Membrane-based separations for solid/liquid clarification and protein purification

Matthew Thomas Aspelund
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Biological Engineering Commons, and the Chemical Engineering Commons

Recommended Citation
Aspelund, Matthew Thomas, "Membrane-based separations for solid/liquid clarification and protein purification" (2010). Graduate Theses and Dissertations. 11771.
https://lib.dr.iastate.edu/etd/11771

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Membrane-based separations for solid/liquid clarification and protein purification

by

Matthew Thomas Aspelund

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Chemical Engineering

Program of Study Committee:
Charles E. Glatz, Major Professor
Peter J. Reilly
Rodney O. Fox
James E. Alleman
Theodore J. Heindel

Iowa State University
Ames, Iowa
2010

Copyright © Matthew Thomas Aspelund, 2010. All rights reserved.
For my family and my wife Sender who makes all things possible
Table of Contents

CHAPTER I. General Introduction 1

CHAPTER II. Improving Permeate Flux and Product Transmission in the Microfiltration of a Bacterial Cell Suspension by Flocculation with Cationic Polyelectrolytes 24

CHAPTER III. Clarification of Aqueous Corn Extracts by Tangential Flow Microfiltration 76

CHAPTER IV. Purification of Recombinant Plant-Made Proteins from Corn Extracts by Ultrafiltration 103

CHAPTER V. General Conclusions 140
Chapter I: General Introduction

Organization

This thesis is divided into five chapters. Chapter I provides an overview of the research discussed in this thesis along with a literature review on membrane separations including factors that influence filtration performance for both microfiltration and ultrafiltration and previous studies involving flocculation and crossflow microfiltration. Chapter II is a paper published in the *Journal of Membrane Science* and describes the results of the experimental work examining polyelectrolyte flocculation as a feed pretreatment to improve the filtration performance during the microfiltration of an industrial fermentation broth. The experiments for Chapter II were performed both at Iowa State and Genencor, International. The Genencor co-authors contributed to the work by technical discussions regarding the design of experiments and by their comments on the manuscript. Chapters III and IV focus on solid/liquid clarification and purification of recombinant proteins from transgenic corn tissues by microfiltration and ultrafiltration using green fluorescent protein (GFP) and rCollagen as model proteins. Chapter III is a manuscript to be submitted to *the Journal of Membrane Science* and examines the effectiveness of microfiltration for the solid/liquid clarification of transgenic corn tissues in both dead-end and tangential flow. Different membrane chemistries were examined for their effect on membrane fouling and product rejection. Chapter IV is a journal article in press to be published in the *Journal of Membrane Science* and describes the use of ultrafiltration as a technique to purify recombinant proteins from aqueous extracts of transgenic corn tissues. The chapter also describes a strategy for the purification of rCollagen from
transgenic corn extracts using only precipitation and ultrafiltration. Finally, Chapter V summarizes the major conclusions emerging from the work described in this thesis.

**Project Objective**

Recombinant protein technology has become increasingly important in recent years. Recombinant proteins can be found in everything from pharmaceuticals to detergents. The increasing demand and the high cost associated with the production and purification of recombinant proteins highlights the need to develop efficient and inexpensive methods for the solid/liquid clarification and purification of these proteins. Membrane technologies, which offer the advantage of high throughput and ever-improving selectivity, have the potential to meet the increased demands placed on the downstream processing of recombinant proteins. The overall objective of this work was to develop methods for the solid/liquid clarification and purification of recombinant proteins using membrane based separations.

First, we explored the use of polyelectrolyte flocculation to improve the microfiltration of an industrial fermentation broth. We evaluated the effectiveness of several cationic polyelectrolytes of differing repeating unit, molecular weight, and charge density for the flocculation of an industrial *Bacillus subtilis* fermentation and the subsequent effect on the permeate flux and product rejection in both dead-end and tangential flow microfiltration. We also investigated the effect of crossflow rate on the permeate flux, product rejection, and the rate of floc breakup. Finally, we used flocculation to examine
particle deposition in the highly variable shear environment that occurs in flow through a spacer filled channel.

Then, we investigated microfiltration for the solids/liquid clarification of transgenic corn extracts. Within this work we characterized the resistance and compressibility of filter cakes formed in dead-end microfiltration of endosperm- and germ-rich extracts. In tangential flow microfiltration the effects of crossflow rate and transmembrane pressure (TMP) on the permeate flux and rejection of corn host cell proteins (HCP), recombinant Type 1 human collagen (rCollagen), and GFP were investigated. We also examined both a ceramic and a poly (vinylidene fluoride) (PVDF) membrane for the extent of membrane fouling and the subsequent effect on the rejection of HCP, GFP, and rCollagen.

Finally, we examined ultrafiltration for the purification of recombinant proteins from transgenic corn extracts. We investigated the effects of membrane pore size, crossflow rate, TMP, filtration pH, and corn tissue (i.e. endosperm or germ) on the permeate flux and protein sieving in ultrafiltration. We also developed a purification strategy to purify rCollagen from transgenic corn extracts using only acid salt precipitation and ultrafiltration.

**Literature Review**

Here we review the state and the limits of the current knowledge relevant to microfiltration and ultrafiltration conditions and their effect on the filtration performance.
The literature review introduces several parameters that can be manipulated to improve permeate flux, product transmission, and selectively of these membrane separation processes.

**Microfiltration**

A key early step in the production of recombinant proteins is the removal of cells and other large particulates from the production medium. Several techniques have been developed to accomplish this separation, including centrifugation, depth filtration, rotary drum vacuum filtration (RDVF), expanded bed adsorption, and microfiltration [1].

Microfiltration is a pressure driven membrane separation technique, using microporous membranes that have average pore sizes that range between 0.05 µm and 10 µm [2]. It is performed using low transmembrane pressures, typically less than 50 psi. High permeation fluxes are achieved compared to other pressure driven separation techniques such as ultrafiltration and reverse osmosis. Some advantages in using microfiltration for the removal of suspended solids from a proteins production medium are (1) the same equipment can be used for different production mediums, (2) low energy requirements [3], (3) elimination of filter aids, (4) relatively mild operating conditions that minimize protein denaturation, and (5) using a 0.2 µm filter results in a particle-free permeate that can be used in subsequent purification processes without further clarification [4]. However, there are several drawbacks associated with microfiltration. Chief among these is the development of large transport resistances that can severely reduce the permeate flux and product transmission [5], making microfiltration an uneconomical option. The
following review focuses on the sources of these transport resistances and general changes that can be made to improve the microfiltration process.

**Sources of Transport Resistance**

The two main transport resistances that occur in membrane filtration are caused by the membrane \( R_m \) and the formation of a layer of rejected particles deposited on the membrane surface \( R_c \) (i.e. the cake layer). The resistance of the membrane and the cake layer can be treated as a resistance in series with the permeate flux described by Darcy’s law:

\[
J = \frac{dV_{perm}}{Adt} = \frac{\Delta p}{\mu_0(R_m+R_c)}
\]  

(1)

where \( J \) is the permeate flux rate, \( V_{perm} \) is the permeate volume, \( A \) is the membrane area available for filtration, \( \Delta p \) is the transmembrane pressure drop, and \( \mu \) is the permeate viscosity. Permeate flux is typically expressed in terms of liters/ square meter – hour (LMH). The resistance of the cake layer can be expressed in terms of a specific cake resistance shown in equation 2,

\[
R_c = \alpha\delta_c
\]  

(2)

where \( \alpha \) is the specific cake resistance and \( \delta_c \) is the height of the cake layer. The specific cake resistance is a convenient way to compare the intrinsic transport resistance of filter cakes independent of the height of the cake layer [6].

**Membrane Resistance**
The resistance due to the membrane depends on the membrane thickness, pore size, and other morphological features of the membrane [3]. Membrane resistance can increase due to internal fouling of the membrane pores. The adsorption of feed components onto the internal surface of the membrane pores can reduce the effective radius of the pore (constriction) or completely block the pore (blockage) [2, 3]. Typically internal membrane fouling is caused by small particles and multilayer adsorption of soluble feed components (i.e. proteins and proteins aggregates) on the pore walls [7-9]. Internal fouling of the membrane pores reduces the permeate flux and the transmission of the desired product. Membrane chemistry and pore size can influence the amount of internal membrane fouling that occurs [10]. In general, hydrophilic membranes absorb less protein than hydrophobic membranes [11].

Cake Layer Formation and the Effect of Particle Size on Cake Resistance

Particles are transported to the membrane surface in a manner depending on the type of filtration module used. Dead-end filtration is operated in batch mode with the bulk flow directed perpendicular to the membrane. As shown in Figure 1, the bulk flow carries particles to the membrane surface which results in the continual growth of the cake layer. [3].
For dead-end filtrations, the cake resistance is expressed in terms of the specific cake resistance as shown in equation 3,

\[ R_c = \alpha \rho_c \frac{V_{\text{perm}}}{A} \]  

(3)

where \( \rho_c \) is the mass of dry filter cake per unit volume of permeate. The effect of particle size can be related to the specific cake resistance using the Carmen-Kozeny equation [12],

\[ \alpha = \frac{k_2(1-\varepsilon)}{(\Phi_s d_p)^2 e^3 \rho_c} \]  

(4)

Figure 1. Schematic representation of fluid flow and particle deposition in dead-end filtration
where $\varepsilon$ is the porosity of the cake, $\rho$ is the density of the filter cake, $k_2$ is the Kozeny constant associated with the morphology of the feed particles and is typically assumed to be $5$ [13], $\Phi_S$ is the shape factor, and $d_p$ is the particle diameter, which shows that the specific cake resistance decreases with the square of the particle size.

In crossflow filtration, the bulk feed flow is tangential to the membrane surface and a pressure differential across the membrane drives the permeate flow as shown in Figure 2. Particles are convected to the membrane surface by the pressure driven flow through the membrane and swept from the membrane surface by the tangential feed flow.

![Figure 2. Schematic representation of fluid flow and particle deposition in crossflow filtration. Mass transfer directions C: convective, B: back diffusive, and A: axial.](image)

The presence of the tangential feed flow causes the particles to be transported away from the membrane by shear-moderated back-transport, or axially along the surface of the membrane [3]. Proposed back diffusion mechanisms for crossflow filtration are shear-
induced diffusion [14] and inertial lift [15] for larger particles (>0.1µm), and Brownian diffusion for small particles (< 0.1 µm) such as proteins [16]. The rolling or sliding of particles along the membrane surface in the presence of the axial crossflow is described by surface transport [17].

The four transport mechanisms above can be used to examine the parameter dependence of the permeate flux as shown in equation 5, assuming that the feed consists of dilute non-adhesive spherical particles [3],

\[
J = c \gamma_o^n d_p^m \phi_b^q L^r \eta_o^z
\]  

(5)

where \( \gamma_o \) the wall shear rate, \( d_p \) is the particle diameter, \( \phi_b \) is the particle concentration, \( L \) is the length of the flow channel, and \( \eta_o \) is the dynamic viscosity of the feed. The values of the constants \( c, n, m, q, r, z \) vary depending on the particle transport mechanism. The models for surface transport, inertial lift, and shear-induced diffusion mechanisms have \( m \) equal to or greater than 1 (1, 3, and 1.33 respectively), and predict a positive correlation between permeate flux and particle size, whereas Brownian diffusion predicts a declining flux with increasing size [3, 14, 15]. Therefore, in both dead-end and crossflow filtrations, the particle size has an integral role in determining the flux, with larger particle size leading to higher flux when \( d_p \) exceeds ca. 0.1 µm.

**Effect of Operating Conditions**

*Transmembrane Pressure*
In crossflow filtration, the effect of transmembrane pressure (TMP) on the permeate flux is dependent on whether the filtration is operated in the pressure dependent or pressure independent part of the flux curve (Figure 3).

Figure 3. Schematic representation of the effect of transmembrane pressure on permeate flux during microfiltration. Region 1. cake consolidation and formation; Region 2. Pressure independent flux.

In the pressure dependent region, the permeate flux varies linearly with the TMP because the low value of the TMP results in a permeate flux that is not sufficient to transport the particles to the membrane surface in the presence of the bulk feed flow. This results in either no cake formation or sparse deposition of particles on the membrane surface (Figure 3). Operation in the pressure independent region results in the formation of a cake.
layer, where increasing the TMP no longer results in an increase in the permeate flux rate (Figure 3) [18, 19].

 Typically, in both dead-end and crossflow filtrations increasing the TMP will increase the resistance of the cake layer for biological feeds [20-23], due to an increase in the solids packing density. Specific cake resistance and TMP are most commonly correlated using the following equation,

$$\alpha = \alpha_0 \Delta p^n$$

where $\alpha_0$ is the zero pressure specific cake resistance, and $n$ is the compressibility index. A value of zero for $n$ represents an incompressible cake and increasing value of $n$ represents increasing cake compressibility [13]. For both dead-end and crossflow filtrations of these compressible feeds, the porosity of the cake layer varies with position within the cake. The minimum porosity of the cake occurs near the filter cake-membrane interface and a maximum porosity occurs at the surface of the cake layer. The porosity decreases near the membrane surface because of an increase in compressive pressure caused by drag on the deposited particles in the direction of the permeate flow [24-26]. The presence and packing density of the cake layer may reduce protein transmission [27-29].

**Crossflow Rate**

In general, increasing the crossflow rate tends to increase permeate flux and decrease the protein rejection by reducing the height of the deposited cake layer for monodisperse
suspensions [5, 30-33]. The reduction in the cake height and subsequent increase in the permeate flux rate are caused by the increased sweeping of particles from the cake layer. Increasing the crossflow rate does not always increase the permeate flux and, in fact, can increase the transport resistance of the cake layer [33-35]. In polydisperse suspensions, cake resistance increases with increasing crossflow rate because the high crossflow rate decreases the size of the particles that deposit on the cake layer due to greater shear-mediated back diffusion and inertial lift of the larger particles [33, 36, 37]. In filtrations of rod-shaped particles, the presence of the crossflow results in the alignment of the particles in the direction of the bulk flow, increasing the resistance of the cake layer [38, 39]. The increased shear stress associated with high crossflow rate can cause cells to break, which can increase the transport resistance by decreasing the average particle size of the suspension [40, 41]. The formation of a gel layer of proteins and polysaccharides on the cake surface increases the resistance significantly. Raising the crossflow rate increases the rate at which this gel layer forms [34]. Protein rejection increases with high crossflow velocity despite the decrease in the height of the cake layer during the filtration of yeast cells and BSA. This increase in BSA rejection is attributed to the increased sweeping of the crossflow, transporting proteins out of the concentration polarization layer and into the bulk feed [42].

Module Design and Operation

Changes in filtration module design and operation to improve permeate flux and product transmission are aimed at disrupting the cake layer by increasing shear and back transport of particles from the membrane. Some mechanical changes that have been shown to
improve filtration performance are the addition of a rotating disc above the membrane [43, 44] and vibration of the membrane [45, 46]. In spiral-wound membrane modules, a mesh of non-woven crossed cylinders is placed between the adjacent membrane layers with the purposes of creating the flow path, promoting unsteady flow, and enhancing the mass transport to disrupt the formation of the cake layer [47]. Disruption of the cake layer can also be achieved by the introduction of Taylor and Dean vortices created in the feed flow using a helically shaped flow path [48, 49]. Back pulsing (i.e. temporary reversal) of the permeate flow [50, 51] also improves the permeate flux and product transmission by disruption of the cake layer.

Effect of Feed Conditions

The pH and ionic strength of the feed can have a strong effect on filtration behavior by modulating the charge and electrostatic interactions of the feed components and the membrane. Change of pH can improve permeate flux by increasing the surface charge of the cells in the suspension, increasing the pore size of the cake layer by electrostatic repulsion of like charged particles [52]. Changes in pH can also induce particle aggregation, increasing the particle size in the feed, leading to an improved permeate flux as shown by equation 4 for dead-end and equation 5 for crossflow filtrations [6, 21]. The effect of pH changes on the cell surface properties is strongly influenced by the other media components. In a filtration of Bacillus subtilis grown in complex media, Graves et al. found that cell aggregation was not dependent on pH, while in the filtration of Bacillus subtilis grown in defined media, pH-dependent cell aggregation was observed [6]. High ionic strength in the feed suspension can shield the charges of the particles,
decreasing electrostatic interactions. This charge shielding can aid in aggregation of the feed particles [21, 53, 54] and decrease rejection of the desired product [55]. Changes in pH can decrease protein rejection by reducing cell-cell, protein-cell, and protein-membrane electrostatic interactions [54, 56]. Rejection of desired proteins is significantly reduced by performing the filtration near the isoelectric point (i.e. net neutral charge; pI) of the desired protein [55, 57]. Adjustment of the pH can reduce the rejection of the desired product by aggregating the feed particles, forming a cake layer with larger pores [42].

*Polyelectrolyte Flocculation in Crossflow Microfiltration*

Flocculation is the process by which particles in suspension form aggregates with the addition of a flocculating agent. It has become an increasingly important step in solid/liquid separation processes. Flocculation is employed extensively in the waste water treatment and biotechnology industries to aid in separation techniques such as filtration, flotation, sedimentation [58], and centrifugation [59], which become more effective with larger suspended particle size. Flocculation can occur naturally in some microbial suspensions such as yeast cultures [60-62], aggregation of which is influenced by various genetic and environmental factors and synthetically by the addition of flocculants such as metal salts [63] and polyelectrolytes.

Previous studies have shown that the addition of a polyelectrolyte flocculants increases permeate fluxes for the crossflow microfiltration of yeast [64, 65] and CHO cells [66] in simple defined media and titanium oxide suspensions in DI water [67], but not in
complex microbial media. In the experiments with yeast cells, the flocculated particles were exposed to average wall shear rates as high as 6000 s$^{-1}$; however, the flocculated particles were not disrupted and the particle size distribution of the bulk feed remained the same throughout the course of the filtration [66]. The effect of operating conditions on the microfiltration of flocculated yeast particles was examined using the direct observation through the membrane technique and deposition of the larger flocculated yeast particles increased with higher permeate flux at a constant crossflow rate. This result indicates that operation at high transmembrane pressures (i.e. high initial permeate flux) and low crossflow rates produces the greatest flux improvements with flocculant addition in the microfiltration of yeast suspensions by increasing the size of the depositing particles [64]. Wickramasinghe et al. found that flocculation increases the rejection of a viral contaminant [68], and studies to examine the effect of flocculation on the rejection of a desired protein product in microfiltration are yet to be reported.

**Ultrafiltration**

Ultrafiltration (UF) has been widely used in pharmaceutical processes for many years primarily for concentration and buffer exchange. Recent work has focused on the use of UF for protein purification [69]. Typically retention of the desired protein and subsequent purification of proteins are based on size. Studies have shown that careful control of filtration conditions (i.e. crossflow rate and TMP) and exploitation of electrostatic interactions can be used to enhance the ultrafiltration process [70-73]. Traditional ultrafiltration processes are usually limited to separation of solutes that differ by ten-fold in size due to the high pore size distribution in the UF membranes. Careful control of the
operating conditions (i.e. crossflow rate and TMP) and exploiting electrostatic interactions can take advantage of solute membrane and solute concentration polarization layer interactions to accentuate the passage of neutral solutes and the retention of the charged solutes [71]. The fractionation of proteins by ultrafiltration can be optimized in the following three ways: 1) controlling the filtration operation conditions (i.e. crossflow rate and TMP), 2) optimizing the pH and ionic strength of the filtration buffer, and 3) using charged membranes [70-73].

Crossflow and Transmembrane Pressure

Rejection of the solutes convected towards the membrane by the permeate flow during ultrafiltration results in the development of a region of elevated solute concentration at the membrane surface. Development of this layer of increasing concentration is referred to as concentration polarization. Operation of the filtration under conditions of high concentration polarization may result in the formation of a gel layer, which can reduce the permeate flux and selectivity of an ultrafiltration process as well as cause solute aggregation [74-76]. The extent of concentration polarization and gel layer formation is controlled in UF by varying the crossflow rate and TMP such that operation is in the pressure dependent part of the filtrate flux curve (Figure 3) [71, 75, 77, 78]. Proper control of the extent of concentration polarization significantly improves the selectivity of a UF process [73, 77-80]. Several studies have shown that the effects of crossflow rate and TMP can be combined to yield high transmission of the larger molecular weight solute and high rejection of the low molecular weight solute (i.e. reverse separation) [55, 75, 77, 81]. Operation below the critical TMP reduces the internal membrane fouling [82,
Internal fouling of the membrane pores reduces the permeate flux and the transmission of the desired product [84]. Also, to reduce the effect of the change in concentration polarization and solute permeation with variations in TMP, filtrations may be operated with a co-current filtrate flow to maintain constant TMP along the length of the membrane [70, 78].

**Electrostatic Interactions**

Protein-protein, protein-concentration polarization layer, and protein-membrane interactions significantly affect protein separation by UF [75, 77, 78, 81]. Solution pH and ionic strength can drastically alter the hydrodynamic volume, charge, and diffusion coefficients of proteins in solution. Operating the filtration with low ionic strength has the potential to accentuate the electrostatic effects [78]. Filtration at low ionic strength and a pH close the pI of the protein that is to permeate enhances selectivity by reducing the hydrodynamic volume of the permeating solute [71, 73, 75, 85]. However, operating under the above conditions may aggregate proteins and increase membrane fouling [78]. The charge of the membrane can also selectively increase protein retention by increasing repulsive electrostatic forces between the membrane and solute of the same charge [71, 78, 86].

Microfiltration and ultrafiltration are useful methods for the solid/liquid clarification and purification of proteins when sufficiently high permeate flux, product transmission/rejection, and protein selectivity are attained.
References:


Chapter II: Improving Permeate Flux and Product Transmission in the Microfiltration of a Bacterial Cell Suspension by Flocculation with Cationic Polyelectrolytes


Matthew T. Aspelund¹, Glenn Rozeboom³, Meng Heng², Charles E. Glatz¹*

1. Department of Chemical Engineering, Iowa State University, Ames, IA 50011
2. Genencor, A Danisco Division, Palo Alto, CA 94304
3. Genencor, A Danisco Division, Cedar Rapids, IA 52404

Abstract:

Microfiltration is an attractive method for the clarification of fermentation broths provided that sufficiently high permeate flux and product transmission can be achieved. Several polyelectrolytes were screened for flocculated particle size and permeate flux enhancement in unstirred and stirred dead-end filtrations. Larger flocculated particles were formed by flocculation with polyelectrolytes of longer chain length. Increase in particle size of the feed generally resulted in an increase in the permeate flux, but the largest particle size did not result in the greatest permeate flux enhancement. In crossflow filtrations, long-term permeate flux improvements occurred despite complete disruption of the floc in the recycled feed and the extent of the improvements were dependent on both the polyelectrolyte dose and crossflow rate. The size of the particles comprising the cake layer closely resembled that of the particles in the initial flocculated feed, indicating that the particles that initially deposited on the cake surface avoided disruption during filtration. Additionally, a decrease in subtilisin rejection was observed with filtration of
the flocculated feed. Thus, flocculation with cationic polyelectrolytes increased permeate flux and reduced product rejection in the microfiltration of a complex industrial fermentation broth.

**Keywords:** Microfiltration; Flocculation; Fouling; Rejection; Flux

**Introduction:**

One of the most costly steps in the production of industrial enzymes by fermentation is the removal of cells and other particulates from the fermentation media. Traditionally, this separation has been performed using centrifugation, depth filtration, or rotary drum vacuum filtration [1]. Microfiltration is an attractive alternative to these methods because of its low energy requirements, ability to treat different feeds with the same equipment, generally nondenaturing operating conditions, and relatively contained and safe operation. Additionally, unlike centrifugation, membrane filtration using a pore size less than 0.2 \( \mu \text{m} \) requires no further clarification. Although microfiltration offers many advantages over other clarification methods, fouling of the membrane by soluble feed components and the buildup of a compressible layer of rejected biomass on the membrane surface (i.e. cake layer) can lead to decreases in permeate flux and increased product rejection, consequently making microfiltration uneconomical compared to other clarification options [2, 3].

The cake resistance, \( R_c \), influences permeate flux \( J \), as seen in Darcy’s law,
\[ J = \frac{dV_{perm}}{Adt} = \frac{\Delta p}{\mu_0(R_m + R_c)} \]  

(1)

where \( V_{perm} \) is the volume of the permeate, \( \mu_0 \) is the permeate viscosity, \( R_m \) is the resistance of the membrane, \( \Delta p \) is the transmembrane pressure, and \( A \) is the area available for filtration. The resistance of the cake layer can be expressed in terms of the specific cake resistance, \( \alpha \), where

\[ R_c = \alpha m \]  

(2)

and \( m \) is the mass of the cake layer per membrane area.

For unstirred dead-end filtrations, the cake resistance is expressed in terms of the volume permeated, \( V_{perm} \),

\[ R_c = \alpha \rho_c \frac{V_{perm}}{A} \]  

(3)

where \( \rho_c \) is the mass of dry filter cake per unit volume of permeate. The specific cake resistance is a convenient way to compare the intrinsic transport resistances of filter cakes independently of the mass of the cake layer [4]. Combining Eqns. 1 and 3 and integrating for constant pressure drop give [5]
The specific cake resistance for unstirred dead-end filtrations can be obtained from the slope of a plot of $tA/V_{perm}$ versus $V_{perm}/A$. For compressible feeds, the specific cake resistance varies with position in the cake [6]; therefore, use of equation 4 gives a specific cake resistance averaged over the entire thickness of the cake layer.

For unstirred dead-end filtrations, the mass of the deposited cake layer is continually increasing during the filtration, while in the absence of settling, the remaining suspension has constant solids concentration. In stirred dead-end filtrations, the concentration of the retained solids in the feed increases as the filtration progresses. This increase in the mass of the deposited cake layer or the solids concentration of the feed will result in a decrease in the permeate flux rate. Therefore, comparisons of the permeate fluxes are best done at the same feed concentration factor, $CF$, where

$$CF = \frac{V_{feed}}{V_{feed} - V_{perm}}$$

and $V_{feed}$ is the initial volume of the feed. Differences in permeate flux at the same concentration factor can be attributed to differences in transport resistances.
The cake layer is often the largest hydraulic resistance in the microfiltration of bacterial suspensions because of the highly compressible nature of the cell cake [4, 7]. Several methods have been developed to reduce specific cake resistance, mass of the deposited cake layer, and product rejection. These methods include changes to hydrodynamics via filtration module design [8-10], introduction of periodic back pulsing to break up the cake layer [11, 12], and feed pretreatments such as pH adjustment [4, 13].

We have previously reported on the effects of pH, temperature, and transmembrane pressure (TMP) on the dead-end filtration of a *Bacillus* fermentation broth where the cake layer was the dominant flux resistance [4]. Specific cake resistance increased with TMP and pH in a complex medium. Interestingly, in simple defined medium, increase of pH caused cell aggregation, which was not observed in the complex medium, resulting in a large decrease in the specific cake resistance. This result is expected as the particle size of the feed suspension has been shown to affect the permeate flux in both dead-end and crossflow filtrations.

The effect of particle size can be related to the specific cake resistance using the Carman-Kozeny equation [14],

\[
\alpha = \frac{k_2 (1 - \varepsilon)}{(\Phi d_p)^2 \varepsilon^3 \rho_p}
\]  

(7)
where \( \varepsilon \) is the porosity of the cake, \( \rho_p \) is the density of the particles, \( k_2 \) is the Kozeny constant associated with the morphology of the feed particles and typically assumed to have a value of 5 [15], \( \Phi_S \) is the shape factor, and \( d_p \) is the particle diameter/size. In crossflow microfiltration, the same relationship would be expected for the cake that forms; however, which particles are deposited depends on particle size as well as crossflow hydrodynamics resulting in ‘quasi’-steady state flux relations of the following form [2];

\[
J = c \gamma_o^n d_p^m \phi_b^q L^n \eta_o^r \tag{8}
\]

where \( \gamma_o \) is the wall shear rate, \( \phi_b \) is the particle concentration, \( L \) is the length of the flow channel, and \( \eta_o \) is the feed viscosity. The values of the constants \( c, n, m, q, r, z \) depend on the dominant particle transport mechanism. The models for surface transport, inertial lift, and shear-induced diffusion mechanisms have \( m \) equal to or greater than 1 (1, 3, and 1.33, respectively) and predict a positive correlation between permeate flux and particle size. Therefore, in both dead-end and crossflow filtrations, the particle size has an integral role in determining the flux, with larger particle size leading to higher flux, all else being equal. This suggests flocculation could be an effective treatment for the complex broth [4].

Flocculation has found extensive use in the waste water and biotechnology industries to aid in separation techniques, such as filtration, flotation, sedimentation [16], and
centrifugation [17], where rate improves with the size of the suspended particles. Previous studies have shown that the addition of a polyelectrolyte floculant results in increased permeate flux rates for the crossflow microfiltration of yeast cells [18, 19], CHO cell suspensions [20] in simple media, bentonite [21], and titanium oxide in deionized water [22], but microfiltration of a complex media broth has not been examined.

However, flocculation of cell suspensions by cationic polyelectrolytes also leads to a widening of the particle size distribution that can negate benefits of increased size. The reduced benefit is likely due to selective deposition of smaller particles because of greater shear-induced back diffusion of larger particles [18, 21, 23-25].

The factors that can affect flocculation by polyelectrolyte addition are polyelectrolyte properties, such as structure, charge density, and molecular weight; polyelectrolyte dose; solution conditions, such as pH, ionic strength, and solution composition; and the shear environment [26-28]. In studies using yeast and mammalian cell suspensions, cationic polyelectrolytes have been shown to be the most effective floculants [20, 29] due to the negative charge on the cells. The amount of polyelectrolyte that must be added to the suspension to obtain the desired degree of flocculation depends strongly on the characteristics of the suspension [26-28] and the polyelectrolyte. The primary mechanism for flocculation with low molecular weight polyelectrolytes is charge neutralization, where flocculation occurs due to neutralization of charges on the surface of the suspended particles [16]. Higher molecular weight polyelectrolytes generally require
lower doses compared to lower molecular weight polyelectrolytes, most likely because the flocs form via a bridging mechanism [30-33], where a single polyelectrolyte chain is absorbed onto two or more particles. Increasing the polymer charge density can result in a decrease in optimal polyelectrolyte dose [26].

Flocculated particles are very sensitive to their shear environment both during and after their formation. Initial high-shear mixing is sometimes required to ensure even distribution of polyelectrolyte. The duration of this period of intense initial mixing can have a strong effect on the final floc formed [34]. However, after the initial mixing, continued high shear can cause floc breakage that could lower permeate flux.

In microfiltration, the presence of the cake layer can also result in an increase in product rejection [35, 36] defined as

\[ R = 1 - \frac{C_{\text{perm}}}{C_{\text{feed}}} \]  

(9)

where \( C_{\text{feed}} \) is the concentration of the product in the feed and \( C_{\text{perm}} \) is the concentration of the product in the permeate. Wickramasinghe et al. [37] found that flocculation increased the rejection of a viral contaminant; however, no studies have examined the effect of flocculation on the rejection of a protein during microfiltration.
In this study, several cationic polyelectrolytes were screened to determine their effectiveness in increasing permeate flux via flocculation for a complex industrial fermentation broth containing an engineered protease, subtilisin. Simultaneous effects on subtilisin rejection were also investigated. To examine the effect of shear on the filtration performance of the flocculated particles, the filtrations were performed in three filtration modules with differing shear rates. These were the no-shear environment of an unstirred dead-end filter cell, the relatively low shear environment of a stirred dead-end cell, and the relatively high shear environment of a lab scale, crossflow filtration unit.

**Materials and Methods:**

*Membrane Preparation and Cleaning:*

The membranes were flat-sheet polyethersulphone (PES) with a nominal pore size of 0.2 µm (Koch Membrane Systems, Wilmington, MA). Circular (~4.45 cm diameter) and rectangular membranes (~22 x 17 cm) were cut from a larger sheet for dead-end and crossflow filtrations, respectively. New membranes were initially cleaned by permeation at 240 kPa using consecutive washes of 40 ml Ultrasil 11 (pH 11.5) (Ecolab, St. Paul, MN), 20 ml DI water, 40 ml Ultrasil 76 (pH 1.5) (Ecolab, St. Paul, MN), 20 ml DI water, 40 ml Ultrasil 11, and 20 ml DI water for each dead-end cell filtration. Before and after each crossflow filtration, wash steps were performed using the same series of cleaning fluids as used for the dead-end cell membrane washes as follows: Ultrasil 11 for 30 min, DI water for 15 min, Ultrasil 76 for 30 min, DI water for 15 min, Ultrasil 11 for 30 min, and water for 15 min at a crossflow rate of 2.4 m/s and a TMP of 240 kPa in total recycle.
Membranes were reused for subsequent filtrations and clean water flux was taken before each filtration to determine cleaning efficiency. Typical cleaning efficiencies (i.e. recovery of clean water flux compared to new membrane) were 99-100% and the membranes were replaced after 15 filtrations if this criterion was still being met.

*Feed Preparation:*

Industrial *Bacillus subtilis* broth (supplied by Genencor, A Danisco Division, Cedar Rapids, IA) from production of engineered subtilisin from a complex medium containing soymeal, glucose, and salts was used for all filtrations. The broth and enzyme were stabilized via pH adjustment and calcium chloride addition and screened through a #250 mesh screen to remove any large residual media components. The solids concentration of the fermentation broth after screening through the #250 mesh was approximately 15 g/L on a dry basis, broth conductivity was equivalent to that of a 0.15 M NaCl solution. Under these conditions the cells had a zeta potential of ~-24 mV. Batch to batch variability in the fermentation broth was controlled by using broth from the same fermentation for all of the experiments where direct comparisons were made; however, several batches of fermentation broth were used with similar results obtained for different broths at the same experimental conditions. The engineered subtilisin has a molecular weight of 28 kDa and a pI of 6.8 giving it a positive charge at the filtration pH of 6.5 [38].

*Flocculation:*
Table 1 gives the characteristics of the cationic polyelectrolytes used for these experiments (Kemira Water Solutions, Lakeland, FL). C-577, C-581, and C-591 were provided as liquid cationic polymers, and C-4224 was provided as an oil-free emulsion. For the same dose all polyelectrolytes provided, within a factor of two, the same charge equivalences (Table 1). One part fermentation broth was added to three parts polyelectrolyte in deionized water and mixed for 10 minutes at 300 rpm on a magnetic stir plate during which the pH of the flocculated broth was adjusted to pH 6.5 using calcium hydroxide (Fisher Scientific, Itasca, IL). The extended chain length of the polyelectrolyte was estimated from the bond lengths and the number of repeating units determine if the extended conformations of these polyelectrolytes were sufficient to flocculate these particles by a bridging mechanism (Table 1). The polyelectrolyte doses ranged from 0 to 0.035 g polyelectrolyte/g undiluted broth (henceforth expressed as 0 to 3.5 wt%). The reproducibility of floc formation was determined by measuring the particle size before the filtration. Flocculated broth was rejected if the volume average particle diameter ($D_{4,3}$) did not fall within 10% of the average $D_{4,3}$ (determined experimentally) obtained with a given polyelectrolyte at a specific dose.

*Unstirred and Stirred Dead-end Filtrations:*

All dead-end filtrations were performed in an Amicon dead-end filtration cell (Model 8050, Millipore Corp. Billerica MA) with an effective membrane area of 13.4 cm$^2$. The filtration cell’s stir bar was removed for the unstirred dead-end filtrations. Fifty milliliters of diluted broth was added to the filtration cell, which was then pressurized with nitrogen gas. All filtrations were performed with a 240 kPa TMP. Samples of the feed and final
retentate were collected for determination of the particle size distribution at the beginning 
and end of the filtration.

Permeate mass was measured using an electronic balance (Mettler-Toledo PG 6002-S, 
Columbus, OH). For unstirred, dead-end filtrations, flux data were collected during the 
linear range of the plot of $tA/V_{perm}$ versus $V_{perm}/A$ following the procedure of Graves et al. 
[4]. The mass of permeate was recorded at a frequency of 0.5 to 5 minutes depending on 
the flux (higher frequency for higher flux). Filtrations were run for two hours or until the 
mass of permeate collected reached 20 g. The filter cake was collected and allowed to air 
dry overnight to determine the dry cake mass.

For stirred, dead-end filtrations, the stirring rate was set to 200 rpm, which corresponds to 
a shear rate of approximately 300 s$^{-1}$. The average shear rate ($\gamma$) was estimated as being 
equivalent to fully developed, laminar flow in a rectangular slit with the top surface 
moving at the velocity of the tip of the stir bar, using equation 10.

$$\gamma = \frac{2\pi R \Omega}{d} \quad (10)$$

where $R$ is the radius of the stir bar (16 mm), $\Omega$ is the rotational velocity, and $d$ is the 
distance between the stir bar and the membrane surface (1.15 mm). Permeate flux was 
determined by measuring the change in permeate mass over time at 1, 2, 3, 4, 5, 8, and 10
minutes and then every 5 minutes thereafter, up to one hour or when air began to permeate the membrane from the bottom of the vortex (i.e. 27 g of collected permeate).

The viscosity of the cumulative permeate was measured using an Ostwald viscometer. After the filtration, the membrane was gently washed with DI water to remove all visible deposited cake, and a water flux was taken to determine the resistance of the fouled membrane.

Crossflow Membrane Experiments:
Crossflow experiments were performed in a Sepa CF II flat sheet crossflow filtration unit (Sterlitech Corporation, Kent, WA) with an effective membrane area of 140 cm². A 65 mil (1.6 mm) thick zig-zag (alternating spacer filaments touching opposite channel walls) spacer was placed in the feed channel as an eddy promoter. C-581 polymer doses were 0, 0.5, 1, and 2.0 wt%. The crossflow velocities (reported as the maximum superficial velocity occurring at the constriction between the spacer filament and the membrane surface) were 1.75 and 2.4 meters per second (m/s) and were controlled using a positive displacement diaphragm pump (Hydracell, Wanner Engineering, Minneapolis, MN). Cao et al. found by simulation of two dimensional flow around similar spacer filaments that the wall shear stresses vary widely with position for such configurations, with the peak shear stresses located in the constriction between the spacer filaments and the channel wall and the lowest shear stress located between the channel walls [39]. The shear rate magnitudes estimated from their results vary between approximately 1,000 and 80,000 s⁻¹ for the 1.75 m/s crossflow rate, and between approximately 2,000 and 200,000 s⁻¹ for the
2.4 m/s crossflow rate [39]. The TMP was maintained at 240 kPa using needle valves on the outlet retentate and permeate streams. The retentate and permeate were recycled to the feed tank to maintain constant feed concentration. The permeate flux was determined using an electronic balance (Mettler-Toledo PG 6002-S, Columbus, OH) to measure the change in permeate mass over time. A 10 ml sample of the 2 L feed was collected at the beginning of the filtration and 10 ml samples of permeate were collected at 10, 20, 40, and 55 minutes. The volume of permeate removed never exceeded 2% of the total feed volume; therefore, the operation was considered to be constant volume. Several 1 ml samples of the feed were taken at different times throughout the filtration for particle size distribution and zeta potential determination.

Upon completion of the filtration, the membrane was removed from the filter housing without any washing. The membrane was imaged using a Canon Digital Rebel XTi (Canon INC., Lake Success, NY). The particles of the cake attached to the membrane surface were then collected for particle size determination by gently rinsing the membrane with deionized water into a 50 ml centrifuge tube so as not to change particle size during cake transfer. The zeta potential and particle size distribution were then determined.

**Polyelectrolyte and Particle Size and Particle Zeta Potential:**

The hydrodynamic diameters of the polyelectrolyte flocculants were measured by dynamic light scattering at conditions similar to that of the flocculation (pH of 6.5, 0.1 M NaCl to match conductivity; 25 °C) using the Zetasizer Nano ZS90 (Malvern
Instruments, Southborough, MA). The polyelectrolyte solutions were prepared by adding 0.5 g of polyelectrolyte to 50 ml of a 0.1 M NaCl solution and mixing for 1 hour. The pH was then adjusted to 6.5 with NaOH addition.

To determine the particle size distribution and zeta potential during the filtration, 1 ml of the sample was diluted in 50 ml of DI water. The particle size distribution was measured using the Malvern Mastersizer E laser diffraction particle size instrument (Malvern Instruments, Southborough, MA) and the zeta potential was measured using a Zetasizer Nano ZS90 (Malvern Instruments, Southborough, MA). It was found that no change in the measured particle size or zeta potential occurred when the dilution was performed using a diluent with the pH and ionic strength adjusted to match that of the fermentation broth. The mass fractal dimension was calculated from the light scattering data obtained from the Mastersizer E, using the procedure outlined by Bushnell et al. [40] in order to determine the morphology of the flocculated particles.

_Rejection Coefficient and Enzyme Assay:_

For stirred and unstirred dead-end filtrations, 3 ml samples of the initial feed and the final cumulative permeate were collected for determination of subtilisin activity, from which the average membrane rejection was calculated using equation 6. In the crossflow filtrations, 10 ml samples of the initial feed together with permeate samples taken at 20, 40, and 55 minutes were analyzed for subtilisin activity. Reported crossflow times were corrected for the approximately 5 minute (~ 25 ml holdup volume) permeate hold up time. Permeate samples collected in the first 20 minutes of the actual filtration were
inaccurate due to dilution by the water in the hold up volume. Since the crossflow filtration was operated in total recycle mode, the subtilisin activity in the retentate was constant and equation 6 was used to determine the instantaneous membrane rejection.

Subtilisin activity was determined using spectrophotometric detection of the enzymatic cleavage of p-nitroanalide from the substrate N-succinyl-Ala-Ala-Ala-p-nitroanalide (AAA, Bachem Bioscience, King of Prussia, PA) at 405 nm. The substrate for the assay was prepared from a stock solution containing 30 mg/ml AAA in dimethyl sulfoxide (DMSO). All permeate and feed samples were prepared in triplicate with a dilution of 1:1 in Tris buffer (0.1 M Tris, 0.01 M CaCl$_2$, 0.005% Triton X-100, pH 8.6). Cells were removed from the feed samples by centrifugation for 2 minutes at 12,000 rpm. Feed supernatants and permeate samples were loaded onto the COBAS FARA II centrifugal analyzer (Roche, Basel Switzerland) for determination of subtilisin activity. All chemicals for the enzyme assay were purchased from Fisher Scientific (Itasca, IL), with the exception of Tween 80 and DMSO (Sigma Chemical Company, St. Louis, MO).

**Results and Discussion:**

**Floc size and Polyelectrolyte Properties:**

The addition of C-4224, C-577, and C-581 resulted in a significant increase ($p < 0.05$) in volume-averaged particle size ($D_{4,3}$) of the flocculated broth (Table 2). Flocculation with the addition of C-591 did not result in a significant increase in $D_{4,3}$ relative to the unflocculated broth; however, there was a significant increase in the fractal dimension of
the particles in the flocculated suspension ($D_f \sim 1.6$) compared to the unflocculated broth ($D_f \sim 1$) indicating that some degree of flocculation occurred. The largest flocs were formed by flocculation with C-4224, with C-581 forming the second largest flocs. This result is consistent with findings from the flocculation of other suspensions where high molecular weight polyelectrolytes caused the highest degree of flocculation [26, 30-33, 41, 42]. The higher molecular weight of C-4224 and C-581 provided a large extended chain length relative to the diameter of the Bacillus particles (~ 2 µm), providing the potential for bridging flocculation. However, higher polyelectrolyte molecular weight did not always result in larger flocs. The addition of C-577, the lowest molecular weight polymer tested, resulted in a higher $D_{4,3}$ than that of the flocs formed with the addition of C-591, a much higher molecular weight polyammonium flocculant. Smaller floc sizes were obtained by flocculation with C-577 and C-591. Since the extended chain lengths of C-577 and C-591 (Table 1) are smaller, charge neutralization, rather than bridging is likely the dominant flocculation mechanism, resulting in the smaller floc size obtained by flocculation with these polyelectrolytes. These results indicate that the structure of the repeating unit and the conformation that the polyelectrolyte acquires in solution has a greater influence on the final size of the flocculated particles than the molecular weight [26, 32, 43].

A typical particle size distribution of the unflocculated feed and the feed flocculated with a 0.5 wt% dose of C-581 is shown in Figure 1. Unflocculated particles had a fairly narrow distribution with the majority of particles between 1 and 8 µm in diameter, similar to the typical ~2 µm size of Bacillus subtilis cells. Additionally, a small amount of larger
particles, as large as 150 \( \mu \text{m} \), were also observed. These were likely residual soymeal solids that passed through the #250 mesh screen. The size range of was considerably wider (Figure 1). At the low shear level of the stirred dead-end cell module, there was no significant decrease in floc size over the time (1 h) of filtration (Table 2).

Based on these three polymers, polymer size was more important than charge density in determining particle size. This was confirmed when C-581, having the same chemistry and charge density as C-577 but higher molecular weight, was evaluated. It also resulted in larger flocs (Table 3).

**Stirred and Unstirred Dead-End Filtrations:**

The average permeate fluxes in dead-end filtration for feeds flocculated with three polyelectrolytes are shown in Figure 2 (unstirred) and Figure 3 (stirred). All flocculated broths showed higher flux to varying degrees, but the enhancements declined with time in the stirred cell. C-577, a low molecular weight, high charge density, linear quaternary polyamine, with the charged group located in the polymer backbone, provided the greatest improvement. The addition of C-4224, a high molecular weight, low charge density, polyacrylamide, resulted in the smallest increase in permeate flux of the polyelectrolytes tested, despite the fact that it resulted in the largest initial flocculated particle size (Table 2). Flocculation with C-591, a mid-range molecular weight, quaternary polyammonium, with the charged group located in a ring structure in the repeating unit, resulted in greater permeate flux than that obtained with the larger floc-producing C-4224 but lower flux than with C-577. In the stirred cell, only the addition of
C-577 resulted in a significant increase ($p < 0.05$) in the permeate flux relative to that of the unflocculated suspension.

Fouling, as measured by the reduction in clean water flux, was the same for all broths (ca. 50% reduction), so the enhancement is attributed to cake properties. Of these, the increase in the particle size of the feed suspension is likely the dominant factor. However, as shown in Figures 2 and 3, the filtration with the initial largest flocculated particle size (C-4224) resulted in the smallest increase in the permeate flux. Comparison of average sizes could overlook increased resistance from greater numbers of smaller particles present or higher permeate viscosity. However, permeate viscosity was unchanged and examination of the number distribution (more sensitive to the presence of smaller particles) also showed no significant difference at the small particle end. Based on the fractal dimension of the flocs (Table 2), particle morphology was also similar for all flocculants. It may be that the chain length of C-4224 increases cake resistance. Polyelectrolyte doses in all cases were much less than required to neutralize particle charge so levels of unbound polyelectrolyte should be low, but extended chains may have added an additional sieving resistance within the cake offsetting the benefit of the increased particle size.

**Polyelectrolyte Molecular Weight and Dose:**

Since the polyamine repeating unit of C-577 showed the greatest increase in the permeate flux of the polyelectrolytes tested, the effects of the polyelectrolyte molecular weight and dose were examined further by flocculating the feed with C-577 and C-581, a high
molecular weight polyelectrolyte of the same repeating unit and charge density as C-577 (Table 1).

Flocculation with either C-577 or C-581 increased the permeate flux for all doses tested both in stirred (Figure 4 as reduced cake resistance) and unstirred (Figure 5 as flux enhancement) dead-end filtrations. The flux enhancement \( \eta \) is defined as

\[
\eta = \frac{J_{\text{floc}}}{J_{\text{nofloc}}}
\]  

(11)

where, \( J_{\text{floc}} \) is the permeate flux resulting from the flocculated feed and \( J_{\text{nofloc}} \) is that of the unflocculated feed. Highest fluxes were obtained with the higher molecular weight C-581. Since the flux enhancement changes with extent of filtration, Figure 5 is presented for a CF of 1.18 at which point the relative fluxes have obtained a fairly constant value.

For these two polyelectrolytes, differing only in molecular weight, there is a correlation between increased floc size with the higher molecular weight and decreased cake resistance and greater flux enhancement. The correlation of flux measures with floc size persists for dosages up to 1 wt\% but not beyond. The fractal dimension of the flocs remains close to 1.8 for all nonzero dosages. This is similar to the observation with C-4224 flocculation of low permeate flux despite a larger particle size, although this occurs at higher polyelectrolyte doses for C-577 and C-581 than C-4224.
This eventual decrease in flux at the highest dosages was not accompanied by increases in permeate viscosity or internal membrane fouling. However, the zeta potential of the flocculated particles was \(~\text{21 mV}\) for all doses of C-581, greater than 0.2 wt\% indicating that some excess flocculant may be free in solution which could add to the cake and increase the transport resistance.

**Product Rejection:**

Subtilisin rejection averaged over the entire filtration was measured for all of the stirred and unstirred dead-end experiments by comparing the subtilisin activity of the initial feed to that of the final cumulative permeate. The subtilisin rejection averaged over all of the polyelectrolytes and doses tested was higher in the stirred dead-end filtrations \((R = 0.24)\) than the unstirred filtrations \((R = 0.09)\). There were no significant difference \((p < 0.05)\) in the rejection among the different polyelectrolytes and doses. The lack of any polyelectrolyte/dosage effect in the unstirred filtrations would indicate that the cake itself is not a factor in rejection. More puzzling is the increased rejection for the stirred dead-end filtrations. The only factor that would seem to lead to increased rejection would be that preferential deposition of smaller particles seen in crossflow \([44, 45]\) leading to a cake or pore plugging capable of rejection.

**Crossflow Experiments:**

The crossflow filtration experiments were performed using C-581 as this polyelectrolyte flocculant resulted in the greatest increase in the permeate flux in the stirred and unstirred dead-end filtrations. In the crossflow experiments, which varied flow rate and
polyelectrolyte dose, declines in clean water flux were approximately 50% as they had been in the earlier dead-end experiments.

**Particle Size:**

For all of the polyelectrolyte doses, the size of the flocculated particles was reduced to that of the unflocculated suspension within the first 30 minutes of the filtration (Figure 6). The flocs were exposed to much higher shear rates in this device than in the stirred cell where there was little breakage. The evolution of the particle size distributions provides evidence for a dosage-dependent breakage mode. For the 0.5 wt% dose, the decreasing intensity with filtration time of the peak associated with the large flocs and the increasing intensity with time of the peaks associated with smaller particles (Figure 7A) is characteristic of erosion. For higher doses, the movement of a monomodal distribution to progressively smaller sizes is indicative of fragmentation (Figure 7B). Similar results were obtained for the 2.4 m/s crossflow rate (results not shown); however, the flocs formed by the 2 wt% polymer dose break down faster at the higher crossflow rate and their size reaches that of the unflocculated suspension after about 20 minutes.

**Permeate Flux:**

Flocculation by the addition of C-581 increased in permeate flux compared to the unflocculated feed for all of the polyelectrolyte doses and crossflow rates tested (Figures 8 - 10). Improvements in $\varphi$ were small during the period of rapid flux decline (the first 5-10 min) then increased until the permeate flux became ‘quasi’ - steady approximately 30 minutes into the filtration (Figure 10). The longer-term flux improvements persisted well
beyond the point (ca. 5-10 min) at which the flocculated particles in the recycled retentate were fully disrupted (Figure 6). At the lower crossflow, flux enhancement increased with each dosage increase; however, at the higher crossflow, a dosage of 1% was optimal. Though the relative enhancements were greater at lower crossflow (Figure 10), the actual fluxes were greater at the higher crossflow (Figures 8 and 9).

While flocs suspended in the retentate were disrupted well before completion of the filtration, flocs remaining on the membrane at the end of the run were nearly the same size as in the initial feed both as regards $D_{4,3}$ (Table 4) and size distribution (Figure 11). For the unflocculated feeds, the deposited particles were larger than the feed particles (Table 4 and Figure 12). While the initial unflocculated feed had little total volume in the 10-100 µm range (and that likely residual soy meal), most of the deposited cake was of that size (Figure 12). Thus this final cake likely consists of cell aggregates formed by cake compression and residual soy meal. As with the flocculated feed cake, the unflocculated feed cake had somewhat larger particles at the lower crossflow. While the particles were larger, it is also likely that the cake was thicker at lower crossflow and the latter factor would have caused the lower fluxes. However, gravimetric measurement of cake mass was not sufficiently accurate to verify this conclusion.

The large particles in the cake likely deposited early in the lower shear regions of the spacer-filled feed channel, while the high shear region coverage would have been thinner and populated by small particles less mobilized by shear-induced back diffusion and more prevalent as the filtration proceeded. This is seen in the pattern of deposited cake
(Figure 13), which is very similar to the pattern seen in the simulation of flow through a spacer of similar geometry by Koutsou et al. [46]. The locations of the thicker deposits (the dark regions of Figure 13) correspond to the low shear regions in their simulation. The higher shear regions would be expected to have deposits renewed from the lower size particles that increasingly predominate with time. Hence, a fraction of the membrane area would permeate with the initial higher flux while the remainder would provide declining flux.

For the crossflow experiments, the changes in flux (Figures 8-10) and deposited particle size (Table 4) do correspond well providing an explanation for dosage-dependent trends. The observed presence of the larger particles characteristic of the filtration feed in the cake layer is in contrast to the result expected from microfiltration of widely polydisperse feeds, where smaller particles preferentially deposit in higher shear regions [23-25]. The larger particles in the cake layer are likely a result of both the early deposition of the full ranged flocculated feed particles in the low shear regions and compaction of the particles on the membrane surface. The decrease in the size of the particles in the cake layer with increasing crossflow rate indicates that, at the low crossflow the shear-induced diffusion in the low shear regions is insufficient to keep the larger particles off the membrane; whereas, at the higher crossflow rate the increased shear-induced diffusion is able to prevent deposition. Additionally, the zeta potential of the cake layer particles of the flocculated feeds were lower than that of the original feed (Table 4) indicating that some rearranging of the flocculated particles or the adsorbed polyelectrolytes occurred in the particles that are compacted on the membrane surface.
**Product Rejection in Crossflow:**

Flocculation was advantageous for recovery of subtilisin in the permeate. Flocculation decreased the rejection of subtilisin for all flocculant doses and crossflow rates examined, with a greater decrease in rejection for flocculant doses greater than 0.5 wt% (Figures 14 and 15). Possibly relevant factors affecting rejection would be the cake pore structure (smaller particles/pores increasing rejection), electrostatic interactions of proteins with the cells in the cake (assisting rejection) [35], and flux-dependent polarization (decreasing apparent rejection). Since flux (Figure 8 and 9) and particle size (Table 4) vary oppositely with crossflow, the particle size effect must have dominated to account for the lower rejection at lower crossflow. This flow-dependent aspect of rejection behavior is the same as that observed in the dead-end filtrations where crossflow increased rejection.

Given the lower cake particle zeta potential after flocculation (Table 4) reduced electrostatic interaction may also be reducing rejection [47, 48]. Rejection of subtilisin was roughly equivalent at flocculant doses greater than 0.5 wt% for both the 1.75 and 2.4 m/s crossflow rate.

A final advantage of flocculation is that subtilisin rejection did not increase with filtration time as did happen when no polyelectrolyte flocculant was used (Figure 14 and 15). At the 2 wt% dose there is actually a decrease between 20 and 40 minutes. These
experiments did not allow for determination of whether the changes with time resulted from changes in the membrane or changes in the cake.

Conclusions:

Flocculation with cationic polyelectrolytes increased the permeate flux in unstirred dead-end, stirred dead-end, and crossflow filtrations, and decreased product rejection in crossflow filtration. Larger flocs were formed with longer-chain polyelectrolytes. In unstirred and stirred dead-end filtrations the optimal polyelectrolyte dose was 0.5 wt% for both C-577 and C-581. While particle size plays a significant role in the permeate flux, larger particle size did not always result in an increase in the permeate flux. The different polyelectrolyte repeating units influenced flux in ways that could not be accounted for by floc size, polyelectrolyte molecular weight, membrane fouling, or solution properties.

In crossflow filtrations, long-term increases in the permeate flux were observed with flocculation, despite complete disruption of the flocculated particles in the recycled retentate, a result explained by the apparent persistence of the initially formed cake in lower shear regions of the spacer-filled feed channel. The optimal polyelectrolyte dose for flux enhancement was dependent on the crossflow rate and flux increased with crossflow rate.

Acknowledgments:

This work was funded by Genencor, A Danisco Division. The authors thank Kevin Graves, Iowa State University graduate student, for performing the unstirred dead-end
polyelectrolyte screening filtrations and Pete Keyes and Marci Baldwin from Genencor, A Danisco Division for providing assistance with the subtilisin assay.

**Symbols:**

$C_{perm}$, activity of the permeate

$C_{feed}$, activity of the feed

$CF$, concentration factor

$J$, permeate flux (LMH)

$J_{floc}$, permeate flux of the flocculated suspension

$J_{nofloc}$, permeate flux of the unflocculated suspension

$A$, membrane area ($m^2$)

$\Delta p$, transmembrane pressure (kPa)

$\mu_0$, permeate viscosity (cP)

$\mathcal{R}$, overall product rejection

$m$, mass of the cake layer in dead-end filtrations (g)

$\mathcal{F}$, Flux enhancement

$R_m$, membrane resistance ($cm^{-1}$)

$R_c$, cake resistance ($cm^{-1}$)

$\alpha$, specific cake resistance ($cm/g$)

$\rho_c$, mass of dry cake per volume of permeate ($g/cm^3$)

$t$, filtration time (s)

$V_{perm}$, volume of permeate (ml)

$V_{feed}$, volume of the feed (ml)
ε, porosity of the cake layer

d_p, particles diameter (µm)

γ_0, wall shear rate (s^{-1})

φ_b, particle concentration in the bulk

Φ_s, particle shape factor

L, length of the filter channel (m)

η_0, viscosity of the feed (cP)

R, radius of the stir bar in the stirred dead-end filtration unit (m)

d, gap distance between the stir bar and the membrane in the stirred dead-end filtration unit (m)

Ω, rotational velocity of the stir bar (rev/s)

References:


<table>
<thead>
<tr>
<th>Polyelectrolyte</th>
<th>Repeating Unit $^a$</th>
<th>Molecular Weight $^a$</th>
<th>Charge Density $^a$</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>Extended chain length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superfloc</td>
<td>AMD$^b$/Q9$^c$/QE9$^d$</td>
<td>8000000</td>
<td>40</td>
<td>87.2</td>
<td>3</td>
</tr>
<tr>
<td>C-4224</td>
<td>Polyacrylamide</td>
<td>80000</td>
<td>100</td>
<td>21.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Superfloc</td>
<td>DMA$^e$/EPI$^f$</td>
<td>2500000</td>
<td>100</td>
<td>78.9</td>
<td>1.6</td>
</tr>
<tr>
<td>C-581</td>
<td>Polyamine</td>
<td>5000000</td>
<td>100</td>
<td>37.7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

$^a$ Properties reported by manufacturer  
$^b$ AMD: acrylamide  
$^c$ Q9: acryloyloxyethyltrimethylammonium chloride  
$^d$ QE9: acryloyloxyethyltrimethylammonium chloride  
$^e$ DMA: dimethylamine  
$^f$ EPI: epichlorohydrin  
$^g$ DADMAC: diallyldimethylammonium chloride  
$^h$ molecular weight reported by manufacturer from viscosity measurements
Table 2. Particles size ($D_{4,3}$) and mass fractal dimension at start and finish of stirred-cell filtrations. The polyelectrolyte doses were 0.2 wt% (g polymer/ g undiluted broth). The error represents 95% confidence intervals.

<table>
<thead>
<tr>
<th>Polyelectrolyte</th>
<th>Initial $D_{4,3}$ (µm)</th>
<th>Mass Fractal Dimension initial</th>
<th>Final $D_{4,3}$ (µm)</th>
<th>Mass Fractal Dimension final</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Poly</td>
<td>7 [±1.6]</td>
<td>1.01</td>
<td>7 [±0.6]</td>
<td>1.01</td>
</tr>
<tr>
<td>C-4224</td>
<td>97 [±16]</td>
<td>1.59</td>
<td>80 [±23]</td>
<td>1.6</td>
</tr>
<tr>
<td>C-577</td>
<td>15 [±0.4]</td>
<td>1.61</td>
<td>13 [±0.8]</td>
<td>1.58</td>
</tr>
<tr>
<td>C-581</td>
<td>45 [±3.1]</td>
<td>1.81</td>
<td>40 [±2.5]</td>
<td>1.73</td>
</tr>
</tbody>
</table>
Table 3. Initial particle size by volume ($D_{4,3}$) measured for C-577 and C-581 at different polymer doses used for unstirred dead-end, stirred dead-end, and crossflow filtrations.

<table>
<thead>
<tr>
<th>Polyelectrolyte Dose (wt%)</th>
<th>$D_{4,3}$ ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-577</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>0.2</td>
<td>15</td>
</tr>
<tr>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>3.5</td>
<td>80</td>
</tr>
</tbody>
</table>

$^a$ For these values a portion of the by volume particle size distribution was larger than the limit of detection of the instrument (600 $\mu$m) so the actual $D_{4,3}$ values is larger than the reported value.
Table 4. $D_{4,3}$ for particles collected from the membrane surface after crossflow filtration with C-581 at two crossflow rates (1.75 and 2.4 m/s). Initial feed particle sizes can be found in Table 3. Error represents a 95% confidence interval.

<table>
<thead>
<tr>
<th>Polyelectrolyte Dose (wt%)$^a$</th>
<th>$D_{4,3}$ (µm)</th>
<th>Zeta Potential (mV)</th>
<th>$D_{4,3}$ (µm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.75 m/s</td>
<td>2.4 m/s</td>
<td>1.75 m/s</td>
<td>2.4 m/s</td>
</tr>
<tr>
<td>0</td>
<td>50</td>
<td>-24.8 [±1.06]</td>
<td>37</td>
<td>-24 [±1.1]</td>
</tr>
<tr>
<td>0.5</td>
<td>166</td>
<td>-19.3 [±1.01]</td>
<td>148</td>
<td>-19.6 [±1.2]</td>
</tr>
<tr>
<td>1</td>
<td>190</td>
<td>-19.4 [±1.3]</td>
<td>137</td>
<td>-19.4 [±1.04]</td>
</tr>
<tr>
<td>2</td>
<td>&gt;203$^b$</td>
<td>-18.4 [±1.07]</td>
<td>128</td>
<td>-18.7 [±1.1]</td>
</tr>
</tbody>
</table>

$^a$Zeta potential of the initial flocculated feed was -21 mV ± 1 for all of the polyelectrolyte dose levels from 0.5 – 2 wt% and -24 mV ± 1 for the broth before flocculation

$^b$A large portion of the by volume particle size distribution was larger than the limit of detection of the instrument (600 µm) for this measurement so the actual $D_{4,3}$ values is larger than the reported value.
Figure 1. Typical particle size distribution of the unflocculated suspension and the suspension flocculated with a polyelectrolyte dose of 0.5 wt% C-581.
Figure 2. Comparison of different polyelectrolytes for their enhancement of permeate flux rates for unstirred dead-end filtrations.
Figure 3. Effect of polyelectrolyte properties on the permeate flux in stirred dead-end filtration. Error bars represent a 95% confidence interval.
Figure 4. Effect of concentration of C-577 and C-581 on the average specific cake resistance in unstirred dead-end filtrations.
Figure 5. The effect of polymer dose on the flux enhancement of the flocculated permeate flux to the unflocculated permeate for C-577 and C-581 at a concentration factor (CF) of 1.18 in stirred dead-end filtrations.
Figure 6. The effect of filtration time on the by volume-averaged particle diameter $D_{4.3}$ ($\mu$m) in crossflow filtration for polyelectrolyte doses of, 0.5, 1.0, and 2.0 wt% C-581 polymer doses C-581 at a crossflow rate of 1.75 m/s.
Figure 7. The development of the particle size distribution during microfiltration of the flocculated feed at a crossflow rate of 1.75 m/s. A: 0.5 wt% C-581 and B: 2 wt% C-581.
Figure 8. Average permeate flux at a crossflow rate of 1.75 m/s at 0, 0.5, 1.0, and 2.0 wt% C-581 polymer doses. Experiments were replicated three times with a 95% confidence interval of ±0.5 LMH.
Figure 9. Average permeate flux at a crossflow rate of 2.4 m/s at 0, 0.5, 1.0, and 2.0 wt% C-581 polymer doses. Experiments were replicated three times with a 95% confidence interval of ± 0.5 LMH.
Figure 10. Observed flux enhancement during crossflow microfiltration after flocculation of the feed with the addition of C-581. Solid lines represent 1.75 m/s crossflow rate and dashed lines represent 2.4 m/s crossflow rate.
Figure 11. Typical particle size distributions of the feed and the cake layers formed during a filtration with a 1.75 and 2.4 m/s crossflow rate at a 0.5 wt% dose of C-581.
Figure 12. Typical particle size distributions of the feed and the cake layers formed during a filtration with a 1.75 and 2.4 m/s crossflow rate of the unflocculated feed.
Figure 13. Images (with background digitally reduced to improve contrast) of a segment of the membrane surface showing the distribution of particles deposited on the membrane surface at the end of the filtration. Arrows indicate the direction of the bulk crossflow and the lines indicate the approximate location of the spacer filaments, solid and dashed lines represent where spacer filaments contacted the membrane surface and the opposite channel wall respectively. A: Unflocculated feed with a crossflow rate of 2.4 m/s. B: Flocculated feed with a 0.5 wt% C-581 dose and a crossflow rate of 2.4 m/s.
Figure 14. Rejection of subtilisin over short time intervals throughout the filtration for a crossflow rate of 1.75 m/s and 0, 0.5, 1, and 2 wt% C-581 doses. Error bars represent a 95% confidence interval.
Figure 15. Rejection of subtilisin over short time intervals throughout the filtration for a crossflow rate of 2.4 m/s and 0, 0.5, 1, and 2 wt% C-581 doses. Error bars represent a 95% confidence interval.
Chapter III: Clarification of Aqueous Corn Extracts by Tangential Flow Microfiltration

A paper to be submitted to the Journal of Membrane Science

Matthew T. Aspelund¹ and Charles E. Glatz¹*

Abstract

The effectiveness of tangential flow microfiltration for the solid/liquid clarification of aqueous corn endosperm and germ extracts was examined using recombinant type I human collagen (rCollagen) and green fluorescent protein (GFP) as model proteins. We identified the effects of transmembrane pressure (TMP), crossflow rate, protein molecular weight, and membrane chemistry on permeate flux, protein rejection, and internal membrane fouling in microfiltration. In dead-end filtrations, both endosperm and germ extracts formed highly compressible cakes that provided the dominant hydraulic resistance. For tangential flow filtration using a ceramic membrane, increasing the crossflow rate had a significant beneficial effect on the permeate flux for all TMPs examined. High fouling of the ceramic membrane occurred during the filtration and this was likely due to the presence of soluble corn starch in the feed. Filtration with a ceramic membrane resulted in low rejection (<10%) of both the host cell proteins (HCP) and GFP and very high rejection (~90%) of rCollagen. In contrast to the ceramic membrane, tangential flow filtration using a poly(vinylidene fluoride) membrane resulted in much less internal fouling and no measurable rejection of HCP, GFP, or rCollagen. Microfiltration was an effective method for the solid/liquid clarification of corn protein
extracts, except in cases where high internal membrane fouling resulted in increased rejection of large molecular weight proteins.

**Keywords:** Tangential flow filtration; Microfiltration; Clarification; Transgenic corn; Protein rejection; Membrane chemistry; Recombinant collagen

**Introduction**

Production of high-volume, lower-value recombinant proteins in transgenic corn seed offers an economic alternative to protein production from cell culture and animal sources. Plant-based production avoids the risk of contamination from mammalian-associated infectious agents (i.e. prions, and viruses) and retains considerable capability for post-translational modifications similar to those occurring in mammalian cell cultures [1]. Plant hosts also have the ability to target the expression of recombinant proteins to specific tissues or organs, including those which allow for prolonged storage (seeds). Extraction of only the targeted tissue reduces the extraction volume and impurity level [1].

Among plant-based recombinant-protein production systems, corn seed offers the advantages of low water-soluble protein content and well-established methods for genetic manipulation, transformation, cultivation, and processing by bio-refining into multiple industrial products. Corn has been explored as an expression host for several recombinant proteins including dog gastric lipase [2], monoclonal antibodies [3], vaccines [4], aprotinin [5], and, more recently, collagen-related proteins [6, 7], which
have also been expressed in tobacco [8, 9] and barley [10]. Corn seed consists largely of endosperm (83% dry weight), which is comprised mainly of starch and water-insoluble proteins, along with germ (11% dry weight), which contains most of the seed’s oil and water-soluble protein. Combining endosperm-targeted expression with dry-milling to isolate an endosperm rich fraction provides an extract with the lowest host cell protein (HCP) burden [1]. While issues of contamination of non-GMO corn with transgenes has delayed large-scale use of corn for recombinant protein production, methods of risk reduction that would enable future adoption are being developed [11].

The low cost that is associated with the production of recombinant proteins in plants and the potential for coupling protein production to current crop-based biorefineries makes the plant host system ideal for the production of low-value/high-volume proteins (e.g. protein-based materials, industrial enzymes) [7]. However, the purification process becomes the major cost, comprising up to 90% of the overall production cost of recombinant proteins in plants [12]. Thus, development of efficient and inexpensive methods for the clarification of plant extracts and purification of recombinant proteins from the host plants is crucial.

A major step that is required in the purification process is the solid/liquid clarification of the protein extracts. Currently, most of the work regarding the clarification of recombinant proteins from corn extracts has used centrifugation [2, 5], rotary drum vacuum filtration (RVDF), or expanded bed chromatography [13, 14] as the solid/liquid clarification step. Impediments to the alternative of microfiltration include membrane
fouling by soluble feed components and the buildup of a compressible layer of rejected particles on the membrane surface (i.e. cake layer), both of which can decrease permeate flux and increase protein rejection [15, 16]. However, microfiltration offers the advantages of low cost operation, the elimination of filter aids [17], the ability to handle the high solids content (10 - 25% w/w) and large particle size range (sub-micron to millimeter) associated with corn extracts [18], and relatively mild operating conditions, which minimize protein denaturation.

Formation of a cake layer often provides the greatest hydraulic resistance to permeate flux in microfiltration. In tangential flow microfiltration, the formation of the cake layer is affected by the filtration operating conditions (crossflow rate and TMP) as well as the feed conditions (particle size, pH, and ionic strength). Several methods exist to reduce the resistance and formation of the cake layer. Increasing the crossflow rate generally increases the permeate flux by reducing the mass of the deposited cake layer due to increased sweeping of the particles from the membrane surface [19-23]. Also, larger feed particle size can help form a more porous cake layer, thus, increasing the permeate flux [15, 24, 25]. Reduction of cake layer formation and solute rejection by the cake layer can also be achieved by operating the filtration below the critical TMP (i.e. in the pressure-dependent permeate flux regime) [26-28].

In this study, the suitability of tangential flow microfiltration for the solid/liquid clarification of corn protein extracts from endosperm- and germ-rich dry milling fractions was examined. We used triple helical type I recombinant human collagen (rCollagen, 265
kDa) and green fluorescent protein (GFP, 27 kDa) as model proteins to assess the effect of protein molecular weight on rejection during microfiltration of corn protein extracts. The effect of crossflow rate, TMP, and membrane chemistry on the permeate flux, product transmission, and membrane fouling during tangential flow microfiltration of corn extracts was examined. In addition, the compressibility of the filter cake that formed during the filtration of corn extracts was determined in dead-end microfiltration.

**Materials and Methods**

**Materials**

All chemicals were reagent grade and purchased from Fisher Scientific (Itasca, IL) unless noted otherwise. Preparation of rCollagen, expression of GFP in transgenic corn, and methods for rCollagen spiking, dry milling, protein extraction are described by Aspelund and Glatz [29].

**Corn Defatting**

GFP-expressing germ- and endosperm-rich fractions were milled a burr-type coffee mill (KitchenAid model KPCG100) with 400 grams of fractionated grain in each batch. The resultant flours had particle sizes ranging from 0.01 mm to >1 mm for both the endosperm-and germ-rich fraction, as determined by dry sieving. Oil was extracted from the milled endosperm- and germ-rich fractions as described in Aspelund and Glatz [29].

**Solids Settling and Clarification**
All dead-end and tangential flow filtrations described in this work were performed using either the suspension that remained after settling or clarified extracts. To prevent clogging of the filtration modules used in these experiments, a solids settling step was performed as follows. Subsequent to extraction, the corn slurry was allowed to settle for 30 seconds, from a maximum settling distance of 15 cm, and the supernatant was decanted to separate the rapidly settling solids from the solids that remained in suspension. Alternatively, the extracts were clarified by centrifugation at $3000 \times g$ and 4 °C for 15 minutes, followed by filtration through a 0.2 µm syringe filter (Sigma-Aldrich; St. Louis, MO).

**Unstirred Dead-end Filtration**

Dead-end filtrations were performed using a 0.2 µm pore size flat-sheet polyethersulfone (PES) membrane (Koch Membrane Systems, Wilmington, MA) in a dead-end filtration cell (Model 8050, Millipore Corp. Billerica MA) with an effective membrane area of 13.4 cm². Circular (4.45 cm diameter) membranes were cut from a larger sheet. Fifty ml of endosperm or germ extract was added to the filtration cell, which was then pressurized to the desired filtration pressure using compressed nitrogen. Dead-end filtrations were performed at several TMPs between 0.34 and 3.4 bar. Permeate mass was measured using an electronic balance (Mettler-Toledo PG6002-S, Columbus Ohio). The specific cake resistance, $\alpha$, for unstirred dead-end filtrations can be obtained from the slope of a plot of $tA/V_{perm}$ versus $V_{perm}/A$ using the following equation:
\[
\frac{tA}{V_{\text{perm}}} = \frac{\rho_c \alpha_0 \mu_0}{2 \Delta p} \left( \frac{V_{\text{perm}}}{A} \right) + \frac{\mu_0 R_m}{\Delta p}
\] (1)

where \(V_{\text{perm}}\) is the permeate volume, \(\mu_0\) is the dynamic viscosity of the permeate, \(R_m\) is the membrane resistance, \(\Delta p\) is the transmembrane pressure, \(A\) is the filtration area, \(m\) is the mass of the cake layer per membrane area, and \(\rho_c\) is the mass of dry filter cake per unit volume of permeate [30]. For compressible cakes, the specific cake resistance varies with position in the cake [31]; therefore, equation 1 estimates a specific cake resistance averaged over the entire thickness of the cake layer. The compressibility index, \(n\), of the filter cake was calculated using equation 2

\[
\alpha = \alpha_0 \Delta p^n
\] (2)

where \(\alpha_0\) is the zero-pressure specific cake resistance. A value of zero for \(n\) represents an incompressible cake and an increasing value of \(n\) represents increasing cake compressibility. Flux data were collected during the linear range of the plot of \(tA/V_{\text{perm}}\) vs. \(V_{\text{perm}}/A\), determined experimentally, for both endosperm and germ extracts. Samples of the feed and final permeate were collected and assayed for HCP, GFP, and rCollagen concentration. The filter cake was collected and allowed to air dry for 24 hours to determine the dry cake mass. Each filtration was performed in duplicate.

The protein rejection coefficient was calculated as,
\[ R = 1 - \frac{C_{\text{perm}}}{C_{\text{feed}}} \]  

(3)

where \( C_{\text{feed}} \) is the concentration of the protein in the feed and \( C_{\text{perm}} \) is the concentration of the protein in the permeate [32, 33].

**Tangential Flow Filtration**

Tangential flow microfiltration was performed using a Membralox 0.2 µm pore size tubular (7 mm diameter) ceramic membrane with an effective membrane area of 50 cm\(^2\) (Pall Filtration, Port Washington, NY). The crossflow rate and inlet and outlet pressure on the feed side of the membrane were adjusted using a peristaltic pump (Masterflex, 7518, Cole Parmer, Chicago, IL) and a pinch valve that was located downstream of the filtration module. Pressure on the feed side was monitored using two pressure gauges (glycerin filled, 0 – 60 psi, Pall Filtration, East Hills, NY), located upstream and downstream of the filtration module. Additionally, filtrations of clarified extracts were performed using both the ceramic filtration module and a lab-scale tangential flow filtration system (Millipore; Billerica, MA) with a 0.2 µm Durapore poly(vinylidene fluoride) (PVDF) Pellicon XL membrane cassette that has a membrane surface area of 50 cm\(^2\) (Millipore; Billerica, MA). Only clarified extracts were used with the Pellicon filtration module because the filtration cassettes were clogged by the corn solids. Prior to filtration, the membranes were conditioned by filtering 500 ml of a 0.1 M sodium phosphate / 0.15 M sodium chloride buffer at pH 2. Permeate flux was obtained by
measurement of the change in the permeate mass over time using an electronic balance (Mettler-Toledo, PG6002-S, Columbus, OH) interfaced with a computer using LABVIEW (National Instruments, Austin, TX).

Filtrations were performed in total recycle mode at three crossflow rates (0.5, 0.8, 1.2 m/s) and three TMPs (0.2, 0.8, and 1.6 bar). The uppermost crossflow rate and TMP were the highest attainable in the filtration module used for this work. Desired combinations of crossflow rate and TMP were set and operated in total recycle mode for 20 minutes or until 50 ml of permeate passed through the membrane, upon which time the permeate flux was measured and 1-ml samples of the recycled feed and permeate were collected for determination of HCP, GFP and rCollagen concentration. Protein rejection was calculated using equation 3. Subsequently, the permeate flow was stopped to allow for the disruption of the deposited cake by the crossflow. The process was repeated for the next condition in the set of randomly ordered operating conditions. All filtrations were performed in duplicate.

The ceramic membrane was cleaned using reversed permeate flow with 4 L of 0.5 N sodium hydroxide and 250 ppm bleach at 55 °C and a TMP of 0.3 bar. The PVDF membrane was cleaned by washing the membrane for one hour with 250 ppm bleach at 25 °C and a TMP 0.3 bar. The clean-water flux was measured pre and post filtration and upon completion of membrane cleaning. After membrane cleaning, clean-water flux recoveries of greater than 90% were achieved for all of the filtrations.
**Particle Size Distribution**

The particle size distribution of the corn solids was measured using a Malvern Mastersizer 2000 laser diffraction particle size instrument (Malvern Instruments, Southborough, MA).

**Protein and Soluble Starch Determinations**

The concentration of rCollagen, total HCP, and GFP were determined using size exclusion chromatography (SEC-HPLC) and fluorescent spectrophotometry as described by Aspelund and Glatz [29] using a BioBasic SEC-300 size exclusion column 300 x 7.8 mm and a BioBasic guard column 30 x 7.8 mm (Thermo Scientific) for the SEC-HPLC assay.

The presence of soluble starch in the clarified filtration fractions was determined by the addition of 0.3 v/v% iodine. The development of a blue black color indicated the presence of soluble starch.

**Results and Discussion**

**Filtration Feed Particle Size**

Dry milling of the GFP grain resulted in a germ-rich fraction consisting of 38% germ, 22% endosperm, and 40% bran, and an endosperm-rich fraction containing >99% endosperm [29]. The particles in both the germ- and the endosperm-rich fractions had a very wide particle size distribution, ranging from smaller than 1 µm to greater than 1000
µm (Figure 1). The majority of the particles (~82 % by mass) before extraction were smaller than 710 µm, with 23% of the endosperm-rich and 19% of the germ-rich fractions by mass having a particle size less than 250 µm as determined by dry sieving. Removal of the rapidly settling particles in the extract reduced the solids concentration from 0.1 g/ml extract to 0.051 g/ml extract for the endosperm extracts and to 0.026 g/ml in the germ extracts. The settling step also resulted in the removal of a large portion of the particles that had sizes greater than ~500 µm from both the endosperm and the germ extracts (Figure 1). The volume averaged particle size ($D_{4,3}$) of the endosperm and germ extracts were 57 and 53.4 µm, respectively. The majority of the particles in the endosperm extract after settling were between 10 and 100 µm, in which the germ extract had considerably more small particles < 10 µm (Figure 1). Both extracts contained particles that were smaller than 1 µm, thus a small pore size membrane is required to prevent passage of these small particles. Particles that remained in suspension after the settling step stayed in suspension over the experimental period for all of the subsequent filtrations of both the endosperm- and germ-rich extracts.

**Protein Extraction**

The amounts of HCP and GFP extracted from both the endosperm- and germ-rich fractions were similar to what was reported previously by Aspelund and Glatz [29]. Further extraction of the endosperm and germ solids after the initial 2 hours added negligible amounts of protein, indicating that complete extraction of soluble corn proteins from the endosperm- and the germ-rich fraction was achieved in the first 2 hours. Extraction from the germ-rich fraction resulted in greater extraction of HCP (18 mg/g
solid) and GFP (0.018 mg/g solid) compared to 7 and 0.006 mg/g solid, respectively, for extraction from the endosperm-rich fraction. The proteins extracted from the germ-rich fraction had a higher molecular weight range (10 - 80 kDa) compared to the proteins from endosperm extracts (10 - 45 kDa) [29].

**Dead-end Filtration and Cake Compressibility**

In dead-end filtrations, the filter cake provided the greatest hydraulic resistance for both the post-settling endosperm and germ extract suspensions. The resistance of the fouled membrane after a 90-minute filtration at 1.4 bar was $9.06 \times 10^9$ cm$^{-1}$, compared to resistances of $6.19 \times 10^{10}$ cm$^{-1}$ and $7.43 \times 10^{10}$ cm$^{-1}$ for the endosperm and germ filter cakes, respectively. This indicates that treatments should be made to reduce both the formation and resistance of the cake layer. The filter cakes that formed during the filtration of the endosperm extracts were notably stable compared to the loose and fragile cakes that formed during the filtration of germ extracts.

The average specific cake resistance was higher for germ than for endosperm extracts for all of the TMPs examined (Figure 2). This is likely due to the presence of a greater amount of smaller particles (< 10 µm), which tend to form a cake of lower porosity, in the germ extract suspension after settling (Figure 1). The average specific cake resistance of both corn tissues increased with rising TMP (Figure 2). Fitting the data in Figure 2 to equation 2 resulted in compressibility indices of 1.28 for the germ material and 1.05 for endosperm material, showing the germ to be the more compressible of the two materials. These compressibility indices correspond to the higher end of the compressibilities
reported for microbial and clay suspensions, which typically range from 0.5 to 1.2 [34-36].

The rejection coefficient of rCollagen was between 0.85 and 0.90 for all of the conditions studied in dead-end filtration. rCollagen rejection was not affected by either the corn tissues used for extraction or the TMP. On the other hand, the rejection of HCP and GFP were found to depend on the tissue from which the proteins were extracted and the TMP at which the filtration was operated (Figure 3 A and B). The rejection coefficients of HCP and GFP were similar for the filtration of both the endosperm- and germ-rich extracts. Despite the high compressibility of the filter cake that formed during the filtration of the endosperm extract, increasing the filtration TMP resulted in a decrease in the rejection of HCP and GFP from the endosperm extract (Figure 3 A and B). The decrease in the rejection at the higher TMP is likely a result of the higher permeate flux increasing the concentration of the proteins in the concentration polarization layer. Rejection coefficients of both HCP and GFP were lower for the germ extracts compared to the endosperm extracts and had no dependence on the filtration TMP (Figure 3 A and B), indicating that rejection is controlled by the membrane rather than the filter cake.

**Tangential Flow Filtration**

The effect of the filtration TMP and crossflow rate on the permeate flux of endosperm and germ extracts using a ceramic membrane is shown in Figures 4 and 5. At the two low crossflow rates (0.5 and 0.8 m/s), the critical TMP was approximately 0.85 bar as above 0.85 bar, no significant dependence of the permeate flux on the TMP was observed for
either the endosperm or the germ extracts (Figures 4 and 5). The observed critical TMP resulted in permeate flux values of approximately 20 and 25 LMH for the 0.5 and 0.8 m/s crossflow rates, respectively, for both the endosperm and germ extracts (Figures 4 and 5). Filtrations at the higher crossflow rate were operated below the critical TMP for both the endosperm and germ extracts as the permeate flux improved with increasing TMP for all of the TMP values examined (Figures 4 and 5). However, the non-linear increase in the permeate flux with TMP at the highest crossflow rate indicates that the high TMP was close to the critical TMP of the endosperm and germ material.

Increasing the crossflow rate resulted in a rise in the permeate flux for all of the TMPs studied, suggesting that a significant cake layer formed in all of the tested filtration conditions. The crossflow rates that were tested in the tubular ceramic membrane channel correspond to Reynolds numbers of 3640, 5810, and 8540. This indicates that the filtration flow was in the transition between laminar and turbulent flow at the low crossflow rate (0.5 m/s), whereas at the higher crossflow rates (0.8 and 1.22 m/s), the filtration flow was likely turbulent due to the presence of solids and the rough channel wall [37]. Increasing the crossflow rate at high TMP resulted in a greater increase in the permeate flux than that observed at low TMP due to enhanced cake layer formation at high TMP (Figures 4 and 5). Raising the crossflow rate from 0.8 m/s to 1.2 m/s improved the permeate flux greater than when the crossflow rate was raised from 0.5 to 0.8 m/s for each of the TMPs examined in endosperm and germ extracts (Figures 4 and 5). This improvement in the permeate flux at the highest crossflow rate is likely caused by the higher turbulence in the crossflow sweeping the particles from the cake layer.
Under the same filtration conditions, permeate flux of the endosperm extract was similar to the permeate flux of the germ extract (Figures 4 and 5) despite the endosperm extract having a solids concentration that is approximately twice that of the germ extract. This result is consistent with the germ material being more compressible and forming a cake layer with higher specific resistance than the endosperm material.

**Membrane Fouling**

Filtration of post-settling endosperm and germ extract suspensions with rCollagen using a ceramic membrane was followed by measurement of the clean water flux. The resulting clean water flux was only 8% of the pre-filtration value, indicating that significant internal membrane fouling occurred. Similar flux reductions were observed with the ceramic membrane when the extracts did not contain rCollagen and/or were clarified by high speed centrifugation and filtration with a 0.2 µm cellulose membrane, suggesting that the membrane fouling was caused by soluble compounds in the corn extract rather than corn solids or rCollagen. Since HCP and GFP transmission were also unaffected, the fouling was most likely caused by non-protein components. Under the same conditions, the clean water flux through the PVDF membrane was up to 70% of the pre-filtration value, indicating that the PVDF membrane fouled less than the ceramic membrane. Hence, we tested for the presence of soluble starch in the feed and the permeate for both membranes. Starch transmission was significant through the PVDF membrane but was negligible through the ceramic membrane, suggesting that the presence of starch in the corn extracts may be responsible for the internal fouling of the ceramic membrane. Singh
and Cheryan reported similar fouling caused by soluble starch components during microfiltration with a ceramic membrane for clarification of a corn starch hydrolysate and found that the fouling was reduced by increasing the pH of the feed from 4.2 to 10 [38]. However, due to the low solubility of rCollagen at high pH, filtration at higher pH was not attempted, but alkaline cleaning was used to remove fouling.

**Protein Rejection in Tangential flow filtration**

Rejection of HCP and GFP was low in the filtration of post-settling endosperm and germ extract suspensions with a ceramic membrane. To examine the dependence of rejection on the presence of the solids and the formation of a cake layer, clarified extracts were prepared by high speed centrifugation coupled with filtration through a 0.2 µm filter. With clarified extracts, rejection of HCP and GFP by the ceramic membrane was also low. Average HCP rejection coefficient was ~ 0.10 and GFP rejection coefficient was ~0.12 from both the post-settling and the clarified endosperm and germ extracts. HCP and GFP rejection was not affected by either the TMP or crossflow rate. Analysis of permeate samples by SEC-HPLC showed that the rejection of HCP was not dependent on the molecular weight of the HCP for either the endosperm or germ extracts. The rejection of the high molecular weight rCollagen was much greater (0.85 < R < 0.95) and was also independent of crossflow rate and TMP. The lack of dependence of rejection on TMP, crossflow, and presence of corn solids indicates that rejection is not caused by a cake layer of solids and is likely controlled by the membrane and its fouling. The combination of flux reduction and rCollagen rejection may indicate internal fouling that reduces pore size.
In contrast to the ceramic membrane, filtration with the PVDF membrane had much lower flux reduction and negligible rCollagen rejection. The PVDF membrane also had negligible rejection of HCP and GFP. These results are consistent with lower internal fouling of the PVDF membrane.

Conclusions

This study is the first to examine the solid/liquid clarification of aqueous corn extracts by tangential flow microfiltration. Microfiltration of corn protein extracts produces the formation of a highly compressible cake layer that has the potential to form the controlling resistance to permeate flux. Our results indicate that microfiltration with a PVDF membrane was most suitable for the recovery of rCollagen during the solid/liquid clarification of endosperm and germ extracts as the PVDF membrane had lower internal membrane fouling and rCollagen rejection when compared to the ceramic membrane. Permeate flux was greatly improved with increasing crossflow rate during tangential flow filtration of endosperm and germ extracts with a ceramic membrane. Rejection of HCP, GFP, and rCollagen was independent of the filtration operating conditions and rejection by the cake layer was negligible. High rejection of the large molecular weight rCollagen that was observed during filtration with a ceramic membrane was likely caused by soluble corn starch fouling the membrane. Filtration at higher pH may reduce the fouling of the ceramic membrane. Microfiltration was an effective method for the solid/liquid clarification of corn extracts when a ceramic membrane was used for the recovery of
lower molecular weight proteins (< 80 kDa) and a PVDF membrane was used for the recovery of a larger molecular weight protein.

Acknowledgements

We thank Julio Baez and Sheri Almeda of FibroGen Inc. for several technical discussions and supplying the recombinant collagen and a protocol for the collagen HPLC assay. J. Baez also made useful comments on the manuscript. The authors would also like to thank Ken Wiley from Enpro Inc., Rick Mumley from Pall Filtration for lending the Membralox ceramic membrane filter holder as well as technical discussions in support of this study, Paul Scott for development and production of the transgenic corn lines used in this study, and the Center for Crops Utilization Research at Iowa State University for the use of the seed processing equipment. Funding for this work was provided by the USDA CREES Grants #2008-34496-19348 and #2009-34496-19899.

References

3 J. Baez, Biopharmaceuticals derived from transgenic plants and animals, in Modern Biopharmaceuticals, J. Knäblein (Eds.). 2005, Wiley-VCH Verlag GmbH & Co. KGaA: Weinhiem, Germany. p. 833-892.

Figures

Figure 1. Particle size distributions of the endosperm- and germ-rich material for both the original extract suspension and the material remaining in suspension after settling.
Figure 2. Dependence of the average specific cake resistance of both the endosperm and germ extracts on the transmembrane pressure in dead-end microfiltration.
A bar chart shows the rejection coefficient at different TMP (bar) values for endosperm extracts and germ extracts. The rejection coefficient is defined along the y-axis, and the TMP (bar) values are 0.3, 1.4, 2.4, and 3.5. The chart indicates that the rejection coefficient increases with increasing TMP for both endosperm and germ extracts.
Figure 3. Rejection coefficients of A) total HCP and B) GFP during the dead-end filtration of both the endosperm- and germ-rich extracts. Rejection coefficients of rCollagen were between 0.85 and 0.90 for all of the filtrations and were not affected by the filtration conditions.
Figure 4. The effect of TMP on the permeate flux of the unclarified endosperm-rich extract at several crossflow rates using the ceramic membrane. 95% confidence interval for the permeate flux was ± 1 LMH.
Figure 5. The effect of TMP on the permeate flux of the unclarified germ-rich extract at several crossflow rates using the ceramic membrane. 95% confidence interval for the permeate flux was ± 1 LMH.
Chapter IV: Purification of Recombinant Plant-Made Proteins from Corn Extracts by Ultrafiltration

A paper in press to be published in the Journal of Membrane Science

Matthew T. Aspelund¹ and Charles E. Glatz¹*

1. Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011

Abstract:

The effectiveness of ultrafiltration for the purification of recombinant proteins from aqueous corn endosperm and germ extracts was examined using model proteins of two different sizes, recombinant type I human collagen (rCollagen, 265 kDa) and green fluorescent protein (GFP, 27 kDa), to evaluate the effects of membrane pore size, transmembrane pressure (TMP), crossflow rate, and filtration pH on permeate flux and protein sieving. Using a 300 kDa MWCO membrane resulted in a significant loss of rCollagen, whereas a 100 kDa MWCO membrane completely retained rCollagen. Increasing the filtration crossflow rate and TMP resulted in a higher permeate flux without significantly altering the sieving of the host cell proteins (HCP) or GFP. The greatest HCP sieving was observed in the endosperm extract filtration at low pH and, compared to endosperm, the filtration of germ extracts had lower HCP sieving. GFP exhibited similar sieving as the average HCP for all filtration conditions. rCollagen purity of 89% was achieved with only diafiltration of endosperm extracts and, when preceded by precipitation, a purity of >99% was attained. Thus, ultrafiltration is a valuable method to separate and purify corn-hosted recombinant proteins >100 kDa, particularly when the expression is targeted to the endosperm.
Keywords: Tangential flow filtration; Ultrafiltration; Protein purification; Transgenic corn; Concentration polarization; Protein precipitation; Collagen

Introduction:

The low cost associated with the production of recombinant proteins in corn seed and the potential for coupling protein production to current crop-based biorefineries makes the corn host system ideal for the production of low-value and high volume proteins (e.g. protein-based materials, industrial enzymes) [1]. Purification steps comprise up to 90% of the overall production cost of recombinant proteins from plants [2]. Therefore, there is a need to develop efficient and inexpensive methods for the purification of recombinant proteins from corn seed.

Corn seed consists largely of germ (11% dry weight), which contains most of the seed’s oil and water-soluble protein, and endosperm (83% dry weight) comprised mainly of starch and water-insoluble proteins. Combining endosperm-targeted protein expression with dry-milling to isolate an endosperm-rich fraction has been shown to provide an extract with the lowest host cell protein (HCP) burden [3]. Several techniques have been applied for the recovery of recombinant proteins from corn germ and endosperm extracts, including packed bed chromatography [1, 4], expanded bed chromatography [5, 6], and aqueous two phase partitioning [7]. Compared to these methods, ultrafiltration offers the advantages of lower cost, generally non-denaturing operating conditions, and easy scalability. Additionally, the selectivity of protein fractionation by ultrafiltration has improved recently due to improvements in the design of both membranes and filtration
modules [8] as well as an enhanced understanding of the factors that can affect protein retention [9].

Ultrafiltration/diafiltration (UF/DF) has been widely used in pharmaceutical processes, primarily for concentration and buffer exchange. Recent work has focused on the use of UF/DF for protein purification [10]. The protein retention characteristics of the UF operation are affected by filtration conditions (crossflow rate and transmembrane pressure (TMP)), solution conditions (pH and ionic strength), and membrane characteristics (pore size and membrane charge) [11-14]. Crossflow rate and TMP control the extent of gel layer formation. The formation of a gel layer can reduce the permeate flux and the selectivity of a membrane by changing the solute concentration at the membrane surface [11, 13]. Co-current flow of retentate and permeate enables operation at constant TMP along the length of the membrane, reducing the effects of the changes in solute permeation and gel layer formation with changes in TMP [15]. Protein transmission can be improved by performing the filtration at a pH close to that of the pI of the proteins, limiting the interactions between the protein and the membrane as long as precipitation of the proteins in the feed and increased membrane fouling can be avoided [16, 17].

Protein transmission can be characterized by the instantaneous observed membrane sieving coefficient of a protein species, \( i \) (\( S_i \));

\[
S_i = \frac{C_{i,\text{permeate}}}{C_{i,\text{bulk}}} \quad (1)
\]
where $C_{i,\text{permeate}}$ is the concentration of species, $i$, in the permeate, and $C_{i,\text{bulk}}$ is the bulk concentration of species, $i$, in the retentate. For constant volume diafiltration a mass balance on species $i$ is given by:

$$V \frac{dC_{i,\text{bulk}}}{dt} = -J A C_{i,\text{permeate}}$$  

(2)

where $V$ is the volume of the feed, $J$ is the permeate flux, $A$ is the membrane area, and $t$ is the filtration time. Combining equations 1 and 2 and integrating, with assumption of constant $S$ gives [18]:

$$S_i = -\frac{1}{N} \ln \left( \frac{C_{i,t}}{C_{i,0}} \right)$$  

(3)

where $C_{i,t}$ is the concentration of species, $i$, in the feed at time $t$, during the filtration, $C_{i,0}$ is the concentration of species, $i$, in the initial filtration feed, and $N$ is the number of diavolumes used in the filtration given by:

$$N = \frac{J A t}{V}$$  

(4)

The selectivity ($\psi$) of the preferably permeated protein $i$, relative to that of protein $j$, is

$$\psi = \frac{S_i}{S_j}$$  

(5)

The fractional purity, $p_i$, of a protein component, $i$, in any stream is calculated as:

$$p_i = \frac{c_i}{c_{\text{total}}}$$  

(6)

where $c_{\text{total}}$ is the total protein concentration. The purification factor, $PF$, is the ratio of initial and final purities:
\[ PF_i = \frac{p_{i,t}}{p_{i,0}} \]  

(7)

In this study, the product-size-dependent suitability of ultrafiltration for the purification of recombinant proteins from corn germ and endosperm extracts was examined using as model products, triple-helical type I recombinant human collagen (rCollagen, 265 kDa) and green fluorescent protein (GFP, 27 kDa). The effects of membrane pore size, TMP, crossflow rate, and pH on permeate flux and protein sieving were assessed. Additionally, a purification process utilizing UF/DF was developed for recovery of rCollagen that was spiked into corn germ and endosperm fractions, as gelatin derived from collagen could be made as a co-product of corn biorefining.

**Materials and Methods:**

**Materials:**

All chemicals used in these experiments were reagent grade and were purchased from Fisher Scientific (Itasca, IL) unless otherwise noted.

**Collagen:**

Because available early generation corn-based expression levels have been low [4] and grain with higher levels of GFP expression was available, for this study yeast-derived rCollagen was spiked into endosperm and germ extracts of transgenic corn expressing GFP. The collagen used in the spiking studies was produced by *Pichia pastoris* fermentation and consisted of two \( \alpha_1 \) subunits and one \( \alpha_2 \) subunit forming a triple-helical
collagen molecule with molecular weight of 265 kDa and pI of 9.2, as calculated from the amino acid sequence. rCollagen was purified to >95% purity by pepsin digestion of *Pichia* extracts followed by two acid salt precipitation steps. The rCollagen was provided in its purified form by FibroGen Inc. (San Francisco, CA) at a concentration of 2.53 mg/ml in 10 mM hydrochloric acid.

**GFP Grain:**

The preparation of transgenic corn expressing the S65T variant of GFP was described by Shepherd *et al.* [19]. This GFP variant has a molecular weight of 27 kDa and a pI of 5, as calculated from the amino acid sequence. Germ-targeted expression of GFP was obtained using the maize Glb1 promoter and endosperm-targeted expression was obtained with the maize 27zn promoter [19]. The grain used for this study was produced in a 2007 field trial at Iowa State University. After harvest, the seeds were stored at 4±1 °C in sealed and moisture-proof containers. GFP expression levels determined by extraction at pH 7 were 115 µg GFP/g grain and 42 µg GFP/g grain for the germ- and endosperm-targeted grain, respectively.

**Dry Milling:**

Kernels were dry milled into germ- and endosperm-rich fractions. Kernels were first tempered in a sealed bag for 2.5 hours with deionized water to achieve a moisture content of 21%, then ground using a laboratory Beal-type drum degermer and a series of corrugated Witt mills (Witt Corrugating Inc., Wichita, KS) using the method and mill settings optimized for fractionation of recombinant dog gastric lipase-containing corn
[20], and finally separated into fractions by screening. The purity of the fractions was calculated using the methods reported by Shepherd et al. [21, 22], but with GFP being assayed after extraction from the solids at pH 7 rather than directly on the solids. The germ-rich fraction contained 38% germ, 22% endosperm, and 40% bran while the endosperm-rich fraction was >99% endosperm.

Corn Defatting:
GFP-expressing germ- and endosperm-rich fractions were milled for 1 min in a household coffee mill (GE model 169028) with 150g of fractionated grain in each batch. The resultant flours had particle sizes ranging from 0.01 mm to >2 mm for the endosperm-rich fraction and 0.1 mm to >3 mm for the germ-rich fraction, as determined by dry sieving. Oil was twice-extracted from both germ- and endosperm-rich fractions with hexane at a ratio of 1:5 flour:hexane on ice for 1 h. Flour was separated from the hexane by centrifugation at 3000 × g at 4 °C. After the second extraction, flour was dried overnight in a fume hood to remove residual hexane.

Protein Extraction:
Proteins were extracted from the defatted flours with continuous stirring on a magnetic stir plate for 2 hours at 4 °C with a ratio of 1:10 (w/v) flour:buffer. The extraction buffer consisted of a 0.1 M phosphate buffer, with 0.15 M sodium chloride, and 0.1 mg/ml of rCollagen. Collagen inclusion in the extraction buffer allowed simulation of any partitioning that might occur between the solids. The rCollagen spiking level was in the range that could be expected for established transgenic lines (1 g of rCollagen/kg dry
grain) and sufficient to accurately assay throughout the experiment. Extracts were clarified by centrifugation at 3000 \( \times \) g and 4 °C for 15 minutes, followed by filtration through a 0.2 µm syringe filter (Sigma-Aldrich; St. Louis, MO). Extract pH was adjusted to the desired pH for UF/DF by adding 5 M sodium hydroxide slowly with mixing.

**Collagen Precipitation:**

One volume of 5 M sodium chloride was added to four volumes of clarified extract (final salt concentration of 1.12 M and pH 2.1). The solution was mixed on a magnetic stir plate for 2 hours at 4 °C before separation of precipitated proteins by centrifugation (30 minute at 14,600 \( \times \) g and 4 °C). The precipitate was dissolved in the same volume of rCollagen-free extraction buffer as the original corn extract. Samples of the corn extract, supernatant, and dissolved precipitate were assayed for collagen, GFP, and total protein concentration.

**Ultrafiltration/Diafiltration:**

UF/DF was performed using a Millipore lab-scale, tangential-flow filtration system (Millipore; Billerica, MA) and Pellicon XL membrane cassettes. The membranes were 100 kDa (PXB100C50) and 300 kDa (PXB300C50) molecular weight cut-off (MWCO) flat sheet polyethersulfone (PES) membrane cassettes with a total membrane surface area of 50 cm\(^2\) (Millipore; Billerica, MA). UF/DF was operated in a co-current flow configuration with a carrier fluid on the permeate side of the membrane. Inlet and outlet pressure on the permeate side of the membrane was adjusted by the permeate pump (Masterflex, 7518, Cole Parmer, Chicago, IL) speed and a needle valve that was located
downstream of the permeate outlet in order to maintain a constant TMP along the length of the membrane. Pressure on the permeate side was monitored using two glycerin filled pressure gauges with a 0 – 60 psi range (Pall filtration, East Hills, NY), located upstream and downstream of the filtration module. The permeate loop was initially filled with 100 ml of rCollagen-free extraction buffer that was adjusted to the filtration pH.

Diafiltrations were kept at constant volume with the continuous addition of a 0.1 M phosphate/0.15 M sodium chloride buffer, which was adjusted to the pH of the feed. Samples of feed and permeate were collected at each half diavolume or each fold concentration factor and were analyzed for collagen and total protein concentration. Permeate flux was calculated from the change in the permeate mass over time with an electronic balance (Mettler-Toledo, PG6002-S, Columbus, OH) and recorded to a computer using LABVIEW (National Instruments, Austin, TX).

Membranes were cleaned by flushing for 30 minutes with 0.5 N sodium hydroxide at 45 °C, followed by recycling 0.2 % Terg-a-Zyme (Alconox, White Plains, NY) at 45 °C for one hour and by a final rinse with 500 ml of 0.5 N sodium hydroxide at 45 °C. The clean water flux was measured before and after the filtration and after the membrane cleaning.

*Experimental Design and Statistical Analysis:*

UF experiments were designed as a single replicate of a randomized, full factorial design combining 4 treatment variables at 2 levels each (see Table 1). Crossflow rates were selected to represent the upper and lower limits of the desired operating conditions.
suggested by Millipore for the Pellicon XL cassettes [23]. The 4th order interaction was taken as the measure of variability in the ANOVA. The Bonferroni correction was used to adjust the required $p$-value for significance for measurement of multiple outcomes [24]. Statistical analysis was performed using the statistical software package JMP (SAS, Cary, NC) to determine the effect of the treatments on the permeate flux, and sieving of rCollagen, HCP, and GFP.

**Protein Determinations:**

The concentration of rCollagen was determined using a denaturing size exclusion chromatography HPLC assay with 2 M guanidine HCl as the mobile phase. Samples were prepared by equivolume dilution with 4 M guanidine HCl. For HPLC, a BioSilect size exclusion column 300 mm x 7.8 mm (Bio-Rad, Cat. #1250477) and a BioSilect guard column 50 mm x 7.8 mm (BioRad, Cat. #1250480) was used at a flow rate of 1 ml/min on an Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA). The protein elution was monitored at 220 and 280 nm. Collagen’s negligible aromatic amino acid content results in no significant absorbance at 280 nm but absorbance is observed at 220 nm due to the peptide bonds. Thus, a peak with very low $A_{280}/A_{220}$ indicates collagen.

Injections of samples of known rCollagen concentration between 0.01 and 1 mg/ml established the correlation between the $A_{220}$ peak area and collagen concentration. Injection of a known concentration (between 0 and 2.5 mg/ml) of corn extract proteins (determined by BCA assay using BSA as the standard; Pierce Biotechnology, Rockford,
IL) provided the corresponding correlation of the A$_{220}$ area and the concentration for HCP. The peaks integrated for HCP concentration included the co-eluting GFP, which was separately assayed so that its concentration could be deducted to calculate the HCP concentration. The standard curves for both rCollagen and corn proteins always showed a high linear correlation with an R$^2 > 0.99$ for concentration ranges of both proteins.

The concentration of GFP in each sample was determined by fluorescence (Versafluor spectrofluorometer; BioRad, Hercules, CA) with an excitation wavelength of 390 nm and an emission wavelength of 520 nm. A standard curve was prepared using recombinant GFP of identical adsorption and emission spectra as the S65T GFP variant expressed in the transgenic corn (Clontech, Cat. #632373; Mountain View, CA). Because GFP fluorescence can depend on the solution pH, a standard curve was generated for each pH values used for filtration (i.e. 2.1, 3.1, and 3.5). The fluorescence yield was 0.2% greater at pH 3.5 than at pH 2.1. The GFP standard curves were linear (R$^2 > 0.97$) over the concentration range 0 - 2 µg/ml.

SDS – PAGE:

Samples were characterized by one dimensional SDS-PAGE using a Mini – PROTEAN II electrophoresis cell and precast 4 – 20 % Tris-HCl gels from Bio-Rad (Bio-Rad; Hercules, CA). Samples were mixed with loading buffer and heated to 100 °C for 30 seconds. Loss of the rCollagen bands occurred when heating the samples at pH 2-3.5 for longer than 30 seconds. Samples were loaded using the same loading volume (20 µL) in each well and gels were stained using Coomassie stain (G-250, Pierce Biotechnology,
Rockford, IL) or silver stain (Silver Stain Plus; Bio-Rad, Hercules, CA). Coomassie-stained gels were analyzed by densitometry using ImageJ (NIH, Bethesda, MD).

Results and Discussion:

Protein Extraction:
Similar to previous studies [1, 25], HCP showed a lower solubility in low pH extractions. Low pH preferentially extracts the acid-soluble collagen [26] over corn HCP (esp. germ fraction) and GFP, which have higher solubility at neutral pH. Extractions performed at pH 7 resulted in HCP concentrations of 8 and 44 mg/g grain from endosperm and germ, respectively. At pH 2, extractions of endosperm and germ resulted in HCP concentrations of 7 and 18 mg/g grain, respectively. GFP likewise showed better extraction at neutral pH for both the germ and endosperm tissues. At pH 7, the GFP concentration was 0.043 mg/g grain from the endosperm and 0.115 mg/g from the germ, while extractions at pH 2 resulted in GFP concentrations of 0.006 and 0.018 mg/g grain, respectively. The quantity of GFP extracted at pH 2 from both tissues was sufficient to accurately assay throughout the experiments. Higher molecular weight HCP were extracted better at higher pH for both germ- and endosperm-rich extracts (Figure 1). The acid-soluble corn proteins had a molecular weight range from 10 to 45 kDa for the endosperm extracts and between 10 and 80 kDa for the germ extracts (Figure 1: lanes A and C). Additionally, previous work has shown that acid-soluble germ HCP have pI values that range between 3 and 10 with a significant number of proteins with pI’s between 3 and 5 [27]. The higher molecular weight proteins would be harder to separate from collagen using UF, a situation
exacerbated if any of these bands are multimeric subunits. Therefore, all UF/DF experiments were performed using pH 2 extractions from both germ- and endosperm-rich fractions.

Inclusion of 0.1 mg/ml of rCollagen with the pH 2 extractions resulted in clarified extracts with an initial rCollagen purities of ca. 17 % and 7 % in the endosperm and germ extracts, respectively. GFP comprises only 0.12% of the total protein extracted from both the germ and endosperm at pH 2 and was evidenced in the band appearing at 27 kDa in the SDS-PAGE gel (Figure 1: lanes A and C). Based on densitometry, however, only 1% of this band was GFP in the endosperm extract and 1.8% in the germ extract with the remainder being corn proteins of similar molecular weight. Adjusting the pH of the proteins that were extracted at pH 2 resulted in some protein aggregation at a pH of approximately 3.6 for the germ extracts and approximately 3.2 for the endosperm extracts. Thus, a filtration pH of 3.1 for endosperm extracts and 3.5 for germ extracts were used as the upper limit of pH adjustment for the all subsequent experiments.

**Diafiltration - Screening of pore size and critical TMP:**

Initial experiments were performed to determine the conditions that are necessary for operation of diafiltrations above and below the critical TMP and to screen membranes of different pore sizes for the retention of rCollagen and transmission of HCP.

**Critical TMP:**
Whether operation of the filtration was above or below the critical TMP was determined by observing the pressure dependence of the permeate flux during diafiltration for each filtration. The pressure on the feed side of the membrane was adjusted throughout the filtration to maintain a consistent TMP either above or below the critical TMP. Figure 2 shows a typical permeate flux curve for a filtration operated below the critical TMP. At an average TMP of about 0.5 bar, the permeate flux curve showed significant dependence on the TMP fluctuations, and permeate flux did not exhibit the initial period of rapid flux decline commonly associated with the formation of a gel layer (Figure 2). On the other hand, at an average TMP of 1.1 bar, a period of rapid flux decline was observed during the first 10 minutes of the filtration and permeate flux was independent of the TMP fluctuations, indicating gel layer formation (Figure 3). These results of filtrations under conditions that were expected to yield the lowest and the highest critical TMP established that filtrations operated at a TMP of 0.5 bar were below and 1.1 bar were above the critical TMP.

**Membrane Pore Size:**

Two membranes, with MWCO of 300 and 100 kDa, were initially screened for their effect on the retention of rCollagen in an endosperm extract. Significant sieving of rCollagen occurred during diafiltration with the 300 kDa membrane, whereas, the 100 kDa membrane provided complete retention of rCollagen over three diavolumes (Table 2). The complete retention of rCollagen observed with the 100 kDa MWCO membrane showed that the filtration can be performed for many diavolumes with no decrease in the rCollagen yield.
The sieving coefficients of HCP and GFP were approximately equivalent for both membrane pore sizes, resulting in similar collagen purities and purification factors (Table 2). Although the 300 kDa membrane had a greater permeate flux, the improved retention of rCollagen in the 100 kDa membrane provided higher collagen yield without loss of purity as relatively minor differences were observed between the sieving coefficients of HCP for the two MWCO membranes. Hence, the 100 kDa MWCO membrane was used in the remainder of the experiments.

**Factorial Design Experiments:**

Experiments were performed to determine the effect of the filtration conditions on the retention of rCollagen and the permeation of GFP and the HCP. The operational variables described in Table 1 were combined in a full factorial design and examined for their effect on permeate flux and sieving of HCP and GFP.

**Permeate Flux:**

The permeate flux had significant dependence on the crossflow rate, TMP, and their interaction. No significant dependence of the permeate flux on the tissue from which the HCP were extracted was found (Table 3). Additionally, significant 2nd order interaction effects between the filtration pH, the crossflow rate, and TMP on permeate flux were found, although no first order dependence of the permeate flux on filtration pH was observed (Table 3).
Increasing both the TMP and the crossflow rate increased the permeate flux with the greatest flux improvement occurring above the critical TMP (Table 4 and Figure 4). The interaction between the crossflow rate and TMP (Table 3) indicated that the highest permeate flux was achieved above the critical TMP at high crossflow rate (Table 4). This improvement in the permeate flux was caused by the greater TMP increasing the driving force for fluid flow across the membrane and the larger shear on the feed side of the membrane reducing the thickness of the gel layer.

The permeate flux was greatest above the critical TMP and at the lower pH. Operating the filtration at the higher pH resulted in a slight decrease in the permeate flux at the low crossflow rate. However, at the high crossflow rate, this effect of high pH on permeate flux was not observed. Such effect of pH on permeate flux cannot be attributed to changes in the feed protein concentration because extractions were all performed under the same conditions and subsequently adjusted to the filtration pH. The results indicate that operating the filtration at a pH close to the pI results in aggregation of some of the HCP worsening the fouling/gel layer flux resistance.

**Protein Sieving:**

Operating the filtration below or above the critical TMP did not significantly alter the retention of rCollagen, which was completely retained in all conditions with the 100 kDa MWCO membrane (Table 2). Previous studies found that filtrations above critical TMP increase the sieving of partially retained proteins as a result of the greater concentration of the proteins accumulating at the membrane surface [16]. However, in our study,
increase in the TMP did not result in a significant change in the sieving of the HCP or GFP (Table 3). Since rCollagen, HCP, and GFP sieving did not depend on TMP, our study suggests that the optimal TMP value would be close to the critical TMP, where a high permeate flux can be achieved with the lowest pressure and pumping requirements.

In contrast to its effect on flux, the crossflow rate did not have a significant effect on the sieving of either the HCP or GFP (Tables 3). Decrease in the gel layer associated with the increased crossflow rate may result in a diminished sieving of proteins through the membrane by lowering the protein concentration at the membrane surface. However, no effect of either the TMP or crossflow rate on the sieving of the HCP and GFP was noted, indicating that the presence of the gel layer does not have a strong effect on the protein sieving over the range of operating conditions examined.

The sieving of the endosperm HCP was significantly higher than that of the germ HCP (Table 3). The average corn protein sieving was 0.64 (±0.05, 95% CI) from the germ extracts and 0.77 (±0.05) from the endosperm extracts (Table 4). Densitometry showed that the sieving coefficient of the proteins ≤ 25 kDa in the germ extract was similar to that in the endosperm extract. Therefore, the reduction in HCP sieving from germ extracts compared to the endosperm extracts is due to the significantly greater concentration of large molecular weight proteins (≥ 25 kDa) in the germ extract (Figure 1, lane C) compared to the endosperm extract (Figure 1, lane A).

While the filtration pH alone showed no significant effect on HCP sieving, a significant interaction between the filtration pH and the extracted corn tissue was observed (Table
3). At the low pH, the endosperm had a significantly greater sieving coefficient than the germ extract. At the higher pH, the germ HCP sieving coefficient increased to the level of the endosperm. This effect of pH was not observed for the endosperm extracts, which had similar HCP sieving coefficients for both the low and higher pH filtrations.

Sieving coefficients GFP and HCP were similar for all filtration conditions (Table 4). Thus, we propose GFP to be good representative model protein for sieving of acid-soluble corn proteins. The similar sieving of GFP and HCP also indicates that separation of low molecular weight proteins (>25 kDa) from corn extracts by ultrafiltration is not feasible under the conditions in this study. However, use of a lower MWCO membrane (i.e. 30 or 50 kDa) may allow for better selectivity of GFP or other recombinant proteins of similar size.

The greatest impact of the examined filtration conditions was seen on the permeate flux, which showed significant dependence on the crossflow rate, TMP and 2nd order interactions between the crossflow rate, TMP, and filtration pH. The greatest permeate flux was achieved during filtration at the 0.28 m/s crossflow rate above the critical TMP. The highest HCP sieving coefficient was observed during the filtration of endosperm extracts at low filtration pH. The desired operating conditions require high permeate flux and HCP sieving, which entails a short processing time. Therefore, the experiments performed to determine the extent of rCollagen purification that is attainable by UF/DF were conducted using endosperm extractions at low filtration pH and high crossflow rate and TMP.
Purification of rCollagen:

**Diafiltration:**

Diafiltration resulted in the removal of 96% of the HCP from the retentate of endosperm extracts and 91.3% from the germ extract. This resulted in an average final purity of rCollagen in the retentate of 84.3% in the endosperm extracts and 38.1% in the germ extracts after only three diavolumes. The greater initial purity of the endosperm extracts results in higher final rCollagen purity for the same purification factor (Table 4). The rCollagen yield for all filtrations was >95%. The yield of GFP in the permeate was comparably high for both endosperm (92.7%) and germ extracts (82.2%). Targeted expression of recombinant proteins to the endosperm would be the most advantageous for the purification of recombinant proteins because UF/DF of the endosperm extracts had lower initial concentration of HCP and greater permeate flux, HCP sieving coefficients, and rCollagen final purity as compared to the purification from germ extracts.

In an effort to increase the final purity of rCollagen, diafiltration was performed up to eight diavolumes. The average permeate flux of 84.4 LMH over the eight diavolumes was similar to that obtained in the factorial experiment under similar conditions (Table 4). Major improvement in rCollagen purity (from 17% to 75%) was achieved during the first three diavolumes and similar results were obtained in the factorial experiment (17%-83%). However, we observed only a small improvement in the rCollagen purity from 75% to 89% over the last five diavolumes as a result of reduced HCP sieving in the latter
part of the filtration. The HCP sieving coefficient was 0.65 for the first three diavolumes and dropped to approximately 0.2 after the fourth diavolume (Figure 5). Under similar conditions of the factorial experiment, HCP sieving coefficient was 0.77 for three diavolumes (Table 4). At these later stages (>3 diavolumes), HCP sieving is largely governed by the larger molecular weight HCP, since the smaller HCP are, for the most part removed, in the early stages. Such selective removal of smaller proteins was confirmed by densitometric analysis of the Coomassie stained SDS-PAGE gels (Figure 6). After eight diavolumes, bands >20 kDa were reduced by only 40 % in the retentate, whereas, bands <20 kDa were reduced by as much as 80 % in the retentate and were present in the permeate. This demonstrates that the application of UF to the purification of proteins larger than 100 kDa from corn extracts can result in high purities of the large molecular weight protein. Extending the application of UF to lower molecular weight proteins will likely yield significant purification of proteins between 20 and 100 kDa, particularly if a lower MWCO membrane is used.

**Collagen Concentration by Ultrafiltration:**

Complete retention of rCollagen over a 10X concentration of the initial feed was observed in a filtration operated in concentration mode. The average permeate flux and HCP sieving coefficient (equation 1) were 64.7 LMH and 0.55, respectively. However, co-precipitation of rCollagen and corn proteins occurred once the feed was concentrated to approximately 6X, at which point, the total protein concentration was 2.8 mg/ml. This limits the greatest achievable concentration factor to approximately 5X. Diafiltration with three diavolumes following 10X concentration produced a final purity of 44.4%. An
initial diafiltration before concentration could avoid the precipitation limitation by reducing the initial HCP concentration and this could lead to purity higher than that achieved with diafiltration alone. However, the larger diavolumes required for non-concentrated feed will increase the processing time; therefore, this alternative was not attempted.

**Collagen Precipitation and Diafiltration:**

Diafiltration of a resuspended rCollagen precipitate from an endosperm extract resulted in >99% purity of rCollagen based on HPLC, BCA, and densitometry of Coomassie stained SDS-PAGE gels (gel not shown) and an 87% yield after just three diavolumes (Table 5). After precipitation, 97% of the total HCP remained in the supernatant and the majority of these proteins were >10 kDa (Table 5 and Figure 7: lanes B-D). The resuspended pellet had a rCollagen purity of 85%. During diafiltration, the average permeate flux was 137.5 LMH and the average corn protein sieving coefficient for the first three diavolumes was 0.62, which was consistent with the sieving coefficient for the low molecular weight HCP. After three diavolumes, the recycled feed contained no detectable amount of HCP when assayed by HPLC, BCA, and Coomassie stained SDS-PAGE (gel not shown). Similarly, GFP was completely removed after the precipitation step. The high permeate flux (137.5 LMH) during diafiltration of the precipitated feed most likely resulted from the low protein concentration of the feed and flux could decrease if the feed were concentrated by resuspending the precipitate in a smaller volume than used in this study.
Silver staining was used to reveal the remaining impurities and showed the most prominent band in the retentate at about 10 kDa (Figure 7). The low molecular weight HCP contaminant may be removed by further purification steps. Overall, our results demonstrate that high rCollagen purity can be achieved using salt precipitation followed by diafiltration.

Conclusions:

The 100kDa MWCO membrane was most suitable for purification of rCollagen from corn extracts. Permeate flux was greatly improved when high crossflow rates (0.25 m/s) and a higher TMP were employed. The low filtration pH (2.1) resulted in the greatest sieving of HCP from endosperm extracts. The sieving of GFP and HCP were similar for each of the filtration conditions examined indicating that ultrafiltration is suitable only for the purification of proteins rejected by the 100 kDa MWCO membrane under the filtration conditions studied. Use of a lower MWCO membrane would likely give improved selectivity for GFP. Initial DF may be a necessary step before concentration of rCollagen by UF from an endosperm extract, because precipitation occurs with direct six-fold concentration. Diafiltration alone resulted in rCollagen purities of as high as 89% and an even greater purity of >99% was achievable when an initial precipitation of collagen from the endosperm extract was introduced. Taken together, our results show UF/DF can be an effective purification strategy when coupled with targeted expression of a large, acid-soluble recombinant protein, such as rCollagen, to corn endosperm.
Acknowledgements:

We thank FibroGen Inc. for supplying the recombinant rCollagen used in this study with special thanks to Julio Baez and Sheri Almeda for providing the collagen HPLC assay and for technical discussions throughout this work, Ryan Nielsen for help conducting experiments, Chris Setina for advice on the selection of precipitation conditions for the recovery of collagen from corn extracts, Paul Scott for development and production of the transgenic corn lines used in this study, and the Center for Crops Utilization Research at Iowa State University for the use of the seed processing equipment. Funding for this work was provided by the USDA CREES Grants #2006-34496-17122 and #2008-34496-19348.

References:


<table>
<thead>
<tr>
<th></th>
<th>Author(s)</th>
<th>Title and Source</th>
</tr>
</thead>
</table>
### Tables:

Table 1. Treatments and levels explored for the full factorial diafiltration experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Levels</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Tissue</td>
<td>Endosperm</td>
<td>Germ</td>
<td></td>
</tr>
<tr>
<td>Transmembrane Pressure (bar)</td>
<td>0.5 (±0.1)</td>
<td>1.1 (±0.1)</td>
<td></td>
</tr>
<tr>
<td>Crossflow Rate (m/s)</td>
<td>0.12</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>2.1</td>
<td>3.1 and</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Table 2. Diafiltration performance for endosperm extracts. Conditions: crossflow velocity 0.12 m/s, TMP 0.45 bar, and a pH of 2.1. Values are averages of 2 runs over three diavolumes.

<table>
<thead>
<tr>
<th>Membrane Pore Size (MWCO)</th>
<th>Permeate Flux (LMH)</th>
<th>rCollagen Sieving Coeff.</th>
<th>HCP Sieving Coeff.</th>
<th>GFP Sieving Coeff.</th>
<th>rCollagen Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Final rCollagen Purity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>rCollagen Purification factor&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>27.7</td>
<td>0</td>
<td>0.80</td>
<td>0.70</td>
<td>95.0</td>
<td>88</td>
<td>5.3</td>
</tr>
<tr>
<td>300</td>
<td>50.9</td>
<td>0.31</td>
<td>0.77</td>
<td>0.71</td>
<td>61.7</td>
<td>76</td>
<td>4.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Retentate values.
Table 3. ANOVA Results for factors affecting the diafiltration of corn extracts with 100 kD MWCO membrane.

<table>
<thead>
<tr>
<th>Source</th>
<th>Permeate Flux</th>
<th>HCP sieving Coeff.</th>
<th>GFP sieving Coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMP</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16</td>
<td>0.22</td>
</tr>
<tr>
<td>Crossflow rate</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74</td>
<td>0.25</td>
</tr>
<tr>
<td>pH</td>
<td>0.39</td>
<td>0.34</td>
<td>0.08</td>
</tr>
<tr>
<td>Tissue</td>
<td>0.03</td>
<td>0.018&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.036</td>
</tr>
<tr>
<td>TMP*Crossflow rate</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.89</td>
<td>0.59</td>
</tr>
<tr>
<td>TMP*pH</td>
<td>0.011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51</td>
<td>0.53</td>
</tr>
<tr>
<td>TMP*Tissue</td>
<td>0.091</td>
<td>0.79</td>
<td>0.86</td>
</tr>
<tr>
<td>Crossflow rate*Tissue</td>
<td>0.114</td>
<td>0.73</td>
<td>0.17</td>
</tr>
<tr>
<td>Crossflow rate*pH</td>
<td>0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55</td>
<td>0.86</td>
</tr>
<tr>
<td>Tissue*pH</td>
<td>0.17</td>
<td>0.016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.065</td>
</tr>
<tr>
<td>Crossflow rate<em>pH</em>TMP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.018</td>
<td>0.59</td>
<td>0.72</td>
</tr>
</tbody>
</table>

<sup>a</sup> Statistically significant treatments using the Bonferroni correction for multiple comparisons (α = 0.05/the number of responses = 0.017)

<sup>b</sup> Included as significant treatment because of proximity to the Bonferroni p-value

<sup>c</sup> Other 3<sup>rd</sup> order interactions not reported as they showed no significant effect on any of the responses.
Table 4. Summary of results for the factorial experiment.

<table>
<thead>
<tr>
<th>Corn Tissue</th>
<th>Crossflow rate (m/s)</th>
<th>Average TMP (bar)</th>
<th>pH</th>
<th>Average Permeate Flux (LMH)</th>
<th>Average HCP Sieving Coeff.</th>
<th>Average GFP Sieving Coeff.</th>
<th>Yield (%)</th>
<th>Final Purity (%)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosperm</td>
<td>0.12</td>
<td>0.5</td>
<td>2.1</td>
<td>27.7</td>
<td>0.90</td>
<td>0.82</td>
<td>94</td>
<td>87.9</td>
<td>5.31</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.12</td>
<td>1.1</td>
<td>2.1</td>
<td>49.1</td>
<td>0.74</td>
<td>0.68</td>
<td>82</td>
<td>87.8</td>
<td>5.10</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.28</td>
<td>1.2</td>
<td>2.1</td>
<td>71.2</td>
<td>0.77</td>
<td>0.69</td>
<td>91</td>
<td>85.6</td>
<td>4.44</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.28</td>
<td>0.5</td>
<td>2.1</td>
<td>37.0</td>
<td>0.90</td>
<td>0.82</td>
<td>107</td>
<td>89.8</td>
<td>4.83</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.12</td>
<td>0.5</td>
<td>3.1</td>
<td>28.5</td>
<td>0.70</td>
<td>0.62</td>
<td>105</td>
<td>72.4</td>
<td>3.96</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.28</td>
<td>1.3</td>
<td>3.1</td>
<td>78.2</td>
<td>0.69</td>
<td>0.68</td>
<td>100</td>
<td>86.3</td>
<td>5.77</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.12</td>
<td>1.1</td>
<td>3.1</td>
<td>37.1</td>
<td>0.77</td>
<td>0.72</td>
<td>91</td>
<td>85.3</td>
<td>4.78</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.28</td>
<td>0.5</td>
<td>3.1</td>
<td>46.0</td>
<td>0.75</td>
<td>0.67</td>
<td>94</td>
<td>81.5</td>
<td>4.61</td>
</tr>
<tr>
<td>Germ</td>
<td>0.12</td>
<td>0.5</td>
<td>2.1</td>
<td>23.3</td>
<td>0.62</td>
<td>0.64</td>
<td>100</td>
<td>42.0</td>
<td>4.21</td>
</tr>
<tr>
<td>Germ</td>
<td>0.12</td>
<td>1.3</td>
<td>2.1</td>
<td>52.7</td>
<td>0.58</td>
<td>0.60</td>
<td>93</td>
<td>45.9</td>
<td>4.18</td>
</tr>
<tr>
<td>Germ</td>
<td>0.28</td>
<td>1.3</td>
<td>2.1</td>
<td>70.4</td>
<td>0.51</td>
<td>0.53</td>
<td>101</td>
<td>26.8</td>
<td>3.67</td>
</tr>
<tr>
<td>Germ</td>
<td>0.28</td>
<td>0.6</td>
<td>2.1</td>
<td>34.9</td>
<td>0.52</td>
<td>0.64</td>
<td>104</td>
<td>27.1</td>
<td>3.64</td>
</tr>
<tr>
<td>Germ</td>
<td>0.12</td>
<td>0.5</td>
<td>3.5</td>
<td>24.7</td>
<td>0.76</td>
<td>0.61</td>
<td>99</td>
<td>40.8</td>
<td>6.15</td>
</tr>
<tr>
<td>Germ</td>
<td>0.12</td>
<td>1.1</td>
<td>3.5</td>
<td>37.0</td>
<td>0.67</td>
<td>0.61</td>
<td>97</td>
<td>50.3</td>
<td>7.02</td>
</tr>
<tr>
<td>Germ</td>
<td>0.28</td>
<td>1.3</td>
<td>3.5</td>
<td>71.5</td>
<td>0.72</td>
<td>0.62</td>
<td>94</td>
<td>32.0</td>
<td>5.05</td>
</tr>
<tr>
<td>Germ</td>
<td>0.28</td>
<td>0.5</td>
<td>3.5</td>
<td>36.5</td>
<td>0.78</td>
<td>0.54</td>
<td>93</td>
<td>39.6</td>
<td>5.94</td>
</tr>
</tbody>
</table>
Table 5. Purification of rCollagen from corn endosperm extract by acid salt precipitation followed by diafiltration.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>rCollagen Concentration (mg/ml)</th>
<th>rCollagen Purity (%)</th>
<th>Step Yield (%)</th>
<th>Step Purification Factor</th>
<th>Overall Yield (%)</th>
<th>Overall Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>60</td>
<td>0.12</td>
<td>17.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resuspended Precipitate</td>
<td>60</td>
<td>0.11</td>
<td>85</td>
<td>88</td>
<td>4.9</td>
<td>88</td>
<td>4.9</td>
</tr>
<tr>
<td>Diafiltration Retentate</td>
<td>65.5</td>
<td>0.1</td>
<td>99</td>
<td>98</td>
<td>1.2</td>
<td>87</td>
<td>5.66</td>
</tr>
</tbody>
</table>
Figures:

Figure 1. Coomassie stained SDS-PAGE of corn proteins extracted at pH 2 and pH 7 without the addition of rCollagen loaded with 20 µL of sample in each lane. Lane A: endosperm extract at pH 2, lane B: endosperm extract at pH 7, lane C: germ extract at pH 2, and Lane D: is germ extract at pH 7. Lanes A and C are the extraction conditions used in the UF/DF experiments.
Figure 2. The permeate flux for a filtration of an rCollagen containing germ extract at low TMP and a crossflow rate of 0.12 m/s. Change of permeate flux with TMP shows the filtration was operated below the critical TMP. Permeate flux data was smoothed using a 4 period moving average.
Figure 3. The permeate flux for a filtration of an rCollagen containing endosperm extract at high TMP and a crossflow rate of 0.25 m/s. The rapid permeate flux decline with filtration time at constant TMP indicates that the filtration is operated above the critical TMP. Permeate flux data was smoothed using a 4 period moving average.
Figure 4. Effect of filtration conditions on the average permeate flux observed during diafiltration. Level 1 corresponds to low crossflow rate and TMP (0.12 m/s and ~0.5 bar, respectively) and endosperm extract. Level 2 corresponds to high crossflow rate and TMP (0.25 m/s and ~1.1 bar, respectively) and germ extract.
Figure 5. Change in the average corn protein sieving coefficient with the number of diavolumes during the filtration of a corn endosperm extract at high crossflow and TMP, and a filtration pH of 2.1.
Figure 6. Coomassie stained SDS-PAGE gel of filtration samples collected during an eight diavolume filtration of rCollagen in an endosperm extract. Lane A: 0.5 mg/ml rCollagen standard used for spiking, lane B: initial filtration feed, lane C: retentate after 1 diavolume, lane D: retentate after five diavolumes, lane E: retentate after three diavolumes, lane F: retentate after seven diavolumes, lane G: retentate after eight diavolumes, lane H: final retentate, lane I: final permeate. Each lane was loaded with 20 µL of sample.
Figure 7. Silver stained SDS-PAGE gel of fractions from the purification of rCollagen by acid salt precipitation followed by ultrafiltration. Lane A: 0.1 mg/ml rCollagen standard used for spiking, lane B: rCollagen containing endosperm extract, lane C: resuspended precipitate, lane D: supernatant, lane E: retentate after one diavolume, lane F: retentate after 2 diavolumes, lane G: retentate after three diavolumes, lane H: retentate after five diavolumes, and lane I: final permeate. Each lane was loaded with 20 µL of sample.
Chapter V: General Conclusions

The high priority placed on the downstream purification processes increases the demand for high throughput and high yield purification methods. The overall objective of the research presented in this thesis was to develop membrane-based separation methods for solid/liquid clarification and purification of recombinant proteins to meet this demand.

The work presents a framework that can be used in the implementation and evaluation of membrane-based separation methods. The effect of polyelectrolyte flocculation on the permeate flux and product rejection during the microfiltration of an industrial fermentation was examined, including a detailed analysis of the effect of the shear environment on the flocculated particles in the filtration. For the first time, a comprehensive study on the solid/liquid clarification of aqueous corn extracts by microfiltration was carried out, incorporating an analysis of the compressibility of the filter cake and the effect of filtration conditions on the permeate flux and product rejection. Finally, purification of recombinant proteins from aqueous corn endosperm and germ extracts by ultrafiltration was assessed.

In the work described in chapter II, polyelectrolyte flocculation was examined as a feed pretreatment to improve the permeate flux and product transmission during the microfiltration of an industrial fermentation broth. The use of cationic flocculation as a feed pretreatment has applications in many areas of the waste water treatment and biotechnology industries. Flocculation is likely to become an important step for
improving not only microfiltration but also centrifugation in the biotechnology industry, particularly in the recovery of protein inclusion bodies from bacterial lysates. This work establishes a method to efficiently screen many flocculants and provides a framework for conducting a detailed analysis of the factors affecting the microfiltration of flocculated particles. In our study, several polyelectrolytes were screened for flocculated particle size and permeate flux enhancements in unstirred and stirred dead-end filtrations. Larger flocculated particles were formed by flocculation with polyelectrolytes of longer chain length. Increase in particle size of the feed generally resulted in an improvement in the permeate flux, but the largest particle size did not result in the greatest permeate flux enhancement. In crossflow filtrations, for the first time it was observed that long term improvements in the filtration performance can be achieved despite complete floc disruption that was observed early in the filtration. The results indicate that flocculation of the filtration feed can significantly reduce the rejection of a recombinant protein. This work provides a foundation for the application of cationic flocculation to other filtration feeds and filtration modules (e.g. flat-sheet, hollow-fiber).

The use of plants as recombinant protein production systems is rapidly moving from the early development stages to large scale implementation, with several companies on the verge of introducing plant-derived recombinant protein products. Plants provide unique advantages over traditional cell-culture production systems, such as established and inexpensive methods for transformation, cultivation, and processing, as well as reducing the risk of contamination by mammalian pathogens. However, the purification process remains the most expensive step in the production of recombinant proteins in plants.
Membrane-based separations have the potential to reduce the cost of the purification process. In the work presented in chapters III and IV, the effectiveness of tangential flow microfiltration and ultrafiltration for the solid/liquid clarification and purification of aqueous corn endosperm and germ extracts was examined using recombinant type I human collagen (rCollagen, 265 kDa) and green fluorescent protein (GFP, 27 kDa) as model proteins.

Traditionally, the solid/liquid clarification of plant processing streams has been carried out by depth filtration or rotary drum vacuum filtration. However, both of these techniques rely heavily on the use of filter aids, which significantly add to the cost of the purification process. Additionally, effluents from these operations typically still contain some solids and require further clarification before the feed is ready for the subsequent purification steps. Microfiltration has the potential to produce a solids-free effluent while simultaneously eliminating the use of filter aids. The effectiveness of tangential flow microfiltration for the solid/liquid clarification of aqueous corn endosperm and germ extracts was examined in chapter III. In this study, microfiltration is established as an effective method for the solid/liquid clarification of aqueous corn extracts. The characteristics of the filter cakes formed during the filtration of aqueous extracts of corn endosperm and germ fractions were identified as well as analyzed the effect of the filtration operating conditions on the filtration performance. Despite high fouling of the ceramic membrane, high permeate flux and transmission of the low molecular weight proteins were achieved. Product transmission of the higher molecular weight proteins was improved by filtration with a poly(vinylidene fluoride) (PVDF) membrane, which
showed significantly less fouling compared to the ceramic membrane. This result illustrates the importance of selecting a membrane chemistry that is compatible with the filtration feed and reduces internal membrane fouling. This study provides an excellent approach for the characterization and evaluation of microfiltration for the solid/liquid clarification of plant systems, as well as a starting point for the implementation, optimization, and extension of microfiltration to other plant or cell culture host systems.

Ultrafiltration has the potential to provide an inexpensive, robust, and scalable purification method in the production on recombinant proteins. In the work presented in chapter IV, the effectiveness of ultrafiltration for the purification of recombinant proteins from aqueous corn endosperm and germ extracts was examined. We developed an approach to evaluate ultrafiltration as a technique for the purification of recombinant proteins produced in transgenic corn. Identification of the effects of membrane pore size, transmembrane pressure (TMP), crossflow rate, filtration pH, and targeted corn tissue on permeate flux and protein sieving in the ultrafiltration of aqueous corn extracts was performed. A method was developed to purify rCollagen to very high purity (>99%) using only precipitation and ultrafiltration. This work could be extended to other larger molecular weight larger recombinant proteins (>100 kDa) to provide an inexpensive purification scheme for proteins from corn extracts using ultrafiltration. Our work could be used as a model to evaluate the purification requirements of potential recombinant proteins and production host combinations to identify a system to minimize the downstream purification cost.
Taken together, successful development of several methods using membrane-based separations for solid/liquid clarification and purification of recombinant proteins from a bacterial fermentation broth and aqueous corn extracts was carried out. Filtration performance was improved for microfiltration of both an industrial fermentation broth and aqueous corn extracts and conditions were identified where ultrafiltration can be used to produce a highly purified recombinant protein from transgenic corn. The methods presented in this work can be used as models to develop more inexpensive and efficient membrane-based separations.

On a broader note, the development of membrane-based separation processes is extremely important to the biotechnology industry. Membrane-based separations can decrease the cost of the purification process by decreasing the processing time and increasing product yields. Future improvements in membrane materials, filtration modules, and understanding of the factors that affect protein retention and permeate flux will continue to drive membrane separations to the forefront of the separation techniques used in the biotechnology industry.

**Future Directions**

Through our work using flocculation to improve microfiltration we gained significant insights into the mechanisms that underlie the increases in permeate flux. Development of a model to elucidate the precise role of the feed spacer in the deposition of particles in a polydisperse feed might lead to significant advances in the design of feed spacers. Additionally, extension of this study to different fermentation feeds and examination of
this process in a scaled-up system are necessary for adoption of this technique at the production scale.

The use of membrane separations for the purification of proteins from transgenic plants shows great potential. Extensions of the studies described in this thesis to other available host systems would provide valuable insight into the purification requirements for each system and may allow for selection of a host organism that minimizes the purification requirements.