PRELIMINARY STUDY ON PARAMETERS OF LYSOZYME PURIFICATION USING ION EXCHANGE CHROMATOGRAPHY

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A report submitted in fulfillment of the requirement for the award of the Degree of Bachelor of Chemical Engineering

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DECLARATION

‘I declare that thesis is the result of my own research except as cited references. The thesis has not been accepted for any degree and is concurrently submitted in candidature of any degree.”

Signature	: …….
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Date	: 27 November 2006

DEDICATION

Special Dedication of This Grateful Feeling to My…

Beloved parent;
Mohd Nawi Namat
Isye Washilah Moch Romlie

Supportive families;
All members of families

Understanding friends;
Mohammad Faizal Abidin Zainal Abidin
Noor Adilah Md Arifin

For all your love, care, support, and believe in me.Thank you so much.

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Last but not list, my sincere thankful is also extends to all my colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. And last, but not least I thank my mother's and other family members for their continuous support while completing this thesis.

ABSTRACT

Lysozyme is high demand bioproduct in market nowadays produced by protein purification process. Chromatography is a multicomponent separation technique which primarily used in the biotechnology sector especially in protein purification. The physical method of chromatography separation in which the components to be separated are distributed in stationary phase and mobile phase. One of the chromatography techniques that widely used is ion exchange chromatography (IEC) which based on the electrostatic interaction. The basic theory of the IEC separation process is depending on the isoelectric of the protein (pI). The pI of the protein is influence to determine the media of IEC whether anion or cation exchanger been used. In the experimental work, the automated liquid chromatography system from Amersham-Pharmacia Biosciences is designed for method development and research application whereas calibrated and controlled by a computer using UNICORN software. Preliminary study on the parameters is based on pH and salt concentration which to achieve the high purity of lysozyme. Some purification experiments were done to find the optimal operation condition to purify lysozyme from protein mixture by changing buffer pH and salt concentration in elution buffer to elute the interest protein. Based on the result and discussion; by manipulating pH value, the highest height detected at pH 8.5 which is 2.680. Using calibration curve, concentration of lysozyme is 0.0315 mg/ml. When NaCl concentration was manipulated, the concentration was optimizing at 0.3 M with is the result is 0.0284 mg/ml. As the conclusion, the optimum condition in purification step at pH buffer 8.5 and elution buffer at 0.3 M which is almost to the expected result refer to previous study.

ABSTRAK

Pada masa kini, lysozyme merupakan produk biologi yang mendapat permintaan tinggi dalam pasaran. Kebanyakan protein tersebut dihasilkan daripada proses penulenan protein. Kromatografi mempunyai kepelbagaian teknik pengasingan yang mana banyak digunakan dalam sektor bio-teknologi khususnya dalam penulenan protein. Secara fizikal, proses pengasingan oleh kromatografi adalah terbahagi kepada dua fasa iaitu fasa statik dan fasa bergerak. Salah satu teknik kromatografi yang digunakan secara meluas dalam industri adalah pertukaran cas kromatografi. Pertukaran cas kromatografi adalah proses pengasingan yang berdasarkan perhubungan cas-cas elektrostatik oleh komponen-komponen yang bergerak dan juga media kromatografi yang statik. Pada asasnya, pertukaran cas kromatografi sangat bergantung kepada titik isoelektrik yang ada pada setiap protein. Ketepatan media kromatografi yang digunakan samada media yang bercas positif ataupun bercas negatif adalah ditentukan oleh titik isoelektrik pada protein. Automatik kromatografi daripada Amersham-Pharmacia Bioscience telah direka bentuk bagi memudahkan eksperimen dijalankan sekaligus untuk pembangunan dan aplikasi dalam penyelidikan. Oleh kerana kromatografi direkabentuk secara automatik, maka segala proses kawalan ditentukan oleh komputer berdasarkan perisian UNICORN yang telah didatangkan khas bersama-sama sistem tersebut. Kajian secara utama dilakukan terhadap parameter-parameter penting iaitu pH penampan dan kepekatan garam untuk mencapai ketulenan lysozyme yang maksimum. Oleh yang demikian, beberapa eksperimen penulenan dilakukan bagi mendapatkan keadaan operasi yang paling optimum dalam proses pengasingan lysozyme daripada campuran protein. Eksperimen dilakukan dengan mengubah nilai pH bagi penampan yang digunakan dan juga kepekatan garam bagi menyahktifkan ikatan ionik di antara lysozyme dan media. Berdasarkan keputusan dan perbincangan daripada eksperimen yang dijalankan, didapati penampan pada pH 8.5 memberikan bacaan yang paling tinggi iaitu 0.0315 mg/ml. Sementara, Kepekatan

NaCl pada 0.3 M memberi bacaan yang paling tinggi iaitu 0.0284 mg/ml. Secara kesimpulan daripada eksperimen yang dijalankan didapati nilai pH 8.5 dan 0.3 M NaCl memberikan bacaan yang optimum untuk proses penulenan lysozyme yang mana menghampiri nilai jangkaan melalui kajian-kajian terdahulu.

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LIST OF SYMBOLS

Abs	-	Absorbance
CEW	-	chicken egg white
g	-	gram
g/ml	-	gram per mililiter
IEC	-	Ion Exchange Chromatography
kg	-	kilogram
L	-	liter
ml	-	mililiter
mm	-	milimeter
pI	-	Isoelectric point
rpm	-	rotation per minute
UV	-	ultraviolet
wt %	-	weight percent
µg/ml	-	microgram per mililiter
°C	-	°Celcius
%	-	percent

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CHAPTER 1

INTRODUCTION

1.1 Overview of Research

Nowadays, the sectors of biotechnology have achieved the successful for bulk product manufacturing. The ever-growing demands for high-quality bioproducts give an impact to the downstream processes. Mostly, products of biotechnology are proteins and these proteins must be prepared in large volumes in purified form. Today's technology, bioseparation processes for life sciences require different approaches from those used in traditional chemical industries (Keller *et al*, 2001). The most efficient bioseparation processes are needed to achieve high demand in marketing such as pharmaceutical, agrichemical, nutrition and biotechnology bioproducts industries.

The word 'bioproduct' means chemical substances must be extracted and purified to some degree before being suitable for market. Separation technologies also play a key role in waste minimization and energy conservation and bioseparation method can be broadly considered as consisting of four sequential steps; removal of insoluble, isolation, purification and polishing of the products (Singh & Singh 1996). On protein purification, separation is usually accomplished by chromatography. There are two mechanisms for chromatography;

i. Adsorption in which the sample molecules are adsorbed onto the chromatographic medium through binding (such as ion exchange chromatography). Ion exchange chromatography is one of the liquid chromatography techniques which based on electrostatic interactions. For IEC, the concept is interaction between opposite charges whereby charged group on the proteins (sample) interact with charged groups on the ion exchanger. Since, proteins vary from each other based on their size, shape, charge, hydrophobicity, solubility and biological activity. Different proteins have different charges and interact differently in ion exchange chromatography.

ii. Nonadsorption concept such as gel filtration chromatography, affinity chromatography, size exclusion and others related techniques.

1.2 Problems Statement

Protein is one of the bioproducts that must be prepared in purified form. The separation and purification process of the proteins become complicated because of the diversity of the proteins. Furthermore, the product is in high demand because of the limitation especially in processes which is time consuming and expensive. Besides, development, scale up and optimization of the process depends on trial and error which is not suitable to apply in large scale. Therefore, bioseparation step is important in order for bioproducts to enter the market in an acceptable time and purity.

1.3 Objective

The objective of this research is to study the critical parameters for protein purification using ion exchange chromatography (IEC) process.

1.4 Scopes of Research

Based on the objective above, several scopes has been outlined which are to:

1) Screen for the correct pH for buffer solution to optimize production.

2) Screen for NaCl concentration used in elution buffer.

CHAPTER 2

LITERATURE REVIEW

2.1 General Bioseparation

Bioseparation is a separation techniques which on biological material. Bioseparation processes may be grouped into multiphase systems, membrane-based separation, analytical and isolation techniques, high resolution purification techniques, chromatography, foam-based separation, and conventional and supercritical extraction.

Techniques of bioseparation can be broadly considered as consisting of four sequential steps. The first step is removal of insoluble which involves the filtration or centrifugation process. Second step of process is isolation of the product that involves extraction or adsorption. Process is proceed to the purification of the product which is accomplished by chromatography and last sequential step is polishing of the product which consists of removing water, solvent or traces of impurities by drying or crystallization. (Singh & Singh, 1996)

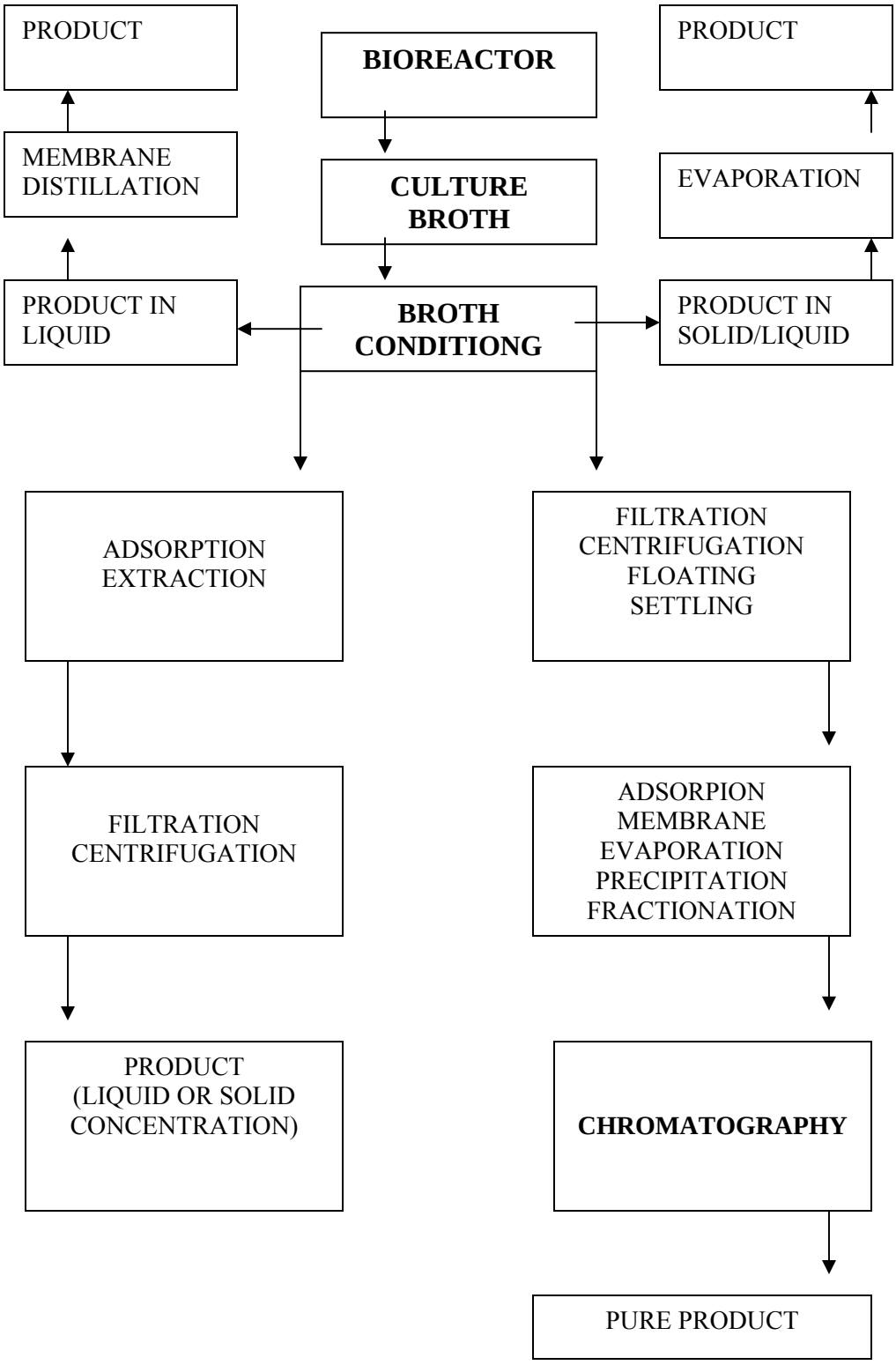

Figure 2.1: Schematics diagram of bioseparation process from Department of Chemical and Biological Engineering

2.2 Lysozyme

Lysozyme is a small, stable enzyme, making ideal for research into protein structure and function that is widely distributed in many tissues in plants and animals. Lysozyme or mucopeptide N-acetyl-muramoylhydolase is a very commercially valuable because of the versatile of applications in many ways. It was discovered in 1922 by Alexander Flemming while he was suffering from a cold and let some of his nasal secretions fall on a plate of bacteria and later noticed that the bacteria in the vicinity of the mucus from his nose had dissolved. He was able to establish that the anti-bacterial activity in the mucus was due to an enzyme, the enzyme he called lysozyme because it breaks down the cell walls of bacteria and causes them to lyse (Southern California Biotech, 2002). Lysozyme can protect human from bacterial infection. It is a small enzyme that attacks the protective cell wall of bacteria.

Lysozyme is a single chain polypeptide of 129 amino acids cross-linked with four disulfide bridge (Jolles & Chemie, 1969). It hydrolyzes β (1-4) linkages between N-acetyl-muraminic acid and N-acetyl-D-glucosamine residues in peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrin (Rupley, 1964; Holler et al., 1990). The molecular weight is 14 307 Da (amino acid sequence) (Canfield., 1963). The isoelectric point (pI) is 11.35 (Wetter, 1951). Activity of lysozyme is a function of both pH and ionic strength. The enzyme is active over a broad pH range (6.0 to 9.0). At pH 6.2, maximal activity is observed over a wider range of ionic strengths (0.02 to 0.1 M) than at pH 9.2 (0.01 to 0.06M) (Davies R.C, 1963).

Lysozyme is inhibited by indole derivatives, which bind to and distort the active site, and imidazole, which induces the formation of a charge-transfer complex (Swan, 1972). It is also inhibited by surface-active agents such as sodium dodecyl sulfate, sodium dodecanate, and dodecyl alcohol. Other compounds of these types with carbon chains of 12 or more carbons in length will also inhibit lysozyme (Smith et al., 1949). The natural substrate for lysozyme is the peptidoglycan layer of bacterial cell walls. However, a variety of low molecular weight substrates including murein degradation

products as well as synthetic compounds have been used for various photometric, isotopic and immunological lysozyme assays (Holtje, 1996).

2.2.1 Application of Lysozyme

Lysozyme is responsible for breaking down the polysaccharide walls of many kinds of bacteria and thus it provides some protection against infection. It is commonly use as an antimicrobial agent in various foods. Besides the bacteriolytic action by destroys bacterial cell walls by hydrolyzing the polysaccharide component of the cell wall, many other biological functions of lysozyme is constructed.

These include antiviral action by forming an insoluble complex with acidic viruses (Jolles & Jolles, 1984), enhanced antibiotic effects (El-Nimr et al., 1981), antiinflammatory and antihistaminic actions (Siwicki et al., 1998), direct activation of immune cells (Rinehart et al, 1979), and antitumor action (Sara, 1989). Sugahara et al. (2000) observed that lysozyme accelerates the translation process to enhance immunoglobulin production. A number of chemical modifications of lysozyme have been undertaken to increase its efficacy as an antimicrobial agent, as reported by Takahashi et al., 2000 who conjugated a fatty acylated saccharide with the protein through the Maillard reaction.

2.2.2 Sources of Protein

Proteins from animal sources such as meat, fish, dairy products, egg white are considered high biological value protein or a complete protein because all nine essential amino acids are present in these proteins. While proteins from plant sources like grains, legumes, nuts and seed generally do not contain sufficient amounts of one or more of the essential amino acids. These proteins are considered to have intermediate biological value

or to be partially complete because, although consumed alone they do not meet the requirements for essential amino acids, they can be combined to provide amounts and proportions of essential amino acids equivalent to high biological proteins from animal sources. According to Lauritzen, Table 2.1 showed the content of proteins in typical food.

Table 2.1: Chart shows protein content in some typical foods (Lauritzen, 1992)

Food	Amount	Protein in Grams
Chicken	3 oz	20
Ground beef	3 oz	21
Pork chop. Lean	2 oz	15
Milk	1 cup	9
Egg	1	6
Cheddar cheese	1 oz	7
Beans	¾ cup	11
Peanut butter	2 Tbsp	8
Bread	1 slice	2
Cooked cereal	½ cup	2
Dry cereal	1 oz	1-4
Rice	½ cup	5
Nuts	2 Tbsp	5
Soybeans	½ cup	10
Cooked vegetables	½ cup	1-2

Because proteins have different distributions in biological materials, it is important to make the right choice of starting material from which to purify the protein (Hames, 1998). The selections go through to the source that relatively rich in the protein of interest and which is readily available.

2.2.3 Chicken Egg White

Chicken egg white (CEW) is a primary source of protein which consist 87% water and other 13% of CEW are proteins. It has many kinds of proteins that very important and valuable as shown in Table 2.2. One of the most valuable proteins in market is lysozyme (3.4%), the fourth abundance protein in CEW. This protein becomes very valuable in world market because of the variety of application and a most important in medical industries for breaking down the polysaccharide walls of many kinds of bacteria.

Table 2.2: Properties of egg white protein (Bedi, 2001)

	% of total	Isoelectric Point	Molecular Weight
Ovalbumin	54	4.5	45 000
Ovontransferrin	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
Ovomacroglobulin	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 Albumin	-	4.9	66 500

2.3 Purification Process

Purifying biomolecules can be divided into two parts. The first part is in a commercial situation which the final goal is to deliver material which fulfills certain criteria. Most purification protocols require more than one step to achieve the desired level of product purity. While the second part is purification protocols are using in a research project, the final goal is to gain information about the biomolecules mechanism and structures of the biomolecules involved. (Winter & Lindblom, 2004).

Purification has three phases which are capture, intermediate purification and polishing. Capture phase is to isolate, concentrate and stabilize the target of product while during the intermediate purification phase is to remove most of the bulk impurities such as other protein. The last phase is polishing which to achieve high purity by removing any remaining trace impurities, solvent unused or closely related substances.

The combination of purification techniques for capture, intermediate purification and polishing is crucial to ensure fast method development, minimize time to pure product and good economy (Amersham BioSciences, 2004).

Dealing with purification process will involves a series of procedures that selectively separate the target protein from contaminating proteins and other material. Otherwise, the cost of protein purification will depend on the nature of the source of material and the number of purification steps required. Instead of proteins may inevitably in each steps of purification, the smallest number of purification steps can provide a satisfying purity and yield of target protein.

2.3.2 Techniques of Purification Process

Protein purification is a process that involves methods to enrich or purify a single protein from a homogenate or extract. Proteins have their own behaviors which vary from each other especially in size, charge, shape, hydrophobicity, solubility and biological activity. Proteins have to be obtained in solution prior to its purification. Some precautions have to be taken during purification by physical or biological factor to prevent the denatured or inactivated process of proteins. The stabilization of proteins includes buffering the pH solutions, the procedures at low temperature and protease inhibitors in order to avoid unwanted proteolysis.

Protein purification is a multi-steps procedure which needs to establish specific method identification of enzymatic, binding or activity assay. By each step, the purification level and specific activity increases and the yield decrease. The basic aim in protein purification is to isolate one particular protein or interest from other contaminating proteins so that its structure and/or other properties can be studied (Hames et al., 1998).

Chromatographic techniques are used to these separations such as ion exchange, gel filtration or affinity chromatography. The process fractionation of the protein from their crude is time consuming and expensive. As the consequences, there are some continued improvements used in the separation techniques appeared such as study of the ion exchange chromatography (Bedi, 2001; Ming *et al*.,1993), affinity chromatography (Sharma, 2002), purified bovine serum albumin (BSA) using expanded bed adsorption (Ramat, 2004; Ryan O. *et al*., 1997), membrane chromatography and so on. Differences of these methods they used to purify one protein from another.

The purpose of standard protocol is the combination of steps with selectivity and a minimum of inter step conditioning. The standard purification protocol for proteins is based on the combination ion exchange chromatography, hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC). The standard protocol of the flow step is shows in the figure 2.2.

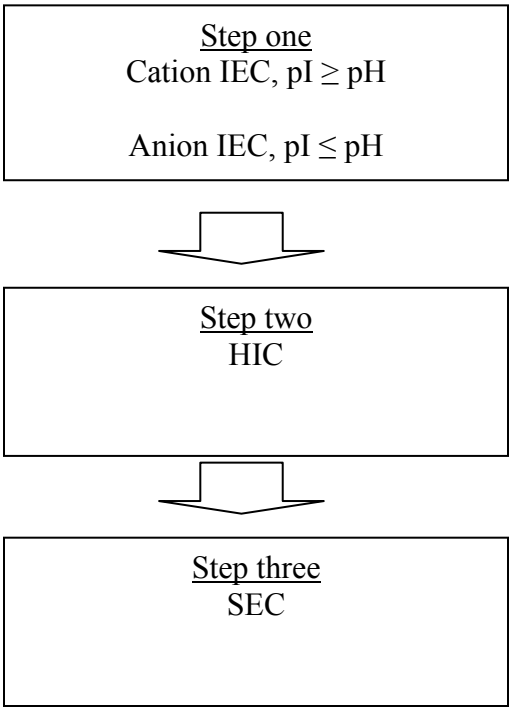

Figure 2.2: Standard protocol of purification

The techniques with selectivity those are highly independent of each other. The order of steps is chosen to fully utilize the high loading capacity of IEC at the beginning of the purification and to avoid any inter-step treatments other than adding salt to the sample prior to the HIC step. Figure 2.3 illustrates the purification strategy of the process.

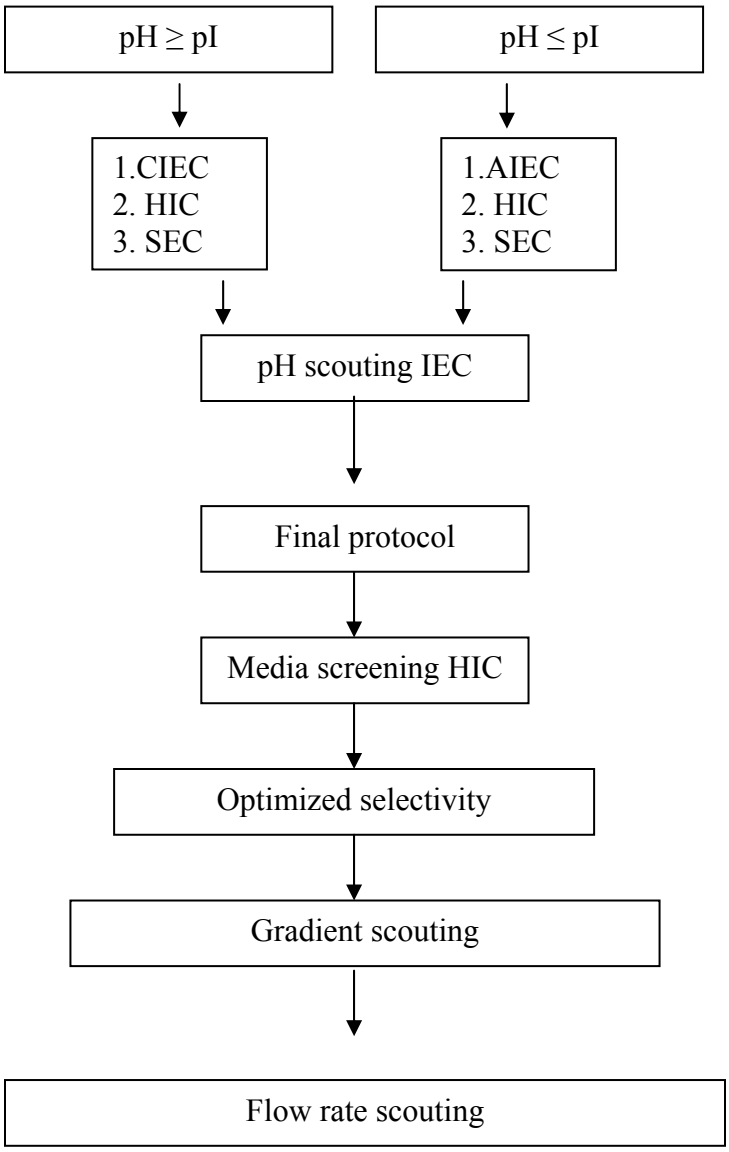

Figure 2.3: Flow path for purification strategy (Winter & Lindblom, 2004**)**

2.4 Chromatography

Chromatography is the separation method which distributed in stationary phase and mobile phase to separate the components in a mixture. The process takes place within a chromatography column. A packed bed column contains particles which make up the stationary phase. Open tubular columns are lined with a thin film stationary phase. The center of the column is hollow. The mobile phase can be either liquid or gas depends on the process which carries the mixture to be separated. A mixture which enters a column is called the eluent and the product outflow of the column is called the eluate. While the process of flowing liquids or gases through the column are terms of elution.

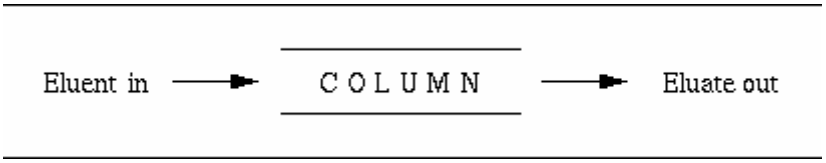

Figure 2.4: Basic concept of chromatography

Chromatography plays an important process step in the downstream purification and the overall goal is to achieve a pure product (protein) from a complex mixture with reasonable product yields.

In a chromatographic system, a mixture of compounds is introduced or “injected” onto the column. The solvent delivery system then pumps liquid mobile phase through the column. After passing through the column, the separated fraction is passed through a detector. The data the detector produces is in the form of peaks, the amplitude of which is based on the amount of light absorbed in the flow cell (Isco.Inc, 2003). Generally, chromatography can be classified by two parts which are gas chromatography and liquid chromatography.

2.4.1 Ion-Exchange Chromatography (IEC)

Ion exchange chromatography is the most useful and most popular for all protein purification and concentration method. IEC is a form of liquid chromatography, using ion-exchange resins to separate atomic and molecular ions for analysis. The basic theory of ion exchange chromatography separation is molecular difference of charges at a given pH.

The chromatography materials for the separation of both cations (cation exchange media) and anions (anion exchange media) are available which a cation exchange medium contains anionic functional group while an anion exchange medium is cationic. Then the charged proteins will adsorbed by the charge adsorbents which the different protein is bounded differently by the adsorbent according to the differences of interaction forces. The used of buffer solution to replace the proteins which the proteins will be washed out of the adsorbents in different velocity. Proteins bind to ion exchangers by electrostatic forces between the adsorbent charged beads and the charged groups of the protein.

In ion exchange chromatography, proteins are separate on the basis of their overall (net) charge (Hames, 1998). The charges need to be balanced by counterions such as metal ions, chloride ions and sometimes, buffer ions. A protein has to move the counterion to be attached; the net charge of the protein will usually be the same as the counterions displaced. That is why this type of chromatography is called “ion exchange”. Net charge is defines the protein’s isoelectric point (pI).

Separation and purification using ion exchange chromatography is based primarily on differences in the ionic properties of surface amino acids. Isoelectric points of proteins can be define when the positive charge equal to the negative charge at certain pH or in other words is the net charge of the protein is zero. The isoelectric point indicates how strongly charged a protein will be at given pH.

In order to avoid conditions under which the protein interest bind too tightly or too weakly to the column, a pH approximately one unit from the pI is the best choice for attempting a purification (Bollag et al., 1996). Refer to the protein's isoelectric point, when the buffer pH is larger than pI protein, the protein will bind to anion exchanger while if pH buffer is smaller than pI, the protein will bind to cation exchanger. Proteins can be separate based on the washing and eluent steps in ion exchange chromatography by using different buffer pH and different salt concentration in elution to optimize the resolution.

2.4.1.1 Theory of Ion exchange

Separation in ion exchange chromatography depends upon to reversible adsorption of charge solute molecules to immobilized ion exchange groups of opposite charge. There are five stages that showing the principles of ion exchange chromatography as showed in Figure 2.5. In starting conditions, the terms of pH and ionic strength are in equilibration which allows the binding of the desired solute molecules.

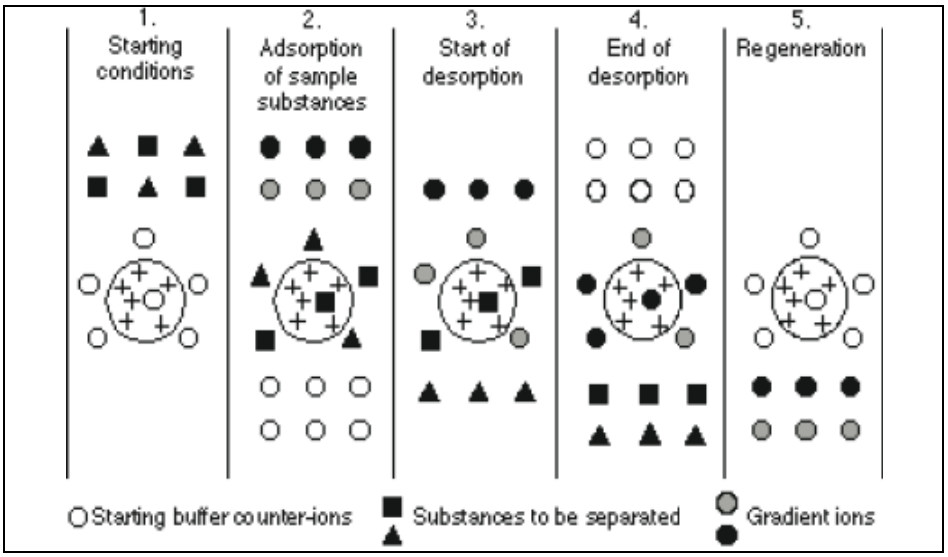

Figure 2.5: The principle of ion exchange chromatography (salt gradient elution). (Amersham BioSciences, 2004)

The second stage is sample application and adsorption which solutes molecules carrying the appropriate charge displace counter-ions and bind reversibly to the gel. The sample buffer should have same pH and ionic strength as the starting buffer in order to bind all appropriately charged protein. Unbound substances can be washed out from the exchanger bed using starting buffer (Amersham BioSciences, 2004). Then, proceed to the third stage which the removal of substances from the column by increasing the ionic bonding of the eluting buffer or changing its pH.

In figure 2.5, desorption is achieved of an increasing salt concentration gradient. Protein is desorbed relative to the number of charged group on their surface. Protein that having weakly bound substances being eluted first followed by strongly bound substances. The fourth and fifth stages are the last step which the phase of regeneration. The substances that not eluted under the previous experimental conditions are removal and re-equilibration at the starting conditions for the next purification.

2.4.1.2 Buffer Solution

Buffer is a solution which is a mixture of an acid and its conjugate base that can reduce change in solution pH when acid or alkali are added. Maintain a protein at the desired pH is quite important which is to ensure reproducible experimental results whereas the selection of appropriate buffer must be imperative.

There are eight important characteristics to consider when selecting a buffer (Scopes, 1982);

- pKa value
- pKa variation with temperature
- pKa variation upon dilution
- Solubility

- Interaction with other components
- Expense
- UV absorbance
- Permeability through biological membranes

Ideally, different buffers with a similar pKa should be tested to determine whether there are undesired interactions between a certain buffer and the protein under investigation (Blanchard, 1984). The buffer of choice also depends on the method employed which for gel filtration chromatography, almost any buffer can be chosen that is compatible with the protein of interest. For anion exchange chromatography, cationic buffers such as Tris are preferred. For cation exchange or hydroxyapatite chromatography, anionic buffers such as phosphate are preferred (Blanchard, 1984). The useful buffering range diminishes significantly beyond 1 pH unit on either side of the pKa. Note that many enzymes are irreversibly denatured at extreme pH values (Tipton & Dixon, 1979).

2.4.2 Protein Concentration

All the purification or isolation of a protein will be follow by determination of protein concentration to measure the concentration of the protein that is carried out from the experiment. There are six methods in determining the protein concentration which are Absorbance at 280 nm (A_{280}), Bradford Assay, Lowry Assay, Bicinchoninic Acid (BCA) Assay and Dot Filter Binding Assay. The progress of purification may be tracked by comparing the concentration of the desired protein with the concentration of protein after each fractionation step.

CHAPTER 3

METHODOLOGY

3.1 Research Design

Research is design based on the modeling system that shows the flow of the processes path. Starting with the sample, is shows the flow steps by steps as guidance to the research development. Besides, this flow path is the summary of overall project development. The overall design of the flow research is showed in figure 3.1 in next page.

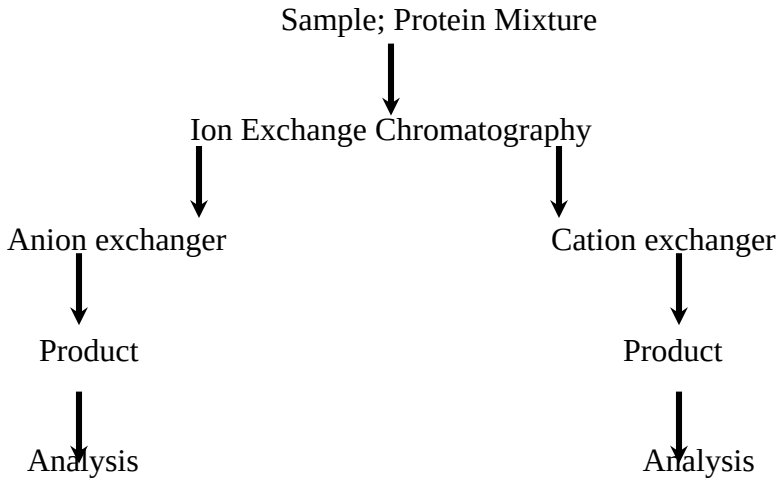

Figure 3.1: Design of flow research

3.2 Sample Preparation

Protein sample was obtained from chicken egg white. Sample is prepared from chicken egg while egg white is separated from yolk. The egg white separated is stirred slowly in a beaker until it seems homogenous. Chicken egg white was diluted with phosphate buffer solution 5X dilution. The mixture was then centrifuged at 30 000 rpm and 4˚C. The supernatant was then stored at 4˚C for further works. Ten percentages of egg white gives 3 to 3.5 g of protein obtained (Bedi, 2001).

3.2.1 Buffer Solution

Buffer solution was prepared based on the type of exchangers used. Tris-base buffer was used for anion exchangers while the phosphate buffer was used for cation exchangers. In this work, the buffer was prepared shown in Table 3.2.

Table 3.2: Buffer formulations for IEC (Coa, 2002)

Ion Exchanger	Sample buffer solution
Anion exchanger	0.95g Tris + HCl
Cation Exchanger	0.95g Phosphate + HCl

For the buffer of anion exchanger (0.95g of Tris) was added into 200 mL deionized water with a few drops of concentration HCl or NaOH. For the buffer of cation exchanger (0.95g of kalium diphosphate) was added into 200 mL deionized water with a few drops of HCl or NaOH to increase or decrease to get desired pH value.

The elution buffer was prepared by adding an amount of sodium chloride (NaCl) to the buffer prepared previously. In this works, 0.1 M to 0.5 M NaCl was used. Elution buffer was used to elute sample protein which was bonded to the column by irreversible charge.

3.2.2 Effect on pH on lysozyme purification

The purification of lysozyme was done by using a different pH value of buffer solution. For cation exchanger, the pH values of 7.5 to 10.5 were used. Meanwhile, pH value of 11.5 was used for the anion exchanger. In pH study, the concentration NaCl for elution was kept constant at 0.2 M.

3.2.3 Effect on NaCl concentration on lysozyme purification

When pH is obtained, different concentration of NaCl was manipulated. In the elution step, NaCl was used to change the ionic strength of buffer in order to desorb the bound proteins. In this work, a range of 0.1 M to 0.5 M NaCl was used.

3.3 AKTAexplorer Cromatography System

ÄKTAexplorer is a fully automated liquid chromatography system which designed for method development and research applications. This equipment is from Amersham-Pharmacia Biosciences. This unit consists of 4 main modules which are pump module, flow rate up to 100ml/min and pressure up to 10MPa, monitor for UV which

monitoring 3 wavelength simultaneously in the range of 190 – 700 nm, monitor for pH and conductivity and fraction collector for fraction collections of samples.

The separation unit is controlled by a computer using UNICORN software which is coming with the system. Figure 3.2 shows the flow path of the automated purification system.

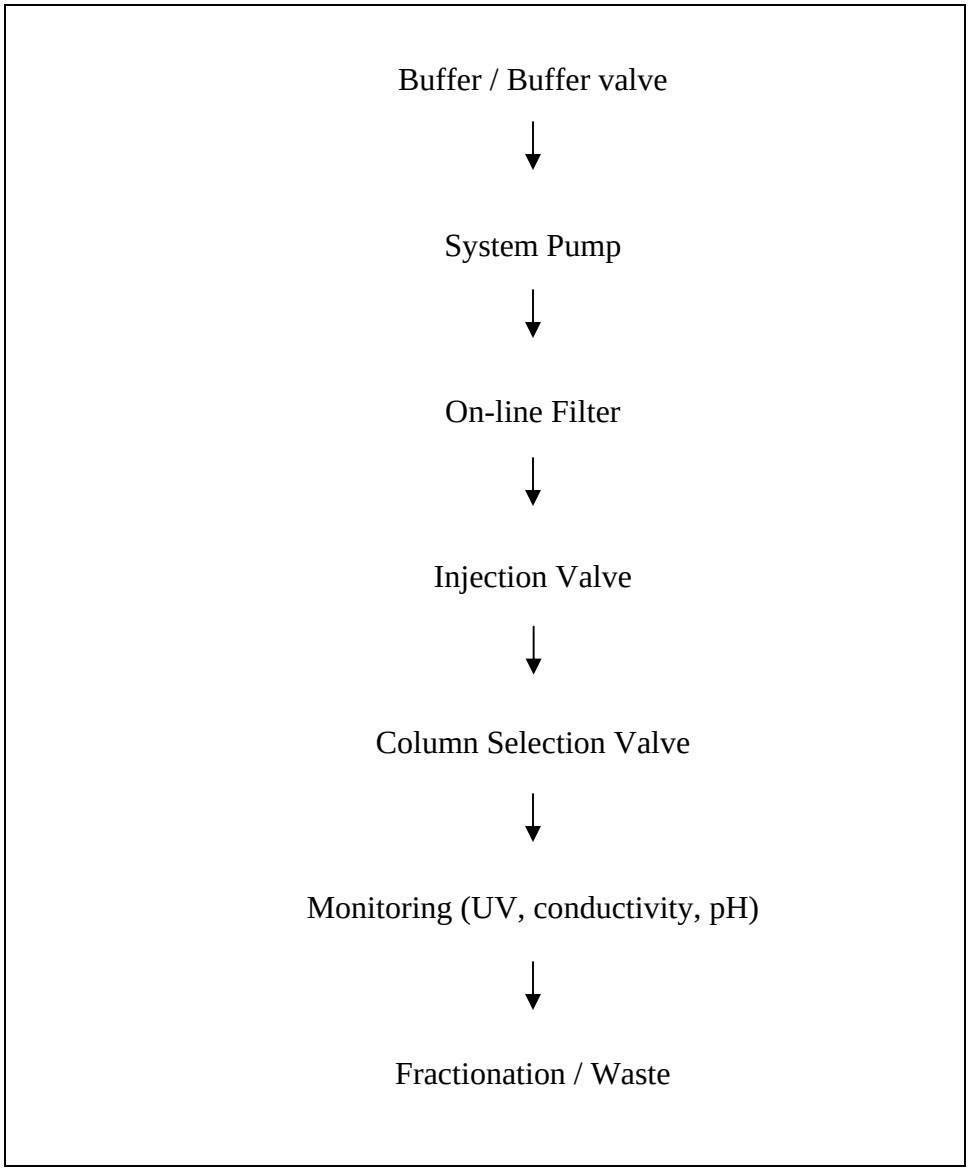

Figure 3.2: Flow path of the System