). Dynamic binding capacities measured at 5% breakthrough with a linear velocity of 150 cm/h and reported as mg lysozyme loaded /mL resin.

Effect of Rice and Egg-white Proteins on Lysozyme Adsorption. To understand how background impurities would impact the binding capacity and purification of both lysozymes, we first compared the binding capacity of HuLZ and spiked HewLZ in pH 4.5 and pH 6 rice extracts. Secondly, HewLZ adsorption from spiked control rice extract was also compared to the binding of HewLZ from egg-white. The latter comparison allows direct comparison of host impurities, i.e. rice extract versus egg-white. Because egg-white is a natural source of lysozyme, the reverse scenario of HuLZ binding from spiked egg-white was not possible.

Effect of Rice Extract on Binding and Elution of Human Lysozyme and Spiked Hen Egg-white Lysozyme. In Chapter II, we measured a 30% reduction in DBC of

HuLZ in rice extract compared to buffer and thought it would be prudent to compare the effect of rice extract impurities (native protein, phytic acid, starch, etc.) on the binding capacities of HuLZ and HewLZ. We were concerned about the presence of complexes between HuLZ and extract components that may have reduced the ability of HuLZ to effectively interact with the cation exchange. For this reason, HewLZ was spiked into filtered rice extract.

pH 4.5 Binding and Elution. To assess the effect of pH 4.5 rice extract on the adsorption of lysozyme to the cation exchange resin, the DBC of HuLZ and HewLZ was determined in pH 4.5 and 50 mM NaCl rice extracts. For this investigation, 50 mM NaCl (10 mS) was selected since this ionic strength was determined to be most compatible for both extraction and adsorption (Wilken and Nikolov, 2006b). The DBC of HuLZ was reduced from 61 mg/mL to 42 mg/mL (31%) in the presence of pH 4.5 rice extract compared to purified HuLZ in acetate buffer of the same ionic strength (conductivity). Similarly, the DBC for HewLZ was reduced from 69 to 55 mg/mL (21%). The measured reduction of DBCs for the two enzymes seems to indicate similar interference irrespective of the enzyme source (spiked or rice extracted). However, the percent reduction of DBC for HuLZ was slightly greater which supports the findings from our studies with pure lysozyme that HuLZ interactions with the cation exchange resin are weaker than HewLZ interactions. This was also evident by overlapping the adsorption-conductivity elution profiles from each experiment (Figure 3.4). HuLZ started to elute at a slightly lower conductivity than HewLZ (14 mS compared to 16 mS), indicating weaker interaction with the cation exchange resin.

The elution profile of bound rice proteins from pH 4.5 control extract was compared to the elution profile of pH 4.5 HuLZ rice extract and HewLZ spiked control extract by overlapping the adsorption-conductivity profiles in Figure 3.4. The total bound protein from the control and HuLZ rice extracts eluted in 20 column volumes (CV) and a conductivity range of 10 to 84 mS. The majority of the native rice proteins eluted between 10 to 50 mS (in 10 CV), a conductivity range which overlaps with that of HuLZ and HewLZ eluates. In other words, unless HuLZ and HewLZ have greater affinity for the SP-Sepharose^{TM} than native rice proteins then little purification should be expected at pH 4.5 with 50 mM NaCl.

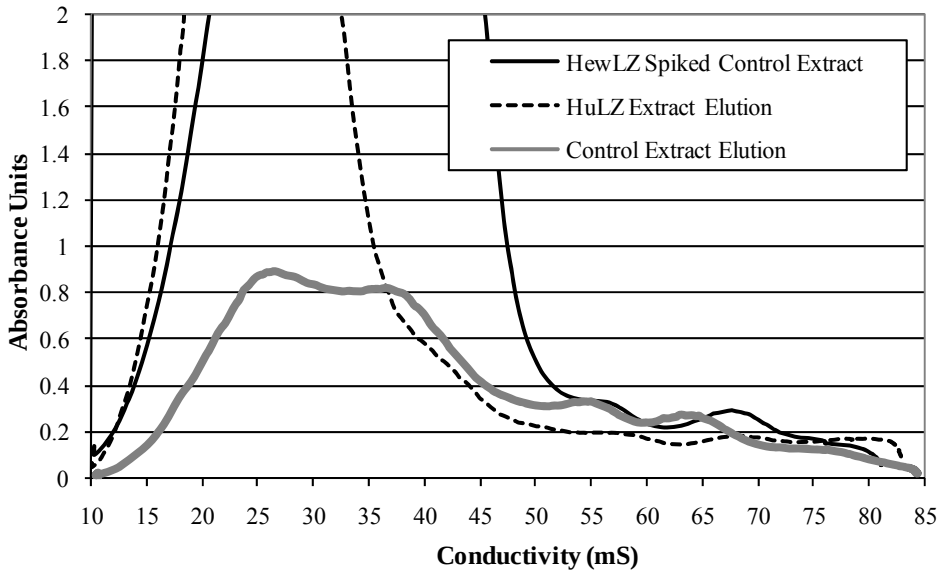


Figure 3.4. Elution profiles for pH 4.5 hen egg-white lysozyme (HewLZ) spiked extract and human lysozyme (HuLZ) extract compared to control (nontransgenic) rice extract. Absorbance at 280 nm monitored throughout the linear gradient elution.

To test lysozyme purification, pH 4.5 HuLZ rice extract and HewLZ spiked control rice extract were loaded on a SP-Sepharose^{TM} FF column to 80% of the saturation binding capacity. The purpose of this experiment was to determine if the eluted HewLZ would have greater purity than the eluted HuLZ. The elution peak fractions were individually analyzed and combined according to lysozyme purity into three pools (Figure 3.5a and 3.6a) to assess purity and yield of eluted lysozyme as a function of eluant conductivity. Pool 1 was formed from fractions that maximized yield without sacrificing lysozyme purity. Pool 1 consisted of fractions that eluted from 2 to 11 CV for HewLZ extract which corresponded to the eluant conductivity range of 10 to 44 mS. For HuLZ extract, pool 1 consisted of eluted lysozyme fractions from 3 to 12 CV, corresponding to the eluant conductivity range of 9.4 to 40 mS. The elution chromatogram and corresponding gel analysis of HewLZ spiked pH 4.5 control extract (initial purity of 67%) are presented in Figure 3.5. HewLZ purity (based on activity) and yield in pool 1 were >98% and 95%, respectively. Pool 2 contained primarily rice proteins with a lysozyme purity of 35%. By comparing the SDS-PAGE analysis of the pH 4.5 control extract elution pools (Figure 3.5c) with the three HewLZ elution pools (Figure 3.5b), HewLZ was more competitive in interacting with SP-Sepharose^{TM} than native rice protein. Nearly 80% of the loaded total protein in the control extract was bound under these conditions (pH 4.5 and 10 mS).

The elution chromatogram and corresponding gel analysis for adsorption from pH 4.5 HuLZ rice extract are presented in Figure 3.6. Lysozyme content in the extract was 70% of the total soluble protein. HuLZ purity (by HPLC) and yield in pool 1 were

91% and 87%, respectively. Pool 2 contained 19 mg of total protein with a lysozyme purity of 50%. The achieved purity of 91% may not be sufficient for some applications and additional improvement in lysozyme purity by optimization of the ion-exchange step is unlikely since rice proteins eluted in the same conductivity range (Figure 3.6). Compared to HewLZ purification from rice extract at pH 4.5, the purification of HuLZ from rice resulted in a lower lysozyme purity and yield. The lower purity and yield of HuLZ compared to HewLZ indicates that the interaction of HuLZ with the cation exchange resin is weaker, a conclusion that was also made from the binding capacity studies with purified lysozyme.

pH 6 Binding and Elution. The capacity and purities of lysozyme were evaluated under saturation conditions at pH 6 to further evaluate and compare the interactions of HuLZ and HewLZ with SP-Sepharose^{TM} FF. Purification experiments (Chapter II) with pH 6 HuLZ extract in the presence of 50 mM NaCl (10 mS) resulted in low lysozyme purity. In this study, pH 6 extracts were loaded at a higher conductivity (15 mS) in an attempt to reduce the amount of rice protein bound and improve lysozyme purity. Using a higher ionic strength for HuLZ extraction was also beneficial because the extracted lysozyme concentration increased 2-fold if 100 mM NaCl (15 mS) was used instead of 50 mM (10mS).

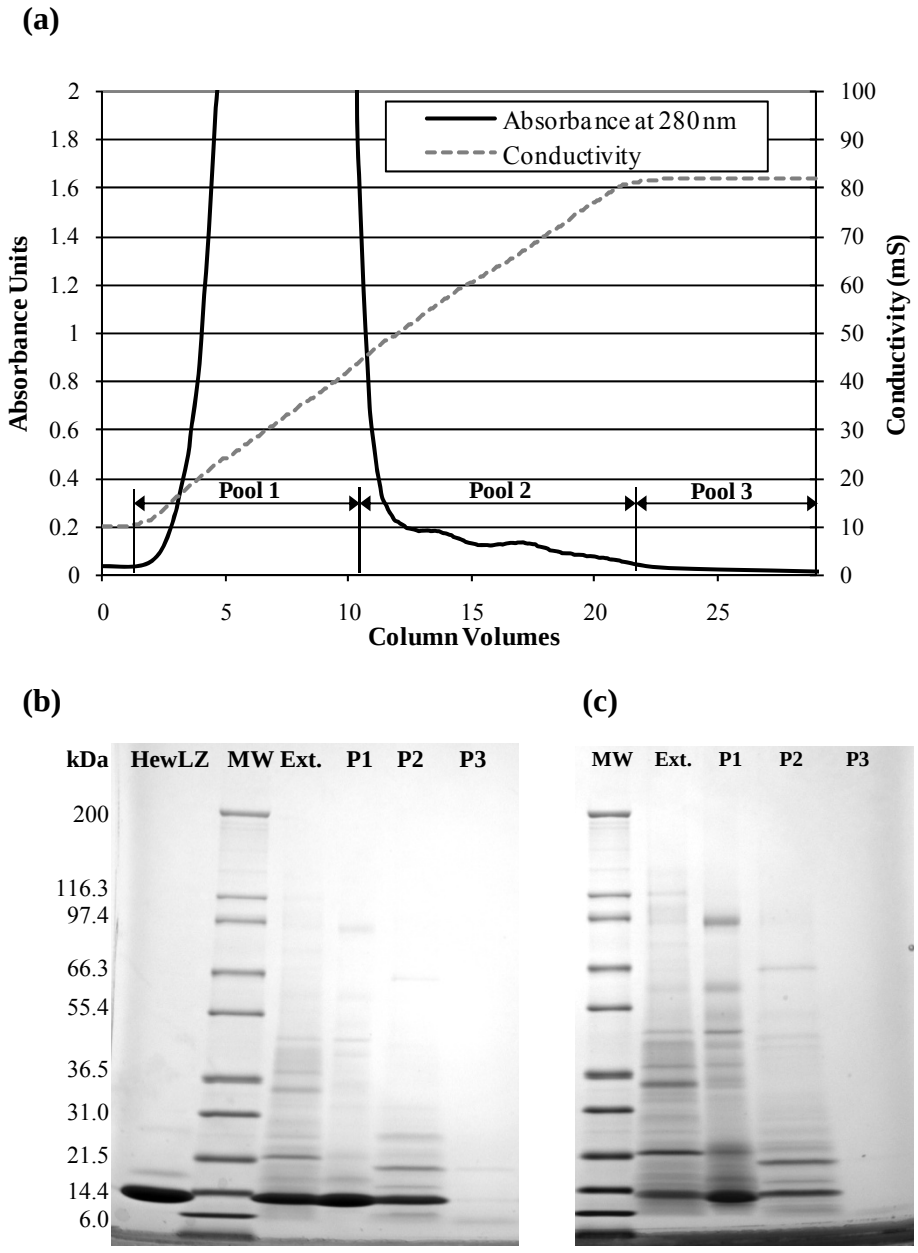


Figure 3.5. (a) pH 4.5 HewLZ spiked control extract elution profile and (b) SDS-PAGE analysis of extract and elution pools. P1, P2, and P3 correspond to the pools 1, 2, and 3 labeled on chromatogram with 10 μg of protein loaded in each lane except for Pool 3 (P3). (c) SDS-PAGE analysis of control extract elution pools (P1, P2, P3) with collection times and volumes identical to the HewLZ extract elution pools.

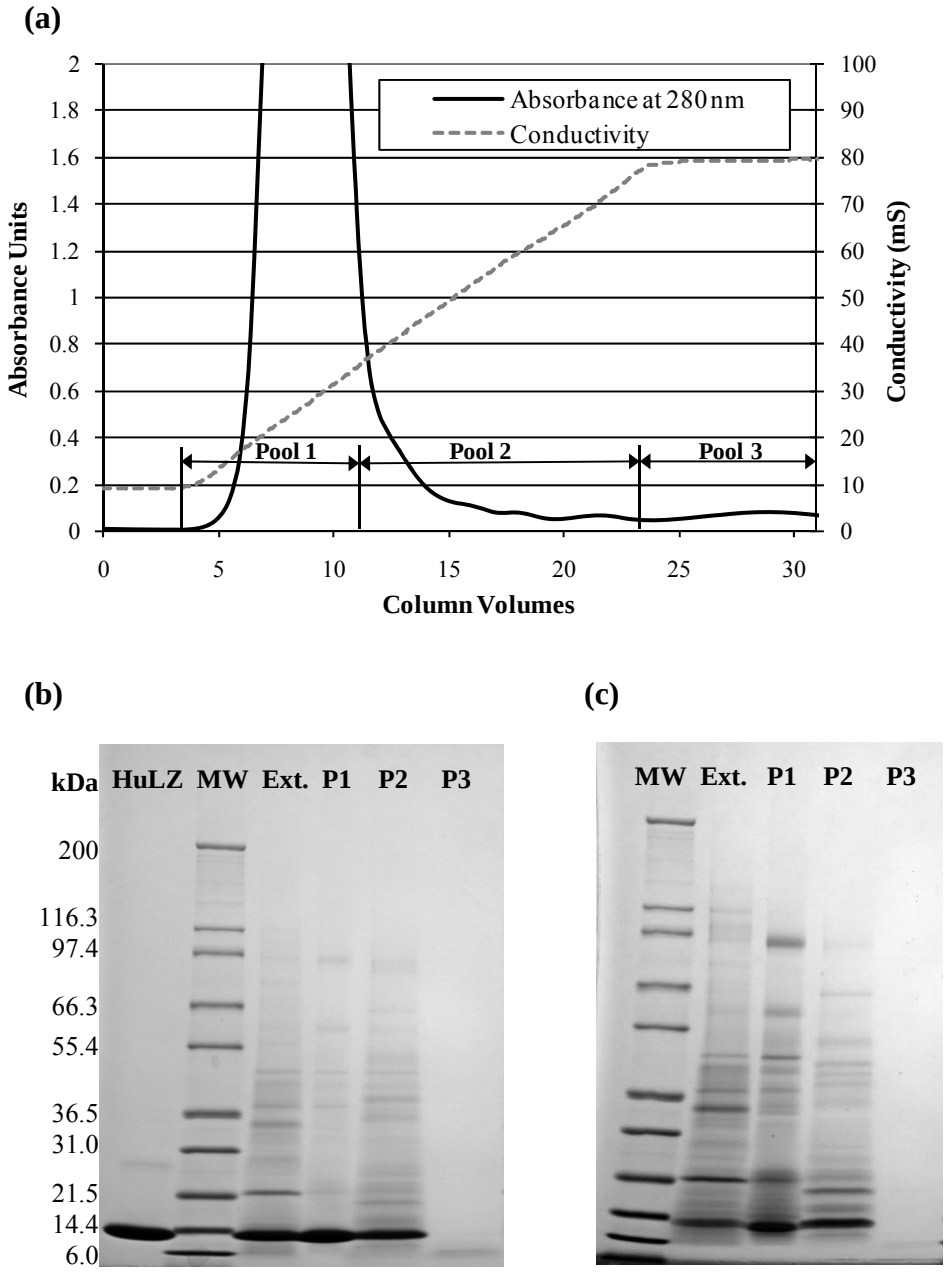


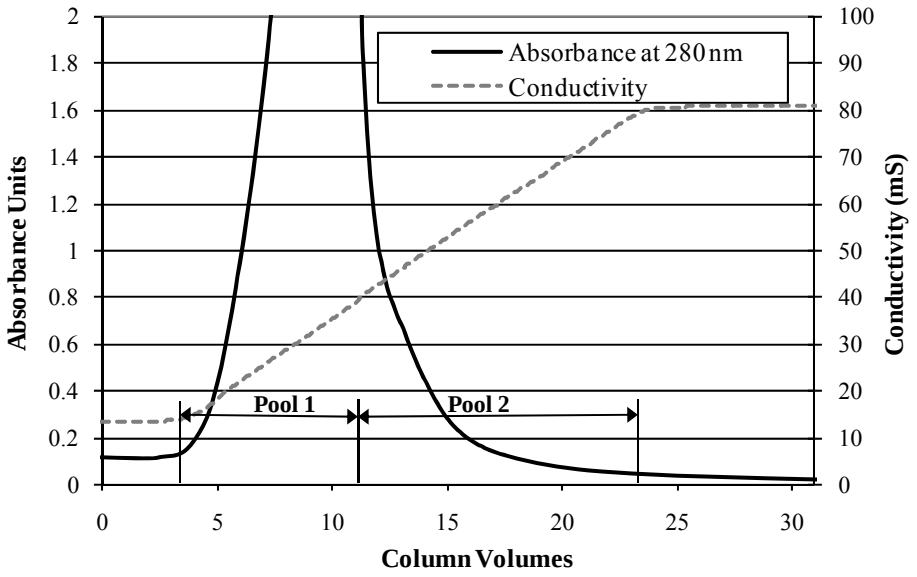
Figure 3.6. (a) pH 4.5 HuLZ rice extract elution profile and (b) SDS-PAGE analysis of loaded extract and elution pools. P1, P2, and P3 correspond to the pools 1, 2, and 3 labeled on chromatogram with 10 µg of protein loaded in each lane except for Pool 3 (P3). (c) SDS-PAGE analysis of control extract elution pools (P1, P2, P3) with collection times and volumes identical to the HuLZ extract elution pools.

The elution chromatogram and corresponding SDS-PAGE analysis of HewLZ adsorption from spiked pH 6 control extract are presented in Figure 3.7. HewLZ was 18% of the total soluble protein in the spiked control extract. The saturation capacity for HewLZ was reduced from 38 mg/mL to 26 mg/mL (32%) in the presence of rice extract compared to phosphate buffer of the same ionic strength. Pool 1 had a purity of 82% with an 87% yield (based on the total amount of eluted lysozyme (pool 1 and 2). Pool 2 contained primarily rice proteins with a lysozyme purity of 24%.

The elution chromatogram and SDS-PAGE analysis of HuLZ adsorption from pH 6 transgenic extract are given in Figure 3.8. HuLZ was 26% of the total soluble protein present in the extract. The saturation capacity of HuLZ in rice extract was 9.5 mg/mL, 39% lower than the capacity (15.6 mg/mL) of purified HuLZ in buffer. The purity of lysozyme, based on activity, in pool 1 was 60%.

The percent reductions in binding capacities for both HewLZ and HuLZ at pH 6 were slightly higher than the reductions observed at pH 4.5. As observed with pH 4.5 adsorption experiments, HewLZ purity was greater than the HuLZ purity.

(a)



(b)

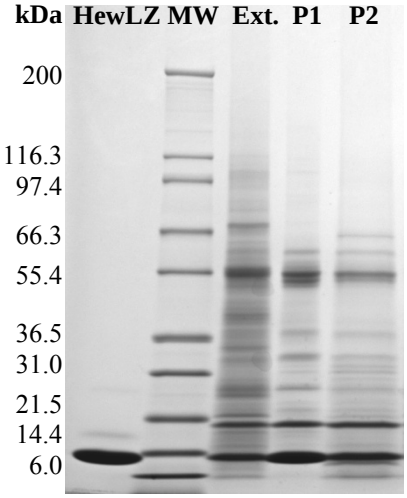
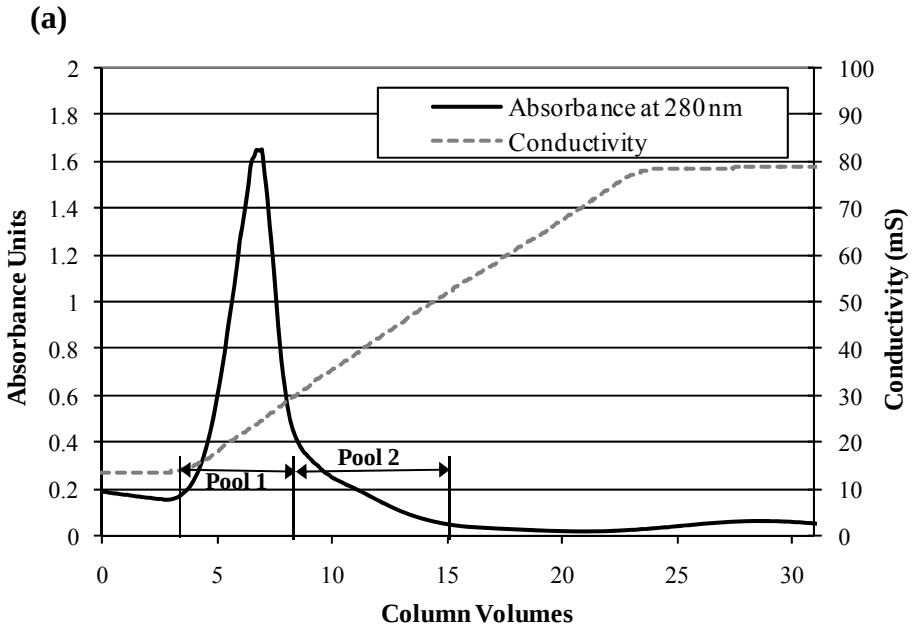


Figure 3.7. (a) pH 6 HewLZ spiked control extract elution profile and (b) SDS-PAGE analysis of extract (ext.) and elution peak fractions which were pooled (P1, P2) as indicated on the chromatogram. HewLZ standard was included for reference and each lane was loaded with 10 μg total protein.



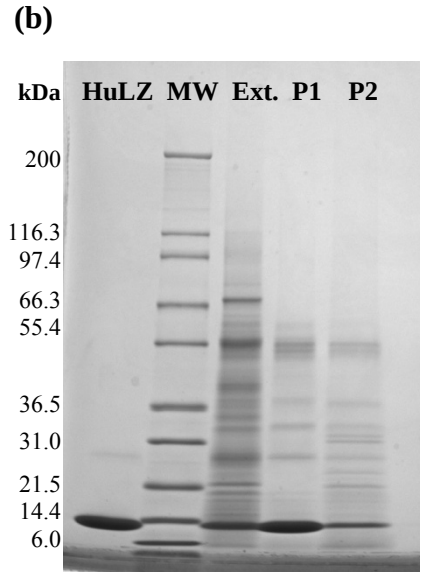


Figure 3.8. **(**a) pH 6 HuLZ rice extract elution profile and (b) SDS-PAGE analysis of extract and elution peak fractions which were pooled (P1, P2) as indicated on the chromatogram. HuLZ standard was included for reference and each lane was loaded with 10 μg total protein.

Effect of Egg-white Proteins on Binding and Elution of Hen Egg-white Lysozyme. To understand the impact of native rice impurities (protein) on lysozyme purification by ion-exchange, we compared the binding capacity and purity of HewLZ purified from egg-white to HewLZ purified from spiked control rice extract. The objective, as stated earlier, was to evaluate rice as a host system against egg-white and to determine the processing conditions that will be used for process simulation of HewLZ.

The binding capacity and purity of hen egg-white lysozyme eluted from SP-Sepharose^{TM} FF were measured as a function of load (egg-white) conductivity to determine the best adsorption conditions for cation exchange purification at pH 6. Adsorption at pH 6 was advantageous for this system since ovomucin precipitation (first step in egg-white process) is optimal at pH 6. In addition, the major protein impurity (ovalbumin) present in egg-white has a pI of 5.1 and theoretically should not bind to the cation exchange. After precipitation of ovomucin from egg-white, sodium phosphate buffer was added with 0, 50, or 100 mM NaCl corresponding to conductivities of 5, 10, and 15 mS. The HewLZ purity increased and binding capacity decreased as the conductivity of the load solution increased. The highest lysozyme purity of 89% was achieved when the load conductivity was 15 mS (50 mM buffer with 100 mM NaCl), with a binding capacity of 24 mg/mL.

The average amount of total protein in the cation exchanger load (clarified egg-white) was 21 mg/mL, which was significantly higher than the protein concentration of 1.4 mg/mL in pH 6 HewLZ spiked control rice extract. However, the lysozyme binding capacities were comparable, i.e. 26 mg/mL for HewLZ spiked in the rice extract

compared to 24 mg/mL for HewLZ from egg-white. The initial HewLZ protein purity in egg-white was 3% and 18% in control rice extract. The purity of eluted HewLZ was 66% for rice (3.9-fold purification) and 89% with egg-white (30-fold purification). The higher purity of eluted lysozyme for the egg-white experiments suggests that egg-white proteins are less competitive than rice ones for the cation exchange binding sites. Most hen egg-white proteins have more acidic isoelectric points (Stevens, 1991) than rice proteins (Menkhaus et al., 2004a) and thus, are less competitive under the conditions evaluated.

To address the final objective of comparing downstream processing costs, we also needed to determine best adsorption conditions for HewLZ purification from egg-white. Since SP-Sepharose™ purification resulted in only 89% purity, we tested a weak cation exchange resin, CM-Sepharose™, which was reported as a resin of choice for HewLZ. The binding capacity and HewLZ purity using the weak cation exchanger was determined at a single ionic strength of 10 mS (50 mM NaCl). The HewLZ saturation capacity was 30 mg/mL for CM-Sepharose™. The elution fractions with the majority of HewLZ were pooled and resulted in a purity of 94% and yield of 95%. The highest purity achieved with SP-Sepharose™ FF was 89% at the highest ionic strength evaluated (15 mS). Elution peak fractions from both exchangers had similar protein profiles, but the lysozyme purity was higher using CM-Sepharose™ FF (Figure 3.9). CM-Sepharose™ FF was selected for process simulation analysis because of the higher purity, slightly higher binding capacity, and lower ionic strength requirement. CM-Sepharose™ FF was evaluated as an option for HewLZ purification but not for HuLZ

purification due to the reduced binding capacity of HuLZ at pH 6, which was the minimum pH required for the weak exchanger to be fully charged.

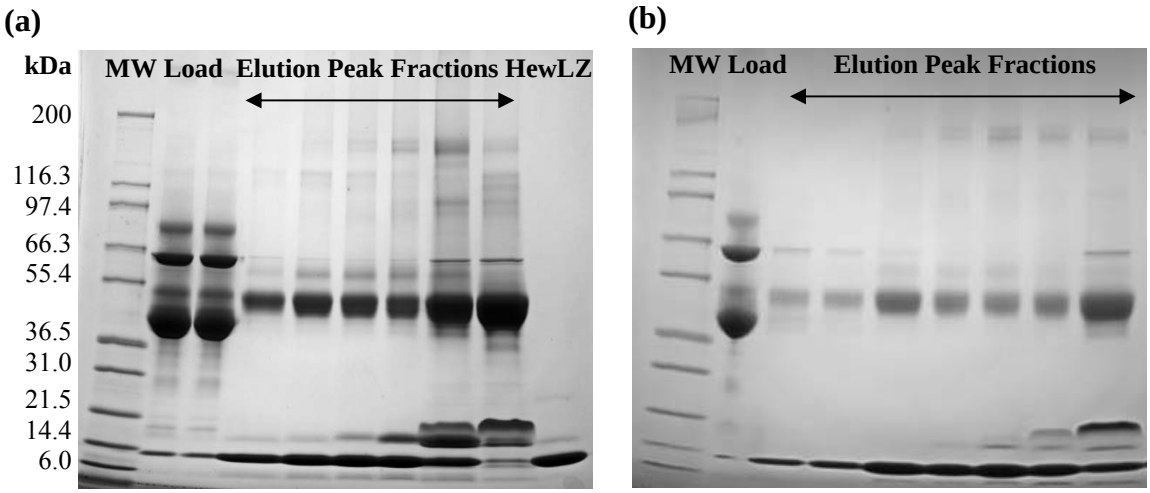


Figure 3.9. SDS-PAGE of pH 6 adsorption experiments using (a) SP-Sepharose™ FF resin or (b) CM-Sepharose™ FF resin. Molecular weight markers (MW), load (egg-white clarified by precipitation and centrifugation), elution peak fractions, and HewLZ reference standard. Load conductivity was 15 mS for the SP-Sepharose™ experiment and 10 mS for CM-Sepharose™ experiment.

Process Simulation & Economics. The two purification processes, depicted in Figures 3.10 and 3.11, were simulated to determine if the inferior binding properties of human lysozyme and the effect of rice extract on the cation exchange adsorption would translate into higher manufacturing costs of rice-derived HuLZ than HewLZ. The objective of process simulation was to compare the relative processing costs under the best adsorption and purification conditions. For this comparison, only downstream processing related costs were considered. Raw material cost (transgenic rice and egg-

white) were not included because of their inherent variability in pricing and cost drivers external to processing such as co-product value, production location, and labor cost.

The selected processes analyzed were pH 4.5 extraction and purification of HuLZ from rice using SP-Sepharose™ FF and pH 6 purification of HewLZ using CM-Sepharose™ FF. The equipment cost and total capital investment comparison (TCI) for HuLZ and HewLZ purification are given in Table 3.1. The equipment costs were comparable for both processes because of similar volumetric throughputs, which was the result of the HuLZ concentration in pH 4.5 rice extract being similar to that of HewLZ in clarified egg-white. The direct fixed costs (DFC) and TCI were also similar as both are directly proportional to the equipment cost.

Table 3.1. Comparison of capital costs for pH 4.5 HuLZ extraction and purification and pH 6 HewLZ purification. Direct fixed capital (DFC) was calculated using a Lang factor of 3. Values reported in $ per g lysozyme.

	Capital Cost ($/g)	
	HuLZ	**HewLZ**
Equipment Cost	1.4	1.2
Direct Fixed Capital	4.1	3.7
Working Capital	0.12	0.11
Start-up Cost	0.20	0.18
Total Capital Investment	4.4	4.0

Table 3.2. Total manufacturing cost comparison and itemized cost comparison of direct and indirect manufacturing costs for pH 4.5 HuLZ extraction and purification and pH 6 HewLZ purification. Maintenance was assumed to be 3% of DFC for each process.

	Direct Manufacturing Cost ($/g)	
	HuLZ	**HewLZ**
Reagents and Consumables	0.21	0.21
Labor and Operating Supplies	0.99	1.0
Utilities and Waste Disposal	0.10	0.06
Total Direct Manufacturing Cost	1.3	1.3
	Indirect Manufacturing Cost ($/g)	
	HuLZ	**HewLZ**
Maintenance	0.12	0.11
Depreciation	0.40	0.36
Total Indirect Manufacturing Cost	0.52	0.47
Total Manufacturing Cost	**1.8**	**1.8**

The total manufacturing costs reported in Table 3.2 include both the direct and indirect costs. The direct cost includes the cost of reagents and consumables, labor and labor-related operating supplies, utilities, and waste disposal. The indirect cost includes maintenance and depreciation. Maintenance was estimated as 3% of the equipment cost and depreciation was estimated using the straight line method over 10 years (Peters and Timmerhaus, 1991). The analysis showed that the total manufacturing cost incurred by the downstream processing for HuLZ was the same as that of HewLZ ($1.8/g). Therefore, purifying lysozyme from rice rather than egg-white does not appear to be different for the proposed processes.

The HuLZ and HewLZ processes were divided into sections to better understand the process cost drivers. The process diagrams with unit operations included in each section are shown in Figures 3.10 and 3.11. The production cost distribution (Figures 3.12a and 3.12b) indicates that ion-exchange chromatography is the major cost

contributor that accounts for 41% of the total cost for HuLZ and 36% of the total production cost for HewLZ. The solid-liquid separation and diafiltration sections are the next most expensive steps for both processes.

Because the ion-exchange section dominated the production cost, we report the individual cost items that contribute to the total production cost for this section. The itemized cost distribution identified facility and labor costs as the dominant factors (Figure 3.13). The cost of the ion-exchange step could be reduced by using a lower cost resin, increasing the number of cycles the resin can be used, and improved the binding capacity. The binding capacity dictates the size of the chromatography column and the number of columns needed for a given process.

Ultimately, the use of a chromatography step such as the cation exchange in HuLZ purification presents a barrier to further process cost reduction. A bind and elute process such as packed-bed chromatography is volume driven with almost no economy of scale (Gottschalk, 2008). The use protein precipitation combined with ultrafiltration may further reduce manufacturing cost of recombinant human lysozyme from rice but probably at the expense of yield and purity.

Sensitivity analysis by individually varying binding capacity (10-60 mg/mL), purity (75-95%), and rice flour cost ($0.25-4/kg) was performed but the variation in total production cost was minimal compared to effect of the production rate and the economy of scale.

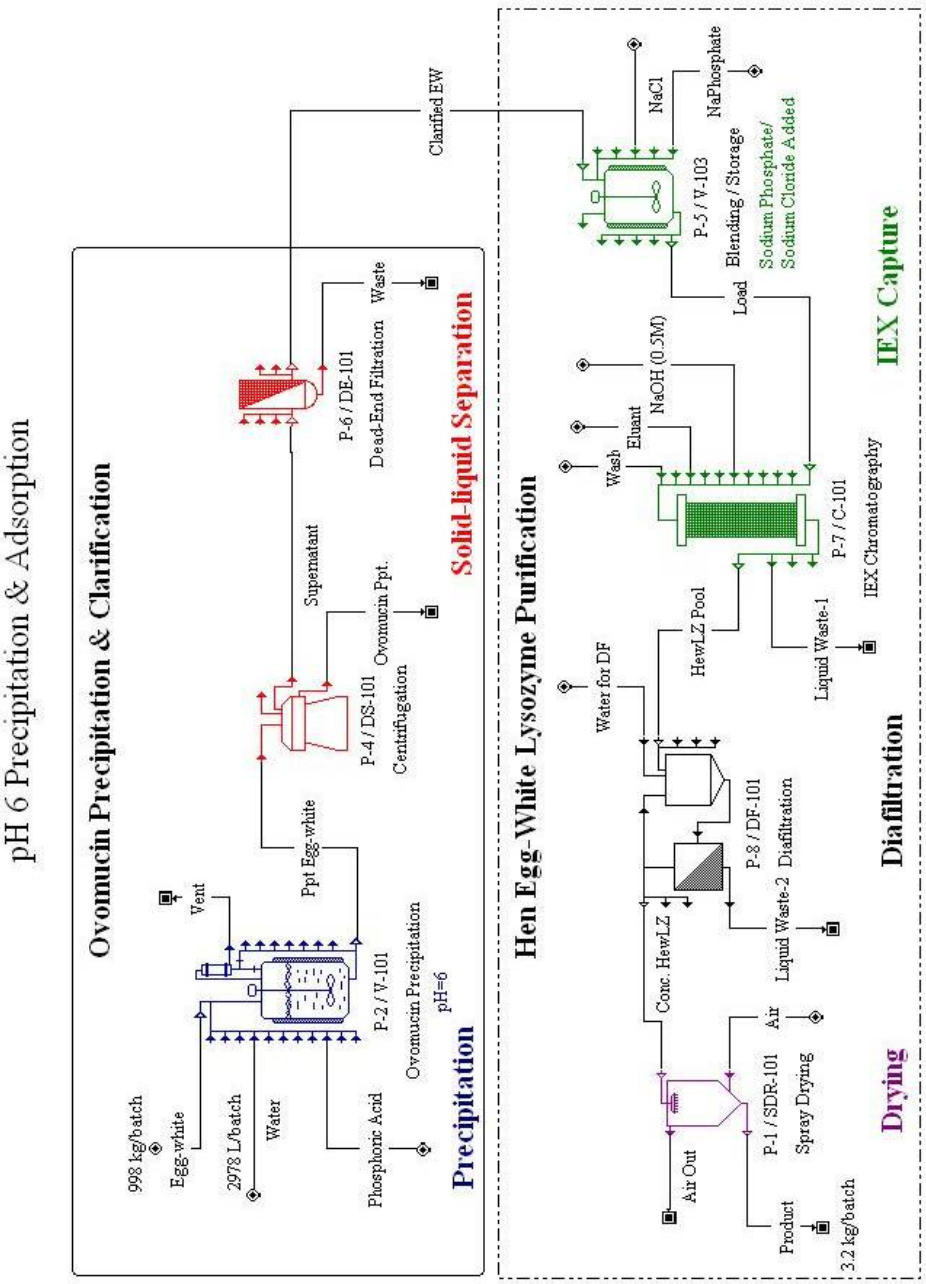


Figure 3.10. Process flow sheet showing the simulated process for purification of hen egg-white lysozyme from egg-white. Unit operations are colored coded to distinguish between sections.

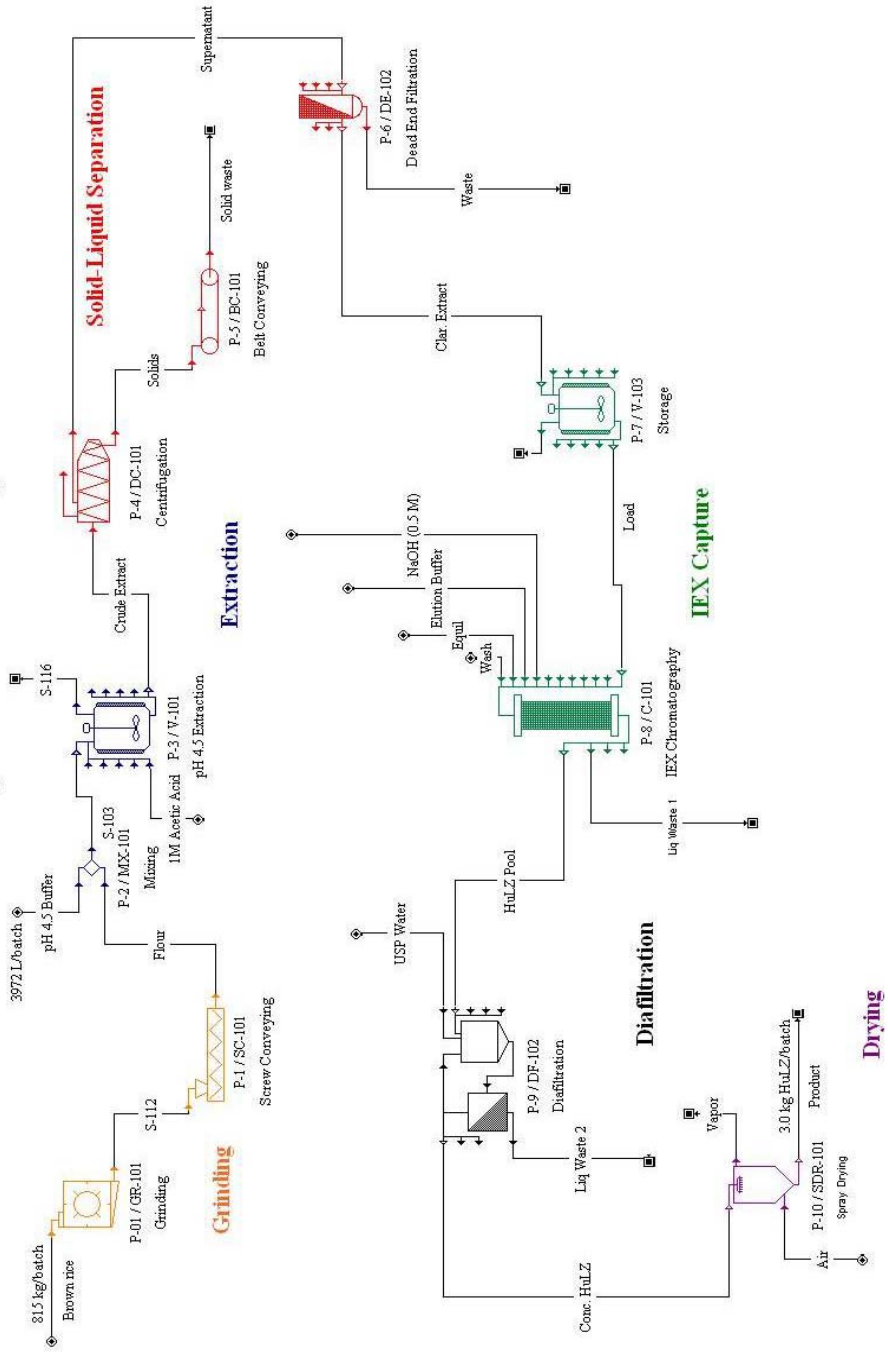


Figure 3.11. Process flow sheet showing the simulated process for purification of human lysozyme from rice extract. Unit operations are colored coded to distinguish between sections.

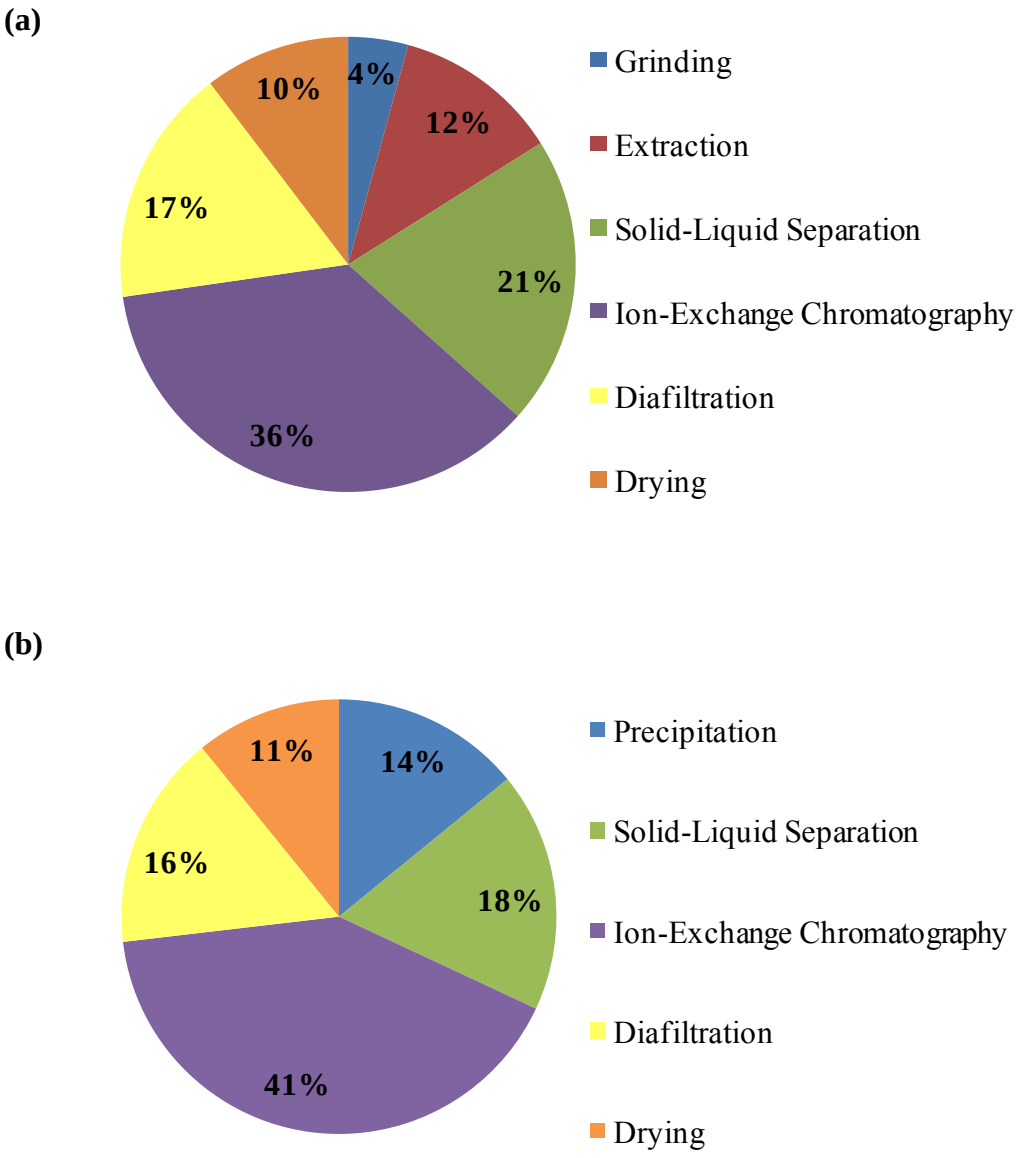


Figure 3.12. Distribution of total production cost by section for (a) human lysozyme extraction and purification at pH 4.5 and (b) hen egg-white lysozyme purification at pH 6. The raw material costs (transgenic rice or egg-white) were excluded from section cost distribution.

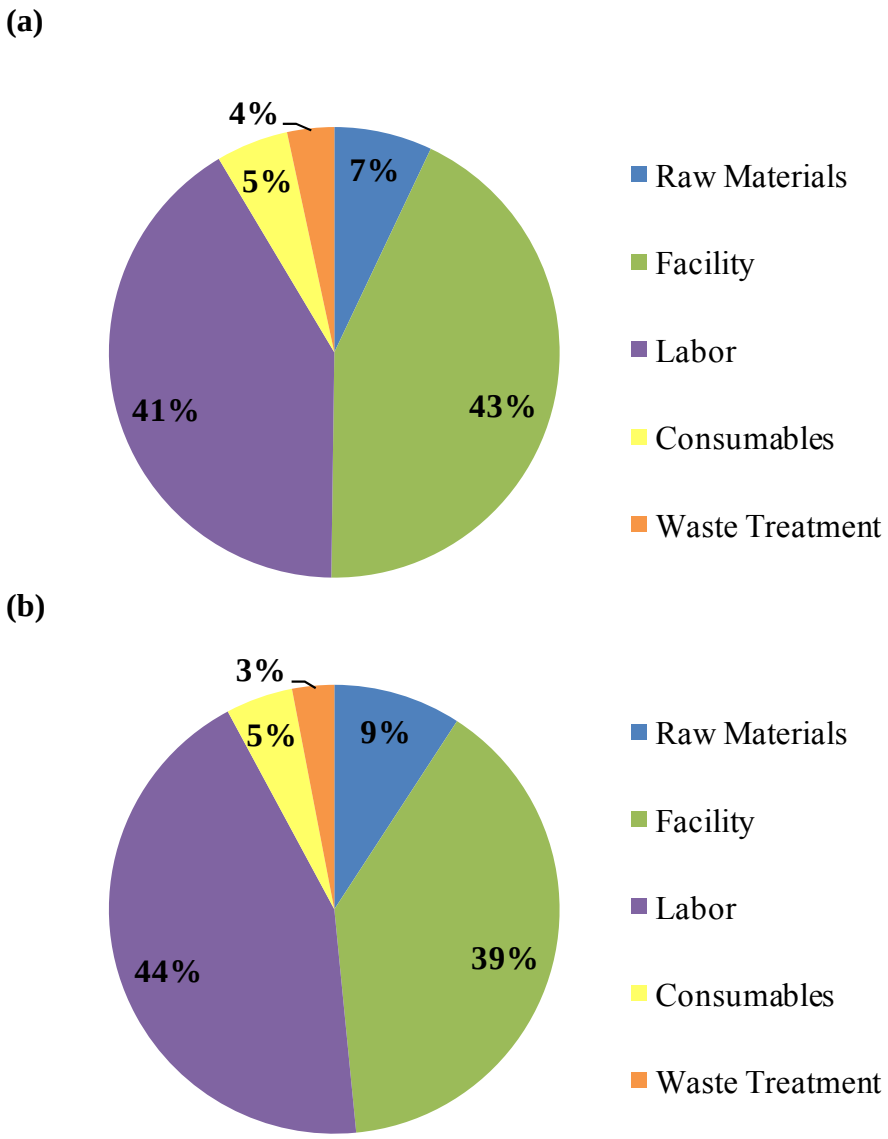


Figure 3.13. Itemized cost distribution of ion-exchange chromatography for (a) human lysozyme extraction and purification at pH 4.5 and (b) hen egg-white lysozyme purification at pH 6.

Transgenic rice will have an additional cost beyond the typical production cost for nontransgenic rice, incurred by identity preservation and containment for regulatory compliance. Estimation for raw material cost as a percent of the total manufacturing cost can range from 5-10% for highly purified pharmaceutical protein to 35-40% for an industrial protein (Nikolov and Hammes, 2002). If the cost of transgenic rice was assumed to be 10 times greater than the production cost of nontransgenic rice, the raw material cost would account for 13% of the total production cost for the simulated process. For the egg-white process, the raw material cost was 6% of the total cost when egg-white was assumed to be $0.33/kg. Human lysozyme would likely be sold on a unit activity base which gives it a significant advantage since it has 3-fold higher N-acetylmuramoyl specific activity compared to HewLZ. The downstream processing costs per 1 x 10^6 units of activity would be $0.039 for HuLZ and $0.013 for HewLZ. Another consideration is that HuLZ from rice can be sold at a premium since it is from a hypoallergenic and animal-free production system.

SUMMARY

This study demonstrated that even though human lysozyme (HuLZ) and hen egg-white lysozyme (HewLZ) have similar physico-chemical properties, purified HuLZ has inferior binding properties to SP-Sepharose^{TM} FF. HuLZ DBCs were lower than hen egg-white lysozyme DBCs at pH 4.5, 6, and 7.5 with ionic strengths from 0 to 100 mM NaCl (5-20 mS), except at pH 4.5 and 5 mS. The DBC of HuLZ was 74% lower than that of HewLZ at pH 7.5, even though the pH was well below the reported HuLZ pI of 10.2. Rice extract components present in pH 4.5 extract reduced the DBC 31% for HuLZ and 21% for HewLZ compared to purified lysozyme in buffer of the same pH and ionic strength. The effect of rice extract on the lysozyme binding capacity was slightly higher at pH 6, with reductions of 39% for HuLZ and 32% for HewLZ. The effect of egg-white proteins on HewLZ adsorption was compared to the effect of clarified rice extract at the same pH and ionic strength. The lysozyme binding capacities were comparable; a capacity of 26 mg/mL for HewLZ was measured from spiked control rice extract compared to 24 mg/mL for HewLZ in egg-white. Thus, purification of lysozyme from rice was similar to that from egg-white using the proposed cation exchange method. Furthermore, process simulation of the best processes for HuLZ purification from rice and HewLZ from egg-white showed that the downstream processing costs were comparable. Therefore, in spite of somewhat weaker interactions with the cation exchange resin and rice extract complexity, the overall process and product potential of HuLZ are promising.

CHAPTER IV

EFFECT OF PHYTIC ACID ON RECOMBINANT HUMAN LYSOZYME PURIFICATION FROM TRANSGENIC RICE

OVERVIEW

Recombinant human lysozyme derived from transgenic rice requires an optimized single chromatography step process to produce an economically viable product for non-pharmaceutical applications such as nutraceuticals or antimicrobial food and processing agents. Developing a process with a single chromatography step requires careful consideration of the compatibility of the extraction conditions with the subsequent purification step and optimization of the processing conditions. Therefore, identifying and understanding critical rice extract components and their interactions are imperative to designing an optimized process. In Chapter II, the processing conditions which gave the highest purity significantly reduced the cation exchange binding capacity. We hypothesize that the rice impurity, phytic acid, was primarily responsible for the unacceptably low binding capacity. In addition, our data suggests that phytic acid may also affect the extraction efficiency of human lysozyme at neutral pH. In this study, we evaluated alternative extraction and purification methods in an attempt to reduce the interference of phytic acid on lysozyme purification. Degrading phytic acid in extracts using phytase enzyme, adding buffer ions that minimize phytic acid interactions with lysozyme, and applying extraction conditions which reduce phytic acid extractability were all effective methods in reducing phytic acid interference. Besides

phytic acid, we also found that phosphate salts and extraction buffer ions can affect human lysozyme purification by cation exchange chromatography.

INTRODUCTION

The use of plant systems to produce pharmaceutical and nutraceutical proteins is novel with the first commercial plant-made pharmaceutical product projected to reach the market in 2009 (Sparrow et al., 2007). Optimization of processing conditions is imperative for purification of protein products for non-pharmaceutical applications and requires careful consideration of the compatibility of the extraction conditions with the subsequent purification step. Human lysozyme from transgenic rice is one example; attractive pricing of human lysozyme would allow it to take a share of the current hen egg-white lysozyme market and generate its own niche market.

Human lysozyme is a 15 kDa, glycosidic enzyme that has numerous potential therapeutic applications (Huang et al., 2006a; Huang et al., 2006b) based on its antibacterial, antiviral, and antiinflammatory properties (Jollès, 1996; Lee-Huang et al., 2005). High expression levels of human lysozyme in transgenic rice have been achieved (Hennegan et al., 2005; Huang et al., 2002a) so additional reduction of manufacturing costs will likely come from improvements in the efficiency of downstream processing (purification).

Aqueous extracts of transgenic rice can contain starch, protein, ash (phosphorus, potassium, magnesium, chlorine, calcium), fiber, and lipid impurities which must be removed during purification (Juliano, 1980). Previous work suggests that besides native proteins, phytic acid, phosphate salts, and extraction buffer may be impediments to

human lysozyme purification by cation exchange chromatography (Menkhaus et al., 2004b; Wilken and Nikolov, 2006a; Wilken and Nikolov, 2006b). In Chapter II, lysozyme extraction and cation exchange adsorption were independently optimized. We were able to obtain high purity human lysozyme with a single chromatography step by combining extraction at pH 4.5 with extract loading at pH 6 so that the slightly acidic and neutral proteins (pI 6-7) extracted at pH 4.5 would not bind to the cation exchange resin, SP-Sepharose^{TM} FF. Although lysozyme purity was as high as 95%, the binding capacity was reduced by 80% compared to pure lysozyme. In other cases (pH 4.5 extraction and adsorption or pH 6 extraction and pH 6 adsorption), rice extract impurities only reduced binding capacity by 30% but additional purification steps would be required to achieve lysozyme purities greater than 95%.

On the basis of these data, we hypothesize that phytic acid was primarily responsible for the unacceptably low capacity when pH 4.5 extract was adjusted to pH 6 for adsorption. Phytic acid (myo-inositol hexakisphosphate) is the primary storage form of phosphate that accounts for at least 80% of the total phosphorus content of brown rice (Juliano, 1980). Brown rice typically contains 0.84-0.99% of phytic acid which exists primarily as potassium, magnesium, and calcium phytate salts that are concentrated in the germ and aluerone layer (Reddy, 2002). Structurally, phytic acid is the phosphoric ester of inositol (hexahydroxycyclohexane) with 12 ionizable protons with pKa values from 1 to 12 (Turner et al., 2002). Therefore, negatively charged phytic acid could electrostatically bind positively charged cations, amino acids, and proteins (Weaver and Kannan, 2002) or indirectly associate with negatively charged proteins, minerals, and

starch through polyvalent cations (Dendougui and Schwedt, 2004). Phytic acid-lysozyme complexes are known to form between pH 6 and pH 9.5. Therefore, phytic acid has been used as a buffer additive to reduce electrostatic interactions of lysozyme with negatively charged silanol groups of the column wall in capillary electrophoresis (Okafo et al., 1994; Okafo et al., 1995; Rodriguez and Li, 1999). In pH 4.5 transgenic rice extracts, human lysozyme is the major protein present (>50% of total soluble protein) and its highly basic nature makes it particularly susceptible to complex formation with phytic acid. By binding phytic acid, the overall surface charge of lysozyme would be reduced affecting the extent of lysozyme interaction with the negatively charged cation exchange resin.

We also observed a similar interference with nontransgenic (control) rice flour extract which suggested that the reduction of capacity and differential protein binding (SDS-PAGE data) was not solely due to lysozyme interactions with phytic acid. Because only low molecular weight (6-14 kDa) rice proteins were adsorbed from pH 4.5 control flour extract adjusted to pH 6, a possible blockage of the resin pores by other non-protein phytate complexes seemed plausible. Siener et al. (2001) observed the formation of insoluble calcium phytate by buffering to pH 6 and a drastic decrease of phytic acid solubility in rice at pH 6 compared to pH 4.5. Formation of insoluble phytate salts may explain the reduced adsorption of higher molecular weight proteins by the cation exchange column. The adsorption behavior could also be manipulated with the addition of various buffers suggesting other ionic interactions. For example, adjusting pH 4.5 lysozyme rice flour extract to pH 6 with 1 M NaOH led to the

immediate precipitation of an extract compound in the chromatography column. The addition of sodium phosphate buffer reduced precipitation and slightly improved the lysozyme binding capacity. We hypothesized that phytate salts and/or phytic acid/lysozyme complexes formed during pH adjustment precipitated on top of the resin during loading which resulted in column plugging. A sample of the precipitate removed from the column was analyzed and confirmed the presence of phytic acid and a protein band of the same molecular weight as lysozyme was detected by SDS-PAGE (unpublished data).

In protein purification, the composition and concentration of buffer ions should also be taken into consideration (Newcombe et al., 2008). The isoelectric point (pI), calculated by the amino acid sequence, is often relied upon to select chromatography conditions (resin charge, buffer type, pH). However, the actual protein charge depends on pH, ionic strength, and buffer type (Winzor, 2004) and specifically adsorbed buffer ions (by electrostatic and non-electrostatic interactions) could lead to a reduction of the net protein charge or even a protein charge reversal.

From the evidence presented above, we believe that a further investigation of the effect of the rice impurity, phytic acid, and buffer ions on the purification of human lysozyme by cation exchange adsorption is warranted. In this study, we evaluate alternative extraction and purification methods to determine if phytic acid and/or buffer ions contribute to the reduction of lysozyme binding capacity and to identify the conditions that minimize the interference of rice extract impurities and/or buffer ions on lysozyme extraction and purification.

MATERIALS & METHODS

Materials. Transgenic rice flour, control rice flour, and purified recombinant human lysozyme were provided by Ventria Biosciences (Sacramento, CA). The control flour was of the same genetic background as the transgenic flour (cultivar Taipei 309). Crude phytase from wheat, phytic acid dodecasodium hydrate from rice, 2,2`-bipyridal, ammonium iron sulfate dodecahydrate, and thioglycolic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Analytical Methods. ***Total Protein and Lysozyme Quantification.*** Total soluble protein (TSP) was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Lysozyme concentrations were determined by enzyme activity assay (for extracts) and UV absorbance (for highly pure lysozyme solutions). The enzymatic assay measures the decrease in turbidity of a *M. luteus* cell suspension (Shugar, 1952). To measure lysozyme content, a modified microtiter plate method was used with a 0.03% (w/v) cell suspension and 0-15 μg/mL human lysozyme standards. Control flour extract was used in the preparation of standards to account for the interference of extract components. Cell suspension (250 μL) was added to each 50 μL sample and activity was monitored at 450 nm for 4 min with a Versamax plate reader (Molecular Devices). When lysozyme was the only UV absorbing solution component, lysozyme content was determined by absorption at 280 nm (Beckman DU 600) using an extinction coefficient of 2.46 mL/mg-cm.

Protein Analysis by SDS-PAGE. The protein molecular weight profiles of chromatography fractions were evaluated by electrophoresis as described by Laemmli

(1970). Samples were loaded on 8-16% tris-glycine gels under non-reducing conditions with Mark12™ protein standards (Invitrogen).

Phytic Acid Quantification. The phytic acid (myo-inositol hexakisphosphate) content of rice extracts was analyzed using an indirect detection method, which is based on the strong iron chelating ability of phytic acid and measures the amount free iron in solution (Haug and Lantzsch, 1983; Reichwald and Hatzack, 2008). The method was adapted for use in a microplate assay format using sodium phytate as a standard.

Experimental Methods. ***Preparation of Control and Lysozyme Rice Flour Extracts.*** To prepare extracts, 20 g of control or transgenic rice flour was added to 100 mL extraction buffer and mixed continuously for 1 hr. The extraction buffers used were 50 mM sodium acetate (pH 4.5), 50 mM sodium phosphate (pH 6), or 50 mM sodium carbonate (pH 10) with 50 mM NaCl. Extracts were centrifuged at 12,000 x g for 15 min and then filtered through No.5C filter paper (Micro Filtration Systems) followed by a 0.45 μm cellulose acetate filter (Nalgene).

Phytase-treated Extracts. Lysozyme rice flour extract was prepared as described above. After clarification by centrifugation and filtration, 177 mg of phytase in 25 mL of pH 4.5 buffer was added to 250 mL of extract and mixed overnight. The amount of phytase added and treatment time was selected based on phytase kinetics. After mixing, the treated extract was filtered with 0.2 μm surfactant-free cellulose acetate filter (Nalgene), adjusted to pH 6 with 1 N NaOH or 1 M sodium phosphate, and filtered with a 0.2 μm Acrodisk syringe filter (Pall). In some cases, clarified extract was diluted with RO water to reduce conductivity prior to cation exchange adsorption.

TRIS-adjusted Extract. Lysozyme rice flour extract was prepared as described. After clarification by centrifugation and filtration, the extract was adjusted to pH 6 by adding 5 mL of 1 M TRIS for each 95 mL of clarified extract (final TRIS concentration was 50 mM) and 1 M NaOH. The pH adjusted extract was filtered with a 0.2 μm surfactant-free cellulose acetate filter (Nalgene) prior to cation exchange adsorption.

Acidic Precipitation of pH 10 Extract. After extraction at pH 10, clarified extracts were adjusted to pH 4.5 with 5 M acetic acid and mixed slowly for 45 min to precipitate rice protein, centrifuged at 12,000 x g for 15 min at room temperature, and then filtered through No. 5C filter paper (Micro Filtration Systems) followed by a 0.45 μm cellulose acetate filter (Nalgene). The clarified extract was either loaded directly at pH 4.5 or adjusted to pH 6 with 3 N NaOH. For the extract adjusted to pH 6, RO water was added to reduce the conductivity from 16 mS to 12 mS.

Binding and Elution of Transgenic Rice Extracts. The effect of various alternative processing methods on the binding capacity, purity, and yield of lysozyme was evaluated. Each lysozyme rice flour extract was prepared as described previously with 50 mM NaCl in either 50 mM sodium acetate, pH 4.5, or 50 mM sodium carbonate, pH 10. Extracts at pH 4.5 were adjusted to pH 6 using 1 N NaOH or 1 M sodium phosphate, pH 7, before loading onto the ion exchange column. Extracts at pH 10 were adjusted to pH 4.5 or pH 6 (with 1 M NaOH) before loading. Wash and elution buffers were prepared and pH adjusted using the same procedures as the extracts to ensure the same buffer composition and ionic strength. Chromatography experiments were conducted using a Bio-Rad (Hercules, CA) BioLogic LP chromatography system with

LP DataView Software. Extracts were filtered through filter paper (Micro Filtration Systems No. 5C) followed by a 0.45 μm cellulose acetate filter (Nalgene) to produce a clarified extract. Clarified extracts were loaded at a flow rate of 1 mL/min (300 cm/h) onto a 0.5 x 5.5 cm SP-Sepharose™ FF column (Tricorn, GE Healthcare) until lysozyme saturation. The column was washed with 20 column volumes (CV) of the respective binding buffer and the bound protein was eluted by linear gradient from 0.05 M to 1 M NaCl over 40 CV. Elution peak fractions (3 mL) were collected and analyzed for lysozyme and total protein content and by SDS-PAGE. Lysozyme yields were calculated as a percent of total bound lysozyme. Lysozyme purity in elution peak fractions or pooled fractions was calculated by lysozyme activity and total protein.

RESULTS & DISCUSSION

The overall objective for lysozyme purification from transgenic rice was to obtain greater than 95% purity lysozyme using a single chromatography step. Our previous work demonstrated that using pH 4.5 extraction followed by pH 6 adsorption (Table 4.1) was the only way to achieve greater than 90% purity in one step (Wilken and Nikolov, 2006b). Therefore, we focused this study on finding process conditions that could improve lysozyme binding capacity while maintaining purity. The previous data suggested that phytic acid affected the cation exchange binding capacity and final lysozyme purity, so three process alternatives were evaluated to reduce its interference. The approaches investigated were 1) hydrolysis of phytic acid to inositol with phytase, 2) extraction of lysozyme at a pH which reduces phytic acid content, and 3) shielding of phytic acid with TRIS counter ions.

Table 4.1. Effect of extraction and adsorption pH on the binding capacity and purity of lysozyme after cation exchange adsorption. Capacity was determined from the breakthrough curve and given in mg lysozyme/mL SP-Sepharose™ FF resin.

Extraction pH	Adsorption pH	Capacity (mg/mL resin)	Lysozyme Purity (%)
4.5	4.5	43	89
4.5	6	8.6	95
6	6	24	50

Adsorption and Purification from Phytase-treated Extracts. To demonstrate the process benefits of reducing the phytic acid concentration, pH 4.5 extracts were first treated with phytase (an enzyme that cleaves phosphate groups from myo-inositol). The results of phytic acid degradation by phytase in control and lysozyme rice flour extracts are summarized in Figure 4.1. The degradation rate of phytic acid was much greater in the control extract with phytic acid concentration reaching the detection limit after 5.5 h. There were no measurable changes in phytic acid concentration during the first 6.5 h of incubation of lysozyme rice flour extracts. It took an overnight incubation to reduce the phytic acid content in the lysozyme rice flour extract to the same level as the control extract. Because control extract was quickly degraded, we believe that the slower rate of degradation was probably due to phytic acid/lysozyme complexes that hindered enzyme access to phosphate moieties on the inositol ring.

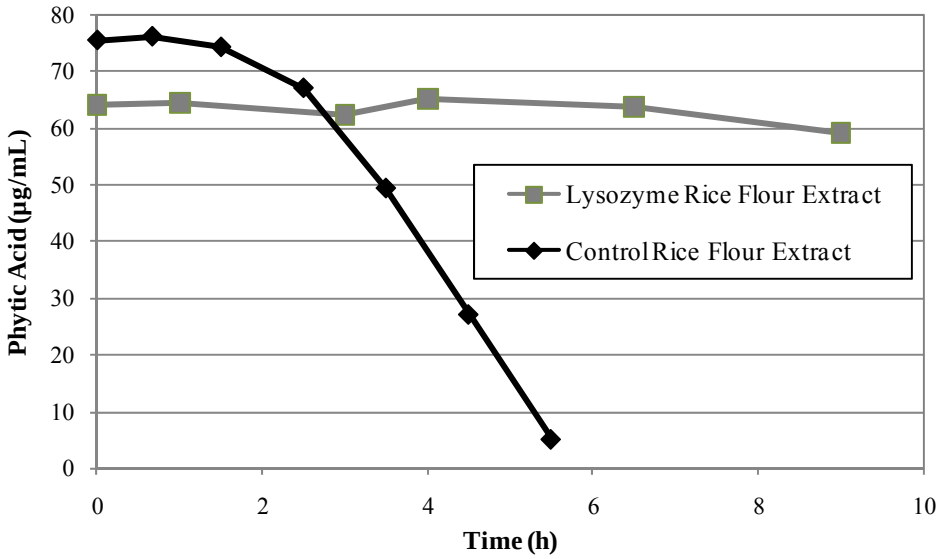


Figure 4.1. Kinetics of phytic acid (µg phytic acid/mL extract) degradation by phytase in pH 4.5 lysozyme rice flour extract and pH 4.5 control rice flour extract.

Phytase-treated extracts were then adjusted to pH 6 with either 1 M sodium hydroxide or 1 M sodium phosphate (Figure 4.2). These two cases were of particular interest because their impact on lysozyme binding was significant and would allow us to quantify process-relevant changes (binding capacity and purity).

Effect of Extract Adjustment with 1 M NaOH. When loading lysozyme rice flour extracts without phytase pretreatment, a white precipitate formed on the top of the cation exchange resin and plugged the column. Overnight treatment of the extract with phytase, prevented column plugging during loading of the extract (adjusted to pH 6 with sodium hydroxide). The mere fact that no precipitate was seen and no column plugging occurred confirmed our suspicion that phytate was the cause of precipitation. Elution peak fractions with the greatest amount of lysozyme were pooled, resulting in a pool

purity of 97% (Table 4.2). A total of 15.2 mg lysozyme (97% of eluted lysozyme) was recovered in the lysozyme pool. The resin adsorption capacity in this case was 77% greater than in the previous best process (pH 4.5 extract adjusted to pH 6 with phosphate for cation exchange adsorption), which resulted in the highest lysozyme purity of 95% (Table 4.2). These results support our hypothesis that phytic acid contributed to the plugging of the chromatography column.

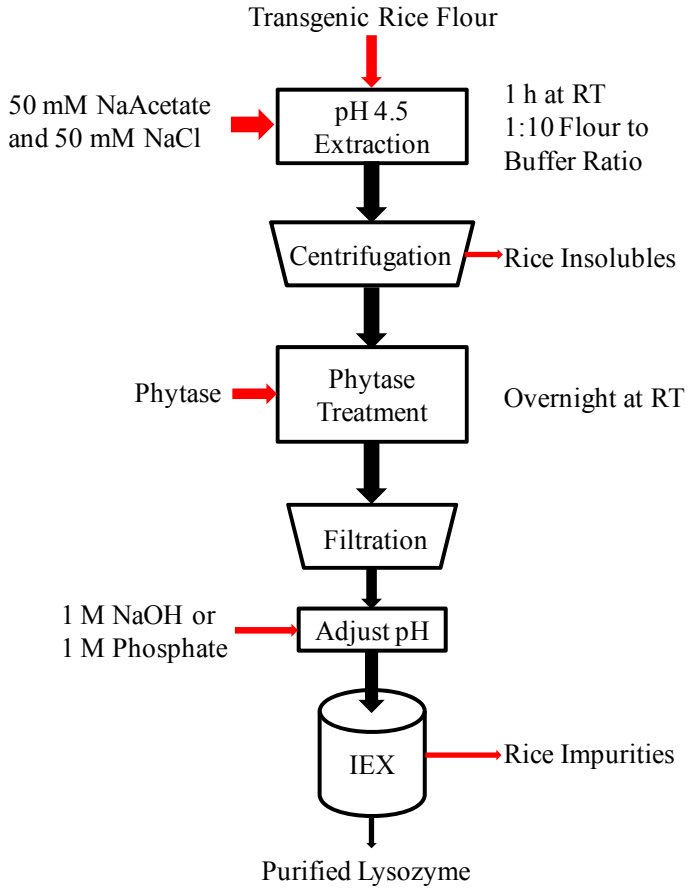


Figure 4.2. Process diagram for extraction, phytase treatment, and purification of lysozyme from the treated extract. After phytase treatment and clarification by centrifugation, the pH of the extract was adjusted to 6 with either sodium hydroxide (NaOH) or 1 M phosphate buffer. RT (room temperature) was 25°C.

Effect of Extract Adjustment with 1M Phosphate. Since phytase treatment improved binding capacity for the NaOH adjusted sample, we wanted to determine whether phytase treatment would also improve the binding capacity for pH 4.5 extract adjusted to pH 6 with phosphate buffer. Phosphate buffer was originally used in the process to prevent precipitation during pH adjustment.

After phytase treatment at pH 4.5, the extract was pH adjusted and loaded directly on the cation exchange column. The amount of eluted lysozyme and lysozyme purity were significantly lower than that of NaOH-adjusted extracts (Table 4.2) and also lower than phosphate-adjusted untreated extract. The lysozyme pool for the phosphate-adjusted extracts experiment contained 4.8 mg of lysozyme with a purity of 85%, lower than the 14.7 mg of lysozyme eluted with 97% purity for NaOH-adjusted extracts and the 8.6 mg with 95% purity eluted for untreated extracts. We believe that inferior binding and lysozyme purity resulted from a combined effect of higher ionic strength and phosphate ion concentration. Therefore, we conclude that 1 M NaOH is the best option for pH adjustment of phytase-treated extracts. The effect of extract conductivity and buffer counterions on lysozyme purification will be discussed later in this chapter.

Table 4.2. Summary of lysozyme purification from phytase-treated extracts. The reported amount of lysozyme eluted was calculated by pooling elution peak fractions collected between 14 and 30 mS. For each experiment, the same elution peak fractions were pooled.

Extraction pH	Adsorption pH	Solution Used to Adjust pH	Conductivity (mS)	Lysozyme Eluted (mg)	Lysozyme Purity (%)
4.5	6	NaOH	13	14.7	97
4.5	6	PO_4	16	4.8	85
4.5 (Not treated)	6	PO_4	16	8.6	95

Effect of pH 10 Extraction on Lysozyme Adsorption and Purification. The treatment of extract with phytase to reduce the phytic acid content clearly improved lysozyme adsorption from NaOH-adjusted extract and lysozyme purity upon elution from the cation exchange column. Therefore, it appeared prudent to test if extraction pH could be used to reduce the amount of extracted phytic acid and also reduce lysozyme/phytic acid interactions. Extraction at pH 10 was selected for two reasons: 1) the amount of phytic acid extracted was lowest at pH 10 compared to pH 2, 4, and 6 with 0.3 mg/g extracted at pH 10 compared to 0.5 mg/g flour at pH 2 and 2) near zero net charge of lysozyme at pH 10 (pI 10.5). The main concern for pH 10 extraction was whether lysozyme could be readily extracted at this pH since protein solubility is typically minimized close to the isoelectric point (pI).

pH 10 Extraction. To assure that pH 10 could lead to a commercially viable lysozyme recovery process, we compared the amount of lysozyme extracted at pH 10 with 50 mM sodium carbonate in the presence of NaCl to previous extraction data at pH 4.5 and 6.5 (Figure 4.3). At pH 10, the amount of extracted lysozyme varied from 2.7 (±0.1) to 3.5 (±0.3) mg/g flour as the NaCl concentration varied from 0 to 300 mM. The pH 10 extracts had less lysozyme than pH 4.5 extracts for all NaCl concentrations. Lysozyme extractability was less dependent on the salt concentration at pH 10 than at pH 6, which was similar to the salt effect observed at pH 4.5.

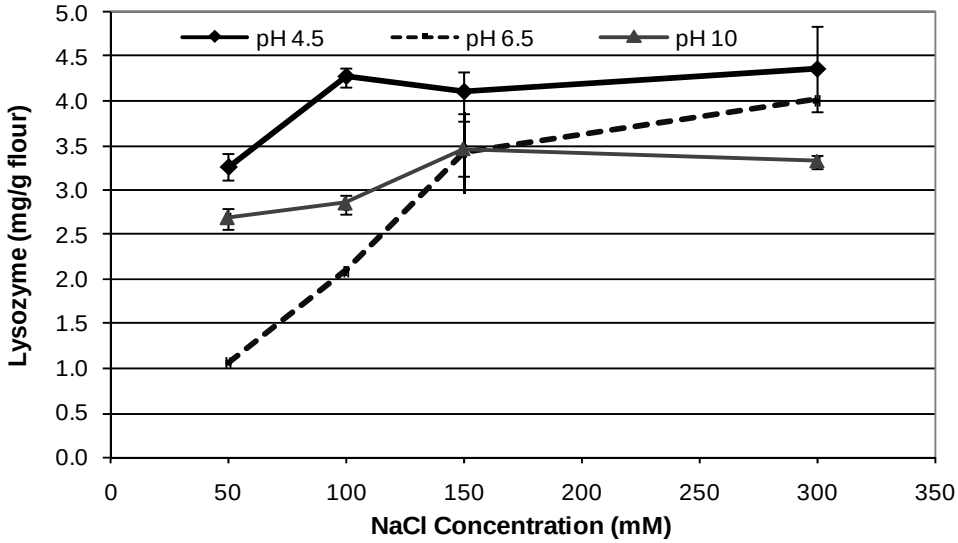


Figure 4.3. Extraction of human lysozyme at pH 10 as a function of salt concentration compared to previous data reported in Chapter II. Error bars indicate standard deviation of triplicate extractions.

Since human lysozyme adsorption to SP-Sepharose^{TM} FF was highly dependent on load conductivity, 50 mM NaCl was typically the maximum salt concentration acceptable for adsorption at pH 6 (Wilken and Nikolov, 2006b). Therefore, the lower salt dependency at pH 10 provides more flexibility in selecting optimal conditions for adsorption (50 mM NaCl) without sacrificing the extraction yield. Interestingly, lysozyme extractability at 50 mM NaCl (10 mS) was higher at pH 10 than at pH 6.5 and pH 7.5 (Figure 4.4). This was unexpected because protein extractability is typically lowest when the pH is close to the pI of a protein. A disadvantage of pH 10 extraction was the drastic increase in total protein extracted compared to the other extraction pHs evaluated (Figure 4.4). For pH 10 and 50 mM NaCl (10 mS), the amount of total soluble protein extracted was 15.4 (±0.6) mg/g flour. Lysozyme only accounted for 17% of total soluble protein, which was comparable to pH 6.5 but significantly lower than pH 4.5

where 60% of the total protein extracted was lysozyme. These extraction results suggest that at neutral pH, electrostatic interactions with plant cell components reduced the extractability of lysozyme. Improved extractability of human lysozyme at pH 10 compared to pH 6.5 indicates that the lower net charge of lysozyme may have reduced interactions with negatively charged plant cell components. Thus, except for the total protein content, extraction at pH 10 could be a reasonable alternative to pH 4.5 extraction.

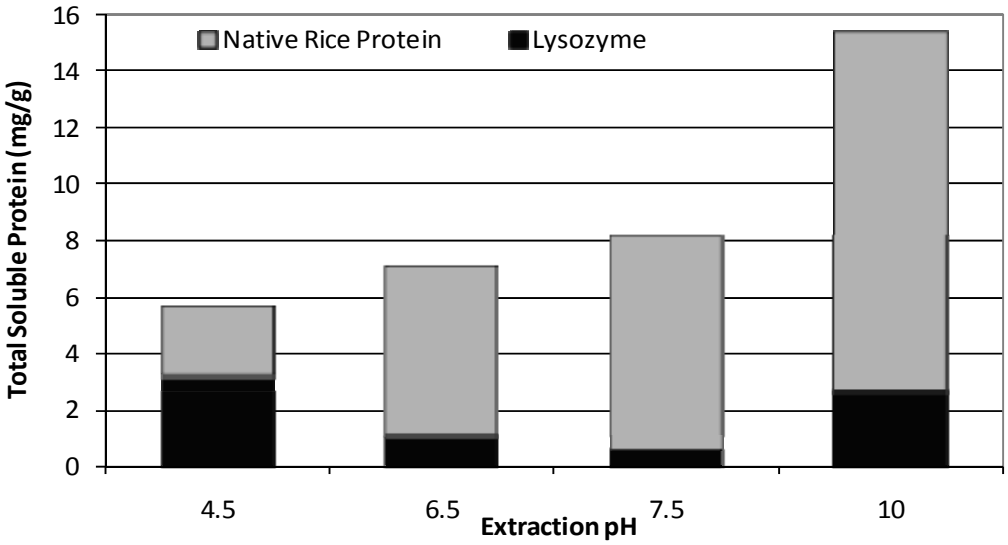


Figure 4.4. Effect of pH on the extraction of human lysozyme and total soluble protein from transgenic rice. Each extraction was performed with 50 mM buffer with 50 mM NaCl and results are reported in mg total soluble protein/g flour.

The addition of a protein precipitation step was considered necessary to reduce native protein content and to compare lysozyme adsorption and purification under conditions similar to phytase-treated extracts. Precipitation of pH 10 extracts reduced

the total soluble protein from 3.8 mg/mL to 1.0 mg/mL for lysozyme rice flour extract and from 1.8 mg/mL to 0.5 mg/mL for control rice extract. Lysozyme was only 1% of the total protein present in the precipitate. Thus, using acidic precipitation was an effective way to eliminate >70% of the total protein from pH 10 extract without losing lysozyme in the precipitate.

Adsorption and Purification from Acidified pH 10 Extracts. Although the overall goal of this paper was to evaluate process alternatives for lysozyme purification at pH 6, the effect of pH 10 extraction and precipitation on lysozyme adsorption was not previously investigated and warranted evaluation at both pH 4.5 and pH 6. We compared the adsorption and purification of lysozyme from clarified pH 4.5 extract and the same extract adjusted to pH 6 (Figure 4.5).

The data for binding capacity and lysozyme purity are given in Table 4.3. A total of 44.9 mg of lysozyme was adsorbed from pH 10 clarified extract that was loaded at pH 4.5 compared to the 26.7 mg of lysozyme from the same extract adjusted to pH 6. The amount of total protein that was bound to the cation exchange column was also greater for pH 4.5 load (81 mg) than the pH 6 load (31 mg). The elution peak profiles shown in Figure 4.6 also indicate that there was a significant reduction in bound protein at pH 6 compared to pH 4.5. For adsorption at pH 4.5, lysozyme purity in the pooled fractions was 84% with a yield of 81%. At pH 6, lysozyme purity was 95% for the pooled fractions with a yield of 95% (Table 4.3). Based on these results, there was better separation between lysozyme and native rice proteins if the extract was adjusted to pH 6 for adsorption than if the extract was loaded onto the cation exchange column at

pH 4.5. The elution profiles for pH 10 control extract and lysozyme rice extract loaded at pH 6 compared in Figure 4.6 indicate that native rice proteins eluted at a higher conductivity than lysozyme which resulted in higher lysozyme purity.

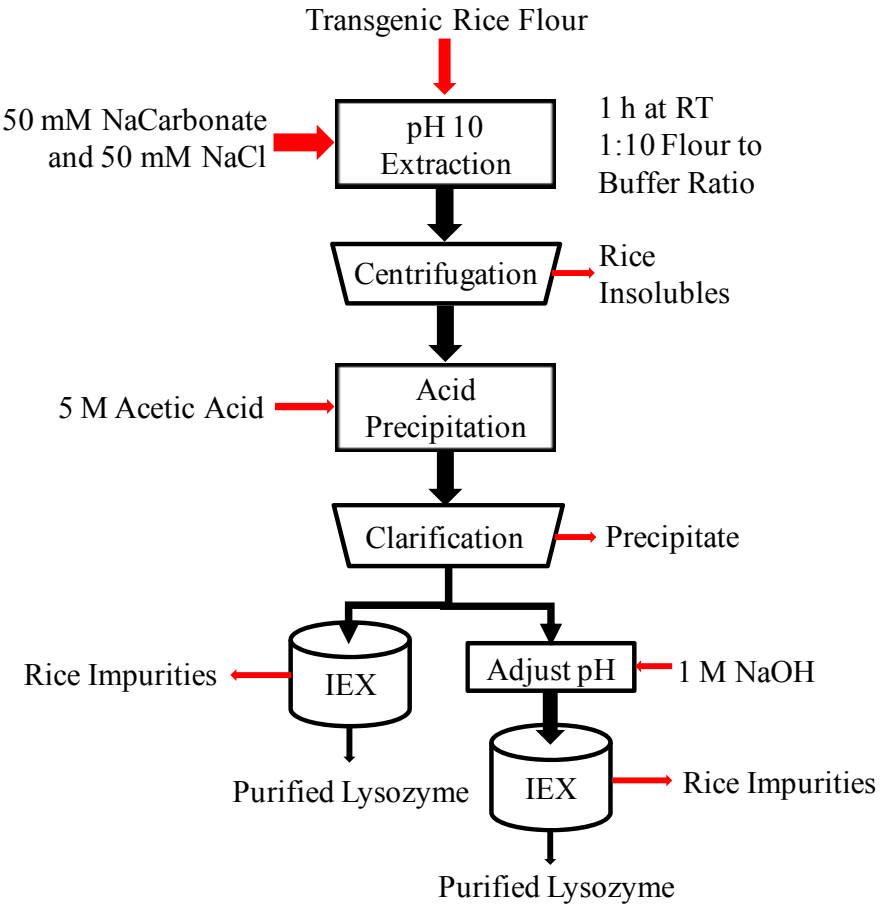


Figure 4.5. Process diagram for pH 10 extraction followed by acid precipitation and cation exchange adsorption. The proposed method was evaluated to try to reduce the effect of phytic acid on lysozyme purification. After precipitation and centrifugation, clarified extract was loaded at pH 4.5 or adjusted to pH 6 before loading onto the cation exchange column.

Table 4.3. Data summary for pH 10 extraction followed by acidic precipitation and cation exchange adsorption at pH 4.5 or pH 6. Extracts were pH adjusted with pH 4.5 and loaded directly or adjusted to pH 6 for adsorption with 1 M NaOH. The amount of lysozyme eluted between 12 and 36 mS (6-18 CV) was compared.

Extraction pH	Adsorption pH	Conductivity (mS)	Lysozyme Eluted (mg)	Lysozyme Purity (%)
10	4.5	11	36.2	84
10	6	12	25.5	95

For pH 10 extract loaded at pH 4.5, the lysozyme binding and purity were similar with the data from pH 4.5 extract loaded at pH 4.5 reported previously (Wilken and Nikolov, 2006b). For applications that require lysozyme of greater than 90%, adsorption at pH 6 would be a better alternative even though the amount of eluted lysozyme was reduced compared to pH 4.5. Most importantly, the acidified and clarified pH 10 extract could be adjusted to pH 6 with sodium hydroxide without the formation of a precipitate or reduction in binding capacity observed previously with pH 4.5 extracts (Wilken and Nikolov, 2006b). This indicates that the non-protein compound, presumably phytic acid, which caused precipitation on the column for pH 4.5 extracts was not extracted at pH 10. The SDS-PAGE protein profiles in Figure 4.7 appear to support this conclusion as eluted fractions from pH 4.5 extract adjusted to pH 6 contained only low molecular weight proteins. The same behavior was not observed for pH 10 extract that was precipitated at pH 4.5 and then adjusted to pH 6, as higher molecular weight proteins were bound and then eluted from the cation exchange column. In other words, in the absence of pore plugging high molecular weight proteins were able to diffuse into the resin pores and interact with the cation exchange ligand.

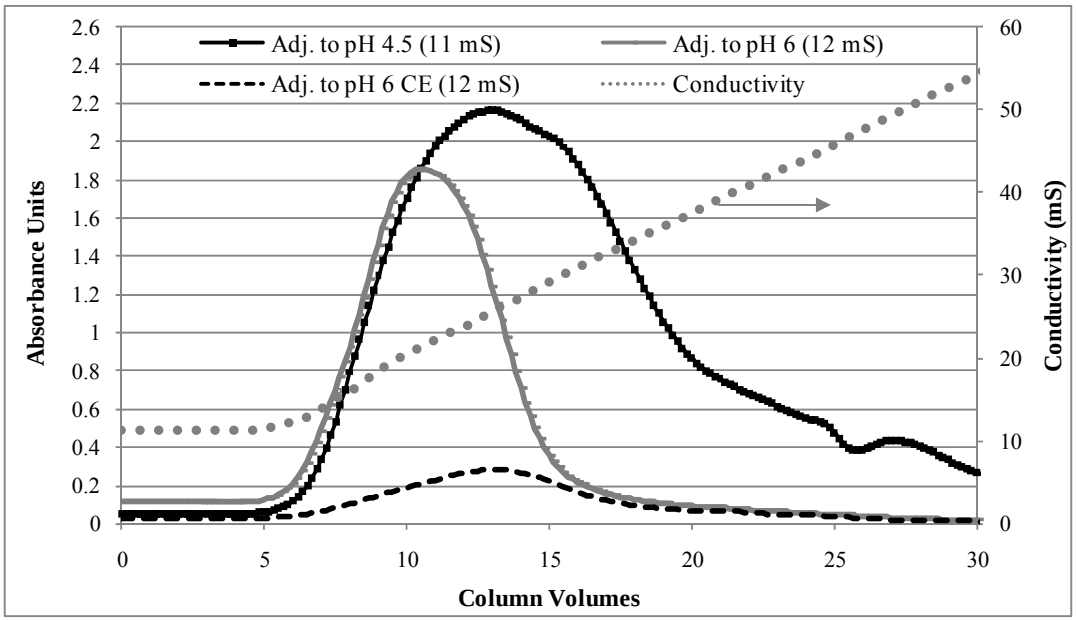


Figure 4.6. Elution profile comparison for pH 10 lysozyme rice flour extract and pH 10 control extract (CE) loaded at pH 4.5 or pH 6. The profiles show the reduction of bound protein for pH 10 extract loaded at pH 6 compared to extract loaded at pH 4.5. The control extract (CE) elution peak indicates that native rice proteins eluted at higher ionic strength than lysozyme.

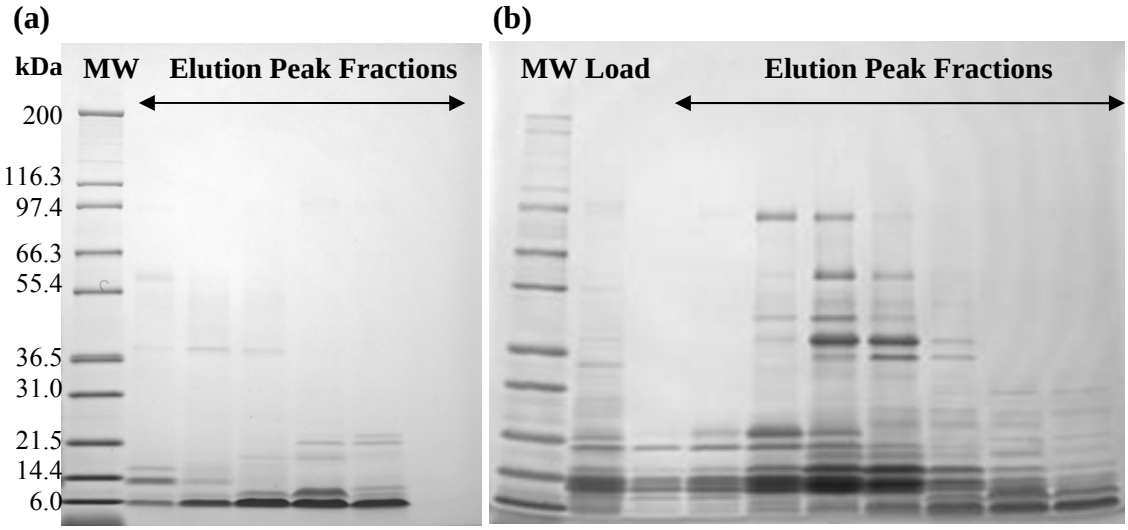


Figure 4.7. Protein profiles for elution peak fractions for (a) pH 4.5 control extract adjusted to pH 6 for cation exchange adsorption with phosphate and (b) pH 10 control extract precipitated at pH 4.5 and adjusted to pH 6 with sodium hydroxide. For (a), only low molecular weight proteins were eluted which indicated pore blockage by a phytate complex which was not seen with pH 10 extract.

Adsorption and Purification from TRIS-adjusted Extracts. TRIS buffer (TRIS(hydroxymethyl)aminomethane), a primary amine with a pKa of 8.1, was evaluated as an alternative to using phosphate buffer for pH adjustment of pH 4.5 extracts or phytase treatment of extract before adsorption. The working hypothesis was that the positively charged TRIS cations would reduce electrostatic interactions between phytic acid and lysozyme and eliminate precipitate formation with pH adjustment. The elution chromatogram and corresponding gel analysis of the TRIS-adjusted extract that was loaded onto the SP-Sepharose™ cation exchange column are presented in Figure 4.8. The pooled peak fractions 1-6 contained 25 mg of lysozyme with 98% purity. Eluted fractions from pH 4.5 extract adjusted to pH 6 with TRIS also had similar protein molecular weight profiles as those from pH 10 extract loaded at pH 6 after precipitation at pH 4.5. This seems to indicate that TRIS eliminated the effects of phytic acid which prevented the cation exchange adsorption of higher molecular weight proteins from pH 4.5 extracts adjusted to pH 6 with phosphate.

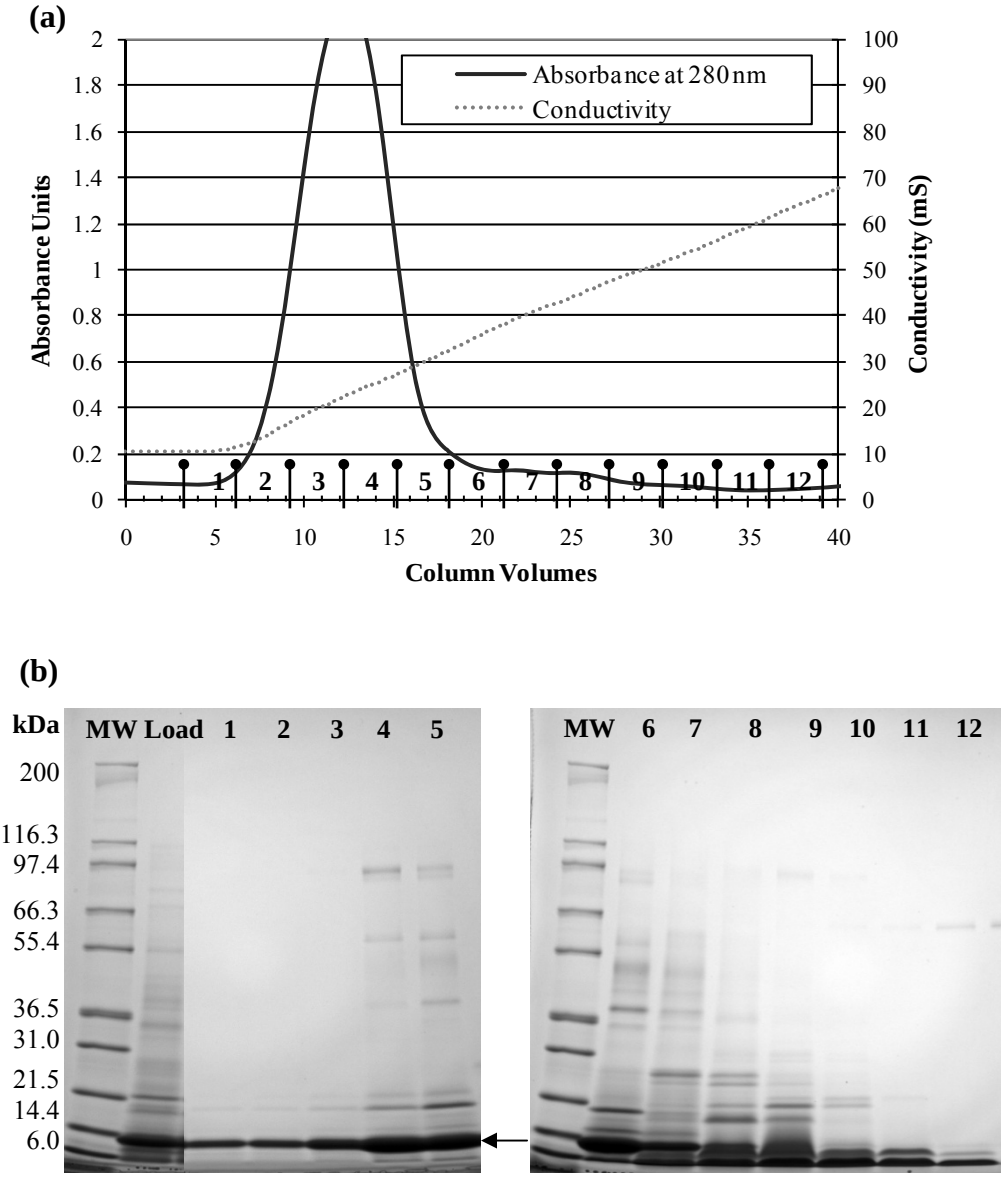


Figure 4.8. Elution profile (a) showing peak fractions analyzed by SDS-PAGE (b). Load solution was clarified pH 4.5 extract adjusted to pH 6 with 1 M TRIS for adsorption. Protein profile for molecular weight markers (MW), load, and elution peak fractions (1-12). Numbered lanes correspond to fraction number in elution peak chromatogram. The arrow shows the position of human lysozyme.

Table 4.4. Summary of lysozyme purification from extracts adjusted from pH 4.5 to pH 6 for cation exchange adsorption on SP-Sepharose™ FF. For pH 10 extract, an acidic precipitation (Ppt) step was used before adsorption.

Extraction pH	Adsorption pH	Solution Used to Adjust pH	Lysozyme Eluted (mg)	Lysozyme Purity (%)
4.5 Phytase-treated	6	NaOH	14.7	97
4.5	6	TRIS	25.0	98
4.5	6	PO_4	8.6	95
10 Acidic Ppt	6	NaOH	25.5	95

Lysozyme adsorption data presented in Table 4.4 compare TRIS-adjusted extract to phytase-treated and acidified pH 10 extracts. TRIS adjustment was clearly advantageous for human lysozyme purification from rice extract, as we measured an almost 3-fold increase in eluted lysozyme from extract adjusted to pH 6 in the presence of 50 mM TRIS instead of 50 mM sodium phosphate. The 3-fold improvement by substituting phosphate buffer with TRIS buffer was somewhat surprising. TRIS cations are not typically used in cation exchange chromatography since the buffer cations can shield the negatively charged resin binding sites (Jacob and Frech, 2007). However, Azzoni et al. (2005) also found that the binding capacity of another basic protein, aprotinin (pI=10.5), on SP-Sepharose™ FF was higher in 20 mM TRIS than in 30 mM sodium phosphate. Thus, TRIS ions at a concentration of 50 mM or less have a beneficial effect on lysozyme adsorption. Apparently, positively charged TRIS ions at pH 6 reduce the effective phosphate charge of phytic acid and, thus, reduce the formation of lysozyme-phytic acid binary complexes.

The amount of eluted lysozyme from TRIS-adjusted extract was greater than that from phytase-treated extract (sodium hydroxide adjusted) but both processes gave similar final lysozyme purities (Table 4.4). TRIS-adjusted extracts had similar capacity and purity to acidified pH 10 extract. In summary, the three approaches we evaluated could be used to produce lysozyme with higher than 95% purity with a single chromatography step. These results further indicate that electrostatic interactions between lysozyme, phytic acid, and rice extract impurities affect both the adsorption and purity of lysozyme.

Effect of Phosphate on Lysozyme Adsorption and Purification. After phytase treatment of pH 4.5 extract, the extract that was adjusted to pH 6 with 1 M phosphate showed inferior binding and lysozyme purity than sodium hydroxide adjusted extract. We attributed that difference to the combined effect of higher conductivity (ionic strength) and phosphate ion concentration. The effect of reducing both the phosphate ion concentration and conductivity was determined by diluting phosphate-adjusted extracts from 16 mS to 13 mS or 11 mS prior to cation exchange adsorption. The amount of bound and eluted lysozyme and total protein for extracts loaded at 16, 13, and 11 mS were then compared.

Comparing the elution peaks for each experiment (Figure 4.9) demonstrates the effect of load (pH adjusted extract) conductivity on the protein binding capacity, which is reflected by the peak area. The largest elution peak corresponded to extract loaded at the lowest conductivity (11 mS). The elution peak fractions were split into two pools based on elution conductivity. Results for pooled fractions (eluted between

conductivity of 14 and 30 mS) from the adsorption experiments of phytase-treated extracts are summarized in Table 4.5. When the pH adjusted extract was loaded at 16 mS (without dilution), only 4.8 mg of lysozyme was recovered and the purity was 85%. Diluting the extract from 16 mS to 11 mS increased the amount of adsorbed lysozyme from 4.8 mg to 19.7 mg and increased the purity of recovered lysozyme from 85% to 98%. The lower lysozyme purity (85%) at the highest conductivity (highest phosphate concentration) suggests that the negatively charged phosphate ions are shielding positively charged moieties on the surface of lysozyme, reducing cation exchange binding sites. This is further supported by comparing the amount of lysozyme bound from extracts loaded at the same conductivity (13 mS) in the presence and absence of phosphate buffer ions (Table 4.5). The amount of lysozyme bound to the cation exchange column from phytase-treated extract adjusted to pH 6 with only sodium hydroxide was greater than the extract adjusted with phosphate. This indicates that phosphate ions in addition to the conductivity affect the binding capacity of lysozyme. For example, Rabiller-Baudry and Chaufer (2001) have estimated that phosphate interactions with hen egg-white lysozyme reduced the effective charge from +5 to +3 when 1-20 mM phosphate buffer is replaced with 50-500 mM phosphate buffer. Therefore, regardless of the solution used to adjust extract pH (NaOH or phosphate), treating extracts with phytase may not be advantageous because release of additional phosphate ions could also reduce the binding capacity of lysozyme.

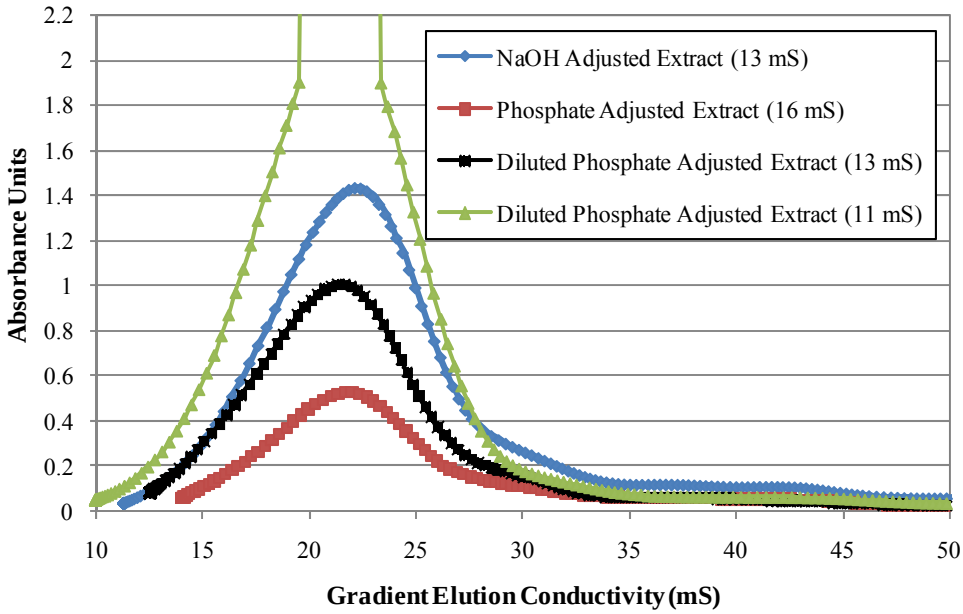


Figure 4.9. Elution peak comparison for phytase-treated pH 4.5 extracts adjusted to pH 6 with NaOH or phosphate which shows the effect of load conductivity on protein binding capacity. Conductivity given in parenthesis represents the extract conductivity after pH adjustment.

Table 4.5. Summary of lysozyme purification from phytase-treated extracts (NaOH or phosphate adjusted) as a function of load conductivity. The reported amounts of lysozyme were calculated by pooling elution peak fractions collected between 14 and 30 mS. For each experiment, the same elution peak fractions were pooled.

Extraction pH	Adsorption pH	Solution Used to Adjust pH	Conductivity (mS)	Lysozyme Eluted (mg)
4.5	6	NaOH	13	14.7
4.5	6	PO_4	16	4.8
4.5	6	PO_4	13 (diluted)	11.8
4.5	6	PO_4	11 (diluted)	19.7

SUMMARY

This work demonstrates that careful consideration of the compatibility of extraction and chromatography conditions such as pH, conductivity (ionic strength), and the type and concentration of buffer ions can lead to a more efficient purification process by improving binding capacity and product purity. Phytase treatment, elimination or reduction of phosphate ion concentration, and pH 10 extraction were effective methods for improving lysozyme adsorption from transgenic rice extract to SP-Sepharose™ FF. Phytase treatment of pH 4.5 extract prior to adjusting the pH to 6 with NaOH eliminated the formation of a precipitate and column fouling. However, phytase treatment releases phosphate ions which were found to reduce the binding capacity of human lysozyme. The adjustment of pH with phosphate further augmented the phosphate counter-ion effect and required extract dilution prior to cation exchange adsorption. Diluting the phytase-treated extract from 16 mS to 11 mS resulted in a 4-fold increase in binding capacity to 20 mg/mL and highly pure lysozyme (98%). The use of TRIS buffer to adjust pH 4.5 extract to pH 6 eliminated the need for phytase treatment and resulted in a further increase of binding capacity to 25 mg/mL and highly pure lysozyme (98%). A similar binding capacity (26 mg/mL) and lysozyme purity (95%) was achieved by extraction at pH 10 followed by acidic precipitation and cation exchange adsorption at pH 6. The results provided further evidence that phytic acid and phytate salts in rice were responsible for the drastically reduced binding capacity reported in Chapter II. By reducing the effects of phytic acid/phytate using phytase treatment, TRIS ions, or pH 10

extraction, lysozyme can be purified in a single chromatography step with an improved binding capacity.

Based on data presented in this chapter, TRIS would be the best and most efficient process because it does not require additional unit operations and would be easier to execute. The process which uses pH 10 extraction would require an acidic precipitation step which would increase equipment cost, labor cost, and total processing time. Extraction at pH 4.5 is also more efficient than at pH 10. The amount of lysozyme extracted at pH 10 with 50 mM NaCl was 35% lower than that at pH 4.5 with 50 mM NaCl, which is used for the TRIS process.

CHAPTER V

PROCESS DEVELOPMENT OF HIGH PROTEIN PRODUCTS FROM DRY-MILLED CORN GERM

OVERVIEW

To improve economics, dry-grind corn ethanol plants are transitioning to fractionation processes to produce higher value co-products from non-fermentable fractions. Dry-milled corn germ is one non-fermentable fraction that is a potential source of higher value food protein. However, low oil and protein purity currently restrict the use and value of dry-milled germ. Germ wet milling is a novel method developed to improve germ purity (protein, oil) by water soaking followed by wet processing of dry-milled germ. In this study, we determined the effect of soaking conditions (pH, temperature, time) on germ composition to identify the best conditions for producing germ (HPHPG) with high protein and high protein dispersibility index (PDI). The soaking and subsequent processing of dry-milled germ increased protein content from 15 to 21%, reduced starch from 33 to 9%, and increased oil content from 16 to 39%. HPHPG was used as a starting material for developing corn protein concentrate, a high protein content product. The extraction studies indicated that protein recovery was directly related to the PDI value of defatted germ flour. Controlling the field conditions, the germ defatting process, and protein extraction were important for maintaining the PDI throughout processing. Corn protein concentrate (CPC) with >80% protein purity was achieved using isoelectric precipitation or membrane filtration.

INTRODUCTION

Corn is the dominate feedstock for fuel ethanol production in the United States, with corn accounting for 97% of ethanol production, utilizing 20% of the U.S. corn crop (Nichols and Bothast, 2008) . The addition of newly constructed ethanol plants is expected to expand the U.S. production capacity from 7.8 billion gallons to 13.4 billion gallons after 2008 (Nichols and Bothast, 2008). Nearly 80% of the corn ethanol plants in operation use a dry-grind process (Erickson and Carr, 2009). In the dry-grind process, the entire kernel is ground into coarse flour prior to saccharification and fermentation. During fermentation, the starch and sugar components are converted to ethanol and the residuals (protein, fiber, oil, ash, minerals) are marketed as distillers dried grain with solubles (DDGS) or wet distillers' grains (Kim et al., 2008). DDGS is used for cattle, poultry, swine, aquaculture, and pet foods (Davis, 2001). However, the use and value of DDGS is limited by the presence of various undesirable components such as fiber, phosphorus, and unsaturated fats. Co-products of the traditional ethanol fermentation from dry grind corn are typically used for lower value applications compared to wet milling co-products (Murthy et al., 2009). As the production of ethanol continues to increase, the value of DDGS is projected to decrease and market saturation is estimated to occur by 2010 for the current applications of DDGS (Wyant, 2006). Therefore, various other options are being explored to improve the long-term outlook and economic stability of corn processing plants.

Since the overall process economics for a corn to ethanol plant depends on composition, quality, and yield of each co-product, various wet and dry milling

(fractionation) alternatives have been proposed to make corn (endosperm starch) a more cost-competitive feedstock for biofuel production. One of the currently explored options is the removal of non-fermentable seed parts (germ and fiber) to increase fermentation efficiency (Ponnampalam et al., 2004; Wahjudi et al., 2000) and the development of higher value co-products from fiber and germ. Fiber oil and gums are potential co-products that would require further attention and development. For example, recent studies suggest fiber oil can be used for neutraceutical applications (Hicks, 1998; Moreau et al., 1996) and/or biofuels (Dien et al., 2005) and fiber gum for food and industrial applications (Singh et al., 1999). Corn oil and germ meal are traditional co-products from wet-milled germ with a market value dictated by the price of corn oil and to a lesser extent, corn germ meal (Johnston et al., 2005). Refined corn oil is typically sold for food applications at $800-1000/ton whereas germ meal (residues after oil extraction containing the germ protein) is sold for animal feed at $90-100/ton. There is an opportunity to further improve corn-to-ethanol economics by producing protein products for food applications instead of using the protein as a component of germ meal for feed.

The objective of this research is to investigate and develop viable process options to produce high protein co-products from corn germ. Based on the protein composition, amino acid distribution, and functional properties, corn germ is a good source of protein for products aimed at food applications. Although the majority of corn protein is contained in the endosperm, the low water solubility of endosperm proteins (zeins) makes them unsuitable for aqueous food applications. In addition, the protein

concentration (g protein/g fraction) in the germ is higher than that in the endosperm (Inglett and Blessin, 1979). The germ represents 11% (dry basis) of the corn kernel (Watson, 1994) and contains most of the water and salt-soluble proteins. Corn germ protein is nutritionally superior to endosperm protein based on the amino acid distribution (Landry and Moureaux, 1980) with a protein efficiency ratio similar to soybeans (Lusas et al., 1989). Although numerous studies on the composition, functionality, and applications of corn germ flours are available, little work has been published on producing protein-rich concentrates from the germ. Furthermore, few studies investigated the effect of processing conditions on the protein quality of corn germ.

Corn germ can be separated from the kernel by wet or dry milling processes. In general, corn wet milling methods provide good fractionation of germ as measured by yield and oil content but usually at the expense of protein quality. In traditional wet milling, entire kernels are soaked at high temperatures (125°F), low pH, for extended periods of time (>20 hours), and in the presence of reducing agents to increase starch and protein separation efficiency. These conditions lead to a high oil content (>48%) but also cause proteolysis and protein aggregation which reduce the levels of water-soluble albumins and salt-soluble globulins (Parris et al., 2006). As a consequence, the protein dispersibility index, an important indicator of food functionality, is reduced. Thus, corn germ prepared by the traditional wet milling process is not an ideal substrate for soluble protein extraction. Modified wet milling processes (enzymatic milling, quick germ, quick germ/quick fiber, HydroMilling) were recently developed to create high oil and

higher protein germ (Johnston et al., 2005; Johnston and Singh, 2001; Johnston and Singh, 2004; Johnston and Singh, 2005; Lohrmann, 2006; Puastian et al., 2007; Singh and Eckhoff, 1996; Wahjudi et al., 2000). Although these processes were able to produce higher protein germ than traditional wet milled germ, the effect of processing conditions on protein isolation/extraction and quality (water solubility/dispersibility) has not been addressed.

Corn dry milling is an alternative germ fractionation method that does not require soaking before degermination and thus, preserves important germ protein properties. Corn is first tempered (moisture adjusted to 20-25%) at room temperature for up to 2 hrs and then degerminated. Dry milling yields relatively low purity germ (≤ 23% oil and 15% protein) with significant residual endosperm starch attached to the germ. The extraction of oil from dry-milled germ by pressing/expelling reaches temperatures above 120°C which affects corn protein properties. Therefore, pressed germ is primarily used for feed with a rather low price of $90-100/ton. Starch and oil loss during processing is an impediment to the development of cost effective protein co-products from dry-milled corn.

The market for dry-milled germ traditionally has been limited to corn grits, meal, and flour for brewing and cereal food (Duensing et al., 2003). The recent transition from dry grinding to dry milling in corn-to-ethanol plants is fueled by public objection to using corn for bioenergy rather than food. The transition is expected to significantly expand the dry-milled germ supply and challenge ethanol producers to increase food protein output while maintaining a competitive processing cost for ethanol. Recent

developments in this area include improved dry fractionation methods to yield high purity germ (Foster, 2008; Giguere, 1993) and wet processing of dry-milled germ (Lohrmann et al., 2008). However, recovery of high quality protein from high purity germ has not been addressed.

There are limited published studies for extraction and purification of germ protein. Conventional methods for extraction of corn protein for food applications include alcohol and/or alkali (pH 8.5-11) extraction followed by protein recovery using protein precipitation by pH adjustment or alcohol containing solutions. Extraction from whole corn or endosperm requires alcohol since these are primarily water insoluble storage proteins, but this is not applicable or desirable for germ protein extraction. Corn protein isolate has been produced directly from dry-milled germ (Nielsen et al., 1973) derived from air-dried corn that was defatted by eight extractions with room temperature hexane. In addition, two extractions were used for protein isolation and dialysis was used to remove ash. The conditions and methods used are difficult to reproduce on a large scale at a cost that would be competitive to soy protein isolate which dominates the food market today.

Membrane filtration has been evaluated as an alternative to protein precipitation primarily for soy protein enrichment (Muralidhara et al., 2003; Thomas et al., 2001) but also for corn protein extracts (Kampen, 1995; Lawhorn, 1986). Membrane filtration is more expensive but may be a good alternative for producing a higher value food protein product. Rao et al. (2002) showed that membrane concentrated protein had a different composition and functionality and retained protein structure better than acid precipitated

protein concentrate. Membrane filtration has not been evaluated as a method to prepare corn protein concentrate or isolate from high protein alkali germ extracts.

The goal of this study was to investigate and develop economically viable processes for producing higher value protein co-products from corn germ. The specific objectives were 1) to determine the effect of processing conditions (pH, temp, time) for producing high protein, high PDI germ (HPHPG) from dry-milled germ and 2) to develop a process to produce a corn protein concentrate from HPHPG.

MATERIALS & METHODS

Materials. Dry-milled corn germ was provided by Quality Technology International, Inc. (Chicago, IL).

Analytical Methods. ***Total Soluble Protein Quantification.*** Total soluble protein (TSP) was determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. Samples and standards were run in duplicates on each microtiter plate.

Protein Analysis by SDS-PAGE. The protein profiles of extracts, precipitate supernatants, washes, and membrane filtration samples were evaluated by electrophoresis as described by Laemmli (1970) . Samples were loaded on 8-16% or 4-20% tris-glycine gels under non-reducing conditions.

Phytic Acid Quantification. Phytic acid content of corn protein concentrates was determined since this compound affects protein solubility and is an antinutritional factor (Maga, 1982). The phytic acid content of concentrates was analyzed using an indirect detection method, which is based on the strong iron chelating ability of phytic

acid and measures the amount free iron in solution (Haug and Lantzsch, 1983; Reichwald and Hatzack, 2008). The method was adapted for use in a microplate assay format using sodium phytate as a standard.

Composition Analysis. Protein by combustion, moisture by forced draft oven, ash, starch, PDI, crude fiber, and amino acid composition was analyzed by Eurofins Scientific, Inc (Des Moines, IA). Crude fat was determined by Dairyland Laboratories, Inc. (Arcadia, WI).

Experimental Methods. ***Lab-scale Studies: Soak Water Analysis.*** The amount of total protein and the protein profiles of soak water samples were determined over 10 h for 50°C soak water and for 24 h for 25°C soak water at pH 4.5 ($4.5 \leq pH \leq 5.0$) and pH 7.5 ($7.0 \leq pH \leq 7.5$). For each experiment, 100 g of dry-milled full-fat corn germ was added to 200 mL of RO water. The soak water pH was maintained throughout the experiment using 1 M HCl or 1 M NaOH. At each time point, a 1 mL soak water sample was taken, centrifuged, and the supernatant was removed for TSP and SDS-PAGE analysis. The volume of soak water remaining at each time point was also measured to calculate the amount of protein lost (g/100g germ).

Lab-scale Studies: Germ Composition Analysis. Lab-scale soaking experiments were conducted at the Bioseparations Lab at Texas A&M University. Dry-milled germ (SunRich Food, Cresco, IA) with 33% starch, 15% protein (PDI of 56%), 16% oil, and 3.8% fiber (% db) was used as the starting material for all experiments. The germ was from corn that was field dried to maintain a high PDI. The effect of three different soak water pHs (pH 3, 6, 9), two temperatures (25°C, 60°C), and four soaking times (0.5, 2, 4,

and 8 hr) on the composition of the soaked germ was determined. Another set of experiments was conducted at pH 6 and 60°C with 0.01% α-amylase to determine if the addition of this enzyme would reduce starch.

For each experiment, 200 g of the dry-milled germ was added to a 1 L Erlenmeyer flask and either 400 mL (25°C soaking) or 425 mL (60°C soaking) of tap water was added to the flask and placed in a water bath. The pH of the soak water was adjusted with either 1 M NaOH or 2 M HCl and once the desired pH was reached, the soak time started. The suspension was mixed periodically by shaking and the pH was maintained at the respective value throughout soaking. After soaking, the wet germ mass and soak water mass were individually measured. The germ was rinsed with tap water and then fresh water was added for grinding (sufficient water to cover the germ in the flask). The germ was mixed with an overhead mixer (Arrow 850, Arrow Engineering Co.) to knock off any loosened starch. After grinding, the germ was removed with a mesh strainer and placed in a starch solution (8 Bé) to separate the clean germ from the low purity germ. The clean germ (floaters) was recovered with a strainer, rinsed with water, and weighed. The clean germ was placed in an aluminum dish and dried at a temperature of 37-43°C to avoid protein denaturation. The mass of the germ was measured throughout drying and once the mass was constant, the germ was removed and stored at 4°C for later analysis. The germ samples were analyzed for protein, PDI, starch, ash, and crude fat.

Pilot-scale Studies: Soak Water Analysis. Pilot plant trials were conducted at the Food Protein Research & Development Center at Texas A&M University (College

Station, TX). Dry-milled germ, prepared by Didion Milling (Cambria, WI), was used as the starting material and had 16.4% protein (58.3% PDI), 26.9% starch, 22.3% oil, and 4.9% crude fiber (% db).

For each pH 4.5 and 25°C soaking experiment, 10 kg of germ was added to 33 L of water (with 0.01% α-amylase) in a stainless steel tank equipped with a pump and circulating loop. The pH was adjusted to 4.5 with 1.5 M phosphoric acid and soak water circulation started immediately. For pH 7.5 and 60°C (50°C ≤ T ≤ 60°C) experiments, the same soaking procedure was used except the germ was added to 39 L of preheated water. The additional 6 L of water was needed at the higher temperature to completely submerge the germ. Soak water samples were taken at 1, 2, 4, and 6 h for total soluble protein, SDS-PAGE, and phytic acid analyses.

Pilot-scale: Germ Composition Analysis. After soaking, the steep water and germ were transferred to a Bauer hammer mill. After grinding, the Baumé level of the steep water was checked and then a hydroclone (Dorr-Oliver) was used to separate the clean germ (low-density) from the high-density fraction (lower oil purity germ and germ fractions). The high-density fraction was passed through the Bauer hammer mill until no additional clean germ was recovered. The clean germ and high-density fraction were rinsed, dried, and weighed. After drying, the clean germ was aspirated to remove fiber and reweighed.

Extraction of Defatted Corn Germ Flour. Extraction and precipitation methods were based on those proposed by Nielsen et al. (1973). Defatted corn germ flour (30 g) was gradually added to 300 mL pre-heated water (T ≤ 50°C) as pH was adjusted to 8.7

with 1 N NaOH. The mixture was blended with a Silverson L4RT high shear mixer at 4500 rpm until the temperature reached 50°C. Once this temperature was reached, the extraction time of 30 min was started. The required temperature and pH were maintained throughout the extraction. The extract was then clarified by centrifugation (Beckman Coulter Allegra 25R) at 10,000 x g and 20°C for 15 min. Exhaustive extraction experiments (3-stage and 5-stage) of defatted corn germ (DCG) flour were performed. In this study, only a single stage extraction was used because the main objective was to determine the effect of multiple processing steps and conditions on protein recovery.

Corn Protein Concentrates by Precipitation. The protein was precipitated from the clarified extract by adjusting the pH to 4.7 with 1.5 M phosphoric acid or 1 M HCl and mixing slowly for 1 hr. The protein precipitated was removed by centrifugation at 10,000 x g and 20°C for 15 min and then washed with 50 mL of water. After 15 min centrifugation (10,000 x g at 20°C), the wash water was decanted, and the precipitate washing procedure was repeated. To resolubilize the precipitate, RO water was added and the pH was adjusted to 7 with 1 M NaOH. The slurry was added to a glass baking dish, placed in a freezer overnight, and freeze dried (Labconco Freezedry 5) for 72 h. The clarified extract, the precipitate supernatant, and the wash supernatants were analyzed for total soluble protein and by SDS-PAGE.

Corn Protein Concentrate by Membrane Filtration. HPHPG (high protein, high PDI germ) was prepared at Iowa State University (Ames, IA) by soaking dry-milled germ (SunRich Food, Cresco, IA) for 30 min at neutral pH and room temperature.

Conditions to prepare HPHPG were selected to maintain PDI during soaking and drying of germ. Full-fat germ flakes were ground and defatted at the Bioseparations Lab (Texas A&M University) using 5, 30 min hexane extractions (1:5 germ flour: hexane) at room temperature. The defatted germ flakes were dried at room temperature. HPLPG (high protein, low PDI germ) was used to prepare corn protein concentrate by membrane filtration. The wet HPHPG was stored for 4 months in the freezer, dried, and then defatted using liquid propane (Ambient Temperature Extraction Partners, Santa Barbara, CA).

Clarified corn germ extracts were prepared as described previously. All extracts were pre-filtered (VWR Grade 410 filter paper) prior to membrane filtration. The extracts were then processed by cross-flow filtration using a Millipore Labscale TFF System with a 0.45µm PVDF, 10 kDa PES, or 30 kDa PES membrane (Millipore Pellicon XL) with a surface area of 50 cm^2. For the 10 kDa membrane experiments, filtration was performed with clarified corn germ extract and also with resolubilized protein precipitate (after isoelectric precipitation of extract at pH 4.7). Constant volume diafiltration was done using 1-2 volumes of RO water followed by UF concentration. Corn protein concentrate was also prepared by direct membrane filtration of clarified extract with a 30 kDa membrane and also by a 2-stage process (membrane filtration followed by isoelectric precipitation of 30 kDa retentate at pH 4.7). After the 1 hr precipitation step, the solution was clarified for 15 min by centrifugation at 10,000 x g and 20°C. The precipitate was washed with 50 mL RO water, centrifuged (15 min at

10,000 x g), resolubilized with 100 mL of RO water (adjusted to pH 7.5 with 3 N NaOH), and freeze dried.

Another option evaluated for preparing CPC involved a 2-stage membrane filtration process that started with a 0.45 μm membrane to remove higher molecular weight impurities from the clarified extract. Permeate from the 0.45 μm filtration was diafiltered and concentrated using a 30 kDa membrane.

Clarified extract, retentates, and permeates were analyzed by Bradford and SDS-PAGE and the conductivity and pHs were monitored throughout filtration. The permeate flux was also measured throughout filtration. The retentates (corn protein concentrates) were freeze dried and sent to Eurofins Scientific Inc. (Des Moines, IA) for analysis.

RESULTS & DISCUSSION

Producing a protein product using germ wet milling consists of soaking dry-milled germ to enhance protein and oil content (% basis) by reducing starch content and recovery of protein from the germ. To produce an economical protein product using germ wet milling requires 1) optimization of germ soaking conditions and 2) development of an efficient protein extraction and concentration recovery method.

Optimization of Germ Soaking. The primary objective of germ soaking is to increase germ purity by releasing or leaching undesirable impurities such as phytic acid, starch, and salts while retaining both the quantity and quality of germ oil and protein. One concern that we initially had was the potential for excessive protein loss during soaking on a pilot-scale since the feasibility of soaking dry-milled germ had not been

previously reported. For this reason, we investigated the effect of soak water temperature, time, and pH on protein content in the soak water. Leaching kinetics were determined to further optimize the soaking conditions, and the molecular weight distribution of leached protein was analyzed to evaluate the potential use of soak water for other applications including as a nitrogen source for ethanol fermentation.

Lab-scale Studies: Soak Water Analysis. The amount of germ protein leached into the soak water at two temperatures (25°C, 50°C) and pHs (4.5 and 7.5) was determined over a 10 h period. The amount of protein leached was the lowest in the pH 4.5 and 25°C soak and highest in the pH 7.5 and 50°C soak water (Figure 5.1). For all soak conditions, the amount of protein leached over the first 2 h increased linearly with time and leveled off by 6 h of soaking. After 6 h, no additional protein was leached for all conditions except for pH 4.5 and 25°C. At pH 4.5 and 25°C (Figure 5.2a), the soak water contained primarily low molecular weight proteins (6-14 kDa). Some higher molecular weight proteins can be seen in the 24 h sample, but they only represent a small fraction of the total protein present in the soak water. Increasing the soak temperature to 50°C increased the amount of higher molecular weight proteins and total protein lost in the soak water (Figure 5.2b). Based on Bradford assay, leached protein was 65% higher at 50°C than 25°C (average difference over 10 h) at pH 4.5. Soaking at pH 7.5 resulted in the loss of proteins with molecular weights from 6 to 60 kDa in the 25°C soak (Figure 5.3a) and 50°C soak (Figure 5.3b). Loss of lower molecular weight proteins into the soak water increased with soak temperature and time. The amount of protein solubilized in the soak water was almost 60% higher at 50°C than at 25°C (average difference over

10 h). The increase of protein leached by soaking at a higher temperature was similar to pH 4.5 results.

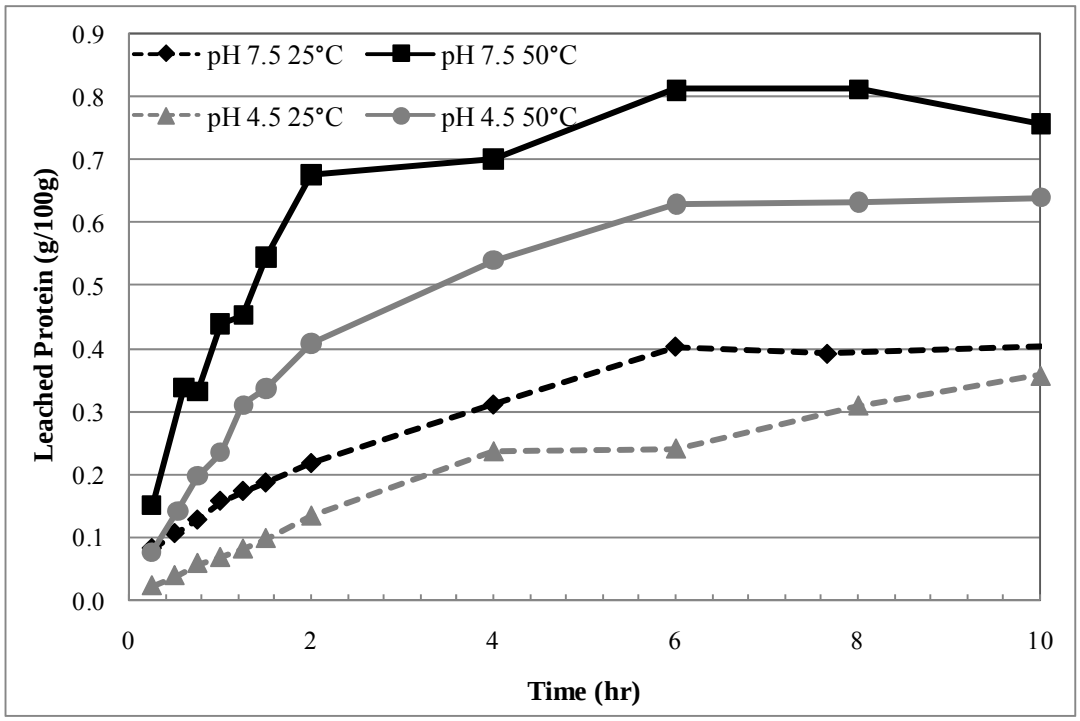


Figure 5.1. Lab-scale soak water analysis: effect of temperature, time, and pH on total protein loss from dry-milled corn germ. Leached protein reported as g total soluble protein (TSP) lost in soak water per 100 g of dry-milled germ.

The phytic acid content in the soak water was also determined because the presence of this anti-nutritional compound in a germ product would be undesirable. Leaching of phytic acid at pH 4.5 after 1 hr soak was 4.3 mg phytic acid/g germ for both 25°C and 50°C, which represents a removal of 15% of total phytic acid present in dry-milled corn germ (O'Dell et al., 1972). For pH 7.5 soaking, the amount of phytic acid extracted from germ was 20% higher at 50°C (4.3 mg/g) than at 25°C (3.3 mg/g). At pH

7.5, a higher temperature was required to extract the same amount of phytic acid compared to pH 4.5 soaking.

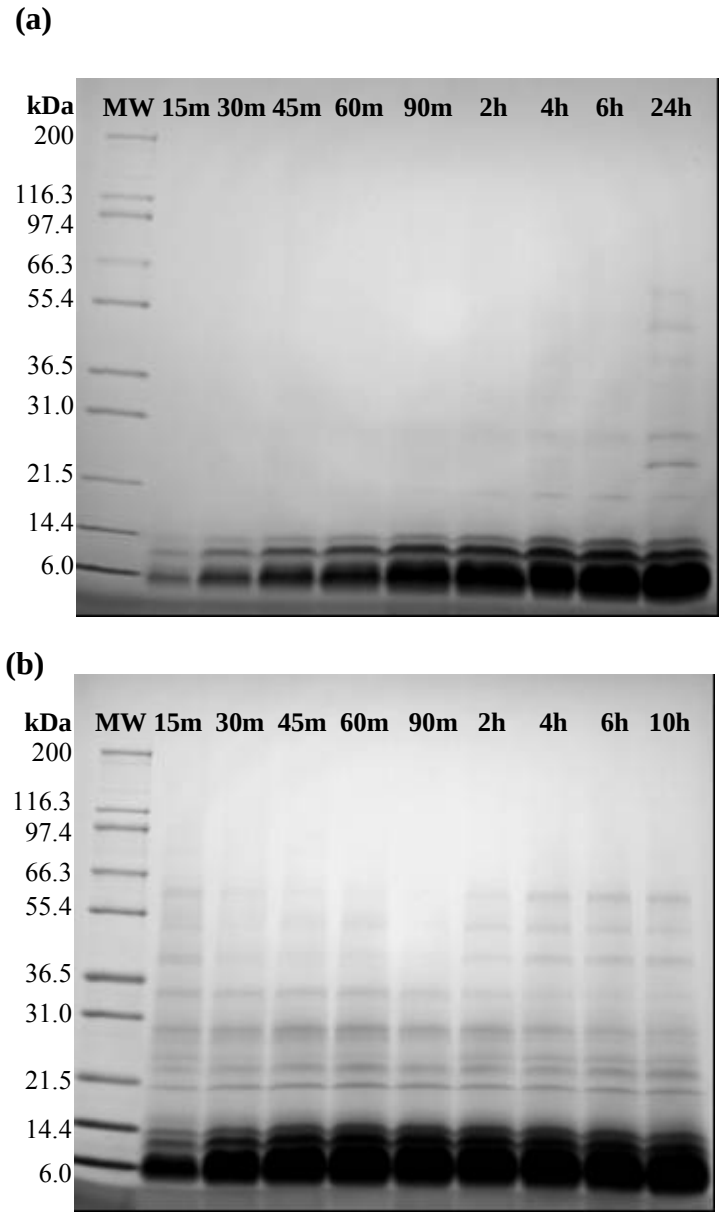


Figure 5.2 Non-reduced SDS-PAGE images showing protein profiles of selected (a) pH 4.5 and 25°C soak water samples from 15 minutes (15 m) to 24 h and (b) pH 4.5 and 50°C samples from 15 m to 10 h. Samples were loaded based on protein content (12.5 µg/well). For extraction kinetics, refer to Figure 5.1.

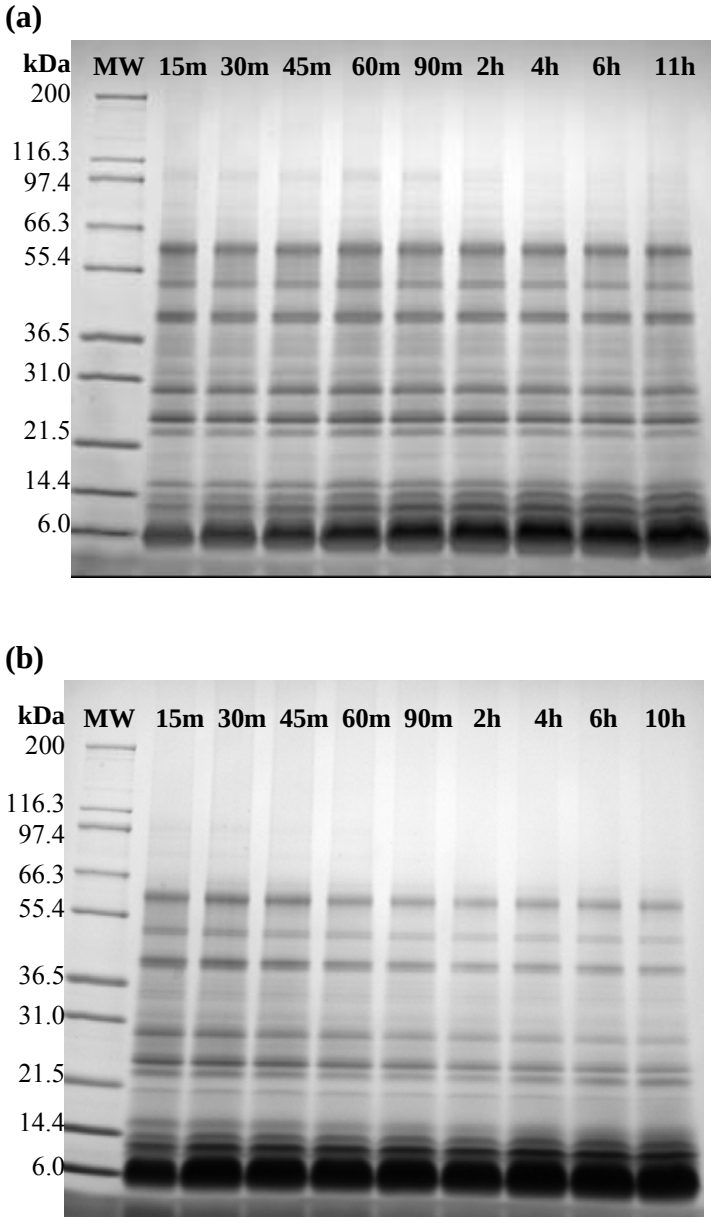


Figure 5.3. Non-reduced SDS-PAGE images showing protein profiles of selected pH 7.5 (a) 25°C soak water samples and (b) 50°C soak water samples from 15 minutes (15 m) to 11 h. Samples were loaded based on protein content (12.5 µg/well). The gel shows the distribution of proteins by molecular weight and not the amount extracted over time.

Pilot-scale Studies: Soak Water Analysis. Based on lab-scale studies, two soaking conditions were selected for scale-up to confirm the effect of the soaking conditions on protein and phytic acid leaching into the soak water. Soaking conditions that minimized protein leaching (pH 4.5, 25°C) and maximized protein and phytic acid leaching (pH 7, 60°C) in the lab-scale studies were further evaluated.

Protein leaching kinetics (Figure 5.4) for the pilot-scale studies were compared to lab-scale data which showed similar trends for both conditions. Loss of germ protein (as % of initial germ protein) due to leaching in the soak water ranged from 0.3% at 0.5 hr to 1.3% at 6 hr for pH 4.5 and from 3 to 6% for pH 7 soaking. The percent of germ protein leached at pH 7 and 60°C was 83% higher than that at pH 4.5 and 25°C for the pilot-scale soaking experiments and similar to the 75% difference measured for the lab-scale experiments. The proteins present in the soak water were predominately 6-14 kDa (Figure 5.5). However, higher molecular weight proteins were observed in the pilot-scale pH 4.5 soak water samples (Figure 5.5a) but not in lab-scale samples.

The amount of initial phytic acid content of germ leached ranged from 0.3% (0.1 g/kg germ) after 0.5 hr to 23% (8.3 g/kg) after 6 hr for the pH 4.5 soak and from 17% (6.3 g/kg) to 20% (7.5 g/kg) during the pH 7 soak. The amount of phytic acid leached was higher at pH 7 than pH 4.5 for the shorter soak times (1, 2, and 4 h) but after 6 h, the results were similar for both pHs. For the lab-scale experiments, phytic acid was only measured at a single time point (1 h) and showed the same amount of phytic acid leached for pH 4.5, 25°C and pH 7, 60°C which was different than the pilot-scale results. As determined in the lab-scale experiments, soaking at high temperature enhanced

phytic acid extractability. The ratio of phytic acid to total soluble protein (TSP) in the soak water was also determined for both soak conditions (Figure 5.6). The ratio could be used as an indirect measurement of the effectiveness of the soaking conditions. To obtain the highest germ protein purity, the ratio of phytic acid to total soluble protein in the soak water should be maximized. Figure 5.6 shows that increasing the soaking time increased the phytic acid to total soluble protein ratio for soaking at pH 4.5 but decreased the ratio at pH 7. The highest ratio of leached phytic acid to TSP was 4.2, which was obtained after soaking for 6 hr at pH 4.5 and 25°C. Thus, pH 4.5 appears to be the best choice unless soak time is limited to 1.5 h or less.

The pilot-scale studies indicated that the lab-scale experiments adequately reflected the results expected for larger scale processing.

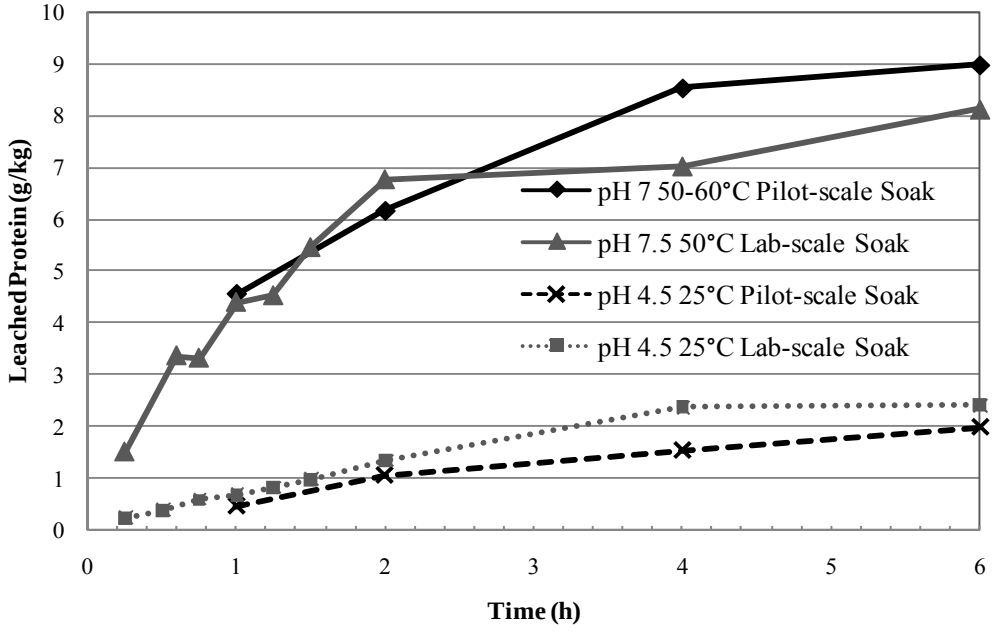


Figure 5.4. Comparison of pilot-scale and lab-scale data for protein leached into soak water throughout the soaking of dry-milled germ. Data reported as g total soluble protein per kg of germ soaked.

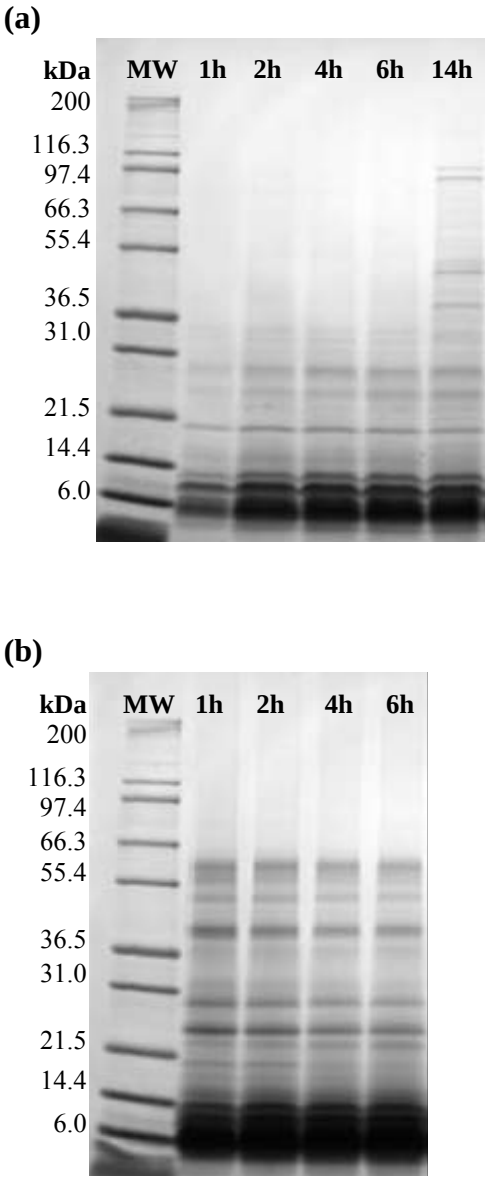


Figure 5.5. Pilot-scale soak water analysis: non-reduced SDS-PAGE image showing protein profiles of selected (a) pH 4.5 and 25 °C soak water samples from 1 h to 14 h and (b) pH 7 and 60°C soak water samples from 1 h to 6 h. Samples were loaded based on equal protein content with (a) 8.5 µg protein and (b) 12.5 µg protein. Compared to the lab-scale experiments, higher molecular weight protein were leached during pH 4.5 soaking for the pilot-scale experiments.

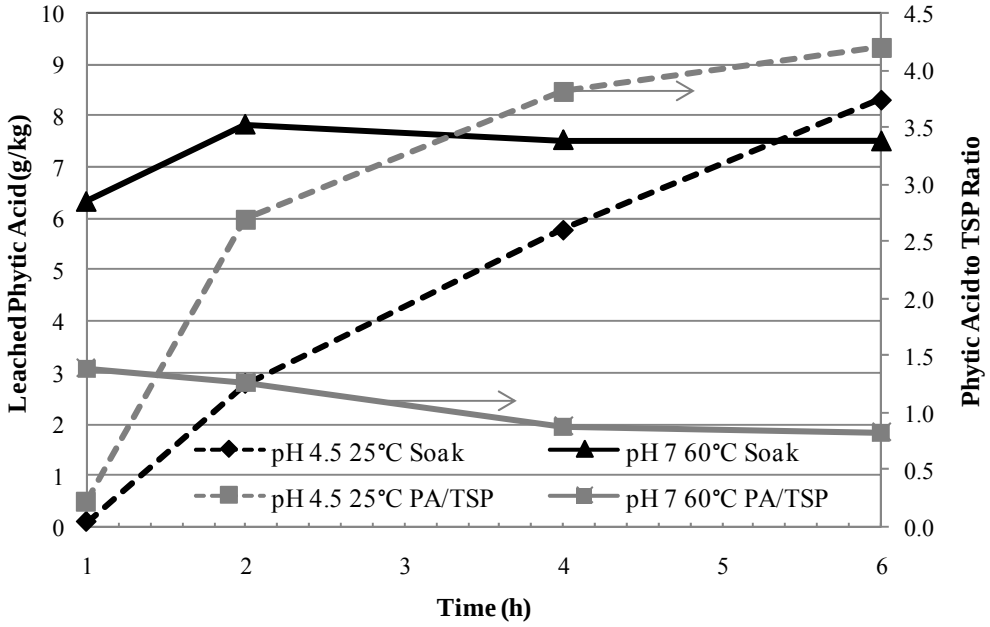


Figure 5.6. Phytic acid and the ratio of phytic to total soluble protein (PA/TSP) leached into soak water as a function of time.

Lab-scale Studies: Germ Composition Analysis. As mentioned before, one of the desirable outcomes of germ soaking is to increase germ purity. The increase of germ purity is usually reflected in increased oil and total protein content. In this set of experiments, a broad range of soaking conditions was evaluated by following the protein, starch, and oil content of the clean germ (soaked and separated by density).

The protein content of the clean germ was increased from 15% to an average of 21% and with only slight variations between soaking pH, temperature, and time. The percent increase in germ protein content ranged from 24 to 37% (Figure 5.7) and increased over time at 25°C but decreased at 60°C. Final protein content on a moisture-free and oil-free basis varied between 27 to 31% for all soaking conditions.

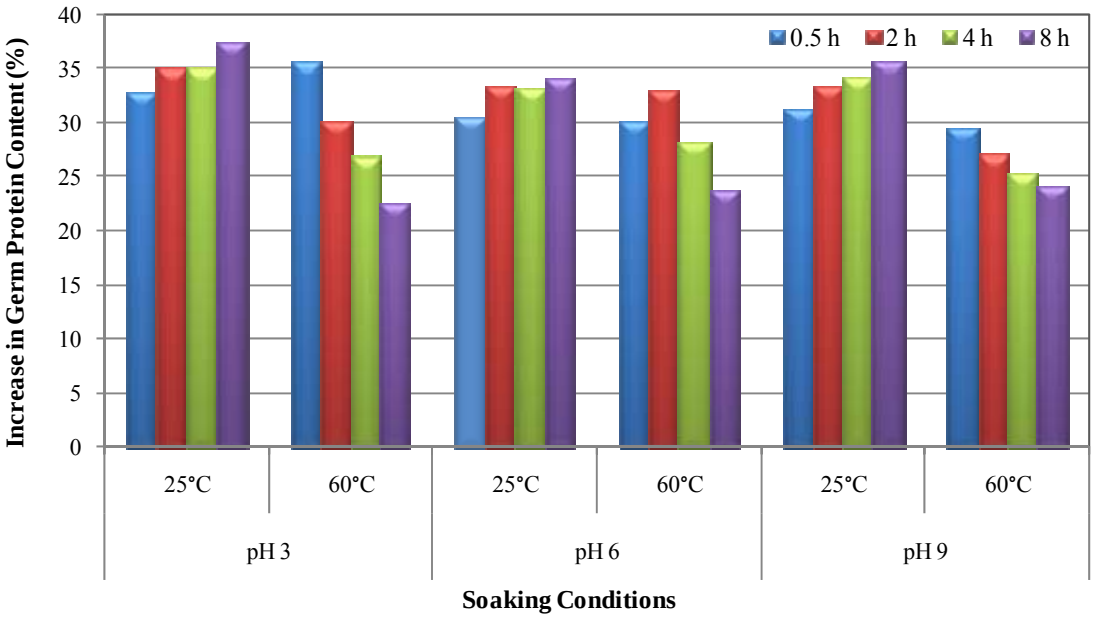


Figure 5.7. Effect of soaking pH, temperature, and time on the % increase of germ protein content (protein determined by Kjehdahl total nitrogen). Results are given in % increase in protein content of germ after germ wet milling (soaked and separated).

If the yield of clean germ or total protein recovered in the clean germ compared to the dry-milled germ is also considered, differences in soaking conditions are apparent. The total protein recovered (Figure 5.8) for germ soaked at 60°C was generally lower than that at 25°C since clean germ yields were lower at 60°C.

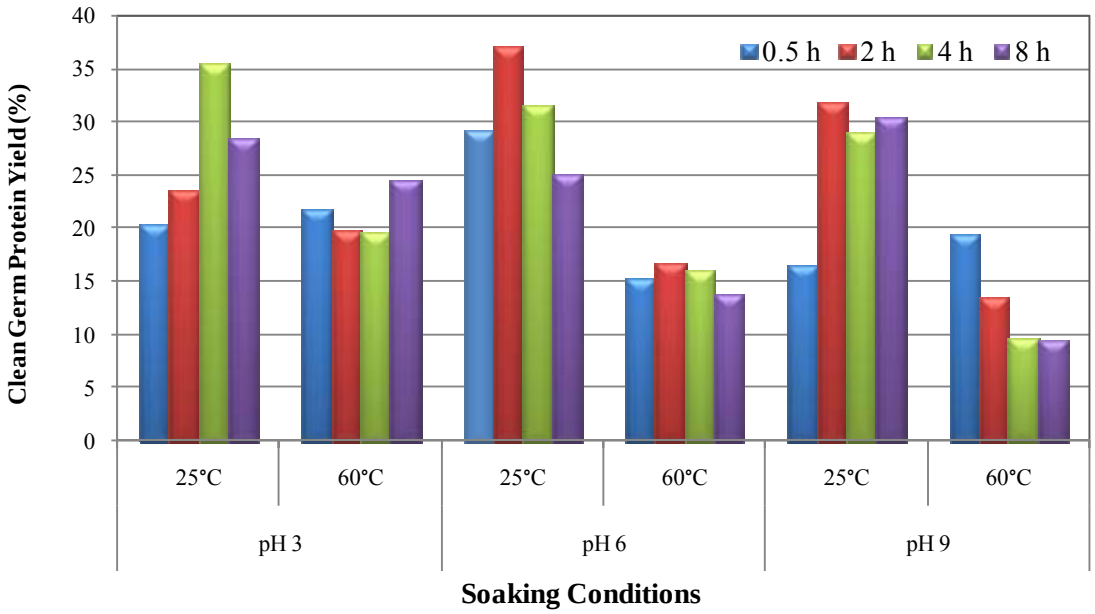


Figure 5.8. Effect of soaking conditions (temperature, pH, time) on germ protein yield (%). After soaking, the clean germ was separated by density, rinsed, dried, and weighed. Protein yield describes the total amount of protein in clean germ as a % of the protein in the starting material (dry-milled germ).

Reducing starch content of dry-milled germ by soaking is beneficial for two reasons: 1) it improves hexane extraction efficiency and 2) it increases ethanol production since starch released into the soak water can be recovered with leached protein and salt and used to supplement fermentation media. The starch content of the germ was reduced from 33% to an average of 9% for all conditions and was independent of soak pH, temperature, and time. The final starch content was within the range of 5-10% expected for clean germ (Watson, 1994). The high starch content in the starting material indicates that a significant amount of endosperm remained attached to the germ after degermination, which is characteristic of the dry milling process. In this regard, soaking was an effective way to remove starch and the shortest soak time evaluated (0.5

h) was sufficient for starch reduction. However, adding amylase enzyme to the soak water did not further reduce starch content in the germ.

Soaking the germ improved oil content from 16% to 28-39% and depended on the soaking conditions. In general, the % increase in germ oil content (Figure 5.9) was improved as the soaking time increased. Using a higher temperature soaking was more effective for producing higher oil germ with an average increase of 120% at 60°C compared to 94% at 25°C. Achieving higher oil content in the germ is desirable because higher oil content improves the efficiency of the subsequent oil extraction step and increases protein purity of the defatted germ.

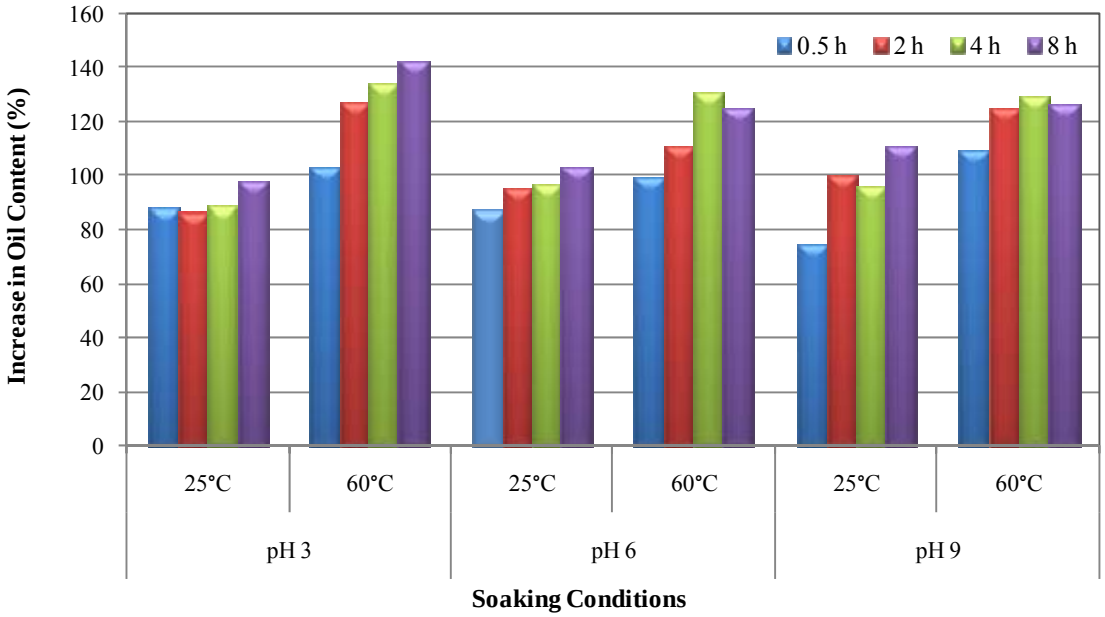


Figure 5.9. Effect of soaking pH, temperature, and time on the % increase of germ oil content (oil determined by acid hydrolysis). Results are given in % increase in oil content after soaking dry-milled germ.

Pilot-scale Studies: Germ Composition Analysis. Two soaking conditions (pH 4.5 at 25°C and pH 7 at 60°C) were selected for scale-up to confirm the effect of the soaking conditions on germ protein and oil content and to produce germ for subsequent corn protein concentrate (CPC) development. The germ soaking conditions (pH 4.5 and 25°C) were selected to minimize the amount of protein lost in the soak water. Two different soaking times (2 h and 6 h) were evaluated since we wanted to reduce the total processing time and still get high germ protein content. Dry-milled germ was also soaked at neutral pH (pH 7) and high temperature (60°C) for either 2 h or 6 h and compared to the pH 4.5 soaking experiments.

The composition of the dry-milled germ used as the starting material and clean germ (soaked and separated) are given in Table 5.1. Soaking improved the germ protein content from 16% to 19-20% protein (db) and was independent of soak time, temperature, and pH. Although the pH 7 and 60°C soaking conditions resulted in 75% more protein leached than at pH 4.5 and 25°C, the loss was not sufficient to reduce final protein content of the clean germ. However, differences could be seen if protein content was expressed on an oil-free basis. Since defatted corn germ flour is used for preparing corn protein concentrate, protein content on an oil-free basis is good way to determine the best starting material (highest protein content). Germ protein content (oil-free basis) was 31% (2 h) and 32% (6 h) for pH 4.5 and 25°C samples and 35% (2 h) and 37% (6 h) for pH 7 and 60°C samples. The higher protein content (oil-free basis) at pH 7 and 60°C was primarily a reflection of the higher germ oil content which was 46% compared to 37% for germ soaked at pH 4.5 and 25°C.

Table 5.1. Comparison of the composition of the starting material (dry-milled germ) to the clean germ (soaked at specified conditions, separated, and dried) from pilot-scale experiments.

Sample	Soaking Conditions			Composition (% db)			
	pH	Temp (°C)	Time (h)	Protein	Protein (oil-free)	Oil	Starch
Dry-milled Germ	NA	NA	NA	16	21	22	27
Clean Germ	4.5	25	2	20	31	36	13
Clean Germ	4.5	25	6	20	32	38	12
Clean Germ	7	60	2	19	37	46	11
Clean Germ	7	60	6	19	35	45	11

Soaking at 60°C also resulted in higher oil content of clean germ in the lab-scale studies. Pilot-scale data were also similar to lab-scale in terms of starch content since soaking was very effective in reducing initial starch concentration, even at the shorter soak times. Starch content was reduced by 50-60% for all soak conditions (Table 5.1).

An additional set of pilot-scale experiments was completed to determine germ composition after soaking at pH 7 and 20°C, conditions that can be maintained with little or no adjustment. Germ composition was compared after soaking one batch at room temperature for only 0.5 h and the other batch for 16 h at 60°C. Even at these two extremes, the protein contents (Table 5.2) were comparable and the slight improvement in oil purity and decrease in starch content probably would not justify using the longer soaking time (16 h) from an economical or operational point of view.

Table 5.2. Germ composition from pilot-scale dry-milled germ soaking at pH 7 for either 0.5 h at 20°C or 16 h at 60°C. Clean germ was analyzed after soaking, separation, and drying.

Sample	Soaking Conditions			Composition (% db)			
	pH	Temp (°C)	Time (h)	Protein	Protein (oil-free)	Oil	Starch
Dry-milled Germ	NA	NA	NA	14	17	18	41
Clean Germ	7	20	0.5	20	30	35	11
Clean Germ	7	60	16	19	33	42	7

The pilot-scale experiments further supported the lab-scale soaking results, i.e. little variation in final protein and starch composition and higher oil content from higher temperature soaking. The pilot-scale studies suggest that soaking germ for 2 h at pH 7 and 60°C would be the preferred conditions if protein isolated from the germ was used for feed or food applications where protein quality (reduced solubility due to denaturation) was not important. If the ultimate goal is to maintain protein water solubility, then the effect of process conditions on the protein dispersibility index of defatted germ must also be taken into consideration.

Corn Protein Concentrates. Dry-milled germ goes through several processing steps to make defatted germ flour (Figure 5.10) and each step warrants consideration. Since our goal is to use defatted germ as the starting material for developing protein concentrates, we evaluated the effect of processing conditions on germ protein quality. Protein quality in this work was evaluated by measuring the protein dispersibility index (PDI). PDI is one of the main methods used to determine protein solubility (Dubois and Hoover, 1981) and one factor known to have a significant effect on protein functionality (Heywood et al., 2002; Zayas, 1997; Zayas and Lin, 1989).

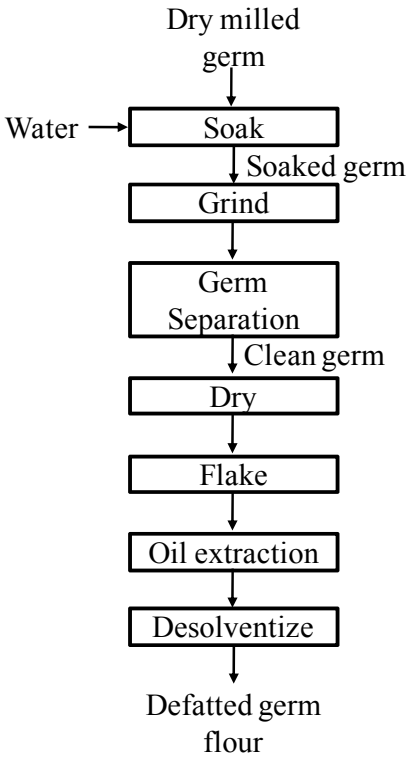


Figure 5.10. Process diagram for preparing high protein defatted germ flour from dry-milled germ.

Lab-scale and pilot-scale studies resulted in a similar protein content of clean germ for all soaking conditions evaluated. Data from lab-scale soaking experiments indicated that the PDI depended on soaking time, temperature, and pH (Table 5.3). PDI decreased as the soaking time increased under most conditions. In addition, germ PDI was generally greater when soaked at 25°C instead of 60°C, unless soaking time at 60°C was limited to 0.5 h, in which case the PDI did not change. The soaking time was particularly detrimental at pH 3 and 60°C due to protein denaturation since the PDI value was reduced 77% when germ was soaked 8 h instead of 0.5 h. Based on the germ

PDI value, soaking at 25°C at either pH 6 or pH 9 appeared to be the best conditions for improving germ protein content and maintaining protein solubility.

Table 5.3. Protein dispersibility index (PDI) of soaked dry-milled germ as a function of soak pH, temperature, and time. PDI of starting material was 56%.

Soak	**pH 3**		**pH 6**		**pH 9**	
Time (h)	**25°C**	**60°C**	**25°C**	**60°C**	**25°C**	**60°C**
0.5	61	53	62	63	65	70
2	53	27	62	44	70	60
4	54	16	61	43	71	61
8	47	13	58	43	69	58

Pilot-scale experiments confirmed the effect of processing on PDI. The PDI values were reduced 19 to 28% during soaking, grinding, separation, and drying of the wet germ. The PDI was further reduced 28 to 35% after hexane defatting and drying. The drying temperature and time as well as the hexane temperature during defatting likely caused the decrease in PDI.

Malumba et al. (2008) found that high drying temperatures (54-130°C) adversely affected protein solubility, with the water-soluble albumins being the most heat-denatured class of protein. Oil extraction with hexane has also been shown to affect the PDI of soybean flakes (Milligan and Suriano, 1974). In a subsequent lab-scale experiment, room temperature hexane was used for defatting clean germ. The PDI value of this germ was only reduced 8% which indicates that reducing solvent temperature was effective for maintaining germ PDI.

Extraction of Defatted Corn Germ Flour. Protein extraction was evaluated using single-stage extraction followed by protein concentration using isoelectric

precipitation as proposed by Nielsen et al. (1973). In general, protein precipitation yield was greater than 95% based on Bradford protein assay. SDS-PAGE showed that primarily small molecular weight proteins (MW <20 kDa) remained in the supernatant (Figure 5.11). After precipitation, the solids were washed with water to remove the liquid with impurities that remained in the interstitial volume. The protein lost in the wash of the precipitated solids was only 0.2% of the total soluble protein in the initial extract.

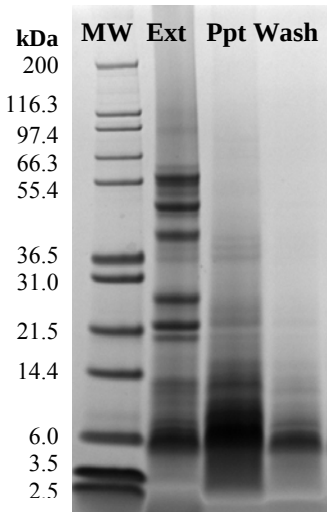


Figure 5.11. Typical molecular weight profiles for defatted corn germ extract (Ext), supernatant after precipitation and centrifugation (Ppt), and precipitate wash solution.

Phytic acid content in the corn protein concentrates (CPCs) was 2% which is within the reported range of 1.4-2.1% for soy protein isolates (Reddy and Sathe, 2002). Protein yield was 16% and much lower than the reported yield of 38% with corn white flakes from dry-milled germ (Nielsen et al., 1973). The defatted germ flour used in this

experiment had a low PDI value (15%) which led us to hypothesize that PDI was also critical for protein extraction yield.

Effect of Germ PDI on Protein Yield. Preliminary data indicated that maintaining PDI may be an important factor for corn protein concentrate yield and quality. The effect of PDI was evaluated further by comparing the yields (by mass) and protein contents of CPCs prepared from defatted corn germ (DCG) flours with different PDI values. CPC yield ranged from 7 g per 100 g DCG to 16 g per 100 g DCG (Table 5.4). The amount of protein recovered ranged from 3.7 g to 11.4 g. The differences in CPC yield and protein content are probably a reflection of the differences in the amount of water soluble protein among the different defatted corn germ flours. Further evidence of the importance of PDI on protein yield (% of protein present in DCG recovered in CPC) was demonstrated as CPC prepared from DCG flour with protein contents of 25-26% had very different protein recovery yields with values ranging from 15% to 26%. The higher protein yield was achieved using DCG with a 52% PDI and the lower yield using DCG with a 15% PDI (Table 5.4).

To confirm these results and separate the effect of initial germ protein content from PDI values, two batches of defatted germ flour with similar protein contents but different PDIs were used to prepare CPC. Corn protein concentrate was prepared from high protein, high PDI germ (HPHPG) with a PDI of 52% and high protein, low PDI germ (HPLPG) with a PDI of 26% to determine if a higher PDI would give higher protein yield. The starting material (corn germ) and soak conditions were identical in both cases.

Corn protein concentrate prepared by isoelectric precipitation from the defatted HPHPG flour had a yield of 16 g CPC per 100 g flour compared to 10 g per 100g for the HPLPG flour (Table 5.5). The fraction of protein recovered from the higher PDI (52%) germ flour was 70% higher than that from the lower PDI (37%) germ flour.

Table 5.4. Effect of defatted corn germ (DCG) PDI on the amount of protein recovered in corn protein concentrate (CPC). The amount of water soluble protein was calculated by multiplying protein content by PDI of defatted corn germ flour. Protein yield was calculated by dividing the total protein in CPC by the total protein in DCG.

Defatted Germ Flour PDI (%)	DCG Protein (%)	Water Soluble Protein (g) in 100 g DCG Flour	CPC(g) per 100 g DCG Flour	Protein (g) in CPC from 100 g DCG Flour	Protein Yield (%)
15	25	3.7	7.0	3.7	15
26	26	6.9	15	7.2	27
52	25	13	16	11	46

Table 5.5. Effect of germ flour PDI on CPC yield (by mass), protein content, and protein yield. Protein yield represents the amount of protein in CPC as a % of the total protein in the DCG.

DCG PDI (%)	DCG Protein (%)	Water Soluble Protein (g) in 100 g DCG Flour	CPC(g) per 100 g DCG Flour	Protein (g) in CPC from 100 g DCG Flour	Protein Yield (%)
37	30	11	10	8.0	27
52	25	13	16	11	46

Evaluation of Three Methods for CPC Preparation. From an economic standpoint, maximizing CPC yield while maintaining protein quality is very desirable. Membrane filtration is a proven and popular method for concentrating protein solutions in food and biotechnology industry. Protein precipitation requires less initial capital

investment than membrane filtration technologies but typically suffers from lower yields than membrane filtration due to protein loss in the precipitation and centrifugation steps. In this section, we report the initial evaluation of membrane filtration methods for CPC production and compare the results to the standard precipitation method. Because productivity of membrane concentration is determined by protein recovery (yield) and flux, the comparative evaluation of these methods included protein yield, composition of the concentrate, and average flux during the membrane concentration process.

The three processes for CPC preparation that were compared are given in Figure 5.12 and included the precipitation method, membrane filtration method, and a combination method using both precipitation and membrane filtration. The CPC yield using HPLPG was increased from 10 g to 15 g CPC per 100 g of DCG flour when membrane filtration was used instead of isoelectric precipitation (Table 5.6). Membrane filtration (30 kDa ultrafiltration) or precipitation alone produced CPC with a similar protein content and composition (based on SDS-PAGE). Protein loss in the permeate for both the 10 and 30 kDa ultrafiltration membranes was 6% of the total protein compared to 10-16% lost if isoelectric precipitation was used. SDS-PAGE showed that the proteins present in the permeates were primarily small ones, around 6 kDa in size. The supernatant after isoelectric precipitation, in addition to 6 kDa protein, contained larger MW proteins anywhere from 14 to 36 kDa. Therefore, SDS-PAGE data support the measured difference in CPC yield between the two methods and explains the probable cause of yield difference, i.e. precipitation efficiency.

Although the two membranes produced similar amount of concentrate, the permeated fluxes were different; 30 LMH with the 30 kDa membrane and slightly lower at 26 LMH with the 10 kDa membrane.

Table 5.6. Effect of CPC preparation method on protein recovery from defatted germ flour. A 30 kDa ultrafiltration membrane was used for membrane filtration experiments.

CPC Preparation Method	Protein (g) in 100 g DCG Flour	CPC (g) per 100 g DCG Flour	% Protein	% Protein Recovered
Precipitation	30	10	77	27
Membrane	30	15	71	36
Precipitation and Membrane	30	8.3	78	22

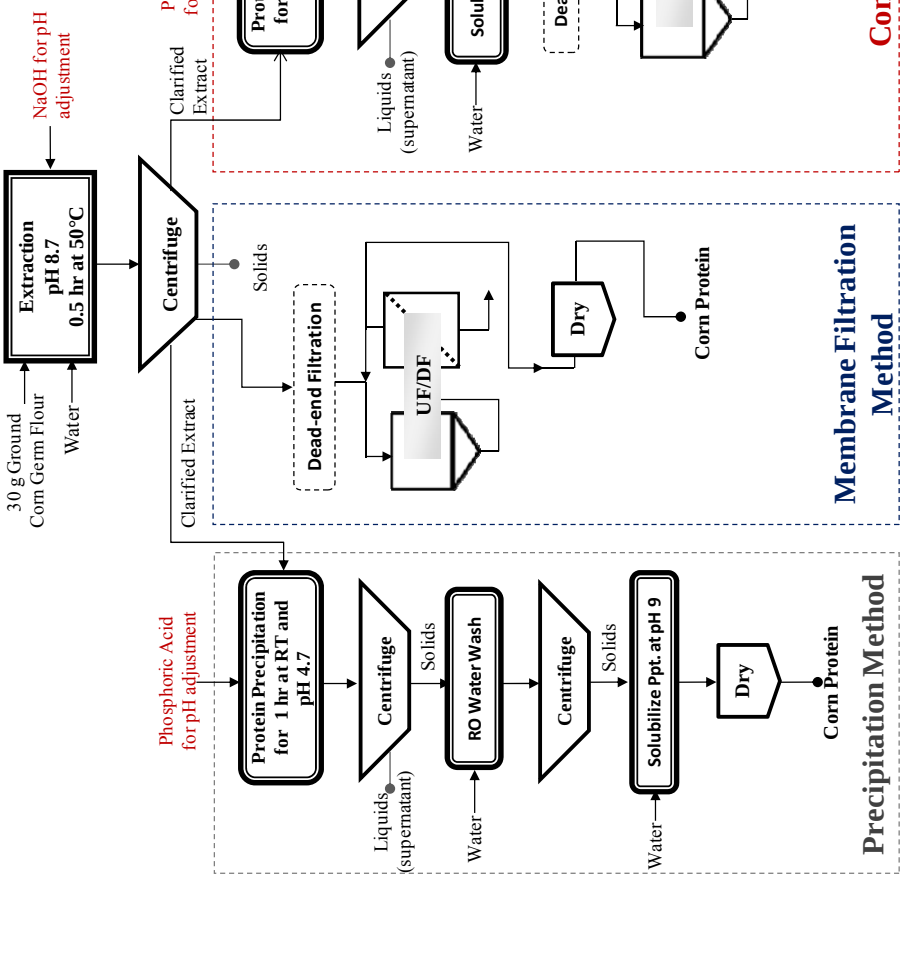


Figure 5.12. Process diagrams for the three different CPC preparation methods evaluated: precipitation, membrane filtration, and the combination method.

To further improve protein purity and/or flux two additional process variations were tested 1) microfiltration (0.45µm) followed by a 30 kDa ultrafiltration step and 2) a combination of 30 kDa ultrafiltration and isoelectric precipitation. The attempt to remove higher molecular weight impurities with a 0.45µm microfiltration membrane before concentration of the permeate on a 30 kDa membrane failed. The microfiltration membrane was severely fouled resulting in only 53% product recovery. The fouling likely occurred by protein plugging of membrane pores. SDS-PAGE analysis of the microfiltration retentate showed that retained proteins were 6 to 60 kDa in size which should not be retained by this membrane.

Precipitation prior to membrane filtration did not increase flux with a 10 kDa membrane. Using precipitation after membrane filtration did improve protein purity of the CPC but at the expense of total yield. The yield using precipitation alone was 10 g /100 g DCG flour but only 8 g/100 g for membrane filtration plus precipitation.

The membrane filtration permeates and supernatant from precipitation were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC) as a way to compare extract impurities removed. HPLC showed that the membrane filtration process did remove phenolic compounds which are known to give unpleasant taste to corn germ protein flour products (Huang and Zayas, 1991).

SUMMARY

Soaking dry-milled germ was an effective method for improving germ protein and oil contents and reducing attached endosperm starch. In the lab-scale experiments, starch was reduced from 33% to 9% and protein content (moisture-free, oil-free basis) was increased from 18% to an average of 29% for all soak conditions. Oil content increased from 16% to 28-39%. In general, 60°C soaking was more effective than 25°C for producing germ with higher oil content. Pilot-scale experiments were consistent with the lab-scale studies with little variation in final protein and starch composition and higher oil content with higher temperature soaking. Compared to the lab-scale experiments, the pilot-scale studies resulted in higher oil contents of clean germ. The oil content of clean germ after soaking at pH 7 and 60°C for 2 h was within the range (42 to 50% db) typically seen in traditional wet milling (Johnson and May, 2003) which uses 24 h soaking of whole kernels. Clearly, soaking germ instead of the entire kernel was advantageous since the same oil content could be achieved in only 2 h.

Protein dispersibility index (PDI) was affected by soaking temperature, pH, and time. High temperature (60°C) and longer soaking times (≥ 2 h) reduced PDI values, especially for pH 3. Based on the germ PDI value, soaking at 25°C at either pH 6 or pH 9 appear to be the best conditions for improving germ protein content and maintaining protein solubility. Controlling the temperature during the germ drying and defatting processes was also important for maintaining the PDI throughout processing.

The protein recovery was greater from defatted germ flour with a high PDI (52%). Corn protein concentrate (CPC) with >75% protein purity could be achieved

using isoelectric precipitation or membrane filtration. Corn protein concentrate prepared by both methods had similar protein composition and content, but membrane filtration had a higher product yield.

Based on these results, neutral pH soaking (pH 6-7) at 25°C for a maximum of 2 h would be the best conditions for preparing high protein, high PDI germ that could be used for protein recovery. After extraction of the defatted corn germ flour, the recommended method for preparing corn protein concentrate would include diafiltration and concentration using a 30 kDa ultrafiltration membrane.

CHAPTER VI

CONCLUSIONS

The use of plant systems for the production of recombinant proteins offers many advantages over traditional production systems but also presents new processing challenges as the introduction of a non-native protein reduces the applicability of established processing methods. The use of plant systems to produce biofuels also introduces new downstream processing opportunities. In separate studies, the downstream processing of transgenic rice and dry-milled corn germ, a co-product of corn-to-ethanol processing, were evaluated for the production of novel protein products.

In the first study, the extraction and purification of human lysozyme from transgenic rice were investigated. The optimum extraction conditions and the effect of rice extract impurities on cation exchange adsorption of lysozyme were determined and the rice extract component that interfered with purification was identified as phytic acid.

A combination of pH and ionic strength was used to optimize the extraction of lysozyme and cation exchange adsorption using SP-Sepharose^{TM} FF. Lysozyme extraction was highly dependent on pH as the amount of lysozyme (expressed as a % of total soluble protein) in crude extract was 60% at pH 4.5, 15% at pH 6.5, and 10% at pH 7.5 with 50 mM NaCl. Extraction at pH 4.5 with 100 mM NaCl (15 mS) resulted in the highest lysozyme to total soluble protein ratio, but the saturation binding capacity (SBC) at 15 mS was modest compared to the highest SBC at 5 mS. The selected conditions for extraction and purification, pH 4.5 with 50 mM NaCl (10 mS), were a compromise

between lysozyme extractability and SBC. These conditions are recommended for applications where 90% lysozyme purity is sufficient. A new method was developed (pH 4.5 extraction followed by pH 6 adsorption) to get a lysozyme purity of 95% in a single chromatography step. However, rice extract impurities reduced the SBC almost 80% compared to the SBC of purified human lysozyme at the same pH and ionic strength.

To assess the effectiveness of human lysozyme (HuLZ) adsorption and purification from rice, the adsorption of HuLZ was compared to hen egg-white lysozyme (HewLZ). Except in one case, dynamic binding capacities (DBCs) of HuLZ were lower than HewLZ DBCs at pH 4.5 and 6 with ionic strengths from 5-15 mS (0-300 mM NaCl). The lower DBCs for HuLZ indicated that HuLZ interactions with the cation exchange resin were weaker overall despite similar protein properties. In the presence of rice extract, the capacities of HuLZ was reduced 31% in pH 4.5 extract to 39% in pH 6 extract compared to purified HuLZ. The effect on HewLZ capacities was slightly lower with reductions of 21% and 32% for pH 4.5 and pH 6 extracts, respectively. The purification of HewLZ from rice extract was similar to that from egg-white with similar binding capacities. Process simulation of the best processes for HuLZ (from rice) and HewLZ (from egg-white) showed that the downstream processing costs were similar.

The apparent interference of rice extract impurities on HuLZ adsorption, especially for pH 4.5 extract adjusted to pH 6 for adsorption, indicated that identification of the interfering impurity was critical to designing an integrated, optimized purification process. The rice impurity, phytic acid, was suspected so enzymatic (phytase) treatment

prior to cation exchange adsorption was evaluated. Phytase treatment eliminated column fouling and improved the binding capacity of HuLZ, implicating phytic acid as the primary culprit. The two approaches tested to reduce phytic acid interference were: 1) reducing the amount of phytic acid extracted and 2) shielding phytic acid with counter-ions to reduce interactions between phytic acid and HuLZ. The method for the first approach was pH 10 extraction followed by acidic precipitation and pH 6 adsorption. The second approach was pH 4.5 extraction followed by pH 6 adsorption in the presence of TRIS counter-ions. The binding capacity using either method was ≥ 25 mg/mL, a significant improvement in capacity compared to the previous best process of 8.6 mg/mL. The lysozyme purity with both processes was also ≥ 95%. By reducing the interference of phytic acid with cation-exchange purification, HuLZ could be purified from rice in a single chromatography step with an improved binding capacity.

Based on data presented, the recommended extraction and purification method for obtaining human lysozyme with a purity of 95% would be pH 4.5 extraction (50 mM sodium acetate with 50 mM NaCl) with pH 6 adsorption in the presence of TRIS counter-ions. Unlike the pH 10 process, the TRIS process does not require an acidic precipitation step which would increase the equipment and labor costs and total processing time.

These studies using transgenic rice revealed that purification of HuLZ using a single chromatography step is possible and identified bottlenecks that need to be considered in the future. One bottleneck identified was the packed-bed chromatography step which is the major cost contributor to the downstream processing cost of HuLZ.

Testing the feasibility of using lower cost alternatives such as batch adsorption, precipitation, or ultrafiltration would also be beneficial. Other future work should include the scale-up of the best purification process to demonstrate the process robustness on a production scale. To further address phytic acid, one potential option that could be evaluated would be to remove the aleurone layer through abrasive milling to reduce the phytic acid content 50-80%. Finally, development of general guidelines or heuristics for the purification of basic proteins expressed in transgenic rice could be tested by evaluating the extraction and purification of another basic recombinant protein.

The second study was aimed at improving the value of traditional dry-milled germ by increasing the protein and oil content through germ wet milling and to develop a process to produce corn protein concentrate (CPC) from the higher protein germ. Soaking dry-milled germ for as little as 0.5 h reduced starch content from 33% to ≤ 9% and increased protein content from 18% to ≥ 27% (moisture-free, oil-free basis) for all soak pHs and temperatures in lab-scale experiments. Pilot-scale experiments were consistent with the lab-scale studies and showed the same trends, i.e. little variation in final protein and starch composition between soaking conditions. Germ oil content increased from 16% to as high as 39% in lab-scale experiments and 46% for pilot-scale studies using a high temperature soaking (60°C). High temperature (60°C) and longer soaking times (≥ 2 h) reduced the PDI of soaked germ as much as 75%. Germ soaked using these conditions would be undesirable for CPC preparation since CPC yield was highest using high PDI defatted germ flour. Concentrating and purifying germ protein

using membrane filtration instead of protein precipitation also resulted in higher CPC yields.

The recommended soaking conditions for preparing high protein, high PDI germ that could be used for protein recovery are neutral pH (pH 6-7) at 25°C for a maximum of 2 h. The recommended method for preparing CPC from defatted corn germ flour extract would include diafiltration and concentration using a 30 kDa ultrafiltration membrane. If the protein content of CPC is not sufficient using membrane filtration alone, a protein precipitation step prior to membrane filtration could be added but would reduce the protein yield.

Future work in this area should include optimizing membrane filtration and scaling-up CPC production using the precipitation and/or membrane filtration methods. Larger scale production would be important for producing sufficient CPC to evaluate the functional properties and to identify the best process for the intended application.

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VITA

Name: Lisa Rachelle Wilken

Address: Department of Biological & Agricultural Engineering
201 Scoates Hall
TAMU 2117
College Station, TX 77843

Email Address: lwilken@tamu.edu

Education: B.S., Biological & Agricultural Engineering, Kansas State University, 2003
Ph.D., Biological & Agricultural Engineering, Texas A&M University, 2009