Protein Separation with Ion-exchange Membrane Chromatography

by

Liming Cao

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Liming Cao

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APPROVED:

William M. Clark, Advisor

David DiBiasio, Head of Department

Abstract

Membrane chromatography is a promising process for the isolation, purification, and recovery of proteins, enzymes, and nuclear acids. Comparing with traditional beads column chromatography, membrane chromatography can faster, easier and cheaper to mass-produce. And also, it is easy to set up and scale up. In this thesis, we are trying to study the performance of membrane chromatography, and the mixture of HSA and chicken egg white is used as an example.

We are investigating the purification of Human serum albumin (HSA) from chicken egg white in terms of precondition, dilution, purification method, product recovery, product purity and product cost.

HSA, is a very important clinical protein. In order to obtain low cost, high efficiency and less risk HSA, recombinant DNA technology is used. Many kinds of host organism have been used to produce recombinant HSA (rHSA).

In this thesis, a kind of ion-exchange membrane (Mustang Q membrane capsule) chromatography was used. The membrane capsule is disposable because it is designed for use in pharmaceutical production. For this project, a cleaning method was used which made the membrane capsule reusable. Washing with 4 mL 1 M NaCl and 4 mL NaOH was sufficient for this purpose.

Since the egg white protein solution was very viscous, it needs to be diluted before loaded on FPLC. Dilute experiment was done to find the best dilution level. In this thesis, we found that 5 times dilution was best not only for high efficiency but also for FPLC operation. After getting the basic conditions, some purification experiments were done to

find the optimal operation condition to purify HSA form chicken egg white protein solution by changing buffer pH, salt concentration in elution buffer and gradient used to elute proteins. The best purification condition for loading buffer is Tris-HCl buffer A (4.75g/L, pH 9.5) and the elution buffer is Tris-HCl buffer A + 0.2M NaCl. The purity of HSA recovered was 93% on the Mustang Q membrane capsule at 1 ml/min when the mixture of HSA and chicken egg white was diluted 10 times. And the yield was 85%. The impurity is probably ovoglobulin as suggested by the result of SDS-PAGE, whose molecular weight is close to 40kd.

To characterize the separation capability of the Mustang Q membrane capsules, equilibrium adsorption and breakthrough curve studies were made using bovine serum albumin (BSA). 1mg/mL BSA solution was used to get the breakthrough curve with different flow rate ranging from 1 to 4 ml/min. With a flow rate is 1 ml/min, breakthrough curve were obtained with different concentrations of BSA ranging from 1 to 16 mg/mL. The dynamic binding capacity was found to be from 9.1 to 119.1 mg/mL.

The equilibrium adsorption isotherm showed Langmuir isotherm behavior with dissociation constant and a maximum adsorption capability. According to the result of isotherm adsorption, a multi-plate mathematical model was used to get the theoretical breakthrough curve. By fitting the theoretical breakthrough curve to the experimental breakthrough curve, constants in the multi-plate model were obtained and were used to estimate the axial dispersion coefficient of the membrane capsule. The estimated axial dispersion coefficient of $2.45*10^{-6}$ cm^2/s is very small which means that the axial ispersion is not significant. The adsorption process is therefore controlled by radial radius dispersion or film dispersion.

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I would like to thank my advisor, Prof. William M. Clark, for his guidance, patience and enthusiasm concerning this project. Without his support, this project would not have been finished.

A special thanks to my husband Hai for his support of all of my endeavors.

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Introduction

Human serum albumin (HSA) is a very important protein in the circulatory system of human blood. It is important in maintaining normal osmolarity in plasma and in interstitial fluid[1]. And it can be used to treat severe hypoalbuminemia or traumatic shock and also can be used to treat burns and maintain homeostasis[2,3].

Recombinant DNA technology is widely used to produce recombinant HSA (rHSA) in bacteria, yeast, *E. coli*, and some farm animals including cows, sheep, goats and even pigs in their milk. Among the host organisms, transgenic chickens are promising because of low cost and high efficiency and low risk. One objective of this thesis is to investigate the use of membrane chromatography for large-scale recovery of HSA from chicken egg white.

The main method used to purify recombinant protein is chromatography[4]. Membrane chromatography has very good characteristic for biomolecular purification. It is easy scale up and set up. Comparing with the traditional column, membrane has bigger pores, which makes the proteins can access the binding site on the membrane surface by directly bulk convection and with very little pore diffusion. So the total mass transfer resistance of proteins passing by the membrane is much lower than that of traditional bead column. The benefit of using membrane column is that there is no high pressure drop comparing with bead column. And the total process is much faster than that of bead column. From these points, membrane is much better than traditional bead column to purify proteins. But the disadvantage of membrane is obvious too. The broaden radius comparing with the short length makes the process not uniform which makes the total performance is

close to that of the traditional beads[5]. In this thesis, Mustang Q membrane from Pall

corporation was used as a column.

Objective

Isolation of rHSA from transgenic chicken egg white has not been done commercially on a large scale. However, due to the vast potential of getting rHSA from transgenic egg white, and the big market requirement for HSA, large-scale production of rHSA is highly demanded.

One objective of this thesis was, therefore, to find an efficient purification method to isolate rHSA from chicken egg white, potentially transferable to large scale. In the absence of transgenic chicken egg white, HSA was mixed with chicken egg white and got the mixture of HSA and chicken egg white, then HSA was isolated from the mixture by FPLC. The primary objective was to develop a method for recovering HSA from chicken egg white. We expect that this developed technique can be applicable with some modifications to the large scale HSA purification from chicken egg white.

According to the advantage of membrane, Mustang Q membrane was used to replace traditional bead column. Chicken egg white is highly viscous system. When it meets with Tris-HCl buffer, precipitation comes out. Trying to understand what makes the result of precipitation, and making all of the precipitation out is important for FPLC system. In order to lower cost, the lowest amount of dilution level which can make all of the precipitation come out should be used. The following step, therefore, was to find the optimal method including pH of the running buffer, salt concentration of the elution buffer, and the gradient used to elute the protein. Minimizing cost and maximizing recovery is the basic objective of this thesis. At last, in order to know some basic membrane characteristic, flow rate and concentration effect on the breakthrough curves has been tested by BSA solution. And isotherm adsorption was obtained too.

Background

HSA

Human serum albumin (HSA) is the most abundant protein in plasma and more than 50% of the protein in human blood plasma is HSA[1,3,6]. It is a major antioxidant and transport protein and is important in maintaining normal osmolarity in plasma and in interstitial fluid[7].

The structure of HSA

HSA is a 66.5 Kd single-chain, non-glycosylated polypeptide that organizes to form a heart-shaped protein. It is 67% alpha-helical and contains no beta-sheet structure[8]. HSA is the major transport protein for unesterified fatty acids around the
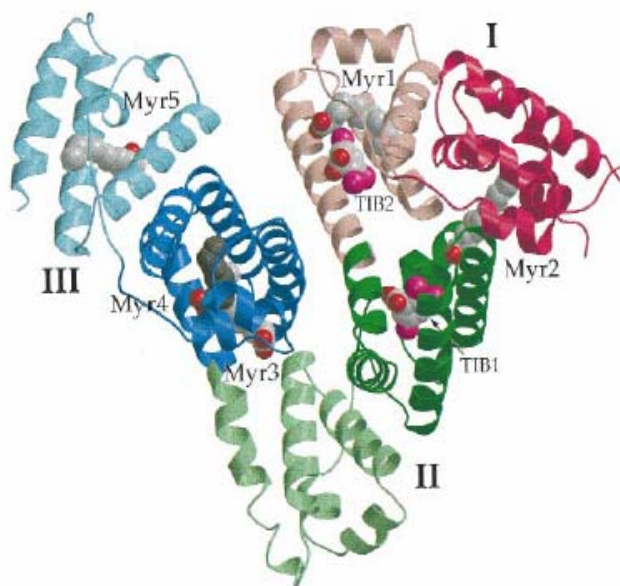
Figure. 1.1[9] Domain structure of HSA and location of myristate and TIB binding sites. The protein secondary structure is shown schematically and the domains are colour-coded as follows: I, red; II, green; III, blue. The A and B sub-domains within each domain are depicted in dark and light shades respectively.

blood-stream. The protein is composed of three homologous domains (I-III), and each domain has two subdomains (A and B) that possess common structural elements. It contains 35 cystrinyl residues, of which 34 form 17 stabilizing disulfide bridges. (Figure.1.1)[9].

The isoelectric point of HSA is 4.9. There are pH-dependent conformational changes named neutral to base or N-B transition. In phosphate buffer at about pH 6, HSA is in the N conformation. And at pH 9 the B form is predominant. Binding of some ligands, especially site I-ligands, are affected by the N-B transition[10].

Application of HSA

HSA can serve as an almost universal transport and depot protein in the circulation since it can bind reversibly a large number of endogenous and exogenous compounds. There are two major binding regions named Sudlow's site I and II in HSA which relative to its binding to aromatic and heterocyclic[11].

The source of HSA

In a long time, the only source of HSA for clinical application is donated human plasma. But, this source is very limited. Also, since the HSA coming directly from human plasma, it has the risk of transmission of pathogenic vira such as hepatitis, HIV, and others[2]. So find another source of HSA is very important. In recent years, several groups and companies try to produce recombinant HSA (rHSA) by recombinant DNA technology by using different host organisms[12]. These organisms include bacteria, yeast, and mammalian cells like cows, sheep, goats and even pigs. These cells are grown and stimulated to express HSA.

The ideal protein production system is one in which the host organism is easily and inexpensively grown and HSA is easily and inexpensively recovered in high purity and high yield. Among the host organisms, transgenic chickens are promising. The main advantage of chicken egg white to produce rHSA is that they can be bred and grown faster and with less expense than diary animals, and with much less capital and operating costs than cell culture techniques comparing with bacterial and E. coli. With low cost and high efficiency, and less risk, chicken egg white is the best source to produce rHSA.

The study of egg-white proteins

Chicken egg white has many kinds of proteins very important and valuable. Table 1.1 lists all the proteins in chicken egg white[13]. The most important thing is that chicken egg white is cheap and easy to get. As a cheaper way to obtain useful protein comparing with the other resource, chicken egg white has been studied widely. Many authors have reported that they use different methods to get the proteins in chicken egg white.

Fractionation of proteins from their crude extract or culture broth was used before, but it was too time consuming and expensive, so in recent years, this method has been given up[14]. As a consequence, there are some continued improvements used in the separation techniques appeared such as ion-exchange chromatography[13,15], affinity chromatography[14], expanded bed adsorption[16,17], membrane chromatography and so on. They are more effective, relatively less expensive and less time consuming.

Bedi[13] has used ion-exchange chromatography with hydrophobic interaction chromatography to get HSA from HSA and egg-white mixture. In this method, the egg-white only needs to be diluted 5 times, then the mixture is centrifuged to get rid of the precipitate. Then the product of the centrifuge can be used for chromatography. Since the precondition is shortened, it can save time and money. The recovery is more then 90%.

Table 1.1. Properties of egg white proteins and Human Serum Albumin[13].

	% of total	Isoelectric Point	Molecular Weight
Ovalbumin	54	4.5	45,000
Ovotransferrin	12	6	77,700
Ovomucoid	11	4.1	28,000
Lysozyme	3.4	10.7	14,300
Ovomucin	3	4.7	220,000
G3 Ovoglobulin	1	4.8	50,000
G2 Ovoglobulin	1	5	47,000
Ovoglycoprotein	1	3.9	24,400
Ovoflavoprotein	0.8	4	66,500
Ovomacroglobin	0.5	4.5	32,000
Avidin	0.05	10	900,000
Cystatin	0.05	5.1	68,300
Thiamin-binding protein	N.D.	N.D.	12,700
Glutamul aminopeptidase	N.D.	4.2	320,000
Minor glycoprotein	N.D.	5.7	52,000
Minor glycoprotein	N.D.	5.7	52,000
Human Serum Albunin	**-**	**4.9**	**66,500**

Immobilized metal ion affinity chromatography (IMAC) relies on the formation of weak coordinate bonds between metal ions immobilized on a column and basic groups on proteins including histidine, cysteine and tryptophan. As we all know, amino acid is the basic structure of proteins. By using different ligands, which can interact with amino acid on the surface of target protein, the target protein can be separated from other proteins. Currently, there has been a dramatic increase in the use of IMAC for the separation and purification of proteins at the laboratory scale. Sadhana et al[14] used IMAC adsorb three chicken egg-white proteins including ovalbumin, conalbumin and lysozyme on Cu (II) and Ni (II) loaded on IMA gels. Also, this method gave some information that the hybrid bioseparation techniques such as metal chelate displacement chromatography and immobilized metal ion-membrane filtration is good at protein separation.

Expanded bed adsorption (EBA) is now widely used too. Ryan et. al[17] used a novel 4-stage system to purify malate dehydrogenase (MDH) continuously from a crude homogenate of Sacharomyces cerevisiae, delivering a fully clarified product stream containing the targeted protein. This method has a high yield and purification factor. In this method, the adsorbent used consisted of a Procion Red HE-7B derivatized perfluorocarbon support. And in his work, hen lysozyme was purified from a solution of egg white, and from a mixture of lysozyme-enriched bovine milk. They purified lysozyme directly from chicken egg white using a continuous, counter-current, expended bed adsorption system. This technique overcame some of the problems associated with packed bed chromatography, such as consolidation of packing material,

formation of channels, and column blocking by particles in the feed solution.

In recent years, natural antibodies are found in chicken egg white proteins[18]. Natural antibodies (NAb) are antigen binding antibodies present in non-immunized individuals. NAb has a lot of functions. It can be involved in the clearance of foreign and dead or catabolic materials. Also, it may enhance antigen uptake, processing and presentation via B cells and it also can provide initial protection against infection. Finally, it may provide tolerance to body-own tissue antigens preventing auto-immune response. So NAb is very important. Henk and Jalkanen with their groups separately found that NAb are present in chicken egg white[18].

Ultrafiltration (UF) processes give a very high throughput of product and can be fine-tuned to give high selectivity in large-scale protein purification process. Ghosh and Cui[19] used ultrafiltration to separate lysozyme from chicken egg white. They found that the separation of lysozyme from chicken egg white by ultrafiltration with 25 kDa MWCO membrane was a simple process since the membrane largely retained the other proteins.

Membrane chromatography was introduced as an integrative technology for the purification of proteins several years ago. The main feature of chromatographic separations based on membranes is the large decrease of pore diffusion. Since its introduction, many successful applications of membrane chromatography have been described[20]. Eli Ruckenstein, Xianfang Zeng[21]employed macroporous chitin membranes for the affinity separation of lysozyme from egg white. Figureure 1.2 is a process using membrane to get lysozyme from egg white. Lysozyme of very high

purity (>98%) was obtained from a mixture of lysozyme, ovalbumin and egg white. The results indicate that the macroporous chitin membranes can be used for the separation, purification, and recovery of lysozyme at large scale.
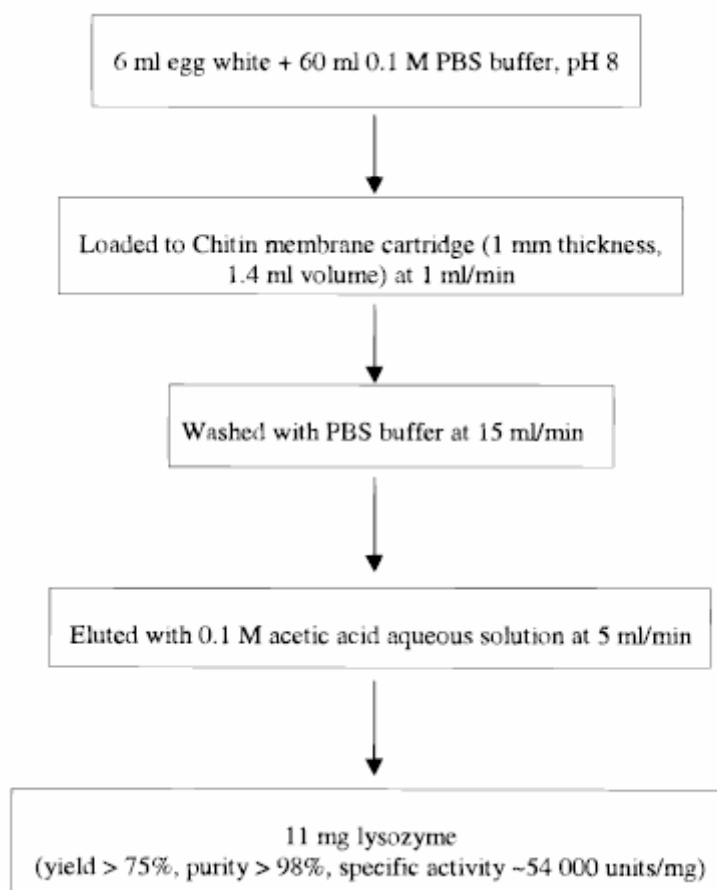
Figureure 1.2[21]   Isolation of lysozyme from egg white. A 6 mL portion of homogenized egg white was diluted with 60 mL of 0.1M PBS buffer, filtered and centrifuged at 1600g for 20 min. The supernatant was loaded to a chitin membrane cartridge (four stacked flat membranes, 1 mm total thickness and 1.4 mL total membrane volume) at 1 mL/min. Washing followed it with 0.1M PBS buffer at 15 mL/min and elution with 0.1M aqueous acetic acid solution at 5 mL/min.

Liquid Chromatography

Liquid chromatography is a commonly used technique to separate mixtures of protein, nuclear acid, and other molecules.

The principle for liquid chromatography to separate proteins is that the molecules in a solution (mobile phase) will interact (bind and dissociate) with the chromatography media (stationary phase). If the solution is allowed to flow across the solid surface, then the molecules that interact frequently with the solid surface will spend more time bound to the surface and thus move more slowly than molecules that interact infrequently with the solid surface.

The interactions between proteins and the chromatographic column are very complicated. Not only because the protein interaction with the solid adsorbent is at more than one site on protein's surface, but also because the types and strengths of interaction at these sites are different. Perhaps even more important is the fact that the adsorbent itself presents a heterogeneous array of binding sites, even assuming that the interacting areas are randomly distributed. Thus, no single parameter can describe adequately the protein-matrix interaction (except in highly specific affinity sites), and chromatographic theory for protein separation must be a compromise of approximations and assumptions[22].

Normally, liquid chromatography is performed in a column packed with spherical beads. The nature of these beads determines whether separation of proteins depends on differences in mass, charge, or binding affinity. However, the traditional packed beds

based chromatography has several major limitations. These limitations include high pressure drop across a packed bed, slow intra-particle diffusion and radial and axial dispersion limitations[23, 24].

Ion-exchange Chromatography

Ion-exchange chromatography is the most popular method for protein purification. The theory of it is to use the difference of charges on proteins at a given pH. The solid adsorbents are charged, positive or negative. Then the charged protein will be adsorbed by the charged adsorbents. According to the difference of the interaction forces between the protein and adsorbent, different protein is bounded differently by the adsorbent. Then, when we use some other buffer to replace the protein, they (the proteins) will be washed out of the adsorbents in different velocity: the less the interaction between the adsorbent and the proteins, the faster they will be washed out. Then, proteins can be separated according to the sequence of their elution.

There are two kinds of ion exchangers: anion exchangers, which have positively charged matrix, and will adsorb the proteins with negative charge; cation exchanger, which have negative charged matrix, and will adsorb the proteins with positive charge. The most common anion exchangers are DEAE- ,TEAE- and QAE-, and the cation exchangers often being used are CM- , S- and SP-[22]. The membrane of Pall Mustang Q cartridge is polypropylene with quaternary ammonium ligand, which is a strong anion exchange adsorbent.

Membrane Chromatography

In order to overcome the limitations of traditional beads column, synthetic microporous or macroporous membranes have been used as chromatography media. This method is called membrane chromatography.

Membrane chromatography can overcome the limitations associated with packed beds based chromatography. In membrane chromatographic processes, the transport of solutes to their binding sites take place predominantly by convection (Figure 1.3) and the pore diffusion is very small comparing with the beads column, thereby the mass transfer resistance is tremendously reduced[5].

The result of this advantage is to reduce process time including adsorption, washing, elution and regeneration time, which save time and improve efficiency[25, 23]. Most importantly, fast process can avoid the inactivity of biomolecules. As we all know, all the biomolecules have activities. The faster is the process, the less possibility for the biomolecules lose activity.
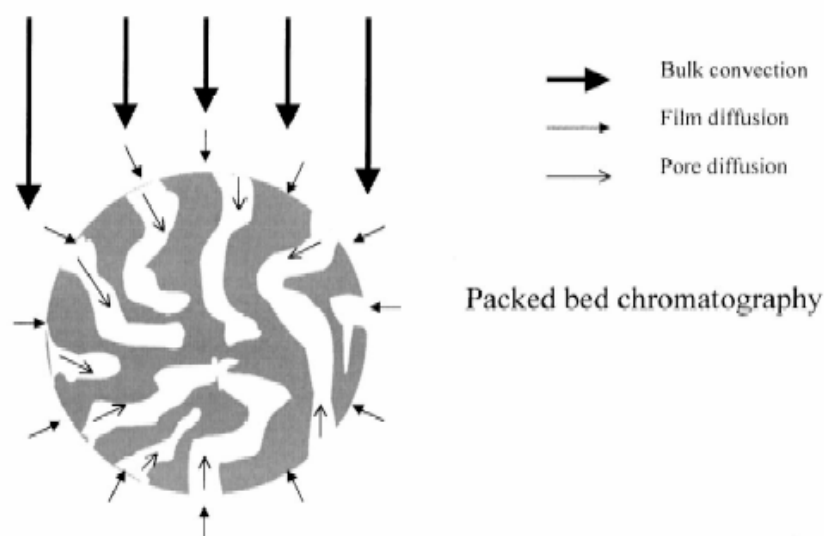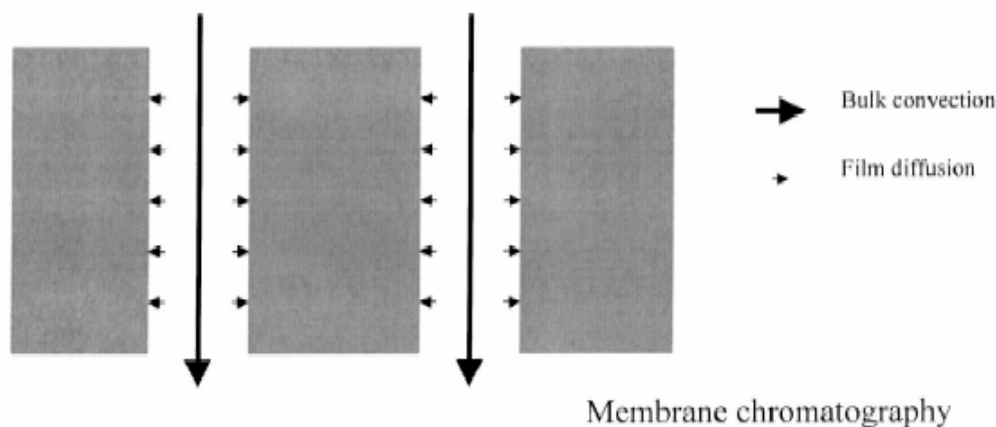
Figure.1.3. Solute transportation in packed bed chromatography and membrane chromatography.

The idea of membrane chromatography is especially suited for large-scale process since the column volume of membrane can be made from less than 0.1 ml and larger than thousands of liters. Due to the macroporous structure of the membrane support, membrane chromatography has a lower pressure drop, higher flow rate and higher productivity[5, 26, 27, 28]. And also, membrane chromatography is particularly suitable for large proteins (MW larger than 250kd). In traditional beads, the pore is too small for large size protein to enter, so large size proteins rarely enter pores and only bind on the externally available surface area. Since the pore size on membrane is rather big comparing with that of beads, large size proteins can bind on it easily. The binding capacity of membrane adsorbents for large size protein is much bigger than that of traditional beads. Membrane chromatography has higher capture efficiency and higher productivity than column chromatography and shows most promising industrial applications for the recovery, isolation and purification of proteins and enzymes. Also, membrane chromatography provides easy set up and scale up. After you buy a

membrane module, there are connections you can choose to set up the membrane on the chromatography and needn't make the column as the traditional chromatography[29, 30]. So, membrane chromatography is really a good separation process for the purification and recovery of proteins, enzymes and nuclear acids.

All I talked above is the advantages of membrane chromatography, which is the reason of its fast development. On the other hand, there are still disadvantages, which need to be overcome. It is difficult to get uniform flow for the short wide beds of the membranes. This happens in many cases and makes the membrane efficiency decrease.[29]. And adequate flow distribution is also necessary to maintain the membrane efficiency when scale-up.

The shapes of the membranes

Here, I want to mention the shapes of the membranes used for membrane chromatography. Membrane chromatography can reduce the mass transfer resistance but diffusion transport is not totally absent. Flow distribution is a major factor in chromatographic process that can affect transport phenomena. There are mainly three shape membranes including flat sheet, hollow fiber and radial flow (Figure 1.4) used for protein purification[5].

Comparing with the other two shapes, flat sheet is simpler and cheaper design of the disk holder. So for lab use, it is better than the other two. But there are many disadvantages too. The application of membrane disks are usually restricted by their inability to deal with crude solutions, the problems of flow maldistribution and disk edge leaking in large-scale separation[25].

A hollow fiber membrane usually consists of a bundle of several hundred fibers potted together within a module in a shell conFigureuration. Hollow fiber membranes are considered better because of their high specific surface area leading to a higher relative adsorption capacity. In addition, the cross-flow cartridge design of hollow fibers is feasible to induce an effective separation for crude solutions. There are problems with hollow fiber membranes too. First, the ligand immobilized onto the fibers are distributed nonuniformly which lower the specific adsorption. Second, if the ligand is immobilized onto the fiber uniformly, it is difficult to assemble the fibers into a cartridge. Radial flow membranes are prepared by spirally winding a flat sheet membrane over a porous cylindrical core.

Radial flow membrane is clearly not suitable for pulse chromatography. It is likely to be more suitable for use in the bind and elute mode. Radial flow membranes are claimed to be suitable for large-scale applications[5].
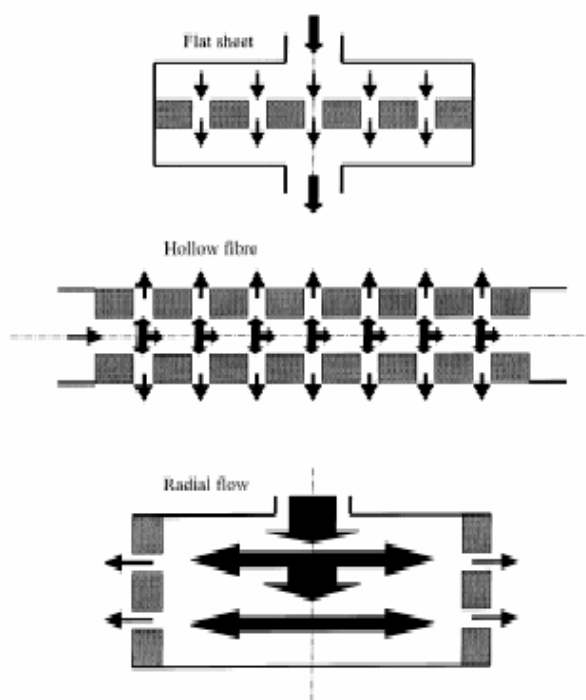
Figure. 1.4. Flow in membrane adsorbers.

Further improvement with proper design and operation of adsorptive membrane is still very important. In this thesis, we use mustang Q membrane capsule. It is a kind of flat sheet membrane with 6 layers flat sheets housed within membrane capsule. It is cheap and easy installed.

Application of membrane chromatography for protein purification

Usually, the membranes used for membrane chromatography have functional ligands attached to their inner pore surface as adsorbents. There are many types of adsorptive membranes including ion-exchange membranes, affinity membranes, reverse-phase membranes and hydrophobic interaction membranes. All these membranes have been developed for the purification of proteins, enzymes, and antibodies from various sources[20].

Membrane cleaning

In some applications, sample molecules may not fully elute or may precipitate on the column which makes membrane fouled.

Methods used to clean the membrane

In the clean up method, reverse the flow direction to help flush out particulates and to prevent contamination of the lower part of the bed. Also, slow the flow rate to expose the column to the regeneration solution for several minutes at each step of the cleaning protocol.

Inazumi[31] used 0.3-2.0 N acid solution at less than 50°C to clean the ion-exchange membrane which is used for electrode. And there is a patent, in which NaClO are used to clean cation exchange membranes. A Neocepta CH-45 membrane was soaked in aq. 500 ppm polyethyleneimine (molecular weight 1000) for 10 hour, and then in aq. HCl-HCHO for 20 hours[32]. When used along with anionic Neocepta ACS membranes in stacks for seawater desalination, the pressure increased from 0.5 to 1.1 kg/cm^2 after 3 mo. Aq. 1000 ppm Na dodecylbenzenesulfonate was circulated through the diln. Chambers for 1 hour and aq. NaClO, contg. 100ppm available Cl, at pH 11.0 for 3 hours to reduce the membrane pressure to 0.5kg/cm^2. Also, Uehara[33] used a mixture of an organic solvent including alcohols and alkali metals and alkalies (including NH$_4$OH) to clean an electrodialyzer consisting of ion-exchange membranes. Heterogenous ion-exchange membranes consist of ion exchange resins dispersed in a

polypropylene matrix. They have good electro properties. They can resist ≤ 3 kg/cm^2 gage pressure and $\leq 50^o$C temperature. Although the membrane can be fouled by organic matter in the raw water, its ion exchange capacity can be fully regenerated by cleaning. Kishi[34] used 3% NaOH solution at 40°C for 1 hour followed by a rinse with HCl for 0.5 hour to clean it.

Mustang Q capsule

The new Pall Mustang® Q product range is designed to remove protein, DNA and other macromoleculars in a flow-through step in downstream bioprocessing by anion exchange filtration[35]. In this thesis, I used Mustang Q capsule to purify HSA from chicken egg white.
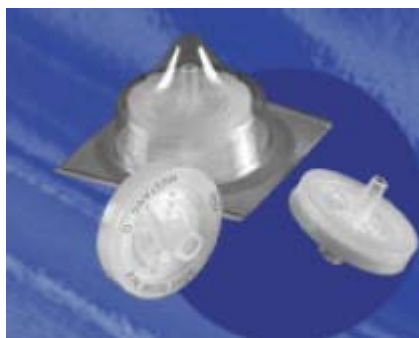
Figureure. 1.5 Mustang Q membrane capsule[33]

On Mustang Q membrane capsule, pendant quaternary amine groups are immobilized on the surface of the membrane. Since pendant quaternary amine groups have positive charges and only negative charged protein can be adsorbed on it, this membrane capsule is anion exchange adsorbent with a crosslinked polymeric coating.

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The pores in Mustang Q membranes are 0.8µm which is very large to allow biomacromoleculars to access all the binding sites by direct fluid convection. So the pore diffusion resistance is very small comparing with traditional column. This produces a very high dynamic protein binding capacity in comparison to beads with large diffusive pores. Combining this high capacity membrane with a unique capsule design resulted in the Mustang Q Capsule. Each Mustang Q Capsule contains 6 layers of pleated membrane that will bind genomic, plasmid, protein and viral DNA. These filters are specifically designed to be single use to eliminate cleaning and cleaning validation.

The membrane used for this thesis is acrodisc unit mustant Q membrane, just as the Figure. 1.5[35]. The membrane bed volume of the acrodisc is 0.18 ml, and the pore size of the membrane is 0.8 µm. The pressure and temperature that can be stand is 5.5 bar (550 kPa, 80 psi) at 21 - 24 °C (70 - 75 °F) and 2.1 bar (210 kPa, 30 psi) at 60 °C (140 °F), the flow rate should be between 1~4 ml/min.

Breakthrough and Breakthrough Curves

If a fluid having an absorbable solute is contacted with a stationary phase which has ligands on, equilibrium between the solute and the adsorbent will be obtained. In order to analysis the mass transfer of the mobile phase in the membrane capsule, breakthrough curves are always used. When continuous sample solution flows into the membrane, a breakthrough occurs by detection of the sample at the system outlet.

Breakthrough is defined as that point where the exit protein concentration in the fluid phase equals 10% of the feed sample concentration[36]. In this thesis, breakthrough is defined that point that there is protein in the exit as described on the manual of the membrane capsule. Actually, this concentration may be taken as the minimum detective concentration. Loading capacity is defined as the amount of protein bound to the membrane at the point of breakthrough. A broadly disperse breakthrough curve causes a decrease in ligand utilization, or a delay in the saturation time, or a waste of feed solution.

Breakthrough curve is the plot of effluent concentration of the sample versus time or throughput volume. Sometimes, dimensionless breakthrough curves are used. Dimensionless breakthrough curves are obtained by plotting dimensionless effluent concentration versus dimensionless effluent volume. The dimensionless effluent concentration is the ratio of effluent sample concentration over feed sample concentration. The dimensionless effluent volume is the ratio of the amount of sample introduced to the system over the total system capacity at equilibrium[37].

Isotherm Adsorption

The mass transfer of the adsorption to the immobilized phase can be described by chase[38]:

$$\frac{dq}{dt} = k_1 C(q_l - q) - k_2 q \qquad (1)$$

here, C is the mobile phase concentration, q is the stationary phase concentration and q_l is the maximum binding capacity of the stationary phase and t is the time. The rate constant k_1 and k_2, are "lumped" parameters that represent rate of adsorption and desorption of the protein to the immobilized ligand as well as contributions from mass transfer limitations.

Langmuir Isotherm[14]

With the assumptions that all binding sites have equal energy, are independent in nature and single-site interaction occurs between protein and ligand, at equilibrium, Eq. (1) can be reduce to:

$$q = \frac{q_l C}{K_d + C} \qquad (2)$$

$$K_d = \frac{k_2}{k_1} \qquad (3)$$

Eq. 2 is called Langmuir isotherm equation.

Multi-plate Mathematical Model Used to Predict Breakthrough Curves

Hao and Wang developed a multi-plate mathematical model to describe the chromatography process as occurring in a series of theoretical plates[39]. In order to understand the model, I cite the equations used for the model in their paper here. In each plate, the amount of the solute entering the plate should equal to the sum of that leaving and the increment in mobile and stationary phase. The mass balance can be expressed as:

$$C_0 dV - C_1 dV = \frac{V_m(1-\varepsilon)}{N} dC_{s,1} + \frac{V_m \varepsilon}{N} dC_1 \qquad (7)$$

$$C_{i-1} dV - C_i dV = \frac{V_m(1-\varepsilon)}{N} dC_{s,i} + \frac{V_m \varepsilon}{N} dC_i \qquad (8)$$

$$C_{N-1} dV - C_N dV = \frac{V_m(1-\varepsilon)}{N} dC_{s,N} + \frac{V_m \varepsilon}{N} dC_N \qquad (9)$$

here, V is the effluent volume, V_m is the membrane volume, N is the theoretical plate number, ε is the porosity, C and C_s are the solute concentration in the mobile and stationary phases.

By adding above equations, we obtained

$$C_0 - C_N = \frac{V_m(1-\varepsilon)}{N} \sum_{i=1}^{N} \frac{dC_{s,i}}{dV} + \frac{V_m \varepsilon}{N} \sum_{i=1}^{N} \frac{dC_i}{dV} \qquad (10)$$

dC_i/dV and $dC_{s,i}/dV$ may be assumed to decrease to zero with increasing effluent volume. Then Eq. (10) can be simplified as:

$$C_0 - C_N \approx \frac{V_m(1-\varepsilon)}{N} \frac{dC_{s,N}}{dV} + \frac{V_m \varepsilon}{N} \frac{dC_N}{dV} \qquad (11)$$

From Langmuir isotherm equation (2),

It can be written as:

$$C_{s,N} = \frac{C_l C_N}{K_d + C_N} \tag{12}$$

so,

$$\frac{dC_{s,N}}{dV} = \frac{dC_{s,N}}{dC_N}\frac{dC_N}{dV} = \frac{C_l K_d}{(K_d + C_N)^2}\frac{dC_N}{dV} \tag{13}$$

Using Eq. (12) into (13),

$$C_0 - C_N = \frac{V_m(1-\varepsilon)}{N}\frac{C_l K_d}{(K_d + C_N)^2}\frac{dC_N}{dV} + \frac{V_m\varepsilon}{N}\frac{dC_N}{dV} \tag{14}$$

Eq. (14) can be rewritten as:

$$\frac{dV}{V_m(1-\varepsilon)} = \frac{C_l K_d}{N}\frac{dC_N}{(C_0 - C_N)(K_d + C_N)^2} + \frac{\varepsilon}{N(1-\varepsilon)}\frac{dC_N}{C_0 - C_N}$$

$$= \frac{C_l K_d}{N(C_0 + K_d)}\frac{dC_N}{(K_d + C_N)^2} + \frac{C_l K_d}{N(C_0 + K_d)^2}\frac{dC_N}{K_d + C_N}$$

$$+ \frac{1}{N}\left[\frac{C_l K_d}{(C_0 + K_d)^2} + \frac{\varepsilon}{1-\varepsilon}\right]\frac{dC_N}{C_0 - C_N} \tag{15}$$

With initial condition $C_N = 0$ at V=0, the analytical solution of Eq. (15) is:

$$\frac{V}{V_m(1-\varepsilon)} = \frac{C_l}{N(C_0 + K_d)}\frac{C_N}{K_d + C_N} + \frac{C_l K_d}{N(C_0 + K_d)^2}\ln(1 + \frac{C_N}{K_d})$$

$$- \frac{1}{N}\left[\frac{C_l K_d}{(C_0 + K_d)^2} + \frac{\varepsilon}{1-\varepsilon}\right]\ln(1 - \frac{C_N}{C_0}) \tag{16}$$

If C replaces C_N to denote the outlet concentration and the following dimensionless groups are introduced to simplify the expression,

$$c = \frac{C}{C_0}$$

$$\lambda = \frac{C_l}{C_0}$$

$$\varphi = \frac{C_0}{K_d}$$

Eq. (16) can be expressed as below,

$$\begin{cases} c = 0, & v \le v_0 \\ v(c) = v_0 + \dfrac{A(c)}{\alpha}, & v > v_0 \end{cases} \tag{17}$$

$$A(c) = \frac{\lambda}{1+(1/\psi)} \frac{c}{(1/\psi)+c} + \frac{\lambda/\psi}{(1+(1/\psi))^2} \ln(1+\psi c) - [\frac{\lambda/\psi}{(1+(1/\psi))^2} + \frac{\varepsilon}{1-\varepsilon}]\ln(1-c) \tag{18}$$

v_0 can be considered as the theoretical dimensionless breakthrough volume at which the solute just reaches the outlet. Parameter α may indicate the chromatographic performance. The value of v_0 and α can be obtained by fitting experimental data Eq. (17).

The author of the model said that, Eq. (16) may be rewritten as[36]

$$v(c) = \frac{A(c)}{N} \tag{19}$$

Comparing Eq. (17) and (19), the value of α and N should be similar, especially when $A(c)$ is very big.

Materials and Methods

Transgenic HSA

Since there is no transgenic egg with HSA(human serum albumin). A model of a recombinant protein mixture is used here. HSA was added to egg white, then we tried to separate HSA from the protein mixture using anion-exchange membrane chromatography. Since the protein mixture was very viscous and it would block the pores on the membrane, increasing the operation pressure and decreasing the flow rate, it was diluted first by the running buffer. There came some precipitate after dilution, so a centrifugation step was used before applying to the membrane. The eggs were bought from the grocery store; HSA is from Sigma (A-1653), the centrifuge is IEC/Damon HT table-top centrifuge. The separation process is shown schematically in Figure 2.1.
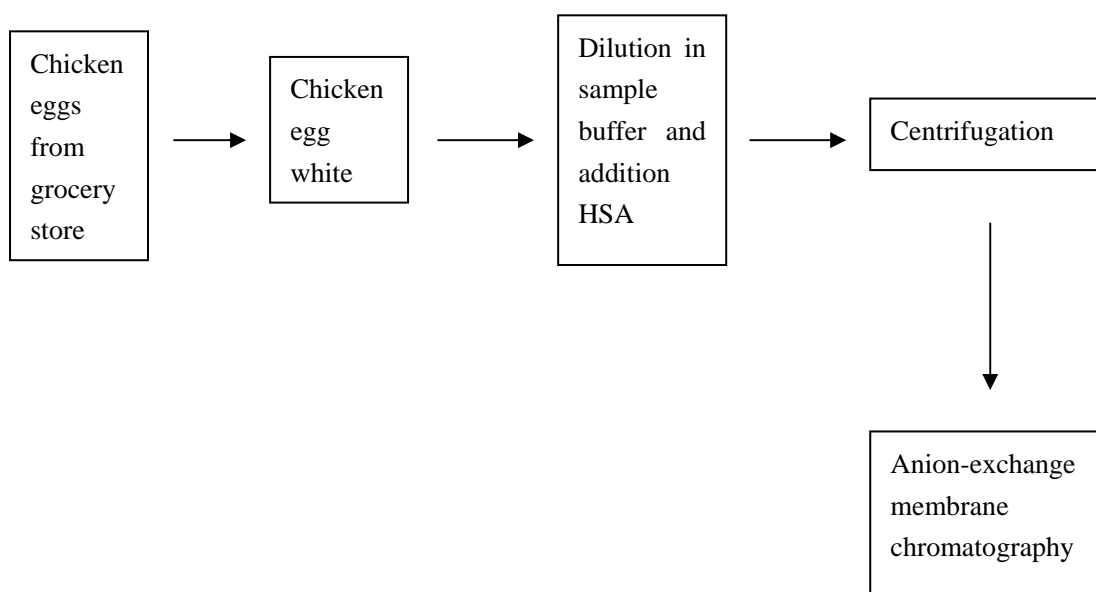
Figure 2.1 Steps for HSA purification from crude chicken egg white

FPLC

Experimental Setup

FPLC (fast protein liquid chromatography) system is from Amersham-Pharmacia Biotech. It has UV detector, pH meter and conductivity meter. All of there were calibrated and controlled by a computer using the UNICORN software coming with the system. And there is also a printer connecting with the system. The details about how FPLC running can be found on a master thesis written by Mandeep Bedi[13].

Ion-exchange Chromatography

Strong anion-exchange adsorption on Mustang Q membrane capsule from Pall was performed. The flow rate used for protein separation was 1 ml/min.

Buffers

All the buffers for anion-exchange chromatography were made in deionized water (Millipore, Milli-Q). Before applying on the FPLC, air bubble were removed by degassing. Buffer was degassed using a vacuum pump by pulling a vacuum while

Table 2.1. Buffer formulations for ion-exchange chromatography in 1 litre deionized water

Name	Composition
Sample buffer A	4.75 g Tris Base + HCl
Elution buffer B	Sample buffer A + NaCl

Stirring. Table 2.1 gives a list of buffers used. For sample buffer A, it was made by adding 4.75 g Tris Base into 1 L deionized water, then added some drops of

concentrated HCl. Different pH needed different drops of HCl. Once sample buffer A was obtained, added NaCl to get elution buffer B. The amount of NaCl used depended on the salt concentration needed in the experiment.

Chicken Egg White Solution Preparation

Egg white was separated from the yolk with care first and put the egg white into a cylinder. Read the volume of the egg whiter and then put the egg white into a beaker. The egg white solution was slowly stirred until it seems homogenous. Separate the CEW solution into 10 tubes, each tube contained about 10 ml, then put the tubes into the refrigerator. In order to keep the protein fresh, a fresh egg only can be stored one week at most before it was used. Each time, three tubes were used.

When one volume egg white was added one volume Tris-HCl buffer A, the solution was called 2X in this thesis. The sample was diluted 10X, then put the solution with precipitation 10mL into a 15mL tube. Centrifuged the tube at the speed of 30,000rpm, got rid of the precipitation, and kept the supernatant into another tube.

Based on a usual 30-35 ml egg white per egg, and assume that 10% of the egg white is protein given 3 to 3.5 g of protein obtained from each egg and a protein concentration of 0.1g/mL before dilution supposing that the chicken egg white protein concentration was 10%.

Procedure

As we all know, each protein has its own PI (isoelectric point). When the buffer pH is larger then PI, the protein has negative charge, and on the other hand, it has positive charge. And when the buffer pH equals to PI, the protein will become precipitation.

Since the PI of HSA is close to 5, and there are some other proteins in the CEW whose PI is very close to that of HSA, the pH of buffer was chosen larger than 5. At this time, the proteins whose PI larger than the buffer pH has positive charge, and they are not bound on the membrane which has positive charge. Then they will be washed out directly without binding. For the other proteins, they have negative charge, so they will bind on the membrane first. But the interaction between the proteins and membrane is different since the amounts of the charges these proteins have are different. So when eluted, they come out at different times. The tighter the interaction between the protein and membrane, the slower the protein comes out.

In this experiment, different buffer pH, and different salt concentration in elution buffer, different gradient were used to optimize the resolution. Columns were washed by 100% buffer B to get rid of any bound protein after the runs.

Protein Assay

Protein concentration was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, 500-0006).

Sample preparation

Protein solutions were made in pH 9.5 Tris-HCl buffer to produce absorbance values between 0.1-1.0 for the standard procedure, and 0.2-1.0 for microassay procedure.

Analysis

For concentrations of 0.2 to 0.9 mg/ml the standard assay was used. 5 mL diluted Bio-Rad dye reagent was placed in a test tube and 100 µL sample was added and vortexed. After 5 minutes, the absorbance of the sample was read by 595 nm. According to the calibration curve for standard procedure made by BSA, the sample concentration can be obtained.

For concentrations of 0.001 to 0.01 mg/mL, the microassay was used. 0.6 mL Bio-Rad dye regent was placed in a test tube and 2.4 mL sample was added and vortexed. After 5 minutes, the absorbance of the sample was read at 595 nm. According to the calibration curve for microassay procedure made by BSA, the sample concentration can be obtained.

SDS-PAGE Analysis

Laemmli sodium dodecyl-polyacrylamide gel electrophoresis(SDS-PAGE) was used to analyze sample and estimated the purity of the product. The gel used is Bio-Rad Tris-HCl 12% precast polyacrylamide gel.

Collect the product

The samples were collected as fractions from the membrane when there was the peak we wanted coming out. Since there was a distance between the UV detector to the exit of the sample, there was a delay from the peak coming out to the sample coming out of the exit. Fill the tube between the UV detector to the exit, then get the solution out, the total volume of the solution was 1.5ml. Since the flow rate was 1 ml/min, the time interval from the peak coming out to the product coming out of the exit was 90 seconds.

$V = vt$,

$$t = \frac{V}{v} = 90 \, \text{seconds}$$

here V is the volume of the solution in the tube from the UV detector to the exit.

v is the flow rate, it is 1 ml/min here, and t is the time interval from the peak coming out to the product coming out of the exit. Using stopwatch to get the product of each peak we wanted. Then collect the sample. From the peak, we can see how much volume the sample has, then collect the same volume of product.

Electrophoresis procedure

Precast Polyacrylamid Gels (12%, Bio-Rad Laboratories) were used. The gels were loaded on the electrophoretic chamber (Bio-Rad Laboratories, Mini Protean III 2-D Cell), in which was filled by electrode buffer. There were 10 lanes on the gel. 20µl of sample was loaded on each lane, 2µl molecular weight marker was loaded and in the first lane. The initial voltage used was 200 V(Hoefer Scientific, PS 500XT) and current was 20mA. Proteins were concentrated into respective bands on the gel. When the bands were out of the concentrated area, the current was changed to 40 mA, till the bands of the proteins run to the end of the gel.

Coomassie Brilliant Blue strain

Staining of the gels was carried out using Coomassie brilliant blue overnight. And the Coomassie brilliant blue can be collected and reused next time. Destaining of the

Table 2.2 Composition of the buffers used for SDS-PAGE [40]

Electrode buffer	3g Tris + 14.4 glycine in 1 L
Solubilization buffer	3.03g Tris in 40ml water, add HCl to adjust pH to 6.8. Diluted to 50 ml.
Loading buffer	2mL solubilization buffer + 1 ml 87%glycerol+ 0.1mg bromophnol blue

gels with 40% methanol and 10% acetic acid until adequate visibility of the bands was

achieved. After destaining, the gel should be dried in the air, and avoiding broken. In the event of excessive destaining the gels were stained back again for best result. The gels were then taken photos by a digital camera.

Dilution Experiment

In order to determine the least possible dilution, dilution experiment was made. When 10ml Tris-HCl buffer was added into 10ml egg white protein mixture, it was called 2 times dilution, and 2X was used to express it. In this experiment, 2X, 3X, 4X, 5X, 6X, 7X, had been used separately.

Since there were precipitate coming out, when the CEW solution was loaded on the FPLC, precipitate will appear again after loading sample when buffer A is used. The precipitate can bound on the membrane unevenly and it will block the pores on the membrane, which will cause the operation pressure to increase greatly. And also, the amount of protein bounded on the membrane as precipitation is unknown, and the interaction between the precipitation and the membrane is unknown too. The precipitation can be washed out in different time comparing with the protein in the solution, which makes the total process complex. So it is very important to find a good dilution level.

When one volume egg white was added one volume Tris-HCl buffer A, the solution was called 2X in this thesis. Added 5mL egg white into a tube first, then added 5mL Tris-HCl buffer, used glass pole to stir the beakers till they are mixed thoroughly to get 2X solution with precipitate. Then added another 5mL Hris-HCl buffer into the beaker to get 3X. Kept doing this to get 4X, 5X, 6X, 7X, 8X, 9X and 10X solution separately. Observe the precipitation in the beakers.

Breakthrough Experiment

In order to test some characteristic of membrane using for protein purification, breakthrough experiment was designed. And since the purpose of this experiment was to test the performance of the membrane capsule used to purify protein, it is no need to use egg white. Because BSA has similar structure as HSA, and HSA is too expensive to do this experiment, BSA was used to do this experiment.

The system was set up as Figure. 2.2. Track 1 is the way the sample loop was filled with buffer. Track 2 is the way that the sample loop was filled with sample. 150 mL sample column was used.
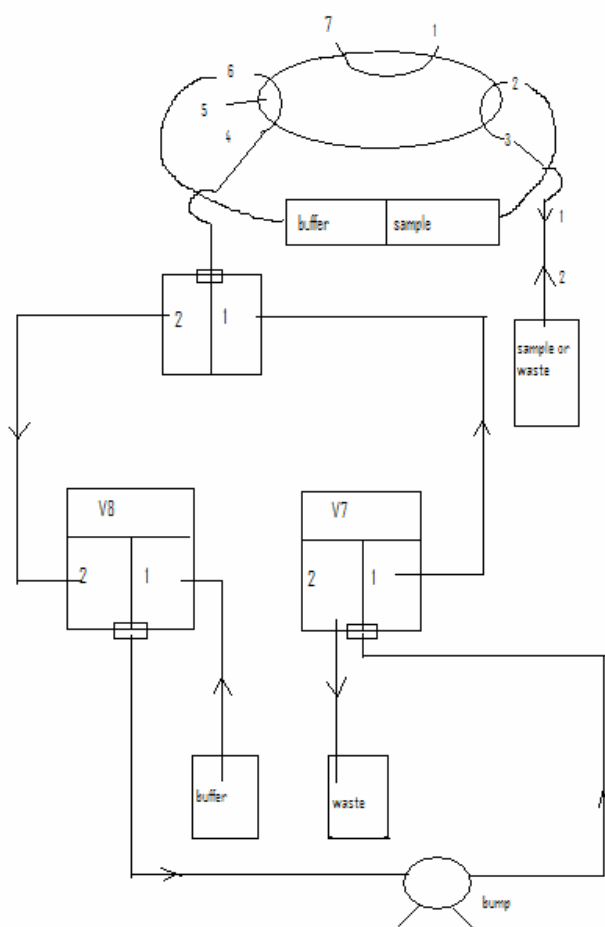
Figure 2.2 Set up for breakthrough experiment

Flow Rate Experiment

This experiment is designed to optimize flow rate. A high flow rate is generally desirable to increase process throughput, however, too high a flow rate will cause excessive pressure drop and may damage the membrane. According to the manual of the membrane capsule, the flow rate that the membrane can stand is 1-4mL/min. In this experiment, 1mg/mL BSA solution was made then used different flow rate 1 mL/min, 2 mL/min, 3 mL/min and 4 mL/min to get breakthrough curve separately. In this experiment, BSA was used rather than HSA since BSA has similar properties but is less costly than HSA.

Process

The membrane was equilibriumed with 15 column volume first, then loaded 17 mL sample using 150mL sample column in which the concentration of BSA is 1 mg/mL, then the breakthrough curve were obtained. Fig. 2.2 showed the set up of this experiment.

Concentration Experiment

In order to test the effect of protein concentration on the breakthrough curve, different BSA concentrations ranging from 1 to 16mg/mL were used to get breakthrough curve when the flow rate was 1mL/min.

Membrane Cleaning

Mustang Q membrane capsule is said to be used once. But it is too wasteful to use it to get the purification condition. So cleaning experiment was designed to find a good way to clean the membrane. If the sample is simple like HSA, it is enough to clean the membrane only use 100% buffer B. If the sample is CEW or the mixture of CEW and HSA, another method should be used since there are so many kinds of proteins in CEW and the interaction is too complicated to use 100% buffer B itself.

Two methods had been tried. One was to use 4 ml 1M NaOH and 4 ml 1M NaCl to clean the membrane each time after it was used. Another one was to use 10 ml 100% Tris buffer B to clean it each time after a membrane was used, and after it was used 5 times, use 4 ml 0.5M NaOH to clean it. Comparing the reproducibility of the graph and the total times a membrane could be used to know which method was better.

Table 2.3 The methods used for cleaning the membrane

Method one	4 ml 1M NaOH + 4 ml 1M NaCl/use
Method two	10 ml 100 Tris buffer B/use + 0.5 M NaOH/5 use

Isotherm Adsorption Experiment

In order to test the isotherm capability, experimental isotherms were obtained for the isotherm absorption of BSA on Mustang Q membrane. Since BSA has similar structure as HSA, and HSA is too expensive to do this experiment, BSA was used to do this experiment.

Process

The sample was buffered by Tri-HCl buffer (pH 9.5). Different concentrations of BSA solution were used in this experiment including 1, 1.2, 1.4, 1.5, 1.6, 1.8, 2, 2.5, 3, 3.5, 3.7, 4, 4.2, 4.4 and 4.5 mg/mL. 5 mL of these solutions was put into small test cells[41,42].

The Mustang Q membrane capsule was preconditioned by 4 mL 1M NaOH and 4 mL 1M NaCl by 10cc syringe. Then the membrane capsule was put on FPLC to desalt by Tris-HCl buffer (pH 9.5). Then Mustang Q membrane capsule was opened by mechanical force and the membrane was taken out. Each capsule has 6 layers of membrane. 3 layers of membrane were put into the BSA solution mentioned above. And a top was used to avoid solution loss. The membrane was kept into the solution for 48 hours. Then test the adsorption at 595 nm. According to protein assay mentioned before, the concentration left in the solution can be obtained by measuring absorbance at 595nm. Then according to mass balance,

$$C_s = \frac{(C_0 - C) * 5}{0.09}$$

The concentration bound on the membrane can be obtained.

C_s is the protein concentration on the membrane, C is the concentration in the

solution after membrane adsorption, C_0 is the original protein concentration. Since

the total membrane volume of the capsule is 0.18 mL, and there are 6 layers membrane,

the volume of 3 layers membrane should be 0.09 mL.

Multi-plate mathematical model

Frontal analysis (FA) is a useful method for chromatography graphic studies especially for preparative chromatography where maximum usage of adsorbent is desirable. In order to test the partition coefficient, multi-plate mathematical model was used. This model was established first by Weiqiang and Junde[39]. It can fit well experimental breakthrough curves from membrane affinity chromatography. Since in this thesis, membrane chromatography is used and breakthrough curve has been obtained, frontal analysis experiment according to this model was used too.

From adsorption isotherm experiment, K_d and C_l is known. For the experimental break through curve, C_0 was 1mg/mL. Then the dimensionless groups are available now. For the experimental break through curve, V is effluent volume which can be got from the curve, and V_m is the volume of the membrane capsule, which is 0.18mL, also the porosity of the membrane capsule ε was assumed to be 0.7. Then v is available. According to the equations, v_0 and α can be obtained. Then the simulated break through curve can be available.

Result and discussion

Calibration curves

For Bio-Rad protein assay, there are two kinds of assay process. For standard process, the linear range of the assay for BSA is 0.2 to 0.9mg/mL. And when the protein
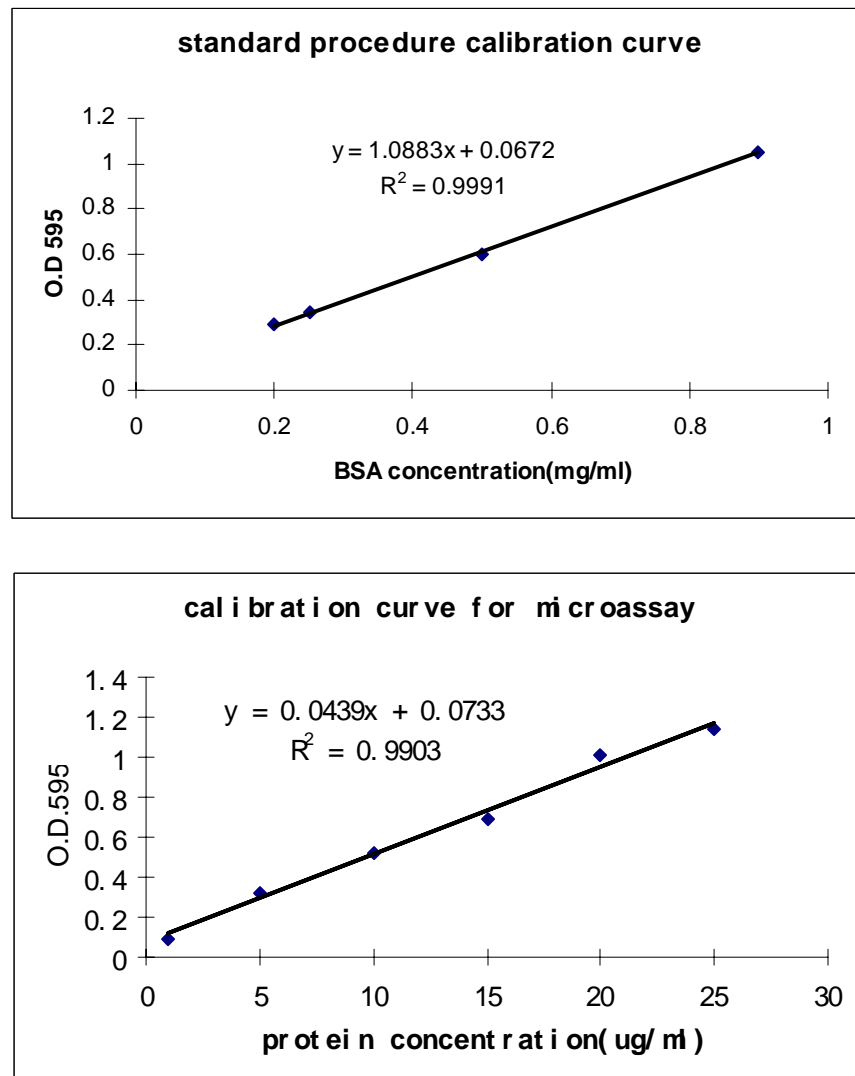
Figure 3.1 Calibration curve for protein assay, a) standard procedure, b) microassay procedure

concentration is smaller then 0.2mg/ml, standard assay is not accurate enough. Then, microassay procedure should be used.

In this thesis, protein concentration can be obtained by spectrophotometer at 595, then according to the calibration curve in Figure 3.1, protein concentration can be obtained.

Purification result

In order to optimize the separation and resolution between HSA and chicken egg white proteins, various buffers with different pH, elution buffers with different salt concentration, and step gradients had been tried. Table 3.1 shows the time profile for 10X sample when loading buffer A was Tris-HCl pH 9.5 and elution buffer B was Tris-HCl pH9.5 + 0.2M NaCl which achieved the best result.

Table 3.1. Parameters for elution of HSA and CEW proteins (10X)

Time(minutes)	Buffer A (percent)	Buffer B(percent)
0	100	0
0.9	100	0
6.3	55	45
10	40	60
20	0	100
29	0	100

This method was separately applied to chicken egg white and HSA, and the results showed the positions of the peaks which were later confirmed by SDS-PAGE as illustrated by Figure 3.5.
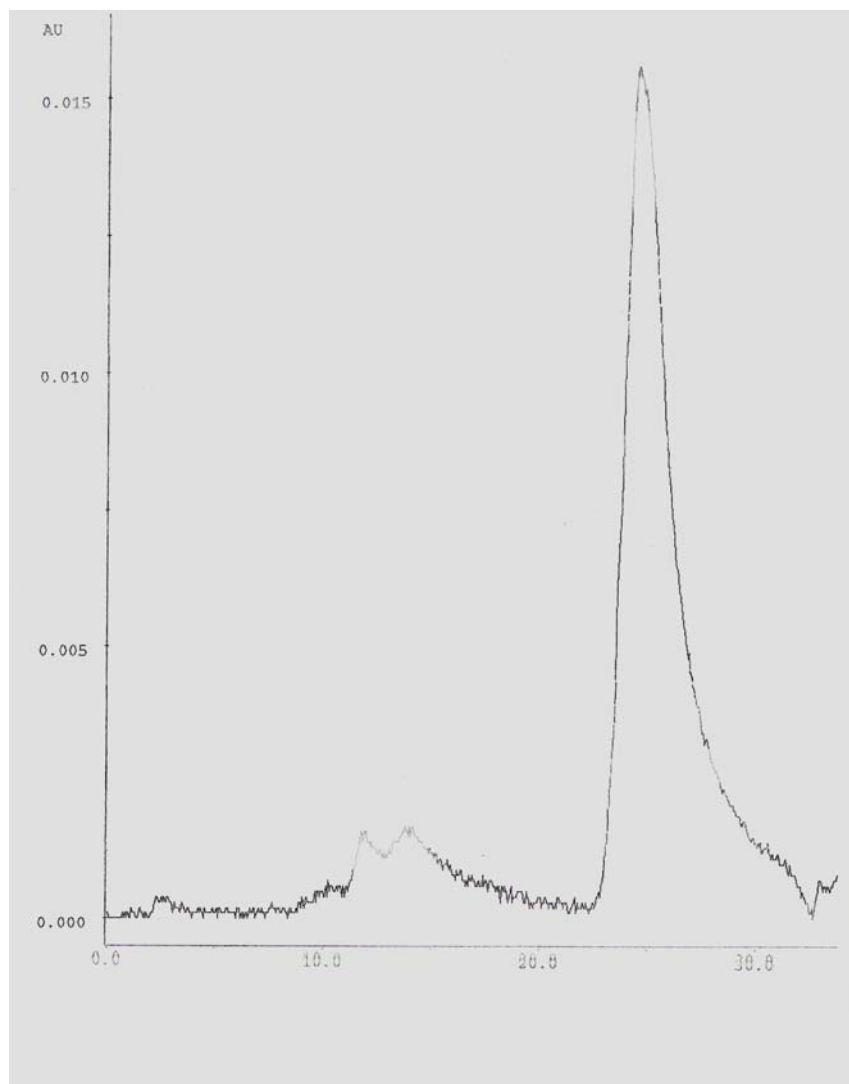
Figure 3.2. HSA elution from Mustang Q membrane capsule at 1 ml/min

In Figure 3.2, there was a small peak coming out when the time interval was from 9 to about 18 mins and a big peak coming out when the time interval was from 20 to 30 mins. The HSA was made in ninetieth, so maybe part of the HSA has denatured or just because the purity of the HSA is 97%, there is just some impurity in the small peak. The HSA peak was eluted when buffer B was 60% and buffer A was 40%.
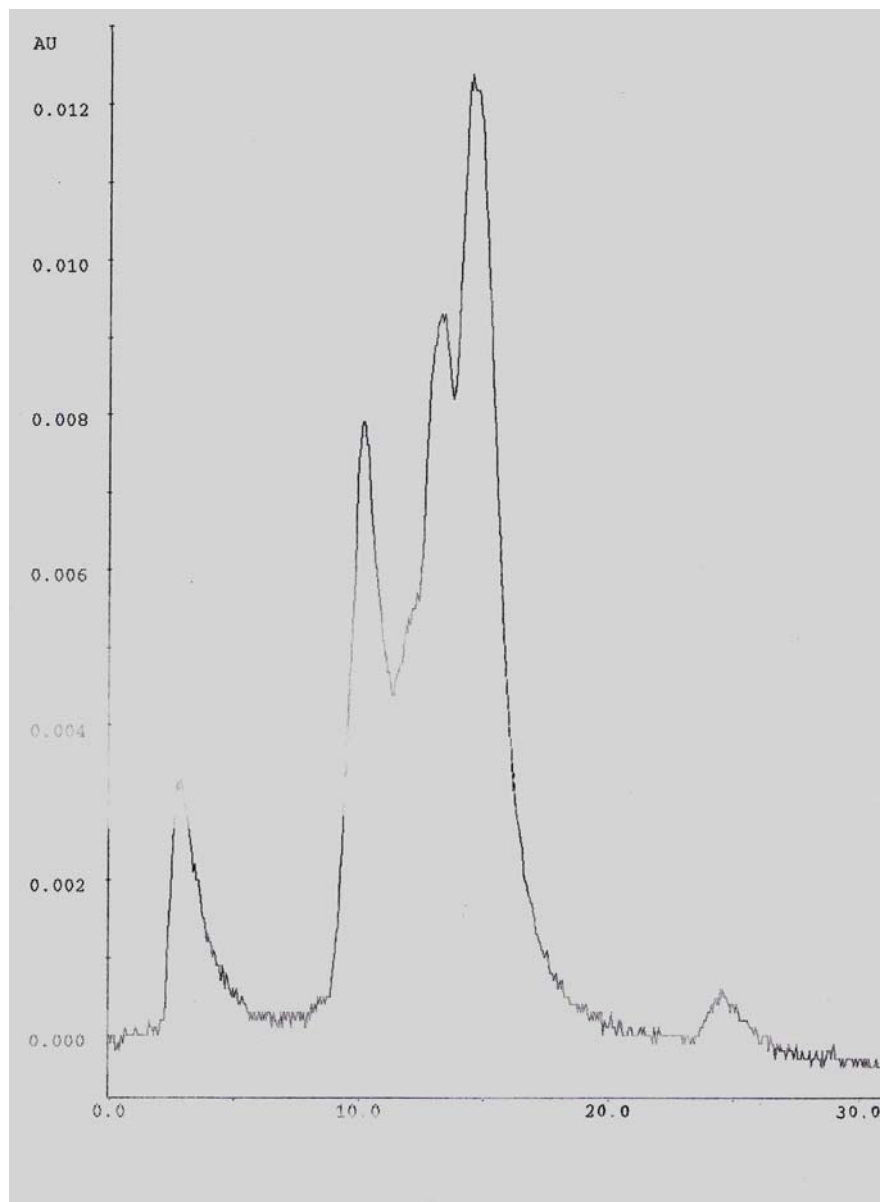
Figure 3.3 Elution of egg white proteins from Mustang Q membrane at 1ml/min.

Egg proteins are shown in Figure 3.3. Egg white proteins were separated to three

peaks, the first one was the proteins including lysozyme and avidin which had positive

charges at this buffer pH and can't be adsorbed by the membrane which has positive

too. The second peak is separated into three small branches, maybe they are different

kinds of proteins which have similar interaction with the membrane. So they come out

almost the same time, but still have some different. The second peak was the proteins

which were eluted when the gradient is from 0 to 45% B buffer. And the time interval

was from about 9 to 20 min. The third peak came out when the gradient is 60% B

buffer. The time interval of it is from 22.5 to 28 min.
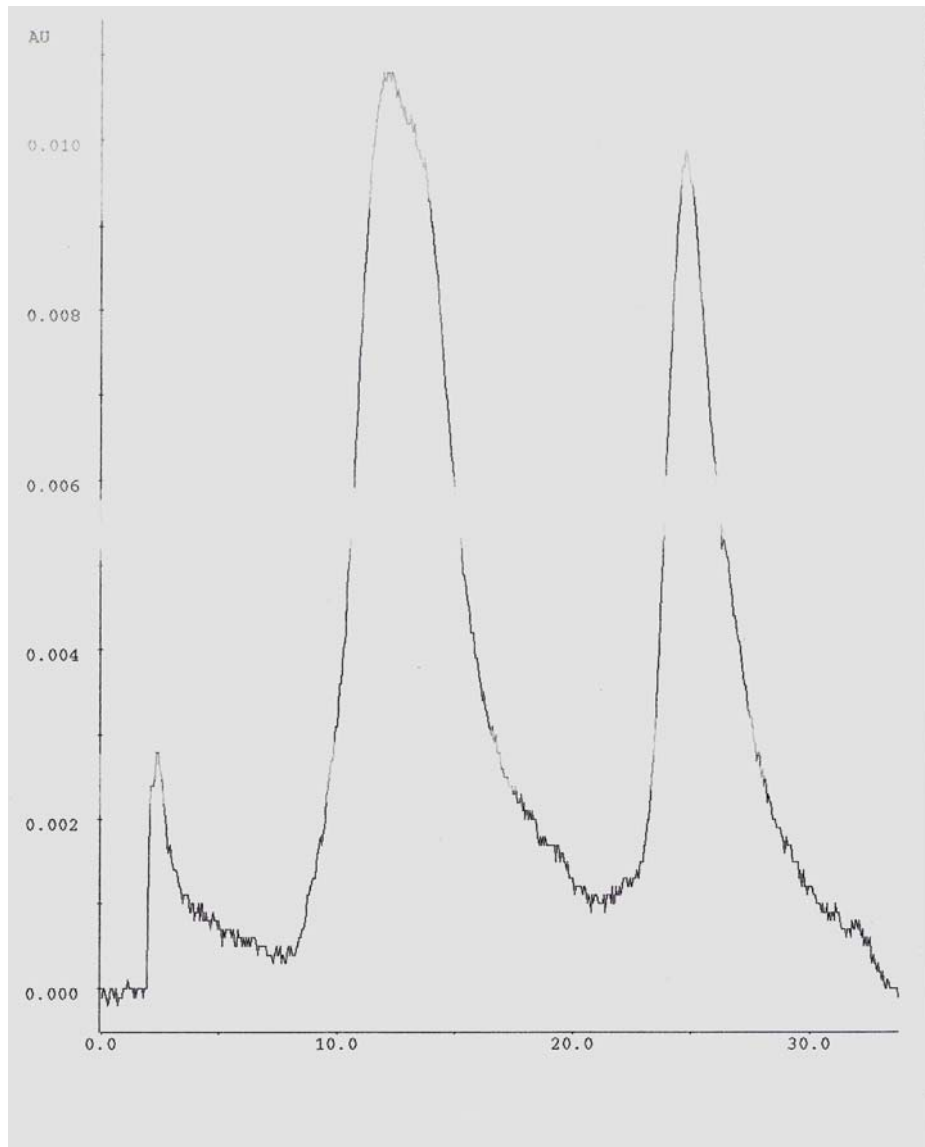
Figure 3.4 Elution of HSA and Egg which proteins from Mustang Q membrane capsule at 1 ml/min

The chromatogram in Figure 3.4 shows egg white proteins and HSA run together. In

this graph, there are three peaks which were similar with the egg white protein curve.

And for the second peak, the small branches do not exist any longer, but a big whole peak. Because the impurity protein in HSA is mixed with the proteins in egg white, and they changed the interaction between the proteins and the membrane, so the little different of the proteins in egg white was almost disappear, and they came out together. The third peak was much bigger, because the HSA was added into the proteins which come out last in the egg white. The small impurity above at 25 mL in the egg chromatogram was visible as in the HSA peak and was difficult to separate from the large HSA peak. So the third peak is not pure HSA. The contaminant may be ovomacroglobulin or one of the ovoglobulins whose MW was close to 40kd as shown by SDS-PAGE. If pure HSA is needed, another process should be used, such as the hydrophobic column used in Bedi's thesis. Further purification was not investigated here.

Fractions were collected for the impurity peak shown in Figure 3.3 and the HAS+ impurity peak in Figure 3.4 and analyzed for protein concentration using a total protein assay. For a 0.05mL sample load of CEW+HSA at 10X dilution and a flow rate of 1 mL/min, the overall yield of HSA is around 85%. The purity is 93% under the similar conditions, obtained by comparing the total protein for HSA+ impurity in Figure 3.4 with the impurity peak along in Figure 3.3 for an identical CEW load in both cases.

SDS-PAGE

Figureure 3.5 shows a gel verifying the presence of different proteins recovered by ion-exchange chromatography of HSA in chicken egg white mixture. The sample from second HSA elution peak was loaded in the second lane; the sample from the third HSA and CEW mixture elution peak was loaded in the 4th lane; the sample of 10X HSA and CEW mixture was loaded in the 6th lane; Molecular weight markers for the range of 6.7 to 209 kD were loaded in the lane 8.
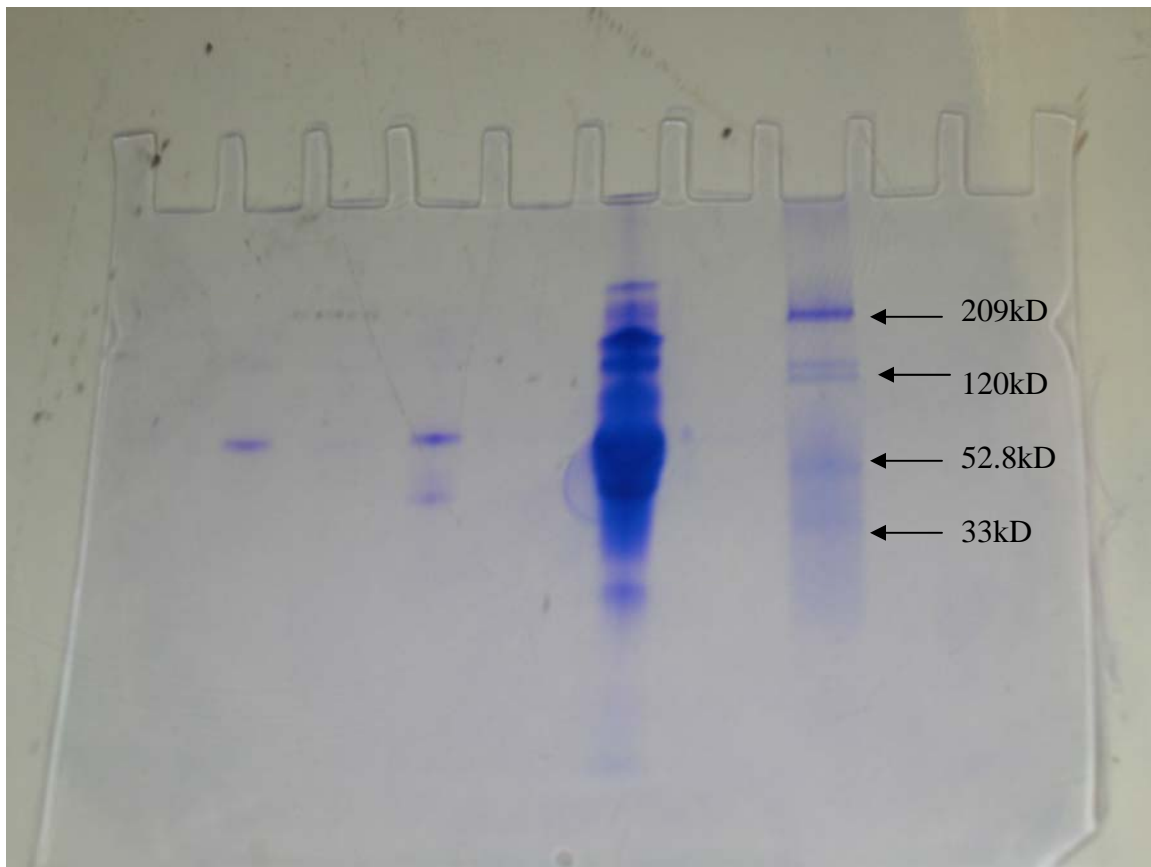
Figure 3.5 SDS-PAGE of chicken egg white proteins and HSA recovered by mustang Q membrane. Lanes: (2) Sample HSA; (4) Recovered HSA; (6) Sample CEW+ HSA; (8) Molecular weight markers.

From the gel result, the molecular weight of the impurity in the elution of HSA and CEW was around 40kD. And the molecular weight of HSA is close to 66kD which matches the published molecular weight of HSA very well.

Dilution experiment

Since the mixture of chicken egg white and HSA is very viscous, it can't be loaded on FPLC directly. Tris-HCl buffer A was used to dilute the mixture. When the mixture met with Tris-HCl buffer, some precipitate came out. Precipitation can't be loaded on the FPLC too. It will cost too much if dilute level is too high, so an optimal dilution level should be found. Various runs were made to study the dilution level.

Figure 3.6 Phenomena for diluted egg white at different dilution level

When Tris-HCl buffer was added into egg white protein mixture, some phenomena were found. At first, when dilution level increased, there were more precipitation came out, then if the dilution level increased to 5, the largest amount of precipitation came out. When the dilution level went on increasing, the amount of precipitation decreased. Like Figure.3.6 showed.There are much more precipitation in the first beaker. The reason for the phenomena was that some kind of protein could change their structure, like folding

when it meets with Tris-HCl buffer. Then it denatures and becomes precipitation. When the protein mixture was diluted 5 times, almost all of this protein has been out as precipitation, so there were the biggest amount of precipitation came out at 5X. But this kind of denature is reversible, when more Tris-HCl buffer was added into the beaker, the amount of precipitation became smaller.

Since the loaded protein meets with Tris-HCl buffer in the FPLC system, all of the precipitation should be got rid of before it is loaded on the system. During the purification experiment, all of the samples were diluted 5 level or more to make sure all of the precipitation had come out.

After diluted, the solution was centrifuged at 3000rpm for 20min, then the precipitation was got rid of and the supernatant was used as sample for FPLC.

Breakthrough experiments

1. Effect of flow rate on breakthrough curves

For Mustang Q membrane, the flow rate can be 1-4 ml/min. Different flow rate has been used to get the breakthrough curves when protein concentration is 1mg/ml.
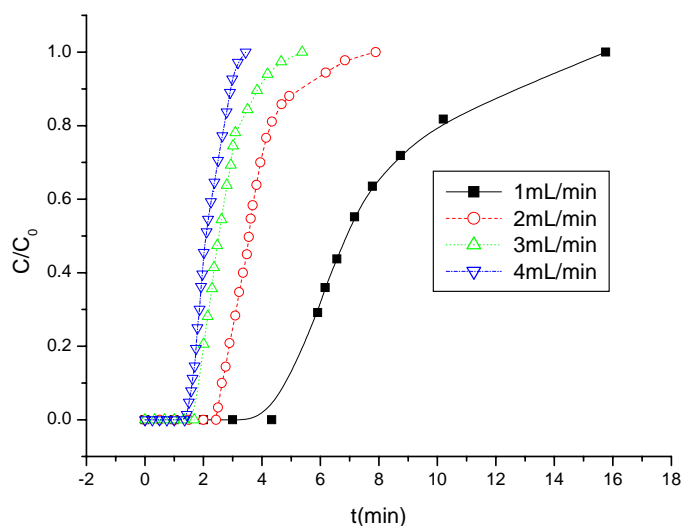
Figure 3.7 Effect of flow rate on breakthrough curves when protein concentration is 1mg/ml

In Figure. 3.7, we can find that, the larger is the flow rate, the larger is the slope of the breakthrough curve. And also, the mobile phase concentration can reach to the original protein concentration in less time interval for the higher flow rate. So decreasing the flow rate can delay the start of breakthrough, steepen the breakthrough curve and complete it in a longer time[43]. The observed effect arises from pore diffusion althrough a slower flow permits a longer contact time of a point concentration in the mobile phase with the stationary phase, resulting in more complete equilibrium. The observed behavior indicated that the pore diffusion resistance still affects the adsorption process although for

membrane it is not very important. When different flow rate was used, it still impacts the adsorption process.

2. Effect of protein concentration on breakthrough curve when flow rate is 1 ml/min

In order to test the effect of protein concentration on breakthrough curves, different concentrations of BSA have been used to get breakthrough curves.
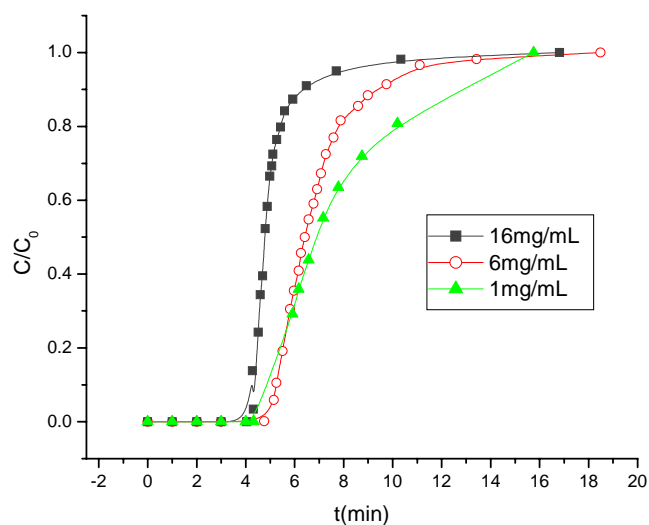
Figure. 3.8 Effect of protein concentration on breakthrough curve when flow rate is 1 ml/min

From Figure. 3.8, we can find that increasing protein concentration with the same flow rate (1 ml/min) can cause a steeper rise and earlier completion of breakthrough curve just as the effect of decreasing the flow rate on the breakthrough curves. Increasing protein concentration causes a increase in pore diffusivity[43]. It is the increased pore diffusivity that contributes to the increase in the initial slope of the breakthrough curve and the early start to the curves with increasing protein concentration.

Membrane fouled and cleaning

When the membrane is fouled, there is no adsorption left. The possible reasons for membrane fouled includes: the membrane is destroyed; the ligands structure is destroyed or lost; the adsorption is irreversible and the membrane is blocked.

Figure 3.9 showed the elution of BSA through a fouled membrane.
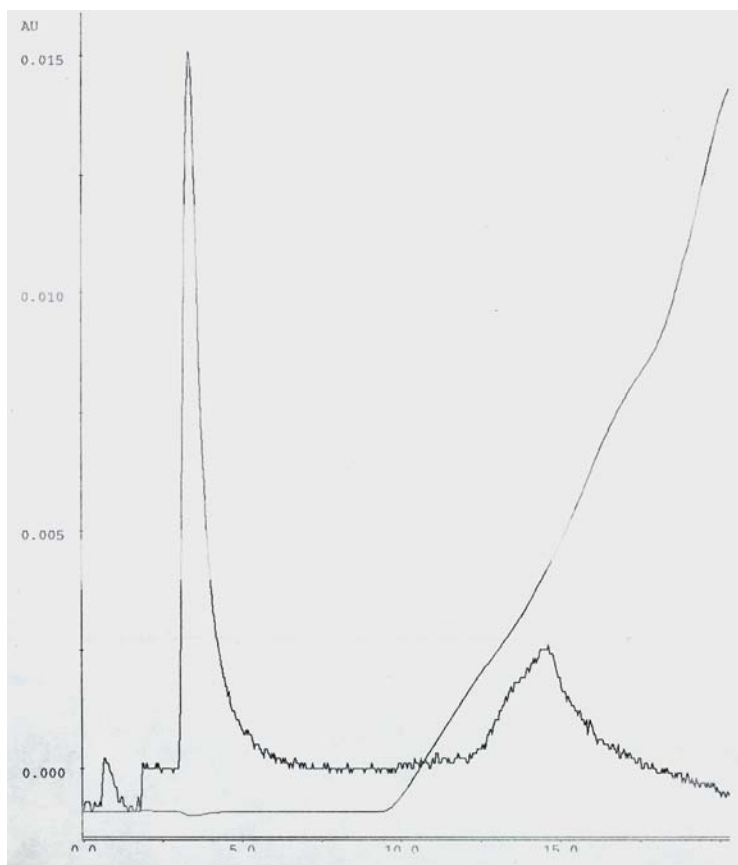
Figure 3.9 Membrane fouled curve.

In this graph, there are two peaks, the first one was much bigger than the second one. And the first one came out before buffer gradient. The first peak was the BSA coming out just after they are loaded on the membrane. It means that they were not bound at all. The second peak is BSA which are bounded on the membrane and washed out later. The

second peak is much smaller than the first peak, which means that almost all of the BSA is washed out without binding. At this time, we say that the membrane is fouled.

The membrane capsule is designed to use once, or to say disposable. To save money, we do membrane cleaning. When 10 ml 1M NaOH was used to clean the membrane, it can be used again. But since 1M NaOH is strong base, it maybe destroy the membrane and leading to the irreversible membrane dead. So the membrane is cleaned in this way: using 100% B buffer washing 20 min each time the membrane is used. Then use 10 ml 0.5 M NaOH to clean the membrane when it has been used 5 times. This method can keep the membrane clean enough to be used. On this experiment, 10 membranes capsules are used hundreds of times by using this cleaning method. Since the membrane can be cleaned, the membrane dead is reversible. The possibility for the membrane dead is that the membrane is blocked.

When the buffer pH is the same as the PI of protein, the protein will precipitate. The precipitation not only can block the membrane pores, which will cause membrane to be fouled, but also it will cause the whole process unrepeatable. Because the precipitate is denatured protein, they are not acted as they are supposed to be eluted on a certain gradient or pH. At this time, the membrane seems not dead, but the result is not accurate. So thorough cleaning is very important.

And also, since there are many kinds of proteins in egg white, the PI of these proteins are wide, so when running egg white the cleaning should be done longer time.

Langmuir isotherm adsorption experiment

According to Langmuir isotherm equation (12):

K_d should be the mobile phase concentration when the stationary phase concentration

equals to the half of the C_l.
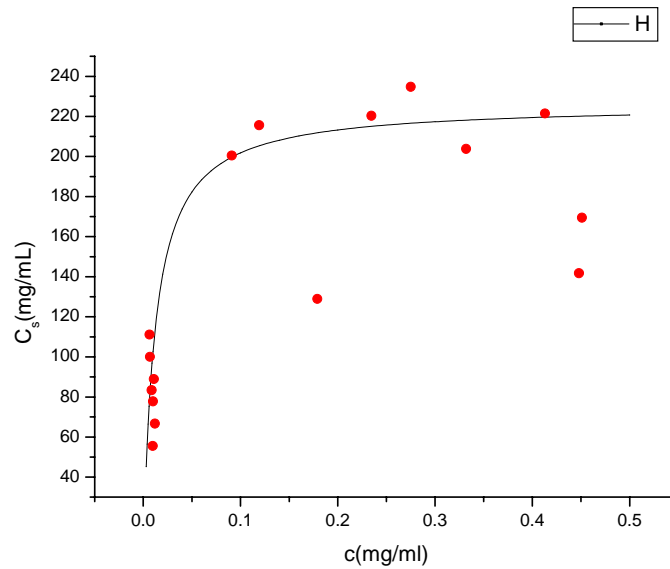
Figure. 3.10 Fitting of theoretical Langmuir isotherm equation (solid line) by experimental data (red point)

From the graph above, the highest stationary concentration C_l is close to 226 mg/ml,

and K_d is close to 0.012mg/ml.

Some points in the graph have very low Cs comparing with the others. There are some

reasons can cause this result: the membranes were not identical; some membranes

especially the are attached to the capsule which was destroyed when it was opened; the

top membrane was dry when they were put into the BSA solution, so the ligand was not

activated as the others which were still wet.

In Figure. 3.10, the maximum capability is 226mg/mL. From Figure. 3.7 and 3.8, the binding capacities were obtained. The binding capacity here was calculated by breakthrough curve and it is the protein concentration on the membrane when there were BSA observed at the exit of the column. Before loading sample, 2.7mL (15 column volume) buffer was used to equilibrium the column. After calculation, the binding capacities for different flow rate and different concentration were listed in Table 3.2. When the sample concentration was 1mg/mL, the four binding capacities with different flow rate were very close, and the higher of the flow rate, the higher of the binding capacity. This result means that pore diffusion still should be counted on although it didn't make too much difference. When the flow rate is 1ml/min, the binding capacity for 6mg/ml sample was much lower than 16 mg/mL. This result meant that when sample concentration was very high, the pore diffusion became much more important. Since the sample concentration used for this experiment was much lower than 16 mg/ml, pore diffusion was still not important.

The binding capacity obtained by breakthrough curve should be lower than isotherm adsorption. The proportion of isotherm adsorption and binding capacity is much different[41, 44, 45, 46, 47, 48]. Sometimes, the isotherm adsorption can be 25 times higher than the binding capacity[49]. For traditional column beads, the maximum capability of BSA solution is much different for different test methods, different salt concentration, pH and different type of beads[50, 51, 52, 53]. The highest q_s I found is 480mg/mL when BSA was merged in hard Dextran DEAE at pH 6.9[50]. This result means that the maximum adsorption capability for BSA on ion exchange adsorbent is in a broad range.

Table 3.2 Binding capacity at different conditions

Concentration(mg/mL)	1				6	16
Flow rate(ml/min)	1	2	3	4	1	1
Binding capacity(mg/mL)	9.1	12	13	15.2	68.3	119.1

After breakthrough point, the protein appeared at the exit of the column. But at this time, the membrane was not saturated. Until the protein concentration of the exit reached to the loading concentration of the protein solution, the membrane still has ability to bind more protein. And for the maximum capability of the membrane, the maximum amount of protein was bound on the membrane after 48 hours, and at this time, the membrane was totally saturated. This is one the reasons that why the stationary maximum capability is bigger than the binding capacity.

The rate of interface mass transfer is dependent the combination of different mass transfer resistance, film transport, intrapartical diffusion and reaction kinetics. The one which is slow among these mass transfer mechanisms might be considered as the rate-limiting tep of the sorption process, and governing the whole saturation performance. The result of isotherm adsorption much higher than binding capacity means that the process of protein passing through the membrane is diffusion control. Permeability, equilibrium capability and mass transfer behavior are all relative for membrane binding capacity[54]. For Mustang Q membrane, a strong anion exchange adsorbent, the adsorption equilibrium between protein and ligand in membrane should be fast. Mass transfer resistance including axial dispersion, radial dispersion, pore diffusion, and film

diffusion. According to the peclet number(from the result of the model fitting), the axial dispersion is very small, which means axial dispersion is not the rate-limiting step too. The size of BSA is 3.0*3.0*14nm [55], and the pore size of the membrane is 0.8μm, which is much bigger than the size of BSA. The large pore size makes the proteins pass through the membrane before it reaches to the pore wall[47]. So the mixture of radial dispersion, pore diffusion and film diffusion must be the main points in mass transfer resistance. Since the pore size on membrane is much bigger than the size of protein, the pore diffusion should be neglect too. So the total mass transfer resistance should be mainly contributed by radius dispersion or film diffusion. And steric hindrance in the lining of ligands may be a factor too.

Multi-plate mathematical model

The result of isotherm experiment has been used for multi-plate mathematical model.
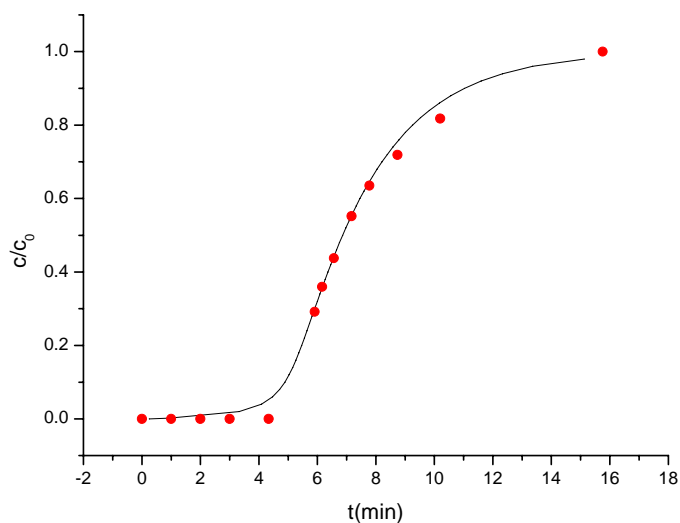
Figure. 3.11 Fitting of simulated data calculated by multi-plate model (solid line) by experimental breakthrough curve (red circle) when the BSA concentration is 1mg/ml and the flow rate is 1 ml/min

When $\alpha=47$, $\upsilon_0=0.25$, the fitting breakthrough curve is showed as Figure.3.11. From this figure, the theoretical breakthrough curve fits that of the experiment one very well when BSA concentration was 1 mg/ml and the flow rate was 1 ml/min.

Parameter α may indicate the chromatographic performance, and it is related to theoretical plate number. The higher is α, the better the chromatographic performance. Parameter υ_0 may be considered as the theoretical dimensionless breakthrough volume. Under this operation, υ_0 is about 0.25. When the mathematical model was used, porosity was needed. Here, porosity was chosen as 0.7. Since the theoretical graph matches the experimental graph very well, the evaluation of porosity is very close to the real data.

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From Equation (17) and (19), we can see that, α has not the same meaning as N. But when $A(c)$ is very big, the value of α is very close to N. So the value of α from this model can be thought the same as that of theoretical plates.

Under the assumption that instantaneous equilibrium between stationary and mobile phases, the solid phase concentration C_s is directly derived from the adsorption isotherm model, $C_{s,i} = f(C1,C2, \ldots ,Cn)$. The contribution of the mass transfer resistances is included in the value of the apparent axial dispersion coefficient[54, 56, 57].

$$D_i = \frac{uL}{2N_i} \qquad (20)$$

Ni the plate number for component i. In practice, it is assumed that all components have the same plate number.

Since peclet number is defined as,

$$Pe = uL/D \qquad (21)$$

Then,

$$N = \frac{1}{2}Pe \qquad (22)$$

So

$$Pe = 2N \approx 2\alpha = 84$$

And here, u is the interstitial flow rate (cm/min), L is the length of the membrane, D is apparent axial dispersion coefficient (cm^2/s).

The radius of the membrane is 2.2cm, and the flow rate is 1ml/min,

So
$$u = \frac{1mL/\min}{area} = \frac{1mL/\min}{\pi(R/2)^2} = \frac{1cm^3/\min}{\pi(2.2/2)^2} = 0.263cm/\min$$

$$L = \frac{V_m}{S} = \frac{0.18mL}{\pi(2.2/2)^2} = 0.047cm$$

$$D = \frac{uL}{Pe} = \frac{0.263*0.047}{84} = 2.45*10^{-6}cm^2/s$$

Such small axial dispersion coefficient means that the axial dispersion is not significant[54].

Conclusions and Recommendations

Mustang Q membranes prove to be a promising technique for the separation of HSA from chicken egg white proteins. For a 50μl sample load of CEW+HSA at 10X dilution and flow rate of 1ml/min, the overall yield of HSA is 85%, and the purity is 93% under similar conditions. However, it is not enough to get the HSA from chicken egg white proteins and HSA mixture only by mustang Q membrane chromatography. It can't separation HSA from chicken egg proteins thoroughly, and there are still some impurity left in the production. The molecular weight of the impurity is close to 40kD according to the result of SDS-PAGE. So the impurity should be one of the ovoglobins according to the Table 1.1. The isoelectric point of the impurity is very close to that of HSA, so they are not able to be separated by ion-exchange chromatography

In order to get rid of the impurity, another method like hydrophobic interaction chromatography should be used. The theory of hydrophobic interaction chromatography to separate proteins is according to different hydrophobic interaction between the proteins and the adsorbent. So hydrophobic interaction column should be able to be used to get pure HSA. Since in Bedi's thesis, this method has been tried and good result has been obtained, we didn't use this method again.

Precondition steps like dilution and centrifugation are required to get rid of egg white precipitate before loading on the membrane. When the mixture of chicken egg white proteins and HSA meets with Tris-HCl buffer, there are precipitation comes out. Since the solution loaded on FPLC meets with Tris-HCl buffer A on the membrane, and there are still precipitation comes out, all of the precipitation should be got rid of before sample

loading. The reason for the precipitation should be a kind of protein folding when meets with Tris-HCl buffer. Then the denatured protein becomes precipitation and comes out of the solution. This denaturation is reversible according to the dilution experiment. After 5X, all of the precipitation comes out. Then with increasing dilution level, the amount of precipitation becomes smaller. Since it costs for more dilution time, 5X should be best. Multi-plate mathematical model has proven suitable to predict breakthrough curve in this experiment.

The method used to get specific pH in the buffer was adding concentrated HCl into Tris buffer in this experiment. This method is not accurate to keep the pH in the accurate number since the pH machine is not very accurate. In the future, if this experiment can be gone on, some other accurate method to get specific pH of buffer should be used.

For Mustang Q membrane capsule used in this experiment, the radius is broad comparing with the length of it, so the process is not uniform. And the radius diffusivity should be very high comparing with the pore diffusivity and axial diffusivity.

For isotherm adsorption experiment, 10mg/mL BSA solution was made first. Then different BSA solution with different concentration was made by diluting the 10mg/mL solution. Since the isotherm adsorption was tested by two parts, and the second part was late than the first part about one week. The same original solution was used to get the diluted BSA solution in order to avoid the operation difference. But the concentration of original solution maybe change since the temperature was so high in the room and the humidity was so low in the refrigerator. Next time, isotherm adsorption experiment should be finished in one time. Or the preexperiment can be done first to know some basic protein concentration needed, then according to the first experiment, the protein

concentrations using for the experiment can be obtained. Then the total experiment should be done in the same time.

From multi-plate mathematical model, the theoretical plate number, peclet number and axial dispersion coefficient can be obtained. Since the axial dispersion coefficient is very small which means that axial dispersion is not important for the adsorption process. And the radius dispersion or film diffusion or both of them is the rate-limiting step for the adsorption process.

Appendix 1

Numerical data for the calibration curve

The data used for the calibration curve for standard procedure was listed in Table A.1.

Table A.1 The data used for the calibration curve for standard procedure

C(mg/mL)	A_{595}			$A_{average}$
0.2	0.291	0.291	0.289	0.290
0.25	0.343	0.340	0.334	0.342
0.5	0.597	0.595	0.589	0.594
0.9	1.052	1.054	1.052	1.053

The data used for the calibration curve for microassay procedure was listed in Table A.2.

Table A.2 The data used for the calibration curve for microassay procedure

C(ug/mL)	A_{595}			$A_{average}$
1	0.087	0.091	0.096	0.089
5	0.322	0.325	0.323	0.324
10	0.518	0.523	0.518	0.521
15	0.690	0.695	0.688	0.693
20	1.009	1.021	1.014	1.009
25	1.133	1.141	1.133	1.137

Appendix 2

Numerical data for the flow rate experiment. Breakthrough curve has been run by different flow rate when the concentration of BSA was 1 mg/mL. Table A.3 listed the data for flow rate experiment graph.

Table A.3 Data for flow rate experiment graph.

1 mL/min		2 mL/min		3 mL/min		4 mL/min	
t(min)	C/C_0	t(min)	C/C_0	t(min)	C/C_0	t(min)	C/C_0
0	0	0	0	0	0	0	0
1	0	1	0	1	0	1	0
2	0	2	0	2	0	2	0
3	0	3	0	3	0	3	0
4	0	4	0	4	0	4	0
4.33	0	2.434211	0	1.684588	0	1.359223	0
5.91	0.292	2.5	0.033	2.007168	0.206	1.432039	0.014
6.16	0.359	2.631579	0.1	2.150538	0.281	1.504854	0.048
6.57	0.438	2.763158	0.144	2.293907	0.357	1.57767	0.079
7.17	0.552	2.894737	0.208	2.365591	0.414	1.626214	0.112
7.78	0.635	3.092105	0.283	2.473118	0.474	1.703883	0.146
8.74	0.719	3.223684	0.347	2.616487	0.544	1.747573	0.194
10.20	0.808	3.355263	0.4	2.795699	0.638	1.796117	0.25
15.76	1	3.486842	0.45	2.939068	0.693	1.868932	0.301
		3.552632	0.497	3.010753	0.745	1.917476	0.362
		3.618421	0.547	3.089606	0.781	1.966019	0.396
		3.684211	0.583	3.512545	0.844	2.014563	0.455
		3.815789	0.639	3.835125	0.896	2.11165	0.511
		3.947368	0.7	4.193548	0.940	2.160194	0.545
		4.144737	0.767	4.659498	0.974	2.257282	0.593
		4.342105	0.811	5.376344	1	2.378641	0.646
		4.671053	0.858			2.5	0.705
		4.934211	0.881			2.645631	0.772
		6.184211	0.944			2.791262	0.837
		6.842105	0.978			2.912621	0.890
		7.894737	1			2.985437	0.927
						3.179612	0.972
						3.446602	1

Appendix 3

Numerical data for concentration experiment. When flow rate is 1 mL/min, 3 different

BSA concentrations have been used to run breakthrough curve. Table A.4 listed the data

for concentration experiment.

Table A. 4 Data for concentration experiment graph.

1 mg/mL		6 mg/mL		16 mg/mL	
t(min)	C/C_0	t(min)	C/C_0	t(min)	C/C_0
0	0	0	0	0	0
1	0	1	0	1	0
2	0	2	0	2	0
3	0	3	0	3	0
4	0	4.75	0.001	4.04	0
4.33	0	5.15	0.059	4.27	0.138
5.91	0.292	5.25	0.105	4.31	0.034
6.16	0.359	5.51	0.191	4.51	0.243
6.57	0.438	5.81	0.305	4.60	0.344
7.17	0.552	5.96	0.355	4.69	0.395
7.78	0.635	6.16	0.409	4.79	0.523
8.74	0.719	6.26	0.457	4.88	0.583
10.20	0.808	6.41	0.5	4.98	0.665
15.76	1	6.57	0.548	5.07	0.693
		6.77	0.591	5.11	0.725
		6.92	0.630	5.26	0.764
		7.07	0.673	5.42	0.798
		7.27	0.725	5.59	0.842
		7.58	0.770	5.92	0.874
		7.88	0.816	6.48	0.910
		8.59	0.855	7.70	0.9500
		8.99	0.884	10.33	0.982
		9.75	0.914	16.81	1
		11.11	0.966		
		13.43	0.982		
		18.48	1		

Appendix 4

Data for isotherm adsorption was listed in Table A.5.

Sample calculation:

For C_0=1.5 mg/mL, by microassay,

$A_{average}$=0.449,

The calibration curve for microassay is

$y = 0.0439x + 0.0733$

So the concentration after membrane adsorption is,

C=(0.449-0.0733)/0.0439=8.558 ug/mL

C_s=(C_0-C)*5/0.09=83.33 mg/mL

For C_0=3.7 mg/mL, use standard procedure,

$A_{average}$=0.166,

And the calibration curve for standard procedure is

$y = 1.0883x + 0.0672$

So the concentration after membrane adsorption is,

C=(0.166-0.0672)/1.0883=0.091 mg/mL

C_s=(C_0-C)*5/0.09=200.5 mg/mL

Table A.5 Data for isotherm adsorption

C_0(mg/ml)	A_{595}			$A_{average}$	C(ug/mL)	C_s(mg/mL)
1	0.515	0.498	0.492	0.502	9.765	55.56
1.2	0.587	0.576	0.573	0.579	11.519	66.67
1.4	0.536	0.532	0.527	0.532	10.449	77.78
1.5	0.457	0.447	0.442	0.449	8.558	83.33
1.6	0.554	0.546	0.543	0.548	10.813	88.89
1.8	0.381	0.377	0.371	0.376	6.895	100
2	0.364	0.364	0.357	0.362	6.576	111.11
C_0(mg/ml)	A_{595}			$A_{average}$	C(mg/mL)	C_s(mg/mL)
2.5	0.266	0.261	0.259	0.262	0.179	128.94
3	0.563	0.553	0.549	0.555	0.448	141.77
3.5	0.566	0.555	0.552	0.558	0.451	169.39
3.7	0.169	0.167	0.162	0.166	0.091	200.5
4	0.430	0.428	0.425	0.428	0.332	203.80
4.2	0.123	0.119	0.115	0.197	0.234	220.3
4.4	0.415	0.414	0.410	0.517	0.413	221.5
4.5	0.364	0.367	0.370	0.367	0.275	234.70

Appendix 5

Data for model

The theoretical breakthrough curve was done according to Table A.6.

Sample calculate:

When $C/C_0=0.2$,

$$\lambda = \frac{C_l}{C_0} = 226$$

$$\varphi = \frac{C_0}{K_d} = 1/0.012$$

then

$$A(c) = \frac{\lambda}{1+(1/\psi)}\frac{c}{(1/\psi)+c} + \frac{\lambda/\psi}{(1+(1/\psi))^2}\ln(1+\psi c) - [\frac{\lambda/\psi}{(1+(1/\psi))^2} + \frac{50\varepsilon}{1-\varepsilon}]\ln(1-c)$$

$$=244.91$$

When

$v_0=0.25$ and $\alpha=47$,

$$v(c) = v_0 + \frac{A(c)}{\alpha} = 0.25 + 197.9/47 = 5.461$$

Table A.6 Data used for multi-plate mathematical model

C/C$_0$	A	V(c$_{th}$)
0	0	0.25
0	0	0.25
0	0	0.25
0	0	0.25
0.002	32.54995	0.942552
0.02	144.5829	3.326231

0.04	180.5384	4.091242
0.06	198.2275	4.467606
0.08	209.5339	4.708168
0.1	217.8788	4.885718
0.12	224.6205	5.02916
0.14	230.4084	5.152306
0.16	235.5933	5.262624
0.18	240.3828	5.364527
0.2	244.9081	5.460811
0.22	249.2573	5.553348
0.24	253.4923	5.643454
0.26	257.6584	5.732094
0.28	261.7902	5.820004
0.3	265.9151	5.907769
0.32	270.0557	5.995867
0.34	274.2312	6.084706
0.36	278.4583	6.174645
0.38	282.7526	6.266014
0.4	287.1286	6.359119
0.42	291.6002	6.454259
0.44	296.1814	6.551732
0.46	300.8865	6.65184
0.48	305.7302	6.754897
0.5	310.7281	6.861235
0.52	315.897	6.971212
0.54	321.2552	7.085217
0.56	326.8229	7.203679
0.58	332.6227	7.32708
0.6	338.68	7.455958
0.62	345.0238	7.590932
0.64	351.6874	7.732711
0.66	358.7096	7.882119
0.68	366.1359	8.040126
0.7	374.0205	8.207884
0.72	382.4286	8.386779
0.74	391.4395	8.5785
0.76	401.1516	8.785141
0.78	411.6887	9.009335
0.8	423.21	9.254469

0.82	435.9249	9.524997
0.84	450.1166	9.826949
0.86	466.1825	10.16878
0.88	484.7039	10.56285
0.9	506.5821	11.02834
0.92	533.3269	11.59738
0.94	567.7683	12.33018
0.96	616.2592	13.3619
0.98	699.0714	15.12386

Table A.7 Data used to calculate coefficient of determination(COD)

v	C(ex)	C(th)
0	0	
1	0	
2	0	
3	0	
4.3333	0	
5.909	0.29167	0.3
6.16162	0.3594	0.36
6.5656	0.4375	0.44
7.1717	0.55208	0.56
7.7778	0.63542	0.66
8.737	0.71875	0.76
10.202	0.8177	0.86
15.75757	1	

Calculation for COD:

The data used here for $c_{ex,j}$ are listed in Table A.7.

$\bar{c}_{ex} = 0.37$

$\sum (c_{ex} - \bar{c}_{ex})^2 = 1.518$

$\sum (c_{th} - c_{ex,j})^2 = 0.004$

$$COD = 1 - \frac{\sum_i (c_{ex,i} - c_{th})^2}{\sum_i (c_{ex,i} - \bar{c}_{ex})^2} = 0.997$$

Appendix 6

Data for HSA purity and recovery calculation is listed in Table A.8.

Table A.8 Data for HSA purity and recovery calculation

Peak	Mean A_{595}	Concentration (ug/mL)	Protein content
HSA+ Impurity	1.049	22.23	0.167
Impurity	0.147	1.67	0.013

Microassay detection limit $\leq$ 25 ug/mL

Sample loaded= 0.05 mL

Total initial load of HSA= 4mg/mL* 0.05mL=0.2mg

Elution volume for HSA+ Impurity=7.5mL

Total HSA in the HSA + Impurity peak= 22.23 ug/mL * 7.5mL=0.167 mg

The recovery of HSA = 0.167/0.2=85%

Total protein content in the elution= 0.167+0.013=0.18mg

The purity of HSA= 0.167/0.18=93%

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