DEVELOPMENT OF MULTIPLE INTERACTIONS MIXED MATRIX MEMBRANE CHROMATOGRAPHY FOR PROTEIN FRACTIONATION IN CHICKEN EGG WHITE

NURUL SHAMSINAR BINTI SHAHIRIN

Thesis submitted in fulfillment of the requirements for the award of the degree of Master of Engineering in Chemical Engineering

Faculty of Chemical and Natural Resources Engineering

UNIVERSITI MALAYSIA PAHANG

JANUARY 2015

ABSTRACT

Fractionation of individual chicken egg white (CEW) proteins such as conalbumin (CNL), ovalbumin (OVL) and lysozyme (LYS) benefits the food, pharmaceuticals or nutraceuticals industry. Although packed bed chromatography is commonly used for protein separation, it still has a few limitations that need to be overcome. Membrane chromatography, which uses adsorptive membrane, is one alternative to overcome the limitation of packed bed column chromatography. Mixed matrix membrane (MMM) preparation concept provides a simple method for the preparation of membrane chromatography. In the current study, several potential low cost cation resins were screened to bind LYS at different pH. At pH 7, Lewatit CNP105 was selected for the preparation of cation exchanger MMM in the first part of study. The Lewatit CNP105 – cation exchanger MMM gave a Langmuir adsorption isotherm with the constant of 223 mg LYS/g membrane for q_m and 0.045 for k_d for single LYS solution. In batch binding of CEW solution at pH 7, both positively charged CEW protein (LYS, pI 10.7) and negatively charged CEW proteins (CNL, pI 6.1 and OVL, pI 4.5) were found to bind onto cation exchanger MMM. However, CNL and OVL were loosely bound and washed out during the washing step. Almost pure LYS can be recovered during the elution step of cation exchanger MMM chromatography.Multiple interactions MMM that combined Lewatit CNP105 cation resin and Lewatit MP500 anion resin, weretested to bind single protein and CEW solution in batch adsorption. In single protein experiment, the binding capacity of LYS, CNL and OVL are 52.3521 mg LYS/g membrane, 91.0453 mg CNL/g membrane and 10.8491 mg OVL/g membrane, respectively. The binding capacity of protein decreased in real CEW solution except for OVL. The reduction of the amount of protein binding might be due to the competitive binding with other impurities in CEW solution. For flow through experiment of multiple interactions MMM, three elution protocols were tested which were isocratic salt elution, linear gradient salt elution and combination of pH 3 and salt elution. Although no single protein fraction was recovered from these elution strategies tested, these fractions can be selected for further purification or used based on their targeted protein composition.

ABSTRAK

Pengasingan proteindaripada putih telur ayamseperti conalbumin (CNL), ovalbumin (OVL) dan lysozyme (LYS) dapat memberi manfaat kepada industri makanan, farmaseutikal atau nutraseutikal. Walaupun kromatografi lapisan terpadat biasanya digunakan untuk pemisahan protein, ia masih mempunyai beberapa kelemahan yang perlu diatasi. Membran jenis kromatografi iaitumembran yang mempunyaisifat penjerapan adalah salah satu pilihan bagi mengatasi kelemahan kromatografi lapisan terpadat.Konsep penyediaan campuran bahan membran (MMM) boleh menyediakan kaedah yang mudah untuk penyediaan membran jenis kromatografi. Dalam kajian ini, beberapa jenis resin penukar cas positif yang berharga lebih murah telah disaring untuk menjerap LYS pada pH yang berbeza. Di bahagian pertama tesis,pada pH 7, Lewatit CNP105 telah dipilih sebagai penukar cas positif untuk penyediaan MMM. Lewatit CNP105 - MMM penukar cas positif memberikan isoterma penjerapan jenis Langmuir dengan nilai 223 mg LYS / g membran untuk q_m dan 0.045 untuk $k_d$menggunakan larutan protein tunggal LYS. Dalam eksperimenpengasinganlarutan CEW secara kumpulan pada pH 7, semua protein bercas positif (LYS, pI 10.7) dan bercas negatif (CNL, pI 6.1 dan OVL, pI 4.5) didapati mengikat pada MMM penukar cas positif. Walau bagaimanapun, CNL dan OVL mempunyai ikatan yang lemah dan tertanggal semasa langkah pembasuhan.LYS berketulenan tinggi diperolehi dalam langkah pelepasan ikatan protein.MMMpelbagai interaksi yang mengabungkan Lewatit CNP105 penukar cas positif dan Lewatit MP500 penukar cas negatif, telah diuji untuk mengikat protein tulen dan larutan CEW. Dalam eksperimenmenggunakan protein tulen, kapasiti pengikatan untuk LYSadalah 52,3521 mg LYS / g membran, CNL-91,0453 mg CNL / g membran dan OVL- 10,8491 mg OVL / g membran. Kapasiti pengikatan protein berkurangan dalam larutan sebenar CEW kecuali OVL.Pengurangan jumlah pengikatan protein mungkin di sebabkan olehpengikatan secara kompetitif protein dengan bahan-bahan lain yang terdapat dalam larutan CEW.Untuk eksperimen secara berterusan menggunakan MMMpelbagai interaksi, tiga protokol elusi telah diuji iaitu elusiisokratik garam, elusi garam secara berkadar terus dan gabungan pH 3 dan elusi garam.Walaupun tiada pecahan protein tunggal yang diperolehi daripada ketiga-tigastrategi elusi diuji, tetapi pecahan ini boleh dipilih untuk penulenan lanjut atau digunakan berdasarkan komposisi protein sasaran mereka.

TABLE OF CONTENTS

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

Wr	Weight resin
W_p	Weight polymer
R	Resin loading
q	Mass of protein bound per gram of adsorbent at equilibrium
c	Equilibrium constant
K	Freundlich constant
n	Freundlich constant
q_m	Maximum binding capacity
k_d	Langmuir dissoaciation
µm	micrometer
mm	milimeter
g	gram
mg	miligram
Da	Dalton
kDa	kiloDalton
mM	milimolar
µl	microliter
R^2	Coefficient of determination
log	Logarithm
g	Unit of graviti

LIST OF ABBREVIATIONS

ACN	Acetonitrile
BSA	Bovine serum albumin
CEW	Chicken egg white
CEX	Cation exchanger
CNL	Conalbumin
CV	Column volume
DAD	Diode Array Detector
DMSO	Dimethylsulfoxide
EVAL	Ethylene vinyl alcohol
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
LYS	Lysozyme
M	Molar
M5	Mixed mode mixed matrix membrane
MMM	Mixed matrix membrane
NaCl	Sodium chloride
OVL	Ovalbumin
pI	Isoelectric point
RPC	Reverse Phase Chromatography
SDS-PAGE	Sodium Dodecylsulfate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscope
TFA	Trifluoroacetic acid

UPLC	Ultra High Performance Liquid Chromatography
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Research Background

Separation process is very critical in downstream processing of biotechnology product. Most of the times, the targeted products have similar chemical and physical properties with the impurities. Separations of biomolecules (proteins, enzyme, etc.) have to be mild and gentle since they are susceptible to denature or degrade in harsh conditions. In addition, it becomes extremely challenging when the targeted products are present at low concentration. The demand for single protein with high purity, to be used in specific application, is the driving force for scientists and engineers to look forward to have more selective separation processes.

Selection of separation techniques normally varies based on size, charge, hydrophobicity and affinity of the proteins. Common bioseparation processes consist of a series of different technique for each step of impurities removal, product isolation, product purification and polishing. Chromatography based separation is among the promising and widely used techniques for each step above. In chromatography, the feed containing the solute of interest flows through a chromatography resin packed into a cylindrical column. The solute diffuses by pore diffusion through the pores of the resin and interacts with the binding sites at the surface of the resin pores.

Conventional packed bed chromatography however suffers several disadvantages such as high pressure drop across the bed and it tends to increase during the operation. In addition, the transportation of solute by pore diffusion within the resin beads is often slow that it increases the processing time and possible degradation of

fragile biological product molecules. The scale up of packed bed chromatography column is also difficult (Wickramasinghe et al., 2006). In some cases, if the column is not packed properly, non-uniform resin distribution can lead to feed flow channeling and give a poor separation.

Membrane chromatography, which uses adsorptive membrane is one of the alternatives to overcome the limitation of packed bed column chromatography. Chromatographic membranes are modified with specific ligands or functional groups similar to the chromatography resin. Membrane chromatography allows fast binding, since the adsorptive ligands are contained directly within the convective flow path through the membrane pores. Generally, membrane chromatography system has pore sizes in microfiltration range. Since there are no separation based on molecular size, the system allows high flow rates, low pressure drops, easy column packing and scale-up, as well as low clogging tendency, which makes the system ideal for large-scale separation of proteins and enzymes (Saufi and Fee, 2009;Kawai et al., 2003; Zou et al., 2001). Various modes of membrane chromatography have been developed such as hydrophobic interaction (Ghosh and Wang, 2006), affinity (Ruckenstein and Guo, 2001) and ion exchange (Rao, 2001).

Chemical modification for preparing membrane chromatography may affects the quality of base membrane since harsh chemical might be involved during the attachment of functional group for specific types of chromatography. Major consequences are undesirable and irreversible changes in the membrane structure (Liu and Bai, 2006). As an alternative approach, Wessling's group had proposed the use of mixed matrix membrane (MMM) concept for preparing membrane chromatography material (Avramescu et al., 2003;Saiful et al., 2006). MMM chromatography was prepared by incorporating any adsorptive resin into a membrane polymer solution prior to membrane casting. This method eliminates the needs for chemical modification to induce specific functional group in the membrane. The development of MMM chromatography in protein separation gives high impression to the downstream process of biotechnology industry. Various types of cation and anion exchanger was applied in the last past years to separate different types of biomolecules (Saufi and Fee, 2010; Graselli et al., 2008; Ulbricht and He, 2008; Suen et al., 2004).

Recently Saufi and Fee (2011b) had expanded the MMM concept to prepare mixed mode mixed matrix membrane (M5) chromatography in a single membrane sheet. They had combined high cost SP Sepharose (cation resin) with low cost Lewatit MP500 (anion resin) in ethylene vinyl alcohol (EVAL) based membrane chromatography. M5 membrane was used for simultaneous binding of major whey proteins (α-lactalbumin, lactoferrin, β-lactoglobulin and immunoglobulin). However, the SP Sepharose cation resin used by them is a specialized resin that is designed for high resolution protein separation and is very costly. Therefore in order to prepare low cost mixed mode MMM with competitive performance, an alternative low cost cation resin was screened in this study. Furthermore, to prove that this kind of mixed mode MMM can be customized for targeted feed stream, the membrane was used for fractionation of major chicken egg white (CEW) protein. CEW proteins consistsof three major proteins which are 54% ovalbumin (OVL), 12% conalbumin (CNL) and 3.4% lysozyme (LYS). These three major proteins are worth to separate due to their own benefit in pharmaceuticals, foods and nutraceuticals industries.

1.2 Problem Statement

An increasing demand for high purity of bioproducts is the driving force to have a more selective separation processes in biotechnology. The separation process contributed to the major cost of bioproducts manufacturing. Although packed bed chromatography is commonly used for protein separation, it still has few limitations that need to be overcome. High pressure drop, slow intra-bed mass transport, long process time and complicated in scale-up are among the limitations of packed bed chromatography. Membrane chromatography, which uses adsorptive membrane is one alternative to overcome the limitation of packed bed column chromatography. However, it is still under developed worldwide and only a few commercial membrane chromatographyis available in the market. It is crucial to develop this membrane chromatography material as alternative separation technique in the protein downstream processing.

Three steps are usually involved in the preparation of membrane chromatography: (1) preparation of the base membrane, (2) chemical activation of the

base membrane and (3) coupling of ligands to the activated membrane (Zeng and Ruckenstein, 1999). However harsh chemical conditions used in the above steps can cause undesirable and irreversible changes in the membrane chromatography structure. MMM preparation concept provides a simple method for the preparation of membrane chromatography. Various types of single interaction MMM chromatography has been developed in the past decade until recently Saufi and Fee (2011b) has expanded this concept to prepare multiple interactions chromatography or mixed mode in MMM. This mixed mode MMM chromatography is able to bind different type of proteins simultaneously in a single run according to the resin functionality in the MMM. This type of MMM is new, still under development and there will a lot of possibility to be explored.

1.3 Objective of the Research

The objectives of this research are:-

a. To obtain low cost cation exchanger resin for MMM preparation.
b. To prepare and characterize cation MMM and multiple interaction MMM
c. To develop elution strategies for multiple interactions MMM chromatography based on EVAL membrane for fractionation of major CEW proteins of LYS, CNL and OVL.

1.4 Scopes of the Research

Several scopes were outlined to achieve the above objectives of the research. There are listed as below:

i. Screening of six potential low cost cation resin (Amberlyst 15 wet, Amberlyst 15 dry, Amberlite IR120, Dowex M-31, Dowex Marathon and Lewatit CNP105) at different pH range of pH 5 to pH 11. The cation resins were tested for binding with pure lysozyme solution.

ii. Preparation and characterization of pure cation MMM chromatography for isolation of LYS in CEW solution. The binding adsorption isotherm of the membrane was determined and batch adsorption experiment was conducted for isolation of LYS from CEW solution.

iii. Preparation and characterization of multiple interactions MMM by incorporating Lewatit MP500 (anion resin) and Lewatit CNP105 (cation resin). The multiple interactions MMM were used for simultaneous binding of acidic and basic proteins from CEW using batch adsorption and flowthrough adsorption.

iv. Three elution strategies were used to recover the bound protein from multiple interactions MMM which are isocratic salt elution, linear gradient salt elution and combination of pH 3 and salt elution.

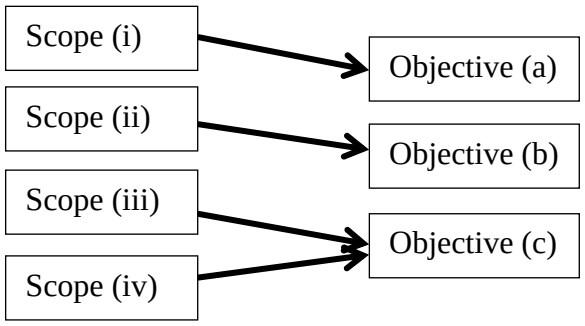

Figure 1.1: Relationship between objectives and scopes for this work.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein

Proteins are often considered to be the central compound necessary for life. Generally, proteins are large, complex ingredients that play varies critical roles in the body. Protein moleculesare made up from a series of amino acids that connected together through peptide bond to form a complex protein structure. The basic structure of amino acid was shown in Figure 2.1 that consist amino group in one end and carboxyl group on the other end. There also alpha carbon that links various types of functional groups (R) to give the different functionality of the amino acid and protein formed.

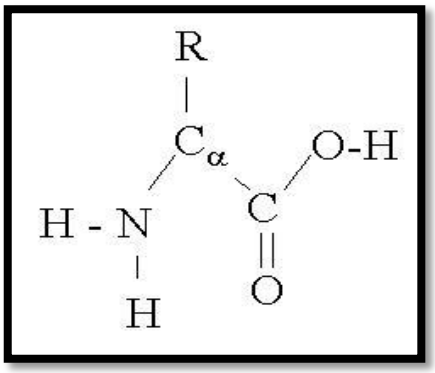

Figure 2.1: Basic structure of amino acid

The protein structure can be divided into four categories which are primary, secondary, tertiary and quaternary structure as shownin Figure 2.2.

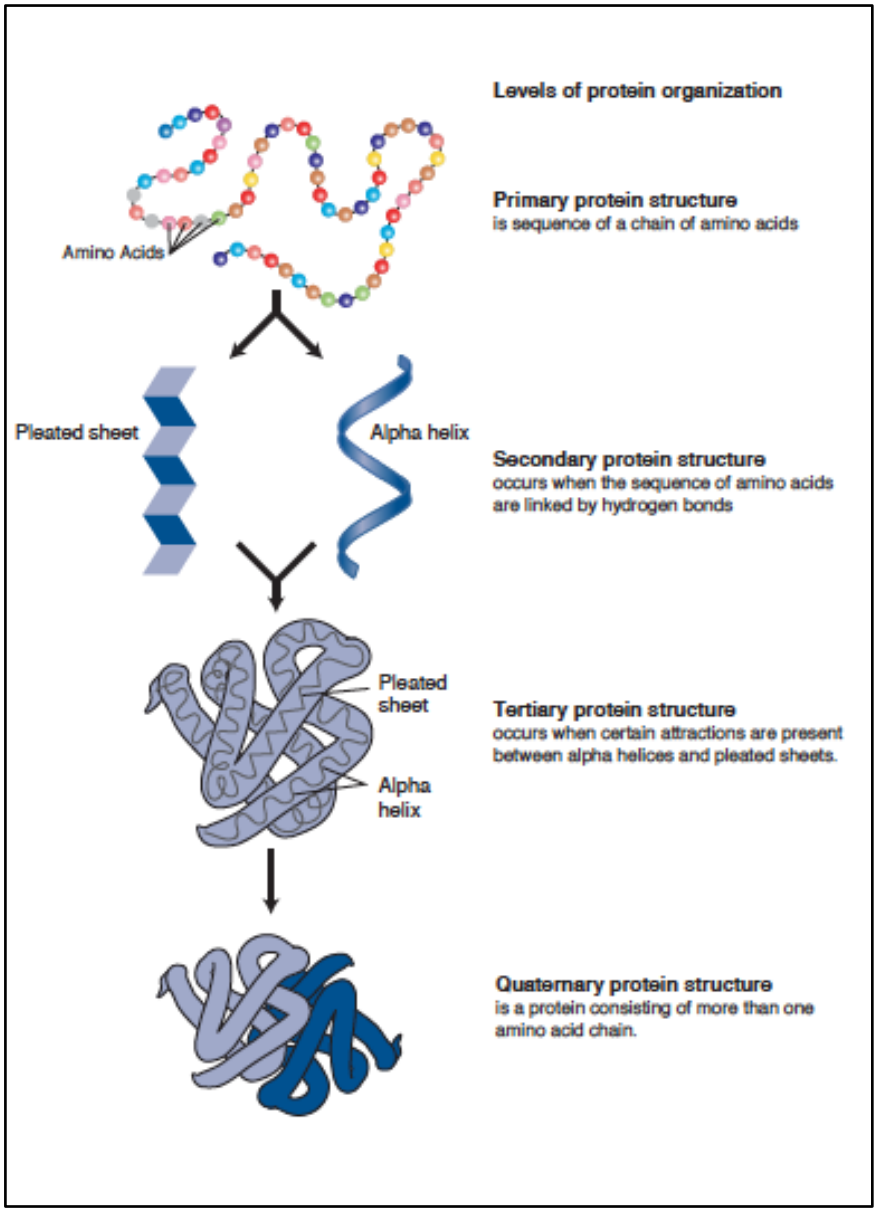

Figure 2.2: Four categories of protein structure

Source: Leja, D., National Human Genome Research Institute

2.2 Protein Separation

Protein can be separated by different techniques based on the manipulation of protein properties such as size, solubility, charge and biological activity. Protein separation or purification is needed due to following purposes: 1) requirement of high purity; 2) concentration enrichment; 3) removal of specific impurities (e.g. toxin from therapeutic products); 4) prevention of catalysis other than the desired type (as with

enzyme); 5) prevention of catalysis poisoning (as with enzyme); 6) recommended product specifications; 7) enhancement of protein stability; and 8) reduction of protein denaturation (Saxena et al., 2009).

In biotechnology industry, high value single proteins are abundant in downstream processing but they were overlooked since they are in bulk mixtures within impurities. Critically, these impurities have almost similar physical and chemical properties with the target protein. Hence, the separation becomes more challenging. A more selective and high performance protein purification is required for the target proteins that ispresent in very low concentrations in the feed stream. If possible extremes physicochemical conditions changes on pH or ionic strengthsneed to be avoided to purify the fragile protein that tends to denature and degrade easily. Table 2.1compared several common techniques that have been applied in bioseparation in terms of resolution and throughput.

2.3 Chromatography Based Separation

In chromatography process, the separation occurred due to the different degree of interaction between the components to be separated with the chromatography media. Normally, the feed mixture (containing analytes) is dissolved in a fluid (gas or liquid) thatis known as mobile phase. The mobile phase transports the mixtures throughchromatography media or called as stationary phase. Normally, the stationary phase was packed into column of plastic, glass or stainless steel. Separation by chromatography depends on differential interactions of solutes onto the stationary phase. Table 2.2 listed several advantages and disadvantages of chromatographicseparation techniques. Chromatography process can be classified into several types of mode interactions as shown in Table 2.3.

Table 2.1: Comparison of common bioseparation techniques

Techniques	High resolution	High throughput	Low resolution	Low throughput
Adsorption		√	√	
Affinity separation	√			√
Centrifugation		√	√	
Chromatography	√			√
Counter current extraction (multistage)	√			√
Electrophoresis	√			√
Filtration		√	√	
Microfiltration		√	√	
Precipitation		√	√	
Solvent extraction (single stage)		√	√	
Supercritical fluid extraction (Singh and Singh, 1996)		√	√	
Ultracentrifugation (Shiragami and Kajiuchi, 1991)	√			√
Ultrafiltration		√	√	

Source: Handbook of Bioseparations (Ahuja, 2000)

Table 2.2: Advantages and disadvantages of chromatography based separation (Curling, 2007)

Advantages	Disadvantages
Operate at low operating temperatures	High operating pressures may be required.
Large scale batch or continuous operation	Over-loading of columns with feed material may cause incomplete separation
Able to separate materials based on size and/or chemical properties	Non-uniform column packing can lead to a significant decrease in separation performance
Variety and flexible in separation method	Process scale-up is complicated
High purity product	Irreversible adsorption of materials creates problems

Table 2.3: Modes of chromatography for protein separation (Curling, 2007)

Chromatographic method	Interaction/separation based
Gel filtration or size exclusion	Size and shape
Ion exchange	Net charge and charge distribution
Chromatofocusing	Isoelectric point
Hydrophobic interaction and reversed-phase chromatography	Hydrophobicity
Immobilized metal ion affinity chromatography	Metal binding
Affinity chromatography	Biospecific affinities for ligands, inhibitors, receptors, antibodies, etc.

2.4 Ion ExchangerChromatography

Ion exchanger chromatography is among the most efficient type of chromatography techniques used for protein separation. It had a broad applicability, gives high resolution separation and applicable in large scale process. In addition,

proteins could maintain their native conformation since elution takes place under mild conditions in ion exchanger chromatography (DePhilips and Lenhoff, 2001; Saiful et al., 2006).

The separation in ion exchange is due to interactions between proteins of different surface charges with oppositely charged groups on the ion exchanger stationary phase as shown in Figure 2.3. Ion exchange mediacan be regenerated and reused by altering the pH or ionic strength of solution to elute the bound protein. Proteins are complex ampholytes that have both positive and negative charges. Proteins charges are depend on isoelectric point (pI) at which the pH that gives the net charge of protein is zero. At pH above the pI, the net protein charges is negative and binding will be occurred to the positively anion resin. Below pI, the protein is positively charges and binds to negatively cation resin.

As shown in Figure 2.3, ion exchange chromatography is operated in four main steps which are equilibrium, sample application and washing, elution and regeneration step. The stationary phase is firstly equilibrated with the binding buffer to the desired start condition. During sample application, oppositely charged proteins bind to the stationary matrix and become concentrated onto the matrix surface. Unbound proteins (i.e neutral or same charges with stationary matrix) are washed out through the column. Elution steps were takes place by increasing ionic strength of buffer to recover the bound proteins onto matrix surface. The sequence of protein eluted depending on their strength of interaction with the matrix. The final step of regeneration removes all molecules that still bound on the matrix and to ensure the full capacity of stationary matrix for the next chromatography run.

Ion exchanger stationary matrix can be divides into several group according on how much the ionization state of the functional groups vary with pH. A strong ion exchanger has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. Table 2.4 shows the classification of ion exchanger matrix.

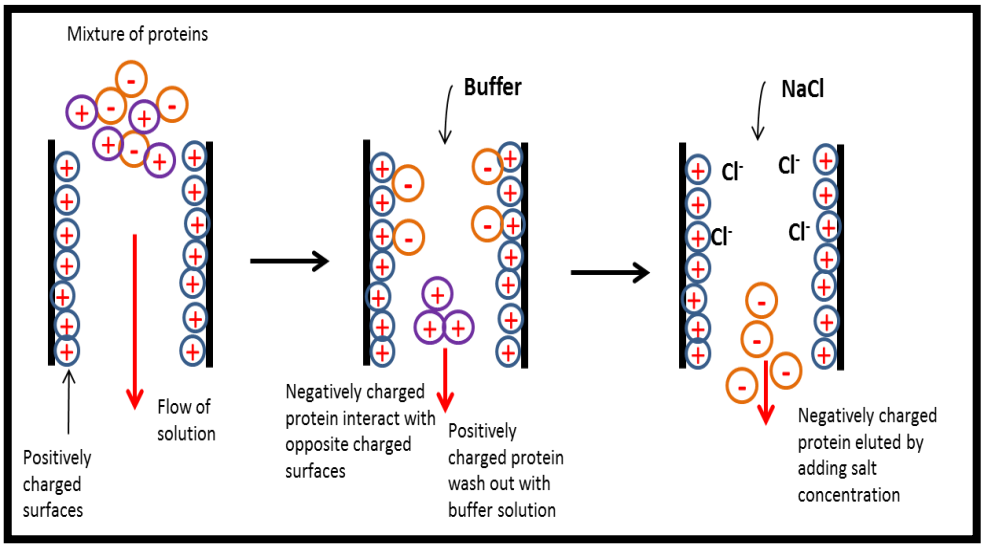

Figure 2.3: Mechanisms of ion exchanger

2.5 Membrane Based Separation

Membrane based separation is widely used in separation process. Some applications consist of product concentration, solute fractionation, solute removal from solution (desalination, demineralization), product sterilization (removal of bacteria and virus particles), purification and clarification.A membrane is a thin semi-permeable barrier that can selectively permit specific component to pass through it. As shown in Figure 2.4, the feed is force through the membrane into a retentate and permeate stream. Specific driving force is required to transport the component through the membrane such as pressure, concentration or electric fielddifference. Table 2.5 shows the membrane classification according to the type of driving force, molecular size or common application.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Separation process is very critical in downstream processing of biotechnology product. Most of the times, the targeted products have similar chemical and physical properties with the impurities. Separations of biomolecules (proteins, enzyme, etc.) have to be mild and gentle since they are susceptible to denature or degrade in harsh conditions. In addition, it becomes extremely challenging when the targeted products are present at low concentration. The demand for single protein with high purity, to be used in specific application, is the driving force for scientists and engineers to look forward to have more selective separation processes.

Selection of separation techniques normally varies based on size, charge, hydrophobicity and affinity of the proteins. Common bioseparation processes consist of a series of different technique for each step of impurities removal, product isolation, product purification and polishing. Chromatography based separation is among the promising and widely used techniques for each step above. In chromatography, the feed containing the solute of interest flows through a chromatography resin packed into a cylindrical column. The solute diffuses by pore diffusion through the pores of the resin and interacts with the binding sites at the surface of the resin pores.

Conventional packed bed chromatography however suffers several disadvantages such as high pressure drop across the bed and it tends to increase during the operation. In addition, the transportation of solute by pore diffusion within the resin beads is often slow that it increases the processing time and possible degradation of

fragile biological product molecules. The scale up of packed bed chromatography column is also difficult (Wickramasinghe et al., 2006). In some cases, if the column is not packed properly, non-uniform resin distribution can lead to feed flow channeling and give a poor separation.

Membrane chromatography, which uses adsorptive membrane is one of the alternatives to overcome the limitation of packed bed column chromatography. Chromatographic membranes are modified with specific ligands or functional groups similar to the chromatography resin. Membrane chromatography allows fast binding, since the adsorptive ligands are contained directly within the convective flow path through the membrane pores. Generally, membrane chromatography system has pore sizes in microfiltration range. Since there are no separation based on molecular size, the system allows high flow rates, low pressure drops, easy column packing and scale-up, as well as low clogging tendency, which makes the system ideal for large-scale separation of proteins and enzymes (Saufi and Fee, 2009;Kawai et al., 2003; Zou et al., 2001). Various modes of membrane chromatography have been developed such as hydrophobic interaction (Ghosh and Wang, 2006), affinity (Ruckenstein and Guo, 2001) and ion exchange (Rao, 2001).

Chemical modification for preparing membrane chromatography may affects the quality of base membrane since harsh chemical might be involved during the attachment of functional group for specific types of chromatography. Major consequences are undesirable and irreversible changes in the membrane structure (Liu and Bai, 2006). As an alternative approach, Wessling's group had proposed the use of mixed matrix membrane (MMM) concept for preparing membrane chromatography material (Avramescu et al., 2003;Saiful et al., 2006). MMM chromatography was prepared by incorporating any adsorptive resin into a membrane polymer solution prior to membrane casting. This method eliminates the needs for chemical modification to induce specific functional group in the membrane. The development of MMM chromatography in protein separation gives high impression to the downstream process of biotechnology industry. Various types of cation and anion exchanger was applied in the last past years to separate different types of biomolecules (Saufi and Fee, 2010; Graselli et al., 2008; Ulbricht and He, 2008; Suen et al., 2004).

Recently Saufi and Fee (2011b) had expanded the MMM concept to prepare mixed mode mixed matrix membrane (M5) chromatography in a single membrane sheet. They had combined high cost SP Sepharose (cation resin) with low cost Lewatit MP500 (anion resin) in ethylene vinyl alcohol (EVAL) based membrane chromatography. M5 membrane was used for simultaneous binding of major whey proteins (α-lactalbumin, lactoferrin, β-lactoglobulin and immunoglobulin). However, the SP Sepharose cation resin used by them is a specialized resin that is designed for high resolution protein separation and is very costly. Therefore in order to prepare low cost mixed mode MMM with competitive performance, an alternative low cost cation resin was screened in this study. Furthermore, to prove that this kind of mixed mode MMM can be customized for targeted feed stream, the membrane was used for fractionation of major chicken egg white (CEW) protein. CEW proteins consistsof three major proteins which are 54% ovalbumin (OVL), 12% conalbumin (CNL) and 3.4% lysozyme (LYS). These three major proteins are worth to separate due to their own benefit in pharmaceuticals, foods and nutraceuticals industries.

1.2 Problem Statement

An increasing demand for high purity of bioproducts is the driving force to have a more selective separation processes in biotechnology. The separation process contributed to the major cost of bioproducts manufacturing. Although packed bed chromatography is commonly used for protein separation, it still has few limitations that need to be overcome. High pressure drop, slow intra-bed mass transport, long process time and complicated in scale-up are among the limitations of packed bed chromatography. Membrane chromatography, which uses adsorptive membrane is one alternative to overcome the limitation of packed bed column chromatography. However, it is still under developed worldwide and only a few commercial membrane chromatographyis available in the market. It is crucial to develop this membrane chromatography material as alternative separation technique in the protein downstream processing.

Three steps are usually involved in the preparation of membrane chromatography: (1) preparation of the base membrane, (2) chemical activation of the

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Ethylene vinyl alcohol (EVAL) polymer with base polymer content of 44 mole % of ethylene was purchased from Sigma Aldrich (St. Louis, USA) and was used as a base membrane polymer. 1-octanol (Merck, Darmstadt, Germany) and dimethylsulfoxide, DMSO (Merck) were used as a solvent for EVAL polymer. Adsorptive cation resins which are Amberlyst 15 wet (Acros Organics, Geel, Belgium), Amberlyst 15 dry (Acros Organics), Amberlite IR120 (Fluka), Dowex M-31(Fluka), Dowex Marathon (Fluka) and Lewatit CNP105 (Fluka) were purchased from Sigma Aldrich.

Fresh phosphate buffer (pH 6 & 7) was prepared by dissolving di-sodium hydrogen phosphate heptahydrate (Merck) and sodium dihydrogen phosphate monohydrate (Merck) in ultrapure water. Acetic acid and sodium acetate trihydrate (Merck) was used to prepare acetate buffer for pH 5. Buffer pH range 8 to 11 was made using Tris-HCl buffer by dissolving appropriated amount of hydrochloric acid (Fischer Scientific, Loughborough, UK) and Tris (hydroxyl-methyl) aminomethane (Merck) in ultrapure water. Sodium chloride (Merck) was added to binding buffer to be used in elution buffer.

Table 3.1: Properties of cation exchanger resin

Resins	Matrix	Functional group	Particle size	*Price per gram (RM)
Amberlyst 15 wet	Styrene-divinylbenzene(macroreticular)	Sulfonic acid	16-50 mesh	4.66
Amberlyst 15 dry	Styrene-divinylbenzene(macroreticular)	Sulfonic acid	16-50 mesh	1.80
Amberlite IR120	Styrene-divinylbenzene(gel)	Sulfonic acid	16-50 mesh	0.24
Dowex M-31	Styrene-divinylbenzene(macroporous)	Sulfonic acid	16-40 mesh	0.20
Dowex Marathon	Styrene-divinylbenzene(macroporous)	Sulfonic acid	30-40 mesh	1.02
Lewatit CNP105	Methacrylate polymer (macroporous)	Carboxylics	100-400 µm	0.15

Source: Supplier, Sigma Aldrich

For ultra performance liquid chromatography (UPLC) analysis, HPLC grade acetonitrile was purchased from Fischer Scientific (Massachusetts, United States) and trifluoroacetic acid was purchased from Sigma Aldrich.

3.2 Methods

Briefly in figure 3.1 shows the overall flow chart of the methodology that used in this work. Details of the each process will be described in next sub-sections.

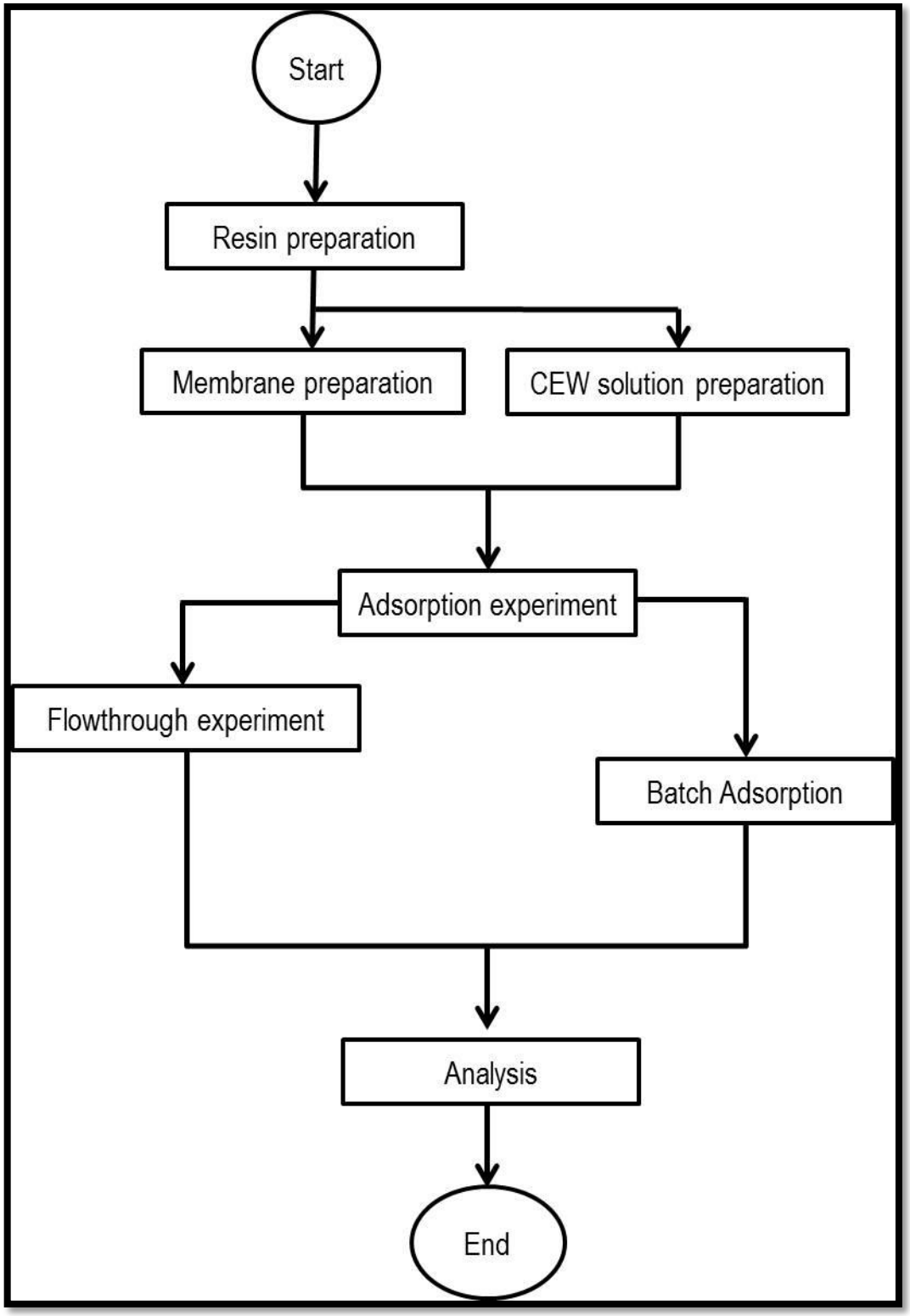

Figure 3.1: Overall work flow