DEVELOPMENT OF MULTIPLE INTERACTIONS MIXED MATRIX MEMBRANE CHROMATOGRAPHY USING LEWATIT MP500 ANION RESIN AND LEWATIT CNP 105 CATION RESIN FOR WHEY PROTEIN FRACTIONATION

CHAN FONG SHIEW

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

FEBRUARY 2013

SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of Bachelor of Chemical Engineering.

Signature :
Name of Supervisor : DR. SYED MOHD SAUFI BIN TUAN CHIK
Position : SENIOR LECTURER
Date :

STUDENTS'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotation and summaries which have been duly acknowledge. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : CHAN FONG SHIEW
ID Number : KA09089
Date :

Special dedication to my parents, my siblings,
especially Kuok Keung and my friend for all
your love, care and supports.

ACKNOWLEDGEMENTS

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

First and foremost, I offer my sincerest gratitude to my supervisor, Dr Syed Mohd Saufi bin Tuan Chik, who has supported me throughout my thesis with his patience, encouragement and knowledge. It is no easy task, reviewing a thesis and I truly appreciate his thoughtful and detailed comments and feedback. He even sacrifices his valuable time in helping me to solve the obstacle faced during the experimentation work which I will never forget. Thank you very much for shaping such a guidance and direction of work with instructive comments.

I am also very thankful to Nurul Shamsinar binti Shahirin, Pang Sook Fun and Nuraini bt Mohd. Yusoff for their guidance, advice and motivation at the FKKSA laboratory. Not forgetting also the technical assistant that constantly provides helpful explanation and guidance in handling equipments which aids in the smoothness of the experimental work.

To the staff at the FIST laboratory, Mr Husaini, I am grateful for his willingness to help me as he always respond to my request whenever I find him there.

I am grateful and glad to have all those friends that offer help whenever I need them. I really do feel thankful for their generosity, time spent and cooperation that they offered as we shared all those laughter, joy and excitement while working together at the laboratory.

And last, but not least, I would like to take this opportunity to express my gratitude from deep heart to my beloved parents and siblings for their love and continuous support – both spiritually and materially.

TABLE OF CONTENTS

	PAGE
SUPERVISOR'S DECLARATION	ii
STUDENT'S DECLARATION	iii
ACKNOWLEDGEMENTS	v
TABLE OF CONTENTS	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xi
LIST OF SYMBOLS	xiii
ABSTRAK	xiv
ABSTRACT	xv
CHAPTER 1 – INTRODUCTION	1
1.1 Background of Study	1
1.2 Problem Statement	3
1.3 Research Objective	4
1.4 Scopes of Study	4
CHAPTER 2 – LITERATURE REVIEW	5
2.1 Whey	5
2.2 Whey Protein	8
2.3 Introduction to Membrane Process	12
2.3.1 Pressure-Driven Based Membrane Process	13
2.4 Chromatography Process	15
2.5 Packed Bed Chromatography for Whey Protein Fractionation	17
2.6 Membrane Chromatography	20
2.6.1 Configuration of Membrane Chromatography	21
2.7 Membrane Technology in Dairy Industry	22
2.8 Mixed Matrix Membrane Chromatography	23

CHAPTER 3 – METHODOLOGY 26
3.1 Materials 26
3.2 Preparation of LF-Spiked Whey 27
3.3 Preparation of Mixed Matrix Membrane 27
3.4 Batch Fractionation of Whey 29
3.5 Gel Electrophoresis 30
3.6 HPLC Analysis 32

CHAPTER 4 – RESULTS AND DISCUSSIONS 33
4.1 Whey Protein Analysis 33
4.2 Batch Fractionation of Whey 33
4.3 SDS-PAGE Analysis 39

CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS 42
5.1 Conclusions 42
5.2 Recommendations 43

REFERENCES 45

APPENDIX
A Mixed matrix membrane preparation 51

LIST OF TABLES

		Page
Table 2.1	Composition of whey protein	8
Table 2.2	Importance functions of protein composition in whey protein fractionation	10
Table 2.3	Column chromatography for whey protein fractionation	18
Table 4.1	Whey protein fractionation using EVAL base polymer MMM chromatography	36
Table 4.2	Whey protein fractionation using cellulose acetate base polymer MMM chromatography	37

LIST OF FIGURES

		Page
Figure 2.1	Annual volume of dairy whey produced globally (1995-2005). Volume increase over this period shows 1-2% annual growth rate, approximately equivalent to the average annual growth in milk output over this same period	6
Figure 2.2	Schematic representation of the relative increase in value of whey protein due to advancement of technology and knowledge on the importance of whey protein composition	7
Figure 2.3	Milestone in development of membrane technologies for protein purification	12
Figure 2.4	Membrane process classification based on the size indication and membrane processes. MF: microfiltration, UF: ultrafiltration, NF: nanofiltration, RO: reverse osmosis	15
Figure 2.5	Illustration of basic interaction modes of chromatographic separation	16
Figure 3.1	Preparation of mixed matrix membrane chromatography	28
Figure 3.2	A thin film membrane was formed on the glass plate using stainless steel block	29
Figure 3.3	Whey protein binding with gentle mixing using tube rotator	30
Figure 3.4	A typical XCell SureLock™ Mini-Cell loaded with samples	31
Figure 4.1	Graph of standard curve peak area versus concentration for (a) BSA (b) α-Lac and (c) β-Lac	34
Figure 4.2	Typical chromatogram of (a) standard mixture of pure protein; (b) LF-spiked whey (4x dilution); EVAL based MMM; (c) After binding fraction (4x dilution); (d) Washing fraction; (e) Elution fraction	35

Figure 4.3	SDS-PAGE of the fractions involve in the batch fractionation of LF-spiked whey using mixed mode interaction MMM using EVAL and cellulose acetate based membrane. Lane 1- LF-spiked whey (4x dilution), Lane 2-Whey (4x dilution), EVAL-based MMM: Lane 3-after binding fraction (4x dilution), Lane 4-Washing fraction, Lane 5-Elution fraction, Lane 6-blank, CA-based MMM: Lane 7- after binding fraction (4x dilution), Lane 8-Washing fraction, Lane 9- Elution fraction, and Lane 10-protein marker	40
Figure A.1	Mixed matrix membrane preparation	51

LIST OF ABBREVIATIONS

α-Lac	α-lactalbumin
β-Lac	β-lactoglobulin
AC	Affinity chromatography
BOD	Biochemical Oxygen Demand
BSA	Bovine serum albumin
CA	Cellulose acetate
COD	Chemical Oxygen Demand
Cys	Cysteine
Da	Dalton
DMSO	Dimethylesulfoxide
EVAL	Ethylene vinyl alcohol
GF	Gel filtration
Hb	Bovine hemoglobulin
HIC	Hydrophobic interaction
HPLC	High Performance Liquid Chromatography
IEC	Ion exchange
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LDS	Lithium dodecyl sulfate
LF	Lactoferrin
LP	Lactoperoxidase
MF	Microfiltration
mM	milimolar
MMM	Mixed matrix membrane
NaOH	Sodium hydroxide
NF	Nanofiltration
NMP	N-Methyl-2-pyrrolidone
PEG	Polyethylene glycol
pI	Isoelectric point

RO	Reverse osmosis
RPC	Reversed phase
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UF	Ultrafiltration
WPI	Whey protein isolate
Wt/vol	Weight/volume

LIST OF SYMBOLS

€	Euro
µ	Micro
Å	Ångström
α	Alpha
β	Beta

PENGHASILAN MEMBRAN KROMATOGRAFI CAMPURAN MATRIK PELBAGAI INTERAKSI MENGGUNAKAN RESIN ANION LEWATIT MP500 DAN RESIN KATION LEWATIT CNP105 UNTUK PENGASINGAN PROTEIN DARIPADA *WHEY*

ABSTRAK

Cara konvensional untuk pengasingan protein dilakukan dengan mengunakan kromatografi turus terpadat. Namun demikian, teknik ini mempunyai beberapa kelemahan seperti kejatuhan tekanan yang tinggi dan kadar aliran operasi yang terhad. Membran kromatografi dapat mengatasi masalah dalam kromatografi turus terpadat tetapi proses penyediaan membran kromatografi ini memerlukan pengubahsuaiankimia yang melampau. Konsep penyediaan campuran membran matrik (MMM) adalah kaedah alternatif kepada penyediaan membran kromatografi iaitu menggunakan kaedah fizikal dengan mencampurkan resin boleh jerap dengan larutan polimer membran. Di dalam kajian ini, MMM pelbagai interaksi telah dibangunkan untuk pengasingan protein daripada *whey* meggunakan 7.5 wt% CNP105 kation resin dan 42.5% wt% MP500 anion resin relatif kepada kandungan polimer asas membran. Berdasarkan analisa HPLC dan SDS-PAGE, kedua-dua protein *whey* bersifat asid dan alkali telah terjerap kepada MMM pelbagai interaksi dalam satu ujian pengasingan protein daripada *whey*. Kadar penjerapan untuk *whey* protein bersifat asid menggunakan MMM berasaskan EVAL adalah 4.255 mg BSA/ g MMM, 60.887 mg α-Lac/ g MMM dan 231.788 mg β-Lac/ g MMM. Bagi MMM berasaskan CA, kadar penjerapan adalah 2.970 mg BSA/ g MMM, 42.392 mg α-Lac/ g MMM dan 179.817 mg β-Lac/ g MMM.

DEVELOPMENT OF MULTIPLE INTERACTIONS MIXED MATRIX MEMBRANE CHROMATOGRAPHY USING LEWATIT MP500 ANION RESIN AND LEWATIT CNP 105 CATION RESIN FOR WHEY PROTEIN FRACTIONATION

ABSTRACT

The conventional method to purify protein is by using packed bed column chromatography. However, this method had several limitations such as high pressure drop and limited flow rate operation. Membrane chromatography can be used to overcome the limitation of packed column but the preparation of adsorptive membrane requires harsh chemical modifications. Mixed matrix membrane (MMM) preparation concept can be used as an alternative route to prepare membrane chromatography by physical blending of adsorptive resin with membrane polymer solution. In the current research, multiple interactions MMM chromatography was developed for whey protein fractionation using 7.5 wt% CNP105 cation resin and 42.5 wt% MP500 anion resin relative to base polymer content. The resins were blend at different composition in EVAL and cellulose base polymer matrix. Based on HPLC and SDS-PAGE analysis, both acidic and basic whey proteins were bound to the multiple interactions MMM in single run of whey batch fractionation. The binding capacity for major acidic whey proteins using EVAL based MMM are 4.255 mg BSA bound/ g MMM, 60.887 mg α-Lac bound/ g MMM and 231.788 mg β-Lac/ g MMM. For CA based MMM, the binding capacity are 2.970 mg BSA bound/ g MMM, 42.392 mg α-Lac bound/ g MMM and 179.817 mg β-Lac/ g MMM.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Proteins molecule are different in each other in term of size, shape, charge, hydrophobicity and affinity towards other molecules. These properties can be exploit to separate protein mixture into its individual components for commercial use in industry. Generally, proteins separation can be done using various techniques such as centrifugation, electrophoresis, membrane filtration and chromatography based separation.

Chromatography is the most favorable used technique for high resolution proteins separation and purification (Ghosh, 2002). Three common types of interactions in chromatography are ion-exchange chromatography, gel filtration chromatography and affinity chromatography. Among them, ion exchange chromatography is the most widely used techniques in protein downstream processing.

Nowadays, different types of ion exchange resins are commercially available in protein capturing, purifying and polishing steps (Zhou et al., 2002). Ion exchanger chromatography is crucial for recovery and purification of proteins, polypeptides, nucleic acid and other biomolecules. It functions based on the concept of reversible electrostatic interaction between a charged molecule and the oppositely charged chromatographic media (Bhattacharjee et al., 2006). Furthermore, it has an advantage as the elution often takes place at a relatively mild condition so that proteins are able to maintain their confirmation during chromatographic processing (Saiful & Wessling, 2006).

Purification of proteins by chromatography conventionally done by using packed bed chromatography configuration. However, there are several limitations of packed bed chromatography. There is an increasing interest in developing membrane chromatography that offer advantages compared to packed bed chromatography such as high flow rate without loss of adsorption capacity, large scale operation, absence of long diffusion path length and low pressure drops. Membrane chromatography is a membrane that acts as a short and wide chromatographic column with a variety of adsorptive mechanisms such as hydrophobic, ion exchange and affinity interactions (Klein, 2000; Zou et al., 2001).

Mixed matrix membrane (MMM) concept offer simple procedure to prepare membrane chromatography instead of using chemical modification process. It is prepared by incorporating an adsorptive resin into a membrane polymer solution prior to membrane casting (Saufi & Fee, 2011). According to Avramescu et al. (2003a), this concept is simple and flexible whereby the geometry, adsorption

capacity and the functionality of the membranes are easily adjusted. Avramescu et al. (2003b), had prepared cationic MMM by incorporating Lewatit CNP80WS cation resin into EVAL polymer in the form of flat sheet, solid fiber and hollow fiber membranes to study the fractionation of bovine serum albumin (BSA) – bovine hemoglobulin (Hb). Later, this concept has been expanded by Saufi and Fee (2011) to prepare multiple interaction membrane chromatography by using SP-Sepharose cation resin and MP500 anion resin in single membrane material. They used this mixed mode MMM for whey protein fractionation. In this study, another alternative cation resin Lewatit CNP105 will be used to prepare mixed mode MMM to replace the SP Sepharose which is very costly cation resin.

1.2 Problem Statement

Traditionally, packed bed column chromatography is used in protein separation. However, there is limitation found in packed bed column chromatography especially related to very high pressure drop occurs across the column. Besides that, the packed bed column is not able to be operating at high flow rate. This problem can be resolve using membrane chromatography. However, the common method to prepare adsorptive membranes requires a complex process and sometimes modifications of membranes using harsh chemicals is required. Thus, the concept of MMM preparation technique has a potential in preparing membrane chromatography material. Besides that, the current ion exchange membrane chromatography normally can be operated with single interaction either as anion or cation exchanger. Hence, the development of multiple interactions in MMM chromatography can offer advantages in binding both acidic and basic protein

simultaneously from single run. Meanwhile, from the past literature works shown that the cost of SP Sepharose cationic resin is high compared to the proposed cation resin, Lewatit CNP105 used in this study. Thus, it is favorable to replace the expensive cation resins to a more affordable, low cost and same efficiency and performance as the SP Sepharose resin.

1.3 Research Objective

The objective of this research is to develop multiple interactions MMM chromatography for whey protein fractionation using Lewatit CNP105 cation resin and Lewatit MP500 anion resin.

1.4 Scopes of Study

In order to fulfill the objectives of this research, the following scopes have been outlined:

i. Development and characterization of multiple interactions MMM chromatography using Lewatit MP500 anionic resin and Lewatit CNP105 cationic resin with EVAL based matrix.
ii. Feasibility study on the development of multiple interactions MMM chromatography using cellulose-based matrix.

CHAPTER 2

LITERATURE REVIEW

2.1 Whey

In dairy industry, whey is a byproduct of cheese-making and casein manufacture. The remaining watery and thin liquids is called whey after the casein curd separate from the milk and undergo coagulation through the action of enzyme or pH adjustment. The whey is yellowish or greenish in color depending on the type and quality of milk (Smithers, 2008). Mostly, whey can be made from a wide range of milk, with cow's milk being the most popular choice in the area of western countries.

Nowadays, whey is a nuisance and major problem to the cheese making and casein manufacture industry. In the production of cheese industry, almost 10 kg of milk produces 1-2 kg of cheese while the remaining 8-9 kg consists of liquid whey (Bhattacharjee, 2006). The increasing quantity of milk production leads to a larger volume of cheese, casein or caseinate and other dairy products and thus increasing

the volume of whey production. From Figure 2.1, the amount of whey production is increasingly due to the growth of milk industry (Smithers, 2008).

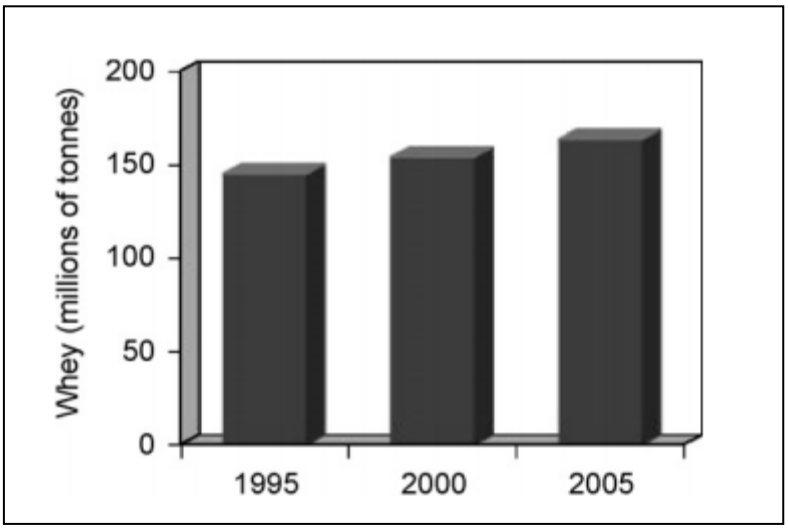


Figure 2.1 Annual volume of dairy whey produced globally (1995-2005). Volume increase over this period shows 1-2% annual growth rate, approximately equivalent to the average annual growth in milk output over this same period (Source: Smithers, 2008)

Whey is a waste product stream and is constantly being disposed and discharged from the industry. There are several disposal method practiced by western country from the previous centuries whereby the cheese-makers and casein manufacturers spray the whey onto fields, discharging through rivers, lakes or ocean, discharging into municipal sewage system or selling it as animal feed (Smithers, 2008). Later, the disposal of whey waste had known to become an issue to environment pollution whereby this waste contaminated the water system. This is proven by an analysis done using biochemical oxygen demand (BOD) and chemical oxygen demand (COD). From the analysis, the BOD value and COD value showed value of 35 000 – 60 000 mg L^{-1} and 80 000 – 100 000 mg L^{-1} each respectively (Bhattacharjee, 2006).

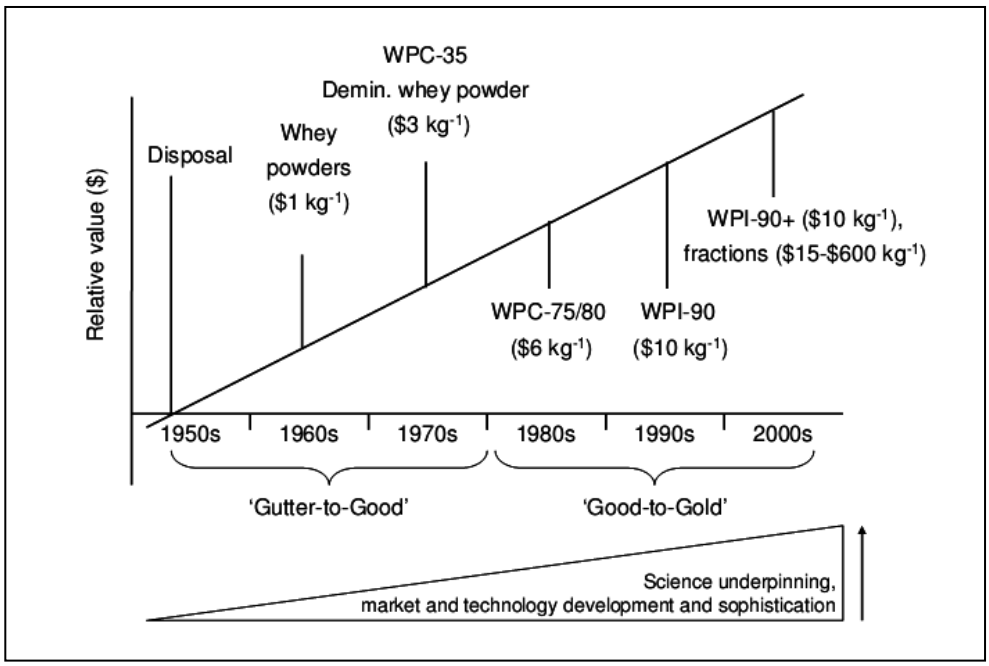


Figure 2.2 Schematic representation of the relative increase in value of whey protein due to advancement of technology and knowledge on the importance of whey protein composition (Source: Smithers, 2008)

In 1950s, whey is considered as waste and is usually being disposed without fully utilization its source of protein. However, with increasing knowledge and advancement of science and technology researchers had found out that whey is an excellent source of proteins that contain all the essential amino acids (Monteiro et al., 2008). Meanwhile, the consumer demand on health product is increasing and thus separation and purification of protein in whey for health supplement is a must. As a consequence, there is an increase in terms of relative price value for whey protein as illustrated from Figure 2.2. The relative value of whey protein for the past 50 years is increasing from $1 per kg at 1960s to $10 per kg at year 2000.

2.2 Whey Protein

The major whey protein components comprises of β-lactoglobulin (β-Lac), α-lactalbumin (α-Lac), bovine serum albumin (BSA), lactoferrin (LF), lactoperoxidase (LP), glycomacropeptide and etc (Bhattacharjee, 2006). Table 2.1 shows the composition and properties of whey proteins.

Table 2.1 Composition of whey protein (Source: Andersson & Mattiason, 2006)

	Concentration (kg m^{-3})	Isoelectric point (pI)	Molecular weight (kDa)
β-Lactoglobulin	2-4	5.2	18
α- Lactalbumin	1.2-1.5	4.5-4.8	14
Bovine serum albumin	0.3-0.6	4.7-4.9	69
Immunoglobulins (IgG, IgA, IgM)	0.6-0.9	5.5-8.3	150-1000
Lactoperoxidase	0.02-0.05	9.5	78-79
Lactoferrin	0.02-0.2	8-9.5	78-92

There are two types of processed whey protein which are whey protein concentrates and whey protein isolates. Whey protein concentrates are rich in whey proteins but contain fat and lactose. This whey protein concentrates are obtained from membrane filtration of whey. Meanwhile, whey protein isolates contain whey protein with low fat and lactose. It is produced through rigorous and complex separations of whey such as a combination of ultrafiltration and microfiltration or ion exchange chromatography (Etzel et al., 2008).

Most of the whey protein components have their own high commercial values in the market and application both in health and food industry. It is known that the world production of cheese whey per year is estimated as 130 million tons. This means that the global production is equivalent to 780 000 tons of proteins which in

turn for separation and protein purification will benefits the economic growth (Monteiro, 2008). Table 2.2 summarizes the importance functions of composition protein found in whey.

Table 2.2 Importance functions of protein compositions in whey protein fractionation

Compositions	Functions	References
β-Lactoglobulin	- Better foam stabilizer used in the production of confectionary due to its food gelling properties - Transport or accumulation of lipid-soluble biological components such as fatty acids and retinols - Rich source of Cys that stimulate glutathione synthesis, an anticarcinorgenic tripeptide produced by the liver for protection against intestinal tunors	Mcintosh et al., 1995; Zydney, 1998; Cowan & Ritchie, 2007; Madureira et al., 2007; Amigo et al., 2008
α-Lactalbumin	- Used in infant formula due to high tryptophan content - Enhanced whippability in meringue like formulations - Potential as contraceptive agents due to strong affinity for glycosylated receptors on the surface of oocytes and spermatozoids - Preventions of cancer, lactose synthesis and treatment of chronic stress-induced disease	Zydney, 1998; Cowan & Ritchie, 2007; Madureira et al., 2007
Lactoferrin	- High iron-binding affinity - Regulation of iron absorption and immune responses - Exhibit antioxidant activity and has both anticarcinogenic and anti-inflammatory properties - Used in skin care cosmetics, special therapeutic diets for the relief of inflammation in dogs and cats - As supplemented infant formula	Tomita et al., 2002; Garcia-Montoya et al., 2012

Table 2.2 (continued)

Compositions	Functions	References
Immunoglobulins (IgG, IgA, IgM)	- Prevents mucosal infections by agglutinating microbes - High valency of antigen-binding sites - Prevent adhesion of microbes to surfaces, inhibiting bacterial metabolism by blocking enzymes, agglutinating bacteria and neutralizing toxins and viruses	Hurley & Theil, 2011; El-Loly, 2007
Bovine serum albumin	- Anti-mutagenic function and fatty acid binding - Ability to inhibit tumor growth	Madureira et al., 2007
Lactoperoxidase	- Catalyzed oxidation of thiocynate by hydrogen peroxidase and generates immediate products with antibacterial properties - Preserve raw milk quality during storage or transportation to processing plant	Zydney, 1998; Watanabe et al., 2000; Seiful et al., 2005

2.3 Introduction to Membrane Process

Membrane process is widely applied for the separation and purification in upstream and downstream processing. Microfiltration and ultrafiltration is the commonly used membrane process in industry. The performance of both type membranes is of high throughput however it is relatively low in terms of resolution and purification basis (Saxena et al., 2009). From years to years, membrane technology begins to evolve and become an emerging tool used mainly in food industry with 20 to 30% of the current €250 million turnover of membranes is used in the manufacturing industry globally (Daufin et al., 2001). The membrane process is now developing and growing rapidly from pressure gradient types to the existing electrical gradient principles as illustrated in Figure 2.3.

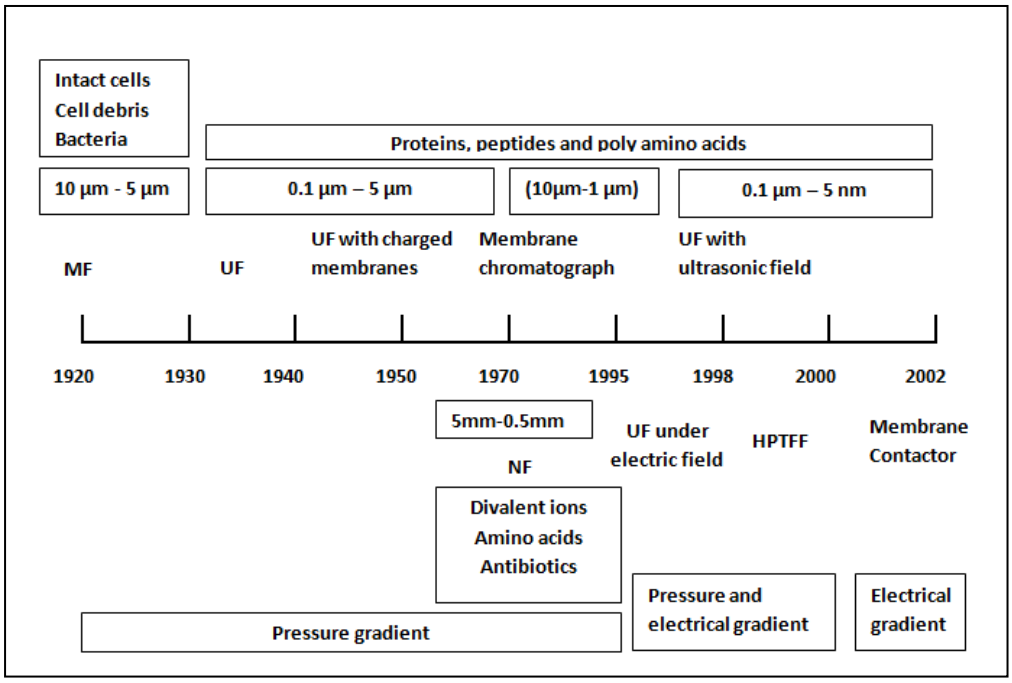


Figure 2.3 Milestone in development of membrane technologies for protein purification (Saxena et al., 2009)

The rapid development of membrane technology is due to its unique separation principle which is selective transport and efficient separation compare with other type of unit operations. A membrane actually acts as an interphase usually in the form of heterogeneous whereby it acts as a barrier to the flow of molecular and ionic species present in the liquid or vapors contacting the two surfaces (Saxena et al., 2009).

There are several advantages of using membrane as the separations process in industry. Membrane does not involve any phase changes or chemical additives, simple and easy to operate. Besides that, it allows for ease of scale up in industry production rate and able to decrease the equipment-size (Drioli, 2004). In addition, the separation process can be performed isothermally at relatively low temperatures with less energy consumption compared to other thermal separation process (Saxena et al., 2009).

2.3.1 Pressure-Driven Based Membrane Process

Membrane process can be classified according to different driving forces which are pressure gradient and electrical gradient. For pressure driven force, the pressure used depends on the pore size of the membrane and has to be adjusted as a function of the concentration rate desired (Langevin et al., 2012). Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) operate principally according to pressure driving force in various applications of both upstream and downstream processing. Figure 2.4 illustrates the membrane process classification according to size of solute to be separated.

MF is one of the oldest filtration technologies. MF membranes are suitable for separation of fine particles with pore size in the range of 0.05 to 10 µm (Reis & Zydney, 2007) and can selectively separate particles with molecular weights of >200kDa using sieve effect. MF is often uses in removal of bacteria or other particulate substances and fractionation of milk proteins (Rosenberg, 1995).

UF membranes have a pore size range within 1 to 100 µm which able to provide high retention of proteins and other macromolecules (Reis & Zydney, 2007). UF is suitable for separation of protein that has molecular weight between 10^3 and 10^6 Da (Atra et al., 2005). This membrane is used for protein concentration and buffer exchange. UF had replaces the role of size exclusion chromatography for buffer exchange at industrial scale (Kurnik et al., 1995). Other than that, UF is used to fractionate proteins from whey and to make cheese from ultrafiltered milk (Atra et al., 2005).

Another type of pressure driven membrane is NF membrane which has the characteristics between RO and UF membrane. The cutoff value of NF is 100-1000 Da with approximate pore size of 1 nm (Oatley et al., 2012). NF can be applied to separate various components such as lipids, bacteria, proteins, sugars and salts. NF is also useful in separating valuable components from sweet whey (Roman et al., 2011).

RO membranes has molecular weight cutoff of ~100 Da and uses transmembrane pressures of 10-50 bar which is 5-10 times higher than UF membrane. RO membrane consists of an ultrathin skin layer superimposed on a coarsely porous matrix (Marcello & Rizvi, 2009). RO applications in dairy industry allow

concentration of milk or whey by removal of water and ionized minerals (Rosenberg, 1995).

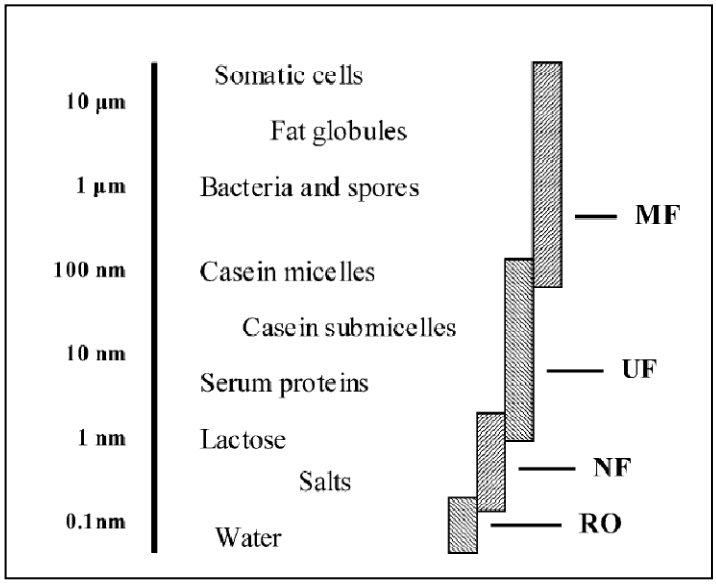


Figure 2.4 Membrane process classification based on the size indication and membrane processes. MF: microfiltration, UF: ultrafiltration, NF: nanofiltration, RO: reverse osmosis (Source: Brans et al., 2004)

2.4 Chromatography Process

In order to purify and separate protein, chromatography technique is of interest. There are several different modes of interactions as illustrated in Figure 2.5. From the figure, it is arranged according to interaction strength with the weakest on top and the strongest on bottom. The interaction modes include gel filtration (GF), hydrophobic interaction (HIC) and reversed phase (RPC), ion exchange (IEC) and affinity chromatography (AC).

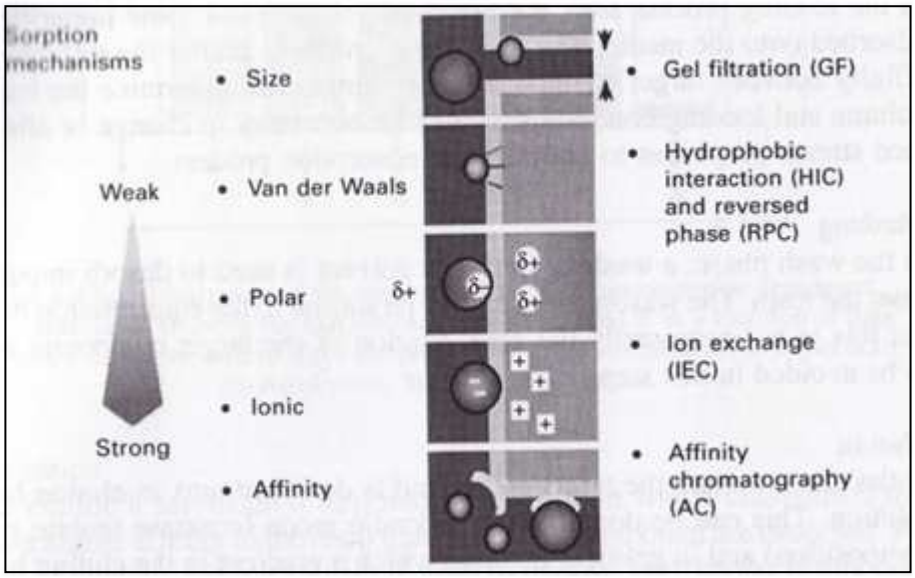


Figure 2.5 Illustration of basic interaction modes of chromatographic separation (Source: Rizvi, 2010)

In the fractionation of whey, selective adsorption and selective elution is the two strategies of interest. However, selective elution is more preferable because this kind of selection both concentrates and fractionates the protein. During the process, all the proteins in a mixture are trapped simultaneously on the adsorbent, rinsed free of contaminants and the eluted one-by-one to manufacture many different purified proteins (Doultani et al., 2004). The bound target proteins can be released separately into several different elution solutions (Etzel, 2004). In short, protein of interest can be obtained easily using selective elution strategy.

In addition, changing the elution buffers will eventually aid in obtaining different protein fractions from the binding of the proteins to the adsorbent. This will certainly reduce the steps of changing equipment if selective adsorption strategy is selected. This means that different composition and types of elution buffer can be prepared to elute the protein solution. The flexibility of changing the buffer solution

enables the manufacturer to meet the demand of the market and saving cost for equipment.

2.5 Packed Bed Chromatography for Whey Protein Fractionation

Column chromatography consists of chromatography beads or ion exchange resins that are packed inside the column (Etzel, 2004) whereby the solutions passed through the column. In whey protein fractionation, both anion and cation exchanger chromatography are use for the separation of whey as summarized in Table 2.3.

Table 2.3 Column chromatography for whey protein fractionation

Protein Source	Protein of interest	Configuration	Mode of interaction	Author
Milk	α – Lac, β-Lg B, β-Lg A	a) Quartenary aminoethyl-Toyopearl (QAE-TP), dimension: 1.5 cm × 18 cm b) Sulphopropyl-Toyopearl (SP-TP), dimension: 1.5 cm × 18 cm	Anion and Cation exchange	Ye et al., 2000
Mozzarella cheese whey	α – Lac, WPI, LP, LF	SP Sepharose Big Beads, bed height 15 cm, column volume 80 mL	Cation exchange	Doultani et al., 2004
Lactic acid whey	WPI, α - Lac	SP Sepharose Big Beads, bed height 15 cm, column volume 80 mL	Cation exchange	Turhan and Etzel, 2004
Whole milk	LF, LP	SP Sepharose Big Beads™ (GE Healthcare), column height 5 cm, column volume 10 mL	Cation exchange	Fee & Chand, 2006
Whey Protein Concentrate (WPC80)	β-Lg, α – Lac, BSA, Ig	Mono Q5/50 GL - Mono Q Column (GE Healthcare, Pittsburgh)	Anion exchange	Santos et al., 2012

For anion exchange column, Gerberding and Byers (1998) observed recoveries of 36% for α-Lac, 94% for β-Lac, 6% for IgG and 21% for BSA by passing whey through the column at pH 5.8. The bound protein was eluted in two peaks. One peak contained α-Lac, however it is contaminated with β-Lac, BSA and IgG. Another peak contained primarily β-Lac contaminated with BSA. Ye et al. (2000) using salt gradient method for selective elution. The process is divided into two which are as followed:

a) Whey at pH 6.5 was passed through cation exchange column to recover LP and LF. The elution gradient used 0-0.55 M NaCl in 50 mM Tris HCl at pH 6.5.
b) The latter effluent was adjusted to pH 8.5 and passed through anion exchange column to recover α-Lac and β-Lac. A-Lac was eluted with 0-0.15M NaCl in 50 mM Tris HCl at pH 8.5. Next, column was adjusted to pH 6.8 and using 0-0.20M NaCl in 50 mM Tris HCl to elute β-Lac.

IgG and BSA fractions were not found during the elution. This process required two column and only different salt gradient is used instead of changing the pH of the solutions. During the resin equilibration steps, salt from the previous steps must be washed out before the elution of protein in sequence is being conducted. This adds to additional steps and time used with waste of buffer and thus increasing the cost.

Mozzarella cheese whey was bound onto SP Sepharose Big Beads with cation exchanger column at pH 4 with bed height 15cm and 80 ml column volume

(Doultani et al., 2004). Different elution buffers are illustrated as below to recover the fractions of whey protein:

a) Single WPI obtained using 10 mM NaOH
b) α-Lac using 100 mM sodium acetate at pH 4.9
c) WPI depleted in α-Lac using 10 mM sodium NaOH
d) α-Lac, WPI depleted in α-Lac, LP and LF using sequence of: 100 sodium acetate at pH 4.9, 50 mM sodium phosphate at pH 6.5, 0.35 M NaCl in 50 mM sodium phosphate pH 6.5 and 1.20 M NaCl in 50 mM sodium phosphate pH 6.5 each respectively.

This work proved the feasibility of selective elution using differ elution buffer to fractionate whey protein. Manufacturer can actually tailor the needs of the changing market and the demands.

2.6 Membrane Chromatography

Another type of membrane processes that can be used for protein purification and separation is membrane chromatography or known as membrane adsorber. Membrane chromatography is another alternative for conventional resin or beads based chromatography column. It separates the protein of interest based on specific functionality in the membrane such as ion exchange, hydrophobic, reversed-phase and affinity interaction.

Membrane chromatography has several benefits and advantageous for bio-molecules separation such as shorter diffusion times, interaction between molecules and active sites based on convective mass transfer and higher flow rates (Charcosset, 2006). Shorter diffusion times mean that transportation of solutes to their binding sites takes place by convection which result in reduces process time and liquid volume (Saxena et al., 2009).

The preparation of membrane chromatography involved the action of grafting specific ligand onto the pore surface in membranes and then adsorbing target biomolecules on these ligands during convective flow through the membrane pores. The properties of large pore size in membranes allow for easy access of protein molecules to the binding sites on the pore wall (Saxena et al., 2009). This implies that for larger protein (molecular weight more than 250 kDa) the surface area available for binding is significantly greater for membranes (Ghosh, 2002).

2.6.1 Configuration of Membrane Chromatograph

Flat sheet, hollow fiber and radial flow are the three types of membrane adsorbers used for protein separation. According to Klein (2000), it is proven that different shapes of membrane adsorbers can link the specific ligate needed which act as an important approach in membrane chromatography. For flat sheet configuration, the liquid was usually introduced normal to the membrane surface in flat sheet membrane adsorbers. Inside the membranes modules, piles of several flat sheets were stacked together (Saxena et al., 2009). Flat sheet is the most commonly used type of configuration for membrane adsorber.

Meanwhile, for hollow-fiber membrane adsorber it has the geometry of tubular shapes with tubes ranging from 0.25 to 2.5mm in diameter. The liquid flows parallel to the membrane surface (Ghosh, 2002). Hydrostatic pressure difference caused the liquid to flow directed towards and through the pores (Saxena et al., 2009). The advantage of using hollow-fiber arrangement is high membrane surface area to volume ratio which will eventually reduce the accumulation of particles near the pore entrance due to cross flow.

For large scale applications, radial flow membrane adsorber is a more appropriate and suitable choice of configuration. The process of spirally winding flat sheet membranes over a porous cylindrical core formed radial flow membrane adsorbers. The membrane area increased in a radially outward direction. Thus, complexities from the drop in superficial velocity of the liquid stream are formed during the flow inside the membrane. It is likely more appropriate for the bind and elute conditions (Ghosh, 2002).

2.7 Membrane Technology in Dairy Industry

For the past 25 years, membrane process technology has become major tools in the food processing industry with its usage of achieving approximately 40%. This is due to each year the growth of market is around 7.5% per year (Daufin et al., 2001). There is need to constantly update the current technologies from conventional to advanced processes that suit certain applications such as in dairy industry, beverages, egg products and other related emerging industries.

There are several technologies that are used in the separations and purifications of proteins such as packed bed column chromatography, ultrafiltration, microfiltration, nanofiltration, reverse osmosis and membrane chromatography. From the list of separation techniques, membrane separation is of the most favorable processes applied in the food industry.

There are a lot of benefits from the utilization of membrane separations process. Firstly, it shortens and simplifies the complex process of the conventional method. Besides that, it also improves the performance of the process and enhances food quality. Considering the environment impact, membrane separations are regarded as clean processes, a substitute for the use of polluting materials (Daufin et al., 2001). The characteristics of the membrane which is easy to put into operation, having great flexibility and compact make it a suitable and attractive method for the industry applications.

2.8 Mixed Matrix Membrane Chromatography

The common route to prepare membrane chromatography by using chemical modification of readymade membrane sometimes requires harsh and excessive chemical solutions. This can damage the membrane structure as well as membrane performance of the membrane. In order to avoid this, Wessling group introduced the concept of mixed matrix membrane (MMM) as alternative to prepare membrane chromatography materials (Avramescu et al., 2003a & 2003b).

The preparation of MMM is simple and involves physical modification. MMMs are prepared by incorporating an adsorptive resin into a membrane polymer solution before proceeding to membrane casting (Saufi & Fee, 2011). By phase inversion process, membrane casting was done by solidifying the dissolved polymers with additives containing ion exchange. This concept is of competitive in terms of adsorption capacity and performance as compared to a chemically modified adsorptive membrane.

Avramescu et al. (2003a) first attempt was to produce various format of the MMM chromatography such as flat sheets, solid fiber and hollow fiber. Later, various types of resins were used to prepare cation exchange MMMs by incorporating Lewatit SP112 resin and anion exchange MMMs by incorporating Lewatit MP500 resin.

Multiple modes chromatography usually combines the hydrophobic and ionic interactions used in resin based chromatography (Brochier, 2008; Gao, Lin & Yao, 2007). The simple preparation of MMM had induced the researchers to study more on preparation of a single multi mode interaction membrane that show comparative performance as compared to two separate anion or cation membrane adsorbers.

Before this, isolation of lactoperoxidase and lactoferrin from bovine whey was conducted by using cation exchange membrane (Chiu and Etzel, 1997). Sartobind membrane adsorbers immobilized by sulfonic acid moieties had proved to show recovery of $73 \pm 6\%$ for LP and $50 \pm 5\%$ for LF. Meanwhile, separation of BSA, α- Lac and β-Lac was achieved by using anion exchange membranes (Kim et

al., 2003) From the studies, three strong anion-exchange membranes, CIMA QA, Q100 and HiTrap Q were used as comparison to determine the potential anion exchanger for the isolation protein of interest. According to the result, HiTrap Q is the most effective in separation of acidic whey protein.

Although there has no any mixed mode matrix membrane available at the market at that time, Freitag et al. (1996) had already tried alternated two layers of anionic membrane with one layer of cationic membrane in a filter holder to bind whey protein in a single chromatography run at pH 6. From the experiment, it proved that the number of cationic and anionic membrane layers used influenced the separation pattern for the protein mixture. Later, Lin and Suen (2002) also mixed a cationic and anionic membrane in a single plate-and-frame for the separation of a binary mixture containing bovine serum albumin (BSA) and lysozyme (LZY). This study demonstrated that membrane with highest saturation capacity should be placed first to enable more binding of target protein.

Recent studies by Saufi and Fee (2011) had showed successful incorporation of two types of resins which are Lewatit MP500 anion exchange resin and SP Sepharose cation exchange resin to prepare multi interaction MMM in fractionation of whey proteins. The MMM is able to bind both basic and acidic whey protein simultaneously with binding capacities of 7.16 $\pm$2.24 mg α – Lac g^{-1} membrane, 11.40 $\pm$0.73 mg LF g^{-1} membrane, 59.21 $\pm$9.90 mg β-Lac g^{-1} membrane and 6.79 $\pm$ 1.11 mg IgG g^{-1} membrane during batch fractionation of LF-spiked whey.

CHAPTER 3

METHODOLOGY

3.1 Materials

EVAL, a random copolymer of ethylene and vinyl alcohol with an average content of 44 mol% and cellulose acetate (CA) were used as a based matrix for preparing MMM. Dimethysulfoxide (DMSO) was used as the solvent for EVAL with 1-octanol acts the non-solvent additive in casting solutions. PEG 400 in liquid form was used as the pore forming agent with NMP as the solvent in CA casting solution. The adsorptive resins incorporated in casting solution are Lewatit MP500 anion exchange resin and Lewatit CNP105 cation exchange. The buffer solution was prepared from sodium phosphate dibasic heptahydrate, sodium dihydrogen orthophosphate 1-hydrate and sodium chloride. β -Lactoglobulin (β -Lac), α -Lactalbumin (α-Lac) and BSA were purchased from Sigma and used without further purification. Two buffer solutions used in HPLC analysis were buffer A: 0.1% (wt/vol) trifluoroacetic acid in ultrapure water and buffer B: 10 vol% of solution 0.085% (wt/vol) trifluoroacetic acid in ultrapure water in HPLC-grade acetonitrile.

3.2 Preparation of LF-Spiked Whey

Fresh milk was bought from nearby dairy farm for feed whey preparation. Fresh milk was centrifuged at 12 000 rpm at 4℃ for 10 min using Eppendorf centrifuge model 5810R for defatting. It was then heated to 40℃ in a water bath under gentle mixing. The milk pH was adjusted between 4.6-4.8 using 0.5M hydrochloric acid to precipitate the casein in milk. The precipitated casein was discarded and whey supernatant was centrifuged at 12 000 rpm at 4℃ for 10 min. Whey was filtered several times with filter paper with the final filtration was achieved with a 0.45 μm membrane filter. The pH of whey was adjusted to the desired pH 6 using 0.5 M NaOH. 6.5 mg LF pure protein was added into the 20 ml whey to prepare LF-spiked whey.

3.3 Preparation of Mixed Matrix Membrane

Two types of polymer based solution were prepared during this study. First solution consist 15 wt% EVAL polymer and 15 wt% 1-octanol in DMSO solvent. Second solution is 10 wt% CA and 20 wt% PEG 400 in NMP solvent. The polymer was partially added into the solvent at certain period of time under continuous stirring at 60℃ for several hours until all the polymers completely dissolved.The adsorbent resins (Lewatit MP500 and Lewatit CNP105) to be incorporated into the polymer solution were grinded into small size about 45μm using an ultracentrifugal mill. Ground resin was added to the prepared polymer solution at certain weight fraction relative to the polymer content in the solution. This mixture was stirred again until homogeneous MMM casting solution is formed.

The total resin loading relative to the mass of based polymer in the polymer solution was fixed at 50 wt%. According to the major proteins compositions in whey, almost 95% are acidic proteins and the rest are basic proteins. This would indicate a mixed mode interaction MMM based on these proportions, should comprise 95% anion and 5% cation exchange resins, neglecting the relative protein binding capacities of each resin. However, for a more practical membrane casting formulations to demonstrate the multiple interaction proof-of-concept, a ratio of 85% acidic protein and 15% basic protein was assumed, which produced a mixed mode membrane with 42.5 wt% of Lewatit MP500 and 7.5 wt% Lewatit CNP105.

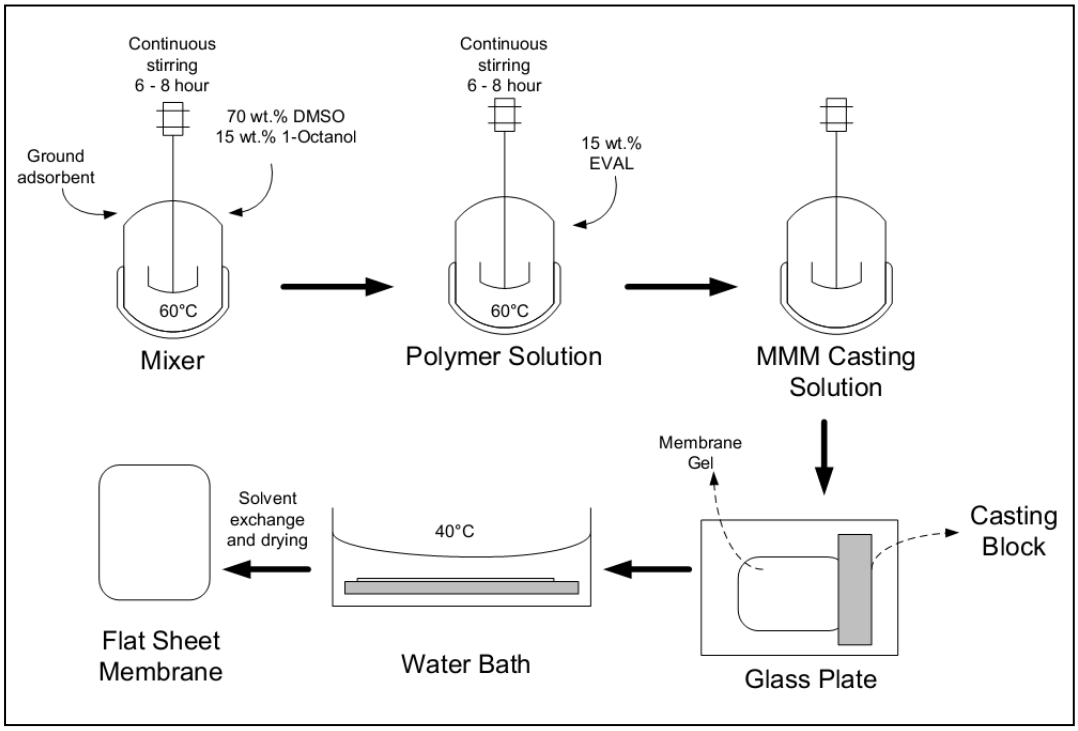


Figure 3.1 Preparation of mixed matrix membrane chromatography

Flat sheet MMM was casted using conventional casting method as shown in Figure 3.1. MMM casting solution was poured onto a glass plate support and then was spread to form a thin film using a stainless steel block with a 600μm recess milled into the bottom surface as shown in Figure 3.2. Immediately after casting, the

glass plate with the film on the surface was immersed to the coagulation water bath at 40℃ until the membrane was completely solidified and detached from the glass. The resulting MMM was washed with water several times and left in the water bath overnight in order to ensure the trace of solvent is completely removed from the membrane structure. Finally, the wet membrane was freeze dried to completely remove water without severely damage the membrane structure.

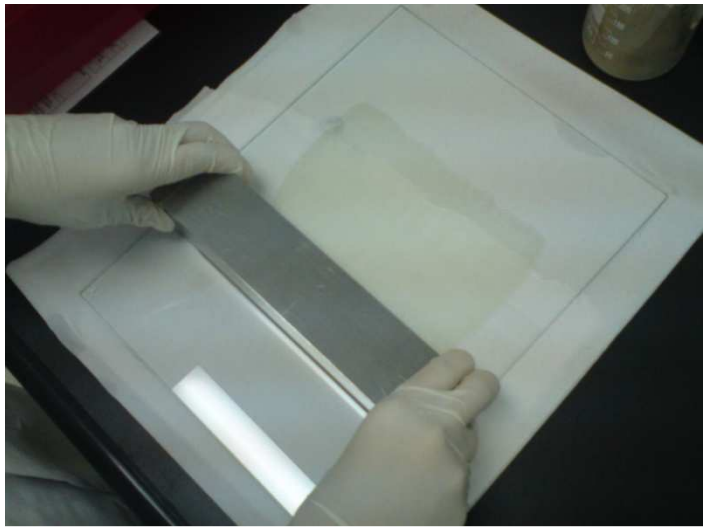


Figure 3.2 A thin film membrane was formed on the glass plate using stainless steel block

3.4 Batch Fractionation of Whey

For batch fractionation, the MMM sheet was cut into a rectangular shape with approximate size of 10 mm × 20 mm size. The MMM was equilibrated for 1 hour in 20 mM sodium phosphate pH 6 equilibrium buffer. Equilibrated MMM was incubated with 2 ml LF-spiked whey solution in small eppendorf tube for binding step. The binding was done for 3 hours under continuous and gentle mixing using rotator at 30 rpm speed as shown in Figure 3.3. The MMM was took out from the whey after binding and was lightly dried by patting with tissue. Dried MMM was

transferred into washing buffer, similar as equilibrium buffer, for washing step about 30 minutes. Then the MMM was removed from the washing solution and the remaining water on the membrane was once again dried by tissue patting. The MMM was then incubated in elution buffer for another 3 hour. 1M NaCl in 20 mM sodium phosphate pH 6 was used as elution buffer. All the solution in each step was further analyzed by HPLC and SDS-PAGE analysis.

Figure 3.3 Whey protein binding with gentle mixing using tube rotator

3.5 Gel Electrophoresis

Qualitative analysis of whey protein fractions from batch fractionation was analayzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For sample preparation, 13 µL protein sample was added with 5 µL Invitrogen NuPAGE® LDS sample buffer and 2 µL reducing agent. Later, the samples were centrifuged for 5 minutes at speed of 13 rpm. Next, samples were heated at 60 ℃ for 10 minutes to denature the proteins.

NuPAGE® Novex® Bis Tris gel was run in XCell SureLock™ Mini-Cell electrophoresis system using NuPAGE® MES SDS running buffer as shown in Figure 3.4. 5 µL of Precision Plus Protein™ Kaleidoscope standards was used as the protein marker and 20 µL of sample were loaded into the gel well. Electrophoresis was run at constant voltage of 200 V with run time of 35 minutes.

After electrophoresis, the gel was stained in a solution of Coomassie Brillant Blue R250, 0.125% (w/v) in 10% acetic acid and 40% methanol. Destaining was done in a solution of 10% acetic acid and 20% methanol in water for overnight. Later, the gel was washed with ultrapure water for 10 minutes. All these procedure is done on the orbital shaker with a speed of 50 rpm.

Figure 3.4 A typical XCell SureLock™ Mini-Cell loaded with samples

3.6 HPLC Analysis

Analysis of protein in solution were carried out by reverse phase HPLC (RP-HPLC) using a C4 Jupiter column (300 Å, 250 × 4.6 mm i.d., 5 μm particle size; Phenomenex, Cheshire, UK) attached to the Waters ACQUITY UPLC H-Class. The flow rate was set at 1 mL/min. Two buffer solutions were prepared as followed: solvent A, 0.1% (wt/vol) trifluoroacetic acid in ultrapure water and solvent B, 0.085% (wt/vol) trifluoroacetic acid in ultrapure water: HPLC-grade acetonitrile (10:90, vol/vol). The column was equilibrated with 20% solvent B. The elution was performed as follows: 0 to 60.0 min, a linear gradient by increasing the concentration of solvent B from 20 to 50%; 60.0 to 65.0 min, 50% solvent B in isocratic mode; 65.0 to 65.5 min, 50 to 100% solvent B; 65.5 to 71.0 min, 100 % solvent B in isocratic mode. Absorbance was recorded at 214 nm. The identity of whey proteins was confirmed by comparison from the standard curve of mixture pure proteins.

CHAPTER 4

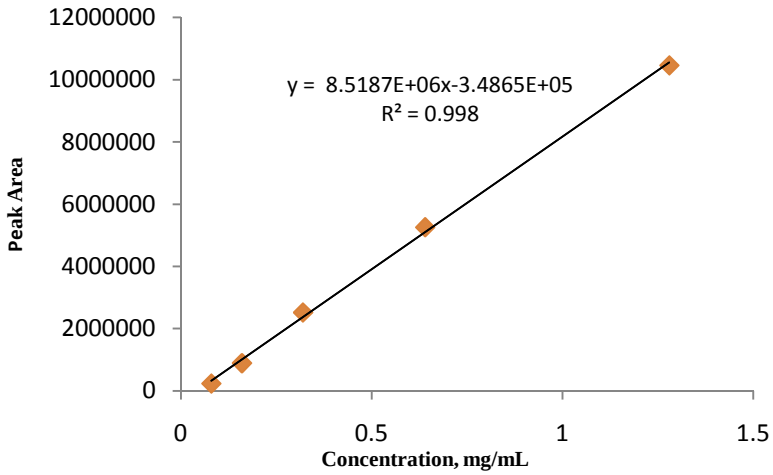
RESULTS AND DISCUSSIONS

4.1 Whey Protein Analysis

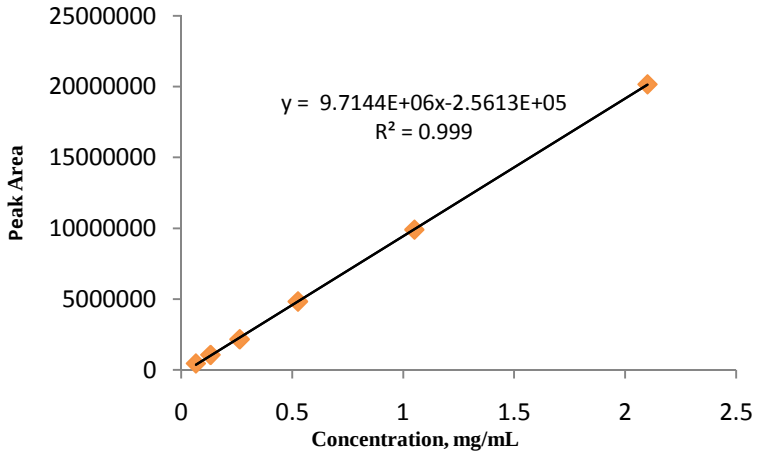
Graph of standard curve were developed for bovine serum albumin (BSA), α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lac), as shown in Figure 4.1, to determine the concentration of the protein obtained from HLPC analysis. Figure 4.2 shows an example of chromatogram for standard mixture of pure protein and protein fractions from whey batch fractionation according to specific protein retention time.

4.2 Batch Fractionation of Whey

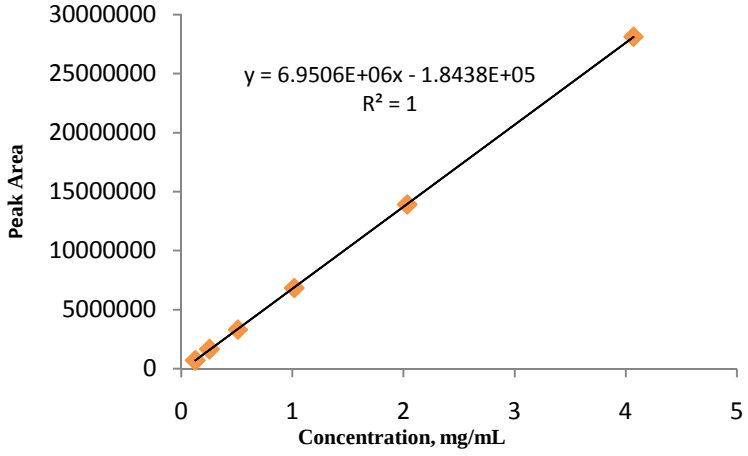
Batch fractionation of whey was performed using EVAL and cellulose acetate based MMM chromatography as shown in Table 4.1 and Table 4.2 respectively. Proteins of interest during this study are BSA, α-Lac, β-Lac and lactoferrin (LF). Both negatively (BSA, α-Lac, β-Lac) and positively (LF) charged proteins are expected to bind to this MMM chromatography since it contains cation (Lewatit CNP105) and anion (Lewatit MP 500) resin.



(a)



(b)



(c)

Figure 4.1 Graph of standard curve peak area versus concentration for (a) BSA (b) α-Lac and (c) β-Lac

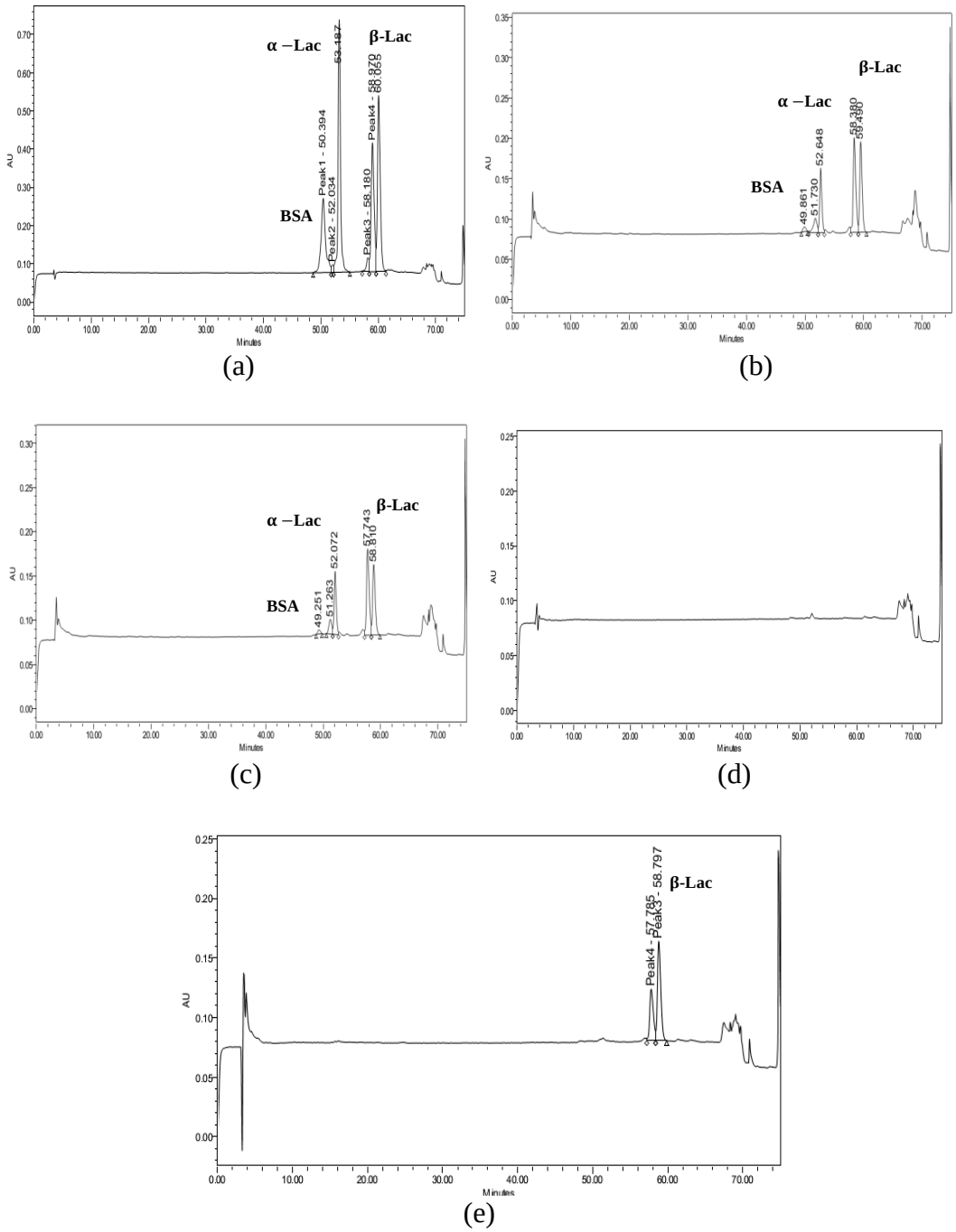


Figure 4.2 Typical chromatogram of (a) standard mixture of pure protein; (b) LF-spiked whey (4x dilution); EVAL based MMM; (c) After binding fraction (4x dilution); (d) Washing fraction; (e) Elution fraction

Table 4.1 Whey protein fractionation using EVAL based polymer MMM chromatography

Set	MMM mass (mg)	Protein type	Equilibrium concentration, (mL)	Total protein in feed solution (mg)	Protein bound to membrane (mg)	mg protein bound/g membrane	mg protein bound / g MP500	Protein elute after elution (mg)	Recovery (%)
1	27.1	BSA	2	0.486	0.116	4.280	10.071	0.000	0.000
		α-Lac	2	1.847	1.717	63.358	149.071	0.000	0.000
		β-Lac	2	8.112	6.798	250.849	590.207	1.219	92.803
2	28.8	BSA	2	0.486	0.124	4.306	10.131	0.000	0.000
		α-Lac	2	1.847	1.778	61.736	145.261	0.000	0.000
		β-Lac	2	8.112	6.683	232.049	545.997	1.275	89.246
3	30.4	BSA	2	0.486	0.127	4.178	9.830	0.000	0.000
		α-Lac	2	1.847	1.750	57.566	135.449	0.000	0.000
		β-Lac	2	8.112	6.459	212.467	499.923	1.414	85.568
Average		BSA			0.122	4.255	10.011		0
		α-Lac			1.748	60.887	143.260		0
		β-Lac			6.647	231.788	545.376		89.206

Table 4.2 Whey protein fractionation using cellulose acetate base polymer MMM chromatography

Set	MMM mass (mg)	Protein type	Equilibrium concentration, mL	Total protein in feed solution (mg)	Protein bound to membrane (mg)	mg protein bound/g membrane	mg protein bound/ g MP500	Protein elute after elution (mg)	Recovery (%)
1	42.0	BSA	2	0.486	0.123	2.929	6.891	0.000	0.0000
		α-Lac	2	1.847	1.760	41.905	98.599	0.000	0.0000
		β-Lac	2	8.112	7.325	174.405	410.364	0.592	75.275
2	41.2	BSA	2	0.486	0.115	2.791	6.568	0.000	0.0000
		α-Lac	2	1.847	1.698	41.214	96.973	0.000	0.0000
		β-Lac	2	8.112	7.316	177.573	417.818	0.651	81.752
3	39.2	BSA	2	0.486	0.125	3.189	7.503	0.000	0.0000
		α-Lac	2	1.847	1.727	44.056	103.661	0.000	0.0000
		β-Lac	2	8.112	7.349	187.474	441.116	0.585	76.624
Average		BSA			0.121	2.970	6.987		0
		α-Lac			1.728	42.392	99.744		0
		β-Lac			7.330	179.817	423.099		77.884

Proteins are positively charged in solutions at pH below it pI value and negatively charged above pI (Bhattacharjee et al., 2006). At running pH 6, BSA, α-Lac and β-Lac is a negative charged protein while LF is a positive charged protein. LF will bind to Lewatit CNP 105, cation exchanger while BSA, α-Lac and β-Lac will bind to Lewatit MP500 anionic resin.

The average percentage elution recovery of β-Lac for EVAL and CA based MMM are 89.206% and 77.884% each respectively. The membrane binding capacity of β-Lac for EVAL and CA based MMM are 231.788 mg protein bound/ g membrane and 179.817 mg protein bound/ g membrane respectively. The low binding capacity of CA showed that some portion of the proteins are bound by non-specific interactions to CA based MMM and this contribute to low recovery of β-Lac during elution. This non-specific binding may refer to hydrophobic interactions whereby once bound the interactions tends to be very strong and often an irreversible process. LF also bound to the mixed mode interaction MMM but is not able to detect by the current HPLC protocol used in this study. The binding of LF to the MMM will be proved by SDS-PAGE analysis, which will be discussed in next section.

There is zero recovery of BSA and α-Lac for both EVAL and CA based MMM. Most α-Lac was lost during the washing step as will be showed later in SDS-PAGE analysis. BSA was not detected by HPLC analysis probably due to the dilute concentration of BSA in elution fraction. Based on the research conducted by Goodall et al. (2008), the binding preference of three whey major proteins on an anion exchanger follows the order of β-Lac > BSA > α-Lac. Both of the MMM show selective binding of β-Lac over other proteins in whey. This is due to the distribution

of electrostatic charges on the protein surface. In α-Lac molecules, the large charge patch consists of a cluster of six positively charged groups which contribute to the strong reduction in electrostatic attraction between α-Lac and the Lewatit MP500 resin. So, it favors the competitive adsorption of β-Lac over other proteins in whey (De Vries, 2004).

Overall, the normalized binding capacities for major acidic whey proteins using EVAL based MMM are 10.011 mg BSA bound/ g MP500, 143.260 mg α-Lac bound/ g MP500 and 545.376 mg β-Lac/ g MP500 while for CA based MMM are 6.987 mg BSA bound/ g MP500, 99.744 mg α-Lac bound/ g MP500 and 423.099 mg β-Lac/ g MP500. This value is higher compared to equilibrium binding capacity of EVAL based MMM of β-Lac for MP500 ground resin of 300 mg β-Lac/ g MP500 by Saufi and Fee (2008). This might contribute to the large portion of small resin particles exist in current MMM compare to that study. Small size resin will contribute to high total surface area and enhance the protein binding in MMM chromatography.

4.3 SDS-PAGE Analysis

Qualitative analysis using gel electrophoresis was carried out to support the binding data above. Figure 4.3 shows the SDS-PAGE of the fractions involve in the batch fractionation of LF-spiked whey using mixed mode interaction MMM using EVAL and CA based membrane.

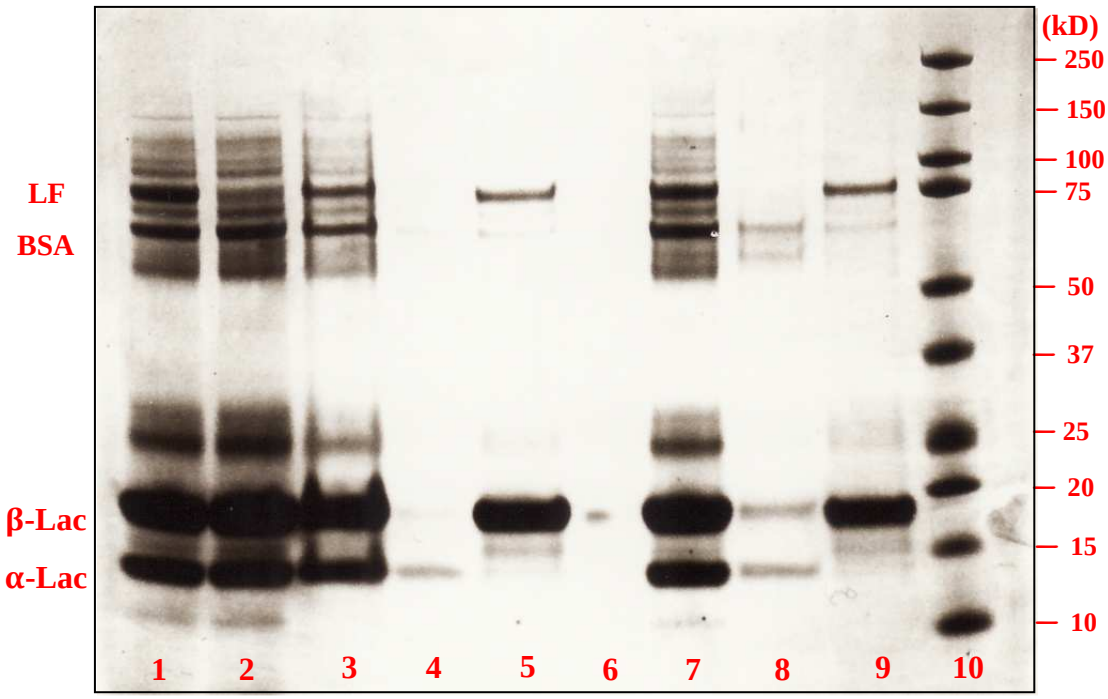


Figure 4.3: SDS-PAGE of the fractions involve in the batch fractionation of LF-spiked whey using mixed mode interaction MMM using EVAL and cellulose acetate based membrane. Lane 1- LF-spiked whey (4x dilution), Lane 2-Whey (4x dilution), EVAL-based MMM: Lane 3-after binding fraction (4x dilution), Lane 4-Washing fraction, Lane 5-Elution fraction, Lane 6-blank, CA-based MMM: Lane 7- after binding fraction (4x dilution), Lane 8- Washing fraction, Lane 9- Elution fraction, and Lane 10- protein marker

Lane 3, 4 and 5 represent the binding, washing and elution step for EVAL based MMM and Lane 7, 8 and 9 represent the binding, washing and elution step for CA based MMM. Both EVAL and CA show a simultaneous binding to acidic β-Lac protein and basic LF protein as showed in elution fraction in lane 5 and lane 9. The LF band can be clearly visualized in elution fraction of both MMM in lane 3 and lane 7. This is the evidence of LF binding to the MMM.

In both MMM, α-Lac was lost during the washing step as seen in lane 4 and 8. No α-Lac band was present in the elution fraction which is consistent with the zero recovery showed by HPLC analysis. BSA band is hardly can seen in elution fraction (lane 9) of CA based MMM while totally absence in EVAL elution fraction (lane 5).

Most probably the amount BSA eluted is very small and is not able to detect by HPLC analysis. Another possibility, the BSA still remains on the membrane after the elution. It is also observed that the binding strength of whey protein to CA is quite low based on the fact a lot of protein leached out during washing step as shown in lane 8.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Protein separations using mixed matrix membrane chromatography is the new emerging technology to replace the conventional packed bed chromatography. The concept of mixed matrix membrane preparation technique is simple with less complex process, reduces cost and no modifications of membranes using harsh chemicals involved during the preparation steps. In fact, it reduced the length of the packed bed column that is required to achieve the separations of protein interest.

In preparation of MMM chromatography, two potential polymer base membranes had been studied to determine feasibility of the membrane in protein separations which are EVAL and CA based polymer. From the result obtained, it can be concluded that EVAL base polymer MMM can recover β-Lac of 89.206% while CA based polymer can obtained a recovery of 77.884%. This shows that both MMM based polymer prepared in this study showed potential in selective binding of β-Lac.

Different polymer can be utilizes as the base polymer for the membrane preparation in whey protein separations. The normalized binding capacities for major acidic whey proteins using EVAL based MMM are 10.011 mg BSA bound/ g MP500, 143.260 mg α-Lac bound/ g MP500 and 545.376 mg β-Lac/ g MP500 while for CA based MMM are 6.987 mg BSA bound/ g MP500, 99.744 mg α-Lac bound/ g MP500 and 423.099 mg β-Lac/ g MP500. The performance of both of the membrane is of comparative in batch fractionation of whey protein.

Major acidic proteins are found bound to the anionic resin while lactoferrin bound to cationic resin as shown in the gel electrophoresis analysis. This is the evidence that simultaneous interactions of anion and cation exchange can be induced in a single membrane to separate protein in a single run

5.2 Recommendations

There are some suggestions or recommendations that need to be take in considerations to improve the future results of the batch fractionation whey and the performance of the MMM chromatography. The list of recommendations is outlined as follows:

1. Membrane casting plays an important role in the preparations of the mixed matrix membrane. A study on casting parameters such as speed of stirrer, temperature, resin loading, type of casting solution and type of resin can be carry out.

2. The size of the resins incorporated onto the membrane should be of finely shape. This will ensure homogenization and structure of the membrane. In the current study, particle size less than 0.45μm was used. Further study in the effect of particle size less than 0.45μm can be conducted to prepare MMM chromatography.
3. Fractionation of whey proteins was done in batch mode. Therefore, there should be a method to scale up for the requirement of industrial scale usage e.g. pilot plant.
4. Elution protocol or strategy can be study to determine the suitable elution gradient to obtain high purity of protein of interest from the bound membrane.
5. A method should be developed to determine the amount of the lactoferrin in sample.

REFERENCES

Amigo et al. 2008. β-lactoglobulin as source of bioactive peptides. *Amino Acids*. **35**: 257-265. DOI: 10.1007/s00726-007-0585-1.

Andersson, J. and Mattiasson, B. 2006. Simulated moving bed technology with a simplified approach for protein purification: Separation of lactoperoxidase and lactoferrin from whey protein concentrate. *Journal of Chromatography A.* **1107**: 88-95.

Atra, R., Vatai, G., Bekassy-Molnar E. and Balint A. 2005. Investigation of ultra- and nanofiltration for utilization of whey protein and lactose. *Journal of Food Engineering.* **67**: 325-332.

Avramescu, ME., Girones, M., Borneman, Z. and Wessling, M. 2003a. Preparation of Mixed matrix adsorber membranes for protein recovery. *Journal of Membrane Science.* **218** (1-2): 219-233

Avramsecu, ME., Borneman, Z. and Wessling M., 2003b. Mixed matrix membrane adsorbers for protein separation. *Journal of Chromatography A.* **1006** (1-2): 171-183.

Bhattacharjee, S., Bhattacharjee, C. and Datta, S. 2006. Studies on the fractionation of β- lactoglobulin from casein whey using ultrafiltration and ion exchange membrane chromatography. *Journal of Membrane Science.* **275**(1-2): 141-150.

Brans, G., Schroen, CGPH., Sman, RGMvd. and Boom, RM. 2004. Membrane Fractionation of milk: state of the art and challenges. *Journal of Membrane Science*. **243**: 263-272.

Brochier, V.B., Schapman, A., Santambien, P., and Britsch, L. 2008. Fast purification process optimization using mixed-mode chromatography sorbents in pre-packed mini-columns. *Journal of Chromatography A.* **1177**: 226-233.

Charcosset, C. 2006. Membrane processes in biotechnology: An overview. *Biotechnology Advance.* **24**: 482-492.

Chiu C.K and Etzel M.R. 1997. Fractionation of lactoperoxidase and lactoferrin from bovine whey using a cation exchange membrane. *Journal of Food Science*. **62**(5)

Cowan, S. and Ritchie, S. 2007. Modified polyethersulfone (PES) ultrafiltration membranes for enhanced filtration of whey proteins. *Separation Science and Technology*. **42** (11): 2405-2418. DOI: 10.1080/01496390701477212.

Daufin et al. 2001. Recent and emerging applications of membrane processes in the food and dairy industry. *Trans IChemE*. **79**:89-102.

De Vries R. 2004. Monte Carlo simulations of flexible polyanions complexing with whey proteins at their isoelectric point. *J.Chem. Phys.* **120** (7): 3475-3481. DOI:10.1063/1.1641003.

Drioli, E. and Fontananova, E. 2004. Membrane Technology and Sustainable Growth. *Icheme*. **82**: 1557-1562.

Doultani, S., Turhan K.N and Etzel MR. 2004. Fractionation of proteins from whey using cation exchange chromatography. *Process Biochemistry*. **3**9:1737-1743

El-Loly, MM. 2007. Bovine milk immunoglobulins in relation to human health. *International Journal of Dairy Science.* **2**(3): 183-195.

Etzel, MR., Helm, TR. and Vyas HK. 2008. Methods involving whey protein isolates. *US Patent 7378123*.

Etzel, MR. 2004. The emerging role of dairy proteins and bioactive peptides in nutrition and health. *The Journal of Nutrition.* **134**(4): 996s-1002s.

Fee, CJ and Chand, A. 2006. Capture of lactoferrin and lactoperoxidase from raw whole milk by ion exchange chromatography. *Separation and Purification Technology.* **48**(2):143-149.

Freitag, R., Splitt, H. and Reif, O-W. 1996. Controlled mixed-mode interaction chromatography on membrane adsorbers. *Journal of Chromatography A.* **728** (1-2): 129-137.

Gao, D., Lin, D. and Yao, S. 2007. Mechanistic analysis on the effects of salt concentration and pH on protein adsorption onto a mixed-mode adsorbent with cation ligand. *Journal of Chromatography B.* **85**: 16-23.

Garcia-Montoya, I.A., Cendon, T.S., Arevalo-Gallegos, S. and Rascon-Cruz, Q. 2012. Lactoferrin a multiple bioactive protein: An overview. *Biochimica et Biophysica Acta.* **1820**: 226-236.

Gerberding, S.J. and Byers CH. 1998. Preparative ion-exchange chromatography of proteins from dairy whey. *Journal of Chromatography A.* **808**: 141-151.

Ghosh, R. 2002. Protein separations using membrane chromatography: opportunities and challenges. *Journal of Chromatography A.* **952**: 13-27.

Goodall, S., Grandison A.S., Jauregi P.J., Price J. 2008. Selective separation of the major whey proteins using ion exchange membranes. *Journal of Dairy Science.* **91**(1):1-10

Hallgren, E., Kalman, F., Farnan, D. and Horvath, C. 2000. Protein retention in ion-exchange chromatography: effect of net charge and charge distribution. *Journal of Chromatography A*. **877**: 13-24.

Hurley, W.L. and Theil, P.K. 2011. Perspectives on immunoglobulins in colostrums and milk. *Nutrients.* **3**: 442-474. DOI:10.3390/nu3040442.

Kim J.I, Choi D.Y and Row K.H. 2003. Separation of whey proteins by anion-exchange membranes. *Korean J. Chem. Eng*. **20**(3):538-541

Klein, E. 2000. Afinity membranes: A 10-year review. *Journal of Membrane Science.* **179**: 1-27.

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

Langevin, ME., Roblet, C., Moresoli, C., Ramassamy, C., and Bazinet, L. 2012. Comparative application of pressure- and electrically-driven membrane processes for isolation of bioactive peptides from soy protein hydrolysate. *Journal of Membrane Science*. **403-404**: 15-24.

Lin, S.Y. and Suen, S.Y. 2002. Protein separation using plate-and-frame modules with ion exchange membranes. *Journal of Membrane Science.* **201** (1-2): 37-51.

Liu, Y., Sun, Xiaomei. & Li, Buhai. 2006. Separation of α- Lactalbumin and β-Lactoglobulin by a convenient liquid-solid extraction system: Application to milk whey. *Chem. Eng. Technol.* **29 No. 12**: 1424-1428. DOI: 10.1002/ceat.200600223

Marcello, P.A and Rizvi, Syed S.H. 2009. Applications of membrane technology in the dairy industry. In: Pabby AK, Rizvi SSH and Sastre AM, editors. Handbook of membrane separations: chemical, pharmaceutical, food and biotechnology applications . Fluorida, US: CRC Press. P 635-669.

Madureira, A.R., Alvarez, S., Menendez, C., Riera, F.A. and Alvarez, R. 2007. Bovine whey proteins – Overview on their main biological properties. *Food Research International.* **40**: 1197-1211.

Mcintosh, G.H., Regester, G.D., Lelue, R.K., Royle, P.J. and Smithers, GW. 1995. Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J Nutr.* **125:**809-816.

Monteiro, P.S., Coimba, JSDR., Minim, L.A., Oliveira, JAd., da Silva, LHM. 2008. Partition of α-lactoalbumin and β-lactoglobulin by cloud point extraction. *Journal of Chromatography B.* **867**(2): 189-193.

Oatley, D.L., Llenas, L., Perez, R., Williams, P.M., Martinez-Llado, X. and Rovira, M. 2012. Review of the dielectric properties of nanofiltration membranes and verification of the single orientated layer approximation. *Advances in Colloid and Interface Science*. **173:** 1-11.

Palmano K.P and Elgar D.F. 2002. Detection and quantitation of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *Journal of Chromatography A.* **947**:307-311.

Reis, R and Zydney, A. 2007. Bioprocess membrane technology. *Journal of Membrane Science*. 297: 16-50.

Rizvi, Syed S.H. 2010. *Separation, Extraction and concentration processes in the food, beverage and nutraceutical industries*. Woodhead Publishing Limited.

Roman, A., Wang, J., Csanadi, J., Hodur, C. & Vatai, G. 2011. Experimental Investigation of the sweet whey concentration by nanofiltration. *Food Bioprocess Technol.* **4**:702-709. DOI 10.1007/s11947-009-0192-0.

Rosenberg, M. 1995. Current and future applications for membrane processes in the dairy industry. *Trends in Food Science & Technology*. **6**: 12-19.

Saiful, Z. Borneman and Wessling, M. 2006. Enzyme capturing and concentration with mixed matrix membrane adsorbers. *Journal of Membrane Science.* **280**: 406-417.

Santos, MJ, Teixeira, J.A and Rodrigues L.R. 2012. Fractionation of the major whey proteins and isolation of β -lactoglobulin variants by anion exchange chromatography. *Separation and Purification Technology*. **90**: 133-139.

Saufi, S.M and Fee C.J. 2009. Fractionation of β-Lactoglobulin from whey by mixed matrix membrane ion exchange chromatography. *Biotechnology and Bioengineering*. **103**: 138-147

Saufi, S.M and Fee, C.J. 2011. Simultaneous anion and cation exchange chromatography of whey proteins using a customizable mixed matrix membrane. *Journal of Chromatography A.* **1218**:9003-9009.

Saxena, A., Tripathi, BP., Kumar, M. and Shahi, VK. 2009. Membrane based techniques for the separation and purification of proteins: An overview. *Advances on Colloid and Interface Science.* **145**: 1-22.

Seifu, E., Buys, E.M. and Donkin, E.F. 2005. Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review. *Trends in Food Science & Technology*. **16**:137-154.

Smithers, G.W. 2008. Whey and whey proteins from 'gutter-to-gold'. *International Dairy Journal*. **18**(7): 695-704.

Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S. and Hayasawa, H. 2002. Bovine lactoferrin and lactoferricin derived from milk: Production and applications. *Biochem. Cell Biol*. **80**: 109-112. DOI:10.1139/O01-230.

Turhan, KN. and Etzel, MR. 2004. Whey protein isolate and α-lactalbumin recovery from lactic acid whey using cation-exchnage chromatography. *Journal of Food Science*. **69**(2): 66-70.

Watanabe, S., Murata, S., Kumura, H., Nakamura, S., Bollen, A., Moguilevsky, N. and Shimazaki, K. 2000. Bovine lactoperoxidase and its recombinant: Comparison of structure and some biochemical properties. *Biochemical and Biophysical Research Communications.* **274:**756-761. DOI:10.1006/bbrc.2000.3217.

Ye, X., Yoshida, S. and Ng, T.B. 2000. Isolation of lactoperoxidase, lactoferrin, α – lactalbumin, β-lactoglobulin B and β-lactoglobulin A from bovine rennet whey using ion exchange chromatography. *The International Journal of Biochemistry & Cell Biology*. **32**: 1143-1150.

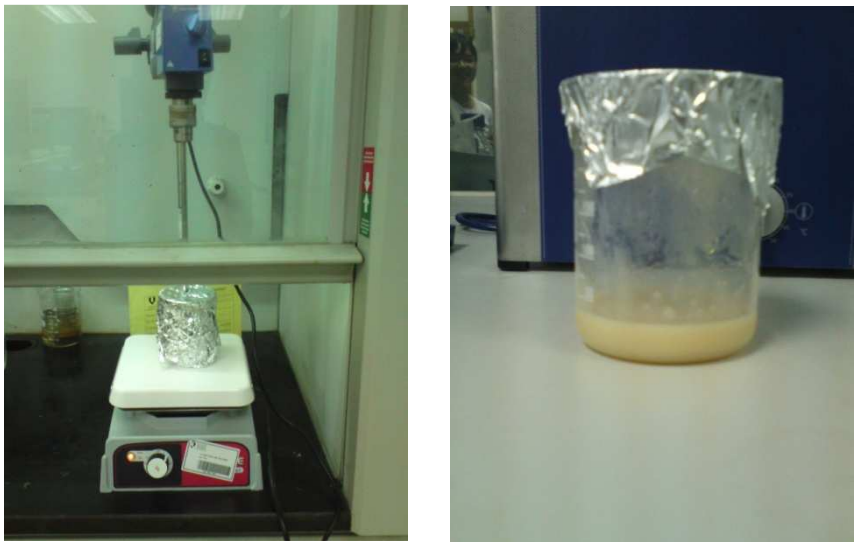
Zadow, J.G. 2004. *Utilization of milk components: Whey*. In R. K. Robinson (Ed.), Modern dairy technology, advances in milk processing, **Vol. 1** (2nd ed.):pp. 313-373. London, UK: Chapman & Hall.

Zhou, X., Xue, B., Bai, S. and Sun, Y. 2002. Macroporous polymeric ion exchanger of high capacity for protein adsorption. *Biochemical Engineering Journal.* **11:** 13-17.

Zou, H., Luo, Q. and Zhou D. 2001. Affinity membrane chromatography for the analysis and purification of proteins. *J. Biochem. Biophys. Methods.* **49**:199-240.

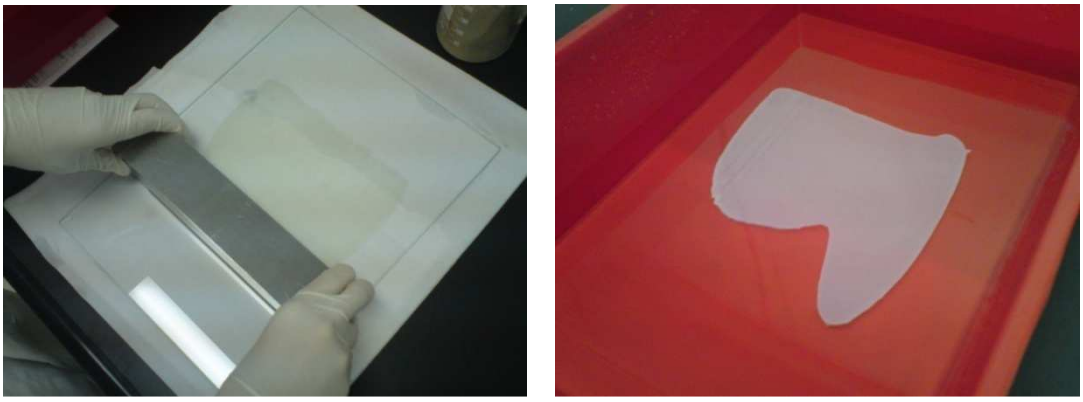
Zydney, AL. 1998. Protein separations using membrane filtration: New opportunities for whey fractionation. *International Dairy Journal.* **8**: 243-250.

APPENDIX A Mixed Matrix Membrane Preparation



(a) (b)

(c) (d)



(e) (f)

Figure A.1 Illustration of mixed matrix membrane preparation with (a) continuous stirring of casting solution under 60°C (b) casting solution readied for ultrasonic (c) casting solution poured onto the glass plate support (d), (e) stainless steel block was used to spread the casting solution and (f) MMM was immersed into the water bath for solvent exchange