Membrane Unit Operations for Dairy Protein Concentration and Fractionation

By

Seyhun Gemili

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Food Science)

at the

UNIVERSITY OF WISCONSIN-MADISON

2012

Date of final oral examination: 05/17/12

The dissertation is approved by the following members of the Final Oral Committee:

Mark R. Etzel, Professor, Food Science

John A. Lucey, Professor, Food Science

Barbara H. Ingham, Associate Professor, Food Science

Regina M. Murphy, Professor, Chemical and Biological Engineering

Thatcher W. Root, Professor, Chemical and Biological Engineering

ABSTRACT

The unique functional properties of different whey proteins and increasing use of these purified proteins as ingredients in food formulations have motivated researchers to develop new methods of purification. Ultrafiltration has been in use for years in the dairy industry mainly to concentrate whey to obtain whey protein concentrate and to produce whey protein isolate by further desalting or diafiltration of the concentrate. However, ultrafiltration is not able to fractionate individual proteins from whey. That is because current ultrafiltration membranes separate proteins based on differences in size only. The size difference between the whey proteins is not large enough to fractionate the proteins. The overall objective of the present research was to develop charged ultrafiltration membranes for the fractionation of whey proteins. The premise of the research is that although the different whey proteins do not differ greatly in molecular weight, the isoelectric points are markedly different. This allows the membrane charge and protein charge to be carefully controlled to increase the selectivity of the fractionation. Furthermore, use of multistage configurations with recycling of the selected streams increases the purity and the recovery of the desired protein. In this study, uncharged and positively charged cross-flow ultrafiltration membranes having a 300 or 30 kDa molecular weight cut-off were used in one, two and three stage configurations to fractionate glycomacropeptide (GMP) from other whey proteins. Sieving coefficients (permeability of the membrane to the protein), concentrations, purities and recoveries of the proteins were experimentally determined. Using sieving coefficients of the proteins in

two different mass balance models, concentrations, purities and recoveries of the proteins were calculated and compared with the experimental results. In conclusion, using positively charged membranes in stage configurations was successful in fractionation of GMP. Purity and recovery were altered using different flow configurations and membranes having different molecular weight cut offs. Calculations obtained from two mass balance models were in close agreement with the experimental results. These mass balance models can be used to design different ultrafiltration systems with different stage configurations.

ACKNOWLEDGMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this dissertation.

First, I would like to express my thanks to my advisor, Professor Mark R. Etzel for his help, suggestions and feedback throughout my Ph.D. education and during the process of dissertation writing.

I am thankful to my committee members; Professor Regina M. Murphy, Professor Barbara H. Ingham, Professor John A. Lucey and Professor Thatcher W. Root for giving valuable suggestions to my research work.

I would also like to thank my lab mate Abhiram Arunkumar for his help, friendship and valuable suggestions.

I am forever indebted to my family, especially to my mother Asiye Gemili, for their unconditional love and support. They receive the most credit for whatever I have accomplished in my life thus far.

Finally, I would like to give my special thanks to my beloved wife and also my lab mate Aysel Yilmaz-Gemili whose patient love, help, support and encouragement kept me going forward through the whole period of this study.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. CHEESE WHEY

In the manufacture of cheese, curds are formed by the action of rennet and/or acid. Cheese whey is the liquid remaining after the recovery of these curds. Cheese whey is classified as sweet whey or acid whey. Sweet whey is manufactured during the production of rennet types of hard cheese like Cheddar or Swiss cheese. Acid whey is manufactured during the production of acid types of cheese such as Cottage cheese. Approximately, manufacture of 1 kg of cheese results in the production of 9 L of liquid whey (Tunick, 2008). Liquid whey consists of approximately 93% water. The main solid compound in liquid whey is lactose which constitutes approximately 50% of the total solids. Proteins represent less than 1% of total solids (Chandan et al., 2008). Table 1.1 shows the physico-chemical properties and content of sweet and acid whey.

Until the 1970s, whey had been considered as waste matter that had to be invariably disposed of. General methods to dispose of whey included feeding to domestic animals, dumping into rivers, application to local farmlands, or discharging into municipal waste water facilities. Because of its high biological oxygen demand (40,000 mg/kg or more), management and disposal of liquid whey has been regulated by strict laws since the beginning of the 1970s (Roginski et al., 2003). These laws encouraged companies and researchers to work on the production of a variety of whey products as an

alternative way to whey disposal. As of December 2011, the United States Department of Agriculture (USDA) reported that 76.5 million pounds of dry whey, 82.1 million pounds of lactose, 36.8 million pounds of whey protein concentrate, and 5.2 million pounds of whey protein isolates were produced in the U.S.A. (USDA Dairy Products Report, 2011). Different technologies have been developed to date to fractionate the proteins from lactose and other minerals and use the fractionated proteins as functional ingredients in food and pharmaceutical formulations.

1.1.1. Major Whey Proteins and Their Application Areas in Food Industry

The most abundant proteins in liquid whey are β-lactoglobulin, α-lactalbumin and glycomacropeptide. The physico-chemical properties of these proteins are listed in Table 1.2. It is important to note at this point that, even though glycomacropeptide is a peptide, it is referred to as a protein to avoid the repetition when proteins in cheese whey are discussed generally, and the word "whey" refers to "sweet whey" unless stated otherwise.

This section of the chapter introduces the major whey proteins and their applications as functional ingredients in the food industry.

1.1.1.1. β-Lactoglobulin

Monomeric β-lactoglobulin (BLG) consists of 162 amino acids and its molecular weight is approximately 18 kDa. Monomeric structure of BLG consists of three short helices, an α-helix, and nine strands of anti parallel β-sheets. BLG can exist in different association states depending on pH, temperature, concentration and ionic conditions (De

Wit, 2009). BLG is an equilibrium mixture of monomers and dimers in the pH range 4.5 – 7.5; and it starts to dissociate into two identical monomers above pH 8.0 and below pH 3.4 (Bonnaillie and Tomasula, 2008). The monomeric structure and the amino acid sequence of BLG are shown in Figures 1.1 and 1.2, respectively.

BLG is the most abundant protein in cheese whey and constitutes more than half of all the whey proteins. Because of this, its properties approximate the properties of whey protein powders. Studies showed that increasing the amount of BLG in food formulations increases gel strength and elasticity (Mehra and O'Kennedy, 2008). Lorenzen and Schrader (2006) showed that whey protein isolate (WPI) gels were stronger and more elastic than the corresponding whey protein concentrate (WPC) gels because of the higher BLG content in WPI. Tomasula and Yee (2001) reported that BLG-enriched whey powders showed enhanced emulsion stability, emulsion activity, viscosity and gelling properties compared to WPC. BLG is sometimes desired to be removed from food formulations such as infant formulae. Human breast milk does not contain any BLG; however, bovine milk does (Bonnaillie and Tomasula, 2008). Because of this, BLG-free infant formulae are produced to reach the consumer's preference.

1.1.1.2. α-Lactalbumin

α-lactalbumin (ALA) is a spherical, glycosylated, compactly folded calcium metalloprotein that consists of 123 amino acids and its molecular weight is approximately 14 kDa. In its amino acid sequence, there are four disulfide bonds and eight cysteine residues, and it is rich in tryptophan (Pritchard and Kailasapathy, 2011). The amino acid

sequence and folded structure are shown in Figures 1.3 and 1.4, respectively. Because of the high tryptophan content in bovine ALA, studies were performed to test the effect of ALA rich diets on cognitive performances; and it has been shown that there was an improved performance in memory tests of stress-vulnerable subjects based on the reaction time and the number of errors (Markus et al., 2002). Studies also showed that ALA incorporated into the diet improves mood and sleep because it is tryptophan-rich (Schmitt et al., 2005). These features make ALA enriched products very attractive to consumers. ALA enriched whey powder is readily available in the market for public consumption. Another important fact about ALA is that human breast milk contains about two to three times more ALA than bovine milk; and it is always desirable to have approximate protein profile of human breast milk in infant formulae (Baxter et al., 2011).

1.1.1.3. Glycomacropeptide

During the enzymatic coagulation of bovine milk, cleavage of a chymosin sensitive bond in κ-casein causes denaturation and destabilization of casein micelles. Cleavage of the bond between the 105^{th} and 106^{th} amino acid residues (Phe^{105} – Met^{106}) in κ-casein forms insoluble para κ-casein and soluble glycomacropeptide (GMP). GMP contains all carbohydrates of κ-casein, which are mainly galactose, N-galactosamine and N-acetylneuramic acid. In nature, there are A and B variants of GMP, and each of these variants has at least seven other forms of glycosylation (Lieske and Konrad, 1996; Kreuß et al., 2008). The amino acid sequence of GMP is shown in Figure 1.5. GMP is the third

most abundant protein in liquid cheese whey. It constitutes 12 – 20% of all the whey proteins (Ney et al., 2008).

Quantification of GMP is challenging work because of the following reasons: 1) GMP does not contain any aromatic amino acid, because of which, it is invisible at 280nm, which is the most common wavelength for the spectrophotometric detection of proteins; 2) GMP retains a net negative charge even at pH 3, and hence, it is not collected on cation exchangers; and 3) GMP does not stain coomassie blue in SDS-PAGE because of the lack of aromatic amino acids (Brody, 2002). The common quantification procedure for GMP requires trichloroacetic acid (TCA) precipitation of the other whey proteins leaving only GMP in solution. However, variations in charge and carbohydrate composition cause variations in solubility of GMP in TCA solutions. It has been found that more negatively-charged and/or carbohydrate-rich components are more soluble in TCA. Hooydonk and Olieman (1982) showed that using 8% TCA solution was optimum for accurate quantification of GMP. Using lower TCA concentrations resulted in insufficient removal of the whey proteins and using higher TCA concentrations caused precipitation of increasing amounts of GMP (Hooydonk and Olieman, 1982). Because of this finding, the most commonly used TCA concentration for purification of GMP from whey has been 8% (Lieske and Konrad, 1996). A 7.2% TCA concentration has been also commonly used in several studies on fractionation of GMP (Lieske and Konrad, 1996, Tek et al., 2005, Bhushan and Etzel, 2009).

GMP has been of interest in the food and pharmaceutical industries because of its unique therapeutic and functional properties. Brody (2002) reviewed the biological

activities of bovine GMP and listed that GMP has physiological functions such as, binding of cholera and *E.coli* entero-toxins, inhibition of bacterial and viral adhesion, suppression of gastric secretions, promotion of bifidobacterial growth, and modulation of immune system responses.

In this project, the goal was to produce highly pure GMP solutions, which can be used in the diets of patients having phenylketonuria (PKU). PKU is a genetic disease which causes deficiency of the hepatic enzyme phenylalanine hydroxylase (PHA). PHA is needed to metabolize the essential amino acid phenylalanine (Phe) to tyrosine. In the absence of PHA, Phe accumulates and is converted into phenylpyruvate (also known as phenylketone). Phenylpyruvate is not metabolized and its accumulation in the blood lowers blood glucose levels and impairs nerve function, causing brain damage (Gazit et al. 2003). Patients with PKU have to follow a very strict lifelong low protein diet that limits Phe intake. PKU patients can tolerate up to 500 mg Phe per day, which is less than 10% of Phe consumed in a normal diet (Ney et al., 2008a and 2008b). The common protein supplement for PKU patients is amino acid enriched beverages in the form of Phe-free formula. However, these beverages have an off-odor and bitter taste. Other than these bitter-taste beverages, there are limited dietary options available for PKU patients because of the unavailability of functional ingredients to produce the desired foods. GMP is the only natural protein having no Phe in its amino acid sequence. It has attractive physico-chemical properties like good solubility, emulsifying properties and foaming ability (Etzel, 2004). The functional properties of GMP are suitable for making low-Phe foods and beverages. Ney et al. (2008b) described the development of food products

made with GMP, including beverages, puddings and a cinnamon crunch bar. Sensory analysis (n= 49, age range 12 - 42 years) showed that these food products are acceptable and palatable. Scores of the products prepared using GMP on appearance, odor, taste, texture, and overall were always significantly higher than the scores of the amino acid beverage and a low protein bar ($P < 0.05$) (Lim et al., 2007).

1.2. ULTRAFILTRATION TECHNOLOGY

Ultrafiltration is commonly used as a concentration step and/or a clarification step in the food and pharmaceutical industries. Common applications are: concentration of milk and whey prior to spray drying, clarification of apple juice to remove the suspended particles (Mohr et al., 1989), and removal of salts by diafiltration in protein purification processes (Ghosh, 2003a). Recent studies showed that ultrafiltration also has potential application in separation and fractionation of functional and therapeutic proteins from different sources (Ghosh, 2003a). In this section, a characterization of physical phenomena in ultrafiltration will be given, and subsequently membranes and different modes of filtration will be explained.

1.2.1. Characterization of Physical Phenomena

1.2.1.1. Concentration Polarization

If a solute in the feed solution is completely or partially rejected by the membrane, the solute starts to accumulate on the upstream surface of the membrane within an ultrafiltration cycle. The first effect of this accumulated solute is that it reduces the flow rate of the permeate, which is the stream that passes through the membrane, because of the creation of an osmotic pressure and a resistance to the hydraulic permeability. The second effect is that the accumulation of the solute may create a gel layer and finally may foul the membrane (Zeman and Zydney, 1996). This accumulation of solute is known as concentration polarization.

Concentration polarization creates a concentration gradient upstream of the membrane (Figure 1.6). Solute concentration increases towards the surface of the membrane. Because of this, the concentration of bulk solute (C_b) is lower than the concentration of solute at the wall (C_w).

1.2.1.2. Sieving Coefficient

Filtration can be defined as a sieving process. An important condition for a particle to pass through the membrane is to have a smaller particle size than the pore size of the membrane. The solution that passes through the membrane is known as the permeate and the solution that is retained by the membrane is known as the retentate. Permeation of a solute in ultrafiltration is characterized by the sieving coefficient. Since concentration polarization creates a concentration gradient in the upstream of the membrane, two different sieving coefficients are defined. The mathematical definitions of the sieving coefficients are given in Equations 1.1 and 1.2 (Ghosh, 2003a).

$$S_{oi} = \frac{C_{Pi}}{C_{Ri}} \qquad (1.1)$$

where, S_{oi} is the observed sieving coefficient, in this study, C_{Pi} is the instantaneous concentration of the solute in permeate and C_{Ri} is the instantaneous concentration of the solute in the retentate (bulk concentration).

$$S_{ai} = \frac{C_{Pi}}{C_{wi}} \qquad (1.2)$$

where, S_{ai} is the actual (intrinsic) sieving coefficient, C_{wi} is the concentration of the solute at the wall of the upstream surface of the membrane.

1.2.1.3. Selectivity

Selectivity is a value showing the preference of a membrane to selectively permeate one solute in favor of another. The mathematical definition of the selectivity is given in Equation 1.3.

$$\Psi = \frac{S_{oi}}{S_{oj}} \qquad (1.3)$$

where, Ψ is the selectivity of the membrane, S_{oi} is the sieving coefficient of solute "*i*" and S_{oj} is the sieving coefficient of solute "*j*." The higher the selectivity better the separation. If the selectivity is 1, the sieving coefficients of two solutes are equal to each other. In this case, no separation occurs.

1.2.1.4. Molecular Weight Cut Off

Membrane manufacturers prefer to use a parameter called the "molecular weight cut-off (MWCO)" to specify the sieving properties of their membranes. MWCO refers to the lowest molecular weight solute (i.e. dextran) in which 90% of the solute is retained by the membrane. However, this method is not an accurate way to characterize a membrane because molecular weights of solutes are not always representative of their actual size. It is possible that two solutes have the same molecular weight values but different sizes or vice versa, especially for biological particles such as proteins (Ghosh, 2003a).

1.2.2. Membranes

In the handbook of membrane separations, a membrane is defined as "*a barrier that separates two phases and selectively restricts the transport of various chemicals*"

(Sastre et al., 2009). The history of membrane technology dates back to the eighteenth century when Nollet discovered that a pig's bladder passes preferentially ethanol when it was placed between a water-ethanol mixture and pure water (Nollet, 1995). Until the twentieth century, scientists focused on understanding the mathematics and morphology of natural membranes. With the beginning of the twentieth century, the first synthetic membranes were developed by Bechhold in 1907. Bechhold made synthetic membranes by impregnating a filter paper with a solution of nitrocellulose in glacial acetic acid (Wickramasinghe, 2008). From then, many studies have been performed to improve membrane technology. Membranes are now stable to wide pH and temperature ranges; and they are available in the form of both hydrophilic and hydrophobic materials.

One general way to classify membranes is to do so by their structural appearances. Membranes can have symmetric or asymmetric structure.

1.2.2.1. Symmetric Membranes

Symmetric membranes have a uniform structure across the thickness of the membrane. They can be porous or nonporous (or dense). Symmetric porous membranes have a highly voided structure with randomly distributed, interconnected pores. These pores dictate the permeability of the membranes. All particles larger than the largest pores are completely rejected by the membrane. Particles smaller than the largest pores, but larger than the smallest pores are partially rejected. Particles much smaller than the smallest pores freely pass through the membrane (Baker, 2004). For symmetric and porous membranes, separation of solutes mainly depends on the molecular size and pore

size distribution. Symmetric nonporous membranes, on the other hand, consist of a dense structure throughout the membrane. For nonporous membranes, separation occurs by diffusion under the driving force of a pressure, concentration, or electrical potential gradient (Mulder, 1991). An important property of nonporous membranes is that even similar size molecules may be separated.

1.2.2.2. Asymmetric Membranes

The first asymmetric membrane was produced from cellulose acetate in the early 1960s by Loeb and Sourirajan (Mohr et al., 1989). This development was a breakthrough in membrane technology. Asymmetric membranes consist of an extremely thin surface layer supported on a much thicker porous structure. They have a graded pore size distribution that varies throughout the membrane (Baker, 2004). Asymmetric membranes can either be formed in a single operation or the skin layer and porous layer can be manufactured separately and then laminated to form the whole asymmetric membrane. Using asymmetric membranes, higher flux can be obtained with the same permeation rate obtained with a symmetric dense membrane. While the skin layer on top of the membrane does the separation job, porous layer increase the mechanical strength of the membrane (Grassi, 2009).

1.2.3. Modes of Ultrafiltration

There are two modes of operation in ultrafiltration systems. These are dead-end and cross-flow modes.

1.2.3.1. Dead-end

In this mode of filtration, the feed stream flows through the membrane perpendicular to the membrane surface. There is only one inlet (feed stream) and one outlet (permeate stream). As the feed stream passes through the membrane, retained species build up on the membrane surface and the permeate stream is collected into another vessel. Dead-end filtration mode is very suitable for small-scale processes. It has a low energy requirement and it is easy to operate (Guizard and Amblard, 2009). However, one drawback of dead-end filtration is that after a short period of time, the build up on the membrane surface has to be removed by backwashing and/or vigorous agitation.

1.2.3.2. Cross-flow

Cross-flow filtration is also known as tangential flow filtration. In this mode of filtration, the feed stream flows parallel to the membrane surface. There is one inlet (feed stream) and two outlets (permeate and retentate streams). As the feed stream passes parallel to the membrane surface, a fraction of the feed stream permeates the membrane and the rest is collected as the retentate stream. Depending on the size of the particles in the feed stream and pore size distribution of the membrane, cross-flow filtration reduces the accumulation of the materials on the membrane surface and creates less concentration polarization than dead-end mode. This allows the filtration to continue longer than it would be using dead-end type of filtration. Because of this, cross-flow filtration is better

than dead-end filtration for the filtration of partially or completely non-permeable particle solutions (Russotti and Goklen, 2001).

1.3. ULTRAFILTRATION TECHNOLOGY IN FRACTIONATION OF CHEESE WHEY PROTEINS

Ultrafiltration is used extensively in the dairy industry for concentration, demineralization, and clarification of liquid milk and cheese whey (Goulas and Grandison, 2008). Recent studies showed that ultrafiltration technology could also be used in the fractionation of milk and whey proteins to produce individual dairy protein fractions such as ALA, BLG and GMP (Xu et al., 2000; Cheang and Zydney, 2003; Bhushan and Etzel, 2009). The scope of the present thesis research is to produce individual cheese whey protein fractions. For this reason, developments in whey protein fractionation using ultrafiltration will be discussed in detail. There are currently three approaches to reach the goal of whey protein fractionation using ultrafiltration. These are adjustment of the physico-chemical conditions (pH and conductivity) of the feed solution, application of multi-stage ultrafiltration using diafiltration (diafiltration and stage configuration), and chemical modification of ultrafiltration membranes (charged ultrafiltration technology).

1.3.1. pH and Conductivity

Careful adjustment of pH and the salt concentration (conductivity) of the protein solution enhances the fractionation of proteins using ultrafiltration. The effects of these parameters on sieving coefficients of different proteins differ largely depending on the properties of each individual protein and the membrane (Ghosh, 2003a). The protein-protein and protein-solution interactions also have an important effect on the sieving

coefficeints of the proteins through the membranes. It is known that protein-protein interactions become more attractive as the salt concentration is increased due to screening of the repulsive double-layer force, and when the net charge of the protein is decreased by adjusting the pH toward the isoelectric pH of the protein (Curtis and Lue, 2006).

Many researchers have reported that the highest sieving coefficient of a protein is obtained when the pH was adjusted to the isoelectric point of the protein (Nakao et al., 1988; Saksena and Zydney, 1994; Balakrishnana and Agarwal, 1996; Lucas et al., 1998; Burns and Zydney, 1999; de la Casa et al., 2007; Bakhshayeshi and Zydney, 2008). Xu et al. (2000) studied the effect of pH and salt concentration on fractionation of bovine immunoglobulin G (IgG) from low molecular weight proteins (ALA, BLG, bovine serum albumin (BSA)) using 100 kDa regenerated cellulose membranes. The results indicated that the rejection of the low-molecular weight proteins increased while pH was increased from 4.3 to 9.0. The isoelectric points of the low molecular weight proteins range from 4.8 to 5.3, therefore, increasing the pH up to 9 increased the negative charge on the proteins. Since ultrafiltration membranes naturally had negative charge, the rejection of the low molecular weight proteins increased. At pH 4.3, drastic decrease was observed in the permeation of the low molecular weight proteins when the salt concentration was increased from 0% to 5% because of the salting out effect of NaCl on major whey proteins. However, no significant change in the permeation of the low molecular weight proteins was observed at higher pH values with an increase in the salt concentration because of the insufficient increase in the salt concentration to affect the permeability of the proteins (Xu et al., 2000). This study showed that the permeation of whey proteins

through ultrafiltration membranes could be regulated by the adjustments of pH and salt content of the whey solution. Cheang and Zydney (2003) fractionated ALA and BLG in binary solutions with high recoveries at pH 5.5 and an ionic strength of 50 mM using 30 kDa composite regenerated cellulose membranes; and they observed significant increase in the sieving coefficients of ALA and BLG but a significant decrease in the selectivity of the membrane when the ionic strength increased from 50 to 150 mM (Cheang and Zydney, 2003). The significant decrease in the selectivity with an increasing ionic strength is because of the greater relative increase in the sieving coefficient of BLG.

1.3.2. Diafiltration and Stage Configurations

One way to improve the effect of ultrafiltration on selectivity is to configure the process in multi-stage configurations. Diafiltration is commonly used in multi-stage configurations to keep the concentration of the feed solution constant, remove low molecular weight solutes (i.e. salts, lactose), improve the selectivity of the fractionation, and improve the recovery of the protein. Diafiltration is a method where a certain amount of buffer solution is added to the feed solution. The amount of the buffer solution is usually kept equal to the amount of the permeate solution so that the volume of the feed solution is constant (Ghosh, 2003a). The amount of buffer solution added to the diafiltration step is defined by the number of diavolumes, which is based on the volume of the diafiltration buffer introduced into the operation compared to the retentate volume.

Simulation and experimental studies have been conducted on fractionation of proteins using multi-stage ultrafiltration processes (Cheang and Zydney, 2004; Mayani et

al., 2009; Mayani et al., 2010; Wang et al., 2010; Ghosh, 2003b). Diafiltration has always been used in these studies to improve the recovery of the proteins. For example, Cheang and Zydney (2004) used a two-stage ultrafiltration system to fractionate ALA and BLG from reconstituted whey protein isolate. They used 30 kDa and 100 kDa membranes in two strategies. In the first strategy, the 30 kDa membrane was used in the first stage and the 100 kDa membrane was used in the second stage. In the second strategy, the opposite approach of the first strategy was used. In both strategies, diafiltration steps were used in both the stages. Purification factors (Purification factor is evaluated as "*the ratio of the mass of the recovered protein in interest to the mass of all impurities in the final product solution divided by the same ratio in the initial feed solution*") and recoveries of ALA and BLG from the two strategies were reported. More than 10-fold purified ALA fractions with 95% and 85% recoveries were obtained from strategy one and two, respectively. 8-fold and 4-fold purified BLG fractions were obtained with overall 70% recovery from the first and second strategy, respectively. Totally 25 diavolumes were used in the experimental study.

1.3.3. Charged Ultrafiltration Membranes

Nakao et al. (1988) published the first study, which reported the use of charged ultrafiltration membranes in separation of two proteins (myoglobin and cytochrome C). Even though these two proteins have nearly the same size, they have different isoelectric points. By adjusting the pH of the protein solution to near the isoelectric point of one of

the proteins, the charged membrane permeated the uncharged protein but rejected the protein that was similarly charged as the membrane (Nakao et al., 1988).

van Reis (2006) patented methods to generate positive or negative charges on ultrafiltration membranes, and their use for fractionation of proteins. Bhushan and Etzel (2007) positively charged regenerated cellulose membranes using the method of van Reis. Different amounts of positive charge (0% to 100%) on the membranes were investigated for separation of GMP from BLG in the pH range 3.0 - 7.5 and at a conductivity of 4 mS/cm. Maximum selectivity values (~55) were obtained for 100% charged membrane at pH 3.0 and 75% charged membrane at pH 4.0. Another study of Bhushan and Etzel (2009) examined the fractionation of GMP from BLG and from Cheddar cheese whey using uncharged and 100% positively charged regenerated cellulose membranes. At pH 3.0, using 100% positively charged membranes increased the selectivity by about 600% compared to the uncharged membrane for fractionation of GMP from binary solution containing GMP and BLG. The increase in selectivity was 220% when liquid cheese whey was used. Both the studies (Bhushan and Etzel, 2007 and 2009) used dead-end ultrafiltration mode in a single stage.

1.4. MASS BALANCE MODEL DEVELOPMENT

In this study, a single-stage ultrafiltration system was used (Figure 1.7). A mass balance model from the literature (Kurnik et al., 1995) was used. Two mass balance boundaries are considered in this treatment (Figure 1.7). For the model system I, there is an accumulation in the retentate beaker and there is an outgoing permeate stream. A general mass balance for the solute "*i*" for this system will assume a form as follows:

$$\frac{dm_{sys,i}}{dt} = -\dot{m}_{out,i} \tag{1.4}$$

Where, $m_{sys,i}$, is the mass of the component *i* in the system, and $\dot{m}_{out,i}$ is the rate of mass loss from the system.

If the density is assumed constant, then we have:

$$\frac{dR}{dt} = -\dot{V}_P \tag{1.5}$$

Where, $\dot{V}_P$ is the volumetric flow rate of the permeate stream and can be expressed as:

$$\dot{V}_P = \frac{dP}{dt}\,. \tag{1.6}$$

Let C_{Pi} and C_{Ri} be the instantaneous concentrations of the solute in permeate and retentate, respectively; *P* and *R* be the volumes of permeate and retentate, respectively; and *t* is time.

Equation 1.4 can be written in terms of concentrations as follows:

$$\frac{d(RC_{Ri})}{dt} = -C_{Pi}\dot{V}_P \tag{1.7}$$

Equation 1.7 can be rearranged using the following substitutions:

1. Observed sieving coefficient (S_{0i}), which was defined as: C_{Pi}/C_{Ri}.
2. Constant total fluid volume in the ultrafiltration system.

$$\frac{dP}{dt} = -\frac{dR}{dt} \tag{1.8}$$

Substitution of S_{0i} and Equation 1.8 into Equation 1.7 gives Equation 1.9.

$$\frac{d(RC_{Ri})}{dt} = S_{oi}C_{Ri}(\frac{dR}{dt}) \tag{1.9}$$

Rearrangement of Equation 1.9 gives:

$$(S_{oi} - 1)\frac{dR}{R} = \frac{dC_R}{C_{Ri}} \tag{1.10}$$

If it is assumed that there is no change in the value of S_{0i} in an ultrafiltration cycle, then the final solution of Kurnik et al. (1995) can be expressed as in Equation 1.11.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (S_{oi} - 1)\ln(1 - f) \tag{1.11}$$

where $f = P/F$ and F is the volume of the initial feed solution. For this model system, the sieving coefficient was measured under total recycle conditions ($f = 0$). With the known constant value of S_{0i} and by knowing C_{Fi}, the value of C_{Ri} can be obtained for any given f using equation 1.11.

To calculate the mixing cup concentration of the solute in permeate ($<C_{Pi}>$), an overall integrated mass balance (based on system II of Figure 1.7) was used:

$$\frac{dm_{sys,i}}{dt} = 0 \tag{1.12}$$

$$At\ t = 0, m_{sys,i} = C_{Fi}F \tag{1.13}$$

$$At\ t = t, m_{sys,i} = < C_{Ri} > R + < C_{Pi} > P \tag{1.14}$$

Using Equations 1.13 and 1.14, an overall mass balance can be expressed as follows:

$$C_{Fi}F = < C_{Ri} > R + < C_{Pi} > P. \tag{1.15}$$

Where $<C_{Ri}>$ is the mixing cup concentration of solute in the retentate. An assumption was made that $<C_{Ri}> \approx C_{Ri}$, and C_{Ri} was obtained using equation 1.11. This assumption is an approximation because C_{Ri} is the instantaneous concentration of solute in the retentate and is always slightly greater than the solute concentration in the beaker $<C_{Ri}>$ due to the concentration effect of withdrawing the permeate stream from the membrane. The permeate flow rate of about 6 mL/min was withdrawn from a feed solution flow rate of about 36-56 mL/min to give a retentate flow rate of 30-50 mL/min. Therefore, the value of C_{Ri} was about 12 to 20% greater than $<C_{Ri}>$. This is because the solution in the beaker was at $<C_{Ri}>$ and the retentate was at C_{Ri}.

In practice, this concentration difference was somewhat less than calculated, and the observed value of C_{Ri} was about 10% greater than the observed value of $<C_{Ri}>$.

1.5. HYPOTHESIS AND OBJECTIVES

Previous work on the fractionation of proteins from cheese whey using charged or uncharged ultrafiltration membranes in staged or unstaged configurations has the following limitation.

1. Commonly dead-end ultrafiltration mode was preferred in previous studies because it is easy to operate and small volumes of feed solution are sufficient. In our study, cross-flow ultrafiltration mode was used. Cross-flow ultrafiltration is the process mode used in the dairy industry; therefore, the findings of our research are more applicable to the industry.
2. To our knowledge, diafiltration step has always been used in previous studies when staged configurations were used. One important drawback of the diafiltration is that the buffer solution which is introduced to the system generates waste because it has to be removed at the end of the process. In our study, no diafiltration step was used.
3. Most of the studies in the literature have been conducted with either dilute pure proteins in buffer or reconstituted whey protein powders in buffer. These systems have no immediate practical value. In our study, we used both protein solutions and real cheese whey.
4. The mass balance model in the literature assumed that the sieving coefficient was constant within an ultrafiltration cycle. In our research, a new mass balance model using inconstant sieving coefficients was developed.

5. The mass balance model in the literature was not used for real protein solutions. We tested the model for real cheese whey.
6. There is no study in the literature which could manage to fractionate proteins having molecular weights of less than $1/10^{th}$ of the molecular weight cut off of ultrafiltration membrane.

1.5.1. Hypothesis

The overall hypothesis of the present research was that highly pure whey protein fractions can be obtained using charged ultrafiltration membranes in staged configurations without diafiltration and at high recovery. Based on this, two specific hypotheses were formulated;

1. Whey protein fractions can be obtained using positively charged ultrafiltration membranes.
2. Multi-stage membrane configurations would increase the purity of the protein.

1.5.2. Objectives

1. Determination of sieving coefficients and selectivity using single and binary protein solutions, and liquid cheese whey.
2. Evaluation of the mass balance models by comparison to the experimental results.

3. Evaluation of positively charged ultrafiltration membranes in one-, two- and three-stage configurations for production of highly pure whey proteins with high recovery.

1.6. REFERENCES

Alpha-lactalbumin. 2011. Available from: <http://en.wikipedia.org/wiki/Alpha-lactalbumin> Accessed on December 25, 2011.

Baker, R.W. 2004. *Membrane technology and applications*. West Sussex, England: John Wiley and Sons Ltd.

Bakhshayeshi, M. and Zydney, A.L. 2008. Effect of solution pH on protein transmission and membrane capacity during virus filtration. *Biotechnology and Bioengineering*. Vol. 100, pg: 108-117.

Balakrishnana, M. and Agarwal, G.P. 1996. Protein fractionation in a vortex flow filter: effect of system hydrodynamics and solution environment on single protein transmission. *Journal of Membrane Science*. Vol. 112, pg: 47-76.

Baxter, J., Dimler, S. and Rangavajala, N. 2011. Dairy ingredients in infant and adult nutrition products. In *Dairy ingredient for food processing*. Edited by Chandan, R.C. and Kilara, A. pg: 515 – 532. Ames, IA: Blackwell Publishing Ltd.

Beta-lactoglobulin. 2011. Available from: <http://en.wikipedia.org/wiki/Beta-lactoglobulin> Accessed on December 25, 2011.

Bhushan, S. and Etzel, M.R. 2007. Charged ultrafiltration membranes for protein separation. *American Society of Agricultural and Biological Engineers Meeting Presentation*. Paper No: 077063

Bhushan, S. and Etzel M.R. 2009. Charged ultrafiltration membranes increase the selectivity of whey protein separations. *Journal of Food Science*. Vol. 74 (3), pg: E131 – E139.

Blaschek, K.M., Wendorff, W.L. and Rankin, S.A. 2007. Survey of salty and sweet whey composition from various cheese plants in Wisconsin. *Journal of Dairy Science*. Vol. 90, pg: 2029 – 2034.

Bonnaillie, L.M. and Tomasula, P.M. 2008. Whey protein fraction. In *Whey processing, functionality and health benefits*. Edited by Onwulata, C.I. and Huth, P.J., pg: 15 – 38. Ames, IA: IFT Press.

Brody, E.P. 2000. Biological activities of bovine glycomacropeptide. *British Journal of Nutrition*. Vol. 84, pg: S39 – S46.

Burns, D.B. and Zydney, A.L. 1999. Effect of solution pH on protein transport through ultrafiltration membranes. *Biotechnology and Bioengineering*. Vol. 64, pg:27-37.

Chandan C. R., Kilara A. and Shah N. (2008). *Dairy Processing and Quality Assurance*. John Wiley & Sons, Inc.

Cheang, C. and Zydney, A.L. 2003. Separation of α-lactalbumin and β-lactoglobulin using membrane ultrafiltration. *Biotechnology and Bioengineering*. Vol. 83, pg: 201-209.

Cheang, B. and Zydney, A.L. 2004. A two-stage ultrafiltration process for fractionation of whey protein isolate. *Journal of Membrane Science*. Vol. 231, pg: 159-167.

Curtis, R.A. and Lue, L. 2006. A molecular approach to bioseparations: Protein-protein and protein-salt interactions. *Chemical Engineering Science*. Vol. 61, pg: 907-923.

De la Casa, E.J., Guadix, A., Ibanez, R. and Guadix, E.M. 2007. Influence of pH and salt concentration on the cross-flow microfiltration of BSA through a ceramic membrane. *Biochemical Engineering Journal*. Vol. 33, pg: 110-115.

De Wit, J.N. 2009. Thermal behavior of bovine β-lactoglobulin at temperatures up to 150 ºC. a review. *Trends in Food Science & Technology*. Vol. 20, pg: 27 – 34.

Etzel, M.R. 2004. Manufactue and use of dairy protein fractions. *Journal of Nutrition*. Vol. 134, pg: 996S – 1002S.

Etzel, M.R. 2009. Charged ultrafiltration and microfiltration in antibody purification. In *Process Scale Purification of Antibodies*. Edited by Gottschalk, U. pg: 325 – 347. Hoboken, NJ: John Wiley & Sons, Inc.

Gazit, V., Ben-Abraham, R., Pick, C.G. and Katz, Y. 2003. β-phenylpyruvate induces long-term neurobehavioral damage and brain necrosis in neonatal mice. *Behavioural Brain Reseach*. Vol. 143, pg: 1- 5.

Ghosh, R. 2003a. *Protein bioseparations using ultrafiltration: Theory, applications and new developments*. London: Imperial College Press.

Ghosh, R. 2003b. Novel cascade ultrafiltration configuration for continuous, high-resolution protein-protein fractionation: a simulation study. *Journal of Membrane Science*. Vol. 226, pg: 85-99.

Goulas, A. and Grandison, A.S. 2008. Applications of membrane separation. In *Advanced Dairy Science and Technology*. Edited by Britz, T.J. and Robinson, R.K. pg: 35 – 75. Ames, IO: Blackwell Publishing Ltd.

Grassi, M. 2009. Membranes in drug delivery. In *Handbook of membrane separations*. Edited by Pubby, A.K., Rizvi, S.S.H., Sastre, A.M. pg: 3 – 5. Boca Raton, FL: CRC Press.

Guizard, C. and Amblard, P. 2009. Current status and prospects for ceramic membranes applications. In *Handbook of membrane separations*. Edited by Pubby, A.K., Rizvi, S.S.H., Sastre, A.M. pg: 140 – 179. Boca Raton, FL: CRC Press.

Gunderson, S.S., Brower, W.S., O'Dell, J.L. and Lightfoot, E.N. 2007. Design of membrane cascades. *Separation Science and Technology*. Vol. 42, pg: 2121 – 2142.

Kilara, A. 2008. Whey and whey products. In *Dairy Processing and Quality Assurance*. Edited by Chandan C. R., Kilara A., and Shah N. pg: 337 – 355. John Wiley & Sons, Inc.

Kreuß, M., Krause, I. and Kulozik, U. 2008. Separation of a glycosylated and non-glycosylated fraction of caseinomacropeptide using different anion-exchange phases. *Journal of Chromatography A*. Vol. 1208, pg: 126 – 132.

Kurnik, R.T., Yu, A.W., Blank, G.S., Burton, A.R., Smith, D., Athalye, A.M. and van Reis, R. 1995. Buffer exchange using size exclusion chromatography, and tangential flow filtration: models, development, and industrial application. *Biotechnology and Bioengineering*. Vol. 45, pg: 149 – 157.

Lim, K., van Clacal, S.C., Nelson, K.L., Gleason, S.T. and Ney, D.M. 2007. Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide

from cheese whey for individuals with PKU. *Molecular Genetics and Metabolism*. Vol. 192, pg: 176-178.

Lorenzen, P.C. and Schrader, K. 2006. A comparative study of the gelation properties of whey protein concentrate and whey protein isolate. *Lait*. Vol. 86 (4), pg: 259 – 271.

Lucas, D., Rabiller-Baundry, M., Michel, F. and Chaufer, B. 1998. Role of the physico-chemical enviroment on ultrafiltration of lysozyme with modified inorganic membrane. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. Vol. 136, pg:109-122.

Markus, C.R., Olivier, B. and de Haan, E.H. 2002. Whey protein rich in α-lactalbumin increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and improves cognitive performance in stress-vulnerable subjects. *The American Journal of Clinical Nutrition*. Vol. 75 (6), pg: 1051 – 1066.

Mayani, M., Mohanty, K., Filipe, C. and Ghosh, R. 2009. Continuous fractionation of plasma proteins HSA and HIgG using cascade ultrafiltration systems. *Separation and Purification Technology*. Vol. 20, pg: 231-241.

Mayani, M., Filipe, C.D.M. and Ghosh, R. 2010. Cascade ultrafiltration systems – Integrated processes for purification and concentration of lysozyme. *Journal of Membrane Science*. Vol. 347, pg: 150-158.

Mehra, R. and O'Kennedy, B.T. 2008. Separation of β-Lactoglobulin from Whey: Its physic-chemical properties and potential uses. In *Whey processing, functionality*

and health benefits. Edited by Onwulata, C.I. and Huth, P.J., pg: 39 – 62. Ames, IA: IFT Press.

Mohr, C.M., Engelgau, D.E., Leeper, S.A. and Charboneau, B.L. 1989. *Membrane application and research in food processing*. Park Ridge, NJ: Noyes Data Corporation.

Morr, C.V. and Ha, E.Y.W. 1993. Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition*. Vol: 33, pg: 431 – 476.

Mulder, M. 1991. *Basic principles of membrane technology*. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Nakao, S., Osada, H., Kurata, H., Tsuru, T. and Kimura, S. 1988. Separation of proteins by charged ultrafiltration membranes. *Desalination*. Vol. 70, pg: 191-205.

Ney, D.M., Hull, A.K., van Calcar, S.C., Liu, X. and Etzel, M.R. 2008a. Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of phenlyketonuria. *The Journal of Nutrition*. Vol. 138(2), pg: 316 – 322.

Ney, D.M., Gleason, S.T., van Calcar, S.C., MacLeod, E.L., Nelson, K.L., Etzel, M.R., Rice, G.M. and Wolff, J.A. 2008b. Nutritional management of PKU with glycomacropeptide from cheese whey. *Journal of Inherited Metabolic Disease*. Vol. 32 (1), pg: 32-39.

Nollet, J.A. 1995. Investigation on the causes for the ebullition of liquids (Reprinted from Histoire De Lacademie Royale Des Science 1748, pg: 57 – 104, 1752). *Journal of Membrane Science*. Vol. 100, pg: 1 – 3.

Pritchard, S.R. and Kailasapathy, K. 2011. Chemical, physical, and functional characteristics of dairy ingredients. In *Dairy ingredient for food processing*. Edited by Chandan, R.C. and Kilara, A. pg: 35 – 57. Ames, IA: Blackwell Publishing Ltd.

Renner, E. and Abd El-Salam, M.H. 1991. *Application of ultrafiltration in the dairy industry*. New York, Elsevier Science Publishers LTD.

Roginski, H., Fuquay, J. W. and Fox, P. F. (2003). *Encyclopedia of Dairy Sciences*. Academic Press, London, Volume 3, pp. 1924–1966

Russotti, G. and Goklen, K.E. 2001. Crossflow membrane filtration of fermentation broth. In *Membrane separations in biotechnology*. Edited by Wang, W.K. pg: 85 – 159. New York, NY: Marcel Dekker, Inc.

Saksena, S. and Zydney, A.L. 1994. Effect of pH and ionic strength on the separation of albumin during ultrafiltration. *Journal of Membrane Science*. Vol. 43, pg: 960-968.

Sastre, A.M., Pubby, A.K. and Rizvi, S.S.H. 2009. Membrane application in chemical and pharmaceutical industries and in conservation of natural resources: introduction. In *Handbook of membrane separations*. Edited by Pubby, A.K., Rizvi, S.S.H., Sastre, A.M. pg: 3 – 5. Boca Raton, FL: CRC Press.

Schmitt, J.A., Jorissen, B.L., Dye, L., Markus, C.R., Deutz, N.E. and Riedel, W.J. 2005. Memory function in women with premenstrual complaints and the effect of serotonergic stimulation by acute administration of an α-lactalbumin protein. *Journal of Psychopharmacol*. Vol. 19 (4), pg: 375 – 384.

Smith, K. 2008. Dried dairy ingredients. *Wisconsin Center for Dairy Research*.

Tek, H.N., Turhan, K.N. and Etzel, M.R. 2005. Effect of conductivity, pH, and elution buffer salinity on glycomacropeptide recovery from whey using anion exchange chromatography. *Journal of Food Science*. Vol. 70(4), pg: E295 – E300.

Tomasula, P.M. and Yee, W.C.F. 2001. Enriched fractions of alpha-lactalbumin (α-La) and beta-lactoglobulin (β-LG) from whey protein concentrate using carbon dioxide. Functional properties in aqueous solution. *Journal of Food Processing and Preservation*. Vol. 25 (4), pg: 267 – 282.

Tunick, M.H. 2008. Whey protein production and utilization: a brief history. In *Whey processing, functionality and health benefits*. Edited by: Onwulata, C.I. and Huth, R.J. pg: 1 – 13. Ames, IA: IFT Press.

USDA Dairy Products Report. 2011. Available from: <http://www.usda.gov/nass/PUBS/TODAYRPT/dary1211.pdf> Accessed on December 17, 2011.

Wang, L., Khan, T., Mohanty, K. and Ghosh, R. 2010. Cascade ultrafiltration bioreactor-separator system for continuous production of F(ab`)2 fragment from immunoglobulin G. *Journal of Membrane Science*. Vol. 351, pg: 96-103.

Wickramasinghe, S.R. 2008. Tangential-flow filtration for virus capture. In *Advanced Membrane Technology and Applications*. Edited by: Li, N.N., Fane, A.G., Ho. W.W. and Matsuura, T. pg: 541- 555. Hoboken, NJ: John Wiley and Sons, Inc.

Xu, Y., Sleigh, R., Hourigan, J. and Johnson, R. 2000. Separation of bovine immunoglobulin G and glycomacropeptide from dairy whey. *Process Biochemistry*. Vol. 36 (5), pg: 393-399.

Zeman, L.J. and Zydney, A.L. 1996. *Microfiltration and ultrafiltration principles and applications*. New York, NY: Marcel Dekker, Inc.

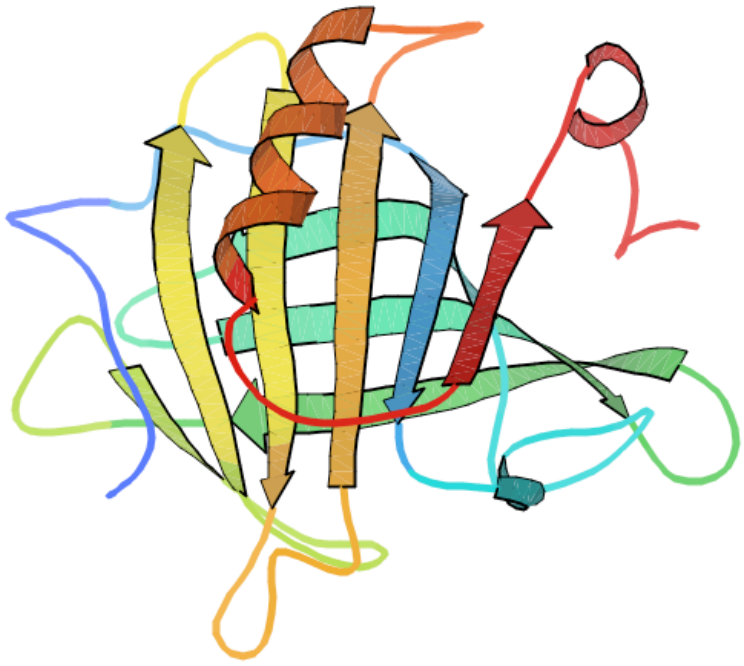

Figure 1.1. Monomeric folded structure of β-lactoglobulin (Source: Beta-lactoglobulin, 2011)

Figure 1.2. Amino acid sequence of β-lactoglobulin (Variant A) (Source: Kilara, 2008)

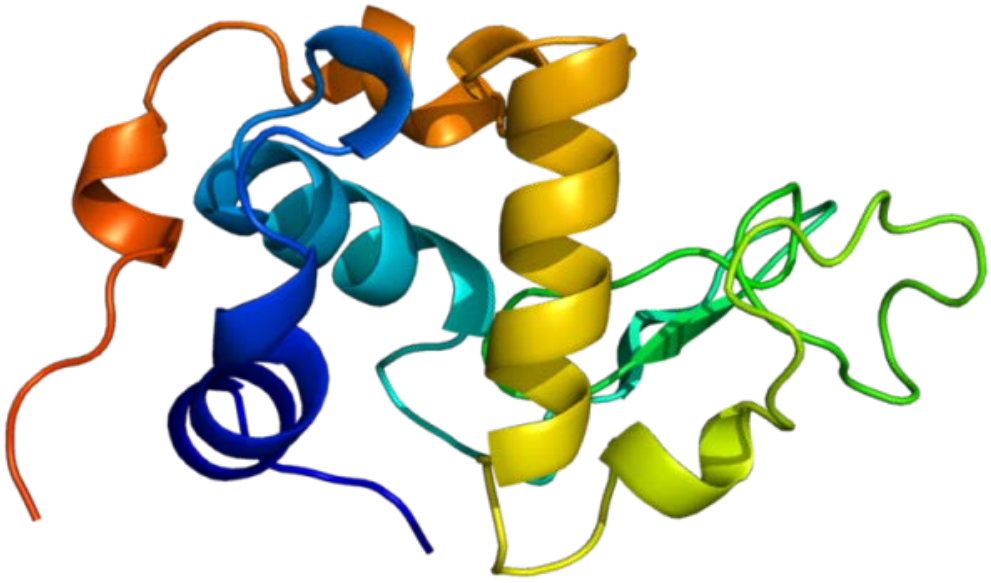

Figure 1.3. Folded structure of α-lactalbumin (Source: Alpha-lactoalbumin, 2011)

Figure 1.4. Amino acid sequence of α-lactalbumin (Variant A) (Source: Kilara, 2008)

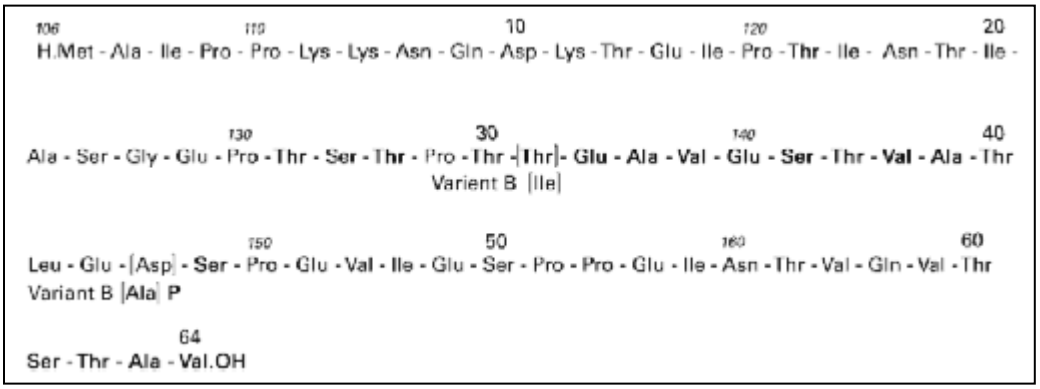

Figure 1.5. Amino acid sequence of glycomacropeptide (Source: Brody, 2000)

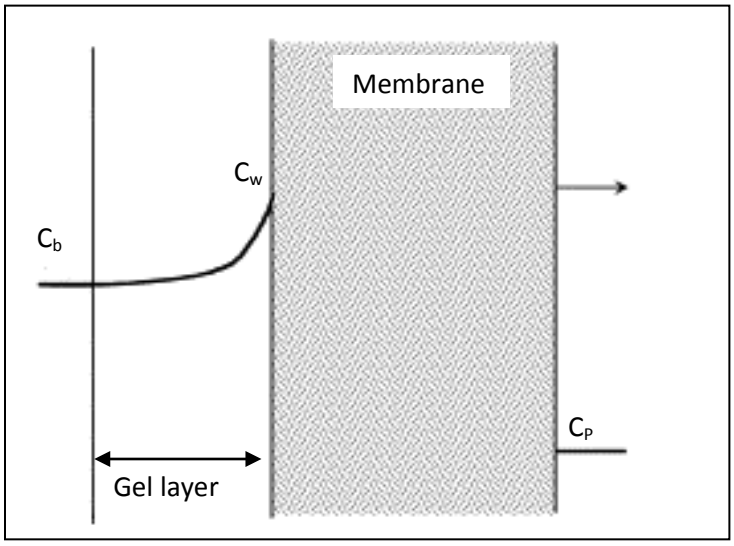

Figure 1.6. Concentration polarization (C_b: bulk concentration, C_w: wall concentration, C_p: permeate concentration)

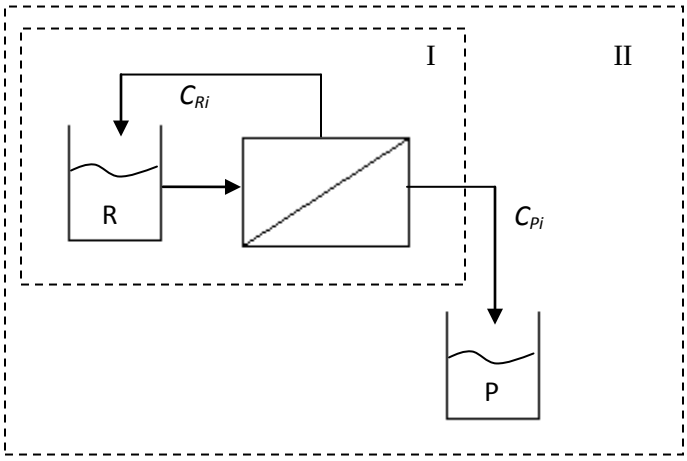

Figure 1.7. Schema of the model system (F: feed, R: retentate, P: permeate)

Table 1.1. Composition of sweet and acid whey (Source: Tunick, 2008; Morr and Ha, 1993; Smith, 2008)

Product	Protein (g/L)	Lactose (g/L)	Minerals (g/L)	Fat (%)	pH
Sweet whey	6 -10	46 -52	2.5 - 4.7	0.3	> 5.6
Acid whey	6 - 8	44 - 46	4.3 - 7.2	0.3	< 5.1

Table 1.2. Physico-chemical properties of major proteins in sweet cheese whey (Source: Bonnaillie and Tomasula, 2008; Etzel, 2004)

Protein	Content		Molecular Weight (kDa)	pI
	% of total protein	g/L		
BLG	48 - 58	3.2	18	5.4
ALA	13 - 19	1.2	14	4.4
GMP	12 - 20	1.5	8.6	< 3.8

BLG: β-Lactoglobulin, ALA: α-Lactalbumin, GMP: Glycomacropeptide, pI: isoelectric point

CHAPTER 2

FRACTIONATION OF GLYCOMACROPEPTIDE FROM β-LACTOGLOBULIN USING POSITIVELY CHARGED CROSS-FLOW ULTRAFILTRATION MEMBRANES

2.1. ABSTRACT

Chromatography is the common technology for fractionation of proteins, and ultrafiltration is commonly used for concentration and clarification of proteins. However, recent studies showed that ultrafiltration could also be used for fractionation of proteins when charge modification was applied on the ultrafiltration membranes. In this study, we examined uncharged and positively charged regenerated cellulose cross-flow type ultrafiltration membranes to fractionate glycomacropeptide from β-lactoglobulin. Placing a positive charge on membranes increased the selectivity of the separation from about 1 to as high as 45. It was found that sieving coefficients of GMP and BLG were not constant throughout the ultrafiltration. A material balance model was developed for ultrafiltration having inconstant sieving coefficients. Calculated results from the new model and from a model taken from the literature using constant sieving coefficient were compared with each other and with the experimental data. Both models gave similar results and fit the experimental data well.

2.2. INTRODUCTION

Recently, protein-based products (therapeutic proteins and food ingredients) have gained more importance due to their increasing application areas in food and pharmaceutical industries. Ion exchange chromatography has been the most popular technique for production of these products. However, it has many disadvantages such as dilute end product, requirements of large amount of buffers, and also desalting (Mayani et al., 2009). These disadvantages cause high cost and environmental waste disposal problems. It has not been possible in the past to find a replacement for chromatography because other methods fail to deliver highly pure and inexpensive products. Ultrafiltration has not been commonly used for protein fractionation because it was thought that at least a 10-fold difference was required between the molecular weights of proteins to achieve a fractionation (Cherkasov and Polotsky, 1996). However, recent work has shown that by using charged ultrafiltration membranes and adjusting the pH and ionic strength of the feed solution, the separation basis is not only from the size but also from the charge, thus making ultrafiltration attractive for protein fractionation.

Many researchers have reported that the highest sieving coefficient (permeation rate of a protein through a membrane) of the protein was obtained when the pH was adjusted to the isoelectric point of the protein (Nakao et al., 1988; Saksena and Zydney, 1994; Balakrishnana and Agarwal, 1996; Lucas et al., 1998; Burns and Zydney, 1999; de la Casa et al., 2007; Bakhshayeshi and Zydney, 2008). Lu et al. (2005) fractionated lysozyme from ovalbumin using a 30 kDa polyethersulfone (PES) membrane and specifically adjusting pH to 10 – 11, NaCl concentration to 100 mM, permeate flux to

26.5 L/m^2h, and stirring speed to 2100 rpm. Wan et al. (2002) showed the effect of pH and salt concentration on fractionation of human serum albumin and human immunoglobulins using PES membranes; and they obtained a selectivity (preference of a membrane to selectively permeate one solute in favor of another) value of more than 300 at pH 4.7 and a NaCl concentration of 1.5 mM. PES is strongly negatively charged at pH values higher than 3 (Wan et al., 2002). These two studies (Lu et al., 2005 and Wan et al., 2002) showed that fractionation of the proteins was possible using PES membranes when one protein had no charge and one protein was negatively charged at pH > 3.

Beside adjusting the experimental conditions, modification of the membrane surface charge increased the selectivity of ultrafiltration to fractionate proteins. van Reis et al. (1999) obtained higher selectivity of separation using charged ultrafiltration membranes for fractionation of bovine serum albumin (BSA) and the antigen binding fragment (Fab) of a recombinant DNA antibody. Bhushan and Etzel (2009) investigated the effect of using positively charged regenerated cellulose membranes to separate glycomacropeptide (GMP) from other whey proteins at different pH and ionic strengths. At pH 3.0, GMP is neutral but other whey proteins are highly positively charged. Because of this charge difference between GMP and the other whey proteins, using positively charged ultrafiltration membranes resulted in a 600% increase in selectivity when pH of the solution was adjusted to 3.0.

Experimental studies with ultrafiltration and proteins are expensive and time consuming. Because of this, simulation studies are important to design the experimental conditions so that the amount of the expenses and time can be lowered. Mass balance

models assuming constant sieving coefficients have been used for ultrafiltration in the past (Kurnik et al., 1994). However, it has been shown that interactions between proteins might change the sieving coefficient during ultrafiltration (Sudareva et al., 1992, Wilharm and Rodgers, 1996). Researchers found that changing concentrations of protein in the feed solution affected the sieving coefficient because of the interactions between proteins. Kanani et al. (2004) showed that addition of BSA and increasing its concentration in a binary mixture of myoglobin and lysozyme increased the sieving coefficient of myoglobin and decreased that of lysozyme and, hence, increased the selectivity. Filipe and Ghosh (2005) found that increasing concentration of BSA in a binary mixture of lysozyme and BSA decreased the sieving coefficient of lysozyme at pH 9.0 where the proteins had net charges of different signs. They also found that the sieving coefficient of lysozyme decreased in the presence of HIgG at pH 4.3 where both proteins were positively charged. Since one would expect to observe increasing concentration of the rejected protein in the retentate during an ultrafiltration process, and to these findings in the literature, assuming a constant sieving coefficient is not always applicable.

In the present study, we investigated uncharged and positively charged regenerated cellulose cross-flow membranes for their ability to fractionate glycomacropeptide (GMP) and β-Lactoglobulin (BLG) at pH 3 and conductivity of 4 mS/cm, at which GMP is neutral or slightly negatively charged and BLG is positively charged. GMP and BLG are the two major dairy proteins of interest in the food industry. BLG is commonly used to improve the strength and the elasticity of food products (Mehra and O'Kennedy, 2008) and GMP can be used to produce food products for the

diets of patients with phenylketonuria (Ney et al., 2008). We determined the sieving coefficient profiles of GMP and BLG for charged and uncharged membranes throughout the ultrafiltration to test if the sieving coefficients were constant or not. Selectivies of uncharged and charged membranes were obtained to investigate the effect of the positive charge on the selectivity of the separation. Beside the experimental work, we developed a new model for inconstant sieving coefficients to fill the gap in the literature; and we compared experimental data with our model and with the model taken from the literature using constant sieving coefficient to test the accuracy of the models.

2.3. MODEL DEVELOPMENT

In this study, a single-stage ultrafiltration system was used (Figure 2.1). Two different mass balance models were used, one of which was obtained from the literature (Kurnik et al., 1995). Two mass balance boundaries are considered in this treatment (Figure 2.1). For the model system I, there is an accumulation in the retentate beaker and there is an outgoing permeate stream. A general mass balance for the solute "*i*" for this system will assume a form as follows:

$$\frac{dm_{sys,i}}{dt} = -\dot{m}_{out,i} \tag{2.1}$$

Where, $m_{sys,i}$, is the mass of the component *i* in the system, and $\dot{m}_{out,i}$ is the rate of mass loss from the system.

If the density is assumed constant, then we have:

$$\frac{dR}{dt} = -\dot{V}_P \tag{2.2}$$

Where, $\dot{V}_P$ is the volumetric flow rate of the permeate stream.

Let C_{Pi} and C_{Ri} be the instantaneous concentrations of the solute in permeate and retentate, respectively; *P* and *R* be the volumes of permeate and retentate, respectively; and *t* is time.

$\dot{V}_P$ can be expressed as:

$$\dot{V}_P = \frac{dP}{dt} \tag{2.3}$$

Equation 2.1 can be written in terms of concentrations as follows:

$$\frac{d(RC_{Ri})}{dt} = -C_{Pi}\dot{V}_P \tag{2.4}$$

Equation 2.4 can be rearranged using the following substitutions:

1. Observed sieving coefficient (S_{0i}), which was defined as: C_{Pi}/C_{Ri}.

2. Constant total fluid volume in the ultrafiltration system.

$$\frac{dP}{dt} = -\frac{dR}{dt} \tag{2.5}$$

Substitution of S_{0i} and Equation 2.5 into Equation 2.4 gives Equation 2.6.

$$\frac{d(RC_{Ri})}{dt} = S_{oi}C_{Ri}(\frac{dR}{dt}) \tag{2.6}$$

Rearrangement of Equation 2.6 gives:

$$(S_{oi} - 1)\frac{dR}{R} = \frac{dC_R}{C_{Ri}} \tag{2.7}$$

If it is assumed that there is no change in the value of S_{0i} in an ultrafiltration cycle, then the final solution of Kurnik et al. (1995) can be expressed as in Equation 2.8.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (S_{oi} - 1)\ln(1 - f) \tag{2.8}$$

where $f = P/F$ and F is the volume of the initial feed solution. For this model system, the sieving coefficient was measured under total recycle conditions ($f = 0$). With the known constant value of S_{0i} and by knowing C_{Fi}, the value of C_{Ri} can be obtained for any given f using equation 2.8.

When S_{0i} is not constant, the solution to Equation 2.7 is:

$$\int_F^R (S_{0i} - 1)\frac{dR}{R} = ln\frac{C_{Ri}}{C_{Fi}} \tag{2.9}$$

Substitution for R using the relation $R = F(1-f)$ yields the equation as follows:

$$ln\frac{C_{Ri}}{C_{Fi}} = \int_0^f \frac{(1-S_{0i})}{(1-f)} df \quad (2.10)$$

During ultrafiltration, S_{0i} declines nearly linearly as f increases. Equation 2.10 can be integrated using this dependence.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (m + b - 1)\ln(1 - f) + mf, \quad (2.11)$$

where, m is the slope, and b is the intercept of the linear decline of S_{0i} versus f. In this case, the instantaneous permeate and retentate tubing concentrations were measured at different f values, and S_{0i} as a function of f ($S_{0i} = mf + b$) was obtained. We can then calculate C_{Ri} for any given f using equation 2.11.

To calculate the mixing cup concentration of the solute in permeate ($<C_{Pi}>$), an overall integrated mass balance (based on system II of Figure 2.1) was used:

$$\frac{dm_{sys,i}}{dt} = 0 \quad (2.12)$$

$$At\ t = 0, m_{sys,i} = C_{Fi}F \quad (2.13)$$

$$At\ t = t, m_{sys,i} = < C_{Ri} > R + < C_{Pi} > P \quad (2.14)$$

Using Equations 2.13 and 2.14, an overall mass balance can be expressed as follows:

$$C_{Fi}F = < C_{Ri} > R + < C_{Pi} > P. \quad (2.15)$$

Where $<C_{Ri}>$ is the mixing cup concentration of solute in the retentate. An assumption was made that $<C_{Ri}> \approx C_{Ri}$, and C_{Ri} was obtained using equation 2.8 and 2.10. This assumption is an approximation because C_{Ri} is the instantaneous concentration of solute in the retentate and is always slightly greater than the solute concentration in the

beaker $<C_{Ri}>$ due to the concentration effect of withdrawing the permeate stream from the membrane. The permeate flow rate of about 6 mL/min was withdrawn from a feed solution flow rate of about 36-56 mL/min to give a retentate flow rate of 30-50 mL/min. Therefore, the value of C_{Ri} was about 12 to 20% greater than $<C_{Ri}>$. This is because the solution in the beaker was at $<C_{Ri}>$ and the retentate was at C_{Ri}.

In practice, this concentration difference was somewhat less than calculated and the observed value of C_{Ri} was about 10% greater than the observed value of $<C_{Ri}>$.

2.4. MATERIALS AND METHODS

2.4.1. Preparation of Protein Solutions

Glycomacropeptide (BioPURE GMP, Davisco Foods International, Le Sueur, MN) and β-lactoglobulin (Cat. No. 100363, 3x crystallized, MP Biomedicals, Irvine, CA) were used without further purification. Composition of GMP is given in Table 2.1. For single protein experiments, 0.5 g/L of GMP or BLG, and for binary protein experiments 0.5 g/L each of GMP and BLG in 25 mM sodium phosphate buffer, pH 3.0, conductivity 4 mS/cm, were prepared using 1M H_3PO_4, or 1M NaOH and granular NaCl. Protein solutions were filtered through 0.45 μm filters and then 0.22 μm filters (Durapore, Millipore Corp., Bedford, MA).

2.4.2. Preparation of Positively Charged Cross-Flow Ultrafiltration Membranes

Regenerated cellulose cross-flow ultrafiltration membranes of 50 cm^2 area (Pellicon XL, 30 kDa, Millipore Corp., Bedford, MA) were charge-modified using the procedure of van Reis (2006) and Bhushan and Etzel (2009) by circulating (3-bromopropyl) trimethylammonium bromide (Cat. No. 347604 Sigma Aldrich, St. Louis, MO) through the membrane for 21 hours at 22 ºC. The clean water permeate flux decreased by 28% after charge modification.

2.4.3. Ultrafiltration Experiments

Membranes were incubated in the protein solution for 15 to 18 h at 22 ºC prior to taking samples for analysis. This step was to equilibrate the membranes with the feed

solution before ultrafiltration. Experiments were conducted at an inlet pressure of 2 bars. 1000 mL feed solution was pumped into the ultrafiltration unit (hold up volume = 3.0 mL) using a peristaltic pump. Retentate flow rate was adjusted to 30 - 50 mL/min (360 – 600 LMH) using a pinch clamp and permeate flow rate was adjusted to 6 mL/min (72 LMH) using another peristaltic pump. During ultrafiltration, retentate was recycled back to the feed solution container and permeate was collected in a graduated cylinder. Samples were taken at different "*f*" values from permeate and retentate tubings, and from permeate and feed solution beakers for measurement of concentration. Experiments were stopped at $f = 0.8$. Two separate replicates were performed for each experiment.

2.4.4. Spectrophotometric measurements

For single protein solutions, the absorbance of GMP and BLG solutions were measured at 214 and 280 nm, respectively. For binary protein solutions, the method of Bhushan and Etzel (2009) was used. GMP does not contain any aromatic amino acids; therefore, it gives little or no absorbance at 280 nm. Bhushan and Etzel (2009) used this feature to distinguish BLG from GMP in binary protein solutions. Absorbance values of the permeate and retentate were determined at 214 and 280 nm; then the absorbance values were converted to concentrations using the Beer-Lambert law for mixtures. Two measurements were made on the same sample and an average was reported. Measurements were nearly identical in each case.

2.4.5. Statistical Analysis

Intercepts and slopes of the plotted data were analyzed by comparing the 95% confidence interval of each value. Values that overlapped the 95% confidence interval were signed with same letters. Selectivity values of uncharged membranes and results of mass balance calculations were analyzed using one sample T-test. Values are significantly different at $P < 0.05$.

2.5. RESULTS

2.5.1. Sieving Coefficients

Sieving coefficients of GMP and BLG were determined individually and for binary mixtures using both charged and uncharged regenerated cellulose ultrafiltration membranes (Figures 2.2 and 2.3). Samples were taken from the retentate and permeate tubings during the ultrafiltration while "*f*" increased from 0.0 to 0.8. In all cases, sieving coefficients decreased with an increase in "*f*." For uncharged membranes (Figure 2.2), the sieving coefficients for BLG and GMP were close to each other when single protein solutions were used. For binary protein solutions and uncharged membranes, the sieving coefficients for GMP and BLG were also close to each other. Sieving coefficients of GMP in single and binary solutions were similar to each other; however, sieving coefficients of BLG in single solutions were greater than the ones in binary solutions.

To correlate the sieving coefficients of GMP and BLG versus "*f*", the intercepts and slopes from linear regression were determined as shown in Figures 2.2 and 2.3, and results were compared with each other using the 95% confidence interval range of each data point (Table 2.2). If 95% confidence interval range of two data points overlapped, they were signed with the same letter. The slopes of the sieving coefficients vs. "*f*" all declined (negative slope). When the two trials of ultrafiltration experiments were compared, one trial frequently fell below the other one. Because of this, slopes from two trials were close to each other. Slopes were not different for GMP using an uncharged versus charged membrane. However, slopes were lower for BLG using the charged membrane compared to the uncharged membrane.

In general, intercepts for the sieving coefficients for GMP were not a function of membrane charge, or whether or not the solution was a single or binary protein solution; the 95% confidence interval range was 0.30 to 0.32. The main effect of adding a charge to the membrane was to strongly decrease the intercept for the sieving coefficient of BLG. Intercepts of the sieving coefficients for BLG compared to GMP when using the uncharged membrane were similar for the binary solutions, but for the single protein solutions the intercept was somewhat (16%) greater for BLG than for GMP.

For the charged membrane (Figure 2.3), there were 14 fold and 19 fold decreases in the sieving coefficient for BLG compared to the uncharged membrane in single and binary solutions, respectively. In contrast, sieving coefficients of GMP for single and binary solutions using charged membranes were nearly the same as those obtained without a charge. Using the charged membrane, sieving coefficients for GMP were always substantially higher than for BLG at any "*f*" for both single and binary protein solutions.

2.5.2. Selectivity

Selectivity was calculated from the ratio of the sieving coefficients for the charged membranes at each value of "*f*" (Table 2.3). For both single and binary protein solutions, selectivity increased as "*f*" increased; reaching a maximum value of 25 at f = 0.8 for the single protein solutions and 45 for the binary protein solutions. Binary solutions had always higher selectivity than single protein solutions.

2.5.3. Mass Balances

The mass balance results were used to determine if there was any loss of the protein during the ultrafiltration process. For the calculations; first, the concentration of the protein (GMP and BLG) was multiplied by the volume of the solution (permeate and retentate) to find the mass of the proteins in the solutions; second, the mass of the proteins in permeate and retentate were summed to find the total mass of the proteins in the whole process, and finally the total mass was divided by the initial mass of the protein in the feed solution. When there was no mass loss in the process, the value of 100% was obtained. Table 2.4 shows the mass balance results for BLG and GMP. Experimental results were compared to 100% using T-test ($P = 0.05$). The mass balance for BLG was not statistically significantly different than 100% using charged or uncharged membranes and for single or binary protein solutions ($P > 0.05$). The mass balances always closed for BLG. However, the mass balance for GMP was significantly less than 100% regardless of the charge and solution type ($P < 0.05$). Some of the mass of GMP in the feed solution did not appear in the sum of the permeate and retentate solutions.

2.5.4. Concentrations

Concentrations of GMP and BLG from tubings and beakers were measured during the ultrafiltration when using single and binary protein solutions, and when the membrane was charged and uncharged. Results for the charged membranes are shown in Figure 2.4. Results for the uncharged membranes are not plotted, because no separation occurred when uncharged membranes were used. For example, for uncharged

membranes, concentrations of GMP and BLG in the permeate were 0.22±0.02 g/L and 0.25±0.03 g/L, respectively; and concentrations of GMP and BLG in the retentate were 1.4±0.2 g/L and 1.6±0.2 g/L, respectively, at $f = 0.8$.

Using the charged membranes, the concentration of BLG in the permeate was small: 0.017±0.004 g/L and 0.011±0.006 g/L for single and binary protein solutions, respectively. These concentrations were untrustworthy because the absorbance values of BLG at 280 nm in the permeate stream were below limit of detection ($A_{280} < 0.03$). These small concentration values of BLG in the permeate stream were observed because of strong rejection of BLG by the positively charged membranes. In the literature, a similar result was observed for separation of ALA from BLG using ultrafiltration (Cheang and Zydney, 2003). The concentration of BLG in permeate was reported as 0.005±0.002 g/L. The very small concentration of BLG in permeate was because of the strong rejection of BLG by the ultrafiltration membrane and the large error was because of the difficulty in evaluating the very small concentration of BLG in permeate (Cheang and Zydney, 2003). The concentrations of GMP in the permeate were higher: 0.20±0.02 g/L and 0.162±0.008 g/L for single and binary protein solutions, respectively. Calculating from the permeate concentrations of GMP and BLG in binary solution, we observed that the purity of GMP increased from 50% to more than 90% using charged membranes; while there was no change in the purity of GMP for uncharged membranes.

Comparison of calculations from the two mass balance models to the experimental observations is shown in Figure 2.4. Two mass balance models were used: (1) the Kurnik et al. (1995) model for a constant sieving coefficient, and (2) the proposed

model (Equation 2.7) where the sieving coefficient is a function of *f*. Sieving coefficients at $f = 0$ were used in the Kurnik model because Kurnik model assumed that the sieving coefficient was constant throughout a cycle of ultrafiltration, and the concentration of feed solution and concentration at the wall are least at $f = 0$; therefore, the sieving coefficient at $f = 0$ is more reliable than the sieving coefficients at other f values. Calculations from both models were reasonably close to experimental results, but the calculated results from Kurnik model was always lower than the proposed model for the retentate, and higher for the permeate, especially for GMP. As a result, for GMP, the Kurnik model was somewhat closer to the experimental observations for the retentate, and the proposed model was closer for the permeate. For BLG, both models matched the experimental observations.

2.6. DISCUSSION

2.6.1. Positively Charged Membranes Increased the Selectivity for Fractionation of GMP from BLG

Placing a positive charge on the ultrafiltration membrane increased the selectivity, hence, enhanced the fractionation of GMP from BLG. At pH 3, BLG is positively charged; on the other hand, GMP is nearly neutral. Due to this fact, we expected that placing positive charge on the membrane would not change the sieving coefficient of GMP but decrease that of BLG, hence, increase the selectivity.

2.6.2. Sieving Coefficients Decreased and Selectivity Increased as "*f*" Increased

There was a mass loss of GMP when the membrane was charged and uncharged. Protein accumulates on the membrane surface during ultrafiltration. The mass loss observed might have been due to the accumulation of GMP on the membrane surface, and hence, creation of a GMP boundary layer. This accumulation might also explain the decreasing sieving coefficients with an increasing "*f*." The protein layer might decrease the permeability of the membrane and, hence, the sieving coefficient. Throughout the ultrafiltration experiment, the concentration of the feed solution increased and the permeability of the membrane decreased as *f* increased from 0.0 to 0.8. Because of this, there was a larger increase in the instantaneous concentration of the protein in the retentate than the permeate as *f* increased from 0.0 to 0.8. For example, the instantaneous concentrations of GMP in retentate and permeate increased 3 times and 2 times,

respectively, when uncharged membranes and single solutions were used (Figure 2.5-A and 2.5-B).

While "*f*" increased, we observed an increasing selectivity profile for both single and binary solutions because of the greater decrease in the sieving coefficients of BLG compared to GMP. We also observed that the selectivity increased more for binary solution than for single solution. The reason of this might be due to the possible protein-protein interactions in binary solutions as observed in different studies (Kanani et al., 2004; Filipe and Ghosh, 2005; Sudareva et al., 1992; Wilharm and Rodgers, 1996). In particular, Filipe and Ghosh (2005) observed that the preferentially retained protein may act to enhance, hinder or not affect the transmission of the preferentially transmitted protein, depending on the type of interaction. At pH 3, GMP is nearly neutral while BLG carries a strong net positive charge. The strong positive charge of BLG might cause to change the transmission of BLG and GMP, causing substantially less transmission of BLG compared to GMP, hence, higher selectivity of separation when binary solutions were used.

2.6.3. Both Mass Balance Models Fit the Experimental Data with Slight Deviations

Both the proposed model and Kurnik's model were reliable for calculating the expected mixing-cup concentrations for a given *f*. The experimentally measured mixing-cup concentrations matched the calculated concentrations using Kurnik's model, even though the sieving coefficient was assumed to be constant in Kurnik's model, and this was not observed in case of our system. In fact, the sieving coefficients declined with an

increasing *f*. The reason why the calculations from Kurnik model were closer to the experimentally measured mixing-cup concentrations for GMP was because the sieving coefficient used for Kurnik model was obtained at $f = 0.0$ when there was total recycle of both permeate and retentate streams back to the feed solution. Because of this, sieving coefficients obtained using the instantaneous concentrations and the mixing-cup concentrations are same at $f = 0.0$. However sieving coefficients obtained using instantaneous concentrations and mixing-cup concentrations at higher f values are different from each other. For example, the sieving coefficient of GMP at $f = 0.0$ is 0.32 and it is same for both instantaneous and mixing-cup conditions, but at $f = 0.8$ sieving coefficients of GMP are 0.25 and 0.16 when mixing-cup and instantaneous concentrations were used for the calculation, respectively. The decrease in the sieving coefficients obtained using mixing-cup concentrations (from 0.32 to 0.25) is less compared to the sieving coefficients obtained using instantaneous concentrations (from 0.32 to 0.16). Because of this, the assumption of constant sieving coefficient is more pronounced in the case of sieving coefficients obtained using mixing-cup concentrations. However, there is still a marginal decrease in the sieving coefficient of GMP obtained using mixing-cup concentrations, and the amount of GMP remaining in the retentate increases more than it would have if the sieving coefficient was constant. We think that the effect of decreasing sieving coefficients was reconciled because of the significant mass loss of GMP throughout the ultrafiltration experiment. For the proposed model, sieving coefficients, obtained using instantaneous concentrations at different f values, were used. Because the instantaneous concentration of the solute in retentate was always

10% higher than the mixing-cup concentration of the solute in retentate, calculated concentrations using the proposed model were slightly higher than the experimentally measured mixing-cup concentrations of the solute in retentate. One could expect to see disagreements between calculated concentrations from Kurnik's model and experimentally measured concentrations when there was no significant mass loss (e.g. for BLG in Figures 2.3-B and 2.3-D) due to the explanation propounded above. The reason for not seeing any disagreement between Kurnik's model and the experimental results might be because of the small sieving coefficients of BLG due to strong electrostatic exclusion throughout the filtration process using charged ultrafiltration membranes. In this case, the difference between the sieving coefficients obtained using instantaneous and mixing-cup concentrations would not be sufficiently different from each other to effect the calculations. This was because the instantaneous and mixing-cup concentrations of BLG in permeate were small; hence, the sieving coefficients of BLG using instantaneous and mixing-cup concentrations were negligible when charged membranes were used. Additionally, there was no significant mass loss of BLG to affect the calculations.

Using the material balance equations 2.8 and 2.15, it is possible to calculate mixing cup concentrations for a given value of f without performing the ultrafiltration up to that f. The expected purity and yield of the product of interest can be estimated using the sieving coefficient at $f = 0.0$ and the initial concentration of the feed solution.

In order to use equation 2.11, an initial ultrafiltration experiment needs to be performed at different values of f to get the dependence of sieving coefficient on f.

Subsequently, equations 2.11 and 2.15 may be used to calculate the mixing cup concentrations for a given *f* by knowing the concentration of the initial feed solution.

2.7. CONCLUSIONS

In this study, we investigated uncharged and charged regenerated cellulose ultrafiltration membranes for the fractionation of GMP from BLG in single and binary protein solutions. Sieving coefficients of GMP and BLG, selectivity of the separation, and concentration of the proteins in the permeate and retentate streams were determined. Experimentally determined sieving coefficients of GMP and BLG were used in two mass balance models to calculate the final concentrations; and experimental and calculated results were compared to each other.

We showed that sieving coefficients were not constant during ultrafiltration for the fractionation of GMP from BLG using both uncharged and charged regenerated cellulose membranes. Adding a positive charge to membranes increased the selectivity up to 45-fold from the selectivity of 1 obtained from uncharged membranes. In the experiments conducted with charged membranes and binary protein solutions, we obtained more than 90% pure GMP in the permeate. Two mass balance models were examined and both these models fitted experimental data with slight deviations.

2.8. REFERENCES

Bakhshayeshi, M. and Zydney, A.L. 2008. Effect of solution pH on protein transmission and membrane capacity during virus filtration. *Biotechnology and Bioengineering*. Vol. 100, pg: 108-117.

Balakrishnana, M. and Agarwal, G.P. 1996. Protein fractionation in a vortex flow filter: effect of system hydrodynamics and solution environment on single protein transmission. *Journal of Membrane Science*. Vol. 112, pg: 47-76.

Bhushan, S. and Etzel, M.R. 2009. Charged ultrafiltration membranes increase the selectivity of whey protein separations. *Journal of Food Science*. Vol. 74, pg: E131-E139

Burns, D.B. and Zydney, A.L. 1999. Effect of solution pH on protein transport through ultrafiltration membranes. *Biotechnology and Bioengineering*. Vol. 64, pg:27-37.

Cheang, C. and Zydney, A.L. 2003. Separation of α-lactalbumin and β-lactoglobulin using membrane ultrafiltration. *Biotechnology and Bioengineering*. Vol. 83, pg: 201-209.

Cherkasov, A.N. and Polotsky, A.E. 1996. The resolving power of ultrafiltration. *Journal of Membrane Science*. Vol. 110, pg: 79-82.

De la Casa, E.J., Guadix, A., Ibanez, R. and Guadix, E.M. 2007. Influence of pH and salt concentration on the cross-flow microfiltration of BSA through a ceramic membrane. *Biochemical Engineering Journal*. Vol. 33, pg: 110-115.

Filipe, C.D.M. and Ghosh, R. 2005. Effects of protein-protein interaction in ultrafiltration based fractionation processes. *Biotechnology and Bioengineering*. Vol. 91, pg: 678-687.

Kanani, D. Ghosh, R. and Filipe, C.D.M. 2004. A novel approach for high-resolution protein-protein separation by ultrafiltration using dual-facilitating agent. *Journal of Membrane Science*. Vol. 243, pg: 223-228.

Kurnik, R.T., Yu, A.W., Blank, G.S., Burton, A.R., Smith, D., Athalye, A.M. and van Reis, R. 1995. Buffer exchange using size exclusion chromatography, countercurrent dialysis, and tangential flow filtration : models, development, and industrial application. *Biotechnology and Bioengineering*. Vol. 45, pg : 149-157.

Lu, J., Wan, Y. and Cui, Z. 2005. Fractionation of lysozyme and chicken egg albumin using ultrafiltration witk 30-kDa commercial membranes. *Industrial & Engineering Chemistry Research*. Vol. 44, pg: 7610-7616.

Lucas, D., Rabiller-Baundry, M., Michel, F. and Chaufer, B. 1998. Role of the physico-chemical enviroment on ultrafiltration of lysozyme with modified inorganic membrane. Colloids and Surfaces A: Physicochemical and Engineering Aspects. Vol. 136, pg:109-122.

Mayani M., Mohanty, K., Filipe, C. and Ghosh, R. 2009. Continuous fractionation of plasma proteins HSA and HIgG using cascade ultrafiltration systems. *Separation and Purification Technology*. Vol. 20, pg: 231-241.

Nakao, S., Osada, H., Kurata, H., Tsuru, T. and Kimura S. 1988. Separation of proteins by charged ultrafiltration membranes. *Desalination*. Vol. 70, pg:191-205.

Saksena, S. and Zydney, A.L. 1994. Effect of pH and ionic strength on the separation of albumin during ultrafiltration. *Journal of Membrane Science*. Vol. 43, pg: 960-968.

Sudareva, N.N., Kurenbin, O.I. and Belenkii, B.G. 1992. Increase in the efficiency of membrane fractionation. *Journal of Membrane Science*. Vol. 68, pg: 263-270.

Van Reis, R. 2006. Charged filtration membranes and uses therefor. U.S. patent 7,153,426 B2.

Van Reis., R., Brake, J.M., Charkoudian, J., Burns, D.B. and Zydney, A.L. 1999. High-performance tangential flow filtration using charged membranes. *Journal of Membrane Science*. Vol. 159, pg: 133-142.

Wan, Y., Ghosh, R. and Cui, Z. 2002. High-resolution plasma protein fractionation using ultrafiltration. *Desalination*. Vol. 144, pg: 301-306.

Wilharm, C. and Rodgers, V.G.J. 1996. Significance of duration and amplitude in transmembrane pressure pulsed ultrafiltration of binary protein mixtures. *Journal of Membrane Science*. Vol. 121, pg: 217-228.

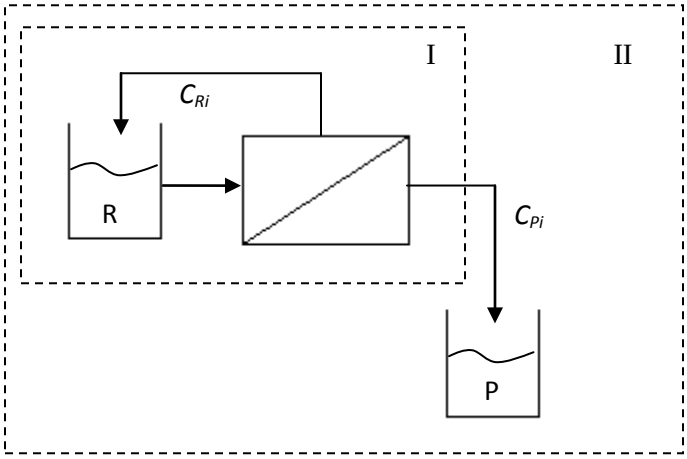

Figure 2.1. Schema of the model system (F: feed, R: retentate, P: permeate)

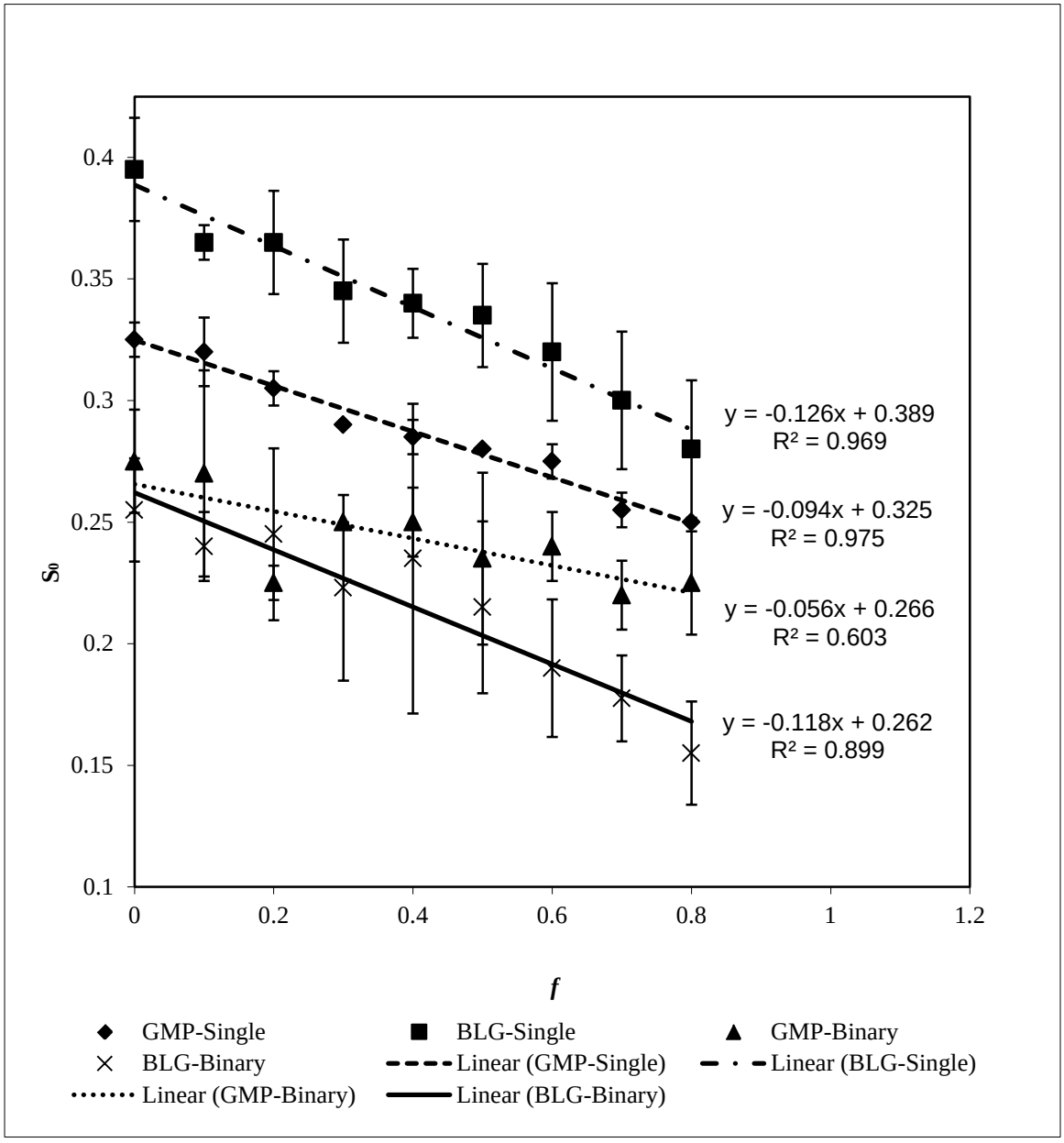

Figure 2.2 Dependence of sieving coefficients of GMP and BLG in single and binary solutions on "*f*" when uncharged membranes were used. Sieving coefficients were determined using instantaneous concentrations in tubings. (Each data point is the average ± range of two separate replicates)

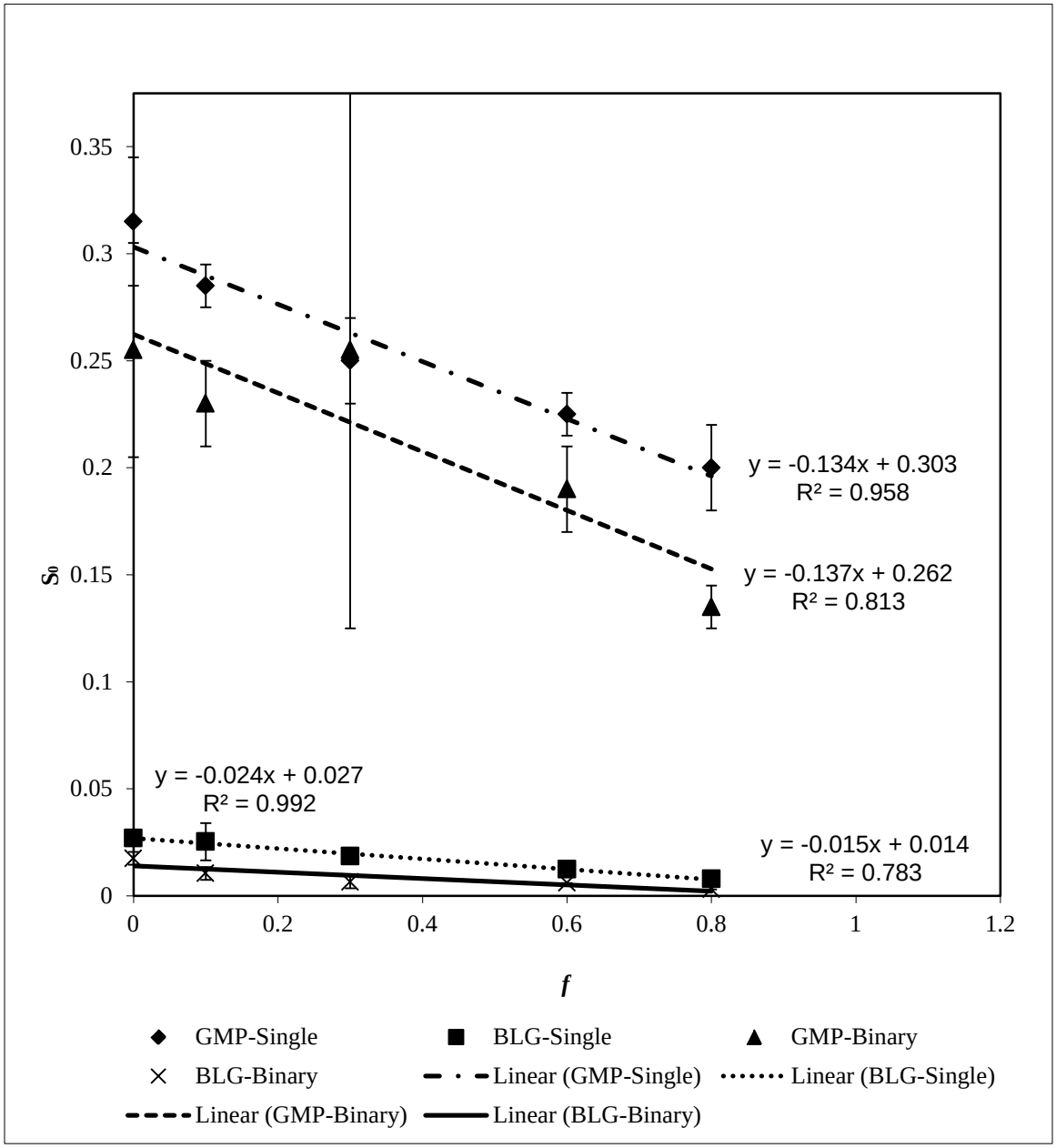

Figure 2.3 Dependence of sieving coefficients of GMP and BLG in single and binary solutions on "*f*" when charged membranes were used. Sieving coefficients were determined using instantaneous concentrations in tubings. (Each data point is the average ± range of two separate replicates)

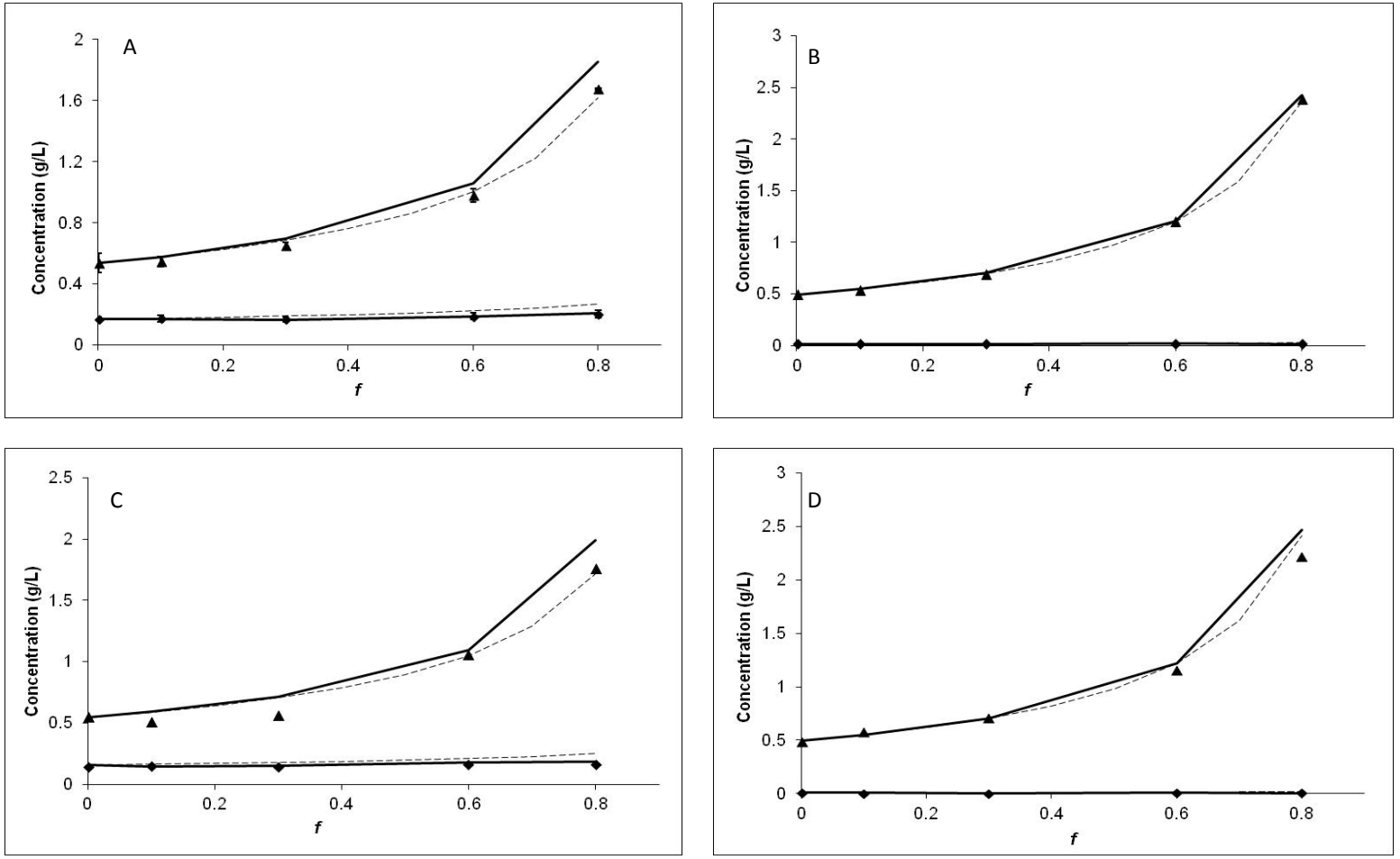

Figure 2.4 Mixing-cup concentration profiles of GMP in single (A), binary (C) solutions; and BLG in single (B), binary (D) solutions when charged membranes were used. (▲) Concentrations in the collection beaker of retentate, (♦) Concentrations in the collection beaker of permeate, (—) Proposed model, (– –) Kurnik model. (Each data point is the average ± standard deviation of two separate replicates)

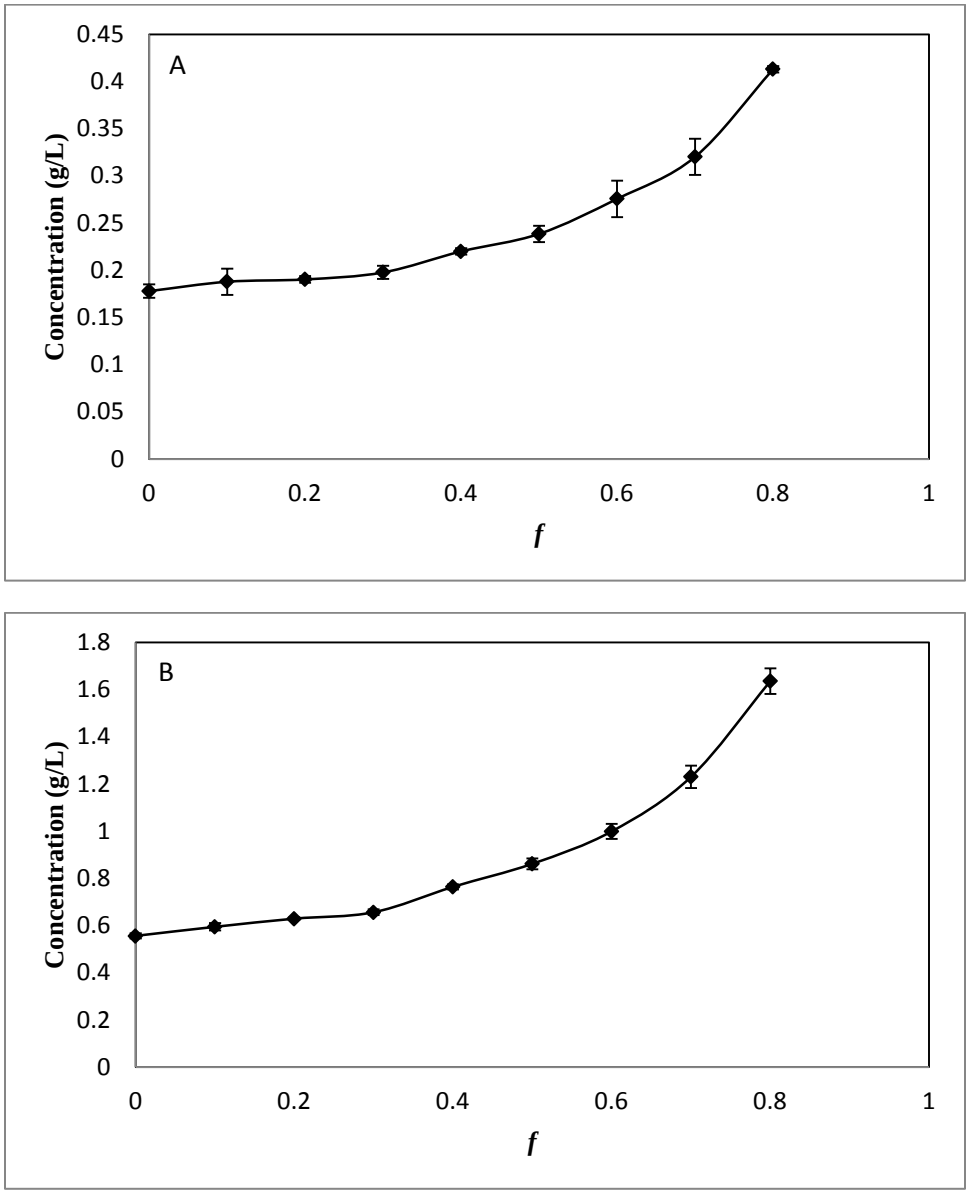

Figure 2.5 Instantaneous concentration profiles of GMP in permeate (A) and retentate (B) when single solutions and uncharged membranes were used. (Each data point is the average ± standard deviation of two separate replicates)

Table 2.1 Composition of Glycomacropeptide (GMP) (BioPURE GMP, Davisco Foods International, Le Sueur, MN)

Analysis	**Specification**	**Typical Range**	**Test Method**
Moisture (%)	7.0 max.	6.0 ± 0.9	Vacuum Oven
Glycomacropeptide (N × 7.07) (% Dry Basis)	90.0 min.	91 ± 1	Leco Combustion
Protein (N × 6.47) (% Dry Basis)	82.5 min.	84 ± 1	Leco Combustion
Fat (%)	1.0 max.	0.5 ± 0.3	Mojonnier
Ash (%)	7.0 max.	6.3 ± 0.5	Residue on Ignition
Lactose (%)	1.0 max.	< 0.5	Enzymatic Assay
pH	7.0 max.	6.4 ± 0.2	10% Sol. At 20°C
Scorched Particles (mg/25g)	15.0 max.	7.5	ADPI

Table 2.2 Intercepts and slopes of the linear regressions of the data in Figures 2.2 and 2.3

Name of the curves	Intercept	Stat analysis for intercepts[a]	Slope	Stat analysis for slopes[a]
GMP-Single-Charged	0.303±0.008	b	-0.13±0.02	ab
GMP-Binary-Charged	0.26±0.02	ab	-0.14±0.04	ab
BLG-Single-Uncharged	0.389±0.005	c	-0.13±0.01	a
BLG-Binary-Uncharged	0.262±0.007	a	-0.12±0.02	ab
GMP-Single-Uncharged	0.325±0.003	d	-0.094±0.006	b
GMP-Binary-Uncharged	0.266±0.008	a	-0.06±0.02	bc
BLG-Single-Charged	0.0270±0.0006	e	-0.024±0.001	c
BLG-Binary-Charged	0.014±0.002	f	-0.015±0.005	c

[a] Same letters indicate the values overlapped in 95% confidence interval

* Average ± standard error

Table 2.3 Selectivity of charged membranes when single and binary protein solutions were used

f	Single	Binary
0.0	11.7±0.2	15±4
0.1	12±3	23±6
0.3	13.6±0.8	40±30
0.6	18.0±0.5	32±2
0.8	25±3	45±2

Each data point is the average ± standard deviation of two separate replicates

Table 2.4 Mass balance results of ultrafiltration experiments when uncharged and charged membranes and when single and binary protein solutions were used

Mass Balance (%)							
Uncharged				Charged			
GMP		BLG		GMP		BLG	
Single	Binary	Single	Binary	Single	Binary	Single	Binary
87±4*	77±5*	100±1	110±10	93±7*	89±8*	100±1	92±4

* Value is significantly different than 100% ($P < 0.05$)

Each data point is the average ± standard deviation of two separate replicates

CHAPTER 3

THREE-STAGE ULTRAFILTRATION IN A BATCH MODE FOR FRACTIONATION OF GLYCOMACROPEPTIDE FROM β-LACTOGLOBULIN

3.1. ABSTRACT

Membrane ultrafiltration is a common method for concentration and diafiltration of protein solutions. However, it is not commonly used for fractionation of proteins. This is because the ability of existing membranes to permeate one protein and not another is not enough. This situation has changed with the development of charged ultrafiltration membranes. However, one stage of ultrafiltration may not be enough to obtain purity comparable to that obtained from chromatography. The configuration of ultrafiltration membranes in stages may solve this problem; moreover, it may increase the distribution of the desired protein into target final stream. This study examined the fractionation of glycomacropeptide (GMP) from β-lactoglobulin (BLG) using three-stage ultrafiltration with recycling of selected streams. Multiple cycles of operation were conducted using uncharged and positively charged ultrafiltration membranes. Using positively charged ultrafiltration membranes increased the selectivity, hence, the purity of the final products. Recycling of the selected streams did not have an effect on the purity (always higher than 95%) but did increase the distribution of GMP in the permeate stream up to 52.6% from 31% when the experiments were conducted with positively charged membranes.

3.2. INTRODUCTION

There have been several attempts to fractionate proteins using different ultrafiltration techniques. Previously, it was thought that fractionation by ultrafiltration could be accomplished only if the proteins had at least a 10-fold difference in molecular mass (Cherkasov and Polotsky, 1996). Adjustment of pH and ionic strength of the protein solutions and use of charged membranes have been investigated to improve the fractionation ability of the ultrafiltration. Many studies have shown that the highest sieving coefficient (permeation rate of a protein through a membrane) of the protein was obtained when the pH was adjusted to the isoelectric point of the protein (Nakao et al., 1988; Saksena and Zydney, 1994; Balakrishnana and Agarwal, 1996; Lucas et al., 1998; Burns and Zydney, 1999; de la Casa et al., 2007; Bakhshayeshi and Zydney, 2008). Lu et al. (2005) fractionated lysozyme from ovalbumin by using a 30 kDa polyethersulfone (PES) membrane and specifically adjusting pH to 10 – 11, NaCl concentration to 100 mM, permeate flux to 26.5 L/m^2h, and stirring speed to 2100 rpm. Wan et al. (2002) showed the effect of pH and salt concentration on fractionation of human serum albumin and human immunoglobulins using PES membranes; and they obtained a selectivity (preference of a membrane to selectively permeate one solute in favor of another) value of more than 300 at pH 4.7 and a NaCl concentration of 1.5 mM. PES is strongly negatively charged at pH values higher than 3 (Wan et al., 2002). These two studies (Lu et al., 2005 and Wan et al., 2002) showed that fractionation of the proteins was possible using PES membranes when one protein had no charge and one protein was negatively charged at pH > 3. Cheang and Zydney (2003) clearly fractionated alpha-lactalbumin (ALA) and beta-lactoglobulin (BLG) in binary solutions with high recoveries at specified

conditions. van Reis et al. (1999) obtained higher selectivity of separation using charged ultrafiltration membranes for fractionation of bovine serum albumin (BSA) and the antigen binding fragment (Fab) of a recombinant DNA antibody. Bhushan and Etzel (2009) investigated the effect of using positively charged regenerated cellulose membranes to separate glycomacropeptide (GMP) from other whey proteins at different pH and ionic strengths. At pH 3.0, GMP is neutral but other whey proteins are highly positively charged. Because of this charge difference between GMP and the other whey proteins, using positively charged ultrafiltration membranes resulted in a 600% increase in selectivity when pH of the solution was adjusted to 3.0.

Multiple stages of ultrafiltration membranes in different configurations have been analyzed for fractionation purposes. Simulation and experimental studies have been conducted (Cheang and Zydney 2004, Mayani et al. 2009, Mayani et al. 2010, Wang et al. 2010, Ghosh 2003). These researchers investigated multi-stage ultrafiltration processes with diafiltration steps using uncharged membranes. Diafiltration has been used in these studies to improve the recovery of the protein of interest. Lightfoot (2005) proposed using three-stage configuration with recycling of the permeate and the retentate streams to the feed stream from the third (bottom) and the second (top) stages, respectively. Lightfoot later showed that purity and yield of the final products could be improved using the proposed configuration rather than using single-stage ultrafiltration (Gunderson et al., 2007; Lightfoot et al., 2008). However, the studies did not have sufficient experimental work to validate the proposed configuration. Only a one-stage ultrafiltration experiment was conducted using ALA and BLG in buffer solutions to find the sieving coefficients. Purity and yield of the proteins in the final streams were

calculated using the models and the experimental sieving coefficients. However, the calculations were not compared with the experimental results; and the effect of recycling of the selected streams on purity and yield was not analyzed.

The present study investigated the fractionation of GMP (a functional peptide used to produce foods for the diets of the patients with phenlyketonuria) from BLG (a commonly used protein to improve the strength and the elasticity of food products) using a three-stage membrane system with recycling of the permeate and the retentate streams to the feed stream from the third and the second stream, respectively. pH and conductivity of the protein solution was set to 3.0 and 4.0 mS/cm, respectively, where GMP is neutral or slightly negatively charged and BLG is positively charged. Uncharged and positively charged ultrafiltration membranes were used. The diafiltration step was omitted to avoid diluted end products. Experimental results were compared with the predicted results obtained using two mass balance models.

3.3. MODEL DEVELOPMENT

In this study, a single-stage ultrafiltration system was used (Figure 3.1). Two different mass balance models were used, one of which was obtained from the literature (Kurnik et al., 1995). Two mass balance boundaries are considered in this treatment (Figure 3.1). For the model system I, there is an accumulation in the retentate beaker and there is an outgoing permeate stream. A general mass balance for the solute "*i*" for this system will assume a form as follows:

$$\frac{dm_{sys,i}}{dt} = -\dot{m}_{out,i} \tag{3.1}$$

Where, $m_{sys,i}$, is the mass of the component *i* in the system, and $\dot{m}_{out,i}$ is the rate of mass loss from the system.

If the density is assumed constant, then we have:

$$\frac{dR}{dt} = -\dot{V}_P \tag{3.2}$$

Where, $\dot{V}_P$ is the volumetric flow rate of the permeate stream.

Let C_{Pi} and C_{Ri} be the instantaneous concentrations of the solute in permeate and retentate, respectively; *P* and *R* be the volumes of permeate and retentate, respectively; and *t* is time.

$\dot{V}_P$ can be expressed as:

$$\dot{V}_P = \frac{dP}{dt} \tag{3.3}$$

Equation 3.1 can be written in terms of concentrations as follows:

$$\frac{d(RC_{Ri})}{dt} = -C_{Pi}\dot{V}_P \tag{3.4}$$

Equation 3.4 can be rearranged using the following substitutions:

1. Observed sieving coefficient (S_{0i}), which was defined as: C_{Pi}/C_{Ri}.

2. Constant total fluid volume in the ultrafiltration system.

$$\frac{dP}{dt} = -\frac{dR}{dt} \tag{3.5}$$

Substitution of S_{0i} and Equation 3.5 into Equation 3.4 gives Equation 3.6.

$$\frac{d(RC_{Ri})}{dt} = S_{oi}C_{Ri}(\frac{dR}{dt}) \tag{3.6}$$

Rearrangement of Equation 3.6 gives:

$$(S_{oi} - 1)\frac{dR}{R} = \frac{dC_R}{C_{Ri}} \tag{3.7}$$

If it is assumed that there is no change in the value of S_{0i} in an ultrafiltration cycle, then the final solution of Kurnik et al. (1995) can be expressed as in Equation 3.8.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (S_{oi} - 1)\ln(1 - f) \tag{3.8}$$

where $f = P/F$ and F is the volume of the initial feed solution. For this model system, the sieving coefficient was measured under total recycle conditions ($f = 0$). With the known constant value of S_{0i} and by knowing C_{Fi}, the value of C_{Ri} can be obtained for any given f using equation 3.8.

When S_{0i} is not constant, the solution to Equation 3.7 is:

$$\int_F^R (S_{0i} - 1)\frac{dR}{R} = ln\frac{C_{Ri}}{C_{Fi}} \tag{3.9}$$

Substitution for R using the relation $R = F(1-f)$ yields the equation as follows:

$$ln\frac{C_{Ri}}{C_{Fi}} = \int_0^f \frac{(1-S_{0i})}{(1-f)}df \tag{3.10}$$

During ultrafiltration, S_{0i} declines nearly linearly as *f* increases. Equation 3.10 can be integrated using this dependence.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (m + b - 1)\ln(1 - f) + mf, \tag{3.11}$$

where, *m* is the slope, and *b* is the intercept of the linear decline of S_{0i} versus *f*. In this case, the instantaneous permeate and retentate tubing concentrations were measured at different *f* values, and S_{0i} as a function of *f* ($S_{0i} = mf + b$) was obtained. We can then calculate C_{Ri} for any given *f* using equation 3.11.

To calculate the mixing cup concentration of the solute in permeate ($<C_{Pi}>$), an overall integrated mass balance (based on system II of Figure 3.1) was used:

$$\frac{dm_{sys,i}}{dt} = 0 \tag{3.12}$$

$$At\ t = 0, m_{sys,i} = C_{Fi}F \tag{3.13}$$

$$At\ t = t, m_{sys,i} = < C_{Ri} > R + < C_{Pi} > P \tag{3.14}$$

Using Equations 3.13 and 3.14, an overall mass balance can be expressed as follows:

$$C_{Fi}F = < C_{Ri} > R + < C_{Pi} > P. \tag{3.15}$$

Where $<C_{Ri}>$ is the mixing cup concentration of solute in the retentate. An assumption was made that $<C_{Ri}> \approx C_{Ri}$, and C_{Ri} was obtained using equation 3.8 and 3.11. This assumption is an approximation because C_{Ri} is the instantaneous concentration of solute in the retentate and is always slightly greater than the solute concentration in the beaker $<C_{Ri}>$ due to the concentration effect of withdrawing the permeate stream from

the membrane. The permeate flow rate of about 6 mL/min was withdrawn from a feed solution flow rate of about 36-56 mL/min to give a retentate flow rate of 30-50 mL/min. Therefore, the value of C_{Ri} was about 12 to 20% greater than $<C_{Ri}>$. This is because the solution in the beaker was at $<C_{Ri}>$ and the retentate was at C_{Ri}.

In practice, this concentration difference was somewhat less than calculated and the observed value of C_{Ri} was about 10% greater than the observed value of $<C_{Ri}>$.

3.4. MATERIALS AND METHODS

3.4.1. Preparation of Protein Solution

Glycomacropeptide (GMP) (BioPURE GMP, Davisco Foods International, Le Sueur, MN) and beta lactoglobulin (BLG) (Cat. No. 100363, 3x crystallized, MP Biomedicals, Irvine, CA) were used without further purification. Composition of GMP is given in Table 3.1. For single protein experiments, 0.5 g/L of GMP or BLG, and for binary protein experiments 0.5 g/L each of GMP and BLG in 25 mM sodium phosphate buffer, pH 3.0, conductivity 4 mS/cm, were prepared using 1M H_3PO_4, or 1M NaOH and granular NaCl. Protein solutions were filtered through 0.45 μm filters and then 0.22 μm filters (Durapore, Millipore Corp., Bedford, MA).

3.4.2. Preparation of Positively Charged Cross-Flow Ultrafiltration Membranes

Regenerated cellulose cross-flow ultrafiltration membranes of 50 cm^2 area (Pellicon XL, 30 kDa, Millipore Corp., Bedford, MA) were charge-modified using the procedure of van Reis (2006) and Bhushan & Etzel (2009) by circulating (3-bromopropyl) trimethylammonium bromide (Cat. No. 347604 Sigma Aldrich, St. Louis, MO) through the membrane for 21 hours at 22 ºC. The clean water flux decreased by 28% after charge modification.

3.4.3. Ultrafiltration Experiments

Membranes were incubated in the protein solution for 15 to 18 h at 22 ºC prior to taking samples for analysis. This step was to equilibrate the membrane with the feed solution before ultrafiltration. Experiments were conducted at an inlet pressure of 2 bars.

1000 mL feed solution was pumped into the ultrafiltration unit (hold up volume is 3.0 mL) using a peristaltic pump. Retentate flow rate was adjusted to 30 - 50 mL/min (360 – 600 LHM) using a pinch clamp and permeate flow rate was adjusted to 6 mL/min (72 LHM) using another peristaltic pump. During ultrafiltration, retentate was recycled back to the feed solution container and permeate was collected in a graduated cylinder. Samples were taken at different "*f*" values from permeate and retentate tubings, and from permeate and feed solution beakers for measurement of concentration. Experiments were stopped when $f = 0.8$. Two separate replicates were performed for each experiment.

The same ultrafiltration procedure was repeated for the second and third stages. Three-stage system was shown in Figure 3.2. The feed solutions for the second and third stages were permeate (P1) and retentate (R1) solutions of the first stage respectively. After one cycle of ultrafiltration, permeate of the second stage (P2) and retentate of the third stage (R3) were obtained as top and bottom products, respectively. Retentate of the second stage (R2) and permeate of the third stage (P3) were recycled to the feed stream to be used for the next cycle. New protein solution (F1) was prepared to mix with R2 and P3 to make up 1000mL F2. Experiments were continued until 6 and 4 cycles of ultrafiltration were completed using uncharged and charged membranes, respectively.

3.4.4. Spectrophotometric Measurements

For single protein solutions, the absorbance of GMP and BLG solutions were measured at 214 and 280 nm, respectively. For binary protein solutions, a method which was reported in a study of Bhushan and Etzel (2009) was used. GMP does not contain any aromatic amino acid; therefore, it gives little or no absorbance at 280 nm. Bhushan

and Etzel used this feature to distinguish BLG and GMP in binary protein solutions. Absorbance values of the permeate and retentate were determined at 214 and 280 nm; then the absorbance values were converted to concentrations using the Beer-Lambert law for mixtures. Two measurements were made in the same sample and an average was reported. Measurements were nearly identical in each case.

3.4.5. Statistical Analysis

Concentration, purity and yield values were compared with predictions from the two mass balance models using one sample T-test. Values are significantly different at $P < 0.05$.

3.5. RESULTS

3.5.1. Sieving Coefficients

Sieving coefficients of GMP and BLG were calculated individually and for binary mixtures using charged and uncharged regenerated cellulose ultrafiltration membranes in a three-stage configuration (Table 3.2). Samples were taken from the permeate and retentate tubings during the ultrafiltration at different "*f*" values as "*f*" increased from 0.0 to 0.8.

A 95% confidence interval (CI) range was calculated for the sieving coefficients of GMP and BLG in the all stages when single and binary solutions and uncharged and charged membranes were used (Table 3.2). When uncharged membranes were used, the 95% CI ranges for the sieving coefficients of GMP and BLG were narrow regardless of the solution type. For single solutions, the sieving coefficients were in the range 0.27 to 0.31, and 0.32 to 0.37, for GMP and BLG, respectively; and for binary solutions, the sieving coefficients were in the range 0.24 to 0.29, and 0.20 to 0.27, for GMP and BLG, respectively, when uncharged membranes were used. When charged membranes were used, the 95% CI range for GMP was 0.22 to 0.29 for single solutions and 0.18 to 0.27 for binary solutions; and for BLG the range was 0.03 to 0.16 for single solutions and 0.04 to 0.32 for binary solutions. However, when the second stage was excluded from the calculations for charged membranes to remove the effect of unexpectedly high sieving coefficients of BLG in the second stage, 95% CI range for BLG was 0.008 to 0.018 for single solutions and 0.002 to 0.009 for binary solutions. Compared to the sieving coefficients obtained from the experiments conducted using the uncharged membranes, BLG had very low sieving coefficients except the ones obtained in the second stage when

charged membranes were used; however, GMP had sieving coefficients close to the ones obtained from uncharged membranes. Sieving coefficients of BLG were always considered as untrustworthy when charged membranes were used. Absorbance values of BLG at 280 nm in the permeate stream were always below limit of detection (A_{280} < 0.03) except at f = 0.6 and 0.8 when single solutions were used and at f = 0.8 when binary protein solutions were used. The coefficient of variation of the sieving coefficients of BLG were in range 20% to 67% at f = 0.6 and 0.8 when single solutions were used and at f = 0.8 when binary protein solutions were used. The large coefficient of variation made the sieving coefficients untrustworthy. These very small sieving coefficients of BLG were obtained because of strong exclusion of the positively charged BLG by the positively charged membranes. In the literature, similar observations were made by different researchers (Cheang and Zydney, 2003, Lu et al. 2005, Wan et al. 2006). Cheang and Zydney (2003) reported the sieving coefficients of BLG as small as 0.005, and sieving coefficients of ovalbumin were reported as small as 0.006 and 0.0008 by Lu et al. (2005) and Wan et al. (2006), respectively.

It is important to analyze the changes in the sieving coefficients of GMP and BLG throughout the ultrafiltration experiment so that the change in the filtration profile can be observed. It is also important to obtain the sieving coefficients at different "*f*" values to be able to use the proposed model, which did not assume that sieving coefficient was constant. When uncharged membranes were used, sieving coefficients of GMP and BLG always decreased with an increasing "*f*" in every stage when single and binary solutions were used. BLG had always slightly higher sieving coefficients than GMP when single protein solutions and uncharged membranes were used; when binary solutions were used,

GMP had slightly higher sieving coefficients than BLG except for four cases which were at $f = 0.2$ in stage 1, and at $f = 0.0$, 0.3 and 0.6 in stage 2.

When charged membranes were used, there was a decrease in the sieving coefficients with an increasing "*f*" for both GMP and BLG when single and binary solutions were used. When charged membranes were used, GMP always had higher sieving coefficients than BLG in the first and third stages; in the second stage, GMP had slightly higher sieving coefficients than BLG when single solutions were used and BLG had slightly higher sieving coefficients than GMP when binary solutions were used.

3.5.2. Selectivity

Table 3.2 shows the selectivity values calculated using the ratio of the sieving coefficients of GMP to BLG. When uncharged membranes and single protein solutions were used, selectivity values were below 1.0 (95% CI range: 0.82-0.85). When uncharged membranes and binary solutions were used, selectivity values were higher (95% CI range: 1.1-1.3). When charged membranes were used, selectivity values highly increased in the first and the third stages compare to those obtained using uncharged membranes. For the first and the third stages, selectivity values obtained from charged membranes and binary protein solutions (95% CI range: 34-129) were higher than those obtained from charged membranes and single protein solutions (95% CI range: 17-26). Selectivity values in the third stage were considered untrustworthy when charged membranes and binary solutions were used because of the large coefficient of variation of the average values, which occurred because of the very small sieving coefficients of BLG.

3.5.3. Concentration

Multiple cycles of ultrafiltration using uncharged and charged membranes were performed to see the effect of recycling of selected streams (Figure 3.2.) on concentrations of BLG and GMP. Since GMP is the product collected in permeate and BLG is the product collected in retentate, only concentration profiles of GMP in permeate and BLG in retentate were shown in Figure 3.3 when single and binary solutions, and charged and uncharged membranes were used. Concentrations of the proteins obtained from the experiments conducted with uncharged membranes reached the equilibrium after the 4^{th} cycle. Figures 3.3-A and 3.3-B are two examples showing concentration profiles in uncharged membranes. Since the equilibrium was reached at the 4^{th} cycle when uncharged membranes were used, all the experiments with charged membranes were performed for 4 cycles. Concentration profiles of GMP were shown in Figures 3.3-A and 3.3-C for uncharged and charged membranes, respectively. As seen in Figures 3.3-A and 3.3-C, the concentration profile of GMP did not change when a positive charge was added on the membrane. On the other hand, concentration of BLG changed from a stable profile to a decreasing profile when a charge was placed on the membranes (Figures 3.3-B and 3.3-D). In Figure 3.3, continuous lines show the calculated results using the two models. Calculated mixing cup concentrations from both models were similar to each other and to the experimental results, but the calculated concentrations correlated better with measured concentrations for charged membranes.

Tables 3.3 and 3.4 show the final mixing-cup concentrations of GMP and BLG from experimental measurements and mass balance calculations for uncharged and charged membranes, respectively. Since the concentrations of GMP and BLG in the last

three cycles of uncharged membranes were similar to each other, the average of these three stages was reported in Table 3.3. As seen in Table 3.3, concentrations of GMP and BLG in permeate and retentate solutions in all three stages are very similar to each other due to the low selectivity of separation in uncharged membranes. For example, when uncharged membranes and single solutions were used, concentrations of GMP and BLG in permeate stream of the first stage were 0.32g/L and 0.28g/L, respectively. Calculated concentrations of GMP and BLG using mass balance models are presented in Table 3.3. Calculated results and the experimental results were compared to each other using one-way T-test ($P = 0.01$); and the statistically significant results were signed with stars. For uncharged membranes, there were significantly different 16 data points out of 24 data points when our proposed model was used ($P < 0.01$); and there were significantly different 12 data points out of 24 data points when Kurnik's model was used ($P < 0.01$).

When charged membranes were used, very low BLG concentrations were obtained in permeate solutions of all stages, and also in the retentate of the second stage (Table 3.4). Concentrations of BLG in the permeate solutions were considered negligible when charged membranes were used because absorbance values of BLG at 280 nm in the permeate solutions were below limit of detection ($A_{280} < 0.03$). A similar observation was made by Cheang and Zydney (2003) for separation of ALA from BLG using ultrafiltration. The concentration of BLG in permeate was reported as 0.005±0.002 g/L. The very small concentration of BLG in permeate was because of the strong rejection of BLG by the ultrafiltration membrane and the large error was because of the difficulty in evaluating the very small concentration of BLG in permeate (Cheang and Zydney, 2003). The present study has similar orders of magnitude for concentration of BLG in permeate

when charged membranes were used. Concentrations of the proteins were calculated using the two mass balance models and statistically different results were signed with stars. Concentrations of GMP and BLG for the charged membranes calculated using the proposed model for the charged membranes were not statistically significantly different than the experimentally obtained mixing-cup concentrations for any of the 24 data points ($P > 0.01$); and, there was one statistically significant difference for Kurnik's model out of 24 data points ($P < 0.01$). The one significantly different data point was obtained from the permeate of the third stage when binary solutions were used. Thus, both models were nearly a perfect match to the experimentally measured mixing-cup concentrations for the charged membranes.

3.5.4. Purity

Concentrations of GMP and BLG obtained by experiments and mass balance calculations were used to calculate purities in each stage and cycle. As explained before, GMP is mostly collected in permeate and BLG is mostly collected in retentate. Because of this, purities of GMP in permeate solutions and BLG in retentate solutions of three stages from the first and the fourth cycles of only charged membranes were shown in Table 3.5. Purities of the proteins were not shown when uncharged membranes were used, because no separation occurred in this case. The reason why both the first and the fourth cycles were reported in Table 3.5 is to show the change in the purity as the cycle number increased. When charged membranes were used, we obtained highly pure GMP (from 93% to 99.96%) from all permeate solutions and around 50% pure BLG in the retentate of the first and the third stages. The purity of BLG in the retentate of the second

stage was very low (4% to 8.4%). Regardless of the type of the solution, the highest purities of GMP and BLG were always obtained from the permeate and the retentate of the third stage, respectively. There is a slight increase in the purity of GMP in the permeate but a slight decrease in the purity of BLG in the retentate from the first to the fourth cycle. In the same table, calculated purity data were shown. Statistically, there was no significant difference between our proposed mass balance model and the experimentally measured mixing cup concentrations ($P > 0.01$). On the other hand, the mass balance model from Kurnik et al. (1995) had two data points out of 36 total data points, which were significantly different from the experimental results ($P < 0.01$).

3.5.5. Distribution

Concentrations of GMP and BLG obtained by experiments and mass balance calculations were also used to calculate the distributions of GMP and BLG into permeate and retentate stream for each stage and cycle (Table 3.5). The calculation of the distribution was performed based on the amount of GMP and BLG collected in the permeate and the retentate streams, respectively. The highest distribution value can be 100%, which means that the protein in the feed stream is completely collected in the corresponding stream (permeate stream for GMP and retentate stream for BLG). Table 3.5 shows distributions of GMP in permeate solutions and BLG in retentate solutions of three stages from the first and the fourth cycles of charged membranes. The highest distribution values of GMP into permeate (26 – 50%) and BLG into retentate (87 – 94.2%) were obtained from the first stage of the system regardless of the cycle number and the solution type; and the lowest distribution values of GMP (13.5 – 23%) and BLG

(0.9 – 1.9%) were obtained from the second stage except for GMP when binary solution was used. When binary solution was used, the distribution of GMP in the second (13.7 – 25%) and the third (12.8 – 22%) stages were similar to each other. When the cycle increased from the first to the fourth, distribution of GMP increased; however, the distribution of BLG remained the same. When binary protein solutions were used, in most cases the distribution was lower than that of single protein solution. In Table 3.5, calculated distribution data were also shown. Statistically, there was no significant difference between the calculated results from two mass balance models and the experimental results ($P > 0.01$).

3.6. DISCUSSION

When charged membranes were used, high selectivity of separation and high purity of GMP were obtained. Moreover, using the charged membranes in the three-stage configuration with recycling of the permeate of the third stage and the retentate of the second stage to the feed stream increased the distributions of GMP and BLG in the permeate and the retentate stream, respectively. Based on these results, this research showed that stage configuration with charged ultrafiltration membranes enhanced the fractionation of GMP from BLG and increased the distributions of the proteins in the final streams. It was also shown that the two mass balance models could be used to calculate the expected mixing-cup concentrations at a given f to test different experimental designs.

When positively charged membranes were used in the first stage, BLG was highly rejected by the membrane and almost all the BLG was collected in the retentate of the first stage. Therefore, we obtained very small sieving coefficients of BLG in the first stage (Table 3.2). Because of the high rejection of BLG in the first stage when charged membranes were used, concentration of BLG in the permeate stream from the first stage was below limit of detection (Table 3.4). The feed solution of the second stage was the permeate of the first stage (Figure 3.1). The permeate of the first stage included a high amount of GMP (0.22 g/L in single solution, 0.19 g/L in binary solution) compared to a very small amount of BLG (0.008 g/L in single solution, 0.006 g/L in binary solution) (Table 3.4). The small concentration of BLG caused absorbance values to be below limit of detection ($A_{280} < 0.03$) when the spectrophotometric analysis was performed to detect BLG in the permeate and retentate streams of the second stage. Because of the small

absorbance values obtained from spectrophotometric BLG detection, high sieving coefficients of BLG were obtained from the second stage of the system when charged membranes were used regardless of the solution type (Table 3.2). The high sieving coefficients of BLG caused small selectivity of the separation for the second stage of the system when charged membranes were used. The feed solution of the third stage was the retentate of the first stage. The retentate of the first stage included high amounts of GMP (1.72 g/L in single solution, 1.6 g/L in binary solution) and BLG (1.6 g/L in single solution, 1.49 g/L in binary solution) compare to the amounts in the permeate of the first stage (Table 3.4). Because the positively charged membranes always rejected high amount of BLG in any stage, the concentration of BLG in permeate of the third stage was untrustworthily small (0.017 g/L in single solution, 0.0002 g/L in binary solution) because the absorbance values of BLG were below limit of detection, and it's concentration was high in the retentate compare to the permeate (6.5 g/L in single solution, 6.4 g/L in binary solution) (Table 3.4). The high difference between the concentrations of BLG in the permeate and the retentate caused very small untrustworthy sieving coefficients of BLG in the third stage (Table 3.2). However, the sieving coefficients of GMP were similar to the ones obtained from the uncharged membranes. Therefore, very high selectivity values in the third stage of the system were obtained when charged membranes were used, especially for binary solution (Table 3.2).

Since BLG was highly rejected by the positively charged membranes, we expected to obtain the highest purity of GMP in the permeate of the second stage of the system, which included almost no BLG in the feed solution, and the highest purity of BLG in the retentate of the third stage, which included almost all BLG. As we expected,

we obtained the highest purity of BLG in the retentate of the third stage (Table 3.5); but unexpectedly we obtained the highest purity of GMP in the permeate of the third stage rather than the second stage (Table 3.5). The reason why we obtained the highest purity of GMP from the third stage can be explained as follows: 1) BLG was highly rejected by the positively charged membranes no matter which stage was performed and no matter how much BLG was in the feed solution, 2) the concentration of GMP in the feed solution of the third stage (1.72 g/L in single solution, 1.6 g/L in binary solution) was much higher than in the feed solution of the second stage (0.22 g/L in single solution, 0.19 g/L in binary solution), 3) the difference between the concentrations of GMP in the feed solutions of the second and the third stages did not affect the sieving coefficients of GMP (95% CI range: 0.22 – 0.29). Since there was no change in the sieving coefficients of GMP and BLG, and there was higher amount of GMP in the feed solution of the third stage than the second stage, higher amount of GMP passed through the ultrafiltration membrane and higher purity of GMP was obtained in the third stage rather than the expected second stage.

We tested the two models statistically to see if there was any significant difference between the calculated results and the experimental results. It was surprising that the two models worked very well for the charged membranes (Table 3.4) but neither of them worked statistically well for the uncharged membranes (Table 3.3). The reason why statistically poor result were obtained from the models for the uncharged membranes might be due to the very small standard deviations of the data points, which required very close predictions from the two models to be considered as non significant difference.

3.7. CONCLUSIONS

Three-stage ultrafiltration with recycling of the permeate of the third stage and the retentate of the second stage has been investigated using uncharged and positively charged ultrafiltration membranes for the fractionation of GMP from BLG in single and binary protein solutions. Uncharged membranes did not have the ability to fractionate the proteins due to low selectivity. On the other hand, because of the high selectivity of the separation, positively charged ultrafiltration membranes cleanly fractionated GMP from BLG, and we obtained GMP solutions with purities up to 99.96%. Recycling of the selected streams to the feed solution did not have a significant effect on the purity of the product, but it increased the distribution of the proteins into target streams. Predictions from the two mass balance models were compared with the experimental results. The two models gave similar predictions and were close fits to experimental observations. However, the predictions for the uncharged membranes were significantly different from the experimental results. Overall, this study showed that the fractionation of GMP from BLG is possible with a three-stage configuration; moreover, it showed that recycling increased the distribution of the final product significantly.

3.8. REFERENCES

Bakhshayeshi, M. and Zydney, A.L. 2008. Effect of solution pH on protein transmission and membrane capacity during virus filtration. *Biotechnology and Bioengineering*. Vol. 100, pg: 108-117.

Balakrishnana, M. and Agarwal, G.P. 1996. Protein fractionation in a vortex flow filter: effect of system hydrodynamics and solution environment on single protein transmission. *Journal of Membrane Science*. Vol. 112, pg: 47-76.

Bhushan, S. and Etzel, M.R. 2009. Charged ultrafiltration membranes increase the selectivity of whey protein separations. *Journal of Food Science*. Vol. 74, pg: E131-E139

Burns, D.B. and Zydney, A.L. 1999. Effect of solution pH on protein transport through ultrafiltration membranes. *Biotechnology and Bioengineering*. Vol. 64, pg:27-37.

Caus, A., Braeken, L., Boussu, K. and van der Bruggen, B. 2008. The use of integrated countercurrent nanofiltration cascades for advanced separations. *Journal of Chemical Technology and Biotechnology*. Vol. 84, pg: 391-398.

Cheang, C. and Zydney, A.L. 2003. Separation of α-lactalbumin and β-lactoglobulin using membrane ultrafiltration. *Biotechnology and Bioengineering*. Vol. 83, pg: 201-209.

Cheang, C. and Zydney, A.L. 2004. A two-stage ultrafiltration process for fractionation of whey protein isolate. *Journal of Membrane Science*. Vol. 231, pg: 159-167.

Cherkasov, A.N. and Polotsky, A.E. 1996. The resolving power of ultrafiltration. *Journal of Membrane Science*. Vol. 110, pg: 79-82.

De la Casa, E.J., Guadix, A., Ibanez, R. and Guadix, E.M. 2007. Influence of pH and salt concentration on the cross-flow microfiltration of BSA through a ceramic membrane. *Biochemical Engineering Journal*. Vol. 33, pg: 110-115.

Filipe, C.D.M. and Ghosh, R. 2005. Effects of protein-protein interaction in ultrafiltration based fractionation processes. *Biotechnology and Bioengineering*. Vol. 91, pg: 678-687.

Ghosh, R. 2003. Novel cascade ultrafiltration configuration for continuous, high-resolution protein-protein fractionation: a simulation study. *Journal of Membrane Science*. Vol. 226, pg: 85-99.

Gunderson, S.S., Brower, W.S., O'Dell, J.L. and Lightfoot, E.N. 2007. Design of membrane cascades. *Separation Science and Technology*. Vol. 42, pg: 2121-2142.

Kanani, D. Ghosh, R. and Filipe, C.D.M. 2004. A novel approach for high-resolution protein-protein separation by ultrafiltration using dual-facilitating agent. *Journal of Membrane Science*. Vol. 243, pg: 223-228.

Kurnik, R.T., Yu, A.W., Blank, G.S., Burton, A.R., Smith, D., Athalye, A.M. and van Reis, R. 1995. Buffer exchange using size exclusion chromatography, countercurrent dialysis, and tangential flow filtration : models, development, and industrial application. *Biotechnology and Bioengineering*. Vol. 45, pg : 149-157.

Lightfoot, E.N. 2005. Can membrane cascades replace chromatography? Adapting binary ideal cascade theory of systems of two solutes in a single solvent. *Separation Science and Technology*. Vol. 40, pg: 739-756.

Lightfoot, E.N., Root, T.W. and O'Dell, J.L. 2008. Emergence of ideal membrane cascades for downstream processing. *Biotechnology Progress*. Vol. 24, pg: 599-605.

Lu, J., Wan, Y. and Cui, Z. 2005. Fractionation of lysozyme and chicken egg albumin using ultrafiltration witk 30-kDa commercial membranes. *Industrial & Engineering Chemistry Research*. Vol. 44, pg: 7610-7616.

Lucas, D., Rabiller-Baundry, M., Michel, F. and Chaufer, B. 1998. Role of the physico-chemical enviroment on ultrafiltration of lysozyme with modified inorganic membrane. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. Vol. 136, pg:109-122.

Mayani, M., Mohanty, K., Filipe, C., and Ghosh, R. 2009. Continuous fractionation of plasma proteins HSA and HIgG using cascade ultrafiltration systems. *Separation and Purification Technology*. Vol. 20, pg: 231-241.

Mayani, M., Filipe, C.D.M. and Ghosh, R. 2010. Cascade ultrafiltration systems – Integrated processes for purification and concentration of lysozyme. *Journal of Membrane Science*. Vol. 347, pg: 150-158.

Nakao, S., Osada, H., Kurata, H., Tsuru, T. and Kimura S. 1988. Separation of proteins by charged ultrafiltration membranes. *Desalination*. Vol. 70, pg:191-205.

Saksena, S. and Zydney, A.L. 1994. Effect of pH and ionic strength on the separation of albumin during ultrafiltration. *Journal of Membrane Science*. Vol. 43, pg: 960-968.

Sudareva, N.N., Kurenbin, O.I. and Belenkii, B.G. 1992. Increase in the efficiency of membrane fractionation. *Journal of Membrane Science*. Vol. 68, pg: 263-270.

van Reis, R. 2006. Charged filtration membranes and uses therefor. U.S. patent 7,153,426 B2.

van Reis., R., Brake, J.M., Charkoudian, J., Burns, D.B. and Zydney, A.L. 1999. High-performance tangential flow filtration using charged membranes. *Journal of Membrane Science*. Vol. 159, pg: 133-142.

Wan, Y., Ghosh, R. and Cui, Z. 2002. High-resolution plasma protein fractionation using ultrafiltration. *Desalination*. Vol. 144, pg: 301-306.

Wan, Y., Lu, J. and Cui, Z. 2006. Separation of lysozyme from chicken egg white using ultrafiltration. *Separation and Purification Technology*. Vol. 48, pg: 133-142.

Wang, L., Khan, T., Mohanty, K. and Ghosh, R. 2010. Cascade ultrafiltration bioreactor-separator system for continuous production of $F(ab)_2$ fragment from immunoglobulin G. *Journal of Membrane Science*. Vol. 351, pg: 96-103.

Wilharm, C. and Rodgers, V.G.J. 1996. Significance of duration and amplitude in transmembrane pressure pulsed ultrafiltration of binary protein mixtures. *Journal of Membrane Science*. Vol. 121, pg: 217-228.

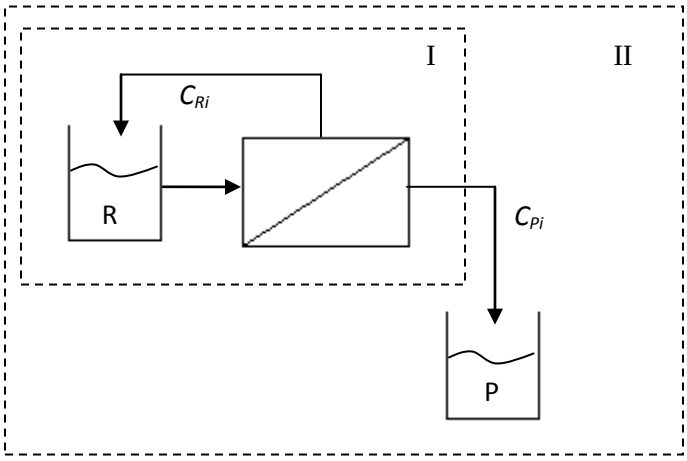

Figure 3.1. Schema of the model system (F: feed, R: retentate, P: permeate)

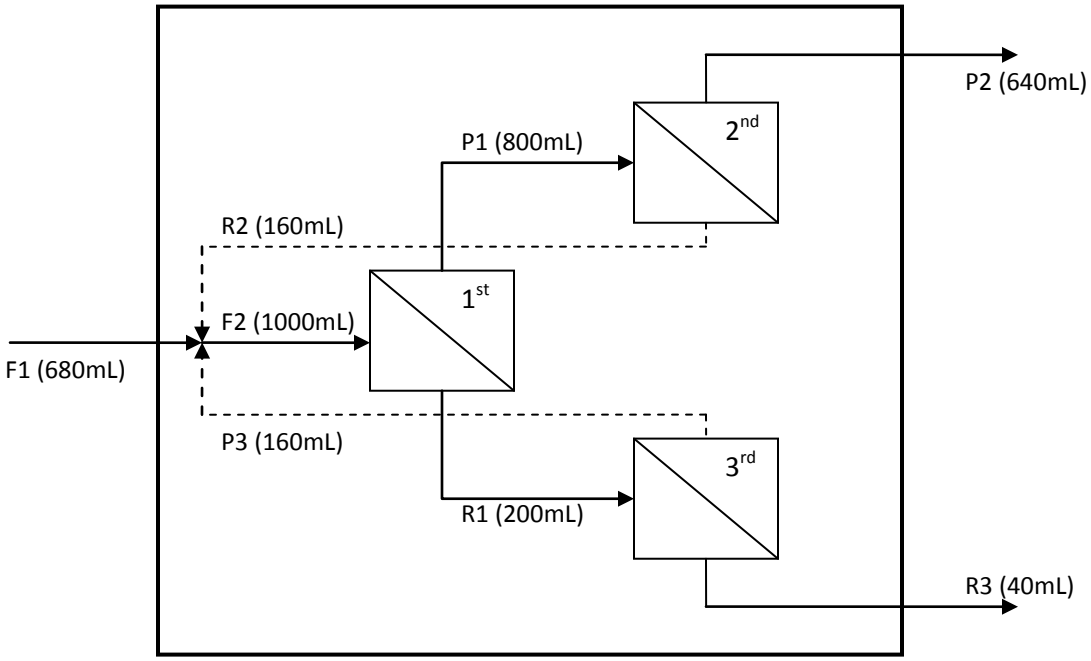

Figure 3.2. Schema of three-stage ultrafiltration

F1: Fresh feed solution, F2: Feed solution used in the first stage, P1: Permeate of the first stage, P2: Permeate of the second stage, P3: Permeate of the third stage, R1: Retentate of the first stage, R2: Retentate of the second stage, R3: Retentate of the third stage.

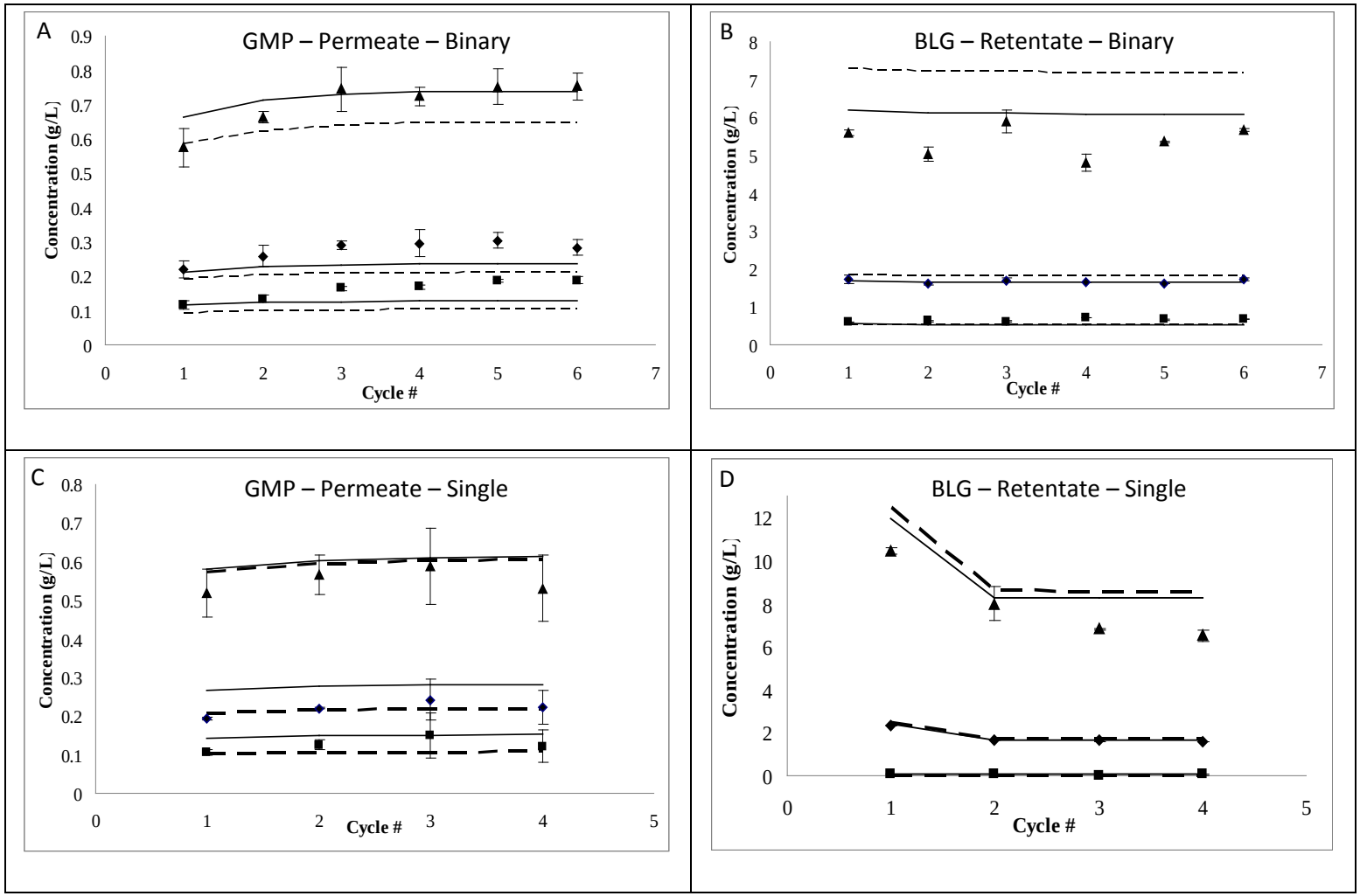

Figure 3.3. Concentration profiles of (a) GMP in the collection beaker of permeate, (b) BLG in the collection beaker of retentate when experiments were performed using binary solutions and uncharged membranes; and (c) GMP in the collection beaker of permeate, (d) BLG in the collection beaker of retentate when experiments were performed using single solutions and charged membranes. (♦) Concentrations in the first stage, (■) Concentrations in the second stage, (▲) Concentrations in third stage, (—) Proposed model, (– –) Kurnik's model. (Each data point is the average of two separate replicates)

Table 3.1. Composition of Glycomacropeptide (GMP) (BioPURE GMP, Davisco Foods International, Le Sueur, MN)

Analysis	**Specification**	**Typical Range**	**Test Method**
Moisture (%)	7.0 max.	6.0 ± 0.9	Vacuum Oven
Glycomacropeptide (N × 7.07) (% Dry Basis)	90.0 min.	91 ± 1	Leco Combustion
Protein (N × 6.47) (% Dry Basis)	82.5 min.	84 ± 1	Leco Combustion
Fat (%)	1.0 max.	0.5 ± 0.3	Mojonnier
Ash (%)	7.0 max.	6.3 ± 0.5	Residue on Ignition
Lactose (%)	1.0 max.	< 0.5	Enzymatic Assay
pH	7.0 max.	6.4 ± 0.2	10% Sol. At 20°C
Scorched Particles (mg/25g)	15.0 max.	7.5	ADPI

Table 3.2. Sieving coefficients and selectivity values obtained from the experiments in three-stage cascade configuration performed using uncharged and charged 30 kDa membranes; and single and binary protein solutions

Uncharged		Single			Binary		
Stage #	*f*	S_0(GMP)[1]	S_0(BLG)[1]	ψ	S_0(GMP)[1]	S_0(BLG)[1]	ψ
1	0	0.33±0.01[a]	0.40±0.02	0.82±0.05	0.28±0.02	0.26±0.02	1.1±0.1
	0.1	0.32±0.01	0.37±0.01	0.88±0.04	0.27±0.04	0.24±0.01	1.1±0.2
	0.2	0.31±0.01	0.37±0.02	0.84±0.05	0.23±0.01	0.25±0.04	0.9±0.2
	0.3	0.3±0	0.35±0.02	0.84±0.05	0.3±0	0.22±0.04	1.1±0.2
	0.4	0.29±0.01	0.34±0.01	0.84±0.04	0.25±0.01	0.24±0.06	1.1±0.3
	0.5	0.3±0	0.34±0.02	0.84±0.05	0.24±0.04	0.22±0.04	1.1±0.3
	0.6	0.28±0.01	0.32±0.03	0.86±0.08	0.24±0.01	0.19±0.03	1.3±0.2
	0.7	0.26±0.01	0.30±0.03	0.85±0.09	0.22±0.01	0.18±0.02	1.2±0.2
	0.8	0.3±0	0.28±0.03	0.9±0.1	0.23±0.02	0.16±0.02	1.5±0.2
2	0	0.33±0.01	0.41±0.06	0.8±0.1	0.35±0.03	0.42±0.01	0.84±0.07
	0.1	0.32±0.01	0.39±0.03	0.81±0.06	0.33±0.04	0.32±0	1.0±0.1
	0.3	0.33±0.01	0.40±0.05	0.8±0.1	0.33±0.01	0.34±0.04	1.0±0.1
	0.6	0.32±0.01	0.40±0.04	0.8±0.1	0.30±0.02	0.35±0.02	0.86±0.08
	0.8	0.3±0	0.38±0.02	0.83±0.04	0.32±0.01	0.31±0.04	1.0±0.1
3	0	0.27±0.01	0.33±0.03	0.80±0.08	0.27±0.01	0.19±0.01	1.4±0.1
	0.1	0.26±0.01	0.32±0.04	0.8±0.1	0.25±0.01	0.19±0.01	1±1
	0.3	0.25±0.02	0.31±0.04	0.8±0.1	0.2±0	0.18±0.01	1.31±0.05
	0.6	0.23±0.01	0.27±0.04	0.9±0.1	0.2±0	0.14±0.01	1.56±0.08
	0.8	0.20±0.03	0.22±0.03	0.9±0.2	0.18±0.01	0.10±0.01	1.8±0.3
95% CI Range		0.27 – 0.31	0.32 – 0.37	0.82 – 0.85	0.24 – 0.29	0.20 – 0.27	1.1 – 1.3
Charged							
1	0	0.32±0.02	0.027±0.001[b]	11.6±0.9	0.26±0.04	0.018±0.002[b]	15±3
	0.1	0.29±0.01	0.025±0.006[b]	11±3	0.23±0.01	0.011±0.002[b]	22±4
	0.3	0.25±0.01	0.019±0.002[b]	14±2	0.26±0.09	0.007±0.002[b]	39±18[b]
	0.6	0.23±0.01	0.0125±0.0007[b]	18±1	0.19±0.01	0.006±0[b]	32±2
	0.8	0.20±0.01	0.008±0.001[b]	25±4	0.135±0.007	0.003±0[b]	45±2
2	0	0.35±0.06	0.35±0[b]	1.0±0.2[b]	0.35±0	0.6±0.1[b]	0.6±0.1[b]
	0.1	0.33±0.02	0.325±0.007[b]	1.00±0.06[b]	0.4±0.1	0.5±0.4[b]	0.7±0.6[b]
	0.3	0.3±0.04	0.29±0.02[b]	1.1±0.2[b]	0.30±0.01	0.8±0.2[b]	0.39±0.09[b]
	0.6	0.32±0.01	0.22±0.04[b]	1.5±0.3[b]	0.3±0	0.4±0.2[b]	0.8±0.4[b]
	0.8	0.31±0.01	0.123±0.004[b]	2.5±0.1[b]	0.2±0	0.4±0.3[b]	0.6±0.5[b]
3	0	0.2±0	0.008±0.002[b]	25±6	0.21±0.09	0.0025±0.0007[b]	80±40[b]
	0.1	0.195±0.007	0.009±0.002[b]	23±6	0.17±0.04	0.006±0.006[b]	30±40[b]
	0.3	0.185±0.007	0.008±0.002[b]	22±6	0.115±0.007	0.0006±0.0005[b]	200±200[b]
	0.6	0.175±0.007	0.006±0.001[b]	28±6	0.125±0.007	0.002±0.003[b]	53±73[b]
	0.8	0.18±0.05	0.005±0.002[b]	40±20[b]	0.095±0.007	0.0003±0.0002[b]	300±200[b]
95% CI Range		0.22 – 0.29	0.03 – 0.16[b] 0.008 – 0.018¥[b]	9 – 21 17 – 26¥	0.18 – 0.27	0.04 – 0.32[b] 0.002 – 0.009¥[b]	12 – 98 34 – 129¥

Each data point is the average of two separate replicates

[1]Sieving coefficients were determined using instantaneous concentrations in tubings in cycle 1

[a] Average ± standard deviation

¥ 95% CI Range excluding the second stage when charged membranes were used

[b] Untrustworthy data point

Table 3.3. Experimental and Predicted Concentrations of GMP and BLG in the collection beakers when $f = 0.8$ obtained from the ultrafiltration experiments performed using 30 kDa uncharged membranes.

Concentrations of GMP and BLG in average of 4th, 5th, 6th cycle (g/L)				
	Single		Binary	
	GMP	BLG	GMP	BLG
	Experimental [a]			
Stage #	Permeate			
1	0.32±0.02[b]	0.28±0.07	0.30±0.03	0.240±0.001
2	0.19±0.02	0.16±0.06	0.18±0.01	0.130±0.006
3	0.75±0.05	0.6±0.1	0.74±0.04	0.50±0.02
Stage #	Retentate			
1	1.69±0.05	1.7±0.1	1.7±0.1	1.66±0.05
2	0.86±0.03	0.8±0.1	0.81±0.03	0.68±0.02
3	4.3±0.3	5.4±1.2	4.4±0.3	5.3±0.4
	Proposed Model			
Stage #	Permeate			
1	0.26*	0.31	0.21*	0.17*
2	0.13*	0.18	0.10*	0.09*
3	0.72	0.78	0.65*	0.45*
Stage #	Retentate			
1	1.9*	1.8	1.8	1.8*
2	0.79*	0.82	0.64*	0.51*
3	6.6*	5.8	6.5*	7.2*
	Kurnik et al. 1995			
Stage #	Permeate			
1	0.30	0.35	0.24*	0.21*
2	0.15*	0.21	0.13*	0.13
3	0.76*	0.82*	0.74	0.55*
Stage #	Retentate			
1	1.7	1.6	1.7	1.7
2	0.88	0.92	0.68*	0.54*
3	5.7*	4.7	5.6*	6.1*

[a] Each data point is the average of two separate replicates

* Value is significantly different from experimental result ($P < 0.01$)

[b] Average ± standard deviation

Table 3.4. Experimental and Predicted Concentrations of GMP and BLG in the collection beakers when f = 0.8 obtained from the ultrafiltration experiments performed using 30 kDa charged membranes.

Concentrations of GMP and BLG in 4th cycle (g/L)				
	Single		Binary	
	GMP	BLG	GMP	BLG
	Experimental [a]			
Stage #	Permeate			
1	0.22±0.05[b]	0.008±0.003[c]	0.19±0.04	0.006±0.005[c]
2	0.12±0.04	0.0085±0.0007[c]	0.13±0.03	0.003±0.001[c]
3	0.53±0.09	0.017±0.004[c]	0.46±0.05	0.0002±0.0001[c]
Stage #	Retentate			
1	1.72±0.04	1.6±0	1.6±0.2	1.49±0.08
2	0.65±0.04	0.042±0.009	0.6±0.1	0.022±0.007
3	6.1±0.2	6.5±0.3	6.0±0.3	6.4±0.1
	Proposed Model			
Stage #	Permeate			
1	0.22	0.007	0.15	0.0005
2	0.11	0.003	0.07	0.0003
3	0.61	0.005	0.33	0.0009
Stage #	Retentate			
1	2.0	1.7	1.7	1.7
2	0.66	0.02	0.49	0.001
3	7.4	8.6	7.0	8.6
	Kurnik et al. 1995			
Stage #	Permeate			
1	0.28	0.02	0.19	0.01
2	0.15	0.009	0.10	0.009
3	0.61	0.03	0.54	0.008*
Stage #	Retentate			
1	1.7	1.7	1.5	1.7
2	0.80	0.05	0.55	0.02
3	6.1	8.3	5.4	8.4

[a] Each data point is the average of two separate replicates

* Value is significantly different from experimental result (P < 0.01)

[b] Average ± standard deviation, n = 2

[c] Untrustworthy data point

Table 3.5. Experimental and calculated purity and distribution values in the collection beakers when $f = 0.8$ obtained from the first and the fourth cycles of the experiments performed using 30 kDa charged membranes.

	Experimental [a]				Proposed Model				Kurnik et al. 1995			
	GMP-Single											
	Purity ¥		Distribution		Purity		Distribution		Purity		Distribution	
	1	4	1	4	1	4	1	4	1	4	1	4
P1	95±1[b]	96.6±0.5	31.0±0.7	50±10	96	97	33	52	91	94	43	66
P2	97±1	93±3	13.5±0.8	23±8	97	98	13	20	91	94	18	29
P3	96.6±0.5	97±1	17±2	25±4	99	99	18	29	94	96	19	29
	BLG-Single											
	Purity ¥		Distribution		Purity		Distribution		Purity		Distribution	
	1	4	1	4	1	4	1	4	1	4	1	4
R1	57.2±0.2	48.2±0.7	94.2±0.3	94.2±0.3	57	47	100	101	60*	50	97	98
R2	8.4±0.7	6±2	1.70±0.02	1.9±0.4	5	3	1.1	1.1	9	6	3	3
R3	67±1	52±0.2	84±1	77±3	64	54	100	101	68	58	96	97
	GMP-Binary											
	Purity		Distribution		Purity		Distribution		Purity		Distribution	
	1	4	1	4	1	4	1	4	1	4	1	4
P1	96±2	97±3	26±2	50±10	100	100	26	36	92	94	32	45
P2	94.7±0.3	97±1	13.7±0.7	25±6	99	100	9	13	89	92	14	19
P3	99±2	99.96±0.02	12.8±0.5	22±3	100	100	11	15	98	99*	18	26
	BLG-Binary											
	Purity		Distribution		Purity		Distribution		Purity		Distribution	
	1	4	1	4	1	4	1	4	1	4	1	4
R1	53.2±0.8	48±2	91±1	87±5	59	51	101	101	61	53	99	99
R2	6±1	4±2	0.9±0.2	1.0±0.3	0.4	0.3	0.07	0.07	6	4	1	1
R3	55±2	51.6±0.7	71±4	75±2	63	55	101	101	69	61	98	98

[a] Each data point is the average of two separate replicates

* Value is significantly different than experimental result ($P < 0.01$)

[b] Average ± standard deviation

¥ Purity was calculated using the concentration results obtained from single protein solutions

CHAPTER 4

FRACTIONATION OF GLYCOMACROPEPTIDE USING POSITIVELY CHARGED ULTRAFILTRATION MEMBRANES IN STAGED CONFIGURATIONS

4.1. ABSTRACT

The current technology to fractionate whey proteins is based on cation exchange chromatography. Ultrafiltration technology, on the other hand, is commonly used for concentration and/or clarification purposes. To date, several studies have proposed the use of charged ultrafiltration membranes and staged-configurations to achieve fractionation of whey proteins using ultrafiltration systems. However, the studies were commonly performed using dilute protein solutions and/or dead-end filtration mode. In addition, studies on investigation of charged membranes in staged configurations are limited. This study is on fractionation of glycomacropeptide and ß-lactoglobulin from binary protein solutions and Swiss cheese whey using positively charged ultrafiltration membranes in staged configurations. Positively charged 30 kDa and 300 kDa regenerated cellulose ultrafiltration membranes in cross-flow mode were used. Two-stage and single-stage configurations of the ultrafiltration process were performed using binary protein solutions and Swiss cheese whey, respectively. 100 % pure glycomacropeptide was obtained using the positively charged 30 kDa membrane in the single-stage configuration with Swiss cheese whey or using positively charged 300 kDa and 30 kDa membranes in the two-stage ultrafiltration with the binary protein solutions. 80 % pure

glycomacropeptide was obtained using the positively charged 300 kDa membrane in the single-stage configuration with Swiss cheese whey. Obtained results showed that the applicability of ultrafiltration processes in production of highly pure whey proteins instead of more expensive chromatographic methods.

4.2. INTRODUCTION

Whey is a by-product of cheese manufacture; and it is produced by the action of rennet and/or acid on casein. Whey consists about 6 g/L protein (Doultani et al. 2004); and there has been an interest in separation of the proteins into individual fractions due to their nutritional and functional attributes. Methods to produce individual whey proteins include selective precipitation, selective adsorption and membrane ultrafiltration (Etzel 2004). Precipitation and adsorption processes have been commercially used for the production of whey protein fractions. Membrane ultrafiltration, however, is still being used as a process of concentration and/or clarification. That is because, membrane ultrafiltration is a size based process; and the size difference of whey proteins is not large enough to be separated using ultrafiltration (Wan et al. 2005).

Adjustment of pH and ionic strength of the protein solutions and use of charged membranes have been investigated to improve the fractionation ability of the ultrafiltration. Cowan and Ritchie (2007) investigated the separation of α-lactalbumin (ALA) and β-lactoglobulin (BLG) using negatively charged polyethersulfone (PES) using a dead-end ultrafiltration mode and single protein solutions; and they observed five times higher selectivity with charged membranes than uncharged membranes at pH 7.2. Bhushan and Etzel (2007) investigated the fractionation of glycomacropeptide (GMP) and BLG using positively charged ultrafiltration membrane in a dead-end filtration mode. The amount of charge changed from 0% (uncharged) to 100% (fully charged). The highest selectivity values were achieved when 100% and 75% charged membranes and the protein solution at pH 3.0 and 4.0 were used, respectively. In another study of

Bhushan and Etzel (2009), the selectivity of the ultrafiltration increased by about 220% when positively charged membranes and liquid cheese whey solution were used.

Staged configurations were also studied to fractionate whey proteins using ultrafiltration (Cheang and Zydney 2004, Mayani et al. 2009, Mayani et al. 2010, Wang et al. 2010, Ghosh 2003). However, applications of charged membranes in staged configurations are limited. Rao et al. (2007) studied the fractionation of bovine serum albumin (BSA) from ovalbumin using negatively charged 100 kDa membrane in the first stage of a two-stage configuration. Initial feed solution included Cibacron Blue as an affinity ligand, which created negatively charged BSA-ligand complex. The first stage of the experiment separated ovalbumin from the BSA-ligand complex, and the second stage of the configuration, which included an uncharged 10 kDa membrane, separated the ligand from BSA. In the retentate of the first stage, 97% pure BSA solution was achieved with 80% of yield. In the second stage, 99.9% of Cibacron Blue was collected in the permeate, which created ligand-free BSA in the retentate.

The present work examines fractionation of GMP and BLG from a binary protein solution in a two-stage ultrafiltration configuration using positively charged 300 kDa and 30 kDa membranes in the first and the second stage, respectively. In the second part of the work, a single-stage ultrafiltration using a positively charged 300 kDa or 30 kDa membrane was used to fractionate GMP from Swiss cheese whey. Cross-flow filtration mode was used in the both parts of the work.

4.3. MODEL DEVELOPMENT

In this study, a single-stage ultrafiltration system was used (Figure 4.1). Two different mass balance models were used, one of which was obtained from the literature (Kurnik et al., 1995). Two mass balance boundaries are considered in this treatment (Figure 4.1). For the model system I, there is an accumulation in the retentate beaker and there is an outgoing permeate stream. A general mass balance for the solute "*i*" for this system will assume a form as follows:

$$\frac{dm_{sys,i}}{dt} = -\dot{m}_{out,i} \tag{4.1}$$

Where, $m_{sys,i}$, is the mass of the component *i* in the system, and $\dot{m}_{out,i}$ is the rate of mass loss from the system.

If the density is assumed constant, then we have:

$$\frac{dR}{dt} = -\dot{V}_P \tag{4.2}$$

Where, $\dot{V}_P$ is the volumetric flow rate of the permeate stream.

Let C_{Pi} and C_{Ri} be the instantaneous concentrations of the solute in permeate and retentate, respectively; *P* and *R* be the volumes of permeate and retentate, respectively; and *t* is time.

$\dot{V}_P$ can be expressed as:

$$\dot{V}_P = \frac{dP}{dt} \tag{4.3}$$

Equation 4.1 can be written in terms of concentrations as follows:

$$\frac{d(RC_{Ri})}{dt} = -C_{Pi}\dot{V}_P \tag{4.4}$$

Equation 4.4 can be rearranged using the following substitutions:

1. Observed sieving coefficient (S_{0i}), which was defined as: C_{Pi}/C_{Ri}.

2. Constant total fluid volume in the ultrafiltration system.

$$\frac{dP}{dt} = -\frac{dR}{dt} \tag{4.5}$$

Substitution of S_{0i} and Equation 4.5 into Equation 4.4 gives Equation 4.6.

$$\frac{d(RC_{Ri})}{dt} = S_{oi}C_{Ri}(\frac{dR}{dt}) \tag{4.6}$$

Rearrangement of Equation 4.6 gives:

$$(S_{oi} - 1)\frac{dR}{R} = \frac{dC_R}{C_{Ri}} \tag{4.7}$$

If it is assumed that there is no change in the value of S_{0i} in an ultrafiltration cycle, then the final solution of Kurnik et al. (1995) can be expressed as in Equation 4.8.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (S_{oi} - 1)\ln(1 - f) \tag{4.8}$$

where $f = P/F$ and F is the volume of the initial feed solution. For this model system, the sieving coefficient was measured under total recycle conditions ($f = 0$). With the known constant value of S_{0i} and by knowing C_{Fi}, the value of C_{Ri} can be obtained for any given f using equation 4.8.

When S_{0i} is not constant, the solution to Equation 4.7 is:

$$\int_F^R (S_{0i} - 1)\frac{dR}{R} = ln\frac{C_{Ri}}{C_{Fi}} \tag{4.9}$$

Substitution for R using the relation $R = F(1-f)$ yields the equation as follows:

$$ln\frac{C_{Ri}}{C_{Fi}} = \int_0^f \frac{(1-S_{0i})}{(1-f)}df \tag{4.10}$$

During ultrafiltration, S_{0i} declines nearly linearly as *f* increases. Equation 4.10 can be integrated using this dependence.

$$\ln\left(\frac{C_{Ri}}{C_{Fi}}\right) = (m + b - 1)\ln(1 - f) + mf, \tag{4.11}$$

where, *m* is the slope, and *b* is the intercept of the linear decline of S_{0i} versus *f*. In this case, the instantaneous permeate and retentate tubing concentrations were measured at different *f* values, and S_{0i} as a function of *f* ($S_{0i} = mf + b$) was obtained. We can then calculate C_{Ri} for any given *f* using equation 4.11.

To calculate the mixing cup concentration of the solute in permeate ($<C_{Pi}>$), an overall integrated mass balance (based on system II of Figure 4.1) was used:

$$\frac{dm_{sys,i}}{dt} = 0 \tag{4.12}$$

$$At\ t = 0, m_{sys,i} = C_{Fi}F \tag{4.13}$$

$$At\ t = t, m_{sys,i} = < C_{Ri} > R + < C_{Pi} > P \tag{4.14}$$

Using Equations 4.13 and 4.14, an overall mass balance can be expressed as follows:

$$C_{Fi}F = < C_{Ri} > R + < C_{Pi} > P. \tag{4.15}$$

Where $<C_{Ri}>$ is the mixing cup concentration of solute in the retentate. An assumption was made that $<C_{Ri}> \approx C_{Ri}$, and C_{Ri} was obtained using equation 4.8 and 4.10. This assumption is an approximation because C_{Ri} is the instantaneous concentration of solute in the retentate and is always slightly greater than the solute concentration in the beaker $<C_{Ri}>$ due to the concentration effect of withdrawing the permeate stream from

the membrane. The permeate flow rate of about 6 mL/min was withdrawn from a feed solution flow rate of about 36-56 mL/min to give a retentate flow rate of 30-50 mL/min. Therefore, the value of C_{Ri} was about 12 to 20% greater than $<C_{Ri}>$. This is because the solution in the beaker was at $<C_{Ri}>$ and the retentate was at C_{Ri}.

In practice, this concentration difference was somewhat less than calculated and the observed value of C_{Ri} was about 10% greater than the observed value of $<C_{Ri}>$.

4.4. MATERIALS AND METHODS

4.4.1. Preparation of Binary Protein Solutions

Glycomacropeptide (GMP) (BioPURE GMP, Davisco Foods International, Le Sueur, MN) and beta lactoglobulin (BLG) (Cat. No. 100363, 3x crystallized, MP Biomedicals, Irvine, CA) were used without further purification. Composition of GMP is given in Table 4.1. 0.5 g/L each of GMP and BLG in 25 mM sodium phosphate buffer, pH 3.0, conductivity 4 mS/cm, were prepared using 1M H_3PO_4, or 1M NaOH and granular NaCl. Protein solutions were filtered through 0.45 μm filters and then 0.22 μm filters (Durapore, Millipore Corp., Bedford, MA).

4.4.2. Preparation of Cheese Whey Solutions

Swiss cheese whey was obtained from the Dairy Plant at the University of Wisconsin-Madison. To prevent microbial growth during storage, 0.05% sodium azide was added to the whey. Cheese fines were removed by centrifugation at 4ºC and 3000g for 1 h. For each experiment, initially 50mL cheese whey was obtained from the stock solution. 0.3 g GMP powder was added into 50 mL cheese whey; and the solution was filtered through 0.7 μm filters (Durapore membrane filters, Millipore Corp.). The cheese whey had initial pH of 6.11 and conductivity of 8.36 mS/cm. pH was adjusted to 3.0 by addition of either 1M H_3PO_4 or 1M NaOH; and the conductivity was adjusted to 4 mS/cm by addition of approximately 120 mL deionized water to the 50 mL of whey. 120 mL of the final feed solution was used for the ultrafiltration experiments.

4.4.3. Preparation of Positively Charged Cross-Flow Ultrafiltration Membranes

Regenerated cellulose cross-flow ultrafiltration membranes of 50 cm^2 area (Pellicon XL, 300 and 30 kDa, Millipore Corp., Bedford, MA) were charge-modified using the procedure of van Reis (2006) and Bhushan & Etzel (2009) by circulating (3-bromopropyl) trimethylammonium bromide (Cat. No. 347604 Sigma Aldrich, St. Louis, MO) through the membrane for 21 hours at 22 ºC. The clean water flux decreased by 28% and 24% after charge modification of 30 kDa and 300 kDa membranes, respectively.

4.4.4. Ultrafiltration Experiments

For all experiments, membranes were incubated using the feed solution for 15 to 18 h at 22 ºC prior to taking samples for analysis. This step was to equilibrate the membranes with the feed solution before ultrafiltration. Experiments were conducted at 2 bar inlet pressure. The feed solution was pumped into the ultrafiltration unit (hold up volume is 3 mL) using a peristaltic pump. Retentate flow rate was adjusted to 30 - 50 mL/min (360 – 600 LMH) using a pinch clamp on the exit tubing. During ultrafiltration, retentate was recycled back to the feed solution container and permeate was collected in a graduated cylinder. Samples were taken at different "*f*" values from permeate and retentate tubings, and from permeate and feed solution beakers for measurement of concentration. Experiments were stopped at f = 0.8. Two separate replicates were performed for each experiment.

For the experiments performed using binary protein solutions, a two-stage configuration was used as shown in Figure 4.2; and for each stage, the permeate flow rate

was adjusted to 6 ml/min (72 LMH) using a peristaltic pump. Stages were performed separately in a batch mode. For the first stage, where 300 kDa membranes were used, 1000 mL initial feed solution (F2) was used, and 800 mL permeate (P1) and 200 mL retentate (R1) were collected. For the second stage, where 30 kDa membranes were used, P1 was used as the feed solution, and 640 mL permeate (P2) and 160 mL retentate (R2) were collected. R2 was stored to be mixed with 840 mL new protein solution (F1) to make up 1000 mL feed solution (F2) for the first stage of the second cycle.

For the experiments conducted using Swiss cheese whey solution, 120 mL feed solution was used instead of 1000 mL to avoid clogging of the membrane because of higher concentration of proteins and salts in the cheese whey solution compared to binary protein solution in buffer. Because of the low volume of the initial feed solution, samples were collected only when f = 0.0 (initial) and f = 0.8 (final) and only a one-stage experiment was performed. The first trials of the ultrafiltration experiments using cheese whey showed that the flow rate of the permeate decreased continuously in one cycle of the experiment. Because of this, no peristaltic pump was used on the permeate side.

4.4.5. Quantitative Analysis of GMP and BLG in Binary Protein Solutions

The method of Bhushan and Etzel (2009) was used to determine the concentrations of GMP and BLG in binary protein solutions. GMP does not contain any aromatic amino acids; therefore, it gives little or no absorbance at 280 nm. Bhushan and Etzel (2009) used this feature to distinguish BLG from GMP in binary protein solutions. Absorbance values of the permeate and retentate were determined at 214 and 280 nm; then the absorbance values were converted to concentrations using the Beer-Lambert law

for mixtures. Two measurements were made in the same sample and an average was reported. Measurements were nearly identical in each case.

4.4.6. Quantitative Analysis of GMP, BLG and ALA in Swiss Whey Solutions

The concentration of GMP was estimated using TCA precipitation adapting the method of Lieske and Konrad (1996). Samples (1 mL) were mixed with 5 mL of 7.2 % TCA solution containing 2.5% Na_2SO_4. Mixtures were held for 30 min at room temperature. Solutions were centrifuged at 6 ºC for 60 min at 3000 g. 3 mL supernatants were applied to desalting columns (Econo-Pac 10 DG, Bio-Rad). Columns were washed with 4 mL of distilled water to elute GMP. 100 µL samples obtained from the desalting columns were injected onto a size exclusion HPLC column (Bio-Sil SEC-125, Cat. No: 125-0060) to determine concentration of GMP. The mobile phase was 50 mM sodium phosphate (pH 3.0) at a flow rate of 0.7 mL/min. Samples were collected from the column when the peak was observed on the monitor (between 11^{th} and 13^{th} minutes of the exclusion). Finally, absorbance values of the samples were measured at 214 nm using a uv-vis spectrophotometer (Pharmacia Biotech, Ultrospec 1000, Cambridge-England). Absorbance values of the samples were converted to concentration values using a calibration curve. The calibration curve was obtained using the same TCA procedure for different known concentrations of GMP solutions, which were prepared with GMP powder (BioPURE GMP, Davisco Foods International, Le Sueur, MN).

The SDS-PAGE using a 4% stacking gel and 15% resolving gel procedure of Laemmli (Cleveland, 1977) was used for determination of BLG and ALA. All the materials for SDS-PAGE were obtained from Bio-Rad (Bio-Rad laboratories, CA).

Dithiothreitol was used to reduce the disulfide bridges. The amount of sample loaded was 20 µL per well. The working buffer was 1X Tris/glycine/SDS buffer and electrophoresis time was 55 min. Protein standards (ALA: Cat. No. L6010, Sigma-Aldrich, St. Louis, MO; BLG: Cat. No. 100363, 3x crystallized, MP Biomedicals, Irvine, CA) were used to identify proteins according to molecular mass. The gel was stained using 1X SYPRO red (Lonza, Rockland, ME) in 7.5% acetic acid solution. After staining, the gels were washed with 7.5% acetic acid for 5 minutes. Each gel contained five samples and three internal standards (a binary mixture of 0.1 g/L of ALA and 0.3 g/L of BLG, 0.2 g/L of ALA and 0.2 g/L of BLG, 0.3 g/L of ALA and 0.1 g/L of BLG). The gels were then scanned on a TYPHOON-9410 laser scanner (GE healthcare, NJ) in the fluorescence mode. The excitation wavelength was 532 nm and the emission filters were at 610 nm. The photomultiplier tube voltage was 590V. The bands were quantified on ImageQuantTL v.2007 (GE healthcare, NJ). Each lane gave peaks corresponding to the concentration; and internal standard calibration was used to determine unknown concentrations quantitatively. The calibration graph was linear for an OD range 0-0.7 (OD measured at 280 nm). Therefore, some samples were diluted prior to analysis.

4.4.7. Statistical Analysis

Concentration, purity and yield values were compared with the predictions from the two mass balance models using one sample T-test. Values are significantly different at $P < 0.05$.

4.5. RESULTS

Fractionation of GMP from BLG, and fractionation of GMP from Swiss cheese whey were studied. Results of binary protein solutions and cheese whey solutions are given in two different sections below.

4.5.1. Binary Protein Solutions

4.5.1.1. Sieving Coefficients and Selectivity

Sieving coefficients of GMP and BLG were determined in binary protein solutions using two positively charged regenerated cellulose membranes having 300 and 30 kDa molecular weight cut offs in a two-stage ultrafiltration configuration (Table 4.2). Preliminary results showed that the sieving coefficients were same for every cycle of the experiment. Because of this, only one cycle of ultrafiltration experiment was performed to determine the sieving coefficients at different "*f*" values. The first stage of the experiment was performed using a 300 kDa membrane. In the first stage, we obtained a sieving coefficient of 0.57 for GMP and a sieving coefficient of 0.16 for BLG at $f = 0$. While the *f* was increasing, the sieving coefficient of GMP decreased to 0.40 but the sieving coefficients of BLG were nearly constant. The second stage of the experiment was performed using a 30 kDa membrane; and the permeate of the first stage was used as the feed solution. In the second stage, sieving coefficients of GMP were smaller than the ones obtained from the first stage; sieving coefficients decreased from 0.353 to 0.244 while *f* increased from 0.0 to 0.8. The sieving coefficients of BLG dropped drastically to very small values in the second stage compared to those in the first stage, and further decreased as *f* increased. Sieving coefficients of BLG in the second stage were considered

as untrustworthy because absorbance values of BLG at 280 nm in the permeate solutions were below limit of detection (A_{280} < 0.03). This is because of the fact that BLG is strongly excluded from the membrane due to electrostatic repulsion, thereby giving a permeate which is very dilute in BLG. In the literature, similar observations were made by different researchers (Cheang and Zydney, 2003, Lu et al. 2005, Wan et al. 2006). Cheang and Zydney (2003) reported the sieving coefficients of BLG as small as 0.005, and sieving coefficients of ovalbumin were reported as small as 0.006 and 0.0008 by Lu et al. (2005) and Wan et al. (2006), respectively.

Selectivities of the membranes were calculated using the sieving coefficients of GMP and BLG (Table 4.2). The selectivity values of 300 kDa membrane varied between 3.0 and 3.5; however, selectivity values of 30 kDa membrane increased from f = 0.0 to f = 0.6 and varied between 9.8 and 29, and reached a selectivity of 170 at f = 0.8. The selectivity of 170 at f = 0.8 was considered as an untrustworthy value because of its large coefficient of variation (35%), and because of the fact that the permeate stream was extremely dilute in BLG.

4.5.1.2. Concentrations, Purity and Distribution

Two cycles of ultrafiltration experiment were performed to determine concentrations, purities and distributions of GMP and BLG. The reason why only two cycles were performed was to see the effect of recycling of the retentate stream (R2) of the second stage back to the feed solution on purity and distribution. No more cycles were necessary for this purpose.

Table 4.3 shows the concentrations of GMP and BLG in permeate and retentate streams of the first and second stages performed in the second cycle; and it also shows their corresponding calculated concentration values obtained using Kurnik's model and the proposed model. Values of sieving coefficients for GMP and BLG were taken from Table 4.2 for calculating the expected mixing-cup concentrations. While only the sieving coefficients at f = 0.0 were used for Kurnik's model, sieving coefficients at every "f" value were used for the proposed model. Both experimental and calculated concentrations are mixing-cup concentrations as collected in the beaker at the end of the experiment when f = 0.8. When the stage level was changed from the first to second, concentration of GMP decreased in the permeate stream (P2 < P1) and increased in the retentate stream (R2 > R1); concentration of BLG reached almost a value of zero in the permeate stream (P2) and decreased approximately 50% in the retentate stream (R2) (Table 4.3). Concentration of BLG in the permeate stream of the second stage (P2) was considered untrustworthy because the absorbance value of BLG at 280 nm was below limit of detection (A_{280} < 0.03). This is because the permeate solution from the second stage was very dilute in BLG.

Calculated mixing-cup concentrations were statistically compared to the experimental results using one-sample T-test (P = 0.05); and the significantly different results were signed with stars in Table 4.3. As seen in the table, our model had two significantly different data points which were the concentrations of GMP and BLG in the retentate stream of the second stage (R2); whereas, Kurnik's model had one significantly different data point for BLG in R2 (P < 0.05). All the differences between experiment

and model occurred on stream R2, which was recycled back to the feed stream. There were no differences between model and experiment for the product streams (P2, R1).

Purity and distribution of mass into each stream was calculated using the results from Table 4.3 and are presented in Table 4.4. Purity of GMP increased from 50% in the feed stream to 99.7% in stream P2 for cycle 2. Distribution into P2 was 37% and is the fraction of the inlet feed stream mass of a given protein that appears in a specific outlet stream. Recycling of the retentate stream (R2) back to the feed stream in cycle 2 did not affect the purity of GMP but increased its distribution in stream P2 compared to cycle 1. For BLG, purity increased from 50% in the feed stream to 62% in stream R1, and was not affected by recycle of stream R2. Distribution of BLG into R1 was affected by recycle of R2, and increased from 64% in cycle 1 to 91% for cycle 2.

Calculated concentrations of GMP and BLG using the mass balance models were used to calculate the purity and distribution. Calculated results were compared with the experimental results using one sample T-test. Significantly different values were signed with stars. As seen in Table 4.4, while our proposed model had 5 significantly different values out of 16, Kurnik's model had 6 significantly different values out of 16 ($P < 0.05$). All the differences were in stream R2 which is not a product stream. There were no differences between model calculations and experimental observations for the product streams P2 and R2 ($P > 0.05$).

4.5.2. Cheese Whey Solutions

4.5.2.1. Sieving Coefficients

Positively charged regenerated cellulose membranes with 30 and 300 kDa MWCOs were investigated for the fractionation of GMP from Swiss cheese whey. Sieving coefficients of GMP, BLG and ALA were determined at f = 0.0 and 0.8 in one-stage experiments (Table 4.5). Using 30 kDa membranes, we determined the sieving coefficient of GMP as 0.09 at f = 0.0 and 0.07 at f = 0.8. Sieving coefficients of BLG and ALA were not available in this condition because the concentrations of BLG and ALA in the permeate stream were below limit of detection. When the fractionation experiments were conducted using 300 kDa membranes, sieving coefficients of GMP increased to 0.43 at f = 0 and 0.26 at f =0.8. Sieving coefficients of BLG and ALA were several-fold lower as reported in Table 4.5. Sieving coefficients always decreased while f increased from 0.0 to 0.8 using either the 30 kDa or 300 kDa membrane.

4.5.2.2. Concentrations, Purity, and Distribution

Concentrations of GMP, BLG and ALA were determined in the collection beaker of permeate and retentate solutions when the experiments were completed (f = 0.8) (Table 4.5). As seen in the table, when 30 kDa membranes were used, we obtained 0.34 g/L GMP and a non-detectable amount of BLG and ALA in the permeate solution. When the 300 kDa membranes were used, the concentration of GMP in the permeate increased 3.3 times to 1.12 g/L; and it contained 0.21 g/L BLG and 0.07 g/L ALA. For the retentate stream, we obtained lower amounts of GMP, BLG and ALA when 300 kDa membranes were used compared to 30 kDa membranes.

Sieving coefficients of GMP, BLG and ALA were used to calculate concentrations using Kurnik's mass balance model (Table 4.5). Statistical analysis of the results showed that there was only one significantly different value out of twelve in the calculated results ($P < 0.05$). Our proposed model does not assume that the sieving coefficient is constant. Because of this, it requires the sieving coefficients at different *f* values. Since we didn't have the sieving coefficients of the proteins at different *f* values, we couldn't use our proposed model for cheese whey experiments.

Experimental and calculated concentrations from Table 4.5 were used to calculate purities and distributions of GMP, BLG and ALA in the collection beakers of the permeate and retentate solutions for a single-stage ultrafiltration using either a 30 kDa or 300 kDa membrane (Table 4.6). Using 30 kDa membranes resulted in 100% pure GMP with 16 % distribution and no BLG and ALA in the permeate. 78% of GMP and all the ALA and BLG were collected in the retentate solution when 30 kDa membranes were used; and the purities were 39%, 43% and 18% for GMP, BLG and ALA, respectively. Calculated concentrations were not significantly different than experimental results ($P > 0.05$) except for the purity and distribution of ALA in the retentate stream ($P < 0.05$) when 30 kDa membranes were used. When 300 kDa membranes were used, 80%, 15% and 5% pure GMP, BLG and ALA were obtained in the permeate stream with 44%, 17% and 12% distributions, respectively; and 39%, 39% and 13% pure GMP, BLG and ALA were collected in the retentate stream with 50%, 84% and 60% distributions, respectively. When 300 kDa membranes were used, there were no statistically significant differences between calculated mixing-cup concentrations and experimentally observed mixing-cup concentrations ($P > 0.05$).

4.6. DISCUSSION

4.6.1. Binary Protein Solutions

We obtained highly pure GMP fractions using positively charged 300 and 30 kDa regenerated cellulose membranes in a two-stage configuration (Table 4.4). Using 300 kDa membrane in the first stage, 64% of the BLG remained in the retentate solution; however, 66% of the GMP passed through the membrane in cycle 1 (Table 4.4). This increased the purity of GMP in stream P1 to 70% compared to 50% in the feed stream. Similarly, for the retentate of the first stage (R1), the purity of BLG increased to 61% compared to 50% for the feed stream. To reach a chromatographic purity of GMP, a 30 kDa membrane was used in the second stage so that BLG could be rejected by the membrane as much as possible. This increased the purity of GMP to 97% in stream P2, and caused 40% of the BLG to be rejected into stream R2. Even though we obtained high purity of GMP with the completion of one cycle of the two-stage ultrafiltration, it was possible to further increase the purity of GMP with increase in the distribution with recycling of the retentate stream (R2) back to the feed solution. GMP purity was 99.7% in stream P2 after cycle 2 and distribution of BLG into stream R1 was 91%. The increase in the distribution of GMP in the permeate stream from cycle 1 to cycle 2 was due to the recovery of the GMP rejected by the 30 kDa membrane in the second stage of cycle 1. As expected, the distribution of GMP in the permeate stream of the first stage (P1) was higher than the second stage (P2) because of the less amount of GMP in the feed stream of the second stage, which was the permeate of the first stage (P1).

Concentrations, purities and distributions of GMP and BLG were calculated using the two mass balance models. Except the retentate (R2) of the second stage, all the

calculations from the models were statistically in agreement with the experimental results. R2 was the stream which was recycled back to the feed stream; therefore, the inaccurate predictions for R2 would not affect the overall design of an ultrafiltration system. The reason why only the calculations for R2 were statistically different than the experimental results might be due to the protein accumulation on the membrane surface for the second time. Neither of the two models takes the protein accumulation into account.

4.6.2. Cheese Whey Solutions

One-stage ultrafiltration experiments were conducted using positively charged 300 and 30 kDa regenerated cellulose membranes and Swiss cheese whey. The experiments conducted using cheese whey resulted in lower sieving coefficients compared to those obtained from the experiments conducted using binary protein solutions. For example, sieving coefficients of GMP at $f = 0$ for a 30 kDa membrane were 0.4 and 0.09 when binary solution and cheese whey solution were used, respectively. This might be the result of the creation of a thicker protein layer on the membrane surface due to the higher protein concentration of cheese whey compared to binary protein solutions in buffer. Without using a diafiltration step, we could fractionate GMP from cheese whey and reach chromatographic purity with one stage of ultrafiltration: we obtained 100% and 80% pure GMP fractions using 30 kDa and 300 kDa membranes, respectively. The reason for obtaining high purities from ultrafiltration membranes with such high molecular weight cut offs was the positive charge on the membranes.

Positively charged BLG and ALA were rejected by the membranes; however, neutral or slightly negatively charged GMP could pass through the membranes with less rejection.

Only Kurnik's model was used to calculate concentrations, purities and distributions. Our proposed mass balance model was not used in this case because the model requires using different sieving coefficients at different *f* values. The calculations from Kurnik's model were not statistically different than the experimental results with one exception which occurred for the concentration of ALA in the retentate stream when the 30 kDa membrane was used. When the 30 kDa membrane was used, the amount of ALA in the permeate stream was undetectable; therefore, the sieving coefficient of ALA was assumed to be 0. There was 0.26 g/L ALA (it was reported as 0.3 in Table 4.5 due to the one significant digit after the decimal point in the standard deviation) in the initial feed stream, and the volume of the feed solution decreased to 24 mL from 120 mL (5 times concentrated) at the end of the ultrafiltration experiment. When the calculations were performed for ALA, the calculated final concentration was found to be 1.3 g/L (5×0.26 g/L). However, the experimental result was 3.1 g/L. The unexpected high amount of ALA in the final retentate stream might be due to an experimental error. The same error also caused significant differences in Table 4.6 for the purity and distribution of ALA in the retentate stream when 30 kDa membrane was used.

Overall Kurnik's model worked very well for the calculation of concentrations, purities and distributions of the proteins for a one-stage ultrafiltration experiment at a given *f* using real cheese whey. The mass balance model can be used to design different ultrafiltration configurations. For example, when calculations were made using Kurnik's model for the two-stage configuration (Figure 4.2) using the 300 kDa membranes in both

the stages, 92% pure GMP with 31% distribution in the final permeate stream (P2) was obtained. The sieving coefficients and the initial concentrations of GMP, ALA and BLG were obtained from the one-stage ultrafiltration experiment using the 300 kDa membrane and the Swiss cheese whey (Table 4.5).

4.7. CONCLUSIONS

Fractionation of GMP from BLG in binary protein solutions and Swiss cheese whey were investigated using positively charged ultrafiltration membranes in staged configurations. Using positively charged regenerated cellulose membranes, we were able to fractionate GMP from binary protein solution and Swiss cheese whey at nearly 100% purity. Using 300 kDa membranes for fractionation of GMP resulted in 70% purity and 66% distribution of GMP into the permeate from binary protein mixtures, and 80% purity and 44% distribution using Swiss cheese whey when one cycle of one-stage ultrafiltration was completed. Considering the very small molecular weights of the proteins (GMP: 8.6 kDa, BLG: 18 kDa) and the large MWCO of the membrane, this result was surprising. Using 30 kDa membranes, on the other hand, resulted in almost 100% pure GMP solutions with no diafiltration when two cycles of two-stage ultrafiltration and one cycle of one-stage ultrafiltration were completed with binary protein solutions and Swiss cheese whey, respectively. Obtained results showed that the applicability of ultrafiltration processes in production of highly pure whey proteins instead of more expensive chromatographic methods. The two mass balance models were used to calculate the expected mixing-cup concentrations at a given f. The calculated concentrations were in agreed with the measured mixing-cup concentrations.

4.8. REFERENCES

Bhushan, S. and Etzel, M.R. 2007. Charged ultrafiltration membranes for protein separation. American *Society of Agricultural and Biological Engineers Meeting Presentation*. Paper No: 077063

Bhushan, S. and Etzel M.R. 2009. Charged ultrafiltration membranes increase the selectivity of whey protein separations. *Journal of Food Science*. Vol. 74 (3), pg: E131 – E139.

Cheang, C and Zydney, A.L. 2004. A two-stage ultrafiltration process for fractionation of whey protein isolate. *Journal of Membrane Science*. Vol. 231, pg: 159-167.

Doultani, S., Turhan, K.N., and Etzel, M.R. 2004. Fractionation of proteins from whey using cation exchange chromatography. *Process Bichemistry*. Vol. 39, pg: 1737 – 1743.

Etzel, M.R. 2004. Manufacture and use of dairy protein fractions. *The Journal of Nutrition*. Vol. 134 (4), pg: 996S – 1002S.

Ghosh, R. 2003. Novel cascade ultrafiltration configuration for continuous, high-resolution protein-protein fractionation: a simulation study. *Journal of Membrane Science*. Vol. 226, pg: 85-99.

Mayani, M., Mohanty, K., Filipe, C., and Ghosh, R. 2009. Continuous fractionation of plasma proteins HSA and HIgG using cascade ultrafiltration systems. *Separation and Purification Technology*. Vol. 20, pg: 231-241.

Mayani, M., Filipe, C.D.M., and Ghosh, R. 2010. Cascade ultrafiltration systems – Integrated processes for purification and concentration of lysozyme. *Journal of Membrane Science*. Vol. 347, pg: 150-158.

Rao, S., Ager, K., and Zydney, A.L. 2007. High performance tangential flow filtration using charged affinity ligands. *Separation Science and Technology*. Vol. 42, pg: 2365 – 2385.

Wan, Y., Ghosh, R., and Cui, Z. 2005. Fractionation of proteins using ultrafiltration: developments and challenges. Developments in Chemical Engineering; Mineral Processes. Vol. 13(1/2), pg: 121-136.

Wang, L., Khan, T., Mohanty, K., and Ghosh, R. 2010. Cascade ultrafiltration bioreactor-separator system for continuous production of $F(ab`)_2$ fragment from immunoglobulin G. *Journal of Membrane Science*. Vol. 351, pg: 96-103.

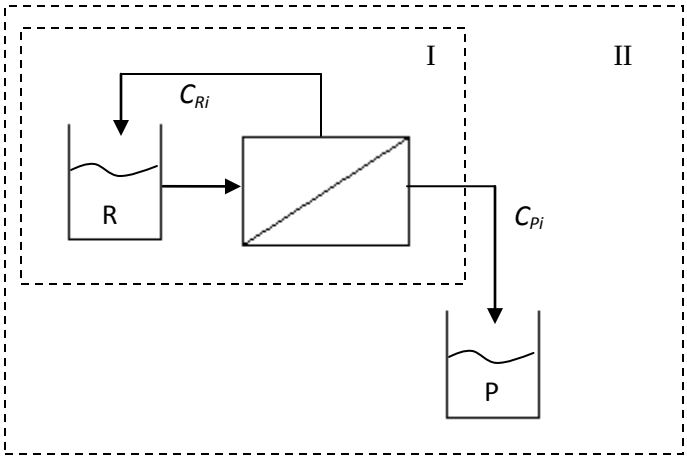

Figure 4.1. Schema of the model system (F: feed, R: retentate, P: permeate)

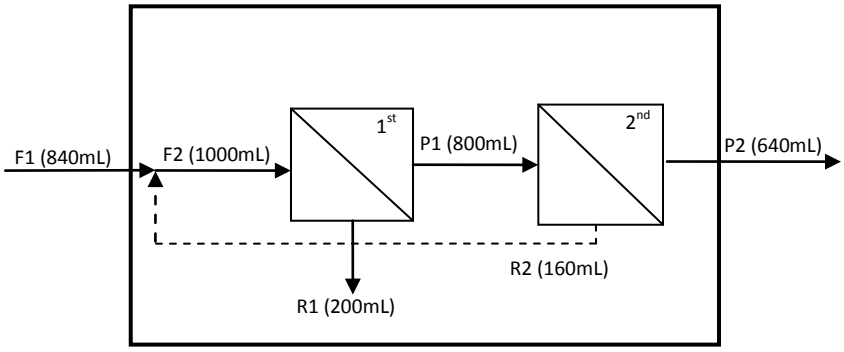

Figure 4.2. Schema of two-stage ultrafiltration configuration

F1: Fresh feed solution, F2: Feed solution used in the first stage, P1: Permeate of the first stage, P2: Permeate of the second stage, R1: Retentate of the first stage, R2: Retentate of the second stage.

Table 4.1. Composition of Glycomacropeptide (GMP) (BioPURE GMP, Davisco Foods International, Le Sueur, MN)

Analysis	**Specification**	**Typical Range**	**Test Method**
Moisture (%)	7.0 max.	6.0 ± 0.9	Vacuum Oven
Glycomacropeptide (N × 7.07) (% Dry Basis)	90.0 min.	91 ± 1	Leco Combustion
Protein (N × 6.47) (% Dry Basis)	82.5 min.	84 ± 1	Leco Combustion
Fat (%)	1.0 max.	0.5 ± 0.3	Mojonnier
Ash (%)	7.0 max.	6.3 ± 0.5	Residue on Ignition
Lactose (%)	1.0 max.	< 0.5	Enzymatic Assay
pH	7.0 max.	6.4 ± 0.2	10% Sol. At 20°C
Scorched Particles (mg/25g)	15.0 max.	7.5	ADPI

Table 4.2. Sieving coefficients of GMP and BLG obtained from the experiments performed using binary protein solutions.

	300kDa (Stage 1)			30kDa (Stage 2)		
f	S_0(GMP)[a]	S_0(BLG)[a]	Ψ	S_0(GMP)[a]	S_0(BLG)[a]	Ψ
0.0	0.57±0.02[b]	0.16±0.01	3.5±0.2	0.353±0.001	0.036±0.003[c]	9.8±0.8
0.1	0.56±0.01	0.17±0.03	3.3±0.5	0.4±0.1	0.04±0.01[c]	10±7
0.3	0.53±0.01	0.17±0.03	3.1±0.5	0.30±0.01	0.021±0.009[c]	16±8
0.6	0.48±0.01	0.16±0.02	3.1±0.4	0.283±0.001	0.0096±0.001[c]	29±4
0.8	0.40±0.04	0.14±0.02	3.0±0.8	0.244±0.001	0.0015±0.0005[c]	170±60[c]

Each data point is the average of two separate replicates

[a] Sieving coefficients were determined using instantaneous concentrations in tubings in cycle 1

[b] Average ± standard deviation

[c] Untrustworthy data point

Table 4.3. Concentrations of GMP and BLG in the collection beakers when f = 0.8 obtained from the second cycle of the two-stage ultrafiltration performed using binary protein solutions.

Concentrations in the second cycle						
	Experimental [a]		Proposed Model		Kurnik et al. 1995	
Stream	GMP	BLG	GMP	BLG	GMP	BLG
P1	0.48±0.03[b]	0.23±0.03	0.4	0.14	0.46	0.15
P2	0.24±0.02	0.0006±0.0005[c]	0.2	0.003	0.2	0.005
R1	1.2±0.1	1.91±0.09	1.4	2.1	1.2	2.1
R2	1.67±0.04	1.05±0.01	1.3 *	0.71 *	1.5	0.73 *

[a] Each data point is the average of two separate replicates

* Value is significantly different from the experimental result ($P < 0.05$)

[b] Average ± standard deviation

[c] Untrustworthy data point

Table 4.4. Purities and distributions of GMP and BLG in the collection beakers when f = 0.8 obtained from the experiments performed using binary protein solutions

	Experimental [b]							
	GMP				BLG			
	Purity (%)		Distribution (%)		Purity (%)		Distribution (%)	
Stream	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2	Cycle 1	Cycle 2
P1	70±3[a]	67±4	66±2	91±5	30±3	33±4	29±4	44±5
P2	97±1	99.7±0.2	24±2	37±3	3±1	0.3±0.2	0.8±0.2	0.09±0.08
R1	40±2	38.0±0.8	42±4	56±5	61±2	62.0±0.8	64±3	91±4
R2	58±3	61.4±0.2	41.90±0.07	64±1	42±3	39±0.2	30±3	40.0±0.5
	Proposed Model							
P1	71	74	54	76	29	26	22	27
P2	98	98	19	27	2	2	0.4	0.5
R1	37	40	46	65	63	60	78	98
R2	62	64 *	34 *	49 *	39	36 *	21	27 *
	Kurnik et al. 1995							
P1	73	75	61	88	27	25	22	29
P2	97	98 *	21	30	3.0	2.5	0.6	0.8
R1	34	38	50	59	66	63	77	98
R2	64	68 *	40 *	58 *	36	32 *	22	28 *

[b] Each data point is the average of two separate replicates

* Value is significantly different from the experimental result ($P < 0.05$)

[a] Average ± standard deviation

Table 4.5. Sieving coefficients and concentration values obtained from the experiments performed using Swiss whey solution

	30 kDa			300 kDa		
	GMP	BLG	ALA	GMP	BLG	ALA
Feed Stream (g/L)	1.72 ± 0.07[c]	1.3 ± 0.5	0.3 ± 0.1	2.1 ± 0.2	1.03 ± 0.09	0.49 ± 0.06
Sieving Coefficients[1]						
S_0 at $f = 0$	0.09 ± 0.01	N/A[b]	N/A	0.43 ± 0.03	0.11 ± 0.05	0.13 ± 0.03
S_0 at $f = 0.8$	0.07 ± 0.02	N/A	N/A	0.26 ± 0.02	0.05 ± 0.02	0.07 ± 0.01
Concentrations obtained from experiments[2]						
Final Permeate (g/L)	0.34 ± 0.06	N/A	N/A	1.12 ± 0.04	0.210 ± 0.002	0.07 ± 0.03
Final Retentate (g/L)	6.7 ± 0.7	7.5 ± 0.5	3.1 ± 0.1	5.3 ± 0.5	4.3 ± 0.4	1.4 ± 0.3
Concentrations obtained from Kurnik's model [2]						
Final Permeate (g/L)	0.29	0	0	1.28	0.2	0.12
Final Retentate (g/L)	7.4	6.5	1.3 *	5.1	4.4	2

Each data point is the average of two separate replicates

[1] Sieving coefficients were determined using instantaneous concentrations in tubings

[2] Concentrations were determined in the collection beaker when $f = 0.8$

* Value is significantly different from the experimental result ($P < 0.05$)

[b] N/A: below limit of detection

[c] Average ± standard deviation

Table 4.6. Purity and distribution of GMP, BLG and ALA in the final collection beakers of permeate and retentate of the ultrafiltration performed using Swiss whey solution

	GMP		BLG		ALA	
	Purity (%)	Distribution (%)	Purity (%)	Distribution (%)	Purity (%)	Distribution (%)
30 kDa	Experimental					
Permeate	100±0[a]	16±2	0	0	0	0
Retentate	38.5±0.9	78±8	43.4±0.2	117±8	18.1±0.8	242±8
	Kurnik's model					
Permeate	100	14	0	0	0	0
Retentate	49	86	43	100	9*	100*
300 kDa	Experimental					
Permeate	80±1	44±2	15.2±0.8	16.6±0.2	5±2	12±4
Retentate	47.9±0.3	50±5	39±1	84±7	13±1	60±10
	Kurnik's model					
Permeate	80	50	13	16	8	20
Retentate	44	49	38	86	17	82

Each data point is the average of two separate replicates

* Value is significantly different from the experimental result ($P < 0.05$)

[a] Average ± standard deviation

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. CONCLUSIONS

In this study, positively-charged regenerated cellulose ultrafiltration membranes in a cross-flow mode were used in fractionation of glycomacropeptide (GMP) from cheese whey proteins. Additionally mass balance models were used for calculation of the experimental results. One model was obtained from the literature. Another model was developed for this study.

The first part of the study was conducted to show the effect of positively charged membranes on fractionation of GMP and BLG from binary protein mixture in buffer. Sieving coefficients of GMP and BLG, selectivity of the membranes, and concentration of the proteins were determined using uncharged and charged membranes. Experimentally determined sieving coefficients of GMP and BLG were used in two mass balance models to calculate the final concentrations; and experimental and calculated concentrations were compared to each other. Adding a positive charge on the membranes increased the selectivity of the fractionation up to 45 times. Selectivity of the uncharged membranes was around one, meaning no separation occured. It has been shown that the sieving coefficients were not constant during an ultrafiltration cycle. Calculations from both the proposed and the existing mass balance models matched the experimental results well.

In the second part, three-stage ultrafiltration configuration with recycling of selected streams was investigated using uncharged and positively charged ultrafiltration membranes for fractionation of GMP from BLG. Staged operations using uncharged membranes did not fractionate GMP from BLG. On the other hand, having positive charge on the membrane surface cleanly fractionated the proteins. Recycling of the selected streams to the feed solution did not have a significant effect on the purity of the product, but it increased the distribution of the proteins into the final product streams significantly. Overall, this study showed that the fractionation of GMP from BLG is possible with proposed staged configuration in a batch mode; moreover, it showed that recycling increased the distribution of the final product in the final target stream significantly.

In the last part of the research, two-stage ultrafiltration with 300 kDa and 30 kDa membranes, and one-stage ultrafiltration with 300 kDa or 30 kDa membranes, were used for fractionation of GMP from BLG and from Swiss cheese whey. Experiments were conducted only using charged membranes. From the two-stage configuration, highly pure GMP was obtained with moderate recovery and moderately pure BLG and high recovery. From one-stage experiments using Swiss cheese whey, 80% and 100% pure GMP solutions from 300 kDa and 30 kDa membranes were produced, respectively.

The overall conclusion of this research is that using positively charged regenerated cellulose membranes in staged configurations allows fractionation of GMP from BLG and from Swiss cheese whey with high purity. Obtained results showed that the applicability of ultrafiltration processes in production of functional proteins instead of

more expensive chromatographic methods. The advantageous of using ultrafiltration instead of a chromatographic method can be listed as the high throughput of the operation, being environmentally friendly due to the absence of using buffer solutions, and ready availability of the process in dairy and pharmaceutical industry.

5.2. RECOMMENDATIONS

Using ultrafiltration membranes with molecular weight cut-offs higher than 10 kDa result in having lactose and minerals in the permeate stream. When the experiments were conducted with liquid cheese whey, the end product contained a high amount of lactose and minerals because the MWCOs of the membranes were 300 kDa and 30 kDa. Concentration and purification of GMP in the end product should be investigated using uncharged and charged membranes with different MWCOs.

Ultrafiltration temperature in the dairy industry is kept around 50°C to limit the microbial growth. Our results were obtained at room temperature (22°C). To be more applicable to the dairy industry, higher temperatures should be investigated for fractionation of GMP from cheese whey.

We showed that it was possible to reach 80% purity with 300 kDa membrane in one stage. Larger pore size ultrafiltration (i.e. 500 kDa) membranes should be tried for fractionation of GMP from cheese whey. Using larger pore size membranes would lead to lower purity; but the purity could be increased using staged configurations. Larger pore size membranes in staged configurations might result in the same purity with higher recovery.

APPENDIX A

This work presents simulations of five different two-stage ultrafiltration configurations for the fractionation of GMP from other two major whey proteins BLG and ALA. Both the stages in the two-stage configurations were considered as positively charged 300 kDa regenerated cellulose membranes. The feed solution was considered as crude sweet cheese whey at pH 3.0 and conductivity 4 mS/cm.

A.1. CONDITIONS, ASSUMPTIONS AND CONFIGURATIONS

A.1.1. Conditions

1. Sieving coefficients of GMP, BLG and ALA were obtained from the experiments performed using positively charged 300 kDa regenerated cellulose membranes and swiss cheese whey at pH 3.0 and conductivity 4 mS/cm (Table 4.4).
2. Initial feed solution composition was obtained from the literature (Table 1.2).
3. Calculations were performed for f = 0.8 and for four cycles in five different two-stage configurations including positively charged 300 kDa regenerated cellulose membranes in both the stages.
4. Calculations were performed using Kurnik's mass balance model, which can be found in Chapter 1.

A.1.2. Assumptions

1. Sieving coefficients for a given protein on a given membrane are constant.

2. Only the three major whey proteins (GMP, BLG, ALA) were taken into account.

A.1.3. Configurations

Five different two-stage ultrafiltration configurations were used for the simulations:

Configuration 1: Retentate of the first stage was recycled back to the feed stream and retentate of the second stage was recycled back to permeate of the first stage. Permeate of the second stage was collected. (Figure A.1 – A)

Configuration 2: Only retentate of the second stage was recycled back to the feed stream. Retentate of the first stage and permeate of the second stage were collected. (Figure A.1 – B)

Configuration 3: Only retentate of the first stage was recycled back to the feed stream. Permeate and retentate of the second stage were collected. (Figure A.1 – C)

Configuration 4: Both retentate of the first stage and retentate of the second stage were recycled back to the feed stream. Permeate of the second stage was collected. (Figure A.1 – D)

Configuration 5: Only retentate of the second stage was recycled back to permeate of the first stage. Retentate of the first stage and permeate of the second stage were collected. (Figure A.1 – E)

A.2. Results

Simulations for fractionation of GMP from whey proteins (BLG and ALA) were performed using five different two-stage ultrafiltration configurations which included positively charged 300 kDa regenerated membranes in both the stages. The calculations were continued until 4 cycles of ultrafiltration were completed. Since the second permeate is the stream which had the highest purity of GMP and GMP was the target protein, only the results from the second permeate were analyzed and presented.

Figure A.2 shows the purities of GMP, BLG and ALA in the second permeate of different configurations from cycle 1 to 4. In all configurations except for the second one, purity of GMP decreased but the purities of BLG and ALA increased with an increasing cycle number. In the second configuration, however, purities were almost constant. Purity of GMP in the second configuration was around 75%.

Figure A.3 shows the recovery of GMP in the second stage of the configurations in all the cycles. As seen in the figure, there is always an increase in the recovery of GMP with an increasing cycle number. In the second configuration, however, the increase stopped after the second cycle and reached a constant value. The highest and the lowest recoveries were obtained in the first and the second configurations, respectively. The second highest recovery of GMP was obtained from the fourth configuration. The recoveries in the configurations three and five were very similar to each other; and they resulted as the third highest recoveries.

Concentrations of GMP in the second permeate of the five configurations with an increasing cycle number were given in Figure A.4. Figure A.4 showed the similar profile

with Figure A.3. The highest and the lowest concentrations were obtained from the first and the second configurations, respectively. The second highest concentration of GMP was obtained from the fourth configuration; and both the third and the fifth configurations resulted as the third highest concentrations of GMP.

A.3. Discussions

In the second configuration, the highest purity was obtained with no decrease in the purity with increasing cycle number. As seen in Figure A.1-B, in the second configuration, only the retentate of the second stage was recycled back to the feed stream. In the first stage, most of the BLG and ALA were rejected by the membrane and the retentate was discarded. When the retentate of the second stage was recycled back to the feed stream, the concentration of GMP increased but the concentrations of BLG and ALA slightly decreased. BLG and ALA in the feed solution was then rejected further twice in the following two stages. As a result of this, there was a slight increase in the purity of GMP in the permeate of the second stage of the second configuration. For all the other configurations, even though concentrations of GMP increased, purity decreased with an increasing cycle number. In configurations 1, 3 and 4, the retentate of the first stage was always recycled back to the feed stream. This caused an increase in the concentrations of BLG and ALA more than the increase in the concentration of GMP; and as a result, purities of GMP in the second permeate streams decreased. In configuration 5, however, the retentate of the first stage was not recycled back to the feed stream, but the permeate of the second stage was recycled back to the permeate of the first stage rather than the feed solution. This caused another cycle of rejection of the BLG and ALA left in the retentate of the second stage from the previous cycle. However, a single rejection of BLG and ALA did not siffice to increase the purity of GMP in the second permeate of the next cycle.

Calculations for the second configuration show that the purity of GMP is the highest compared to other configurations, but recovery is the lowest. This could be attributed to the following facts; first, considerable amount of GMP is lost in the retentate of the first stage; and second, recirculation of GMP to the feed stream resulted in two serial rejections of GMP left in the retentate of the second stage from the previous cycle. As seen in the Figure A.4, concentration of GMP in the permeate of the second stage for configuration 2 is the lowest. On the other hand, around 85% recovery was obtained from the first configuration. This configuration increased the concentration of GMP in the second permeate more than other configurations (Figure A.4).

A.4. Conclusions

Five different two-stage ultrafiltration configurations were analyzed using a mass balance model. The simulation was performed for the ultrafiltration of cheese whey using the 300 kDa positively charged regenerated cellulose membrane.

Even though the membranes had pore size ratings which were at least 20 times more than the proteins, fractionation of GMP from other whey proteins was possible using two-stage configurations. Configuration 1 gave the highest recovery (85%) but the lowest purity (55%), configuration 2 gave the highest purity (75%) but the lowest recovery (35%). In our opinion, the most optimum configuration, which gave considerably high purity and recovery, was the fourth configuration. In the fourth configuration, we observed 65% purity and 70% recovery at the fourth cycle of the two-stage operation.

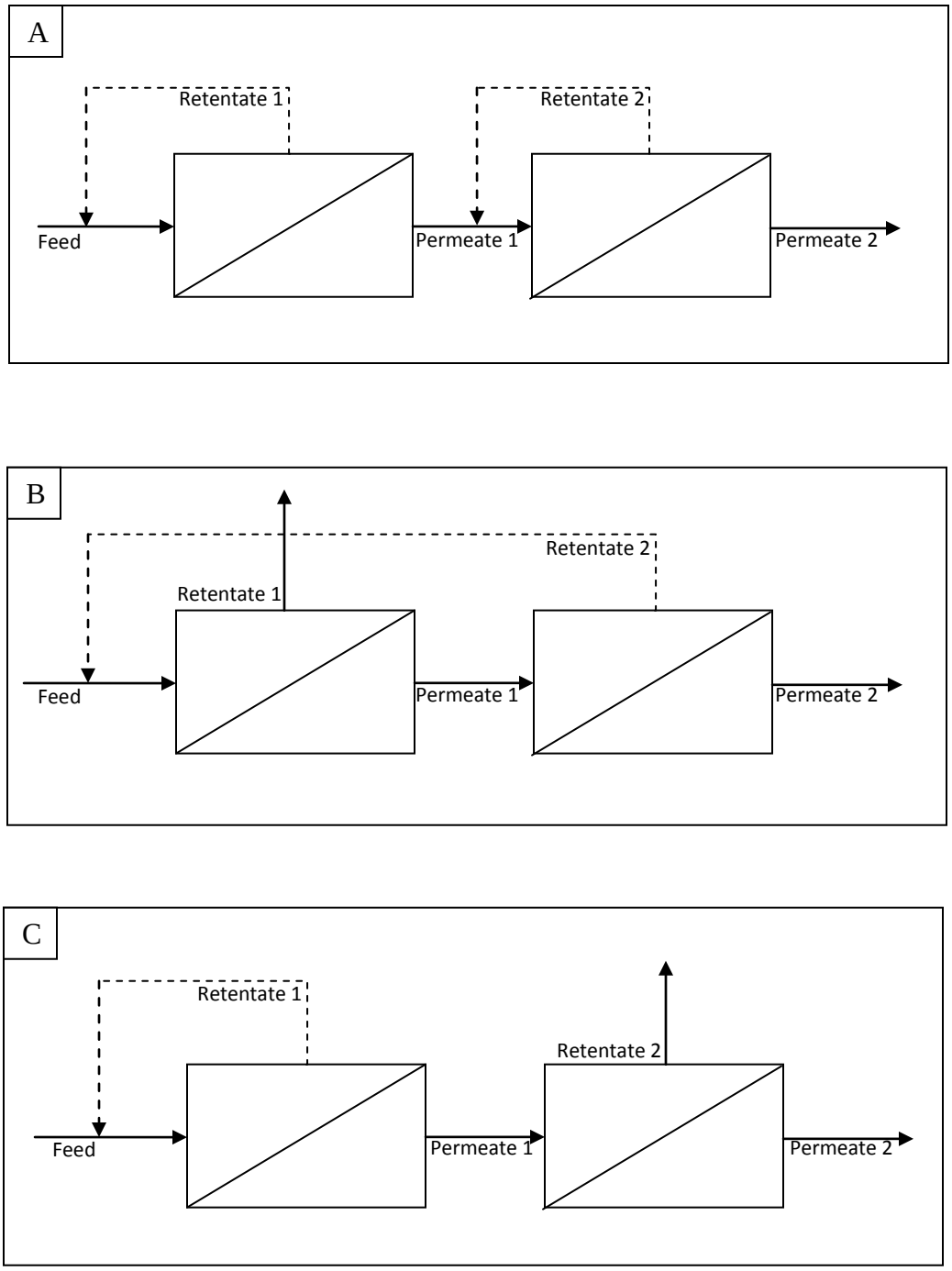

Figure A.1. Two-stage configurations: (A: 1st, B: 2nd, C: 3rd, D: 4th, E: 5th)

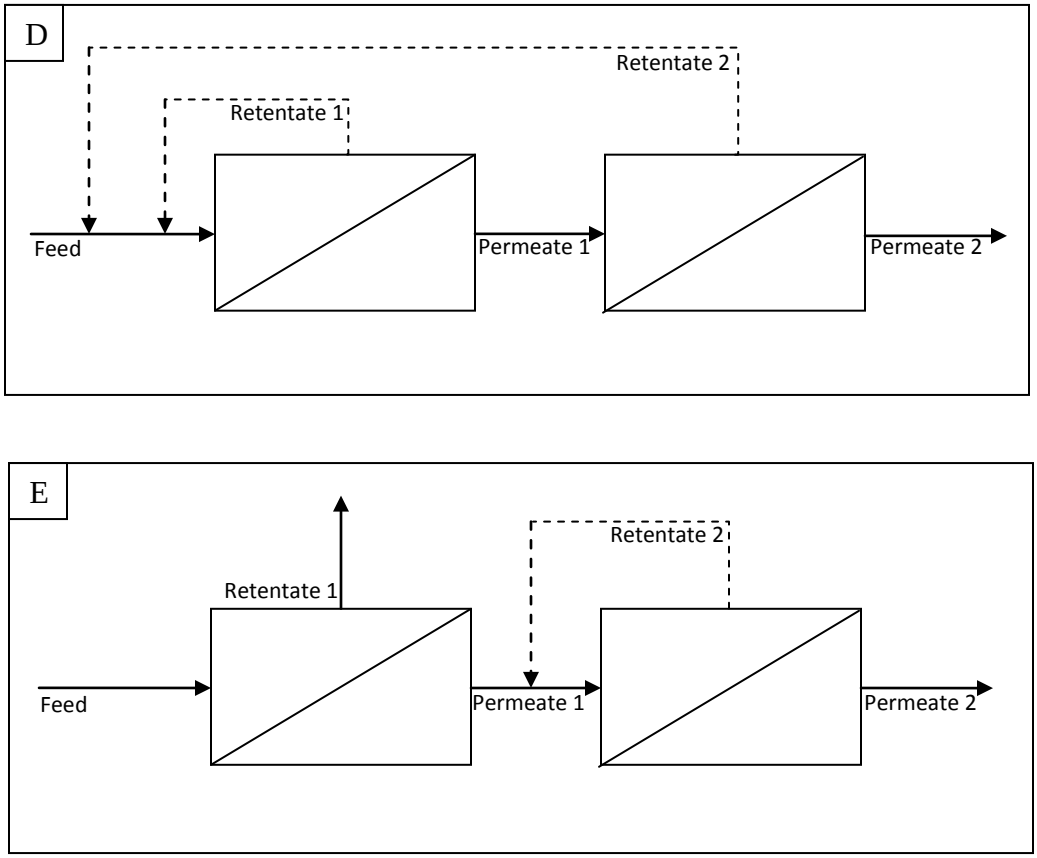

Figure A.1. Continued

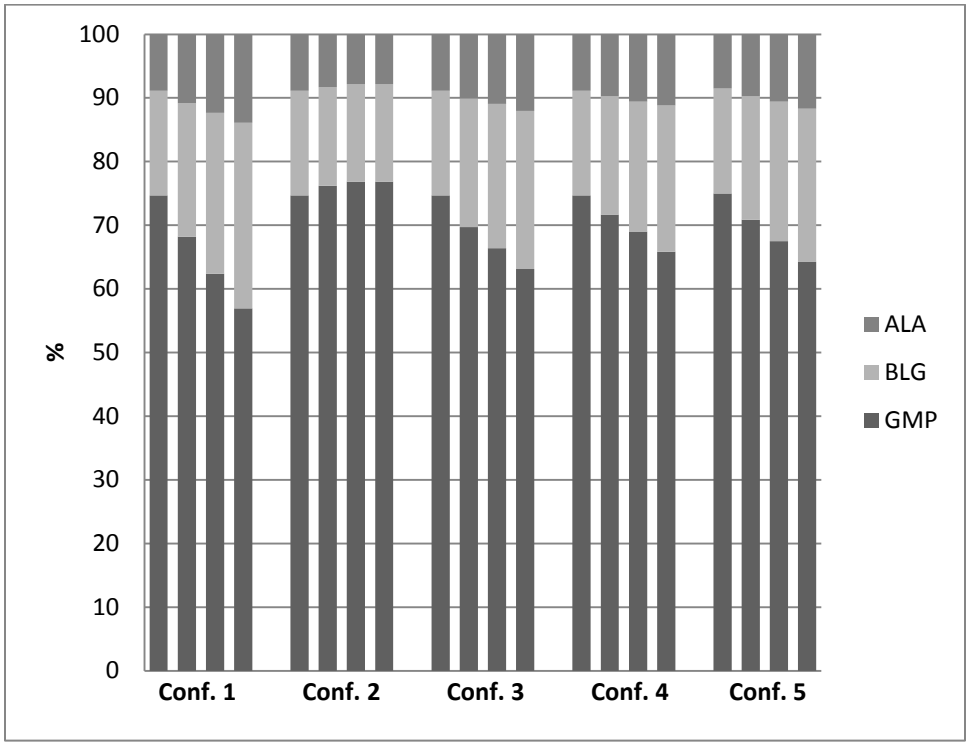

Figure A.2. % Purity of GMP, BLG and ALA in the second permeate stream of different two-stage configurations from cycle 1 to 4 (Cycle number for each configuration increases from left to right)

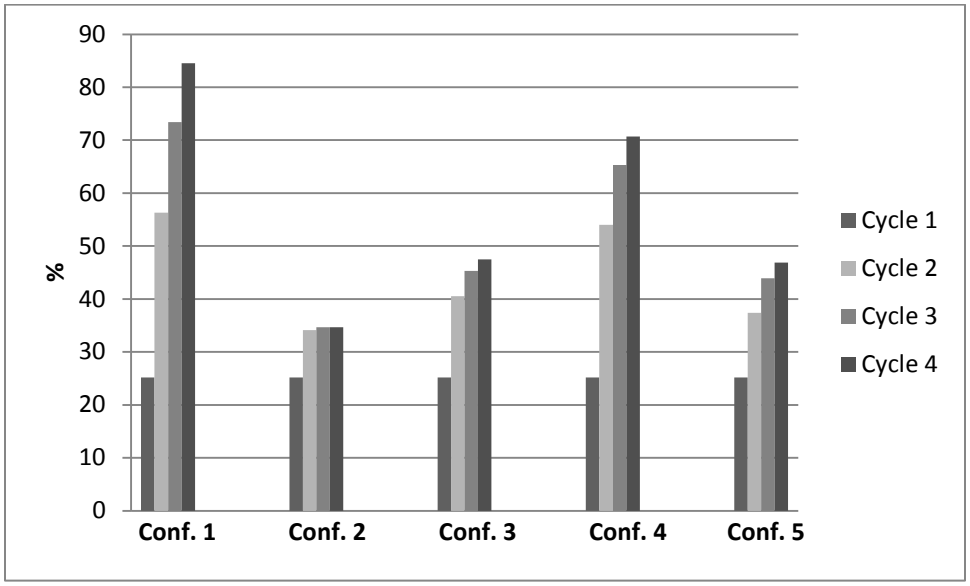

Figure A.3. Distribution of GMP in the second permeate stream of different two-stage configurations

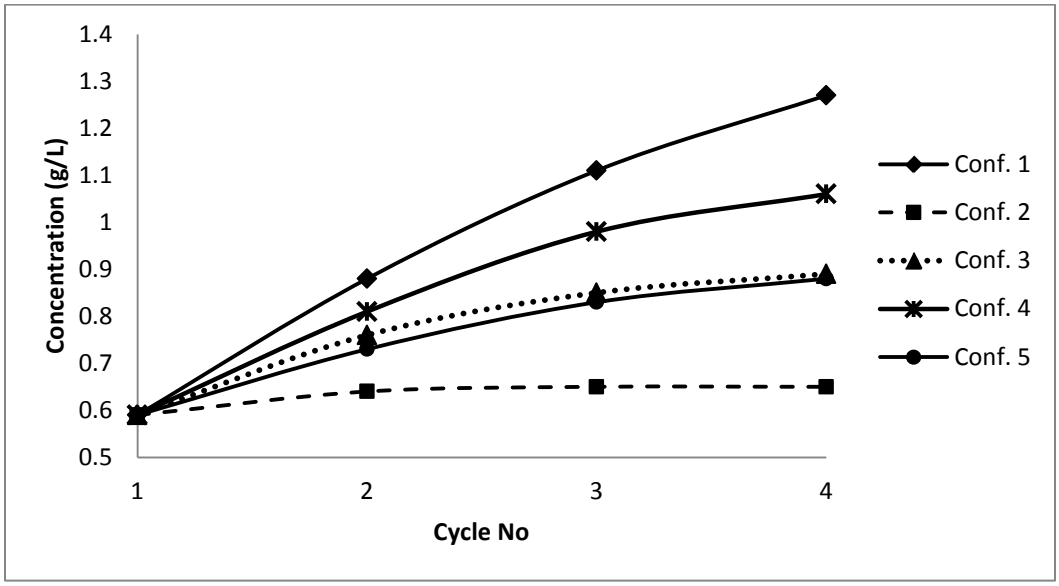

Figure A.4. Concentrations of GMP (g/L) in the second permeate stream from cycle 1 to 4 in different configurations.

APPENDIX B

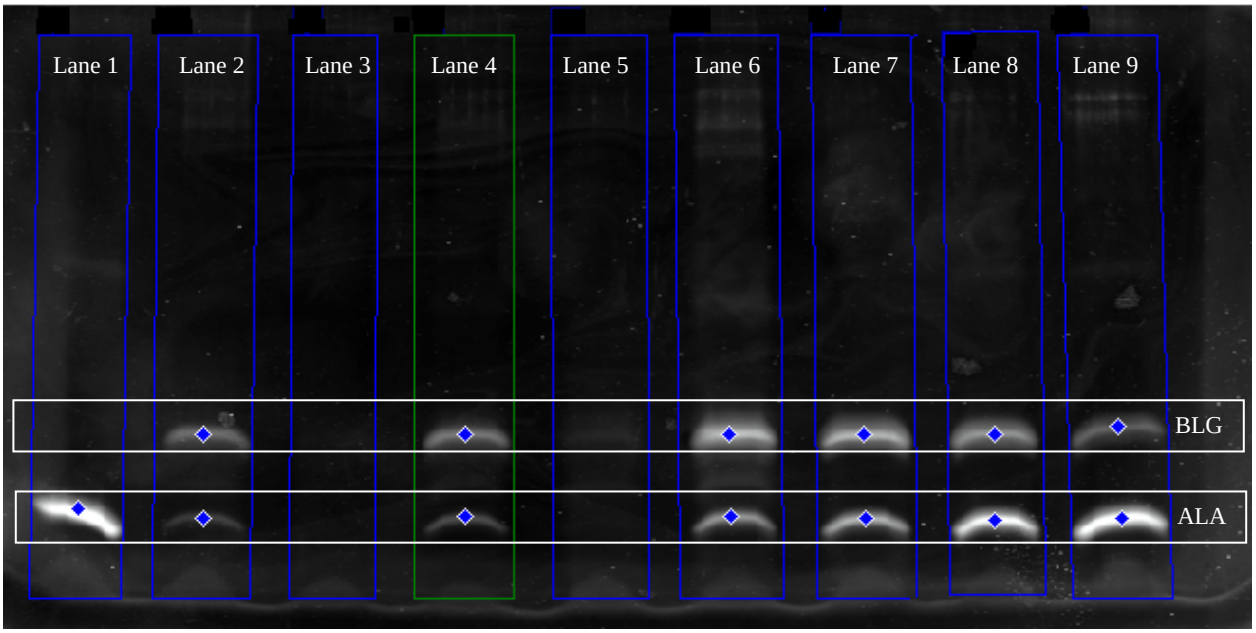

Figure B.1. Picture of SDS-PAGE gel from the experiments performed using 30 kDa charged membrane and Swiss cheese whey at pH 3.0, conductivity 4 mS/cm. (Lane 1: Control BLG solution, Lane 2: Feed, Lane 3: Permeate at *f*: 0.0, Lane 4: Retentate at *f*: 0.0, Lane 5: Permeate at *f*: 0.8, Lane 6: Retentate at *f*: 0.8, Lane 7: Mixture of 0.3 g/L ALA and 0.1 g/L BLG, Lane 8: Mixture of 0.2 g/L ALA and 0.2 g/L BLG, Lane 9: Mixture of 0.1 g/L ALA and 0.3 g/L BLG)

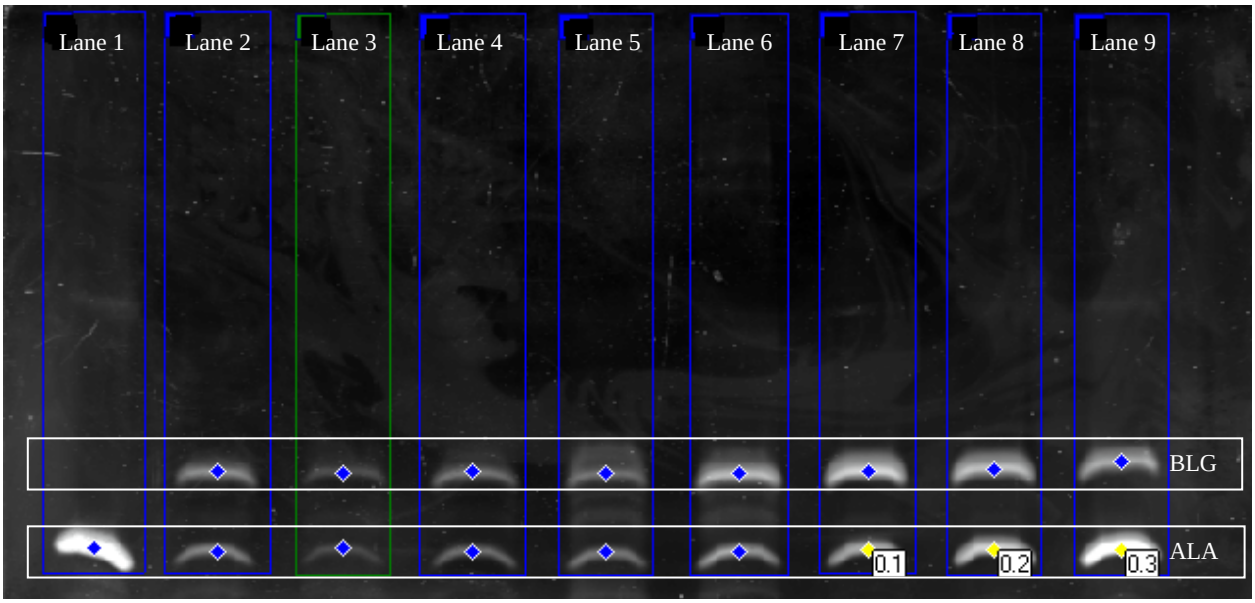

Figure B.2. Picture of SDS-PAGE gel from the experiments performed using 300 kDa charged membrane and Swiss cheese whey at pH 3.0, conductivity 4 mS/cm. (Lane 1: Control BLG solution, Lane 2: Feed, Lane 3: Permeate at *f*: 0.0, Lane 4: Retentate at *f*: 0.0, Lane 5: Permeate at *f*: 0.8, Lane 6: Retentate at *f*: 0.8, Lane 7: Mixture of 0.3 g/L ALA and 0.1 g/L BLG, Lane 8: Mixture of 0.2 g/L ALA and 0.2 g/L BLG, Lane 9: Mixture of 0.1 g/L ALA and 0.3 g/L BLG)

APPENDIX C

Table C.1. Extinction coefficients of GMP and BLG

Wavelength (nm)	GMP	BLG
214	8.659	11.336
280	0.052	0.771

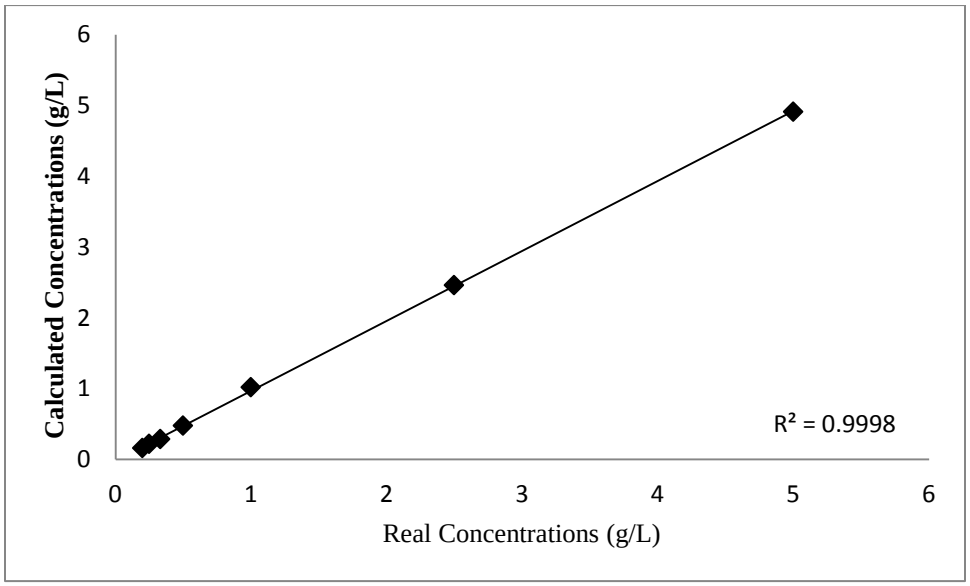

Figure C.1. Comparison of real and calculated concentrations of GMP (g/L) in a binary protein mixture using the extinction coefficients

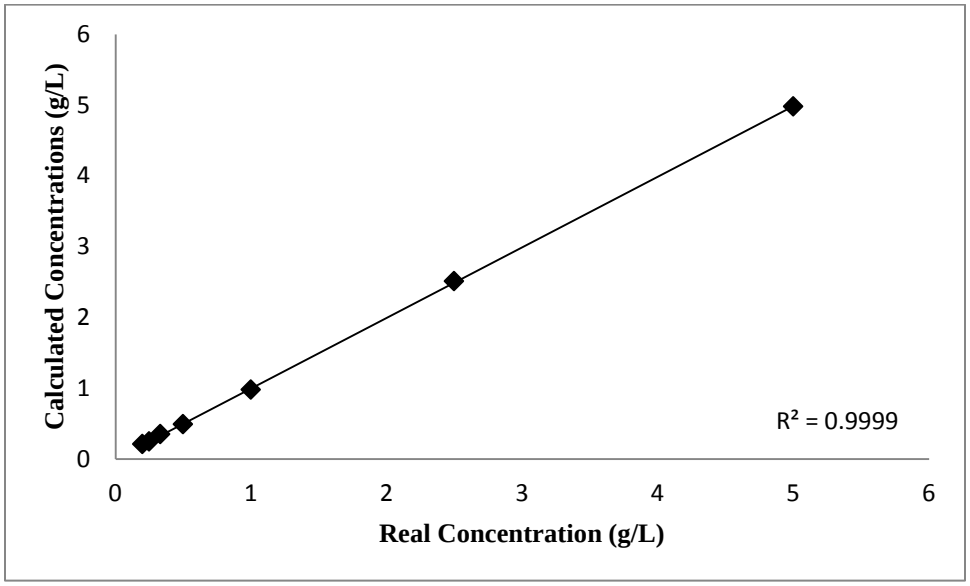

Figure C.2. Comparison of real and calculated concentrations of BLG (g/L) in a binary protein mixture using the extinction coefficients

APPENDIX D

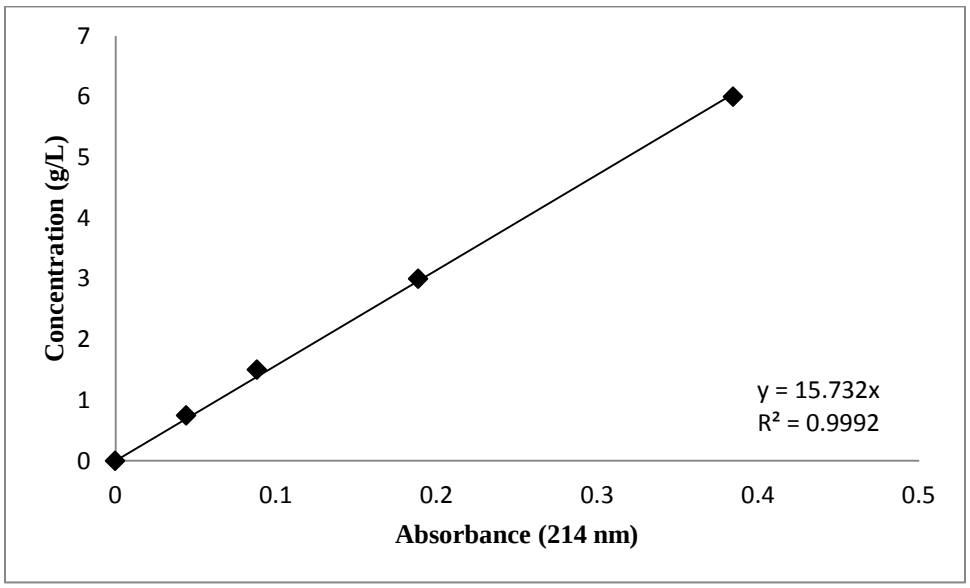

Figure D.1. Calibration curve of GMP using TCA precipitation method