ISOLATION OF ALBUMIN PROTEIN FROM GOAT'S MILK USING ANION EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Milk proteins have been extensively studied for over 90 years. The aim of this study was to introduce a simple, reproducible, and less expensive method which is ion exchange chromatography for isolation of albumin protein from goat's milk. The main whey proteins are β-lactoglobuline and α- lactalbumin for which genetic variants are also known. The fraction of whey also contains substantial amounts of immunoglobulin and serum albumin. Whey (lactoserum) was obtained by isolating casein from defatted milk using hydrochloric acid. The proteins in the supernatant were fractionated using Q-Sepharose column chromatography for separation. This research were conducted to optimize the pH of the milk sample for separation process. The processes of the separation initialized with sample preparation followed by packing process of ion exchange column , buffer preparation, ion exchange running process and ending by analyzing the ion exchange fraction using reverse process chromatography (PRC). The pH that have been analyzed during this study were milk with pH 5.5, 6.5, 7.5 and 8.5. Result shows that ph 8.5 able to collect α-lactalbumin whereas in other pH does not so. Anion exchange chromatography able to isolate BSA and higher concentration was obtained at pH 5.5 with the amount, 0.03616164 mg/ml. RPC method is presented for the determination of albumin method is rapid, reliable and sensitive and gives good accuracy and precision for measurement of the whey proteins. The procedure developed is particularly suited to laboratory-scale applications, but it can also be applied to the purification on a large scale. Separation using simple process and local dairy product which is goat milk able to reduce the cost and time and have potential for commercialization. Standard curve used to analyze the data obtained from RPC analysis.

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ABSTRAK

Susu protein telah dipelajari secara menyeluruh selama lebih dari 90 tahun. Tujuan kajian ini adalah untuk memperkenalkan kaedah yang potensi , sederhana, dan lebih murah pertukaran ion kromatografi untuk isolasi protein albumin dari susu kambing. Whey protein utama adalah β-lactoglobuline dan α-lactalbumin yang variasi genetik juga diketahui. Komposisi whey juga mengandungi sejumlah besar imunoglobulin dan albumin serum. Whey (lactoserum) diperolehi dengan mengasingkan kasein dari susu lemaknya dengan menggunakan asid hidroklorik. Protein dalam supernatan difraksinasi menggunakan Q-Sepharose kromatografi sebagai medan untuk pemisahan. Penelitian ini dilakukan untuk mengoptimumkan pH sampel susu untuk proses pemisahan. The proces pemisahan dianalisasi dengan persiapan sampel diikuti dengan proses pembungkusan medan pertukaran ion, persiapan penyangga, pertukaran ion yang berakhir dengan menganalisis fraksi pertukaran ion dengan proses kromatografi terbalik (PRC). pH yang telah dianalisa dalam kajian ini adalah susu dengan pH 5,5,, 6,5 7,5 dan 8,5. Keputusan kajian menunjukkan bahawa pH 8,5 dapat mengumpulkan α-lactalbumin sedangkan pada pH yang lain tidak. Kromatografi pertukaran anion boleh mengasingkan BSA dan konsentrasi yang lebih tinggi diperolehi pada pH 5,5 dengan jumlah sebanyak 03616164 mg / ml. Kaedah RPC berpotensi untuk penentuan kaedah albumin dengan cepat, handal dan sensitif dan memberikan ketepatan yang baik dan presisi untuk pengukuran protein whey. Prosedur ini sangat sesuai untuk aplikasi skala makmal, tetapi juga boleh digunakan dalam skala besar. Pemisahan menggunakan proses yang sederhana dan produk susu tempatan yang susu kambing mampu mengurangkan kos dan masa dan mempunyai potensi untuk komersialisasi. Graf standard albumin digunakan untuk menganalisis data yang diperolehi.

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Protein purification is a series of processes intended to isolate a single type of protein from a complex mixture which mainly from cow's milk but this this study is to expose goat milk for protein separation. Separation of one protein from all others is typically the most laborious aspect of protein purification. This study is to introduce a simple, reproducible, and less expensive method for isolation of albumin protein from goat's milk. Proteins , well known for their nutritional value and versatile functional properties, are widely utilized in the food industry. The heterogenous nature of protein preparations is typical of many commercial food protein preparations.

Albumin, the most abundant plasma protein, is among the most studied of all proteins, being important from clinical monitoring, physiological, and therapeutic perspectives. Albumin protein has numerous biochemical applications such as used in the bio-manufacture of therapeutic monoclonal antibodies and recombinant proteins , useful in tissue engineering and also important component of many cell culture systems. This study is aim to establish a simple, reproducible, and less expensive method for isolation albumin protein from local goat's milk using anion exchange chromatography and analyze the fractions using reverse phase chromatography (RPC).

The scope of the research is to analyse the product obtained with the exist standard product produced from expensive and complicated methods and to optimize the pH of the milk sample for separation process also to perform the indentification and quantitative analysis of final product. The research will provide empirical evidence by isolating albumin protein from goat's milk in higher content this is because the albumin contents in cow milk is 0.030 per 100 ml whereas in in goat milk is 0.0563 per 100ml. Separation using simple process and local dairy product which is goat milk able to reduce the cost and time and have potential for commercialisation.

1.2 Problem Statement

So far the researches conducted are mainly with cow milk as raw material whereas locally produced goat's milk did not utilized in optimum level by researchers. Because the goats milk contains much more higher valuable nutrients compare with cow milk. For example the albumin contents in cow milk is 0.030 v/v whereas in goat milk is 0.0563v/v per 100ml.

1.3 Objectives

The main objective of the present work is to study on isolation of targeted component which is albumin protein from goats's milk using simple, reproducible, and less expensive method, anion exchange chromatography and analyze using reverse phase chromatography.

1.4 Scope of Research

1.4.1 Analyse the product obtained with the exist standard product produced from expensive and complicated methods.

1.4.2 To optimize the pH of the milk sample for separation process.

1.4.3 To perform the identification and quantitative analysis of final product.

1.5 Rationale and Significance

The rationale of this proposed research project is to provide empirical evidence by isolating albumin protein from goat's milk in higher content this is because the albumin contents in cow milk is 0.030v/v per 100 ml whereas in goat milk is 0.0563 per 100ml. Additional advantages of ion exchange are its high resolving power, high capacity (capable of large scale purification), and the relative ease with which it can be controlled. .

CHAPTER 2

LITERATURE REVIEW

2.1 Albumin

Albumin, the most abundant plasma protein, is among the most studied **of** all proteins, being important from clinical monitoring, physiological, and therapeutic perspectives. Albumin protein has numerous biochemical applications such as used in the bio-manufacture of therapeutic monoclonal antibodies and recombinant proteins , useful in tissue engineering and also important component of many cell culture systems. (K.J. Cross *et al*, 2005)

Figure 1 : Example of Standard Albumin Protein

2.2 Goat Milk

Goat milk is richer in nutrient compare with cow milk. The average casein and albumin were 0.4034, 0.0225 respectively in goat's milk whereas in cow's milk 0.414, 0.0212 respectively. This shows that the albumin content in goat's milk is higher compare with cow's milk. The alpha-casein proteins, including alpha s1-casein, and the beta-casein proteins were both considered in this regard. However, more recent studies suggest that the genetic wiring for these casein proteins is highly variable in both cows and goats. Goat's milk contains around ten grams of fat per eight ounces compared to 8 to 9 grams in whole cow's milk. Therefore, the fat content in goat milk is much higher and during the experimant causes many centrifugation process during the preparation of sample.

Figure 2: Goat milk used in experiment.

2.3 Anion Exchange Chromatography (AEC)

The most popular method for the purification of proteins and other charged molecules is ion exchange chromatography. In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. Ion exchange chromatography separates molecules based on differences between the overall charge of the proteins (G. Bordin *et al*,2001) It is usually used for protein purification but may be used for purification of oligonucleotides, peptides, or other charged molecules. The protein of interest must have a charge opposite that of the functional group attached to the resin in order to bind. For example, immunoglobulins, which generally have an overall positive charge, will bind well to cation exchangers, which contain negatively charged functional groups. Because this interaction is ionic, binding must take place under low ionic conditions. Elution is achieved by increasing the ionic strength to break up the

ionic interaction, or by changing the pH of the protein. (Tatjana Weiss *et al*,2005).

To optimize binding of all charged molecules, the mobile phase is generally a low to medium conductivity (salt concentration) solution. The adsorption of the molecules to the solid support is driven by the ionic interaction between the two moieties and binding capacities are generally quite high. The strength of the interaction is determined by the number and location of the charges on the molecule and solid support. By increasing the salt concentration (generally a linear salt gradient) the molecules with the weakest ionic interactions are disrupted first and elute earlier in the salt gradient. Those molecules that have a very strong ionic interaction require a higher salt concentration and elute later in the gradient (Tatjana Weiss *et al*,2005).

Proteins have numerous functional groups that can have both positive and negative charges. Ion exchange chromatography separates proteins according to their net charge, which is dependent on the composition of the mobile phase. By adjusting the pH or the ionic concentration of the mobile phase, various protein molecules can be separated. For example, if a protein has a net positive charge at pH 7, then it will bind to a column of negatively-charged beads, whereas a negatively charged protein would not. By changing the pH so that the net charge on the protein is negative, it too will be eluted. (G. Bordin *et al,* 2001).

The mechanism of interaction of the solutes with the stationary phase determines the classification of the mode of liquid chromatography. In ion chromatography the basic interaction is ionic. The stationary phase is charged due to fixed anions or cations, which are neutralized by counter ions of the corresponding opposite charge as shown in Figure 3. The counter ions can be exchanged by other ions either from the mobile phase or from the sample, hence the name ion-exchange chromatography.

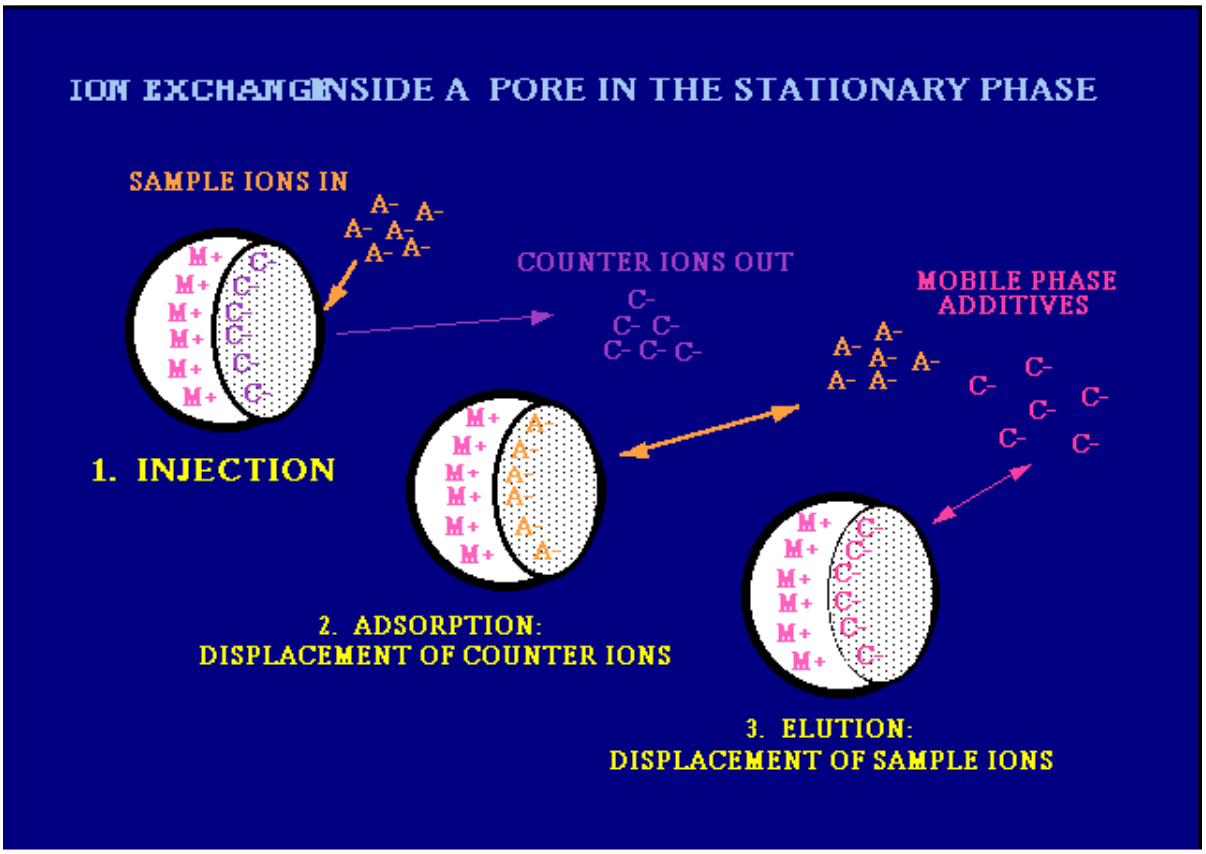

Figure 3: Illustrates the principle of retention by exchange of anions in anion-exchange chromatography.

The functional groups on the stationary phase's surface are fixed positively charged species (M+). At equilibrium these positively charged functional groups are neutralized by the counter ions from the running mobile phase (C-). In the second and the third steps, the anionic sample components (A-) enter the column and distribute between the stationary and the mobile phases by displacing the counter ions, and being displaced by the mobile phase ions back and forth. The distribution equilibrium is determined by the competition between the sample components and the anions of the mobile phase on the charged sites of the stationary phase.

The process can also be described as shown in Eqn 1:

1) M+C- + A- ---> M+A- + C-

The electroneutrality of the solution must be maintained during the ion-exchange process, therefore, the exchange is stoichiometric so that a single monovalent anion A- displaces a single monovalent counter ion C-.

2.4 Centrifugation

A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed. In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. The greater the difference in density, the faster they move. If there is no difference in density (isopyknic conditions) where the particles stay steady. To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "centrifugal force". (D.N. Kelner *et al*, 2007).

Figure 4: Centrifugation process during pretreatment step.

2.5 Filtration

This is the most common method of filtration and is used to remove an insoluble solid material from a solution. During the research whatman filter paper used to filter fat from milk after centrifugation processas shown in Figure 5. The whatman filter paper frequently changed to reduce the blockage. Other than whatman filter paper, 0.45 and 0.20 filters used to filter the milk. But then, this types of filter used after casein precipitation by addition of 1M of HCL.

Figure 5: Filtered milk after centrifugation process.

2.6 Reversed- Phase Chromatography (RPC)

The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phases shown in Figure 6. The actual nature of the hydrophobic binding interaction itself is a matter of heated debate (3) but the conventional wisdom assumes the binding interaction to be the result of a favourable entropy effect. The initial mobile phase binding conditions used in reversed phase chromatography are primarily aqueous which indicates a high degree of organised water structure surrounding both the solute molecule and the immobilised ligand. As solute binds to the immobilised hydrophobic ligand, the hydrophobic area exposed to the solvent is minimised. Therefore, the degree of organised water structure is diminished with a corresponding favourable increase in

system entropy. In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e. solute and ligand. Water adjacent to hydrophobic regions is postulated to be more highly ordered than the bulk water. Part of this structured water is displaced when the hydrophobic regions interact leading to an increase in the overall entropy of the system.

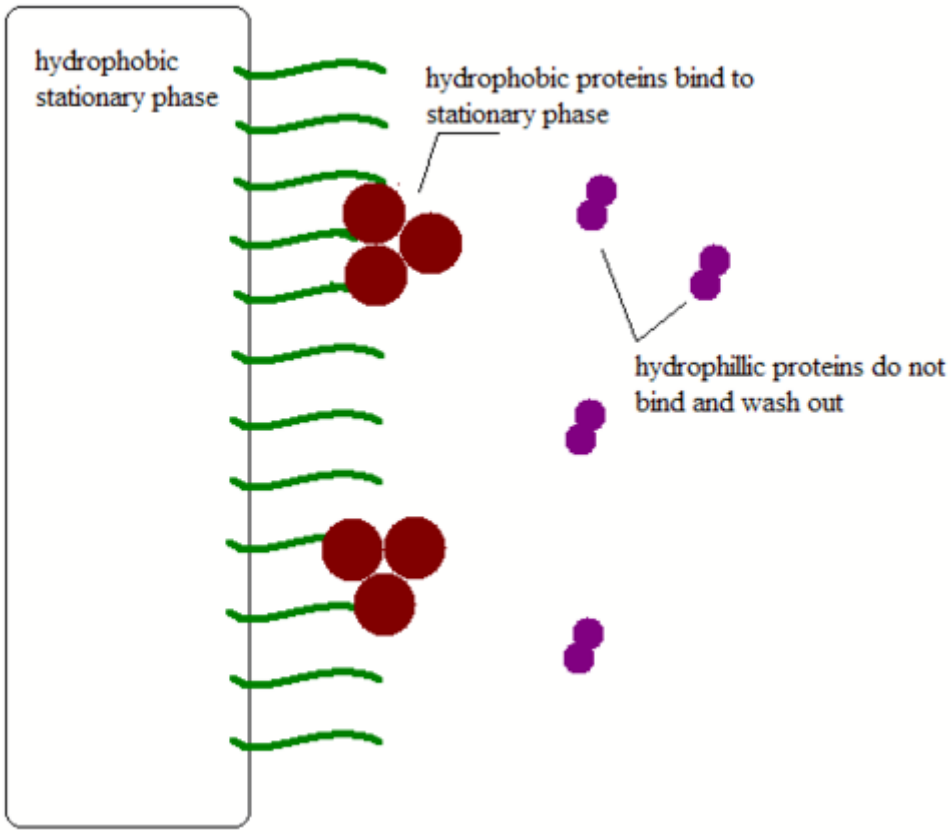

Figure 6: Reversed Phase Chromatography - Proteins with exposed hydrophobic region (red).

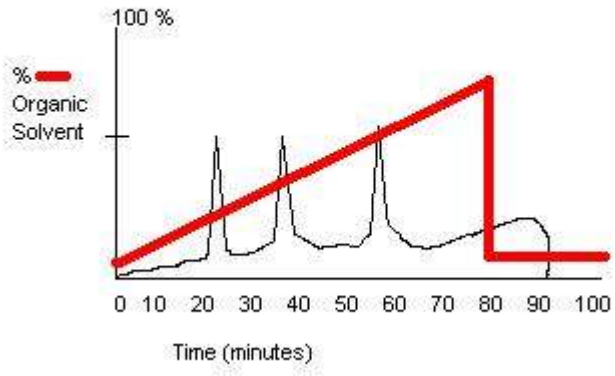

Figure 7: Sample diagram of a reversed phase separation run for 80 minutes.

There is a delay in the Figure 7 and the delay is common to all liquid chromatography gradients, and is caused by a delay in the software telling the gradient to start pumping, and the solvent reaching the column. In addition the organic solvent concentration does not starts at 0%. This is because the bonded hydrocarbons are much less efficient at binding to other hydrophobic molecules without a small amount of organic solvent present. In order to see greater peak separation, need to repeat the same proteins again for a shorter period of time, or lower the maximum amount of organic solvent. Using the above diagram, it appears that the last protein elutes at about 50% organic solvent concentration. After taking into account the delay, the last peak elutes and an adjusted 40% organic solvent concentration. A new separation can then be done, using a 40% maximum organic solvent concentration, which would generate much more widely separated peaks.

CHAPTER 3

METHODOLOGY

3.1 Experimental Work Flow

In order to separate whey protein components, the experimental works are divided into three major sections as shows in Figure 8:

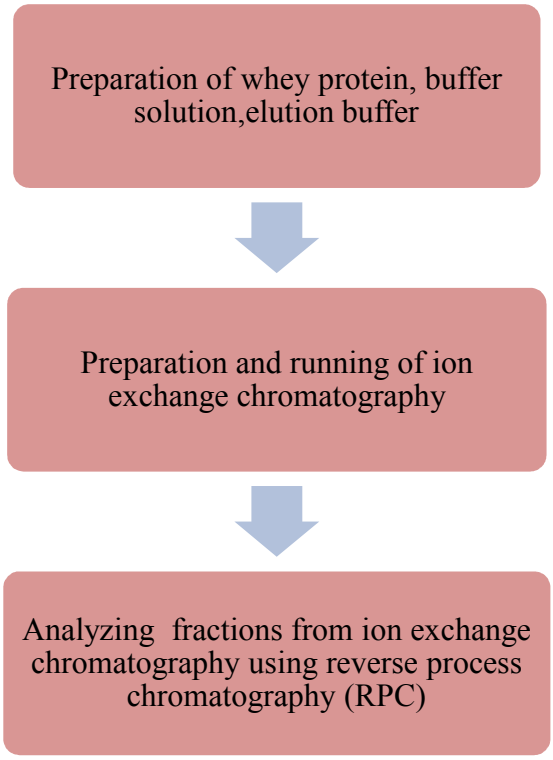

Figure 8: Flow of processes involved in separation

3.2 Preparation of whey protein

Whey will be prepared according to the method described by Hahn et al. (1998). Milk was centrifuged in a Beckmann J2-21 centrifuge equipped with a JA-20 rotor (Beckman instruments, Palo Alto, CA, USA) at 4420*g* at room temperature for 30min for delipidation. The pH of the skimmed milk was adjusted to 4.7 by the slow addition of 1 M HCl. After casein precipitation, the solution was stirred for a further 30min to complete precipitation. Casein was removed by centrifugation at 17700 *g* and 4°C for 30min. The pH was readjusted to the desired pH of the study which is 5.5, 6.5, 7.5, 8.5. Before use in chromatography experiment, the whey was filtered through a 0.45 µm membrane filter followed by 0.20 µm. Figure 9 summarized all the steps involve in whey preparation used in this study.