ISOLATION OF β-LACTOGLOBULIN AND α-LACTALBUMIN FROM WHEY

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ABSTRAK

Dadih adalah produk sampingan dalam pengeluaran keju. Larutan dadih kaya dengan protein; protein utama dalam dadih adalah β-Lactoglobulin (β-Lg) (50%), α-Lactalbumin α-La (20%), bovine serum albumin (BSA) (10%) dan imunoglobulin (Igs) (10%). Protein tunggal memiliki harga yang lebih tinggi, daripada larutan dadih dan mempunyai aplikasi khusus. Oleh kerana itu, kajian ini bertujuan untuk mengasingkan dua protein dadih yang utama iaitu β-Lg dan α-La kerana fungsi dan harga protein tulen adalah lebih tinggi. Dalam kajian ini, dadih telah perolehi semasa koagulasi kasein dari susu skim dengan campuran asid pada pH kurang daripada 5 dengan menggunakan 0.5 M asid hidroklorik. Degan kajian *Fast Protein Liquid Chromatography AKTA (FPLC)*, kedua-dua protein dari dadih dipencilkan dengan menggunakan kromatografi penyisihan saiz (superdex^{TM} 75 gel penurasan media dipek dalam turus XK (60 ml) kosong) dan kromatografi pertukaran kation (HiTrap SP HP, 1ml column) dan SDS-PAGE digunakan untuk mengenalpasti setiap protein daripada langkah pemencilan dan menentukan tahap ketulenan β-Lg dan α-La. Penstainan argentums digunakan untuk menunjukkan jalur protein pada SDS-PAGE. Kesimpulannya, kedua-dua β-Lg dan α-La boleh dipencilkan dari protein lain dalam dadih dengan menggunakan kromatografi penyisihan saiz dan kromatografi pertukaran kation. Kedua-dua protein β-Lg and α-La yang dipencilkan menunjukkan saiz 18 kDa dan 14 kDa pada SDS-PAGE, masing-masing.

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LIST OF SYMBOLS / ABBREVIATIONS

β-lg	-	β-Lactoglobulin
α-la	-	α-Lactalbumin
BSA	-	Bovine Serum Albumin
IgG	-	immonoglobins
pI	-	isoelectric point
ml	-	Milliliter
SDS-PAGE	-	Sodium dedocyl sulfate polyacrylamide gel electrophoresis
M	-	Molarity
rpm	-	Revolution per minute
°C	-	Degree celcius
µm	-	micrometer
µl	-	microliter
kDa	-	kilodaltons
HCl	-	Hydrochloric acid
NaCl	-	Sodium chloride
NaOH	-	Sodium Hydroxide
CMP	-	Casienomacropeptide

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Whey is byproduct in the cheese production over 25 years into a readily available and utilized ingredient category in countless food systems (Schneider, 2006). Whey is usually produced from any type of milk such as cow's milk goat's milk and camel's milk. Traditionally whey was a waste in cheese production and has been effluent directly disposed into rivers and other water resources, until someone decided to exam this waste water product. They found that whey was full with a highly bio-reactive protein that is more similar to the protein found in human milk. These proteins dissolved well in water, were highly digestible and contained an even better amino acid profile than egg white (Abboud *et al,* 2009).

Whey is created when the curds separate from the milk or cream during casein coagulation. It can be produces either by acidic precipitation at pH below than 5 or by using rennet curdling. After the cheese curds are formed, the remaining liquid is called

whey. Whey has a tinge of bluish color, but it depend on type and quality of milk used (Smithers, 2008).

Whey contain various of protein such as, α-La (α-Lactalbumin), β-Lg (β-lactoglobulin), bovine serum albumin (BSA), immunoglobulins (Igs), proteose peptone fraction (PP), osteopontin, vitamin binding proteins, lactoferrin and about sixty indigenous enzymes (Jovanovic, *et al*, 2007). Because of that whey component generally was purified for the specific purpose (E. Casal *et al*, 2006). This work mainly focuses on the isolation of two components exist in whey which are α-La (α-Lactalbumin), β-Lg (β-lactoglobulin).

1.2 Problem statement

Whey contains several of protein mixture. In order to get pure α-La and β-Lg proteins, it must be isolated from other proteins in whey because single proteins have a high value compared to the protein mixture and have their own specific application.

1.3 Objective

The objective of this research is to isolate α-La (α-Lactalbumin) and β-Lg (β-Lactoglobulin) from whey protein by using size exclusion and cation exchange chromatography.

1.4 Scope

The scopes of this research are as follows:

a) To produce whey from cow's fresh milk

b) To isolate α-La and β-Lg from whey by size exclusion and ion exchange chromatography

c) To determine the degree of purity of α-La and β-Lg by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

CHAPTER 2

LITERATURE REVIEW

2.1 Whey

Whey was base of many protein drinks for athletes or others that want build or repair muscle tissues because it has an excellent source of protein, vitamins, minerals and lactose. Moreover, Whey proteins are well known for their high nutritional value and versatile functional properties in food products. Estimates of the worldwide production of whey indicate that about 700,000 tonnes of true whey proteins are available as valuable food ingredients **(**J.N. de Wit, 1997). During recent decades, interest has grown in the nutritional efficacy of whey proteins in infant formula and in dietetic and health foods, using either native or predigested proteins.

The main products of the protein fraction from whey are whey protein concentrate (WPC) and whey proteins isolate (WPI). WPC protein products having a protein content between 34% and 85% while WPI have at least 90%. Table 2.1 shows

the several products that made from liquid whey, including whey powder, lactose, demineralised whey powder, whey protein concentrate (WPC), whey proteins isolate (WPI), lactose, α-lactalbumin, β- lactoglobulin, whey protein hydrolysates and bioactive peptides (Huffman and Harper, 1999).

Table 2.1: Whey ingredients in food product (Rocha, 2007)

Ingredients	**Food product**	**Function**
Whey powder	Sport nutrition specialties, bakery, meat and fish product, and confectionery	Nutrition (protein supplement); low cost milk solids; emulsification, foaming, filter/water binder and thicker.
Demineralised whey powder	Infant formula	Nutrition
WPC	Infant formula, Sport nutrition specialties, bakery, meat and fish product, and confectionery	Skim milk replacer, emulsification, foaming, adhesion, nutrition
WPI	Infant formula, Sport nutrition, Nutritional product	Nutrition

Edible grade lactose	Infant formulas, meat and fish product, and confectionery	Sweetener, flavor enhancer, texture enhancer, color fixation
Pharmaceutical grade lactose	Pharmaceuticals (nutritional drugs, inhalers, tablets)	Tableting excipient, raw material for lactose derivatives
α-Lactalbumin	Infant formula (baby formula)	Nutrition
β- Lactoglobulin	Meat and fish products, fortified beverages	Nutrition, gelling agent, replacement of egg white
Lactoferrin	Infant formula, meat	Iron-binding, antimicrobial
Lactoperoxidase	Milk, pharmaceuticals, cosmetics	Bacteriocide, antioxidant, anticaries
Immunoglobulins	Nutraceiticals, dietetic food	Immunological, anticancer
Whey protein hydrolysates	Infant formula, sport food, dietetic food, slimming foods, elderly foods	Nutrition, reduce allergenicity, foaming, emulsification

Bioactive peptides	Dairy, nutraceutical, dietetic food	Health promoter and nutrition

2.2 Protein in Whey

Major components in whey are lactose (44–52 g/L), proteins (6–8 g/L) and mineral salts (4–9 g/L) (Gonzalez-Siso, 1996). The main proteins in whey are 50% β-Lg, 20% α-La 10% bovine serum albumin (BSA) and 10% immunoglobulins (Igs) (Lucena, 2006). The main constituents of protein in whey are *β*-lactoglobulin (*β*-lg) and *α*-lactalbumin (*α*-la), two small globular proteins that account for approximately 70–80% of total whey protein (Chatterton, 2006). Besides the major proteins, the whey fraction contains numerous minor proteins such as proteose peptone fraction (PP), osteopontin, vitamin binding proteins, lactoferrin and about sixty indigenous enzymes.

Summary about the main proteins in whey based on their biological function and characteristics is shown in Table 2.2.

Table 2.2: Summary of whey proteins (Rocha, 2007).

Protein	Concentration (g/L)	MW (KDa)	Isoelectric point	Biological function
β-lactoglobulin	2.7-3.0	18.3	5.2	Retinol carrier, binding fatty acids, possible antioxydant
α-lactalbumin	0.7-1.2	14.2	4.5-4.8	Lactose synthesis
Bovine serum albumin	0.3-0.4	69	4.7-4.9	Fatty acid transfer
immunoglobulins	0.6-0.65	150-1000	5.5-8.3	Immunity
Lactoferrin	0.05-0.1	78	9	Antimicrobial ,antioxidative ,immunomodulation, iron absorption, anticarcinogenic

2.2.1 β-Lactoglobulin

β-Lactoglobulin is the main bovine whey protein and generally accounts for 50% of the total whey protein in ruminants and 10% of the total protein in bovine milk. β-Lg, consists of 162 amino acids and has the following composition: Asp10, Asn5, Thr8, Ser7, Glu16, Gln9, Pro8, Gly4, Ala15, Cys5, Val9, Met4, Ile10, Leu22, Tyr4, Phe4, Lys15, His2, Trp2, and Arg3. The calculated formula molecular weight is 18,277 Da (Farrell *et. al*, 2004; Cho *et al*, 1993).β-Lg is the main non-casein protein in bovine milk, but β-Lg has generally been reported to be absent from human breast milk, although some reports have suggested that minor amounts do occur in human milk. Furthermore, β-lg is considered to be the most allergenic protein fraction in cow's milk.

β-Lg associated from changes with pH, temperature, ionic strength and protein concentration. The isoelectric point for β-Lg is 5.2 to 7.5, native β-Lg occurs as a dimer at this point. At pH 3.5 and 5.2 β-Lg reversibly forms tetramers/octamers, whereas below 3.5 and above 7.5 it dissociates into monomers due to electrostatic repulsions. At temperature higher than 30^0C the dimeric form of β-Lg dissociated to monomers and at temperature higher than 55^0C unfolding of the molecule start to occur, which results in an increased activity and oxidation of the thiol group (Caessens *et al*, 1997).

β-lg also function as a fatty acid or lipid binding protein. It is a rich source of cysteine, an essential amino acid that appears to stimulate glutathione synthesis, and an anti-carcinogenic tripeptide produced by the liver as protection against intestinal cancer (Mcintosh et al, 1995). Other than that, β-Lg has a variety of useful nutritional and food functional characteristics that have made this protein, and β-lg-containing whey protein

products, ingredients of choice in the formulation of modern foods and beverages. For example, β-Lg shows high solubility and clarity over a wide pH range, particularly at low pH (>97%, pH 3), and under this treatment it stable to high temperature conditions. The protein has a high nutritional value as reflected in an essential amino acid profile comparable to egg white. These properties of β-lg have facilitated its use as the active agent in various protein-fortified beverages, such as fruit juices and sports drinks, and in varieties of these beverages with long shelf-life (Chatterton *et al*, 2006).

β-Lg shows excellent whip ability and thus provides an alternative to egg albumin (egg white) in some food applications. For example, β-lg shows a foam overrun capacity and heat stability equivalent to egg white, even in the presence of sugar. Thus, an ingredient enriched in β-lg should serve as a cost-effective substitute for egg white in meringues and similar products. The foaming properties of whey and egg white proteins and their performance in food applications has recently been reviewed (Foegeding, 2006).

2.2.2 α-Lactalbumin

α-Lactalbumin is the second major protein in cow's milk that consist of 20 % in total whey protein. It also has been reported present in all mammals'milk that secrete lactose due to its function that modifies the substrate specificity of β-1,4-galactosyltranferase that allowing the formation of lactose from glucose and UDP-glucose. α-Lactalbumin is composed of the following amino acid residues: Ala3, Arg1,

Asn8, Asp13, Cys8, Gln6, Glu7, Gly6, His3, Ile8, Leu13, Lys12, Met1, Phe4, Pro2, Ser7, Thr7, Trp4, Tyr4, Val6 (Brew *et al*., 1970). α-Lactalbumin has a formula molecular weight of 14,178Da (Farrell *et al*, 2004).

α-Lactalbumin have one strong calcium binding site and also several zinc binding sites. This will make the α-lactalbumin have ability to bind metal cations. The binding site of Ca^{2+} to α-lactalbumin causes pronounced changes in tertiary structure and function and can increase its stability. Meanwhile, zinc or other cation binding might induce α-lactalbumin aggregation to forms that have anticancer activity and perform various transport functions (Permyakov and Berliner, 2000).

This protein is used commercially in infant formulas because it is similar in structure and composition to the main whey protein in human breast milk. α-Lactalbumin is also used in sport food formulas because its contains a high amount of the branched-chained amino acids that is L-isoleucine, L-leucine and L-valine, which must be present in the muscle cells to enable protein synthesis (Walzem et al, 2002).

2.2.3 Bovine Serum Albumin

Serum albumin is a major protein found in blood serum that occurs in all body tissues and secretions and play role in transport, metabolism, and distribution of ligands. Bovine serum albumin represents about 1.5% of total milk protein and 10% of total whey protein. Bovine serum albumin is very similar to the human blood serum albumin.

It has 582 amino acids and molecular weight of 69 kDa (Coulson and Stevens *et al*, 1950).

2.2.4 Immunoglobulin

Immunoglobulins (Ig) are glycoprotein molecules which are produced by plasma cells in response to an immunogen and which function as antibodies. Immunoglobulin has "Y-shaped" form, which composed of two identical light chains (23 kDa) and two identical heavy chains (53 kDa). These four chains are joined together with disulphide bonds. The complete immunoglobulin molecule has a molecular weight about 180 kDa. IgG represent 10% of total whey protein and there are consist of three major classes of immunoglobulins (Igs): IgG, IgM and IgA. Immunoglobulin act as antibodies by inhibit bacterial metabolism, agglutinate bacteria, neutralize toxins and viruses (Korhonen *et al*, 2000).

2.2.5 Lactofferin

Lactoferrin or also known as lactotransferin is a family of specific iron-binding protein that occurs in milk. It's a single-chain polypeptide with varying degrees of glycosylation. It consists of 689 amino acids as follows: Asp36, Asn29, Thr36, Ser45,

Glu40, Gln29, Pro30, Gly49, Ala67, Cys34, Val46, Met4, Ile16, Leu66, Tyr21, Phe27, Lys54, His10, Trp13, Arg37. Lactoferrin has a calculated molecular weight of 76,110Da (Farrell, *.et al*, 2004). Lactoferrin function is in iron transport and absorption in the gut of the young. Its also has been suggested that it has a role in the non specific defense against pathogens, being important in antimicrobial defense of the mammary gland and mucosal surfaces, and modulating the inflammatory response (Gupta *et al*, 2000)

2.3 Bioseparation

Development of purification techniques nowadays has become a factor for the biotechnology phenomenal. Bioprocessing is one of major part in biotechnology field, which produced many products for human benefit such as in biochemical, food, agrochemical, biopharmaceutical and nutralceutical. These biotechnology products usually must be purified using bioseparations engineering before they can be applying in their application. Bioseparations engineering is the systematic study of the scientific and engineering principles utilized for the large scale purification of biological product. In early purification step, the target protein is minor component of other proteins and other contaminants. This will lead to the error in calculation, blind-alleys, and wasted effort, to overcome this problem certain rule have been introduce that help in minimize such problem thus the purification process will successful.

There are ten rules in purification steps as proposed by Simon Roe (2001);

1. Keep the purification simple by decrease the purification step and avoid difficult manipulations which will not reproduce.
2. Keep the purification cheap by using cheaper techniques.
3. Adopt a step approach and optimize each step.
4. Speed is important used the faster equipment to avoid time delays.
5. Use reliable techniques and equipment.
6. Spend money on simple bits and pieces such as test tube and pipettes.
7. Before purification start makes sure the procedure have been write out and record the activity accurately.
8. Ensure your assays are developed to monitor the purification
9. Record the data that obtain
10. Lastly, put in mind your objective of purification that is high yield, high purity, final scale of operation, reproducibility, economical used of equipment, convenience and throughput.

2.3.1 Purification Techniques for Proteins in Whey

There are lots of purification techniques that have been used to separate proteins in whey. Based on (Caessens *et al,* 1997) Samples from trichloroacetic (TCA) precipitation (containing β-lg and CMP) were centrifuged, and the supernatant was collected. Diafiltration and ultrafiltration was performed using the indicated membrane system at an average pressure of $1.3x10^5$ Nm^{-2}. During dialiltration the pH of retentate was kept between 3.5 and 3.8 by addition of acetic acid (2 M). During ultrafiltration, the pH was not adjusted. The purification procedure was monitored by reversed-phase high-

performance liquid chromatography (RP-HPLC). During diafiltration CMP was removed. β-Lg recover during ultrafiltation process.

Other method that was used in purification of protein in whey is by pepsin treatment, using this method pH of whey was adjusted to 2.0 by addition of 2N HCl protein concentration was adjusted to 70mg/ml and the solution was preincubated at 37°C for 10 minutes. The enzymatic reaction carried out for a given period with a protein to enzyme ratio of 200:1 (wt/wt). The protein fraction was then collected by ammonium sulfate precipitation, before filtered twice with distilled water by ultrafiltration (30-kDa membrane pore size). The filtrate was centrifuged at 13000 rpm. Size exclusion chromatography was used to separate protein in whey and Native PAGE SDS-PAGE used to determined degree of purity of *β*-lg (Kinekawa and Kitabatake, 1996).

Summary of purification techniques and characterization of protein in whey is shown on Table 2.3.

Table 2.3: Summarizes the purification method and characterization of protein in whey.

Decryption	**Method**	**Characterization**	**reference**
Removal of *β*-lg from whey using chitosan.	• Interaction between *β*-lg and chitosan at pH interval 4.6 to 6.5.	• Reverse-phase HPLC • SDS-PAGE	Casal *et al*, 2006
Biochemical chracterizatio of a novel whey protein from murine milk	• Ammonium sulfate fractionation • Gel filtration chomatography	• DEAE-cellulose chromatography	Piletz *et al*, 1981
Purification of *β*-lg	• Treated with Pepsin	• Native PAGE	Kinekawa

from whey protein concentrate by pepsin treatment	• Ammonium sulfate precipitation • Filtration • Centrifuge • Size exclusion chromatography	• SDS-PAGE	and Kitabatake, 1996
Isolation of bovine *β*-lg	• Centrifuge • Diafiltration • Ultrafiltration	• RP—HPLC	Caessens *et al*, 1997