Regulation of glucagon-like peptide-1 (GLP-1) by milk protein fractions: An in vitro analysis

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Huda Wazzan

2\textsuperscript{ed} March 2018
**Abstract**

Obesity and diabetes are metabolic syndrome associated diseases. Increased body weight has been noticed worldwide and the World Health Organization (WHO) considers this as an epidemic. Reduction in body weight helps to reduce obesity-related issues such as diabetes, fatty liver diseases and certain type of cancers. Stimulating the release of satiety regulation hormones, such as Glucagon-like peptide-1 (GLP-1) has been shown to reduce food intake and help with weight loss. Dietary supplementation with milk proteins has been suggested to be an effective approach to the prevention and treatment of obesity in humans. Other than milk, green tea has also been shown beneficial effect on body weight management. However, the precise mechanisms by which these beneficial effects are mediated are not fully understood.

This research focuses on new areas for the management of obesity and discusses the possibility of using milk proteins and its fraction and epigallocatechin-3-gallate (EGCG) from green tea, to regulate appetite by stimulating the secretion of GLP-1. EGCG conjugates with Apo-lactoferrin (Apo-LF) were synthesized and the effect on GLP-1 studied, to determine if this combination might have a synergistic effect on the hormone production. Human colorectal (NCI-H716) cell line was used as the model cell line to study the effects of the test substances on GLP-1.

The results indicate that, with β-casein showing the greatest effect on GLP-1 up regulation at gene and protein levels, with the least toxicity. The interaction between Apo-LF and EGCG in the conjugates was characterized and it was found to be via hydrogen bonding, with a decreased in α-helix of Apo-LF and parallel increase in β sheets. The thermodynamic parameters suggest the interaction is spontaneous, involving hydrophobic forces at ambient temperature and electrostatic forces above 40 ºC. With regards to toxic effects on the cells,
the conjugates showed an increase in cell death with decrease in EGCG concentration. This could be due to the smaller EGCG molecule being up taken into the LF molecule at higher concentrations, whereas it remains on the surface of the protein at lower concentrations. Further, the conjugates have a higher effect on enhancing of the expression of GLP-1 compared with pristine Apo-LF and EGCG alone and their effect on the regulation of satiety hormones. In conclusion, the Apo-LF-EGCG conjugates shows potential as a new therapy for the management of obesity, by regulation of satiety hormones.
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# Table of content

| Abstract | ii |
| Acknowledgment | iv |
| Table of content | vi |
| List of Figures | viii |
| List of Tables | ix |
| List of Abbreviations | xi |

## Chapter 1: Background and Literature review

1.1. Introduction 1  
1.2. Appetite regulation along the gut-brain axis 3  
1.3. Appetite controls biological responses 6  
1.4. Gastrointestinal (GI) track hormones in appetite control 7  
1.4.1. Hormonal of regulation of food intake 8  
1.4.1.1. Ghrelin 8  
1.4.1.2. Leptin 9  
1.5. Satiety Hormones/Incretins 10  
1.5.1. Cholecystokinin (CCK) 10  
1.5.2. Glucose-dependent insulino-tropic polypeptide (GIP) 12  
1.5.3. Peptide YY (PYY) 13  
1.5.4. Glucagon-like peptide-1(GLP-1) 14  
1.6. Incretin release as an alternative approach to obesity treatment 15  
1.7. Effect of dietary proteins on incretins 17  
1.8. Milk composition 18  
1.8.1. Milk fat and sugar 19  
1.8.2. Milk proteins 19  
1.8.3. Milk vitamins and minerals 20  
1.9. Milk proteins and its role in inducing satiety 20  
1.10. References 23

## Chapter 2: Hypotheses and objectives

2.1. Rationale of thesis 39  
2.2. Research aims 40  
2.3. Outline of thesis 41  
2.4. References 41

## Chapter 3: Materials and methods

3.1. Bio-chemicals and reagents 43  
3.2. Determine protein concentration in milk protein samples using BCA kit 44  
3.3. Selection of in vitro cell models and cell culture conditions 45  
3.4. Cell culture conditions of NCI-H716 cell line 45  
3.5. Cell viability assay 46  
3.6. Gene expression assay 48  
3.6.1. Gene expression studies 49
3.6.2. Ribonucleic acid (RNA) isolation
3.6.3. Complementary deoxyribonucleic acid (cDNA) synthesis
3.6.4. Real-time quantitative polymerase chain reaction (qPCR)
3.7. Enzyme-linked immunosorbent assay (ELISA)
3.7.1. Confocal microscopy
3.7.2. Flow Cytometry analyse
3.9. Atomic emission spectroscopy (AES)
3.10. Synthesis of Apo-LF-polyphenol conjugates
3.11. Characterisation of Apo-LF-polyphenol conjugates
3.11.1. Fourier Transform Infrared (FTIR) Spectroscopy
3.11.2. Circular Dichroism (CD) Measurements
3.11.3. Fluorescence Spectroscopy Measurements
3.11.4. Zeta potential
3.11.5. Electron microscopic analysis
3.11.6. ABTS•⁺ radical scavenging activity
3.11.7. HPLC-MS Analysis
3.11.8. Stability of EGCG
3.12. Statistical analysis
3.12. References

Chapter 4: Effect of milk proteins on GLP-1 expression and extracellular release
4.1. Introduction
4.2. Effect of milk proteins (casein and whey) and its fractions on cells viability and proliferation
4.3. Results
4.3.1. Effect of casein fractions and whey proteins on gene expression of GLP-1
4.3.2. Effect of caseins fractions and whey proteins on protein expression of GLP-1
4.4.1. Fluorescent microscopic analysis
4.4.2. Flow cytometry analyse
4.4.3. Quantitative analysis of GLP-1 protein levels
4.5. Intracellular calcium contributes in the GLP-1 secretion mechanism
4.6. Discussion
4.7. References

Chapter 5: Characterization of milk protein– polyphenols conjugate
5.1. Introduction
5.2. Results
5.2.1. Macular confirmation changes in the conjugates
5.2.2. Effect of phenol on the secondary structure of Apo-LF
5.2.3. Surface charge
5.2.4. Bindings parameters
5.2.5. Measurement of antioxidants activity in the conjugates

5.2.6. Estimation of phenol content in the conjugates

5.3. Discussion

5.4. References

Chapter 6: Effect of lactoferrin, polyphenols and Apo-LF-EGCG conjugates on GLP-1 expression

6.1. Introduction

6.2. Effect of LF protein, EGCG and Apo-Lf-EGCG conjugates on cells viability and proliferation

6.3. Effect of LF proteins, EGCG and Apo-LF-EGCG conjugate on regulation of GLP-1 gene expression

6.4. Study the effect of LF proteins, EGCG and Apo-LF-EGCG conjugate on GLP-1 release

6.5. Discussion

6.6. References

Chapter 7: Summary and future direction

7.1. Summary

7.2. Future direction

7.3. References

Appendix 1

Figure No. | List of figure | Page No.
--- | --- | ---
1.1. | Schematic representation of the regulation of long and short - term energy balance adopted | 5
1.2. | Gut peptides localization and their functions. | 8
1.3. | Schematic functions of Cholecystokinin (CCK) | 12
1.4. | Schematic functions of Glucose-dependent insulinitropic polypeptide (GIP) | 13
1.5. | Schematic representation of the functions of glucagon-like peptide-1 (GLP-1) | 15
1.6. | Milk composition and its molecular protein constituents | 18
3.1. | shows NCI-H716 cell line in humans cell line which extensively validated as models to investigate GLP-1 secretion representing at different magnifications, a: 40X ,b:100X and c: 200X | 46
3.2. | Reduction of MTT to purple formazan crystal in metabolically live cells by enzymatic reduction of a yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide salt (MTT). | 47
3.3. | Schematic representation of gene expression | 49
4.1. | 3D structures of (a) casein and (b) whey protein. These images were obtained from RCSB Protein Data Bank | 65
4.2. | Cell viability assay after 24 h of treatment of NCI-H716 cells. | 68
4.3. | GLP-1 gene expression after 18 h of treatment of NCI-H716 cells. | 70
4.4. | Confocal micrographs depicting increase in GLP-1 protein content in | 72
<table>
<thead>
<tr>
<th>Table No.</th>
<th>List of table</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.</td>
<td>Body mass index categories</td>
<td>2</td>
</tr>
<tr>
<td>1.2.</td>
<td>Gastrointestinal hormones known to affect food intake</td>
<td>11</td>
</tr>
<tr>
<td>2.1.</td>
<td>Effect of different proteins on satiety hormones release</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>3.1.</strong></td>
<td>Preparation of BSA Standards for estimation of total protein content</td>
<td>45</td>
</tr>
<tr>
<td><strong>3.2.</strong></td>
<td>Volume of component to prepare 2x RT Master Mix per 20 µL reaction.</td>
<td>50</td>
</tr>
<tr>
<td><strong>3.3.</strong></td>
<td>Thermal cycle conditions optimized for High-Capacity cDNA Reverse Transcription Kits</td>
<td>50</td>
</tr>
<tr>
<td><strong>3.4.</strong></td>
<td>qPCR samples preparation</td>
<td>51</td>
</tr>
<tr>
<td><strong>3.5.</strong></td>
<td>Concentration depending preparation of Apo-LF-EGCG conjugates in PB at pH 7.0</td>
<td></td>
</tr>
<tr>
<td><strong>4.1.</strong></td>
<td>Protein contents in milk protein fractions α, β and κ-casein, WPI and WPC from 1 to 5% w/v measured by Pierce BCA Protein Assay Kit method (mg/mL).</td>
<td>68</td>
</tr>
<tr>
<td><strong>5.1.</strong></td>
<td>Changes observed in the Apo-LF and EGCG FTIR spectra after conjugation</td>
<td>91</td>
</tr>
<tr>
<td><strong>5.2.</strong></td>
<td>Secondary structure of Apo-LF, and Apo-EGCG conjugates</td>
<td>93</td>
</tr>
<tr>
<td><strong>5.3.</strong></td>
<td>ζ-potential of the conjugate, EGCG and Apo-LF</td>
<td>94</td>
</tr>
<tr>
<td><strong>5.4.</strong></td>
<td>Stern-volmer constants and thermodynamic parameter for Apo-LF-EGCG conjugates</td>
<td>97</td>
</tr>
<tr>
<td><strong>5.5.</strong></td>
<td>Percentage of EGCG found in supernatant of Apo-LF-EGCG complex</td>
<td>100</td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drugs Administration</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide YY</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>CCK</td>
<td>cholecystokinin</td>
</tr>
<tr>
<td>DPP VI</td>
<td>the dipeptidyl peptidase IV</td>
</tr>
<tr>
<td>TPP2</td>
<td>The tripeptidyl peptidase II</td>
</tr>
<tr>
<td>BCAAs</td>
<td>Branched-chain amino acids</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organisation of the United Nation</td>
</tr>
<tr>
<td>WPC</td>
<td>whey protein concentrate</td>
</tr>
</tbody>
</table>
| EGC
t| Epigallocatechin gallate         |
| PP           | pancreatic polypeptide           |
| NHANES       | The Third National Health and Nutrition Examination Survey |
| WPI          | whey protein isolate             |
| BSA          | bovine serum albumin             |
| T2DM         | Type 2 diabetes mellitus         |
Chapter 1: Background and Literature review

1.1. Introduction

The World Health Organization (WHO) defines obesity as “abnormal or excessive fat accumulation that presents a risk to an individual’s health”. Obesity has become an epidemic and a major health problem throughout the world. According to WHO, the incidence has increased by two fold since 1980 especially in developed countries [1]. It is considered the fifth leading cause of death, resulting in at least 2.8 million adult deaths, annually [1, 2]. The WHO statistics also indicate that obesity increases the risk of developing chronic diseases such as diabetes by 40%, heart disease by 23% and cancers between 14 – 41% [3]. Obesity is also linked to high blood pressure and high cholesterol level, especially low density lipoprotein (LDL) and triglyceride levels and has been shown to reduce high density lipoprotein (HDL) levels [4]. Australia has seen a 26.8% increase in overweight and obesity rate in the short span between 2014 and 2015 [5] and projection indicate that incidences will continue rise [6].

Energy balance is a state in which the total intake energy from food consumed is equal to the energy expenditure in the form of cellular metabolism and exercise, resulting in a stable body fat mass and controlled body weight [4, 7]. Therefore, an imbalance between energy intake and energy expenditure leads to weight gain and ultimately obesity [8, 9]. Changes in lifestyle, excessive eating of energy rich foods and a lack of exercise lead to an imbalance between energy intake and expenditure, thus leading to an increase in body weight and/or fat deposition [4, 7].

Body mass index (BMI) is the most commonly used measurement to estimate if a person is overweight or obese. It is calculated by dividing the person’s weight in kilograms by the square of their height in meters [10-12]. Table 1.1 indicates the BMI range for each weight
category. It has been found that people who frequently eat food outside the house tend to have a much higher BMI than those who eat more at home [4] and this has been found to be more common in developed countries [13]. This may account for the higher obesity rates observed in those countries.

Table 1.1: Body mass index categories [3].

<table>
<thead>
<tr>
<th>Category</th>
<th>BMI</th>
</tr>
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<tbody>
<tr>
<td>Under weight</td>
<td>&lt;18.5</td>
</tr>
<tr>
<td>Normal</td>
<td>18.5 – 24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>25.0 – 29.9</td>
</tr>
<tr>
<td>Obese class 1</td>
<td>30.0 – 34.9</td>
</tr>
<tr>
<td>Obese class 2</td>
<td>35.0 – 39.9</td>
</tr>
<tr>
<td>Obese class 3</td>
<td>≥ 40.0</td>
</tr>
</tbody>
</table>

However, using BMI to determine ideal bodyweight is not without its limitation. For example, BMI measures excess weight rather than excess body fat. Therefore, it does not account for muscle mass or fat distribution. In addition, body weight is influenced by other factors including age, gender, ethnicity, and the interactions between hormonal, genetic and metabolic processes, breastfeeding and economic conditions [4, 7, 14-16]. Hence, a clear understanding of factors that contribute to the development of obesity are critical for developing effective prevention and treatment strategies [11].

Over the years, a number of potential strategies have been applied to manage and reduce obesity with a variety of diets and pharmacological agents. The most common strategy employed to prevent obesity and control body weight is increasing the expenditure of energy, resulting in weight loss. Using nutritional information, following a healthy diet and a consistent physical activities regimen are the first steps in the management of obesity [4, 11, 17]. A complementary approach to achieve weight loss is using weight loss drugs or “pharmacotherapy”, some of which have been approved by the Food and Drugs Administration (FDA), such as Orlistat (Xenical) and sibutramine “Meridia” [11, 18].
However, the usage of these drugs is associated with a range of side effects such as increased blood pressure, increased heart rate and vitamin A and E deficiency [11, 17]. Bariatric surgery is considered to be a last resort solution for patients who have a BMI greater than 39. This kind of surgery is performed on the stomach and/or intestine to reduce food intake, which promotes weight loss and reduces health problems such as Type 2 diabetes [11, 17]. Since this invasive surgical approach has many disadvantages, such as adverse events and high costs, the modulation of hunger and satiety regulation hormones using dietary approaches is a safer method to improve metabolic functions and regulate body weight [19].

1.2. Appetite regulation along the gut-brain axis

The gastrointestinal tract (GI) is the first organ system to encounter nutrients ingested in food. In addition, the brain and adipose tissue also play a vital role in maintaining body weight [20]. It is important to understand the mechanisms of regulating food intake and body weight to develop effective treatments for control of obesity and associated disorders [20]. What, when and how much people eat in a day is regulated by a series of signals i.e. hormones, that are produced from the GI tract in response to food which has been consumed [21]. Food provides our body with energy in the form of calories that comes from macronutrients (protein, fat and carbohydrate) that are essential for building cellular structures and maintaining body functions.

Appetite and satiety are influenced by hormonal and nervous signals that control eating behavior. The most common definition of appetite is the demand to eat food and this causes hunger. In some cases, appetite is related to individual behavior. For example, feeling of stress causes increased appetite resulting in increased food intake. However, the demand to eat is decreased in individuals with anorexia [22, 23].
The three main types of signals that influence food intake are:

(i). **Satiety signals** begin with an interaction between ingested nutrients and the GI tract. GI track hormones such as peptide YY (PYY), glucagon-like peptide-1 (GLP-1), pancreatic polypeptide (PP) and cholesystokinin (CCK) constitute satiety signals. These are responsible for maintaining meal size and symmetry with energy expenditure, thereby helping to regulate short-term appetite [24].

(ii). **Adiposity signals** are hormones secreted directly into the blood in proportions dependent on the amount of stored body fat. The two adiposity signals are insulin and leptin and they help to regulate long-term appetite [24]. Insulin is secreted from pancreatic β–cells to regulate glucose levels and leptin, which is secreted from fat cells in direct proportion to the amount of stored fat [24]. There is an inverse relationship between insulin or leptin levels and food intake. Increased levels of these hormones reduces food intake and helps to lose weight through increased energy expenditure [25, 26], in what is referred to as a catabolic response. In contrast, an anabolic response leads to increased body weight by increased food intake and reduced energy expenditure. Hence, in obese people, insulin is secreted at higher level compared to underweight people, since these hormones are released into the blood stream in proportion to body fat mass to inhibit food intake. Figure 1.1 provides an illustration of the regulation of long and short-term appetite by the satiety and adiposity signals. It illustrates the long-term energy balance controlled by adipose tissue, leptin and insulin from the pancreas, whereas the short term energy balance is via satiety hormones released from the gut to regulate food intake and improve the feeling of satiety. If the level of leptin or insulin signal is low, the individual become obese [25, 26].

(iii). the **central effectors** include the hypothalamus and the hindbrain. The hypothalamus contains the arcuate nucleus, within which are two groups of neuronal populations. One
A group residing in the medical arcuate nucleus can stimulate food intake, while the other is found in the pro-opiomelanocortin (POMC) and can inhibit food intake. A balance between the activities of these two nuclei is essential for regulation of body weight. The hindbrain controls satiety through the nucleus of the solitary tract (NTS) and the area postrema, which are reciprocally connected to the two hypothalamic nuclei [27].

Figure 1.1: Schematic representation of the regulation of long and short-term energy balance adopted from Cummings and Shannon [26].

The above two types of signals work in conjunction with the central effectors to regulate satiety, energy balance and body weight reduction. For example, leptin can directly act on the arcuate nucleus of the hypothalamus, which can either stimulate or inhibit food intake. Similarly, the NTS in the hindbrain expresses GLP receptors (GLP-1R), and is directly influenced by GLP-1. Since the NTS is connected to the hypothalamic nuclei, it plays an important role in appetite regulation. These regions of the brain also control emotional, social and learned behavior, hence these can also impact energy balance and body weight [27].
1.3. Appetite controls biological responses

Negative energy intake, continued increases in the rate of energy expenditure, as well as maximizing the satiety level are required conditions to achieve weight loss [28]. Moreover appetite is controlled by external (environment such as cultural and nutritional) and internal factors (storage, utilization of energy and physiological conditions by the brain mechanisms) [29]. A variety of physiological and social factors also influence appetite including age, bodyweight, sex, smoking, alcohol consumption, physical activity, cold and heat [25, 30]. The bio-behavioral control system of appetite can be presented in three main processes. Firstly, psychological events including hunger perception, cravings and hedonic sensations. Behavioral operations include meals, snacks and macronutrient intakes. Lastly, level of peripheral physiology, metabolic events, level of neurotransmitter and metabolic interactions in the brain [31]. Hence, it is important to understand the differences between hunger, satiety and satiation concepts to determine their effects on controlling body weight [29].

Satiation and satiety are two important concepts for controlling food intake. Satiation is a feeling of fullness and a sense of satisfaction, signaling the individual to cease eating. Satiation is intra-meal factor to reduce hunger and meal size; therefore, it influences directly on the energy consumed from that meal. This mechanism is a result of nervous and hormonal signals sent from the gut [31-33]. It is a short term phenomenon which starts early during meal consumption and ends when eating finishes [34].

In contrast, satiety is a biological response in which the sensation of hunger between meals is inhibited so people do not eat for a while [31-33]. It is an inter-meal factor developed after food is digested. Biological responses include stomach distension, gastric emptying rate, and release of pancreatic and gut hormones. Increasing the concentration of glucose, amino acids and fatty acids will helps in releasing of satiety hormones [31].
Characterization of a homeostatic system is an important approach for the treatment of obesity has been discovered [35]. Hunger signals initiate eating and satiety signals inhibit appetite for a period [31]. Hunger is a sensation, which represents a physiological need to eat and usually begins when blood sugar levels results in the motivation to eat food. The biological relationship between leptin and ghrelin hormone levels resulting in the motivation to eat food. Decreasing in leptin levels in blood plasma reduceses motivation to eat. A significant drop in the leptin levels causes the release of ghrelin hormone, which in turn reinitiates the feeling of hunger [36-38].

Taken together, these mechanisms help the human body to maintain body weight over time and act against starvation, preventing the development of obesity.

1.4. Gastrointestinal (GI) track hormones in appetite control

The GI tract is known as the largest endocrine organ in the human body [18, 39]. It releases more than 20 hormones to enhance the process of digestion and absorption of nutrients in the human gut. In addition to the direct impact these hormones have on hypothalamic and brainstem centers for appetite control, they also act as inhibitors of gastric emptying to decrease energy intake [39]. Figure 1.2 illustrates gut hormones and their functions, effects and where they are produce in human body.

Incretins are hormones that are released from the GI tract into the blood stream in the presence of ingested nutrients such as fats, proteins and carbohydrate [40]. These hormones can be classified as orexigenic (hunger hormones) and anorexigenic (satiety hormones or incretins). Anorexigenic hormones include peptide YY (PYY), glucagon-like peptide-1 (GLP-1), pancreatic polypeptide (PP) and cholesystokinin (CCK) [16, 41]. In the last two decades, anorexigenic hormones and their contribution to increasing the feeling of satiety
have taken a prominent place in scientific research. Most of this attention is focused on signals that improve the satiation feeling to control body weight [27, 39, 42, 43].

Figure 1.2: Gut peptides localization and their functions. (Adopted from Murphy and Bloom [44].) *

Central nervous system

1.4.1. Hormonal regulation of food intake

Ghrelin and leptin are two hormones that play a vital role in energy balance. Leptin has its effect on the long-term regulation of energy by suppressing food intake. Whereas ghrelin, known as a fast acting hormone, plays a vital role in initiating feeling of eat. Increasing number of obese people, understanding the mechanisms of leptin and ghrelin hormones and their influences on energy balance have been intensive research.

1.4.1.1. Ghrelin

Ghrelin is a hormone produced mainly in the stomach, from neuroendocrine cells (ghrelin cells) and to a lesser extent, in some areas of the brain such as hypothalamus, hippocampus, and cortex. It was discovered in 1999 [45] and has a unique structure that comprises 28
amino-acids [38, 46]. It plays a key role in meal initiation [38], stimulates appetite, controls gastric motility, influences pancreatic function, impacts sleeping behavior and modulates the immune system [36, 46]. Ghrelin is at higher concentrations before meals, at night and during times of fasting. It then leads to the beginning of the meal and it is rapidly inhibited after food within 1 hour of meal consumption. Therefore, it leads to progressive increase in the regulation of hormones just before the next meal [46, 47]. Higher plasma ghrelin level is associated with early obesity, due to its role in the stimulation of hunger and increased food intake [38, 48]. In 2011, higher levels of ghrelin secretion were observed in teenagers with a greater food intake compared to lean equivalents [38]. Action of ghrelin on eating behavior is controlled by regions in hypothalamus of the brain [47]. Ghrelin has a variety of metabolic functions and is considered a fast-acting hormone, responsible for meal-initiation, while leptin is responsible for suppressing food intake and long-term regulation [49].

1.4.1.2. Leptin

Leptin is a hormone secreted by adipocytes, the placenta and the gastric fundic mucosa in the stomach [50, 51], and is 66 amino acids in length [49]. The obese gene (OB), responsible for the production of leptin was discovered in 1994 by Zhang, et al. [52]. It plays an important role in the regulation of food intake and energy expenditure to control and manage body weight [53]. It acts through two main receptors, leptin receptor (LEPR) or OB receptor. While higher levels of leptin are observed in obese subjects, studies suggest obese individuals exhibit “leptin resistance”, hence there is less regulation of food intake [54]. Interestingly, leptin and cholecystokinin (CCK) receptors, Ob-R and CCKR, respectively are co-expressed on the vagal surface in the gut wall and these two hormones play similar roles in suppressing food intake [55, 56].
1.5. Satiety Hormones/Incretins

1.5.1. Cholecystokinin (CCK)

The CCK hormone is a polypeptide hormone produced by I cells. The peptide is present in high concentrations in the intestinal, duodenal and jejunal mucosa in the upper small intestine [21]. It is a gut-brain hormone that exerts a variety of physiological actions in the GI tract and the central nervous system through cell surface receptors [57]. The concentration of the CCK hormone is increased dramatically after a meal and remains elevated high for up to 5 hours, especially after meals containing long chain fatty acids and proteins [18, 39, 58].

There are many different active forms of CCK which are classified depending on their amino acid content including CCK-5, CCK-22, CCK-8, CCK-33, CCK-39 and CCK-58. All of those forms are derived from 95 amino acids and CCK-8 is the most active form found in human brain. While CCK-58, CCK-33, CCK-22 and CCK-8 sign significantly presenting in human intestine [59]. The physiological actions and satiating effects of CCK are mediated through two main group of receptors, CCK-A (CCK-1) and CCK-B (CCK-2) [33, 42, 60]. Figure 1.3 illustrates the physical action of CCK hormone in human body. This hormone controls gall bladder contraction, slowing of gastric emptying, modulation of gastrointestinal motility, bile and pancreatic secretion, and regulation of appetite [58, 61]. Table 1.2 summaries the effect of incretins release in GI track on food intake, satiety, and satiation and therefore, body weight management.
Table 1.2: Gastrointestinal hormones known to affect food intake [18, 39].

<table>
<thead>
<tr>
<th>GI Hormones</th>
<th>Sites of release</th>
<th>Stimulating factors</th>
<th>Receptors</th>
<th>Sites of action on food intake</th>
<th>Role of hormone in BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>Distal gut (L-cells) of distal small and large intestine; immunoreactivity in hypothalamus, dorsovagral complex, pituitary</td>
<td>↑Macronutrients ↑Bariatric surgery ↓Somatostatin ↓Calorie restriction</td>
<td>GLP-IR</td>
<td>↑Satiation ↑Satiety Potentiates insulin release Possible pathogenic role in obesity ↑promotes pancreatic β-cell growth inhibits gastric emptying inhibits gastric secretion inhibits energy intake effects on cardiovascular system</td>
<td></td>
</tr>
<tr>
<td>CCK</td>
<td>Proximal small intestine (I-cells)</td>
<td>Fat and protein-rich chime ↑Coffee ↓Bile acids</td>
<td>CCK-1 Vagus Brainstem Hypothalamus</td>
<td>↑Satiation Possible pathogenic role in obesity promotes gallbladder contraction increases secretion of pancreatic enzymes and bicarbonate</td>
<td></td>
</tr>
<tr>
<td>GIP</td>
<td>Enteroendocrine K-cells in duodenum and proximal jejunum</td>
<td>Glucose and lipids</td>
<td>GIPR</td>
<td>↑Satiation ↑growth of the pancreatic β-cell ↑bone formation ↑hypothalamus proliferation</td>
<td></td>
</tr>
<tr>
<td>PYY</td>
<td>Enteroendocrine L-cells</td>
<td>Lipids and carbohydrates</td>
<td>GPCR</td>
<td>inhibit food intake reduces body weight</td>
<td></td>
</tr>
</tbody>
</table>

CCK can be inactivated by a single enzyme named tripeptidyl peptidase II (TPP2) [17]. The usefulness of the CCK hormone as a therapeutic agent for obesity treatment is limited. That is because of its short shelf life and its effect on appetite [18].
Glucose-dependent insulinotropic polypeptide (GIP)

Glucose dependent insulinotropic peptide (GIP) plays a vital role in the release of insulin [62]. The peptide is 42 amino acid and is secreted by enteroendocrine K-cells found predominantly in the duodenum and proximal jejunum. It is encoded by the proglucagon gene, which also codes for glucagon-like peptide-1 (GLP-1). Its function is similar to GLP-1, in that, an increase in GIP leads to an increase in insulin production. GIP persists in the blood for approximately seven minutes before it is deactivated by the endoprotease dipeptidyl peptidase IV (DPP-IV) [63, 64]. In addition to its role in the regulation of body weight (Table 1.2), GIP has additional biological actions (Figure 1.4) such as the promotion of growth of the pancreatic β-cell, stimulation of adipogenesis bone formation [65] and increasing cell proliferation in the hypothalamus [66]. Glucose and fat were found to be the most important stimulators of the GIP hormone in human body [63] and the GIP receptors (GIPR) are found in several organs such as the pancreas, stomach, and small intestine.
1.5.3. Peptide YY (PYY)

Peptide YY3-36 is the main form of PYY which belong to the pancreatic polypeptide family [67]. It is secreted by enteroendocrine L-cells in the GI tract, practically in the ileum, colon and rectum and in the upper portion of the small intestine. PYY is deactivated by the DPP-IV enzyme, and activated by the theneuropeptide Y (NPY) receptor family includes Y1, Y2, Y3 Y4 and Y5. PYY has various biological functions including slow gastric emptying, improvement of digestive activities and nutrient absorption and regulation of insulin [68]. It also plays a vital role in inhibiting of food intake and reducing body weight [69].

PYY levels are low during fasting time and increase within 30 min after a meal [70]. Some studies have found that PYY 3-36 hormone is lower in obese subjects during the fasting time and the increase of the hormone during and after a meal is less than in non-obese subjects [67, 71]. Macronutrients can stimulate PYY hormone secretion but to different levels. Researchers have found evidences of a synergistic relationship between PYY and GLP-1, in which they work together to inhibit food intake (Table 1.2) [69].
1.5.4. Glucagon-like peptide-1 (GLP-1)

L cells are open-type epithelial cells [72], that are found in the intestinal tract, particularly in the small intestine and pancreas [20, 39, 73]. GLP-1 is one of the L cell hormones and is produced in two different forms, GLP-1 (7-37) and GLP-1 (7-36) [18, 39]. GLP-1 actions are controlled by the G-protein-coupled GLP-1 receptor (GLP-1R), which is found in the GI track, brain, heart and in pancreatic islets [74]. It contains 30 amino acids and is encoded produced by the proglucagon gene (GCG) [40, 75].

The mRNA produced by the GCG is translated to a 180 amino acid preproglucagon sequence, which is cleaved to produce a 160 amino acid proglucagon sequence. This proglucagon undergoes posttranslational shortening by cleavage of the six N-terminal amino acids, removal of the C-terminal glycine, and amidation of the remaining C-terminal arginine residue. This results in GLP-1, the hormone secreted by L-cells [75].

After meals the concentration of GLP-1 in blood plasma rises immediately within 10 – 15 minutes. The half-life of bioactive GLP-1 in the blood is less than 2 minutes before it is deactivated by dipeptidyl peptidase IV (DPP -IV) [18, 39, 40, 73, 76]. DDP -IV, also known as CD26, is a serine protease and cleaves proteins that contain an alanine or proline residue. GLP-1 hormone contains an alanine residue and therefore is a substrate for DPP –IV enzyme and is broken down rapidly into GLP-1 (9-37) or GLP-1 (9-36) NH₂ [40]. DPP -IV exists as a cell surface, membrane bound peptidase found in many tissues including GI track, liver, kidney and pancreas [77]. The presence of nutrients in the gut and their interaction with the microvilli are the possible reasons for the GLP-1 secretion [78].

GLP-1 inhibits food intake and therefore regulates energy production (Table 1.2) [20, 39]. Figure 1.5 depicts the functions of GLP-1. In human and animal studies, GLP-1 plays a major role in the reduction of food and calorie intake in patients with type 2 diabetes. Recent studies
have shown that the levels of the GLP-1 in the blood plasma are lower in obese people, though the cause has not yet been identified [58, 79]. GLP-1 inhibits the secretion of gastric acid and glucagon release from α-cells in the pancreas [53, 73] improving in pancreatic β-cell mass by inhibiting apoptosis [39, 76]. GLP-1 is also thought to influence learning and memory [80].

Figure 1.5: Schematic representation of the functions of glucagon-like peptide-1 (GLP-1)

1.6. Incretin release as an alternative approach to obesity treatment

Anti-obesity drugs aim to increase metabolic rate and reduce the absorption of macronutrients especially fats and carbohydrates [81]. However, current research interests revolve around controlling appetite by stimulating satiety regulation hormones such as CCK, PYY and GLP-1 [82, 83]. Evidence has been growing regarding gut hormones and their action in the treatment of obesity. Presented below is a short list of commercially available anti-obesity drugs that target hormone regulation.

Exedin-4 is isolated from the venom of a lizard has been approved for type 2 diabetes due to its incretin-like properties [43, 84]. It is a 39 amino acids peptide that shows glucoregulatory activities similar to the GLP-1 hormone. It enhances insulin secretion and decrease glucagon secretion, and decelerates gastric emptying. In animal diabetes studies and cell lines,
exenatide-4 and GLP-1 have been shown to improve in β-cell function by increasing gene expression responsible for insulin secretion [85].

Human GLP-1 hormone is proving to be a promising anti-obesity drug; however, its consumption may cause nausea and vomiting [86]. In addition, it has been found that obese individuals showing decreased GLP-1 responses compared with lean controls, show no improvement in GLP-1 secretion after weight loss [78]. The mechanism whereby obesity lowers GLP-1 secretion is not understood, however studies suggest it might be related to the insulin resistance that accompanies weight gain. Therefore, regulation of gut hormones may be effective in treating obesity, with fewer side effects and reduced weight regain after discontinuation of the treatment [81, 87]. Other incretin mimetic drugs which can have same effect as incretin that the body usually produces naturally are listed below:

**DPP-4 inhibitor** is prescribed to individuals who suffer from type 2 diabetes. The drug regulates blood sugar levels after eating. After a meal, GLP-1 and GIP hormones are released to increase insulin secretion, however they are quickly degraded by DPP-4, and have a short circulation period. DPP-4 inhibitors, such as Sitagliptin and Saxagliptin block the DPP-4 receptors, thereby preventing the release of DPP-4 and hence preventing cleavage of GLP-1 and GIP [88, 89]. Sitagliptin (known commercially as Januvia), has been approved by the Food and Drug Administration (FDA) and is meant mainly for diabetic patients. It is predominantly used to treat diabetic patients. It is prescribed in combination with insulin releasing pills and insulin injections. However, sitagliptin commonly produce side effect in users such as sore throat, runny nose and low blood sugar, especially when it is taken with insulin releasing pills and insulin [88, 89].

**GLP-1 Analogs** are injected therapeutics which control blood sugar levels after eating. GLP-1 analogs act like GLP-1 hormone and were developed to resist cleaving by DPP-4 which allows long lasting effects of GLP-1 [88, 89].
1.7. Effect of dietary proteins on incretins

Significant changes have been shown in hormone concentrations after high protein meals [90]. Studies indicate that protein induced satiety occurs through (1) increased in the concentration of satiety regulation hormones including GLP-1, CCK, PYY or reduction in ghrelin hormone, (2) increased energy expenditure as a result of increase in metabolite reactions, and (3) increased gluconeogenesis. Gluconeogenesis is defined as the synthesis of glucose from non-carbohydrate source.

Proteins form an essential component in the human body and play a role in the bodies structure, repair and functional activities. Amino acid units combine to form protein and to metabolic compounds [91]. Consumption of proteins increases satiety, regulates satiety hormones and decreases food intake and appetite in humans [92-97]. A high protein meal helps to delay the consumption of the next meal by at least 60 min, as compared with 34 min for carbohydrate and fat [98]. A number of studies have been conducted to examine the effects of proteins on hunger, satiety and food intake. For example, amount of proteins play a vital role on the duration of satiety and delay feeling of hunger more than fat or carbohydrate [91, 99, 100]. High protein diets have shown increased levels of satiety within a range of 25 % to 81 % with significant energy intake reduction. Moreover, the source of proteins may play an important role in satiety. For example, meat, egg, soy and milk proteins exert different effects on satiety. [101-103]. Hence, there is a need to understand the effect of protein fractions, to minimize the side effects, while allowing increased satiety regulation.

Researchers have shown considerable interest in the use of dairy proteins to improve metabolic health with greater impact on satiety [28, 104]. Milk proteins and their fractions have been reported to exert differential effects on GI hormone secretion and appetite [90,
105-109]. They have functional properties for controlling body weight, and associated with a decreased risk of developing metabolic syndrome diseases [91, 95, 110].

1.8. Milk composition

In the last two decades milk and individual milk components have gained considerable attention, in terms of their satiation effect [107, 111]. Milk is secreted in the mammary glands of mammals by epithelial cells which line the mammary alveoli and is stored in the alveolar lumina adjacent to those cells [112, 113]. It is a nutrient rich source of high-quality proteins and fats [114]. Therefore, it is considered as an essential part of human diet. In addition, it is inexpensive and readily available for consumption [115, 116]. The major protein fractions of milk; casein and whey (Figure 1.6), have been reported to increase satiety and reduce food intake by improving secretion of gastrointestinal hormones. In addition, milk helps glycemic control through insulinotrophic mechanisms [110, 111, 117, 118]. However, the exact mechanism by which milk contributes to the regulation of satiety, food intake and blood glucose is not well-understood.

1.6: Milk composition and its molecular protein includes. α, β and κ-casein α and β-lactalbumin, Lactoferrin (LF) and Bovine serum albumin (BSA). α= alpha ; β = beta ; κ = kappa.
The composition of milk varies depending upon the breed of dairy animals, stages of lactation and nutritional status [119]. Milk is composed of three main components including fat, carbohydrates and protein and its molecular protein constituents (Figure 1.6) [120]. In addition, it contains significant nutrients which contribute to the required intake of minerals and vitamins such as calcium, magnesium, selenium, riboflavin, vitamin B12 and pantothenic acid [121].

1.8.1. **Milk fat and sugar**

Milk is an oil-in water emulsion [114] containing riacylglycerols, phospholipids, sterols and traces of fatty acids. It contains 65% saturated, 32% monounsaturated and 3% polyunsaturated fatty acids as well as fat soluble vitamins A, D, E, and K [114]. It also contains about 4.5 – 4.9% of lactose, which is the carbohydrate source consisting of two sugars: glucose and galactose, that provides 4 calories/g when metabolized by lactase [112, 122].

1.8.2. **Milk proteins**

The protein content of milk is approximately 3.3% of casein and whey proteins (Figure 1.6). Whey and casein are classified as high quality proteins based on human amino acid requirements, digestibility and their bioavailability functions. They have functional properties for the control of food intake and glycaemia [123, 124]. Furthermore, milk proteins are considered an important source of bioactive peptides which have been found in milk protein hydrolysates and fermented dairy products. Milk proteins contain more than 26 bioactive peptides which have been isolated from either casein or whey protein such as β-casomorphin-5, α-Casein-exorphin, α-Lactorphin, Lactokinin, Immunopeptide and β-lactorphin. The amino acid sequence of the individual peptides ultimately determines their function of those peptide [124]. Various physiological activities have been defined against
pathogens and illnesses for milk bioactive peptides such as anti-oxidative antimicrobial proteins and anti-microbial properties [124, 125].

1.8.3. Milk vitamins and minerals

Dairy products are significant source of micronutrients. They provide more than 10% of nutrients required by humans including vitamins A, B12, calcium, phosphorus, potassium and zinc [126]. The roles of these minerals have been studied in the treatment of metabolic disorders. For instance, milk contains high levels of calcium which plays a vital role in body weight regulation [127] and may also improve lipid profiles [128].

1.9. Milk proteins and its role in inducing satiety

Dairy consumption can be beneficial in achieving lower body weight and decreasing the risk of developing hyperglycemia and diabetes [108], decreasing the prevalence of metabolic disorders [94, 129], and increasing energy expenditure [94]. Milk protein fractions lead to decreased food intake in the short term by regulating food intake hormones stimulating glucose utilization [90, 108, 117]. A high protein breakfast which contains 58% of total energy (mainly dairy products), increase GLP-1 concentrations over 3 hours compared with high carbohydrate breakfast that contains only 19% of protein [107].

While the effect of whey and casein has been addressed particularly in satiating [130], it is not clear which component has a better effect [99, 107]. One study showed similar effects on satiety and food intake between whey proteins and caseins [92], while other studies found that whey protein has a stronger effect on hunger and food intake compared to casein, soy and egg albumin [90, 94, 107, 129]. They suggested that the increased effect observed for whey proteins was due to the high concentration of branch chain amino acids (BCAAs) compared to casein, which leads to an increase in plasma amino acids and levels of plasma
incretins [90, 94, 107, 129]. Similar results have been found relating to the effect of whey protein on the energy intake; where the mean energy intake was significantly lower for whey protein consumption compared with other sources of proteins, including tuna, egg and turkey [103]. A recent study involving whey proteins showed reduction in hunger and food intake compared to casein. The authors showed that a small amount of whey proteins, especially whey proteins isolate, could increase the satiation feeling and prevent the onset of type 2 diabetes mellitus [94]. Consuming 30 g and / or 15 – 20 % of whey protein can reduce food intake for 30 minutes, and that is more than any other sources of protein [131]. Also, diet supplement with whey protein led to increased feeling of satiation compared with carbohydrates and soy proteins in overweight and obese people [132]. Interestingly, skim milk, which contains both proteins – casein and whey, has been found to decrease food intake more than the individual proteins [133].

With regards to hormone regulation, whey proteins have a stronger response of GLP-1 in humans, compared to casein and soy [111]. A human trial by Bowen et al in 2007, found that 50 g of whey protein resulted in a prolonged suppression of ghrelin and raised GLP-1 and CCK levels without changing food intake [105]. The concentration of CCK hormone in plasma increased significantly after a whey protein meal within 15 – 20 min and remained high for more than 3 h [90, 132]. On the other hand, CCK hormone was higher after casein consumption in one study [90]. Plasma GLP-1 concentrations fell substantially after 2 h of consumption of whey, whey hydrolyses and casein hydrolyses solutions, but increased continually with the casein solution. This effect is due to the slow rate of casein digestion [102, 104, 107, 111]. In comparison, casein contains peptides such as casomorphins, which help reduce gastrointestinal motility, gradually increasing amino acid concentrations in plasma and contribute to satiety [134]. However, based on protein absorption rates, there is no clear evidence to suggest that one of the milk proteins is more satiating than the other.
In fact, it has potential impact on increased digestion and absorption rate of the protein. Calbet and Holst in 2004 have confirmed that intact casein and whey proteins were absorbed at lower rate than hydrolyzed. The rate of GLP-1 and PPY responses in blood plasma was higher with hydrolyzed milk proteins solution [135]. Effect of hydrolyzed milk proteins were examined on appetite regulation. Hydrolyzed whey protein had less effect on appetite regulation [136].

It has been also been suggested that incomplete proteins (which lacks one or more essential amino acids) might be more satiating and reduce food intake than complete proteins. The effect of whey and casein has been compared with incomplete proteins on satiety and incomplete proteins have been found to increase satiety more than whey and casein. Diets with low levels of essential amino acids will decrease the concentration of those amino acids in plasma. As a result, the brain will reject consumption of unbalanced diets therefore initiating the feeling of hunger [137].

1.10.References

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94. Anna L.Gillespie, Danielle Calderwood, Laura Hobson, and B. D.Green, *Whey proteins have beneficial effects on intestinal enteroendocrine cells stimulating cell growth and increasing the production and secretion of incretin hormones*. Food Chemistry, 2015. 189: p. 120-8.


101. Manuela PGM Lejeune, Klaas R Westerterp, Tanja CM Adam, N.D.L.-M. and, and M.S. Westerterp-Plantenga, *Ghrelin and glucagon-like peptide 1 concentrations, 24-h satiety, and energy and substrate metabolism during a high-protein diet and


Chapter 2: Hypotheses and objects

2.1. Rationale of thesis

Hence, there are no sufficient studies applied on the effect of milk protein and its fraction on the regulation of satiety hormone. Summary of in vitro studies are provided below which showed the effect of different source of proteins on satiety regulation hormones. Table 2.1 provides a brief outline of the effect of different proteins on satiety hormones released.

<table>
<thead>
<tr>
<th>Aims of study</th>
<th>In vitro cell line</th>
<th>Effect of reagent on satiety hormones</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of glutamine on the GLP-1-secreting cell line</td>
<td>GLUTag.</td>
<td>Increased GLP-1 released more glucose.</td>
<td>Reimann, F., et al. (2004).[1]</td>
</tr>
<tr>
<td>Effect by which milk proteins including leucine, isoleucine, valine, skim milk, casein, and whey improve GLP-1 release</td>
<td>NCI-H716, in dose response 0.5%, 1.0%, 2.0%, and 3.0%.</td>
<td>Leucine, isoleucine, skim milk, and casein stimulated GLP-1 release.</td>
<td>Chen, Q. and R. A. Reimer (2009).[2]</td>
</tr>
<tr>
<td>Effect of olive leaf extract, glutamine, chlorogenic acid α-casein &amp; β-casein</td>
<td>STC-1</td>
<td>All test reagents except α-casein showed significant increase in GLP-1 secretion.</td>
<td>Rafferty, E. P., et al. (2011).[3]</td>
</tr>
<tr>
<td>Investigated which dietary proteins, hydrolysates, or synthetic-peptides most impact on the secretion of CCK and GLP-1</td>
<td>STC-1</td>
<td>Casein and whey showed strongest effects on CCK release, whereas casein affected GLP-1</td>
<td>Geraedts, M. C., et al. (2011).[4]</td>
</tr>
</tbody>
</table>

Based on the review of the literature presented in Chapter 1, it is evident that milk proteins have a promising effect on increasing satiety, however, lactose intolerance, increased blood lipids and cardiovascular disease risks and kidney disease resulting from high calcium content in milk can pose issues for the use of milk proteins to regulate body weight. Hence, this thesis will aim to identify which milk protein and its fraction would have the most effect on satiety hormone regulation. Further, this project will explore the combined delivery of milk protein and polyphenols from green tea, in comparison to the individual components on
satiety hormone regulation. This protein-polyphenol conjugate could provide a new promising approach to be used as anti-obesity treatment.

**This thesis will aim to answer the following research questions**

1. Do milk proteins have the ability to influence the production of GLP 1, the major GI track satiety regulating hormone?
2. Is there a difference in the ability of different milk proteins in influencing regulation and secretion of GLP-1?
3. What are the mechanisms by which milk proteins influence the secretion of satiety hormones?
4. Can milk proteins be used as a platform to deliver bioactive compounds for GLP-1 secretion?

**2.2. Research aims**

In order to answer these research questions through *in vitro* study, this project will comprise of the following four aims:

**Aim 1:** Identification of milk proteins and fractions that are able to stimulate the secretion of GLP-1.

**Aim 2:** Compare efficacy of selected (promising) whey or casein proteins / fractions to stimulate the secretion of GLP-1.

**Aim 3:** Elucidation of molecular mechanisms by which milk protein influence the secretion of GLP-1.

**Aim 4:** Synthesis & characterisation of protein–polyphenol conjugate and its effect on the regulation of GLP-1 hormone. as well as, to elucidate its potential as dietary supplementation to prevent obesity and control body weight.
2.3. **Outline of thesis**

**Chapter 1** provides an introduction and review of existing literature on the use of milk and milk proteins as an approach to control body weight and anti-obesity treatment. It also provides an explanation of the regulation of satiety hormones in the body and how this can provide a solution to formulate diet supplements for controlling body weight.

**Chapter 2** summarize of project background and aims

**Chapter 3** gives details of the methodology employed in this thesis, including synthesis and characterisation techniques and provides an explanation of the *in vitro* assays used to study satiety hormone regulation.

**Chapter 4** deals with identifying the milk protein that has the best effect in regulation of GLP-1 and the molecular mechanism by which this occurs, in human intestinal cells.

**Chapter 5** investigates the formation of a conjugate between a milk protein and a polyphenol from green tea; physio-chemical characterisation of this conjugate which will be used to monitor satiety hormone regulation in human cells.

**Chapter 6** explores the *in vitro* effects of the milk protein-polyphenol conjugate on human intestinal cells and the use of this conjugate as a platform to deliver bioactive compounds to enhance satiety hormone secretion.

**Chapter 7** provides a summary of findings in the conclusion and future direction work possible, based on the results obtained from this thesis.

2.4. **References**


Chapter 3: Materials and methods

3.1. Bio-chemicals and reagents

Casein and whey proteins were gifts from Fonterra Dairy (Australia). Purified (Apo-LF), saturated (LF SAT) and semi saturated (LF SEMI) lactoferrin samples were gifts from Tatura Milk Industries Ltd., a subsidiary of Bega Cheese, (Australia). (−)-Epigallocatechin gallate (EGCG) (purity ≥95.0%), gallic acid (GA, 98%), Sodium phosphate dibasic and Sodium phosphate monobasic dehydrate, Iron (III) chloride hexahydrate, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid ,Pluronic F-127, Krebs ringer bicarbonate Buffer, Adenosine 5′-triphosphate (ATP), Ionomycin calcium salt (INO) disodium salt hydrate, potassium per-sulphate, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid and dialysis tubing cellulose membrane (12 kDa cut-off) were purchased from Sigma-Aldrich (Australia). Sodium phosphate buffer (pH 7.0) was prepared by mixing 30.5 mL of 0.2 M Na₂HPO₄ with 19.5 mL of 0.2 M NaH₂PO₄ and made up to a final volume of 100 mL. Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco’s Modified Eagle (DMEM) medium, Foetal bovine serum (FBS), 1x phosphate buffered saline (PBS), - 0.53 mM Ethylenediamine tetraacetic acid (EDTA), Geltrex™ hESC-Qualified Ready-To-Use Reduced Growth Factor Basement Membrane Matrix, Trizol, High-Capacity cDNA Reverse Transcription Kits, TaqMan fast universal PCR master mix, FAM-MGB probe/primer set for GLP-1 (Hs01031536_m1) and β- Actin (Hs02742610_g1), donkey anti mouse polyclonal antibody tagged with HRP (secondary antibody for the enzyme-linked immunosorbent assay (ELISA)), Hoechst 33342, gold anti-fade mounting medium and RIPA Lysis and Extraction Buffer were purchased from Life technologies (Australia). BCA Protein Assay Kit was purchased from Thermo Fisher (Australia). Anti-GLP1 antibody was purchased from Abacam (Australia). 16% paraformaldehyde was purchased from Electron Microscopy Sciences (Australia). Working solution of 4% was prepared in PBS and chilled to 4 °C right
before use. ELISA reagents including coating buffer, wash buffer, substrate and stop solution were purchased from BD Bioscience (Singapore). MilliQ water was used for all experiments. 75 cm$^2$ flasks, centrifuge tubes and tissue culture grade 96 well, 12 well and 6 well plates were purchased from Corning (Australia). Pipette tips and 1.7 mL tubes were purchased from Eppendorf (Australia).

3.2. Determination of protein concentration in milk samples using BCA kit

Protein content was determined using BCA Protein Assay Kit. This method is based on the reduction of Cu$^{2+}$ to Cu$^{+}$ by protein in an alkaline medium. The resulting purple coloured solution is produced by the chelation of two molecules of bicinchoninic acid (BCA) with one cuprous ion [1]. The protocol was followed according to the manufacturer’s instructions. Briefly, a series of dilutions of known concentrations were prepared with the BSA (0-2 mg/mL) and assayed alongside the samples (Table 3.1). 25 µL each standard or unknown sample in triplicate was taken in a 96 well plate. Additional of 200 µL of the BCA Working Reagent (WR) was added to each well and mixed thoroughly on a plate shaker for 30 seconds. WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. The plates were incubate at 37 ºC for 30 min., after which the plates were cooled to room temperature (RT). Absorbance at 562 nm was read using a Envision Micro plate reader (PerkinElmer, USA). Protein concentrations of the samples were determined using the linear regression equation generated from the BSA standards.
Table 3.1. Preparation of BSA Standards for estimation of total protein content

<table>
<thead>
<tr>
<th>No</th>
<th>Volume of PB (µL)</th>
<th>Volume of Diluent (µL)</th>
<th>Final PB Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
<td>2000</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>25</td>
<td>1500</td>
</tr>
<tr>
<td>3</td>
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<td>1000</td>
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<td>750</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>75</td>
<td>500</td>
</tr>
<tr>
<td>6</td>
<td>12.5</td>
<td>87.5</td>
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<tr>
<td>7</td>
<td>6.25</td>
<td>93.75</td>
<td>125</td>
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<tr>
<td>8</td>
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</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3. Selection of *in vitro* cell models and cell culture conditions

A number of cellular models of animal and human origin have been used to study the regulation of satiety hormones secretion, such as mouse intestine cell lines (STC-1), human colorectal cells (NCI-H716) and murine endocrine cells (GLUTag) [2]. Those modules have provided useful information regarding the signalling pathways that regulate proglucagon gene expression for example GLP-1 secretion [2]. GLP-1 is an incretin hormone that is released by enteroendocrine L cells in the gastrointestinal tract (GI). Since NCI-H716 cells have been extensively validated as models to investigate GLP-1 secretion [3], these cells were chosen to study GLP-1 expression. NCI-H716 cells release GLP-1 not only in response to nutrient stimulation including fatty acids, amino acids, protein hydrolysates, artificial sweeteners and bitter compounds, but also in response to other hormones such as insulin, leptin and selected neurotransmitters [4, 5]. Hence, NCI-H716 cell lines were chosen for this project, to study the effects of milk proteins on regulation of satiety hormones.

3.4. Cell culture conditions of NCI-H716 cell line

According to ATCC product sheet, the NCI-H716 cell line was initiated from ascites fluid of a 33-yr-old male with poorly differentiated adenocarcinoma of the colon. This is a hypotriploid human cell line with a modal chromosome number of 61 occurring in 28% of cells. The cells will be grow in suspension in RPMI 1640 medium supplemented with 10% FBS,
100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37 °C in the presence of 5% CO₂ and 95% humidified air. Figure 3.1 depicts normal cell morphology as observed under a phase contrast microscope (Nikon Eclipse TS100, Nikon Corporation, Japan). RPMI medium was used for subculturing and maintenance of the cells in suspension. However, those cells change to adherent morphology and express GLP-1 only in DMEM media with Matrigel. Hence, to setup the experiments, cells were seeded in complete DEME medium into wells precoated with Matrigel [4].

![Figure 3.1](image)

**Figure 3.1:** shows NCI-H716 human cell extensively validated as models to investigate GLP-1 secretion, represented at different magnifications, a: 40x, b: 100x and c: 200x

### 3.5. Cell viability assay

Measurement of *in vitro* cell viability has been applied further determine if test reagents show cytotoxic effects and to determine subtoxic concentrations for gene and protein expression assays. Different tetrazolium compounds have been used to detect cell viability such as MTT, MTS, XTT, and WST-1. 3-4, 5-dimethylthiazolyl-2-2, 5-diphenyltetrazolium bromide (MTT) assay is a popular method to examine cell viability and was chosen for this project. It was the first cell viability assay developed for a 96-well plate [6]. MTT reagent enters the cells and passes into the mitochondria where it produces purple formazan crystals. Figure 3.2 represents the chemical reaction involved in this conversion. The quantity of formazan refers
to the number of live cells and is measured by absorbance readings at 570 nm Envision Micro plate reader (PerkinElmer, USA) [6].

![Diagram of MTT and Formazan](image)

**Figure 3.2:** Reduction of MTT to purple formazan crystal in metabolically live cells by enzymatic reduction of a yellow 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide salt (MTT).

Cells were seeded at a density of 5×10⁴ cells/well in 96 well culture plates in DMEM complete media and incubated at 37 °C in the presence of 5% CO₂ and 95% humidified air. After 24 h, the test agents at required sub-concentrations starts from 5% for each component added in complete media. After 24 h, 0.5 mg/mL MTT solution (MTT stock solution was 5 mg/mL were dissolved in PBS) was added in serum free media and incubated for 4 h at 37 °C in the presence of 5% CO₂ and 95% humidified air. At the end of incubation period, the formazan crystals generated by live cells were dissolved in 100 µL of acidified isopropanol in each well and absorbance measured at 570 nm. The results are presented as percentage viability, taking average of three independent experiments and each experiment contained three replicates. The percentage viability of cells was determined against cell free control. In order to remove background absorbance and to make sure that test sustained did not interact
with MTT, cell free control at each concentration was used. The absorbance reading from those wells was subtracted before the final calculation of cell percentage viability.

3.6. Gene expression assay

Gene expression involves two main processes. Starts with transcription process inside the nucleus where different types of RNA are produced, including messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). mRNA produced from the nucleus in preparation for protein expression, hence monitoring mRNA levels can provide information on the gene expression associated with a certain protein [7]. Figure 3.3 show a schematic diagram of gene expression production in human cells.

In order to study regulated changes in gene expression, we need a technique to measure how gene activity patterns vary within an organism. Genes encode messenger ribonucleic acid (mRNA) which are translated into proteins, as a result gene expression can be assessed at either the mRNA or the protein level [8, 9]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is an increasingly used powerful method because of its high sensitivity, and wide dynamic quantification range for the detection and quantification of gene expression in tissues [10, 11].

For successful and reliable results in real-time qRT-PCR, RNA needs to be of high quality, and DNA-free. Hence, pure RNA is critical to all gene expression analysis techniques [12-14]. RNA extracting techniques may work differently depending on the type of cells and origin of the tissue, leading to up to 10 fold variations in the total RNA yield [11, 13, 14]. For this study, TRIzol method was used for RNA extraction. RNA extraction using Trizol reagent has been proven to be an effective method for RNAs including microRNAs, piwi-associated RNAs, or endogeneous, small interfering RNAs [15]. Since RNA is easily degraded by RNases even during the RNA purification or storage [16, 17], samples were stored in TRIzol
at -80 °C until the extraction process could be performed and converted to complementary deoxyribonucleic acid (cDNA) on the same day of extraction.

Figure 3.3: Schematic representation of gene expression

3.6.1. Gene expression studies
For gene expression study, cells with total of $2 \times 10^6$ cells were seeded in 12 well culture plates coated with matrix gel in reconstituted DMEM medium. After 24 h, cells were treated with media containing different concentrations of test reagents and 0.2% bovine serum albumin (BSA) instead of FBS. 100 µM glucose was used as positive control [18]. After 18 h of treatment, TRIzol was added to the cells and samples were stored in 1.7mL RNase/DNase free tubes at -80 °C.

3.6.2. Ribonucleic acid (RNA) isolation
After Trizol was added to samples, 200 µL of chloroform per 1 mL of TRIZOL was added to cause phase separation where DNA remains in the interface, and RNA remains in the aqueous phase. Tubes were mixed vigorously. Samples then were centrifuged at 12,000 x g for 15 mins at 4° C. After centrifugation, upper aqueous phase containing RNA was transferred carefully into new tube. 500 µL of isopropyl alcohol was added per 1 mL of TRIZOL precipitation of nucleic acid. Samples were then incubated at room temperature for 10 mins and centrifuged at 12,000 x g for 15 mins at 4° C.
Supernatant was completely discard, and RNA pellet was washed with 75% ethanol. Samples were vortexes and centrifuge at 12,000 x g for 15 mins at 4 °C. All leftover ethanol was removed by air-dry RNA pellet for no more than 5mins. RNA pallet was dissolved in nuclease free water. Dissolved RNA samples was quantified using Nano-Q. The yield was determined based on A260/A280 ratio.

3.6.3. Complementary deoxyribonucleic acid (cDNA) synthesis

After extracting RNA, 2 µg of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit. The procedure was followed as per manufacturer’s instructions. The master mix was prepared as shown in Table 3.2. Thermal cycling was performed on a BioRad T-100 thermal cycler and the running conditions are displayed in Table 3.3. Samples were stored at -20 °C.

Table 3.2: Volume of component to prepare 2x RT Master Mix per 20 µL reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X reverse transcription (RT) Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25X deoxynucleotide triphosphate (dNTP) Mix</td>
<td>0.8</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>2.0</td>
</tr>
<tr>
<td>MultiScriber™ Reverse Transcriptase</td>
<td>1.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>4.2</td>
</tr>
<tr>
<td>2µg of RNA dissolved in nuclease-free water</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 3.3: Thermal cycle conditions optimized for High-Capacity cDNA Reverse Transcription Kits.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Step 2</td>
<td>37</td>
<td>120</td>
</tr>
<tr>
<td>Step 3</td>
<td>85</td>
<td>5</td>
</tr>
<tr>
<td>Step 4</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

3.6.4. Real-time quantitative polymerase chain reaction (qPCR)

cDNA was subjected qPCR to analyse the expression of the target genes, GLP-1 and β-actin was selected as the housekeeping gene. GLP-1 is produced by proglucagon gene in human body (Chapter 1 section 1.5.4), GCG (glucagon) is the Taqman prime used to study GLP-1
gene expression [19]. The reaction mix was set up as shown in Table 3.4, as per manufacturer’s instructions. Samples were run on a 7500 Fast Applied Biosystems instrument. Samples were held at 50 °C for 2 mins, 95 °C for 10 mins, followed by 40 cycles of 95 °C for 15 mins and 60 °C for 1 minute. The threshold cycle values (Ct) were determined at the same fluorescence threshold line for each gene. The fold change in target gene relative to the housekeeping gene and the untreated samples are expressed as fold change of delta delta threshold cycle (ΔΔCt), taking average of three independent experiments and each experiment contained three replicates.

Table 3.4: qPCR samples preparation

<table>
<thead>
<tr>
<th>qPCR</th>
<th>µL/reaction</th>
<th>µL per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x TaqMan gene expression master mix</td>
<td>5</td>
<td>5.5 µL</td>
</tr>
<tr>
<td>20x TaqMan gene expression assay mix</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>cDNA sample</td>
<td>2.5</td>
<td>4.5 µL</td>
</tr>
<tr>
<td>Ultrapure RNase DNase free H₂O</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

3.7. Enzyme-linked immunosorbent assay (ELISA)

For protein expression study, protocol was adopt from Chen and Reimer [4] with minor modifications. In brief, cells were seeded at a total of 2×10⁶ cells/well in 12 well culture plates coated with matrix gel in reconstituted DMEM medium. After 24 h, cells were treated for 3 h with media containing different concentrations of test reagents and 0.2% BSA. After 3 h, culture supernatant was saved for extracellular release and cells were washed with cold PBS twice and 500 µL of radioimmunoprecipitation assay buffer (RIPA) was added to cells with the addition of 50 mg/L of phenylmethylsulfonyl fluoride and 34 mg/L of diprotin A. Cells were incubated for 15 mins on ice. Cells were then collected and centrifuged at 14,000 × g for 15 minutes to pellet the cell debris. Supernatant were frozen at -80 °C and dried using an OPERON (Gimpo, Korea) freeze dryer -55 °C to obtain a concentrate protein content.
powder. The culture supernatant collected for extracellular protein levels was also supplemented with 50 mg/L of phenylmethylsulfonyl fluoride and 34 mg/L of diprotin A.

Protein concentrations were determined using BCA kit as described in Section 2.2. GLP-1 protein standards concentration was 1mg/mL and was dissolved in coating buffer. Wells were coated and allowed to incubate overnight at 4 °C. Uncoated samples were discarded and wells were washed with 1X washing buffer. Wells were blocked with 2% of BSA (blocking buffer) to prevent non-specific binding of antibodies and allowed to incubate for 45 mins at room temperature (RT). Blocking buffer was discarded and 1:40,000 dilution of Anti-GLP1 antibody-10 primary antibody was added to each well and incubated for 1 h at RT. Plate was washed 5 times for 30 seconds each. Secondary antibody of donkey anti mouse polyclonal antibody tagged with Alexa Fluor® 488 was added (1:10,000 dilution) to each well, and plate was incubated for 45 mins at RT and in the dark. Secondary antibody was discarded and the plate was washed 5 times with wash buffer for 30 seconds. 100 µL of the substrate was added and incubated for 25 mins in the dark. Finally, 20 µL of stop solution was added and absorbance read immediately at 450 nm.

3.7.1. Confocal microscopy

The protocol was adapted from Anna L. Gillespie, et al. [20] with minor modifications. Total of 2x10⁶ cells were seeded per well in 6 well culture plates coated with matrix gel in reconstituted DMEM medium. Cells were treated as described in Section 3.7. After treatment, media was removed and cells fixed with 4% cold paraformaldehyde (PFA) for 10 mins at room temperature. Cells were washed three times with PBS and stored in PBS at 4 °C until staining could be performed.

For the staining, the PBS was removed and cells were permeabilised with 0.02% Triton X-100 in PBS for 90 seconds at RT. Cells were washed once with PBS, followed by blocking
with 2% BSA in PBS for 30 min. Cells were then incubated with 1:200 dilution of GLP-1 primary antibody in blocking buffer for 1 h. After washing three times with PBS, cells were incubated with 1:500 dilution of secondary antibody (donkey anti mouse polyclonal antibody) tagged with Alexa Fluor® 488 in blocking buffer, for 1 h, in the dark. Cells were washed 5 times in PBS, followed by staining with Hoechst 33342 (2μg/mL in PBS) for 10 mins. After the final three PBS washes, cells were mounted on to glass slides using ProLong gold antifade mounting medium and imaged on a N–STORM SuperResolution/Confocal microscope (Nikon-Japan). Images were obtained using the NIS Elements 4.0 software.

3.7.2. Flow Cytometry

Flow cytometry has been developed in the late 1960s, and is become a popular analytical technique for cell biology. It utilizes light to count and profile cells in a heterogenous fluid mixture [21]. The histogram plots a single parameter (horizontal axis) against the number of events detected (vertical axis) [21, 22].

For flow cytometry analysis, a total of 2x10⁶ cells were seeded per well in 6 well culture plates coated with matrix gel in reconstituted DMEM medium. Cells were treated as described in Section 2.7. Cells were detached from the wells using 1 mL of trypsin-EDTA for 5 mins. The trypsin-EDTA was neutralised using complete media and cells were centrifuged at 1000 rpm for 5 mins. Media was removed and cells fixed with 4% of for 10 mins at room temperature. Cells were washed in PBS and stored in PBS at 4 °C until staining could be performed. For the staining, the PBS was removed and cells were permeabilised with 0.02% Triton X-100 in PBS for 90 seconds at room temperature. Again, cells were washed 4 times with PBS and 1 µL/mL GLP-1 polyclonal primary antibody was added in 0.1% Triton followed by incubation for 1 h at RT. Cells were washed (×4) with PBS by centrifugation. Secondary antibody of donkey anti mouse polyclonal antibody tagged with Alexa Fluor® 488
(1 µL/mL) was added and incubated at room temperature in the dark for 1 h. Cells were washed thrice with PBS and flow cytometry analysis was performed on a BD Accuri flow cytometer. Forward (FFS) vs side (SSC) scatter was performed and gated to remove cell debris. From within the gated sample, histogram was generated from 10000 events, using the FL-1A (green filter) vs count. Unstained cells and cells stained with only primary and only secondary antibodies were used as controls. The data was analysed using FlowJo software, where fluorescent intensity was normalised to the untreated samples.


Activation of NCI-H716 cells was assessed by monitoring Ca²⁺ signals using a flexstation plate reader (Multi-Mode Microplate Reader). Protocol was adapted from Le Neve B and H. [5] with minor modifications. Briefly, NCI-H716 cells were seeded at 2x10⁵ cells per well in a 96 well plate coated with Matrigel for 24 h. Measurement of Ca²⁺ was achieved after loading the cells for 40 min with the membrane-permeant fluorescent indicator FURA-2A. Dye loading medium composition was the following: 0.25% Pluronic F-127 and 2µM FURA 2AM in Krebs buffer pH7.4 (11 mM: 137NaCl, 5.4KCl, 1.2 CaCl₂ 2H₂O; 1M gSO₄₄ 7H₂O; 0.3 Na₂HPO₄ H₂O; 0.3 KH₂PO₄; 10 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Test reagents were prepared in Krebs buffer and automatically added onto the cells during the assay. Measurements were typically carried out for 350 seconds using Flexstaion 3 Multi-Mode Microplate Reader. Fluorescence signals were read at excitation 340/380 nm–emission 510 nm. Ionomycin (INO) 5 µM was used as a positive control in all experiments. Each effector was tested in triplicate.

3.9. Atomic emission spectroscopy (AES)

Iron content of lactoferrin was determined by Microwave Plasma Atomic Emission Spectroscopy (MP-AES). Samples were prepared by dissolving 0.01 g/mL of lactoferrin in
Milli Q water. Iron standards ranged from 0-10 ppm from which the standard curve was obtained. Three replicates were performed for each sample on an Agilent Technologies 4200 MP-AES instrument.

3.10. Synthesis of Apo-LF-polyphenol conjugates

Apo-LF-EGCG conjugates was synthesized according to Liu et al. (2016) [23], with slight modifications in Apo-LF and EGCG concentration. In brief, stock solution of 20 µM of Apo-LF was prepared by dissolving 0.0016 g/mL in phosphate buffer (PB) at pH 7.0 and stirred for 2 h to ensure the sample is fully dissolve. To prepare LF-EGCG conjugates, 1 mM of EGCG stock solution was prepared by dissolving 0.0004 g/mL in PB. Both Apo-LF and EGCG solutions were mixed to obtain 15.63-500 µM final concentrations of EGCG with a constant Apo-LF concentration of 10 µM. The samples were continuously stirred for 24 h at RT. Table 3.5 depicts the method of sample preparation used for preparation of the conjugates. The samples were dialyzed for 24 h against water to remove free EGCG. Thereafter, the resulting solutions were frozen at -80 °C and dried using an OPERON (Gimpo, Korea) freeze dryer at -55 °C to obtain powder.

Table 3.5: Concentration depending on preparation of Apo-LF-EGCG conjugates in PB at pH 7.0

<table>
<thead>
<tr>
<th>Final Conc. of EGCG (µM)</th>
<th>Apo-LF 20 µM Stock (µL)</th>
<th>EGCG 1 mM stock (µL)</th>
<th>PB (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500.00</td>
<td>1500</td>
<td>1500.00</td>
<td>0.00</td>
</tr>
<tr>
<td>250.00</td>
<td>1500</td>
<td>750.00</td>
<td>750.00</td>
</tr>
<tr>
<td>125.00</td>
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<td>375.00</td>
<td>1125.00</td>
</tr>
<tr>
<td>62.50</td>
<td>1500</td>
<td>187.50</td>
<td>1312.50</td>
</tr>
<tr>
<td>31.25</td>
<td>1500</td>
<td>93.75</td>
<td>1406.25</td>
</tr>
<tr>
<td>15.62</td>
<td>1500</td>
<td>46.88</td>
<td>1453.12</td>
</tr>
</tbody>
</table>
3.11. Characterisation of Apo-LF-polyphenol conjugates

3.11.1. Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR spectra of the samples obtained after freeze dryer were measured using a GladiATR Single Reflection instrument in the 400–4000 cm\(^{-1}\) range, with a resolution of 4 cm\(^{-1}\). For each measurement, 20 scans were taken. The samples analysed were free Apo-LF, free EGCG, and freeze-dried Apo-LF-EGCG complexes. Data were analysed using OPUS Spectroscopy Software version 7.2.139.1294 and Origin software version 16.

3.11.2. Circular Dichroism (CD) Measurements

Samples were used in liquid form before the freeze dryer was applied. The CD spectra results were recorded using JASCO J-1500 CD (Japan) spectrometer in the far-UV (190–300nm) range, under constant nitrogen flush. Path lengths of the cuvette used was 0.1 cm. Ellipticity was recorded at a speed of 100 nm/min with 0.1 nm resolution. Each spectrum presented was the average of three consecutive measurements. Changes in secondary structure of Apo-LF were obtained by online website calculation http://perry.freeshell.org/cgi-bin/raussens.cgi.

3.11.3. Fluorescence Spectroscopy Measurements

Hence fluorescence measurements were conducted to determine the mechanism of interaction between Apo-LF and EGCG in the conjugates. Fluorescence measurements were carried out using a fluorescence spectrophotometer (FluoroMax-4, Horiba). Fluorescence emission spectra of Apo-LF were recorded with the excitation wavelength at 285 nm with emission from 295-500 nm. Both excitation and emission slit widths were set at 1 nm. The fluorescence quenching data were analysed by fitting to the Stern–Volmer equation (eq 1).
\[
\frac{F_0}{F} = 1 + K_{sv}[Q] \quad \text{(eq:1)}
\]

Where \(F_0\) and \(F\) refer to the fluorescence intensities in the absence and presence of the quencher, and \([Q]\) is the concentration of quencher. \(K_{sv}\) is the Stern–Volmer equation constant \([24-26]\). The binding constant \(K_q\) and the number of binding sites \(n\) can be calculated according to a double-logarithmic equation (eq 2).

\[
\log \frac{F_0 - F}{F} = \log K_q + n \log [Q] \quad \text{(eq:2)}
\]

The intercept of the double logarithmic Stern–Volmer plot provides the binding constant \((K_q)\), and the slope yields the number of binding sites \((n)\).

Since the thermodynamic forces responsible for the binding of EGCG to Apo-LF did not follow a linear regression, polynomial fit was employed as described by Galaon and David \([27]\). The dependence of \(\ln K\) on \(1/T\) is represented by the following equation:

\[
\ln K = \alpha_0 + \frac{\alpha_1}{T} + \frac{\alpha_2}{T^2} \quad \text{(eq: 3)}
\]

Where \(K\) is the binding constant at the corresponding temperature \((T)\) in Kelvin.

The thermodynamic parameters can be calculated using the following equations:

\[
\Delta H^\circ = -R(\alpha_1 + 2\frac{\alpha_2}{T}) \quad \text{(eq: 4)}
\]

\[
\Delta S^\circ = R(\alpha_0 - \frac{\alpha_2}{T^2}) \quad \text{(eq: 5)}
\]

\[
\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad \text{(eq: 6)}
\]

Where \(R\) is the gas constant \((8.314 \text{ J/K mol})\), \(\Delta H^\circ\) and \(\Delta S^\circ\) correspond to the changes in enthalpy and entropy, respectively and \(\Delta G^\circ\) corresponds to the Gibbs free energy.

3.11.4. Zeta potential
The ζ-potential of the complex was determined using a Malvern Zetasizer Nano ZS (Malvern, United Kingdom). Samples were loaded in a folded capillary cell and temperature was set at 25 °C. All samples were dissolved in PB (pH 7.0) to 0.001 g/mL. The data were collected over 15 sequential readings. The ζ-potential measurements were reported as the average of three measurements.

3.11.5. Electron microscopic analysis

Scanning electron microscopy (SEM) was used to study the microstructure of lactoferrin (LF) protein before and after its conjugates with EGCG. Freeze dried samples was gently mounted on SEM stubs covered with a double-sided carbon tape and sputter coated with a fine layer of gold. The microstructure of LF samples was examined by means of Philips XL30 digital scanning electron microscope operated at 30 kV accelerating voltage.

3.11.6. ABTS•+ radical scavenging activity

The amount of antioxidant activities in the LF-EGCG complexes were determined using ABTS•+ decolouring assay accordingly to Re et al. (1999) [28] with slight modifications. First stock solution of 7 mM of ABTS•+ was prepared by dissolving 3.9 mg/mL in water. ABTS•+ free radical was produced by reacting ABTS•+ stock solution with 2.45 mM of potassium persulfate. The solution was stored in dark at room temperature for 12 – 16 h before use. ABTS•+ solution was diluted with PBS, pH 7.4 to an absorbance of 0.70 (±0.02) at 734 nm using Envision Micro plate reader (PerkinElmer, USA). Trolox standards were prepared fresh each time in ethanol to final concentrations between 0-15 µM. Appropriate solvent blanks were run in each assay. The reaction mixture of the standard and complexes were obtained by mixing 200 µL of ABTS•+ diluted with 20 µL of each sample. All measurements were run in triplicates. The percentage of antioxidant activity of the conjugates was calculated using the following formula:
ABTS$^{+}$ scavenging activity (%) = \( \frac{(A_C - A_S)}{A_C} \times 100 \) (eq:7)

Where \( A_C \) is the absorbance of the control, and \( A_S \) is the absorbance of the samples.

3.11.7. HPLC-MS Analysis

The aim of this study was to develop an efficient High-performance liquid chromatography (HPLC) method for the measurement of EGCG in Apo-LF-EGCG conjugate supernatant. To perform faster analysis, a conventional HPLC-MS system (Agilent Technologies 1200 Series, 6410 Triple Quad LC/MS) was equipped with a Zorbax Eclipse Plus C18 analytical column (2.1 × 50) mm packed with 1.8 μm particles. EGCG standards ranging from 3.90-500 μM were prepared in PB. EGCG content in the Apo-LF-EGCG complexes was determined by quantifying the EGCG content in the supernatants after centrifuging the complexes at 10,000 rpm for 10 mins. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The linear gradient elution system was 75% of A and 25% of B for 2 min at a flow rate of 0.3 mL/min and temperature maintained at 30 °C.

3.11.8. Stability of EGCG

The effect of different pH on the stability of EGCG was assessed according to Radhakrishnan, et al. [29] with slight modification. EGCG was assessed by preparing 12.50 μM of EGCG dissolved in 1 mL of PB. The pH of EGCG was adjusted using fix amount of 2M NaOH and HCL solution to achieve the following final pH values (3, 5 and 7). In a Pyrex tube open with free exposure to air at RT, EGCG stability assay was applied at different time points (T0, T2h, T4h and T24h). An aliquot (1 mL) of the incubation mixture was tested using Hitachi F-7000 spectrofluorometer (Hitachi High-Technologies Co.) in a 1 cm quartz cell at 20°C. Scan speed was medium at UV of 200 – 500 nm.

3.12. Statistical analysis
The experiments were conducted in triplicate with 3 replicates of each sample. The results were presented as the means ± standard deviation. Statistical analysis were conducted using one-way ANOVA using Graph Pad Prism version 7. Statistical differences was considered significant when \( p* > 0.05 \), \( ** > 0.005 \) and \( *** > 0.0005 \).

### 3.13. References


20. Anna L.Gillespie, Danielle Calderwood, Laura Hobson, and B. D.Green, *Whey proteins have beneficial effects on intestinal enteroendocrine cells stimulating cell growth and increasing the production and secretion of incretin hormones*. Food Chemistry, 2015. 189: p. 120-8.


Chapter 4: Effect of milk proteins on GLP-1 gene expression and extracellular release

4.1. Introduction

Milk proteins are considered as high quality proteins based on human amino acid requirements, digestibility and their bioavailability functions [1]. The two major components of milk proteins are casein and whey. Bovine casein comprises of 80% of total milk proteins [2-4] and whey protein comprises 20% of total milk proteins [2, 5]. There are two types of commercially produced whey proteins, namely, whey protein concentrate (WPC) and whey protein isolate (WPI), WPC contains more fat (5-7%), higher lactose content and higher concentrations of lactoferrin than WPI [6, 7].

**Casein** is a group of phosphoproteins with molecular weight 26,000 to 45,000 (mol. wt.) [2, 4, 8] and is made of polypeptides known as α, β and κ-caseins [8]. Figure 4.1.a illustrates the 3D structure of casein in milk. All three fraction of casein are similar in size, with a molecular weight of 24 kDa and negative charge. However, they differ in their degree of unfoldedness of amino acids, hence it has become an interesting protein due to its unique unfolded chemical structure [8]. Casein proteins are endowed with unique biological and nutritional functions due to its ability to carry a large amount of insoluble calcium phosphate. In addition, casein protein contain citrate, minor ions, lipase and enzymes, in addition to calcium and phosphate [3]. Casein can be obtained from skim milk by precipitation with either acids or rennet [4], whilst whey proteins remain in the supernatant fraction [9]. The high phosphate content of casein in milk allows it to associate with calcium ions to form calcium phosphate salts and casein complexes [9]. It contains a higher proportion of essential amino acid such as histidine, methionine, phenylalanine and valine. It also contains non-essential amino acids such as arginine, glutamic acid, proline, serine and tyrosine [10].
Casein has been characterized as slow protein as a result of delayed gastric emptying and takes longer to increase amino acid profile in blood due to the high acidic conditions in the stomach compared to whey proteins [2, 11].

**Whey** includes a range of proteins that stay soluble in the serum phase of the milk after separation of curd, at pH of 4.6 at 20 °C, during cheese making [4, 12]. Figure 4.1.b shows the 3D structure of whey protein. It contains 50% β-Lactoglobulin, 20% α-Lactalbumin, 10–15% bovine serum albumin and immunoglobulins and 1–2% lactoferrin [2, 5]. Other minor whey protein include lactoperoxidase, β-microglobulin, lysozyme, insulin-like growth factor and gamma globulins [5]. It is rich in water-soluble vitamins and lactose [4]. Caseinomacropeptide, however, is considered as a sweet whey protein even though it is derived from κ- casein during cheese making [5]. Whey protein contains a higher proportion of the branched chain amino acids (BCAA), including leucine, isoleucine and valine in comparison to casein [10, 13]. It is considered as a fast protein, as it is digested and absorbed faster - within minutes in the intestine and gives a high blood amino acid profile [2, 5, 10, 13], hence whey is a good source of amino acids and bioactive peptides generated during its digestion [2].

![3D structures of (a) casein and (b) whey protein. These images were obtained from RCSB Protein Data Bank.](image-url)
Whole casein and whey have been reported to increase the secretion of satiety hormones such as glucagon-like peptide-1 (GLP-1), in the gastrointestinal (GI) tract [14-17]. Since studies indicate that stimulation of satiety hormones may lead to reduction in food intake and control of body weight, consumption of a diet supplemented with milk proteins has been suggested as an approach for the prevention and treatment of obesity and metabolic syndrome-related diseases. Casein and whey proteins are commonly used in pre and post work-out protein shake powders to help with weight loss and building muscle [18]. However, milk proteins are made up of various protein fractions and the exact protein component responsible for enabling weight loss is not known. This information is essential to provide insights into the formulations of more effective dietary supplements and to help with the development of better treatments for obesity and weight loss.

The object of this chapter is to explore which milk proteins or fractions are responsible for the regulation of GLP-1. The human intestinal cell line, NCI-H716, is considered a standard cell model to study the regulation of GLP-1 in humans [19]. Hence, this cell line was chosen to study the effects of various milk proteins and fractions on GLP-1 protein and gene expression.

4.2. Effect of milk proteins (casein and whey) and its fractions on cells viability and proliferation

Referring to Chapter 3 section 3.5, 3-4, 5-dimethylthiazolyl-2-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability in triplicate culture wells per treatment for each of the different concentration of milk proteins and its fractions including α, β and κ-casein, whey protein isolate (WPI), whey protein concentrate (WPC), and bovine serum albumin (BSA) with concentrations ranged from (0-5% w/v).
4.3. Results

The results (Figure 4.2) show % cell viability of NCI-H716 cells in the presence of milk protein at various concentrations (0-5%) over 24 h. The results showed a progressive decline in the cell viability as the concentration of milk proteins was increased. These results indicate that all milk protein fractions show dose dependent toxicity. Among casein fractions, κ-casein (Figure 4.2a) showed a remarkable decrease in cell viability with less than 30% cell viable at 2.5% concentration and cell viability was decreased as κ-casein concentration was increased in compression with other casein fractions (p< 0.0001). On the other hand, β-casein was less toxic to cells at >2.5% concentration. (p< 0.0001). However, at concentration higher than 3% the cell viability was decreased (Figure 4.2a). Similar pattern was observed for α-casein, % cell viability was decreased as the concentration of α-casein was increased with ≈62% cell viability (p< 0.0001) at 2.5% (Figure 4.2a). In contrast, whey protein fractions showed toxicity only above 2.5% as no significant (p < 0.05 and p < 0.001) difference in the toxicity profiles of the two fractions were noticed (Figure 4.2b). On the other hand, BSA showed the least toxicity with 60% viability (p< 0.0001) even at the highest concentration (cell viability >57 % with 5% BSA (Figure 4.2c). This toxicity data suggests that casein protein fractions are more toxic to cells than whey proteins. The in vitro toxicity of milk proteins on mammalian cells has never been reported in published literature. However, Osborne, et al. [20] have tested whole milk proteins on CaCo-2 (epithelial cells) and STC-1 (intestinal neuroendocrine tumor from mouse) cells, stating no toxicity was observed, however no information was provided on the concentrations tested or testing conditions.
Figure 4.2: Cell viability after 24 h of treatment of NCI-H716 cells with milk proteins. Cells $1 \times 10^4$ were incubated for 24 h with Dulbecco’s Minimal Eagle’s Medium plus 0.2% bovine serum albumin with or without one of the treatments (w/v) ranging from 3.13 to 5 mg/mL (0-5 %); (a) caseins fractions, (b) whey protein fractions and (c) BSA. Milk protein fractions all show dose dependent increase in toxicity with κ casein showing the most toxicity. Values are mean ± SD of three replicates per experiments and *p < 0.05, **p < 0.001 and ***p< 0.0001 when compared with the corresponding untreated cells.

For further understanding of the results obtained from cells viability, the protein content was assessed in the various fractions of milk protein at 3.13 to 50 mg/mL (1 to 5%) (Table 3.1). It was found that protein content varied between the various milk proteins. These results further indicate that despite the protein content of all casein fractions being less than the whey protein fractions, they showed higher toxicity to the cells [21]. This result could be caused by molecular weights of each milk proteins which might effect their up take by cells. Casein fractions are smaller in a molecular weight (24 kDa) in compassion with whey protein which has bigger molecular weight of ~40 kDa.

Table 4.1. Concentration of milk proteins from 1 to 5% w/v 9 Pierce BCA Protein Assay Kit method (mg/mL) Protein contents in milk proteins α, β and κ-casein, WPI and WPC from 1 to 5% w/v measured by Pierce BCA Protein Assay Kit method (mg/mL).

<table>
<thead>
<tr>
<th>Milk Proteins</th>
<th>1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Casein</td>
<td>0.11</td>
<td>0.23</td>
<td>0.34</td>
<td>0.45</td>
<td>0.57</td>
</tr>
<tr>
<td>β-Casein</td>
<td>0.53</td>
<td>1.05</td>
<td>1.58</td>
<td>2.10</td>
<td>2.63</td>
</tr>
<tr>
<td>κ-Casein</td>
<td>0.61</td>
<td>1.23</td>
<td>1.84</td>
<td>2.46</td>
<td>3.07</td>
</tr>
<tr>
<td>WPI</td>
<td>0.65</td>
<td>1.31</td>
<td>1.96</td>
<td>2.62</td>
<td>3.27</td>
</tr>
<tr>
<td>WPC</td>
<td>0.79</td>
<td>1.58</td>
<td>2.37</td>
<td>3.15</td>
<td>3.94</td>
</tr>
</tbody>
</table>

4.3.1. Effect of casein fractions and whey proteins on GLP-1 gene expression

Cells were exposed to different concentration of milk proteins at sub-toxic concentrations that showed at least 60 % cell viability after 24 h exposure (Figure 4.2) Accordingly, cells were
cultured in the presence of 10 mg/mL (1%) α and κ-casein, 10 – 40 mg/mL (1-4 %) β-casein, 10 - 30 mg/mL (1-3 %) WPC, 10 – 30 mg/mL (1-3 %) WPI or 30 mg/ml (3%) BSA for 18 h. The reason for choosing 18 h for GLP-1 gene expression was based on the method in which cells secrete and produce hormones. Cells synthesise and store hormones in their cytoplasms and releases these as soon as hormone receptors are stimulated. This is followed by activation of relevant genes (gene expression) to synthesis more hormones [22]. Hence, shorter time of 3h was used for protein expression studies (Section 4.4). It has been reported that 18 h is ideal time period for GLP-1R receptor to get activate (elongated period of time) [23]. Glucose was used as a positive control (+ve) based on the previous studies done by Won Young Song, et al. [24]. Quantitative real time polymerase chain reactions (qRT PCR) for genes of interest were normalized with β-actin as a housekeep gene.

β-casein (Figure 4.3.a) showed the greatest increase in GLP-1 gene expression, overall, with 1.89 fold increase at 1% w/v. Of the casein fractions, it was possible to examine concentration dependant effect of β-casein (up to 3% w/v), since α and κ casein could not be tested above 1% w/v, due to high levels of toxicity (Figure 4.3a). Of the whey proteins, WPC at 2% (Figure 4.3c) showed a significant effect on the expression of GLP-1 (2.3-fold change) in comparison to 3% of WPI (1.5 fold change) as shown in Figure 3.3d. BSA did not show any significant effect at all concentrations tested (Figure 4.3d). Glucose (100 μM), the positive control, showed a two-fold increase in gene expression and this is in agreement with the published data [24].
Figure 4.3: GLP-1 gene expression in NCI-H716 cells after 18 h of treatment with milk proteins. NCI-H716 cells (1.5 × 10^6) were incubated for 24 h with Dulbecco's Minimal Eagle's Medium with the addition of 0.2% bovine serum albumin with or without one of the treatments (w/v); (a) α and κ-casein, (b) β-casein, (c) whey protein concentrate (WPC) and (d) whey protein isolates (WPI). WPC showed a significant effect on the expression of GLP-1 at 20 mg/mL (2%) (2.3 fold change), whereas, WPI showed significant fold change in GLP-1 at 30 mg/mL (3%) by >2 fold change. However, BSA did not show any significant effect at any of the concentrations. On the other hand, casein fractions, β-casein showed concentration-dependent increase in GLP-1 expression relative to other casein fractions (α and κ-casein). P*>0.05.

While the effects of casein and whey have been previously explored [14, 17, 25], an in vitro comparison of casein fractions and whey proteins has not been reported. β-casein at 3% contained 1.58 mg/mL of protein and showed three-fold increase in gene expression, compared with WPC (2.37 mg/mL) at 2% and WPI (1.96 mg/mL) at 3% which showed 2.3- and 1.5-fold increases, respectively in GLP-1 expression. The results of this study indicate that casein fractions have a better effect on increasing GLP-1 gene expression than whey protein fractions, since they are capable of regulating the expression at a lower concentration.
4.3.2. Effect of casein fractions and whey proteins on protein expression of GLP-1

As a preliminary method of identifying changes to intracellular GLP-1 protein expression, confocal microscopy and flow cytometry were used. Cells were exposed to concentrations of milk proteins that showed significant increase in gene expression for 3h, before fixing and staining the cells. BSA was not included since there was no evidence of increase in gene expression.

4.3.3. Fluorescent microscopic analysis

GLP-1 expression in response to milk protein on NCI-H716 cell was studied using confocal microscopy. Hoechst 33342 is a fluorescent dye that binds to DNA in cells forming a fluorescent DNA-DAPI complex. Figure 4.4 displays the confocal images obtained with/without the test reagents at sub-concentration which showed highest cell viability. Nuclear fragmentation and GLP-1 protein were observed at a magnification of 20x with a scale of 100 µm. NIS Elements D 4.1 software was used to quantify the green fluorescence from the images. Figure 4.4 indicates that all milk proteins show increase in green fluorescence in comparison to the untreated cells, which suggests an increase in intracellular GLP-1 protein content. The casein fractions showed the greatest effect on intracellular protein content and these results reflect the increase in gene expression observed with these fractions in comparison with whey protein.
Figure 4.4: Confocal micrographs depicting increase in GLP-1 protein content in NCI-H716 cells. Cells 1 \times 10^6 were incubated for 2 h with sub-concentrations of milk protein after for 3 h. Upper panel is DAPI, middle panel is GLP-1 protein and lower panel is merge of DAPI (blue) and GLP-1 (green) protein. Cells treated with all milk proteins (caseins fraction and whey protein concentrated (WPC) and whey protein isolation (WPI) showed increases in green fluorescence in comparison to the untreated cells. Scale bar 100 µm.

4.3.4. Flow cytometry

Flow cytometry is used to analyse the expression of cell surface as well as intracellular molecules. Cells are incubated with antibodies and analysed using the flow cytometer. Flow cytometry was performed to further quantify the cellular GLP-1 protein content in response to treatment with milk protein fractions. Figure 4.5 depicts the histograms showing shifts in intensity between untreated cells and treatments group with milk proteins. As shown in Figure 4.5a. Positively stained cell population showed shift in fluorescence intensity to the right when cells were treated with milk protein at sub-toxic concentration. The highest shift in the fluorescence intensity observed with β-caseins (2.5 fold change) flowed by α-casein (1.8 fold change) in comparison to the untreated cell (Figure 4.5e&f respectively). This indicates β-casein shows the highest and significant increase in GLP-1 protein levels.
Approximately same increases in fluorescence observed after treatment with κ-casein (1.12 fold change), WPI (1.11 fold change) and WPC (1.17 fold change) in comparison to the untreated cells (Figure 4.5 e&f) which indicates an increase in intracellular GLP-1 concentration. In addition, Figure 4.6 shows the fold change in fluorescent intensity against untreated cells.
Figure 4.5: Histograms showing GLP-1 protein expression level in NCI-H716 cells following treatment with milk proteins. (a) untreated cells, (b) α Casein, (c) β casein, (d) κ casein, (e) WPI and (f) WPC

Figure 4.6b indicates the normalised fluorescence with respect to the untreated cells. All milk proteins show an increase in GLP-1 intracellular protein levels similar to the confocal microscopy results, where the casein fractions showed higher increase in GLP-1 than the whey proteins. This was further quantified using ELISA.
Figure 4.6: Relative fluorescent intensities obtained from the (a) flow cytometry data and (b) confocal micrographs, with respect to the untreated cells and after 3 h treatment with various milk proteins.

4.3.5. Quantitative analysis of GLP-1 protein levels

To quantify the effect of the milk proteins on protein expression of GLP-1 by ELISA, cells were incubated for 3 h with the same concentrations used for gene expression studies (Section 3.7). Extracellular and intracellular GLP-1 protein levels from cell lysate and cell culture medium, are shown in Figures 4.7 a and b, respectively. The casein fractions once again showed the greatest increase with κ-casein fraction (1% w/v) showing the most significant increase in intracellular GLP-1 levels (1.2 x10⁶ pg/mL) and release of GLP-1 (16.7 pg/mL) into the surrounding media.

Interestingly, WPI (3% w/v) showed release of GLP-1 without any significant increases in intracellular protein levels or gene expression (Figure 4.3a). This suggests that WPI has a burst release of satiety hormones and without any sustained effect on satiety. The positive control, glucose also shows an increase in GLP-1, however, the casein fractions had a greater effect of intracellular and extracellular release of GLP-1 protein. Our findings are in
agreement with previous study by Geraedts, et al. [25], where whole casein showed the most pronounced effects on GLP-1 release compared with the whole whey protein.

Figure 4.7: Effects of casein fractions and whey proteins on (a) intracellular GLP-1 levels and (b) GLP-1 release from the NCI-H716 cells. Cells were incubated for 2 h with α-casein (1%), β-casein (3%), κ-casein (1%), WPC (2%) or WPI (3%). Release following cell lysis and in medium was normalized to the total GLP-1 content and expressed as x10^6 pg/mL and pg/mL respectively. Values are means ± SD of three replicate experiments.***p< 0.0001 value compared with the corresponding to untreated cells.

4.4. Intracellular calcium contributes to GLP-1 secretion mechanism

To investigate whether cytosolic calcium is involved in GLP-1 secretion induced by treatment with milk protein, changes in intracellular calcium of NCI-H716 cells were assessed using FlexStation 33 Benchtop Multi-Mode Microplate Reader (Molecular Devices). For this, casein fractions, WPC and WPI were used at 1% concentration. Five μM of ionomycin (INO) was used as the positive control [19]. Briefly, NCI-H716 cells were seeded at 2×10^5 cells/ml in 96-well flat bottom cell culture plates coated with Matrigel for 24 h. Measurements were typically carried out for 25min [Ca^{2+}]i after loading the cells with the membrane-permeant fluorescent indicator FURA-2A for 40 min.

We applied a strategy to investigate the mechanisms by which specific milk proteins may cause an increase in intracellular calcium in NCI-H716 cells. Calcium ions play an important role in a range of cellular activities, including signal transduction pathways that stimulate the
release of neurotransmitters from neurons. Increased levels of [Ca\^{2+}]i is one of the main mechanisms involved in hormone secretion from enteroendocrine cells.

As shown in Figure 4.8, α and β-caseins fraction were able to increase the intracellular calcium [Ca\^{2+}]i response in NCI-H716 at 1% in comparison to κ-casein, WPC and WPI. While treatments with both WPC and WPI showed a slight increase in Ca\^{2+} intake. The result demonstrates that GLP-1 release by casein fractions, might be brought about due to increase in calcium channels activity [19, 26].

![Figure 4.8: Effect of casein fractions, WPC and WPI on [Ca\^{2+}] in NCI-H716 cells determined using Fura-2 fluorescence. NCI-H716 cells were seeded at 2×10^5 cells/ml in 96-well flat bottom cell culture plates (volume or cells/well) coated with matrigel for 24 h. Measurement was typically carried out for 25min of [Ca\^{2+}] after loading the cells with the membrane-permeant fluorescent indicator FURA-2A for 40min. Data were expressed as fluorescence ratio resulting from excitation at 340 and 380 nm. α and β-casein showed the highest/greatest effect on intracellular signalling of [Ca\^{2+}] i in NCI-H716 cells compared to κ-casein, WPC and WPI.](image)

### 4.5. Discussion

Previous studies have found that proteins and milk proteins shown to increase the regulation of GLP-1 hormone in both in vitro and human studies [11, 16, 25, 27, 28]. However, the signaling mechanisms underlying these effects are still unknown.

The results of these studies supported the hypothesis that different milk proteins including α, β and κ-casein, WPI and WPC have different effects on GLP-1 secretion. Existing in vitro
studies have produced contradictory results on the effect of whole casein and whey proteins on the regulation of satiety hormones [16, 25]. The results reported here indicate that casein fractions have the most significant effect on the regulating GLP-1 at the gene expression and protein secretion levels, as well as on the intracellular free calcium response of NCI-H716. There has been a considerable amount of contradiction amongst previous studies that, whey is better than casein or vice-versa. A possible reason for the contradictory results in published in vitro data could be the use of different cell lines for the studies. One of the studies used mouse endocrine cell line STC-1 [25], while the other used the NCI-H716 cells [16]. The results of this study are in agreement with the earlier observations that used the same cell line [16], in that the whole casein was found to be more efficient at increasing GLP-1 levels than whey.

The effect of milk proteins on cell viability and proliferation were tested after 24 h of exposure. The results indicate that all milk proteins showed a dose-dependent increase in the toxicity. Among casein fractions, κ-casein showed the most toxicity on % cell viability with concentrations above >1% showing less than 30% viability (Figure 4.2a). β-casein was the most promising with regards to use as a dietary supplement since α- and κ-caseins showed high levels of toxicity to the cells.

Whey proteins showed less toxicity on cell viability in compression to casein fractions (Figure 4.2b&c). Whey protein fractions showed toxicity only at the concentration above 2.5% with no significant difference in the toxicity profiles of the two fractions (WPC, WPI and BSA). In order to explain the toxicity of milk proteins, the protein content of various fractions at 1 to 5% was assessed. These results showed that despite the protein content of all casein fractions being less than the whey protein fractions, they showed higher cell toxicity. This may have been due to the differences in the up-take of different protein caused by differences in the molecular weight of casein versus whey proteins. Different casein proteins
have lower molecular weight (24 kDa) in comparison to WP (~40 kDa). For instance, κ-casein showed the greatest increase in GLP-1 gene expression (3.1 fold change) at 1% w/v. β-casein have showed promising results in the current study, to be used as anti-obesity due to its effect on the regulation of GLP-1 hormone. It was possible to obtain concentration dependant effect of β-casein (up to 3% w/v), since β-casein exhibited significantly lower toxicity in comparison to α and κ. It is noteworthy that β-casein from non-dairy sources has also been shown to exert similar effects when compared with other casein proteins [19, 26].

Of the whey proteins, WPC showed a significant effect on the expression of GLP-1 at 2% (2.3-fold change) as shown in Figure 4.3a, in comparison to WPI. BSA and WPI did not show significant effect at any concentration tested. Glucose (used as positive control), showed a 2-fold increase in gene expression and it is in agreement with the published data [22]. However, WPI also caused an increase in extracellular GLP-1 release, without an increase in intracellular or gene expression of GLP-1. A possible explanation for this could be the difference in composition of WPC and WPI; WPC contains higher concentration of lactoferrin than WPI [6, 29]. Lactoferrin alone has been shown to increase GLP-1 secretion [30]. Lactoferrin in whey proteins may also play a role in satiety regulation. To explore this further, the effect of the various forms of lactoferrin on GLP-1 is explored further in Chapter 5. This also suggests that WPI might not be of significant value as a dietary supplement, since it does not support sustained regulation of GLP-1. It should be noted that protein structures and composition play a major role in the regulation of satiety hormones. Whey protein is considered as a ‘fast protein’ as it is rapidly digested and absorbed and imparts a high blood amino acid profile [2, 5, 10, 13]. On the other hand, casein is a ‘slow protein’ since it delays gastric emptying [2, 11]. Hence, whey proteins cause a spike in GLP-1, that quickly dissipates, while casein allows a prolonged GLP-1 secretion. This is reflected in the results of this study, where the casein fractions showed sustained GLP-1 regulation at the protein and
gene expression levels. Similar results were reported by Remier R, et al. [19] and Le Nevé and Daniel [26], who showed increased GLP-1 expression with small molecular weight proteins such as tetra-glycine, tetra-alanine.

In conclusion, casein protein fraction show promising effects on the regulation of satiety. From the results of this chapter, β-casein showed the greatest effect on the regulation of GLP-1 with high gene and protein expression and less toxic to cell.

4.7. References


Chapter 5: Characterization of milk protein– polyphenols conjugate

5.1. Introduction

Fluid, especially water, the largest part of human diet, is essential for cellular homoeostasis and life [1]. Beverages are suggested to account for up to 25% of the total daily energy intake and as a result are the focus of attention for their potential role in energy intake and body weight homeostasis [2]. Their consumption has also been associated with the rise in obesity and metabolic diseases [3, 4]. Beverages can be classified into four categories based on their impact on body weight:

i. **Alcoholic beverages**: are considered as low satiety with low impact on body weight as they enhance hunger and decrease fullness ratings and satiation when consumed in moderate quantities, especially with meals. The mechanisms for these effects are not yet clear, however research suggest that this may relate to aspects of ethanol metabolism or psychoactive properties of alcohol.

ii. **Clear beverages**: these are considered as low satiety with high impact on body weight [5-7]. With the increasing popularity of certain types of drinks, especially teas and coffees, studies are focusing on their effect on energy balance and body weight. The mechanisms behind their effect on body weight might be due to the fluid vehicle than beverage type or nutrient composition.

iii. **High viscosity** or energy dense drinks are high satiety: these have high impact on body weight and are the beverages formulated to have high nutrient density and satiety value. Their satiety value mainly comes from protein and fiber content. Examples include pre and post workout drinks.

iv. **Soups**: these exhibit high satiety and have low impact on body weight. Also, these products vary highly in their rheological properties and nutrients content. In short term studies, soups
have been shown to provide strong satiation and satiety properties, enhance weight loss and assist in the maintenance of reduced body weight.

According to the US Dietary Guidelines for Americans, milk is considered a high viscosity beverage since it has high impact on satiety and body weight [8]. Chapter 4 dealt with the effects of milk proteins and fractions on glucagon-like peptide-1 (GLP-1). Based on the findings, whey protein concentrate (WPC) has greater effect on the regulation of GLP-1 than whey protein isolate (WPI) (Chapter 4 Section 4.6) and this was possibly due to the higher concentration of Lactoferrin (LF) in WPC as compared to WPI [9, 10]. Lactoferrin can aid in several biological functions such as defence against infections in the gastrointestinal tract, modulation of cell growth, immune function and antibacterial properties [11, 12]. It exerts its anti-inflammatory effect by inhibiting hydroxyl radical formation and glycosaminoglycans [13, 14]

Lactoferrin (LF) is also known as lactotransferrin. It was first discovered and isolated by Sorensen and Sorensen from bovine milk in 1939 as a red protein in whey [15]. LF is found in high concentrations in colostrum and milk [15, 16], and can be found in healthy bovine milk at concentrations of 1.15 μg/ml to 485.63 μg/ml [16, 17]. It is a glycoprotein comprised of a single polypeptide chain containing 703 amino acids folded into two globular lobes with a molecular weight of about 80 kDa. One lobe is called C – (carboxy) and other is N – (amino) regions, they are connected with α-helix [16, 18]. It comprises of 33-34% helices and 17-18% strands and also contains very low quantities of other metal ions like Al³⁺, Ga³⁺, Mn³⁺, Co³⁺, Cu²⁺, Zn²⁺ [16]. Figure 5.1 displays the 3D structure of LF.

It can be classified into three main forms based on the iron content: (1) apolactoferrin (Apo-LF iron free), (2) monoferric form (mono-LF one ferric ion), and (3) hololactoferrin (holo-LF
binds two Fe\textsuperscript{3+} ions) which has the most compact chemical structure \[16, \ 19\]. Due to iron binding properties, LF plays an important role in iron uptake by the intestinal mucosa \[20\].

![3D structure of Bovine LF that shows the N and C – lobe. Adopted from Arutyunova, et al. \[21\]](image)

Tea is a clear beverage that is also known to have a high impact on the body weight. Based on the fermentation process, tea is classified into three main types green (unfermented tea), black (fully fermented tea), and oolong tea (partially fermented); and the total world production of these different types, of tea is 20\%, 60\% and, 80\%, respectively \[22, \ 23\]. Tea has been found to be rich in polyphenolic compounds, accounting for \textasciitilde30\% of its total dry weight \[24, \ 25\], which are responsible for numerous health benefits. These compounds can be classified into different groups based on either the number of phenol rings or structural elements that bind to these phenol rings. The main groups are phenolic acids, flavonoids, stilbenes, and lignans \[26\].

Green tea, in particular, is a rich source of flavonoids also known as catechins. There are many type of those catechins including (+)-catechin (C), (-)-epicatechin (EC), (-) - epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate
(EGCG) [23, 25, 27]. Figure 5.2 provides the basic structure of the different types of catechins. The catechins are colourless, water soluble compounds that are readily oxidisable [28]. Green tea contains more than 50% EGCG which contributes to its health benefits such as antioxidant effect, cancer prevention, improving cardiovascular health, enhancing weight loss and protecting skin from sun damage and skin cancer [29-33].

Milk is considered as an ideal vehicle for the delivery of bioactive compounds such as phenols for targeting modern lifestyle diseases for example diabetes, cardiovascular diseases and cancer [35]. Polyphenols, including EGCG have been shown to have a higher impact on the regulation of satiety hormones in comparison to sugar, amino acids and fatty acids [36]. Hence, the work in this chapter aims to explore the effects of the addition of polyphenols to milk proteins, in this case Lactoferrin.

In this chapter, we are focussing on improving our understanding of protein-phenol interactions between LF and EGCG, and their effect on protein functionality, as it is important to elucidate the effects of polyphenols on the functional properties of the protein.
5.2. Results

Apo-LF-EGCG conjugates were synthesised with increasing concentrations of EGCG (15.625-500 µM) as described in Chapter 3 Section 3.9. Apo-LF was chosen for its higher antioxidant properties than mono-LF and holo-LF [37], hence would provide better potential as a food supplement. Additionally, Apo-LF has least amount of iron content, therefore when investigating the effect of the conjugate there would be least interference from iron. Iron content was determined as shown in Chapter 3 Section 3.9, and the effect of iron on cells will be dealt with in Chapter 6.

Figure 5.3 displays the scanning electron microscopy (SEM) images of free Apo-LF and the conjugates synthesised with the lowest (15.625 µM) and highest (500 µM) concentrations of EGCG. The freeze dried Apo-LF has a smooth surface morphology (Figure 5.3a), compared to the conjugates. The surface of Apo-LF becomes more uneven when the EGCG concentration is increased (Figure 5.3b and c). These results of SEM micrographs for apo-LF match with previous published reports [37, 38]. These changes in structure were further investigated to gain deeper understanding.

![Figure 5.3](image)

**Figure 5.3:** SEM micrographs of (a) freeze dried Apo-LF, (b) Apo-LF with 500 µM of EGCG and (c) Apo-LF with 15.613 µM of EGCG conjugates. The surface of Apo-LF became uneven as the concentration of EGCG was increased. Scale bar = 2 µm.

5.2.1. Macular conformation changes in the conjugates

Fourier transform Infrared (FTIR) spectroscopy was utilised to study change in secondary and tertiary structure due to side chain interaction of protein with EGCG [39, 40]. In addition,
changes in the EGCG structure were also studied. These changes were analysed using OPUS software (version 7.2.139.1294) and resulting graphs with peak annotations are shown in Appendix 1.

EGCG has main peaks around 3550, 3476, and 3355 cm\(^{-1}\), which represent the O–H linkage of phenolic and hydroxyl groups (represented by * in Figure 5.4a). It also contains two peaks around 1691 and 1616 cm\(^{-1}\) (represented by lines in Figure 5.4b) due to the carbonyl stretching of the gallic acid [41, 42] and the region between 900-700 cm\(^{-1}\) (represented by a circle in Figure 5.4b) contains peaks unique to EGCG [40, 42]. In the conjugate, it was found that at lower concentrations of EGCG (up to 62.50 µM), there was a shift in the 3550, 3476, and 3355 cm\(^{-1}\) peaks to lower wavelength. However, these peaks are replaces by a broad feature around 3300 cm\(^{-1}\), when the EGCG concentration increases, The peaks at 1691 and 1616 cm\(^{-1}\) have also shifted to lower wavelengths, accompanied by a disappearance of the characteristic peaks between 900-700 cm\(^{-1}\). These changes are due to intermolecular H-bonds and O–H stretching modes of the EGCG when conjugated with Apo-LF. This indicates that EGCG interacts with the protein through hydrogen bonds at the O-H and carbonyl groups. These results are in agreement with previous findings [40, 42].

With regards to Apo-LF, the area in the spectra between 2936 and 2875 cm\(^{-1}\) (represented by a * in Figure 5.4c) is associated with anti-symmetric and symmetric CH\(_2\) stretching vibrations of the protein. There is a shift to high wavelength in this region in the case of the conjugates. This suggests that hydrophobic interactions are involved [42] in the formation of Apo-LF-EGCG conjugates. Table 5.1 representing the FTIR spectra for EGCG and Apo-LF after conjugation process.
Table 5.1: Changes observed in the Apo-LF and EGCG FTIR spectra after conjugation

<table>
<thead>
<tr>
<th>Peak</th>
<th>Area represented</th>
<th>Appearance in complex</th>
<th>Indication</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Changes in FTIR spectra of EGCG after the conjugation processing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3550</td>
<td>Vibration of O-H linkage of phenolic and hydroxyl groups</td>
<td>Highest concentrations of EGCG replaced by broad peak at 3300.</td>
<td>Apo-LF-EGCG complex interaction by hydrogen bonds.</td>
<td>43, 44</td>
</tr>
<tr>
<td>3476</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3355</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polypehols is bound to proteins by H-bonds.</td>
<td></td>
</tr>
<tr>
<td>1691</td>
<td>Carbonyl stretching of the gallic acid</td>
<td>Shifted to lower wavelengths</td>
<td>Confirms Apo-LF-EGCG complex by non-covalent interaction</td>
<td>42</td>
</tr>
<tr>
<td>1616</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>700-900</td>
<td>Characteristic of EGCG</td>
<td>Disappeared</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Changes in FTIR spectra for Apo-LF protein after the conjugation processing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2936</td>
<td>Antisymmetric and symmetric CH₂ stretching vibrations</td>
<td>shift to high wavelength</td>
<td>Hydrophobic interaction</td>
<td>42</td>
</tr>
<tr>
<td>2875</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1392</td>
<td>C-O stretching bond of carboxyl groups</td>
<td>Disappeared</td>
<td>Indicates involvement of these groups in the LF-EGCG complex formation</td>
<td>42</td>
</tr>
<tr>
<td>1446</td>
<td>Associated with the C-H deformation of the protein in (R-CH₃)</td>
<td>Shifted to lower wavelength in the conjugates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1234</td>
<td>Associated with N-H of the amide III band</td>
<td>shifts to higher wavelength with higher EGCG concentrations</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Apo-LF has two peaks at 1392 cm⁻¹ associated with the C–O bond stretching of carboxyl groups and 1446 cm⁻¹ associated with the C–H deformation of the protein in (R–CH₃) (represented by * in Figure 5.4d) which have shifted to lower wavelength in the conjugates.

It also has a band at 1234 cm⁻¹ which is associated with N–H of the amide III band [40, 42] (line in Figure 5.4d), which is present at lower EGCG concentrations in the conjugates, however shifts to higher wavelength with higher EGCG concentrations. These changes to the secondary structure are further investigated by circular dichroism (CD) spectroscopy, below.
Figure 5.4: FTIR spectra of Apo-LF-EGCG conjugates, where a&b shows the effect of phenols (EGCG) on Apo-LF, and c&d show the effect of Apo-LF on EGCG structure at pH 7.0

5.2.2. Effect of phenol on the secondary structure of Apo-LF

Circular dichroism spectroscopy data provides information on α-helix and β-sheet structure in proteins [45]. Data analysis found in this study showed that when Apo-LF is conjugated with EGCG, there is a decrease in α-helix of Apo-LF with parallel increase in β-sheet (refer to Chapter 3 Section 3.11.2). This change in secondary structure increases with increasing concentrations of EGCG (Except with 250 µM). These results, together with the FTIR data
reveal that there is a physical interaction between the phenols of EGCG and the Apo-LF secondary structure and this is in agreement with previous studies [40, 42, 46].

Table 5.2. Secondary structure of Apo-LF, and Apo--EGCG conjugates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Apo-LF 10µM</th>
<th>Apo-LF-EGCG conjugates (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>500</td>
</tr>
<tr>
<td>α helix</td>
<td>31.7</td>
<td>16.0</td>
</tr>
<tr>
<td>β strand</td>
<td>13.9</td>
<td>38.8</td>
</tr>
</tbody>
</table>

Figure 5.5: Far-UV CD spectra of Apo-LF-EGCG conjugate at 20°C. Conjugates a polyphenol with protein is causing a decreased in α-helix of Apo-LF with parallel increase in stand and turn.

5.2.3. Surface charge

Previous studies showed that the addition of green tea polyphenols to proteins donot affectthe ζ-potential of proteins [47]. However, in this study, we found that the ζ- potential of the Apo-LF was, in fact, affected by the presence of EGCG. Apo-LF is a basic protein with a high isoelectric point (pH 8.5), and the ζ-potential of the Apo-LF was found to be 8.86 mV, where this charge was changed in the presence of EGCG to -5.88 mV. Interestingly, the change in the ζ-potential of the conjugate with increasing EGCG concentration were not significant
(Table 5.3), which would be expected, since FTIR and CD data exhibited a concentration dependent change in the secondary structure of Apo-LF.

Table 5.3: ζ-potential of the conjugate, EGCG and Apo-LF

<table>
<thead>
<tr>
<th>EGCG concentration (µM)</th>
<th>Zeta potential</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>-7.26</td>
<td>0.33</td>
</tr>
<tr>
<td>250</td>
<td>-6.29</td>
<td>0.02</td>
</tr>
<tr>
<td>125</td>
<td>-6.33</td>
<td>0.33</td>
</tr>
<tr>
<td>62.5</td>
<td>-6.03</td>
<td>0.3</td>
</tr>
<tr>
<td>32.25</td>
<td>-6.29</td>
<td>0.43</td>
</tr>
<tr>
<td>15.162</td>
<td>-5.33</td>
<td>0.10</td>
</tr>
<tr>
<td>EGCG alone (500)</td>
<td>-13.23</td>
<td>0.60</td>
</tr>
<tr>
<td>Apo-LF alone 10</td>
<td>8.86</td>
<td>0.50</td>
</tr>
</tbody>
</table>

5.2.4. Binding parameters

In general, heating a protein causes conformational changes in both secondary and tertiary structures of proteins, which may influence Apo-LF conjugate with EGCG. Aromatic residues of tyrosine (Tyr) and tryptophan (Trp) contribute to hydrophobic interactions that stabilize the main structure of protein interiors because they have relatively large polar surface areas [42, 48]. The tryptophan and tyrosine residues are often found fully or partially buried in the hydrophobic region of protein, at the interface between two protein domains or subdomains [42, 49]. Hence, measuring changes to the inherent tyrosine and tryptophan fluorescence emission spectra will provide information for understanding structural changes during the conjugation of Apo-LF with EGCG [42]. It can also provide insight into the binding mechanism and thermodynamic parameters involved in the conjugation process [50].

The fluorescence of the tyrosine residues in Apo-LF was used for this study and the results are shown in Figure 5.6. To optimise the length of time required to solubilise the Apo-LF in the phosphate buffer (PB), fluorescence spectra were collected at various time points from 0-24 h. The fluorescence reached maximum intensity after 2 h and remained constant up to 24 h (Figure 5.6a); hence Apo-LF was dissolved for 2 h before it was used for further experimentation.
Figure 5.6b indicates a decrease in Tyr fluorescence with increasing EGCG concentrations. This implies that there is concentration dependent binding between Apo-LF and EGCG. When conjugation occurs, the Tyr emission peak can undergo a blue shift, indicating a change in structure towards a hydrophobic environment since the Tyr molecule is buried further into the native protein structure. A red shift indicates a change towards a hydrophilic environment, possibly resulting from the unfolding of the protein [51]. In this case, there is an obvious red shift in the Apo-LF spectra at 339 nm as the EGCG concentration increases. This revealed that there was immediate change in the environment of the tyrosine residues and possible change to the secondary structure of Apo-LF in the presence of EGCG [42, 52]. It should be noted that EGCG at the highest concentration used for conjugation did not show any fluorescence with the excitation wavelength (285nm) used in this study. This indicates that the observed spectra is solely from Apo-LF.

Figure 5.6: (a) Time dependent fluorescence emission spectra of Apo-LF to optimise solubilisation of Apo-LF and (b) Concentration dependent effect of EGCG on Apo-LF-EGCG during conjugation. Apo-LF concentration is 10 µM. The fluorescence intensities were decreased with increasing of EGCG concentrations indicating binding of EGCG with Apo-LF.

The above quenching can occur through two different mechanisms, namely static quenching i.e. ground state complex formation or dynamic quenching i.e collisional quenching. This can be determined from temperature dependent fluorescent studies, from which Stern–Volmer
plots are obtained and the Stern–Volmer constants ($K_{sv}$) determined using equations 1 and 2, as described in Chapter 3 Section 3.10.3. Table 5.4 summarizes the results and Figure 5.7a shows the Stern–Volmer plots. Only concentrations that fall within the linear range of the Stern–Volmer regression curve were used. From Table 5.4, it is evident that the $K_{sv}$ value for Apo-LF-EGCG show a decrease with increase in temperature. This indicates that the quenching mechanism is static binding, where the EGCG forms a ground state complex with Apo-LF [50]. The fluorescence lifetime of the Tyr molecule is $10^8$ s ($\tau_0$) and $k_q$ was calculated using the formula $k_{sv}/\tau_0$ [50]. Since the $k_q$ values we obtained are in the order of $10^{13}$ M$^{-1}$ s$^{-1}$ (Table 5.3) which is above $10^{10}$ M$^{-1}$ s$^{-1}$, confirms that the quenching resulted from static more than dynamic quenching.

The double logarithmic regression curves of log $[(F_0-F)/F]$ versus log $[Q]$ were plotted for the Apo-LF-EGCG conjugates (Figure 5.7a & b), the intercept of which gives $K$, the binding constant. Using this binding constant, the thermodynamic forces responsible for the binding of LF protein to EGCG were determined using eq3 in Chapter 3 Section 3.10.3. Where $K$ is the binding constant at the corresponding temperature (T) in Kelvin, $R$ is the gas constant, $\Delta H^\circ$ and $\Delta S^\circ$ correspond to the changes in enthalpy and entropy, respectively [50]. The Gibbs free energy ($\Delta G^\circ$) was estimated from the equation (eq 6) $\Delta G^\circ = \Delta H^\circ / T \Delta S^\circ$

As listed in Table 5.4, Apo-LF-EGCG conjugates exhibit positive $\Delta H^\circ$ and positive $\Delta S^\circ$ values. This is indicative of hydrophobic forces [50]. The negative $\Delta G^\circ$ also indicates spontaneity of the binding of EGCG to Apo-LP during conjugation [50]. The interactions between Apo-LF and EGCG are driven by hydrogen bonding between phenolic hydroxyl and peptide carbonyl, and hydrophobic interactions between hydrophobic amino acid residues and the aromatic rings of the phenols [42, 53-55]. Furthermore, a study using Apo-LF and EGCG estimates that the binding of the polyphenols of EGCG to Apo-LF seems to be more hydrophobic than hydrophilic [42].
Table 5.4: Stern-volmer constants and thermodynamic parameter for Apo-LF-EGCG conjugates

<table>
<thead>
<tr>
<th>TEP °C</th>
<th>Kelvin</th>
<th>$K_{sv}$ (x 104 M⁻¹)</th>
<th>$K_q$</th>
<th>$K$ (x102 M⁻¹)</th>
<th>$\Delta G^\circ$ kJmol⁻¹</th>
<th>$\Delta H^\circ$ kJmol⁻¹</th>
<th>$\Delta S^\circ$ Jmol⁻¹K⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>293</td>
<td>3.180</td>
<td>3.18026E+12</td>
<td>29.141</td>
<td>-21.511</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>303</td>
<td>3.007</td>
<td>3.00745E+12</td>
<td>35.922</td>
<td>-22.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>313</td>
<td>3.032</td>
<td>3.03172E+12</td>
<td>34.635</td>
<td>-23.291</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>323</td>
<td>2.916</td>
<td>2.91569E+12</td>
<td>25.863</td>
<td>-24.181</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>333</td>
<td>2.823</td>
<td>2.823E+12</td>
<td>20.705</td>
<td>-25.071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>343</td>
<td>2.891</td>
<td>2.89113E+12</td>
<td>17.321</td>
<td>-25.961</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.7 (a) shows Stern - Volmer of F0/F vs. EGCG as per eq1, fig (b) shows the double logarithmic regression curves of log [(F0-F)/F] versus log[Q]. The binding constants (K) for the Apo-LF-EGCG conjugates indicate the binding capacity increases as temperature rises. The K values for the complexes showed an increasing followed by decreasing with temperature; these results revealed that the conjugation of phenolic compounds with proteins might change the net charge of protein molecules, which in turn influenced the hydrophilic, hydrophobic and surface properties of the protein.

5.2.5. Measurement of antioxidants activity of the conjugates

There are some arguments in relation to the effect of milk on the bio-efficacy of polyphenols in tea. It has been found that addition of milk proteins to polyphenols decreased their antioxidant capacity [56, 57], while other reports have found no such effect [58-60]. In order to quantify the antioxidant activity of the Apo-LF-EGCG conjugate, the Trolox Equivalent Antioxidant Capacity (TEAC) (Chapter 3, Section 3.11.6) method was used, using the ABTS⁺ decolourisation assay [61]. The antioxidant potential of the conjugates was
quantified in reference to Trolox standards. A linear regression curve was prepared using different concentrations of Trolox from 0 to 3.84 μm from which the percentage of antioxidant activities of EGCG alone and the Apo-LF-EGCG conjugates were calculated in a time dependent manner.

The results (Figure 5.8a) indicate that EGCG shows concentration dependent increase in antioxidant activity. There is no significant difference in activity between 0 h and 4 h, however there is a significant decrease in activity of EGCG after 24 h. At the higher concentrations (250 µM and 500 µM) there is a 20% decrease in activity after 24 h. It is possible that after 24 h there is a significant degradation that affects the antioxidant activity of EGCG. After 24 h dialysis, some free EGCG is either lost or further degraded, leading to a significant decrease in the antioxidant activity.

Figure 5.8b indicates that the Apo-LF-EGCG conjugate has the same antioxidant profile up to 24 h, as free EGCG. This suggests that the antioxidant properties of the conjugates are from EGCG alone, as free LF shows no antioxidant potential. While studies show that LF does (> 1M) have antioxidant properties [11, 18, 62], the concentration of LF used in this study (10 µM) was much lower and hence showed no effect. The results also indicate that the EGCG antioxidant properties are not hampered during the conjugation process. Interestingly, after dialysis (24 WD), the conjugates show the same development as before dialysis (24 ND) and this was significantly better than the antioxidant activity of the free EGCG. This suggests that the EGCG is firmly bound to the LF structure and protected from degradation and the dialysis process, since no EGCG was lost. Similar preservation of green tea polyphenol antioxidant activity by milk proteins has been previously reported [33, 56, 63], however, none of these studies have shown this behaviour with Apo-LF-EGCG conjugates.
Figure 5.8: Time dependent antioxidant activity of (a) EGCG alone and (b) Apo-LFP-EGCG conjugate at time 0 h, 4 h, 24 h with no dialysis (ND) and 48 h with dialysis (WD) using ABTS•⁺ decolourisation assay. Apo-LF-EGCG complex shows better antioxidant activity after 24 hours in comparison to free EGCG. * < 0.05, ** < 0.001 and *** < 0.0001 in comparison to 0 h. +++ < 0.0001 in comparison to the corresponding concentration of free EGCG.

5.2.6. Estimation of phenol content in the conjugates

The aim of this study was to determine the amount of EGCG associated with Apo-LF in the conjugates. This was achieved by centrifuging the conjugates after synthesis, followed by HPLC-MS analysis of the supernatants to quantify the free EGCG not involved in the conjugation process. The results (Table 5.5) is displayed as percentage of EGCG in the supernatants, calculated with respect to the initial concentrations of EGCG used for synthesis. Less than 1% of EGCG remained in the supernatant, indicating ~99% of EGCG is bound to Apo-LF during the formation of the conjugates. This suggests that, as EGCG concentration increases, the amount of EGCG in the conjugates also increases. The antioxidant activity results (Section 5.2.5) support this finding. This further suggests that the phenols were protected by the protein.
### Table 5.5: Percentage of EGCG found in supernatant of Apo-LF-EGCG complex

<table>
<thead>
<tr>
<th>EGCG concentration in complexes (µM)</th>
<th>% EGCG in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>0.96</td>
</tr>
<tr>
<td>250</td>
<td>0.41</td>
</tr>
<tr>
<td>125</td>
<td>0.10</td>
</tr>
<tr>
<td>62.5</td>
<td>0.10</td>
</tr>
<tr>
<td>31.25</td>
<td>0.04</td>
</tr>
<tr>
<td>15.625</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### 5.3. Discussion

Several studies have shown strong interactions between polyphenol in green tea, such as EGCG, EGC and gallic acid (GA) with milk proteins [13, 14, 64]. However, these studies have focused on milk protein-polyphenols interactions and not on the formation of conjugates. This study demonstrated a successful and effective conjugation between milk protein (Apo-LF) and EGCG. It also confirms that Apo-LF has the potential to be used as a platform for delivery of polyphenols. Proteins are widely used in food applications because of their functional attributes and nutritional value [65]. In particular, LF plays a vital role in human health through antimicrobial activities and defence against infection and inflammation in the gastrointestinal tract. Hence, LF is used in food supplements, functional foods and therapeutic drinks. Proteins–polyphenol conjugates, on the other hand, have their own specific functional attributes and formation of Apo-LF-EGCG conjugates leads to the development of a new ingredient with novel functional properties.

Protein-polyphenol interactions are based on two main mechanisms, either through the hydrogen bonding between hydroxyl group in the phenol and peptide carbonyl of the protein, or hydrophobic interactions between hydrophobic amino acid residues and the aromatic rings of the phenols [66-68]. This study revealed that the Apo-LF-EGCG interactions occurred through both the above mentioned mechanisms and resulted in associated changes to the secondary structure of the protein.
The interaction occurs through static binding with hydrophobic interactions. Furthermore, the hydrophobicity of the milk protein was decreased in the presence of EGCG and this is consistent with earlier reports [69, 70]. Approximately 99% of the EGCG interacts with the protein, and the antioxidant properties of the EGCG are protected by the protein. The EGCG, in turn, brings the protein into a more hydrophilic environment; hence these Apo-LF-EGCG conjugates can be applied in beverages and liquid foods. It is possible that the conjugate may protect degradation of LF by pepsin in the stomach, thereby allowing the delivery to the intestine in its original form [71]. The protein conformational analysis data shown in Figure 5.5 indicates that the addition of EGCG causes changes in the secondary structure of Apo-LF protein similar to a previous report [72]. This suggests that there is successful interaction between Apo-LF and EGCG, with possible changes to the secondary structure of the Apo-LF molecule.

Simultaneously, EGCG is protected from degradation and the antioxidant activity is maintained for a longer duration. Since this conjugate shows potential as a dietary supplement, the effect of this conjugate on intestinal cells and section of satiety hormone, glucagon-like peptide-1 (GLP-1) is investigated in Chapter 6.

5.4. References


71. and, B.L. and S. lyer, Lactoferrin: Molecular strcutre and bilogical function 1995.

Chapter 6: Effect of lactoferrin, polyphenols and Apo-LF-EGCG conjugates on GLP-1 expression

6.1. Introduction

Previous studies and reviews have shown that non-nutrients have ability to induce the secretion of satiety hormones. Neve, et al. (2010), found that the steroid glycoside H.g.-12 from Hoodia gordonii plant induces CCK secretion from HuTu-80 cells, and plant-derived components are associated with loss of body weight [1]. Capsaicin, the main pungent compound in red peppers, has also been found to increase the secretion of GLP-1 in human plasma [2].

Milk is an ideal vehicle for the delivery of bioactive compounds such as polyphenols and can be exploited for targeting modern lifestyle diseases such as cancer and cardiovascular disease [3]. Hence, in chapter 5, the ability of milk protein lactoferrin (LF) conjugated with epigallocatechin gallate (EGCG) from green tea was investigated.

As showed in chapter 3, milk proteins show dose-dependent effect on regulation of glucagon-like peptide-1 (GLP-1) to varying degrees. In addition, a human study performed by Hursel and Westerterp-Plantenga [4] showed that protein combined with green tea can help with weight maintenance in moderately obese subjects [4]. These effects were linked with thermogenesis, fat oxidation, and increases in energy expenditure. Furthermore, study done by Stojadinovic, et al. [5] suggested healthy protein-phenol formulation provides beneficial effects on human health in prevention of cardiovascular disease and cancer. They also considered proteins as good carriers to carry phenols in the gastrointestinal track [5]. However, there is no study that shows the effect of a LF-EGCG conjugates on the regulation of satiety hormones and if it can be used as an anti–obesity formulation. Therefore, this study
was planned to investigate the ability of LF-EGCG conjugate on the release of GLP-1 hormone, in comparison to the individual components. It is important to determine whether these ingredients (tea and milk proteins) will work synergistically and are non-toxic to the intestinal cells.

In this chapter, there is an investigation of the possible effect of individual components of lactoferrin protein (apolactoferrin (Apo-LF), hololactoferrin, monoferric lactoferrin and Epigallocatechin gallate (EGCG) phenol individually and when Apo-LF combined with EGCG forming a conjugation with EGCG concentrations ranged between 15.63 to 500 µM and a constant concentration of Apo-LF of 10 µM..

**6.2. Effect of LF protein, EGCG and Apo-Lf-EGCG conjugates on cells viability and proliferation**

The main focus of this study was to access the effects of LF proteins including apolactoferrin holo-LF, monoferric and hololactoferrin, EGCG, iron chloride hexahydrate and the Apo-LF-EGCG conjugates on the proliferation of human colon cells (NCI-H716) using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay over a period of 24 h. LF protein concentrations used ranged between 3.13 to 50 mg/mL (1 to 5 % (w/v)), to replicate the concentrations found in milk. Various studies have observed that polyphenols inhibit tumorigenesis and proliferation in different cell lines [6-8]. The concentration of EGCG were determined based on a similar study conducted by Won Young Song, et al. [9] and ranged from 0.63 to 10 mg/mL (0-200 µM). EGCG showed toxic effects on the cells for all concentrations above 5 mg/mL (Figure 6.1a). Iron plays an essential role in growth and developments of organisms. However, iron also can be toxic for organisms if it is in excess [10].
Figure 6.1: Cell viability of NCI-H716 cell after 24 h of treatment with (a) EGCG, (b) iron chloride hexahydrate (FeCl₃·6H₂O) (c) lactoferrin (LF) include apolactoferrin (Apo-LF), monoferric (mono-LF) and hololactoferrin (holo-LF) and (d) Apo-LF-EGCG conjugate. LF samples at concentration below 50 mg/mL. Whereas, 200 µM of EGCG showed toxicity to cells, Apo-LF-EGCG conjugates showed toxicity effect on cell growth at concentration > 0.25 mg/mL of each conjugate. Results are the average of three independent experiments and bars represent the standard deviation. *p < 0.05, **p < 0.001 and ***p < 0.0001 when compared with the corresponding untreated cells.

With regards to iron toxicity, Suhr-Jessen and Rasmussen [11] have shown that Fe concentrations > 4 µM can be toxic to Tetrahymena thermophila, a eukaryotic cell that has a similar metabolism to that of mammalian cells. Therefore, iron content of lactoferrin samples
was determined using MP-AES and the concentration of iron were found to be ≤ 0.56 µM, 1.97 µM and 20.23 µM in Apo-LF and holo-LF (20.23 µM), respectively. Hence, to determine the effect of the iron in the LF samples, the effect of FeCl₃ was tested in a range of 0.01 to 0.001 mg/mL concentration, and found to be toxic at ≥0.003 mg/mL (Figure 6.1b). There are no published reports that have considered toxicity of these two components on NCI-H716 cell line.

The results (Figure 6.1c) indicate that LF proteins shows dose dependent toxicity to NCI-H716 cell. Cell proliferation was not drastically affected (~70% viability) with any of the LF samples up to 25 mg/mL, above 50 mg/mL cell viability was dropped. Apo-LF is proven to act as a growth factor [12], hence no toxicity was expected.

With regards to the Apo-LF-EGCG conjugate, toxic effect on cell growth after 24 h was determined and results shown in Figure 6.1d. Surprisingly, the viability of the cells decreased as the amount of EGCG in conjugate decreased. One possible explanation for this effect is that EGCG molecule is much smaller than LF; therefore, EGCG might be inserted into the LF molecule at high concentration and surround the LF molecule surface at low concentration as shown in Figure 6.2.[13]. At higher concentrations ≥250 µM of EGCG, there was less effect on proliferation since the EGCG was contained within the LF structure.

Figure 6.2. Schematic diagram showing predicted Apo-LF-EGCG conjugate and its interaction created by Swissdock (a web service)
6.3. Effect of LF proteins, EGCG and Apo-LF-EGCG conjugate on regulation of GLP-1 gene expression

Figure 6.3 shows the effect of sub-toxic concentrations of Apo-LF, mono-LF, holo-LF proteins and EGCG individually and of Apo-LF-EGCG conjugate, on the regulation of satiety hormone after 18 h (refer to Chapter 4.3). Glucose was used as a positive control (+ve) [9]. When comparing Apo-LF, holo-LF and mono-LF, 10 mg/mL of holo-LF showed the greatest increase in GLP-1 gene expression (2.1 fold) compared to the same concentration of Apo-LF and mono-LF (Figure 6.3 a). Apo-LF also significantly enhanced GLP-1 hormone expression (1.68 fold) at 30 mg/mL, whereas holo-LF showed higher fold change at 10 mg/mL (2.1 fold). There is an apparent decrease in GLP-1 expression at 30 mg/mL holo-LF, however it showed toxicity at this concentration (Figure 6.3 c). Similar effect of LF on the regulation of GLP-1 levels have been reported in an animal study by Yuta Maekawa, et al. [14]. However, their study did not include different forms of LF (level of iron saturation).

Figure 6.3: Effects of (a) lactoferrin, (b) EGCG and Apo-LF-EGCG conjugate on GLP-1 expression after 18 h of treatment. Cells were treated with test reagent, which show less toxicity effect on cells. Values are means ± SD of three replicate experiments. *P>0.05
Apo-LF-EGCG conjugate with 125 µM of EGCG showed the highest effect on the expression of GLP-1 i.e. 2.9 fold increases as compared to 1.8 and 1.35 fold increases at 250 and 500 µM of EGCG in conjugate, respectively (Figure 6.3b).

Apo-LF-EGCG conjugate showed increase in cell death as EGCG concentration was decreased. This result can be explained by the different molecule sizes between Apo-LF and EGCG as referred in section 6.2 [13]. EGCG (5 mg/mL) significantly induced GLP-1 expression (1.9 fold). These results are in agreement with a previous study done by Won Young Song, et al. [9] who reported that EGCG polyphenol conjugate can stimulate secretion of satiety hormones in Caco-2 cells.

Interestingly, iron, at a concentration of 0.0013 mg/mL also showed 1.7 fold changes in GLP-1 gene expression. This effect has not been previously reported. This suggests that the iron in LF could contribute to the increased expression of satiety hormones.

6.4. Study the effect of LF proteins, EGCG and Apo-LF-EGCG conjugate on GLP-1 release

Lactoferrin, EGCG and Apo-LF-EGCG concentrations were selected based on their ability to significantly up-regulate GLP-1 gene expression as shown in Figure 6.4. Effect of LF, including Apo-LF (30 mg/mL), holo-LF (10 mg/mL), EGCG (5 mg/mL) and Apo-LF-EGCG conjugate containing 125 µM EGCG were used to determine GLP-1 hormone levels using an ELISA assay, due to its effect on increases of GLP-1 gene expression as compared to 250 and 500 µM EGCG. Cells (2×10⁶) were exposed to test reagents and incubated at 37°C for 2 h. Secretion of GLP-1 into cells culture medium and on cell lysis were expressed as pg/mL. Milk protein, phenol and protein-phenol conjugate enhanced GLP-1 release in both supernatants following cell lysis (Figure 6.4).
Figures 6.4a-b shows the effect lactoferrin, EGCG and Apo-LF-EGCG conjugate on (a) intracellular GLP-1 and (b) GLP-1 released in medium after 2 h treatment. Cells were exposed to Apo-LF (30 mg/mL), holo-LF (10 mg/mL), EGCG (5 mg/mL) and 125 µM of Apo-LF-EGCG conjugate 0.0125 mg/mL. Values are means ± SEMs of four independent experiments. **p < 0.001 and ***p< 0.0001 when compared to corresponding to untreated cells.

When GLP-1 concentration in cell lysate was determined after treatment with EGCG, Apo-LF and holo-LF, there was significantly increase in comparison to the untreated (UNT) (Figure 6.5a), with Apo-LF showing marginally higher increase (3.61 times) than holo-LF (3 times, Figure 6.4b). The positive control showed 2.65 times higher increase than untreated cells. EGCG also showed 18.9 times higher increase compared to untreated cells. With regards to the conjugate, there was a significant (~27 times) increase in GLP-1 levels compared with the cell lysates from untreated cells. When compared with free Apo-LF and EGCG, the conjugate showed 1.25 times and 1.3 times greater increases, respectively.

Apart from intracellular protein content, the extracellular release of GLP-1 into the surrounding cell media was also assessed. This is an important parameter to consider when observing hormone regulation, since evidence suggests that a cell can store certain quantity of protein in its cytoplasms. This is released in a ‘burst’ when receptors are activated. This is followed by increase in gene expression to replenish the cytoplasmic stores of the hormone [15]. Most studies only consider the extracellular release of satiety hormones, which only
provides information on the “burst” release, and does not consider gene and intracellular, sustained up-regulation of these hormones. Therefore, these studies provide information on short-term satiety regulation and do not consider long-term, sustained increase in satiety regulation.

The results from the extracellular release of GLP-1 (Figure 6.4b) showed that Apo-LF, holo-LF and EGCG increase GLP-1 release in comparison to untreated cells and is similar in trend to that observed for intracellular GLP-1 content. While the release of GLP-1 by the Apo-LF-EGCG conjugate was greater than untreated cells, there was no significant difference when compared to Apo-LF.

6.5. Discussion

As discussed in Chapter 4 Section 4.2, different milk protein affect differently on cell viability and proliferation. The effect of lactoferrin protein samples, EGCG, and Apo-LF-EGCG on cells viability and proliferation were tested after 24 h of exposure. The results indicate that all lactoferrin proteins, EGCG and iron chloride hexahydrate showed a dose-dependent increase in toxicity. However, in case of Apo-LF-EGCG conjugates, cells viability was decreased when EGCG concentration was decreased as well. As discussed in Chapter 5, the changes in the secondary structure of the protein increase with increasing EGCG concentrations. The protein conformational analysis based on CD data showed that when EGCG is conjugated with Apo-LF causes a decrease in α-helix of Apo-LF protein with increase in β sheet (Table 5.2). In addition, Figure 5.6.b also shows a decrease in tyrosine (Tyr) fluorescence with increasing EGCG concentrations which means there is concentration dependent binding between Apo-LF and EGCG. This could indicate that at lower concentrations, EGCG sits on the surface of the LF structure, hence not in the vicinity of many tyrosine molecules. However as the concentration increases, the EGCG gets imbedded
further into the protein, thereby coming in contact and interacting with more tyrosine molecules, leading to a decrease in the tyrosine emission spectra. At the same time at high concentration of EGCG for conjugation there was no fluorescence with the excitation wavelength (285 nm) used. This indicates that the spectra observed are solely from Apo-LF. Therefore, when lower concentrations (≤125 µM) of EGCG were used in the conjugate, there was a significant cell death.

From these studies, we found that lactoferrin in its all three forms (Apo-LF, mono-LF and holo-LF) has a potential impact on the regulation and release of GLP-1 hormone. Additionally, we demonstrated that protein-polyphenol conjugate has greater effect on gene and protein expression of GLP-1 in comparison to the LF and EGCG, individually. In addition, as it has been discussed in Chapter 4, each milk protein has different effect on gene and protein expression of GLP-1, whereas β-casein shows the greatest impact. Similarly, the effect of the different types of LF was assessed. Interestingly, holo-LF (20 mg/mL) showed significant increase in the release of GLP-1 in intracellular protein levels (Figure 6.4.a) and gene expression with less effect in extracellular protein concentration levels (Figure 6.4b) in comparison with Apo-LF (30 mg/mL). As discussed in Section 4.3 with regards to the ‘burst’ release mechanisms for GLP1, Apo-LF showed (Figure 6.4.a) higher intracellular gene expression but a lower increase in extracellular levels of GLP-1.

The polyphenol EGCG showed effects on the intracellular as well as extracellular protein levels (Figure 6.4a&b). As data suggests that the release of GLP-1 from the cells is largely governed by the Apo-LF content of Apo-LF-EGCG conjugate. However, the conjugate had a greater effect on interacellular GLP-1 production, as well as on gene expression. This would suggest that the conjugate not only causes a burst release of GLP-1 hormone, but also has a higher potential to induce sustained satiety, than Apo-LF and EGCG. These results are in
agreement with Won Young Song, et al. [9] and G. Aktas, et al. [16], who reported EGCG induces GLP-1 and Cholecystokinin (CKK) hormones release in another cell line.

Previous studies suggested iron deficiency and obesity are molecularly linked and equally affect each other [17, 18]. Obesity may increase the chances of iron deficiency by reducing absorbance of iron from the duodenum [18]. Won Young Song, et al. [9] and G. Aktas, et al. [16], reported a relationship between iron deficiency and waist circumference, body weight and body mass index (BMI), where people with high body weight and BMI have low iron levels. Additionally, the body weights of twenty-one women were significantly reduced when treated with oral iron supplement [16, 18]. Hence the use of iron supplements can aid in body weight control. Since lactoferrin protein contains iron within its structure, it has been shown to aid in iron uptake from the gastrointestinal tract. Therefore, protein-phenol conjugate could be a new approach to treat and prevent obesity by its effect on the regulation of satiety hormones. Thus, this LF-EGCG conjugate can potentially be used to not only enhance satiety and prevent body weight gain, but also as a method of simultaneously treating iron deficiency resulting from obesity.

6.6. References:


Chapter 7: Summary and future direction

7.1. Summary

Obesity is considered as an epidemic disease and it has increased dramatically in last few years according to World Health Organization (WHO). Increasing body weight leads to increase risk of associated disorder diseases such as type 2 diabetes, cardiovascular disease and the metabolic syndrome [1-4]. The first strategy to prevent obesity and control body weight is to increase energy expenditure and decrease energy intake with a sufficient physical exercise. Also, current dietary recommendations and strategies used to treat and prevent obesity, mainly focus on the energy density and fat content in the diet [5].

Proteins especially proteins from milk including casein and whey proteins have been showed more effect on the satiety and satiation more than fat and carbohydrate by increasing the release of satiety hormones such as glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK) [6-11]. In addition, increasing the consumption of dairy proteins have suggested to be a new way to prevent and treat obesity, due to their effect on regulation of satiety hormone such as glucagon-like peptide-1(GLP-1) and cholecystokinin (CCK) [7, 12, 13]. Nevertheless, there are a limited number of studies that has been done in demonstrating the utility of GLP-1 as anti-obesity agent, and the importance of enhancing GLP-1 levels for obesity treatment [14]. In addition to that, researchers suggest that increasing GLP-1 could be one of the therapeutic approaches for type 2 diabetes, since GLP-1 levels are lower in people with Type 2 diabetes [14, 15], and improves insulin release which leads to lower glycerine [14].
In milk, casein and whey are the main protein found in dairy products which accounts for around 3% of total milk, and contains higher concentrations of essential and non-essential amino acids than other protein sources, hence has a high nutritional value [16, 17]. In previous studies, milk proteins have been shown to suppress short-term food intake, enhance satiety through the stimulation of satiety hormones, therefore lowers body weight and decrease risk of developing hyperglycemia and diabetes [4, 12, 18-21]. However, inconsistent data exists from different studies to specify which dairy protein or fraction has more effect on satiety [9, 18, 19, 21-25].

The main aim of this thesis was to understand which milk protein and its fraction have most impact on appetite and food intake and their effect on the regulation of GLP-1 hormone. We found that each of the milk protein and its fraction has different effect on the regulation of GLP-1 hormone which was confirmed by gene expression through the quantitative real-time polymerase chain reaction (qRT-PCR) and protein expression using the enzyme-linked immunosorbent assay (ELISA) in vitro studies using human adenocarcinoma of the colon cell line (NCI-H716).

The results of qRT-PCR and ELISA studies indicate that different milk proteins, viz. casein fractions (α,β and κ), whey protein concentrate (WPC), whey protein isolate (WPI), lactoferrin (LF) protein including apolactoferrin (Apo-LF), monoferric (mono-LF) and hololactoferrin (holo-LF) have different impact on the GLP-1 gene and protein expression on human colorectal (NCI-H716) cells. β-casein protein had the greatest effect on the regulation of GLP-1 hormone compared with other protein, whereas, no effect was noticed with mono-LF and bovine serum albumin (BSA). However, other milk protein fractions including α and κ casein, WPC, Apo-LF and holo-LF also show increase in
regulation of GLP-1 in both gene and protein expression, but not as much as β-casein. WPI, on the other hand, caused an increase in extracellular GLP-1 release, but no increase in intracellular or gene expression. Thus, these results suggest that each milk protein and fraction would affect differently in short and long term GLP-1 regulation. Hence, would have different application in body weight control.

Milk has been considered as one of ideal vehicle to deliver bioactive compounds such as polyphenols [26]. Therefore, protein-phenol interactions become a new area to investigate and improve our knowledge in different fields such as medicine, toxicology, chemistry and food science. This project focused on the possible use of milk protein-phenol interaction using Apo-LF and epigallocatechin gallate (EGCG) at different concentrations of EGCG with constant Apo-LF concentration to regulate appetite by stimulating the secretion of GLP-1. The results (as showed in Chapter 5 section 5.2.1) of Apo-LF-EGCG is based on two main chemical reactions (1) hydrogen bonding between hydroxyl group of the phenol and the carbonyl group of the peptide and (2) hydrophobic interactions between the amino acid residues and the aromatic rings of the phenols [27-29]. It was also found that addition of milk proteins to polyphenols prevented degradation of the phenol and the antioxidant effect of the phenol was maintained. This suggests that the EGCG was protected by the Apo-LF protein up to 48h as it showed higher antioxidant activity in the conjugate than free EGCG.

The toxicity studies of the conjugate revealed a positive relationship between increasing EGCG concentration and cell viability. The viability of the cells decreased as the amount of EGCG in the conjugate decreased. The speculation on the factors that may contribute to this trend in toxicity with the conjugate include (i) EGCG molecule is much smaller
than Apo-LF, therefore EGCG might be inserted into the Apo-LF molecule at high concentration and surround the Apo-LF molecule surface at low concentration, (ii) the changes in the secondary structure of the protein increase with increasing EGCG concentrations. This could change the conformation of the protein, such that the EGCG at higher concentrations are moved towards the center of the protein structure.

Also, the hypothesis that protein-phenol conjugates have the ability to regulate GLP-1 genes and protein expression was tested by studying the effect of the Apo-LF-EGCG conjugates on GLP-1. Apo-LF-EGCG conjugates show significant effect on enhancing the expression of GLP-1 at gene and protein level, more than the individual components at equivalent concentrations.

This research helps to increase our knowledge of the role of milk protein and phenol as anti-obesity approach. It will bring an understanding to the importance of milk and its proteins in human daily diet and health, which may lead to a decrease in chronic disease rates, and increase in a healthier population. In vitro studies of milk proteins and other sources of proteins will provide the foundation for meaningful intervention studies. Furthermore, the results of this research may lead to increased consumption of milk protein by healthy individuals of various age groups in general, and by overweight and obese individuals in particular. This research adds to the accumulating evidence that milk has potential usage as anti-obesity and provides encouragement to combined milk with other beverages especially tea.
7.2. Future direction

Due to an increasing in obesity rate in the last years, there is more motivation for developing the anti-obesity beverages, which have an impact primarily on satiety hormone such as GLP-1 and thereby reduce energy intake. This research provided biological and food technology explanations for the role of milk protein and its fractions on the regulation of satiety hormone, which might work as anti-obesity approach. Thus, the results add some biological information and inverse associations found between milk proteins and satiety hormone. However, in depth research is required in many areas, some of which have been listed below:

- This project focused on GLP-1 hormone and studies focusing on other satiety regulating hormones are required to fully understand the anti-obesity potential of the conjugate used in this project. *In vivo* effects should also be considered, since this project was limited to *in vitro* investigations.

- As discussed in Chapter 6, the conjugation of lactoferrin with the polyphenols (EGCG) from green tea showed better effect on the regulation of GLP-1 than individual components. Further is investigation needed to study the conjugation of different milk proteins with different polyphenols and investigate their *in vitro* and *in vivo* regulation of satiety hormones.

- The concentrations of macronutrients in whole milk differ from those in low-fat and reduced-fat milks. Therefore, studies should consider and investigate the effects of different types of milk on regulation of satiety hormones.
• Protein will digest in stomach by the acidic gastric juices; *in vitro* assays that stimulate the gastric juices should be performed. This will help to understand if the hydrolyzed forms of those proteins can also regulate the satiety hormones.

• A follow-up study assessing the effect of milk protein on insulin production should be a priority in future research as previous studies show the relationship between the satiety hormone especially GLP-1 and insulin production in human body [30]. More investigations needed on lactoferrin conjugated with polyphenols and using it as iron supplement to increase iron absorption and antioxidant effect.

• Polyphenols, such as EGCG show anticancer properties [31]. However, they can degrade quickly in the digestive system [32]. Hence, it might be worthwhile to study the effect of milk protein-phenols conjugates for anti-cancer properties, since the results from this project suggests that protein can protect the polyphenol from degradation (Chapter 5, Section 5.1.5).

### 7.3. References

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Appendix 1