Effect of α-Lactalbumin and β-Lactoglobulin Hydrolysates on Markers of Metabolic Syndrome

By

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements of the Degree of

MASTER OF SCIENCE

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I hereby declare that I am the sole author of this thesis.

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Acknowledgements

I would like to thank my primary advisor Dr. Susan Arntfield as her expertise, advice, enthusiasm, and optimism were essential to the completion of this project. Many thanks to my co-advisor Dr. Curtis Rempel, for providing the knowledge and background required to understand the aspects of inflammation and metabolism. Without their encouragement and support this thesis would not have been possible and hardly completed.

I would also like to thank my other committee members, Dr. Rotimi Aluko and Dr. Martin Scanlon, for their suggestions and guidance.

I am indebted to the support staff of the department of Food Science, Alison Ser, Michael Stringer, Pat Kenyon, and Yang Qui for sharing their experiences and knowledge. I would also like to thank Quyen Van and Meili Hao, from the Richardson Center for Functional Foods and Nutraceuticals, for their assistance with the murine macrophage cell culture work. I am deeply grateful to Dr. Helen Roche, Deirdre Keane, Niamh Healy, and the other students at University College Dublin for sharing their knowledge and guidance with the adipocyte cell culture work. I am grateful for Sally Diep and Saraphine Liu as their assistance made this project achievable. Many thanks to Davisco Foods International Inc. for providing the whey protein isolate.

I would like to acknowledge the Canadian Dairy Commission and the Agri-Food Research and Development Initiative for their financial support.

Lastly I would like to thank my family, friends, and colleagues for their encouragement and continued support.

Abstract

The effects of peptides derived from β-lactoglobulin and α-lactalbumin on metabolic syndrome were studied. α-lactalbumin and β-lactoglobulin were hydrolyzed with trypsin, alcalase, flavourzyme, or a combination of alcalase and flavourzyme and fractionated. Angiotensin coverting enzyme inhibition of the < 1 kDa fraction of alcalase hydrolyzed β-lactoglobulin was 95 %. Antioxidant activity of the < 1 kDa fraction of β-lactoglobulin hydrolyzed with a combination of alcalase and flavourzyme was 18 %. Stimulated adipocytes incubated with the < 1 kDa fraction of β-lactoglobulin hydrolyzed with either trypsin or alcalase produced 30 pg/mL of interleukin 6. Adiponectin and glucose transporter type 4 secretions increased 1.1 and 0.86 fold respectively during incubation with the < 1 kDa fraction of β-lactoglobulin hydrolyzed with a combination of alcalase and flavourzyme. Results indicate that β-lactoglobulin peptides formed with alcalase and a combination of alcalase and flavourzyme influence markers associated with metabolic syndrome and may be useful as functional foods or nutraceuticals.

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List of Abbreviations

Ala	Alanine
α-La	α-Lactalbumin
ACE	Angiotensin converting enzyme
AMPK	AMP-activated protein kinase
Arg	Arginine
Asp	Aspartic acid
ATCC	American Type Culture Collection
BHT	Butylated hydroxytoluene
β-Lg	β-Lactoglobulin
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CVD	Cardiovascular disease
Cys	Cysteine
DMEM	Dubellco's modified eagle medium
DNA	Deoxynucleic acid
DPPH	(2,2-diphenyl-1-picylhydrazyl)-free radical scavenging activity
ELISA	Enzyme linked immunosorbent assay
FAPGG	N-[3-(2-Furyl)acryoyl]-Phe-Gly-Gly
FBS	Fetal bovine serum
Gln	Glutamine
Glu	Glutamic acid
GLUT	Glucose transporters
GLUT 4	Glucose transporter type 4
Gly	Glycine

HHL	Hippuryl-L-histidyl-L-leucine
HPLC	High performance liquid chromatography
IL1β	Interleukin-1β
IL6	Interleukin 6
iNOS	Inductible nitric oxide synthase
JNK	c-Jun amino-terminal kinase
Leu	Leucine
LPS	Lipopolysaccharide
Lys	Lysine
Met	Methionine
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-κB
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PG	Propylgallate
Phe	Phenylalanine
PKB	Phosphorylating phosphate kinase B
PI 3-K	Phosphoinositide 3-kinase
PPARγ	Peroxisome proliferator-activated receptor gamma
RCF	Relative centrifugal force
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction

Ser	Serine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N’,N’-tetramethylethyledienediamine
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
Trp	Tryptophan
Tyr	Tyrosine
T2DM	Type 2 diabetes mellitus
UV-Vis	Ultraviolet-visible
Val	Valine

1.0 Introduction

Milk is considered to be a complete food product for newborn mammals, capable of maintaining and supporting an infant's immune system while providing the nutrients required for development (Gutierrez-Lopez *et al*, 2007). Bovine milk is composed primarily of water, which contains 13% total solids. These solids are made up of about 27% protein, 20% of which are whey proteins and 80% which are caseins (Driskell, 2007).

Cheese production creates one of the largest industrial effluents in the world, whey. Worldwide production of this byproduct (sweet, acid, and casein whey) is approximately 82 million metric tons per year. This highly polluting material has a biological oxygen demand of 30,000 to 50,000 mg/L. Whey has a high nutritional value and various functional properties, therefore having large potential for economic gain (Oreopoulou & Russ, 2007; Gutierrez-Lopez *et al,* 2007).

In the early 1980's, ultrafiltration became a popular topic in the world of food engineering. This gave new life for the waste product, whey. Ultrafiltration allowed efficient recovery of whey proteins, which reside in the supernatant from cheese making during coagulation of casein at a pH of 3.6. Whey protein concentrates were then made available as food ingredients showing many functional and health promoting effects (Gutierrez-Lopez *et al,* 2007; Hill, 2009). Recently whey protein isolates have become available. Isolates contain between 90-95% protein and insignificant amounts of lactose making them great alternatives to concentrates for those individuals who experience lactose intolerance to dairy products. A small but growing number of hydrolyzed whey protein isolates have also been emerging. It is thought that hydrolyzing these proteins to create small peptides can increase the speed and concentration of absorption through the small intestine (Driskell, 2007).

Metabolic syndrome is a cluster of disorders, namely hypertension, dyslipidemia, hyperglycaemia, and central adiposity (Hansen & Bray, 2008). Metabolic syndrome occurs when disorder occurs between adipose tissues and insulin target tissues such as β-cells and skeletal muscle. Underlying roles of systemic inflammation, plasma free fatty acids, adiponectin, and endothelial function all influence the extent of this disease. Occurence has been rising, affecting approximately 12 million adults over 40 years of age in the United States (Hansen & Bray, 2008).

Dairy products have traditionally been thought to contribute to a number of diseases, including metabolic syndrome. Recently, whey proteins, the byproduct of cheese production, have shown to have a number of positive health effects in individuals (Thompson *et al*, 2009). Scientific studies have been completed which indicate that whey proteins are capable of decreasing fat mass and maintaining lean mass while promoting satiety and thermogenesis. β-Lactoglobulin (β-Lg) and α-Lactalbumin (α-La), the major whey proteins, have shown various bioactivities including opioid, antimicrobial, immunomodulatory, mineral-transport, growth promoting, anticancer, proteinase, and angiotensin converting enzyme (ACE) inhibition (Thompson *et al*, 2009).

In this study, β-Lg and α-La were each hydrolyzed with a number of endopeptidases and exopeptidases followed by fractionation. The bioactivity of the unfractionated and fractionated hydrolysates were then determined using chemical and *in vitro* assays. The objectives of this study were to determine the effect of the hydrolysates derived from β-Lg and α-La on markers of inflammation, blood glucose and blood pressure regulation.

2.0 Literature Review

2.1 Whey Proteins

Whey proteins contain 20 different amino acids including those which are essential. They contain the highest naturally occurring portion of the branched chain amino acids, leucine (Leu), valine (Val), and isoleucine (Ile), which provide energy to muscle tissue and help regulate protein synthesis.

Whey proteins contain a high level of sulfur amino acids which appear to enhance immune function. They increase glutathionone levels in the body by utilizing the amino acid cysteine (Cys). Glutathione (γ-L-Glutamyl-L-cysteinylglycine) is a sulfur containing tripeptide, which acts as an antioxidant within the body by neutralizing the reactive oxygen species (ROS), and thereby provides some degree of protection against cancer. It can also interact with the cell membranes of microorganisms which ultimately cause their death (Driskell, 2007; Gutierrez-Lopez *et al,* 2007).

Whey proteins show various biological activities within humans, some of which are due to the protein itself and not the amino acids from which they are made (Driskell, 2007). Bioactive substances are food components with some kind of regulatory function in humans that goes beyond basic nutrition (Gutierrez-Lopez *et al*, 2007). For example, whey proteins appear to aid in nitrogen retention, increase glutathione production, and possibly prevent osteoporosis from developing in the elderly (Driskell, 2007). Whey proteins contain many bioactive peptides which remain latent until they are released by enzymatic reactions within the gastrointestinal tract. These peptides can have different effects on the body such as opioid,

antimicrobial, immunomodulatory, mineral-transporting, growth promoting, anticancer, proteinase, and angiotensin converting enzyme inhibition effects (Thompson *et al*, 2009).

Whey proteins are more water soluble than casein (Gutierrez-Lopez *et al*, 2007). They show high solubility properties across the entire pH range (acid, neutral, and alkaline). They remain soluble at the acid pH of the stomach whereas caseins coagulate. Their solubility accelerates their transit though the gastrointestinal tract allowing them to reach the small intestine practically completely intact. This makes absorption very slow but allows for interaction between the proteins and the microflora to occur, therefore causing microorganism inhibition. They also interact with minerals in the bollus thus improving their absorption (Gutierrez-Lopez *et al*, 2007).

Whey proteins, particularly β-Lg, exhibit excellent foaming, emulsifying and gel formation properties (Gutierrez-Lopez *et al*, 2007). For β-Lg to retain these desirable functional properties, the protein must remain in its native conformation following processing. Subjecting this protein to a high temperature causes denaturation, which increases the amount of water that it can hold. Heat denaturation of whey proteins does not however cause any change to the nutritional value. On the other hand, functional properties that these proteins exhibit are generally lost due to conformational changes that they undergo during the process of heating (Gutierrez-Lopez *et al*, 2007).

Whey proteins are capable of promoting satiety and provide a source of calories to people (Thompson *et al,* 2009). Amino acids provide less efficient energy than other nutrients such as glucose and fatty acids. This increases thermogenesis within the body, therefore leaving less available energy to the person compared to carbohydrates and fats. This causes short term satiety to occur in people when a high protein meal is ingested by the individual. Whey proteins

are also capable of maintaining or even possibly decreasing fat body mass while increasing lean body mass in people. This is due to a decrease in metabolic efficiency as energy utilization. Energy is lost during the digestion of whey proteins due to incomplete absorption within the gastrointestinal tract. This material is then excreted as urinary metabolites following catabolism (Thompson *et al,* 2009).

There are seven different whey proteins: β-Lg, α-La, bovine serum albumin (BSA), lactoferrin, immunoglobulins, lactoperoixdase, and glycomacropeptide. β-Lg and α-La are the main proteins as they comprise 50-55% and 20-25% of all whey proteins respectively. The other five proteins are founds in levels lower than 15% (Driskell, 2007).

β-Lg is a globular whey protein, which exists as 10 different variants in bovine milk. It contains 162 amino acids which have a high occurrence of branched chain amino acids. It has a molecular mass of 18.4 kDa and is compact and spherical in shape. In each monomer of this protein, 2 Cys molecules are present (Thompson *et al*, 2009). β-Lg contains 2 disulfide bonds and 1 free thiol group. It is very resistant to hydrolysis in its native conformation suggesting its primary function is not nutritional in nature. It exists naturally as a non-covalently bonded dimer with an isoelectric point of about 5.2, in which the dimers form octamers. Below a pH of 3.4, the dimers dissociate into monomers (Hill, 2009). β-Lg is the milk protein in bovine milk that most people find themselves allergic to; it remains completely absent in human milk (Thompson *et al,* 2009).

β-Lg contains some important biological activities in addition to the functional properties it provides to food products. This protein shows some antimicrobial and antiviral activities by inhibiting pathogens within the gastrointestinal tract thereby enhancing the immune response. β-Lg contains highly charged loci due to the presence of amino acids such as

Cys, lysine (Lys), and methionine (Met). This allows minerals to bind to the peptides formed from digestion of the protein and pass through the intestinal wall. It also aids in the binding and absorption of liposoluble substances such as retinol (Driskell, 2007; Gutierrez-Lopez *et al,* 2007). These charged pockets protect the retinol from oxidation. β-Lg transports retinol into the small intestine where it is transferred to retinol-binding protein. It can also bind to fatty acids which help stimulate lipase activity within the gastrointestinal tract (Thompson *et al*, 2009).

α-La is the primary whey protein in human milk and only minor differences exist in this protein across species. It is believed that this protein may have evolved from lysozyme which is found in eggs. In cattle, the bos taurus breed contains 1 variant and zebu cattle contain 2 variants of this protein. This protein is also compact and globular in shape but is heat stable as well. The protein can be denatured by heat but renatures afterwards. This protein is considered to be a metalloprotein in which 1 calcium molecule per mole binds in a pocket of the α-La containing aspartic acid (Asp) (Thompson *et al*, 2009). Thermal denaturation combined with a pH below 4 causes bound calcium to be released (Hill, 2009). α-La also has a high affinity for the metal ions zinc, manganese, cadmium, copper, and aluminum. All of these ions are essential to maintaining health in humans (Gutierrez-Lopez *et al*, 2007). This protein shows very poor gelling and other functional properties compared to β-Lg (Thompson *et al,* 2009).

α-La is particularly rich in branched chain amino acids, which are necessary in muscle cells for protein synthesis as its energy is generated in the muscle and not in the liver. This increases the bioavailability of carbohydrates as an energy source and prevents muscle degradation under extreme conditions (Gutierrez-Lopez *et al,* 2007). It has a molecular mass of 14.2 kDa and is made up of 123 amino acid residues. Its isoelectric point is at pH 4.8. α-La contains 8 Cys groups, all of which are involved in disulfide bonds within the protein. It also

contains 4 tryptophan (Trp) residues (Hill, 2009). This protein also contains some hormone-like stimulating factors (Gutierrez-Lopez *et al,* 2007).

Due to the health effects of β-Lg and α-La, researchers have examined the bioactivity of these proteins and their associated peptides. Although much research has been conducted regarding cardiovascular disease (CVD) and proteins, there have been insufficient studies conducted to show the effects on metabolic syndrome. This topic is long overdue especially since whey is considered to aid in weight loss and lean muscle mass gain.

2.2 Antioxidant Activity

Antioxidant activity in food products is a research topic that is gaining more attention. Antioxidants prevent spoilage of food products and oxidation within the body due to the presence of free radicals. Free radicals are capable of causing lipid oxidation and oxidative decomposition of unsaturated fatty acids. In food products, antioxidants are incorporated mainly to decrease rancidity and increase the shelf life of fat containing products. In humans, free radicals can modify deoxyribonucleic acid (DNA), proteins, and small cellular molecules. These changes can contribute to CVD, T2DM, various neurological disorders, and even Alzheimer's disease (Peng *et al*, 2009).

Common commercial free radical scavengers such as butylated hydroxytoluene (BHT) and propylgallate (PG) are used in foods but controversy exists over the long term health effects of using synthetic scavengers. This has pushed a movement for discovering natural ways to prevent oxidation. The primary mechanism that antioxidants use to inhibit oxidation is radical quenching. Small peptides in the size range of 0.1-2.8 kDa show strong radical quenching properties (Peng *et al*, 2009). Recent exploration of the antioxidant activity in whey proteins has

shown that whey as a whole and β-Lg, in particular, possess antioxidant activity (Liu *et al*, 2007). The antioxidant activity of whey protein generally increases linearly with the degree of hydrolysis, until reaching a plateau when the proteins are approximately 35-40% hydrolyzed (Peng *et al*, 2009). At present, no work has been reported to show whether or not α-La or products of α-La hydrolysis, possess antioxidant activity.

2.3 Metabolic Syndrome

Metabolic syndrome is a combination of medical disorders that occur within humans. It increases the risk of CVD and diabetes in those people who show signs of developing this disorder. People at risk for developing metabolic syndrome are generally overweight or obese, often have high blood pressure and live a relatively sedentary lifestyle. These people are generally older in age and may have T2DM, which is a decrease in glucose tolerance. They may also show signs of coronary heart disease and lipodystrophy. Inflammation is thought to play a role in metabolic syndrome. Little work has been done directly on the subject of dairy products and their effect on metabolic syndrome. Work that has been completed has focused primarily on specific areas of the syndrome such as hypertension or diabetes and not a combination of the two.

2.3.1 Hypertension and CVD

The subject most thoroughly researched has been the effect of ACE inhibitiors on blood pressure. High blood pressure exists when a systolic blood pressure is above 140 mm Hg and/or a diastolic blood pressure above 90 mm Hg. The ACE inhibitor influences both the renin-angiotensin and the kallikrein-kinnin systems. In the renin-angiotensin system angiotensinogen is converted into angiotensin I by renin and further into angiotensin II by ACE (Li *et al*, 2004). When angiotensin II is present within the human body, it can cause increased peripheral

vascular resistance which leads to vasoconstriction and/or increased aldosterone secretion which leads to increased sodium and water retention, both of which cause an increase in blood pressure. However, when an ACE inhibitor is present, angiotensin I is not converted into angiotensin II. Then the kallikrein-kinnin system becomes affected. In this sytem, kininogen is converted into bradykinin by kallikrein which is then normally converted into inactive fragments in the system by ACE. In the presence of an ACE inhibitor, the inactive fragments are not formed. Bradykinin remains present in the system and causes increased prostaglandin synthesis which leads to vasodilation, decreased peripheral vascular resistance, and ultimately leads to a decrease in blood pressure (Li *et al*, 2004).

High blood pressure has traditionally been treated by modifying diet and physical activity. If this fails then an ACE inhibitor is prescribed. Common pharmaceuticals used include captopril and lisinopril (Pfeuffer & Schrezenmeir, 2000) with molecular weights of 217.3 Da and 441.5 Da, respectively. These drugs are very potent; therefore only very small concentrations are needed in order to cause a decrease in blood pressure. Natural sources of ACE inhibitors, including peptides exist. Peptides which are capable of decreasing blood pressure are generally small in size, about 10 amino acids in length. They bind to ACE at the C-terminal tripeptide preferentially binding hydrophobic, aromatic, or branched chain amino acids. Interestingly, the casokinin milk peptides contain amino acids such as arginine (Arg) and Lys that do not fit with this system (Pfeuffer & Schrezenmeir, 2000). This means that mechanisms other than the renin-angiotensin and kallikrein-kinnin system must be involved in blood pressure regulation. Researchers believe that the autonomic nervous, endothelin and nitric oxide (NO) systems may also play roles in decreasing blood pressure (Fitzgerald, & Meisel, 2000).

A number of publications have been released relating dairy products, especially those which contain hydrolyzed proteins, to blood pressure by measuring the amount of ACE inhibition. Peptides commonly found in milk are called casokinins and lactokinins, which are derived from the proteins casein and whey respectively. These peptides are found in products such as sour milk where the proteins have been hydrolyzed using lactic acid bacteria. Bacteria which are commonly used to produce these peptides include *Lactobacillus helvecticus* and *Saccharomyces cerevisiae.* These natural alternatives are generally considered to be milder, safer, and cheaper than drugs because they do not cause the common side effects such as dry cough and angioedema (Li *et al*, 2004; Fitzgerald, & Meisel, 2000; Pihlanto-Leppala *et al*, 1998). The method developed by Cushman and Cheung in 1971 that measures the amount of hippuric acid which is produced from hippuryl-L-histidyl-L-leucine (HHL) in an ACE reaction is widely used to determine ACE inhibition in hydrolyzed products (Cushman & Cheung, 1971).

Since measurement of the amount of ACE inhibition has been discovered, many *in vivo* and *in vitro* experiments on casokinins have been completed. Casein has been hydrolyzed using enzymes commonly found in the gastrointestinal tract of humans and certain proteolytic enzymes produced by specific bacteria. Fractions from these hydrolysates have been isolated and characterized using high performance liquid chromatography (HPLC) and mass spectroscopy (MS). Some of these peptides, particularly the commercially available C12 peptide, have shown potent ACE inhibition (Cadee *et al*, 2007).

Less research has been completed on ACE inhibitors present within whey proteins. Gastric enzymes have been used to hydrolyze these proteins (Mullally *et al*, 1997). The peptides appear to inhibit ACE but not to the extent of the available pharmaceuticals. Exploration of peptides produced using enzymes from bacterial or a plant sources is needed.

It should be noted that natural ACE inhibitors are not a great way to control high blood pressure in people with hypertension. However, by incorporating these inhibitors into food products or making them available as nutraceuticals, people who are in a prehypertensive state may not have to rely on medications such as catopril to control their blood pressure.

2.3.2. Inflammation

Inflammation occurs as a response by the body to harmful stimuli. It has recently been thought that inflammation may be the underlying cause of metabolic dysregulation rather than a side effect. In fact inflammation is thought to trigger symptoms such as elevated blood glucose and hypertension. As a result, looking at markers for inflammation provides some insight into the development of metabolic syndrome. Tumor necrosis factor alpha (TNFα) is a potent paracrine and endocrine mediator of inflammatory and immune functions. It regulates cell growth and differentiation which leads to a large variety of cells. *In vivo,* this mediator leads to necrosis of methyl cholanthrene induced murine sarcomas (Fitzgerald *et al*, 2001). TNFα is secreted by activated monocytes, macrophages, neutrophils, fibroblasts, keratinocytes, tumor cells, NK-, T-, and B-cells (Fitgerald *et al*, 2001; Caligiuri & Lotze, 2007). Primarily it is produced by macrophages upon lipopolysaccharide interaction as an immune response (Caligiuri & Lotze, 2007). Using murine macrophage cell culture lines, the change in inflammation can be measured by monitoring the TNFα.

TNFα is cytotoxic to tumor cells but not to normal cells within the body. There are 20 molecular related members in the TNF superfamily. Several of these members induce cell death in both normal and tumor cells. To measure cytotoxicity, the cells are exposed to a cytotoxin and cell death is measured for a fixed amount of time. If the cytotoxin TNFα is used, two things can happen. First of all, necrosis can occur. This causes swelling of the cells, destruction of

cellular organelles, and lysis. Secondly, apoptosis can occur. This causes the cells to shrink, apoptotic bodies are formed, and specific internucleosomal DNA fragmentation results. In addition to affecting specialized cells, those which are resistant to TNFα can undergo cell death when it is found in combination with either a transcriptional inhibitor such as actinomycin or a translational inhibitor such as cyclohexamide (Ley, 2004).

TNFα mediates the host's response to both acute and chronic inflammation. It aids in protecting the host from infection and malignancy. TNFα can act as both a tumor necrosis factor (TNF) or as a tumor promoting factor. It can cause proliferation, invasion, and metastasis in cells. In high doses, TNFα can be vasculotoxic and cause tumor regression, although uncontrolled and excessive production of this mediator can lead to chronic disease. Hypotension and organ failure can occur when administered in high doses, although in rodents, once the maximum dosage is reached and decreased, an antitumor effect occurs. Such a treatment is available in Europe for treating certain types of cancer, specifically those which reside in a limb of the body (Caligiuri & Lotze, 2007).

Patients who are diagnosed with metabolic syndrome tend to show an increased level of inflammation markers. It is plausible that by inhibiting TNFα, inflammation may be decreased. This can be related to an increased level of antioxidants, such as glutathione, in the diet. Whey is rich in Cys, which is required for glutathione synthesis. Therefore it is important to determine whether α-La and/or β-Lg or peptides from these proteins, are capable of inhibiting TNFα *in vitro*.

2.4 Blood Glucose Regulation and Diabetes

The primary source of blood glucose in humans is from dietary carbohydrates. Carbohydrates are hydrolyzed by α-glucosidase and pancreatic α-amylase within the

gastrointestinal tract to allow for absorption within the small intestine. One strategy to control diabetes is by inhibiting the activity of these two enzymes (Apostolidis *et al*, 2006). Glucose is created due to the conversion of starch and sucrose in the gastrointestinal tract. Inhibition of α-glucosidase causes glucose absorption to be delayed thereby moderating the elevation in blood glucose after ingestion of a meal. People with hyperglycaemia can use medications, such as acarbose and miglitol, to moderate glucose absorption (Apostolidis *et al*, 2006). Large polysaccharides such as starch are further broken down by α-amylase which breaks internal bonds within the starch molecule. Inhibiting this enzyme can therefore cause a decrease in the amount of glucose which is released by starch, reducing hyperglycaemia. Phenolic acids and α-amylase inhibitors have been shown to aid in the management of diabetes (Apostolidis *et al*, 2006). Excessive inhibition of either α-glucosidase or α-amylase can have side effects. Abdominal distension, flatulence, meteorism, and diarrhea are all possible side effects due to starch passing into the colon undigested. Microflora residing in the gastrointestinal tract breaks down the undigested starch and causes these unpleasant effects (Apostolidis *et al*, 2006).

Blood glucose levels in healthy individuals range from 4-7 mM. These levels are controlled by the balance between glucose absorption from the intestine, production by the liver, and uptake and metabolism by peripheral tissues. Insulin is the hormone that increases glucose uptake by muscle and fat cells and decreases hepatic glucose production. It also stimulates the translocation of glucose transporter type 4 (GLUT 4) from intracellular sites to the cell surface (Saltiel & Kahn, 2001). GLUT 4 is a protein in humans that is encoded by the GLUT 4 gene. GLUT 4 and insulin regulated glucose transporters (GLUT) found in adipose and muscle are responsible for insulin-regulated glucose translocation into the cell. Type 2 diabetes mellitus (T2DM) causes an increase in hepatic glucose and a decrease in insulin production. This can

affect the overall glucose transport, glucose and lipid metabolism, growth, protein synthesis, and gene expression (Oliver *et al*, 2010).

2.4.1 Insulin Receptors

Insulin receptors belong to the subfamily of receptor tyrosine (Tyr) kinases (Saltiel & Kahn, 2001). They are tetrameric transmembrane proteins with 2 α and 2 β subunits. They function as allosteric enzymes because the α subunit inhibits Tyr kinase activity of the β subunit. When insulin binds to the α subunit, a decrease in kinase activity in the β subunit occurs followed by transphophorylation of β subunits and conformational changes to cause an increase in kinase activity (Saltiel & Kahn, 2001).

An insulin resistant state reduces the ability to decrease blood glucose levels as a result of a decrease in the uptake of insulin by fat and muscle cells. In an insulin resistant state, insulin binds to receptors on insulin-responsive cells causing the receptors and a number of substrates including insulin receptor substrates to phosphorylate (Wellen & Hotamisligil, 2005; Saltiel & Kahn, 2001). This occurs due to inflammation and results in insulin resistance. TNFα and free fatty acids stimulate the inhibitory phosphorylation of the serine (Ser) residues on insulin receptor substrate 1 in response to insulin and impairs its ability to associate with the insulin receptor leading to inhibition of downstream signalling and insulin action (Wellen & Hotamisligil, 2005). Specifically, insulin resistance causes the signalling of the insulin receptor to become impaired due to a decrease in the activation of targets insulin receptor substrate 1 and protein kinase B, which stimulate translocation of GLUT 4 to the cell surface. Normally insulin binds to the cell surface receptors insulin receptor substrates 1 and 2 when they become phosphorylated by Tyr residues in active insulin receptors, such as insulin-like growth factor 1, but this is diminished in an insulin resistant state. It is thought that macrophages in adipose tissue

contribute to insulin resistance by down-regulating GLUT 4 and insulin receptor substrate 1 which decreases insulin's action (Oliver *et al*, 2010).

2.4.1.2 Inflammation and insulin receptors

As noted above, macrophages are also linked to inflammation. Macrophages express the majority of adipocytes gene products including peroxisome proliferator-activated receptor γ (PPARγ), fatty acid binding protein 4 and proteins such as TNFα, interleukin 6 (IL6), as well as metalloproteinases which degrade extracellular proteins and process bioactive molecules (Wellen & Hotamisligil, 2005). TNFα and IL6, both indicators of inflammation, suppress cytokine signalling molecules 1 and 3. As these molecules inhibit the pathway which transmits chemical signals from outside the cell to go through the cell membrane and interact with gene promoters on the DNA responsible for DNA transcription and cell activity, insulin signalling is sterically hindered by their presence (Oliver *et al*, 2010). TNFα induces the expression of IL6, plasminogen activator inhibitor 1, and monocyte chemotactic protein 1. There is a correlation between an increase in TNFα and a decrease in insulin receptor substrate 1 and GLUT 4 expression (Oliver *et al*, 2010). IL6 causes long term inhibitory effects of gene expression of insulin receptor substrate 1, GLUT 4, and PPARγ, which regulates fatty acid storage and glucose metabolism, and stimulates lipid uptake and adipogenesis. This results in a decrease in their expression which ultimately leads to impaired expression of insulin stimulated glucose transport (Oliver *et al*, 2010).

2.5 Role of Obesity in Metabolic Syndrome

Obesity is a term normally used to describe anyone with a body mass index greater than 30. In metabolic syndrome, the tendency is for extra weight to be seen in the circumference of the waist, resulting in central adiposity. Obesity is correlated with low grade chronic

inflammation characterized by changes in a wide range of cellular metabolites as shown in Table 1. As adiponectin, for example, decreases inflammation and modulates insulin sensitivity, the decrease in adiponectin leads to increased insulin sensitivity resulting in a decrease in hepatic glucogenesis and an increase in lipid oxidation (Oliver *et al*, 2010). The hormone leptin is the main endocrine signal to the hypothalamus in regulating appetite and energy balance (Wang *et al*, 2008). It also plays a role in adaptive and innate immunity. When leptin levels are decreased in the body, immunity becomes decreased (Wellen & Hotamisligil, 2005).

Table 1. Effects of inflammation induced obesity on cellular metabolites.

Factor	Effect on	Function of factor	Reference
TNFα	Increase	Mediates response to inflammation	Oliver *et al* , 2010
IL6	Increase	Stimulation and protection against inflammation, activates signalling through the cell membrane	Oliver *et al* , 2010; Fried *et al*, 1998; Mohamed-Ali *et al*, 1997
NO synthases – inducible isoform	Increase	Immune response, binds calmodulin and produces NO	Oliver *et al* , 2010
Transforming growth factor β1	Increase	Controls cell growth, cell proliferation, cell differentiation and apoptosis	Oliver *et al* , 2010
C- reactive protein	Increase	Binds to phosphocholine on dead or dying cells to activate the clearing of pathogens from organisms	Oliver *et al* , 2010
Intracellular adhesion molecules	Increase	Bind with cells and extracellular matrix	Oliver *et al* , 2010
Monocyte chemotactic protein 1	Increase	Recruits monocytes, T-cells, and dendritic cells to sites of injury, infection, and inflammation	Oliver *et al* , 2010
Plasminogen activator inhibitor-1	Increase	Inhibits tissue plasminogen activator and urokinase, increases risk of thrombosis	Oliver *et al* , 2010
Factor VII	Increase	Involved in blood clotting	Oliver *et al* , 2010
Macrophage migration inhibitory factor	Increase	Regulation of macrophage function by suppression of anti-inflammatory effects of glucocorticoids	Trayhurn & Wood, 2005
Haptoglobin	Increase	Binds free haemoglobin, protects the kidneys from damage by haemoglobin, prevents iron loss	Trayhurn & Wood, 2005
Serum amyloid A	Increase	Associated with high-density lipoprotein in plasma	Trayhurn & Wood, 2005
Adiponectin	Decrease	Hormone that modulates glucose regulation and fatty acid catabolism	Oliver *et al* , 2010; Trayhurn & Wood, 2005
GLUT 4	Decrease	Insulin-regulated glucose translocation to the cell	Oliver *et al*, 2010
C-reactive protein	Increase	IL6 stimulates hepatic synthesis of C-reactive protein in obesity	Trayhurn & Wood, 2005; Yudkin, 2003; Yudkin *et al*, 1999
Interleukin 18	Increase	Expressed by adipose and stimulated by TNFα	Trayhurn & Wood, 2005

2.5.1 Influence of obesity on insulin response

Adipose tissue produces cytokines and bioactive molecules which are also known as adipokines. Adipokines act in an autocrine and paracrine manner or act as signalling molecules (Oliver *et al*, 2010). They are involved in homeostasis, lipid metabolism, blood pressure regulation, insulin sensitivity, and angiogenesis (Trayhurn & Wood, 2005). The overproduction of TNFα in adipose and muscle tissue during obesity contributes to insulin resistance (Wellen & Hotamisligil, 2005). It has also been suggested that the overproduction of NO in obese individuals causes a decrease in muscle cell insulin action and beta cell function. High NO levels have been linked to the ability of pro-inflammatory cytokines, such as TNFα, and lipopolysaccharides to induce insulin resistance in muscle tissue. This effect can be reduced by inhibiting the inductible nitric oxide synthase (iNOS), the enzyme controlling the production of NO (Wellen & Hotamisligil, 2005).

2.5.2 Obesity and inflammation

Preadipocytes are undifferentiated fibroblasts that, when stimulated, develop into adipocytes through a process known as adipogenesis (Wellen & Hotamisligil, 2005; Wang & Zhang, 2008). Preadipocytes possess both phagocytotic and antimicrobial activity and are capable of differentiating into macrophages. As a result they potentially possess roles in the immune response. Both macrophages and preadipocytes colocalize an individual is in adipose tissue when in a state of obesity. Here they contribute both alone and together with adipocytes to produce inflammatory mediators and promote the development of insulin resistance (Wellen & Hotamisligil, 2005).

White adipose tissue, a major component in human adipose tissue, has been previously viewed as an inactive lipid storage site due to its primary function as an energy reservoir and

insulator. As blood vessels and nerves connect the adipose to the rest of the body, it plays a role in metabolism regulation. It has been found to be a source of hormones and acts as an endocrine organ in obese individuals. This affects the distribution of leptin and adiponectin, both of which are associated with metabolic syndrome, insulin resistance, and CVD (Wang *et al*, 2008). If the white adipose mass expands, the clusters of adipocytes distant from the vasculature tissues become deprived of oxygen due to hypoxia. This causes the release of cytokines, chemokines, and angiogenic factors which increase the blood flow causing vascularisation (Trayhurn & Wood, 2005).

Adipocytes exist to accumulate and release fatty acids, store energy as triglycerides during periods of calories abundance, and retrieve energy during periods of calorie deficit. Triglycerides are transported to the adipose tissue as lipoprotein molecules where they are hydrolyzed by lipoprotein lipase and enter the cells as fatty acids (Wang *et al*, 2008; Trayhurn & Wood, 2005). This pathway causes CoA derivatives to be transferred to glycerol in the form of glycerol 3-phosphate which is an essential intermediate in glycolysis. In addition, adipocytes play a role in endocrine function. They are not a major source of cytokines, but increased lipids causes increased cellular stress, which activates inflammatory signalling pathways. This leads to increased levels of TNFα, IL6, and chemoattractant molecules (Wang *et al*, 2008).

When stressed, the endothelium reticulum increases the secretion of ROS resulting in inflammation. Macrophages are also recruited to the adipose to scavenge debris from dead adipocytes. Adipocyte cell death is actually three times higher in obese individuals when compared to healthy individuals (Wang *et al*, 2008).

2.6 Dairy Proteins and Metabolic Syndrome

Dairy products have been widely thought as contributing to metabolic syndrome due to the presence of saturated fatty acids. Recently research has been showing this to be untrue. Milk in general has been shown to decrease glycaemic response compared to mixed meals (Schrezenmeir *et al*, 1989) and lactose equivalent meals (Nilsson *et al*, 2004; Ostman *et al*, 2001). This indicates that milk in general can have a positive effect on diabetes related to metabolic syndrome. In experiments utilizing only the whey fraction of the milk, diabetic subjects showed a lower glycaemic response after consuming lunch but not at breakfast (Frid *et al*, 2005). Additionally the insulin response to whey was significantly higher than for mixed meals (Nilsson *et al*, 2004) but milk was not any different (Nilsson *et al*, 2004; Ostman *et al*, 2001). This may indicate that proteins in the whey fraction can have an effect when concentrated compared to milk as a whole. Additionally, yogurt has been found to significantly lower postprandial glycaemia and insulinaemis compared to a mixed meal (Ostman *et al*, 2001). This suggests that fermentation creates bioactive peptides which can influence the effects of insulin in people. These bioactive peptides may be up to 10 amino acids in length. Peptides derived from milk proteins have been shown to possibly decrease blood pressure by possessing angiotensin converting enzyme inhibitory effects (Fitzgerald & Meisel, 2000) and endothelian release-inhibitory activity (Maes *et al*, 2004). This information combined makes it quite plausible that dairy proteins, in particular whey proteins, are capable of controlling the individual symptoms related to metabolic syndrome.

3.0 Materials and Methods

3.1 α-Lactalbumin and β-Lactoglobulin Extraction

Fractionation was carried out by chemical separation as follows. The whey protein isolate (Davisco Foods International Inc., MN, USA) was dissolved in water (15% w/v). The solution was then acidified to a pH of 3.8 using 1M HCl (Fisher Scientific, ON, Canada) followed by heating to 55°C for 30 min with stirring. The solution was then centrifuged (Sorvall Instruments RC5C, SLA-3000 Rotor) at 2,600 relative centrifugal force (RCF) for 30 min to separate the supernatant and pellet. The supernatant was freeze dried leaving a highly purified fraction of β-Lg. The pellet was then dissolved in water which was acidifed to a pH of 3.8 and brought to a temperature of 56°C. The solution was then centrifuged at 2,600 RCF for 30 min and the supernatant was removed. This was repeated four times. Water was then added to the pellet and dissolved. 1M NaOH (Fisher Scientific, ON, Canada) was added until a pH of 7.3 was reached. The calcium content was then adjusted to 1.2 g/kg using calcium chloride (Fisher Scientific, ON, Canada). The solution was chilled to a temperature of 2°C. The solution was rapidly heated to 50°C and held at this temperature for 8 min. The solution was then centrifuged at 17,500 RCF (Sorvall Instruments RC5C, SS-34 Rotor) for 30 min. The supernatant was transferred to containers and freeze dried providing a fraction enriched with α-La (Maubois *et al,* 1987).

Fractions were analyzed for purity and degree of separation using sodium dodecyl sulphate gel electrophoresis (Laemmli, 1970) and HPLC (Eprogen, Illinois, USA).

3.2 β-Lactoglobulin and α-Lactalbumin Hydrolysis and Degree of Hydrolysis

3.2.1 Hydrolysis

The β-Lg and α-La fractions were hydrolyzed using three different enzymes: trypsin (Sigma-Aldrich, ON, Canada), alcalase (Sigma-Aldrich, ON, Canada), and flavourzyme (Sigma-Aldrich, ON, Canada). Each fraction was dissolved in water (5% w/v). The pH of the solution was corrected to the optimal pH for efficient enzyme activity using 1M NaOH. For trypsin an optimal pH of 9 and temperature of 37°C, alcalase an optimal pH of 8 and temperature of 50°C, and for flavourzyme an optimal pH of 7.0 and temperature of 50°C was required. Hydrolysis was carried out in triplicate as follows. Enzymes were introduced into the solution at 4% w/w and immersed into a water bath at optimal temperature and allowed to incubate for 4 h. The pH was maintained using the appropriate acid or base and a pH meter (Accumet Basic AB15 Plus, Fisher Scientific, ON, Canada). After 4 h the pH of the samples was corrected to a pH of 7 to slow the reaction rate and the samples were immersed into a water bath at 90°C to completely stop the reaction. The samples were filtered using a Whatman No. 4 filter paper and freeze dried (Humiski & Aluko, 2007).

3.2.2 Degree of Hydrolysis

The degree of hydrolysis of the samples was determined by 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS; Pierce, IL, USA) method (Adler-Nissen, 1979). A 0.2125M sodium phosphate buffer at pH 8.20 was made by creating two buffers by dissolving in deionized water, one from sodium phosphate monobasic (Fisher Scientific, ON, Canada) and another from sodium phosphate disbasic (Fisher Scientific, ON, Canada). The monobasic buffer was then added to the dibasic buffer until the appropriate pH was achieved. A solution of 1% (w/v) sodium dodecyl sulfate (Sigma-Aldrich, ON, Canada) in phosphate buffer was used to dissolve the samples and

the standards to their desired concentrations. Samples were dissolved at a concentration of approximately 16.67 mg/mL and a standard curve was made from L-Lys (Sigma-Aldrich, ON, Canada) at the following concentrations: 2.5 mM, 2.0 mM, 1.5 mM, 1.0 mM, 0.5 mM, 0 mM. A 250 µL aliquot of each sample and standard solution was transferred to labeled glass test tubes. To each test tube, 2 mL of sodium phosphate buffer was added. Immediately prior to use, a 0.1% TNBS in sodium phosphate buffer solution was created. The solution was kept in the dark at all times by securely placing tinfoil around the container and working in low lighting conditions. To each sample test tube, 2 mL of 0.1% TNBS solution was added and samples were incubated in a water bath (Julabo SW22 Shaking Water Bath, PA, USA) at 50°C for 60 min. During incubation, the TNBS solution reacts with the primary amine of molecules in the samples and standards producing an orange-coloured derivative which can be measured by ultraviolet-visible (UV-Vis) spectrophotometry. Immediately following incubation, 4 mL of 0.1 N hydrochloric acid (Fisher Scientific, ON, Canada) in deionized water was added to each test tube to stop the reaction. The final concentration of peptides was s0.5 mg/mL.The samples were allowed to cool for 30 min prior to measuring the absorbance at 340 nm using a Novaspec Plus spectrophotometer (Biochrom, England; Model # 80-2117-50). The degree of hydrolysis was calculated as follows:

$$Degree\ of\ Hydrolysis\ (\%) = \frac{AN_2 - AN_1}{N_{pb}}$$

AN_1 is the amino nitrogen content before hydrolysis in mg/g

AN_2 is the amino nitrogen content after hydrolysis in mg/g

N_{pb} is the nitrogen content of the peptide bonds in mg/g; 123.3 mg/g for whey proteins

3.3 Ultrafiltration of Hydrolysate

The resulting α-La and β-Lg hydrolysates were further processed using ultrafiltration membranes (Millipore, MA, USA) in an Amicon stir cell (Millipore, MA, USA) with molecular weight cut-offs of 10 and 1 kDa. The hydrolysates were first separated into retentate and permeate using a 10 kDa membrane. The resulting permeate from the 10 kDa membrane separation was then further fractionated using a 1 kDa membrane to produce a retentate and permeate. This resulted in 3 fractions: >10 kDa, 1 to 10 kDa, and < 1 kDa fractions. The resulting retentates and permeates were freeze dried for further analysis.

Bioactivity measurements were carried out in triplicate using the retentates and permeates of the hydrolyzed and unhydrolyzed material. Samples included 8 hydrolyzed protein samples in triplicate and the corresponding unhydrolyzed protein samples to act as a control. This allowed evaluation of the difference in bioactivity between the hydrolyzed and unhydrolyzed material.

Characterization of the bioactivity of the samples was carried out using the following techniques.

3.4 Angiotensin Converting Enzyme Inhibition

Angiotensin converting enzyme inhibition was determined based on the method by Cushman and Cheung in 1971. The enzyme directly affects the conversion of angiotensin I to angiotensin II in the rennin-angiotensin system which can lead to an increase in blood pressure. Inhibiting this enzyme leads to a decrease in blood pressure. This method measures the amount of hippuric acid produced by the reaction between HHL and ACE. The samples were prepared by dissolving HHL in a 0.3% (w/v) (Sigma Aldrich, ON, Canada) solution of 100 mM borate buffer (Sigma Aldrich, ON, Canada) and 300 mM sodium chloride (Fisher Scientific, ON, Canada). The

pH was adjusted to 8.3 at 37°C with 1 M NaOH (Fisher Scientific, ON, Canada) Sample was dissolved in the buffer solution at a concentration of 3.75 mg/mL to create a final concentration of peptides after dilution with reagents of 1 mg/mL. A 200 µL aliquot of the sample solution was transferred to a glass test tube. The sample solution was preincubated in a water bath (Julabo SW22 Shaking Water Bath, PA, USA) for 3 min at 37°C prior to adding the enzyme. A 50 µL aliquot of 0.33 unit/mL angiotensin converting enzyme from rabbit lung (Sigma Aldrich, ON, Canada) was added to each test tube and incubated for 30 min at 37°C in the water bath. The reaction was terminated by adding 250 µL of 1 M hydrochloric acid (Fisher Scientific, ON, Canada). The hippuric acid produced was extracted by adding 2 mL of ethyl acetate (Fisher Scientific, ON, Canada) to each test tube and vortexing for 30 s. This was followed by centrifugation (Sorvall GLC-1) for 5 min at 1000 RCF. A 1 mL aliquot from the aqueous layer, consisting of ethyl acetate and hippuric acid, of each sample test tube was removed and placed into a vial in a fume hood in a heating block (Thermolyne Dri-Bath, Model # DB28125, Iowa, USA) set to 100°C for 15 min or until all the ethyl acetate was evaporated. The remaining hippuric acid was dissolved in 3 mL of deionized water and mixed by inversion until completely dissolved. The absorbance at 228 nm was measured using a UV-Vis spectrophotometer (Biochrom Ultrospec 1100 Pro, England, UK) and quartz cuvettes. The spectrophotometer was blanked using a test tube which did not contain sample and had the hydrochloric acid added prior to the angiotensin converting enzyme. The positive control consisted of a test tube without sample allowing as much HHL to be converted to hippuric acid as possible. The percent inhibition was calculated as shown below:

$$ACE\ Inhibition\ (\%) = \frac{Control\ Absorbance - Sample\ Absorbance}{Control\ Absorbance - Blank\ Absorbace}\ X\ 100$$

3.5 α-Lactalbumin and β-Lactoglobulin Hydrolysate Characterization

3.5.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Whey protein extracts were examined by SDS-PAGE by the method outlined by Laemmli in 1970 using a Protein 3 Cell® Mini Electrophoresis SDS-PAGE and a 250/2.5 Power Supply (Bio-Rad, ON, Canada). Samples were dissolved at a concentration of 0.01 mg/mL in a sample buffer consisting of 0.0625M Tris-HCl (Sigma-Aldrich, ON, Canada) buffer at pH 6.8 containing 2% sodium dodecyl sulfate (Sigma-Aldrich, ON, Canada), 10% Glycerol (Sigma-Aldrich, ON, Canada), 5% Mercaptoethanol (Sigma-Aldrich, ON, Canada), and 0.01% Pyronin Y (Sigma-Aldrich) as an indicator. Samples were heated on a heating block (Thermolyne Dri-Bath, Model# DB28125, Iowa, USA) to 95°C for 5 min prior to application to the gels. A 5 µL aliquot of each sample was applied to a 12% separating gel and a 4% stacking gel. The separating gel consisted of: 3 mL acrylamide/bis 30% T 2.67% C (Bio-Rad, ON, Canada), 2.5 mL 1.5M Tris-HCl (Sigma-Aldrich, ON, Canada) pH 8.8, 100 µL 10% sodium dodecyl sulfate (Sigma-Aldrich, ON, Canada), 4.4 mL deionized water, 50 µL 10% ammonium persulfate (Sigma-Aldrich, ON, Canada), 5 µL N,N,N',N'-tetramethylethyledienediamine (TEMED) (Sigma-Aldrich, ON, Canada). The stacking gel consisted of: 1 mL acrylamide/bis 30% T 2.67% C (Bio-Rad, ON, Canada), 2.5 mL 0.5M Tris-HCl (Sigma-Aldrich, ON, Canada) pH 6.8, 100 µL 10% sodium dodecyl sulfate (Sigma-Aldrich, ON, Canada), 6.4 mL deionized water, 10µL TEMED (Sigma-Aldrich, ON, Canada) and 50 µL 10% ammonium persulfate (Sigma-Aldrich, ON, Canada). Gels were run for approximately 45 min at 0.1 amp. Gels were visualized by staining with a 0.1% Coomassie Brilliant Blue (Pierce, IL, USA), 40% methanol (Fisher Scientific, ON, Canada), 10% acetic acid (Fisher Scientific, ON, Canada) solution and incubating with shaking (The Belly Dancer, Stovall Life Science Inc, North Carolina, USA) overnight. This was followed by de-staining in a 40% methanol (Fisher Scientific, ON, Canada) and 10% acetic acid (Fisher Scientific, ON, Canada) solution for approximately 1 h with

shaking (The Belly Dancers, Stovall Life Science Inc, North Carolina, USA). The destaining solution was changed every 15 min to facilitate in the process. The gels were scanned to provide a digital record of the results (Epson Perfection Scanner, Epson, Ontario, Canada) and analyzed using Image J (Version 1.43u) image processing and analysis software.

3.5.2 Gel Permeation Chromatography

The peptides were characterized by gel permeation chromatography (Waters LCM1, MA, USA) to determine the approximate size distribution of the peptide fractions. The samples were reconstituted at a concentration of 10 mg/mL in gel permeation chromatography buffer which was a mixture of 0.1M sodium phosphate monobasic and 0.1M sodium phosphate dibasic at a pH of 7.0. The samples were filtered with a 0.22 µm PVDF filter (Fisher Scientific, ON, Canada) and a 10 µL aliquot of each sample was injected onto a glycerol bonded support on 5 µm 50Å spherical silica column (Eprogen GPC-PEP 250 mm x 4.6 mm, Illinois, USA). The column was calibrated using a globular protein low molecular weight gel filtration calibration kit (GE Healthcare, 28-4038-41, Buckinghamshire, UK): Aprotinin (6.5 kDa , Ribonuclease A (13.7 kDa), Ovalbumin (43 kDa) and Conalbumin (75.0 kDa)). The column was also calibrated with a peptide mixture to determine the molecular weight of small peptide (Sigma Aldrich, ON, Canada): GLY-TYR (238.2 Da), VAL-TYR-VAL (379.5 Da), Met Enkephalin Acetate (573.7 Da) and Leu Enkephalin (555.6 Da), Angiotensin II Acetate (1046.2 Da). The flow rate of the buffer through the column was set to 0.4 mL/min and the absorbance of the detector was set to 214 nm to detect peptides or peptide bonds. The system was controlled by Software Millenium[32] Software Version 3.20 (Waters Corporation).

3.6 (2,2-diphenyl-1-picylhydrazyl)-Free Radical Scavenging Activity (DPPH)

Antioxidant activity was analyzed following the DPPH method as outlined by Brand-Williams *et al* (1995). Extraction was carried out by dissolving whey peptide samples in 100% methanol (Fisher Scientific, ON, Canada) at a concentration of 0.1 mg/mL. Samples were inverted for 3 h in the dark followed by centrifugation at 1000 RCF (Sorvall GLC-1) to remove the pellet from the antioxidant containing extract. A 100 µL aliquot of extract was placed into a cuvette followed by 3.9 mL 0.06 mM DPPH in methanol (Sigma-Aldrich, ON, Canada) solution. The absorbance at 515 nm was measured using a UV-Vis spectrophotometer (Biochrom Ultrospec 1100pro, England) at 0 min and at 30 min. Methanol was used as a blank. The percent discolouration to the DPPH solution was calculated as follows:

$$Discolouration\ (\%) = \left(1 - \frac{Absorbance\ at\ 30\ min}{Absorbance\ at\ 0\ min}\right) X\ 100$$

The free radical scavenging activity of the whey peptide extracts in triplicates were expressed as percent antioxidant activity. A standard curve of known concentrations of Trolox (Sigma-Aldrich, ON, Canada) from 0M to 0.1M was used to determine the antioxidant activity of the sample extracts.

3.7 Murine Macrophage Cell Culture and Analysis

3.7.1 Murine Macrophage Cell Culture

Murine macrophage cells from the RAW 264.7 cell line were incubated with samples and media as a negative control to determine the TNFα production in unstimulated cells. The media was examined for the production of TNFα using enzyme linked immunosorbent assay (ELISA) and nitrite production by the Greiss reaction to determine if vascular inflammation of the cells occurred. The cells were examined for cell viability by incubation with MTT (3-(4,5-

dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) to determine whether the mitochondria of the cells were capable of reducing MTT to formazan crystals which is an indication of the viability of the cells.

The following protocol was used for murine macrophage cell culture. Murine macrophage cell line RAW 264.7 was suspended in a dubellco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) all from the American Type Culture Collection (ATCC). The cells and medium were grown in a 25 cm^2 cell culture flask (Fisher Scientific, ON, Canada) in an incubator at a temperature of 37°C and in 5% CO_2. At 80-85% subconfluency the cells were subcultured in 75 cm^2 flasks. At 80-85% confluent, the cells were trypsinized (American Type Culture Collection) and quantified using a haemocytometer. The cells were separated from the trypsin and media by centrifuging the cells in a 15 mL sterile centrifuge tube at 133 RCF for 3 min. The media was removed and fresh media was added. The cells were gently massaged with the media to reconstitute them. The cells and media at a density of $5X10^4$ cells/well were dispersed amongst a 96-well tissue culture plate (VWR International, ON, Canada) and incubated for 24 h. Hydrolyzed peptides were reconstituted in media at concentrations of 50, 100, 200, and 500 µg/mL, applied to unstimulated cells and incubated overnight. Supernatant was collected and frozen at -80°C for future analysis.

3.7.2 Cytotoxicity MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium Bromide) Assay

The cytotoxicity of the samples was analyzed by MTT assay (Van *et al*, 2009). The assay measures the reduction of MTT to formazan by mitochondrial reductase enzymes indicating cell viability. After the media was removed from the cells, 100 µL of fresh media and 10 µL of MTT was added to the wells. The cells were incubated for 4 h at 37°C and 5% CO_2 followed by

removal of the media. The purple formazan crystals formed were dissolved in a 200 µL detergent solution and the optical density was measured at 570 nm.

3.7.3 Cytokine Production Analysis

To determine the effects of whey peptide crude slurries on the inflammatory markers of the murine macrophage cells, the media was analyzed by ELISA (e-Bioscience Inc., OH, USA). The assay was conducted as instructed by the manufacturer of the kits. A 96-well tissue culture plate was coated with 100 µL/well of capture antibody and incubated overnight at 4°C. The wells were washed five times with a wash buffer consisting of phosphate buffered saline (PBS) (Sigma Aldrich, Dorset, UK) and 0.05% Tween-20 (Sigma-Aldrich, Dorset, UK) to remove excess antibodies from the plate. The wells were then incubated at room temperature for 1 h with 200 µL/well 1X assay diluents to block the nonspecific protein binding sites of the capture antibody. The wells were washed an additional five times followed by adding 100 µL of the positive standard TNFα (25, 50, 100, 200, 500, 1000 pg/mL) or 100 µL of the media from the whey peptide slurries and incubated overnight at 4°C. The TNFα molecules bound to the capture antibody during this time. Plates were then washed five times with wash buffer and 100 µL of avidin-horseradish peroxidase, which acts as the detection antibody, was added to each well and incubated for 30 min at room temperature. During this time, TNFα bound to the detection antibody. This was followed by washing the wells seven times with wash buffer to remove any excess detection antibodies. 100 µL/well of substrate solution was added to each well and incubated at room temperature to allow blue colour development to occur. The reaction was stopped after 5 min by adding 50 µL of 1M phosphoric acid stop solution to each well causing a colour change from blue to yellow. The optical density of each well was measured at 450 nm and 570 nm. The values measured at 570 nm were subtracted from those obtained at 450 nm.

3.7.4 Nitrite Production Analysis

Nitrite induced inflammation was quantified by the Griess reaction (Van *et al*, 2009). The reaction quantifies the nitrite levels in the media by adding 100 µL of media previously collected and 100 µL of Greiss reagent (Sigma-Aldrich, ON, Canada) to a 96-well tissue culture plate. A standard curve was created by incubating sodium nitrite at concentrations between 0 and 100 g/L with the Greiss reagent in the same manner as the samples from the tissue culture media. The Greiss reagent consists of a 1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% α-naphthylamine in distilled water. The reagent causes a purple colour to develop due to the reaction with nitrite forming an azo-compound which is quantified using a spectrophotometer. After incubation at room temperature for 10 min, the absorbance was measured at 550 nm.

3.8 Adipocyte Cell Culture and Analysis

3.8.1 Adipocyte Tissue Culture Analysis to Examine the Effect of Peptides on Inflammation and Insulin Sensitivity

Cells were derived from 3T3 mouse fribroblasts and grown in cell culture medium until they reached 100% confluency. Differentiation was initiated to cause the fibroblasts to form fat vacuoles with characteristics similar to those found in humans. Cell viability was assessed followed by stimulation of the cells with IL1β to cause the cells to be in a state of inflammation. Samples and controls were then incubated with the cells. The media was removed and examined by ELISA for IL6 levels to determine if inflammation was up or down regulated by the whey peptides. The cells, which remain after removal of the media, had the ribonucleic acid (RNA) extracted, the concentration adjusted, and were reverse transcribed. This sequence of events allows the genes to be examined for up or down regulation. The genes examined were GLUT 4 and adiponectin because they are both shown to be downregulated during a diabetic

state (Oliver *et al*, 2010). Upregulation therefore suggests a return to normal glucose metabolism.

3.8.2 3T3-L1 Cell Line

Cells used in cell culture were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). The cells were derived from 3T3 mouse fibroblasts by diluting cells and incubating until a single colony was extracted. This process is known as clonal isolation. The fibroblasts are capable of differentiating into adipocytes under specific cell culturing conditions (Green & Kehinde, 1975). The transformation occurs after the cells stop growing. The cells increase the amount of fatty acid precursors which are incorporated into triglycerides. Fat vacuoles begin forming and eventually merge into a single vacuole (Russel & Ho, 1976). After differentiation is complete, the cells exhibit all the characteristics of mouse and human adipocytes including having an increased sensitivity to lipogenic and lipolytic hormones and drugs which are capable of stimulating glucose uptake (Cowheard *et al*, 1999; Novikoff *et al*, 1980).

3.8.3 3T3-L1 Cell Culture

Cells from 3T3-L1 cell culture line were grown in DMEM containing 4.5 g/L glucose and 2 mM of L-glutamine (Lonza Walkerville Inc, MD, USA). The growth medium is rich in nutrients such as inorganic salts, amino acids, vitamins, D-glucose, and energy from sodium pyruvate. Phenol red is a component of the cell culture medium that acts as an indicator of the pH to help identify any problems occurring within the culture, such as an accumulation of wastes or a bacterial infection. The medium was also supplemented with 10% FBS (Gibco®, Invitrogen, Paisely, UK). It is low in antibodies but very rich in growth factors as BSA is a main component. Additionally 100 mg/mL of streptomycin sulfate and 100 µg/mL of potassium penicillin (Lonza

Walkersville Inc, MD, USA) were added as they impart a broad spectrum bacteriostatic and bacteriocidal effect in the medium. They prevent the growth of both gram positive and gram negative microorganisms which can have adverse effects on the cells during experimentation.

The cells and medium were grown in 75 cm^2 filter-vented cap flasks (Iwaki Seiyaku Co, Ltd, Tokyo, Japan) at a temperature of 37°C and in 5% CO_2 and 95% air in a humidified incubator (Thermo Electron Corporation, OH, USA) until they reached 70% sub-confluency, which is a state between the cells being sparse and confluent. The cells were split 1:10 once a week and washed with PBS (Gibco®, Invitrogen). The live cells were trypsinated by applying 0.25% typsin with EDTA (Lonza Walkersville Inc.) in PBS to remove the adherent cells from the flask for transfer. The trypsin activity was halted by adding growth media to the flask. The mixture of cells and growth media was transferred to a sterile centrifuge tube and centrifuged at 1,200 RCF for 5 min to separate the cells from the media. New media was then added and the cells were transferred to new sterile flasks.

3.8.4 Differentiation of 3T3-L1 Cell Culture

Once the experiment was ready to proceed, the cells were transferred to sterile 6 or 12 well plates (BD Biosciences, NJ, USA) instead of sterile flasks. The cells were grown until 100% confluent and allowed to continue growing for an additional 2 days. To cause differentiation to occur, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich,Dorset, UK), 0.25 µM dexamethoasone (Sigma-Aldrich, Dorset, UK) and 1 µg/mL insulin (Sigma-Aldrich, Dorset, UK) was added to DMEM supplemented with 10% FBS and P/S and incubated for 6 days. In preparation for experimentation, the differentiation medium was removed and replaced with DMEM supplemented with 10% FBS and P/S for 24 h.

3.8.5 Cell enumeration and Viability Assessment

Prior to utilizing the cell culture line, it was necessary to ensure the cells were viable. To determine the viability of cells, the cells were stained with tryptan blue solution (Sigma-Aldrich, Dorset, UK) and counted using a haemocytometer under a light microscope (Phillips & Terryberry, 1957). This method turns dead cells blue in color and used to estimate the proportion of viable cells in the population.

3.8.6 Cell Stimulation

To simulate inflammation within the differentiated 3T3-L1 cells, IL1β (R&D Systems, Abington, UK) was added to the cells as it is an activator of Interleukin-1 receptor I which primarily imparts the inflammatory effects of Interleukin-1. IL1β was reconstituted in sterile PBS with 0.1% BSA and was stored at a concentration of 10 µg/mL in 50 µL aliquots at -20°C until further used.

3.8.7 Sample Incubation with 3T3-L1 Adipocytes

Whey protein hydrolysates evaluated with adipocytes were limited to selected 1kDa fractions. Samples were weighed out using a balance (Denver Instruments Company DE Series Model 100A), dissolved in water to a concentration of 25 mg/mL and mixed thoroughly by vortexing. The samples were centrifuged (Micromax Thermomax RF Thermo Electron Corporation) for 5 min at 4,000 RCF to separate any undissolved peptides from the solution. The peptide solution was filtered through a Millex-HV Sterile 0.45µm PVDF syringe filter to remove any contaminants that might be suspended within the solution. The samples were then diluted with media that consisted of DMEM supplemented with 10% FBS and 1% P/S by combining 120 µL of sample with 3000 µL of media. The samples were replaced with water as the negative blank for the experiment. Confluent cells were then incubated with media-containing samples in

sterile 24 well plates. The plate consisted of 4 rows and 6 columns. Columns from 1 through 6 were incubated as follows: column - 1 negative blank, columns 2 and 3 – media only and columns 4, 5, 6 – samples. After 48 h, media was removed from the wells in columns 1-2 and replaced with fresh media. For the remainder of the wells, the media was removed and replaced with 1 ng/mL TNFα and 10 ng/mL IL1β. The 3rd column represented the positive control for the experiment. After 24 h of incubation with the inflammatory factor, the media was removed from the cells and placed into sterile centrifuge tubes and frozen for further evaluation.

The plate, which contained only cells at this point, was blotted to remove as much media as possible. A mixture of phenol and guanidine thiocyanate, also known as the TRI reagent (Sigma-Aldrich, Dorset, UK), was added to the cells and gently agitated to remove the cells from the plate. The solutions were then transferred into sterile centrifuge tubes and stored at -20°C prior to further evaluation.

3.8.8 RNA Extraction

The 3T3-L1 cells in the TRI reagent collected previously were combined with 200 µL of chloroform (Sigma-Aldrich, Dorset, UK) and mixed well for 15 s in centrifuge tubes. This solution effectively disrupts and dissolves the contents of the cells while maintaining the integrity of the RNA by inhibiting ribonuclease activity (Chomczynski & Sacchi, 1987). The samples were allowed to sit for 15 min at room temperature to allow the separation of the aqueous and organic layers to occur. This was followed by centrifugation at 15,000 RCF at 4°C for 15 min. Three distinct layers were apparent within the samples. The aqueous or clear layer contains the RNA, the white layer contains the DNA, and the pink layer contains the waste materials. The clear layer containing the RNA was removed and placed into a sterile centrifuge tube and stored on ice to prevent degradation of the RNA. To each tube, 0.5 mL of 100% isopropanol (Sigma-Aldrich,

Dorset, UK) was added and the samples were inverted multiple times to mix the solutions and cause the RNA to precipitate out of solution. The samples were stored for 30 min under refrigeration to facilitate precipitation of RNA. Samples were then centrifuged at 15,000 RCF at 4°C for 15 min to create a pellet containing RNA. The waste was carefully decanted to avoid disturbing the pellet. A solution of 75% ethanol in RNase-free water (Sigma-Aldrich, Dorset, UK) was prepared and 500 µL was added to the pellets. The samples were centrifuged at 7,500 RCF at a temperature of 4°C for 5 min. The ethanol was carefully decanted to avoid disturbing the pellet. A mechanical pipette and sterile tips were used to remove as much ethanol as possible. The pellets were allowed to dry for 10 min to remove any traces of ethanol. Once dry, 25 µL of RNase-free water was added to the almost clear pellet and stored at -80°C for further evaluation.

3.8.9 Concentration and Purity of RNA

The samples were thawed on ice for 30-60 min prior to determining the concentration and purity of the RNA obtained from the cells. A NanoDrop ND-1000 Spectrophotometer (NanoDrop® UV-vis ND-1000, NanoDrop Technologies Inc., DE, USA) and RNA-40 software were used to determine these parameters. The pedestal of the spectrophotometer was cleaned by applying 2 µL of 0.1N HCl in RNase-free water and removed using a kimwipe. The spectrophotometer was then initialized by applying 1 µL of RNase-free water to the pedestal which was then removed with a kimwipe. The spectrophotometer was then blanked by applying 1 µL of RNase-free water to the pedestal and measuring the absorbance at the following wavelengths, 260nm, 280nm, and 230 nm. The absorbance of 1 µL of each sample was then measured. The pedestal of the spectrophotometer was cleaned between samples using the procedure reported above. The purity of the samples and the concentration of nucleic acids

were then determined using the software that was supplied with the instrument. When the optical density ratio 260/280 falls between 1.8 and 2.0 the sample is considered to contain an acceptable amount of RNA. When the optical density ratio 260/230 exceeds 1.8 the sample is considered to be free of contaminants such as protein, salt, and phenols which absorb at a wavelength of 230 nm. The concentration of the RNA in ng/µL was calculated using the software from the absorbance measurement at 260 nm based on the Beer-Lambert Equation.

The concentration of nucleic acids was used to determine how much RNase-free water needed to be added to each sample prior to further analysis. Based on the results obtained, the volume required for further analysis was 10 µL. The calculations used to determine the amount of water to be added to each sample are shown below.

$$\mu L\ of\ sample = \frac{1000}{Concentration\ of\ Nucleic\ Acids\ (\frac{ng}{\mu L})}$$

$$\mu L\ of\ Nuclease\ Free\ or\ RNase\ Free\ Water = 10\ \mu L - \ \mu L\ of\ sample$$

After the calculations were completed, the RNase-free water was added to each sample and they were stored at -20°C until replication was done.

3.8.10 Complementary Deoxyribonucleic Acid (cDNA) RNA Replication

The samples were thawed along with the cDNA reverse transcription kit (Perkin-Elmer Applied Biosystems, Warrington, UK) on ice. To each sample the following reagents were added while on ice: 48 µL of 10X reverse transcription buffer, 19.2 µL of 100mM 25X dNTP mix, 48 µL of 10X reverse transcription random primers, 24 µL of 50 U/µl multiscribe™ reverse transcriptase, 24 µL of RNase inhibitor, and 76.8 µL of RNase-free water. The samples were gently mixed on ice and placed into the thermal cycler (Techne Touchgene FTG02TD). The samples were cycled as follows, 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and held at

4°C until removed from the thermocycler. The samples were removed from the thermal cycler after reaching 4°C and stored at -20°C until further analyzed.

3.8.11 Real-Time Reverse-Transcription-TaqMan® Polymerase Chain Reaction (RT-PCR)

The reverse transcribed samples were the analyzed using RT-PCR. This method detects and quantifies the polymerase chain reaction amplification while the reaction is occurring. Probes which consist of oligonucleotide with an attached fluorescent dye at the 5' end and a quencher moiety at the 3' end of the chain are added to the reverse transcribed sample. This causes the internal region of the polymerase chain reaction product to hybridize. In an unhybridized state, the location of the quencher and the fluorescent dye prevents the detection of the fluorescence from the probe. The template where the TaqMan® probe is bound is replicated and the 5'-nuclease activity of the polymerase chain cleaves off the probe. This causes fluorescence to occur which increases with each subsequent cycle in a proportional amount to the probe cleavage. The amount of fluorescence emitted due to the cleavage of the probes eventually crosses the fixed threshold during the exponential phase of amplification. This is an inverse relationship between the number of fractional cycles and the amount of the template present.

The samples produced from the cDNA reverse transcription were thawed on ice for 20 min prior to adding the reagents of the polymerase chain reaction. Two master mixes were prepared containing probes (Perkin-Elmer Applied Biosystem, Warrington, UK). Master mix A consisted of 0.5 µL of GLUT 4 probe, 0.5 µL of 18S probe to act as the reference gene for the experiment, 5 µL of TaqMan® polymerase chain reaction mix which contains the flourogenic probe which enables the detection of polymerase chain reaction product, and 3.5 µL of RNase-

free water. Master mix B consisted of 0.5 µL of adiponectin probe, 5 µL of TaqMan® polymerase chain reaction mix, and 4 µL of RNase-free water.

Nineteen microliters of either mastermix A or B were added to 1 µL of the cDNA sample and were centrifuged briefly to aid mixing of the sample and to pull the sample down into the bottom of the centrifuge tubes. Samples were then added in 9.5 µL aliquots to a sterile 384 well plate in duplicate. The well plate was then covered and inserted into an Applied Biosystems 7900HT Sequence Detection System (Perkin-Elmer Applied Biosystems, Warrington, UK) and analyzed using SDS 2.2 Software. The thermal cycling conditions were as follows, 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The samples were thermal cycled until the RNA probes were detected at the threshold level. The data was then analyzed by subtracting the number of cycles for the control 18S gene from the gene of interest which was either adiponectin or GLUT 4. The duplicates were then averaged, the blank was subtracted and the units were converted to fold change.

The purpose of examining the change in gene expression of GLUT 4 and adiponectin was to determine if there was an up or down regulation in the gene due to the presence of bioactive peptides. Upregulation of both GLUT 4 and adiponectin suggests proper glucose metabolism where down regulation suggests a diabetic state.

3.8.12 ELISA Interluekin-6 Production

The media obtained from the 3T3-L1 cells was also evaluated by enzyme- linked immunosorbent assay to measure the activity of the inflammatory cytokine IL6. The 96-well plates were first coated with 100 µL of anti-IL6 capture antibody and incubated overnight at room temperature. This was followed by washing the plate 3 times with a buffer which consisted of 0.05% Tween 20 in PBS (Sigma-Aldrich, Dorset, UK). The wells were then blocked by

applying 300 µL of blocking buffer which contained 1% BSA in PBS and incubated for 1 h at room temperature. This was done to remove any excess antibody and to prevent any non-specific binding from occurring. This was followed by washing the plate with wash buffer 3 times. The media from the samples and serially diluted standards were added in 100 µL aliquots and followed by incubation at room temperature for 2 h. This was followed by washing the plate an additional 3 times with the wash buffer. Biotinylated anti-cytokine detection reagent was then added in 100 µL aliquots to each well and incubated at room temperature for 2 h. Plates were washed an additional 3 times with the wash buffer. The detection reagent, strevidin conjugated horseradish peroxidase was diluted (1:200) and added to each well in 100 µL aliquots and incubated for 20 min at room temperature while protected from light. This was followed by washing the plate 3 more times with the wash buffer. A substrate solution which consisted of a 1:1 mixture of hydrogen peroxide and tetramethylnezidine was added to each well in 100 µL aliquots and incubated for 20 min at room temperature to aid in the color change. The reaction was finally stopped by adding 50 µL of sulphuric acid to each well. The absorbance of each well was then measured at 450 nm with a wavelength correction at 570 nm using a SPECTRAmax®M2 microplate reader with SoftMax Pro® software. A standard curve was created by plotting the absorbance against the concentration of the standards. This was used to calculate the concentration of the cytokine of interest, IL6.

3.9 Statistical Analysis

The results show the mean ± standard deviation. Statistical analysis was performed using SAS (version 9.0) using PROC GLM followed by a least significant difference test of significance. In all cases, the probability (p) value of the significant difference was set to $p < 0.05$.

4.0 Results and Discussion

4.1 α-Lactalbumin and β-Lactoglobulin Hydrolysate Characterization

4.1.1 Purity of α-Lactalbumin and β-lactoglobulin Extracts

The extracts containing α-La and β-Lg were examined by SDS-PAGE and gel permeation chromatography to determine the extent of purification of these two proteins. SDS-PAGE is used to determine the molecular weight of proteins in a solution by comparing the unknown proteins to a standard solution of known molecular weight. The proteins are initially denatured so they only retain their primary structure. When an electric field is applied the proteins move towards the positive pole but the larger proteins move more slowly through the polyacrylamide network. This is due to the channels in the polyacylamide and the larger proteins having more difficulty to pass through them. This causes the proteins to be separated on the gel based on size. Approximate concentration of the protein as a percentage can be determined from gels by making an electronic copy of the gel and processing it using software to determine the intensity of the scanned band. Purity was calculated as the band density for the desired protein compared to the total band density for the lane. Figure 1 shows the resolution of samples and standards following SDS-PAGE. α-La extracts showed two contaminating proteins, β-Lg and immunoglobulins, and had an approximate purity of 64.7 ± 3.0 %. β-Lg showed one contaminating protein, α-La, with an approximate purity of 92.8 ± 1.3 %. Purities of both samples were lower compared to gel permeation chromatography and are not in accordance with the HPLC data obtained by Maubois *et al* (1987) which showed 80 % pure α-La and > 98 % pure β-Lg. This is possibly due to the number of parameters, such as background noise and distortion of optical density during scanning, which need to be controlled in order to analyze the lanes (Gassmann *et al*, 2009).

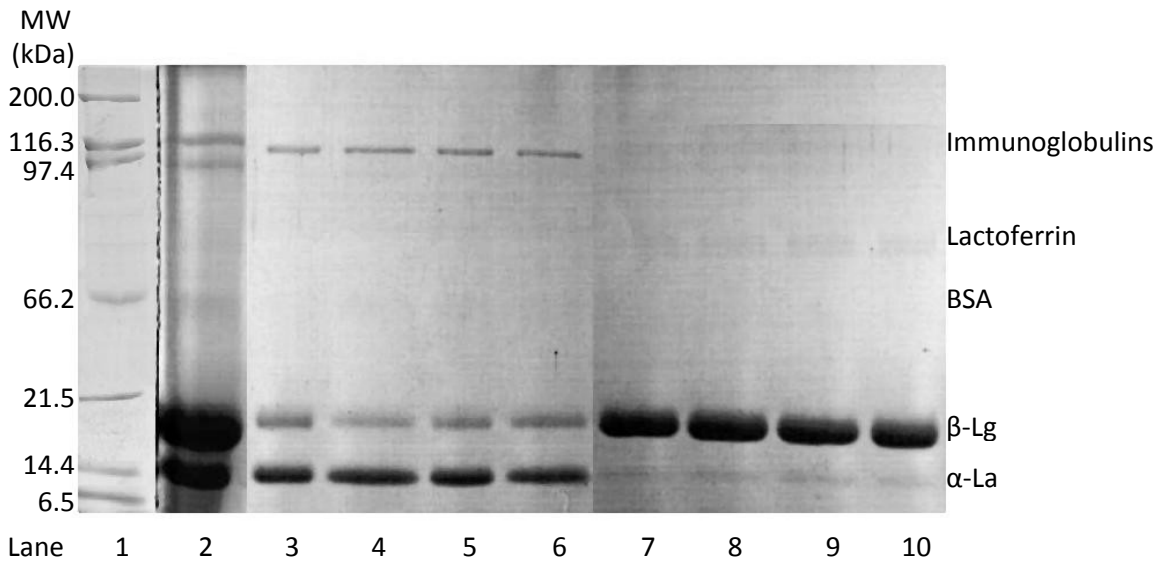


Figure 1. SDS-PAGE gel of standards and samples to determine the approximate molecular weight and concentration of the whey protein of interest. Lane 1 broad range molecular weight standards, 2 starting material, 3-6 α-La fraction, 7-10 β-Lg fraction.

Gel permeation chromatography utilizes a similar concept except in reverse. Small proteins elute later due to access to many of the small pores which make up the stationary phase of the column. Due to the pH limitations of the available columns, considerations must be made to determine if proteins are interacting with each other causing their molecular weights to be larger than anticipated. For example, at a neutral pH β-Lg acts as a dimer. Therefore its molecular weight after being resolved on a column at a pH of 7.0 is twice that of its theoretical molecular weight. α-La extracts, as resolved on a gel permeation chromatography column, show 90.8 ± 2.3 % purity as seen in Figure 2.

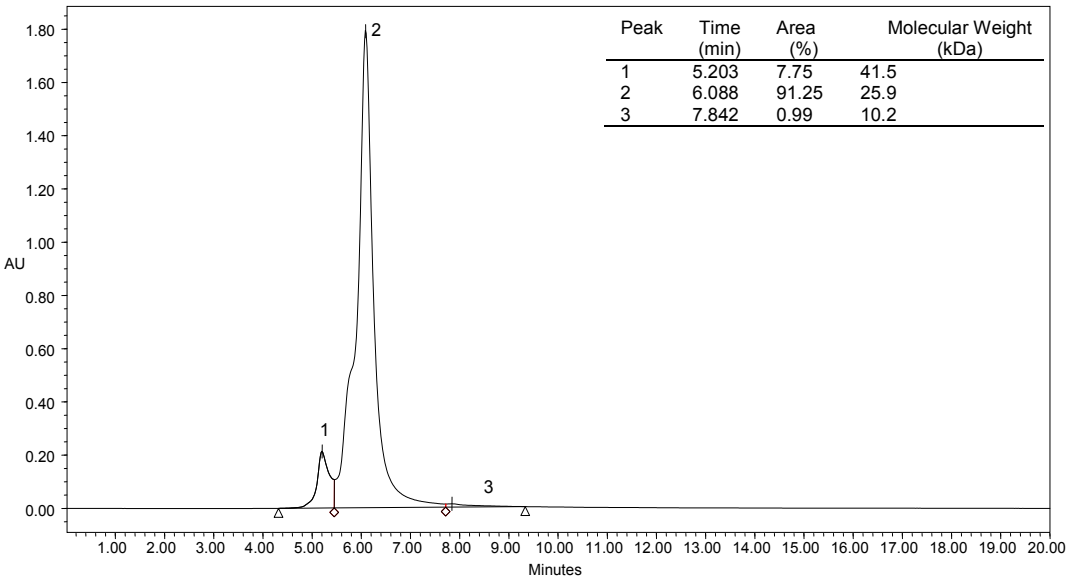


Figure 2. Gel permeation chromatography chromatogram at pH 7.0 of an extracted α-La fraction, indicated as peak 2, at a concentration of 10 mg/mL.

Contaminants in this extract as shown by SDS-PAGE are immunoglobulins and β-Lg. The immunoglobulins are identified in the chromatogram as a single peak. The molecular weight is much lower, 41.5 kDa compared to approximately 100 kDa by SDS-PAGE. This is due to the stationary phase limitations of the column. It is unable to resolve proteins outside of the 0.8 to 30 kDa range due to the size of the pores. The contaminating β-Lg is not seen as a single peak on this chromatogram. This contaminant elutes at a similar time to α-La due to both proteins acting as dimers at this particular pH causing a shoulder to form to the left of the α-La peak. This means that the 91 % α-La peak also includes β-Lg. This problem could be resolved by using a pH which causes both proteins to act as monomers, however due to the limitations of this column this could not be done. Another alternative would be to resolve these larger molecular weight proteins on a column which could handle proteins with a molecular weight between 10 and 100 kDa. The chromatograms for β-Lg extracts show 99.7 ± 0.1 % purity as seen in Figure 3.

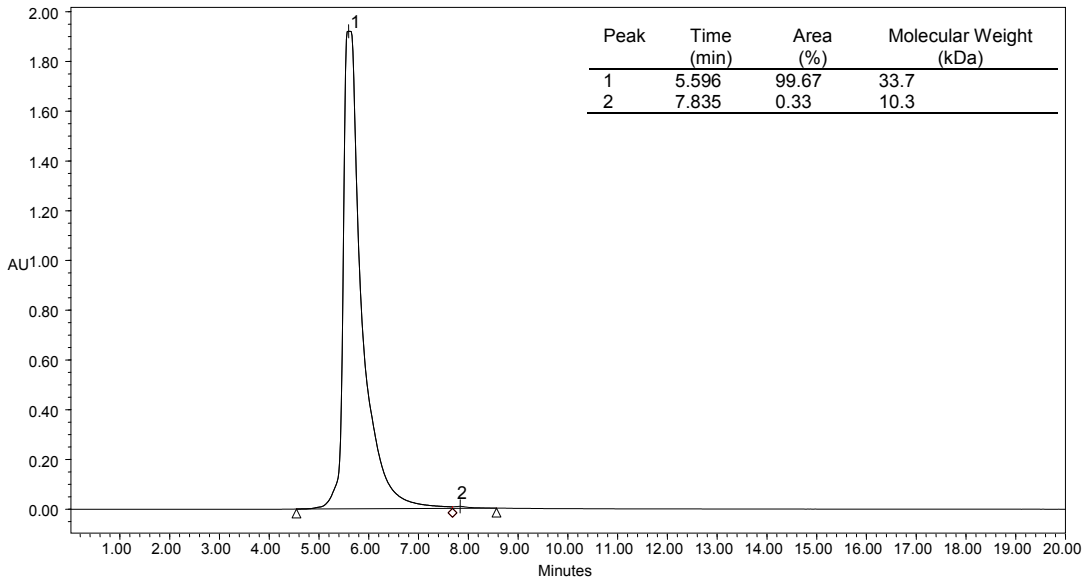


Figure 3. Gel permeation chromatography chromatogram at pH 7.0 of an extracted β-Lg fraction, indicated as peak 1, at a concentration of 10 mg/mL.

According to the SDS-PAGE gels there is a small amount of contaminating α-La included in this β-Lg peak. The contaminating α-La could be removed with further washing of the extract. Other researchers who utilized this method of extraction for these two proteins found that β-Lg extracts were > 98 % pure and α-La extracts were approximately 80 % pure by HPLC (Maubois *et al*, 1987). The contaminants were identified by HPLC but were not quantified. My results are in accordance with this research for the β-Lg extract however their research shows higher purity for the α-La extract. The contaminating proteins were the same proteins in both sets of experiments. To remove contamination, it has been advised to increase the pH to 8.0 prior to calcium chloride addition to remove immunolgobulins from the α-La extract. Additionally immunoglobulins can be removed by dialfiltration using a high molecular weight cut-off following the α-La extraction. Washing of the α-La extract will increase the purity of the extract by removing contaminating β-Lg but will, at the same time, reduce the amount of protein of interest in the sample as some α-La is lost with each washing step (Bramaud *et al*, 1997). Extracts were hydrolyzed without further purification in the current work.

4.1.2 Degree of Hydrolysis

The degree of hydrolysis was determined by incubating a sample with TNBS which reacts with primary amino acids. This causes the solution to turn yellow and the darker the yellow the higher the degree of hydrolysis of the sample (Adler-Nissen, 1979). Hydrolyzed samples had varying degrees of hydrolysis from 7 % to 40 % as seen in Table 2 and Figure 4. In general, α-La samples had lower hydrolysis levels when compared to β-Lg samples for all of the enzyme combinations. α-La samples incubated with flavourzyme alone had a higher degree of hydrolysis compared to the hydrolsates formed from a combination of alcalase and flavourzyme, with hydrolysis levels of 36 % and 32 % respectively. This phenomenom does not hold true for the β-Lg hydrolysates formed with flavourzyme and the combination of alcalase and flavourzyme both with a degree of hydrolysis of approximately 40 %. Hydrolysates formed by the incubation with alcalase had lower levels of hydrolysis than the combination of alcalase and flavourzyme; α-La hydrolysates had a degree of hydrolysis of 18 % and 32 % and β-Lg hydrolysates with a degree of hydrolysis of 22 % and 40 % for alcalase and the combination of alcalase and flavourzyme, respectively. Hydrolysates formed using trypsin caused the lowest degree of hydrolysis for both α-La and β-Lg with degrees of hydrolysis of 7 % and 10 % respectively.

Table 2. Enzyme reaction conditions of α-Lactalbumin and β-Lg hydrolysates.

	Reaction Conditions	
	pH	Temperature (°C)
α-Lactalbumin		
Alcalase	8.5	50
Flavourzyme	7.0	50
Trypsin	8.0	37
Alcalase/Flavourzyme	8.5 and 7.0	50
β-Lactoglobulin		
Alcalase	8.5	50
Flavourzyme	7.0	50
Trypsin	8.0	37
Alcalase/Flavourzyme	8.5 and 7.0	50

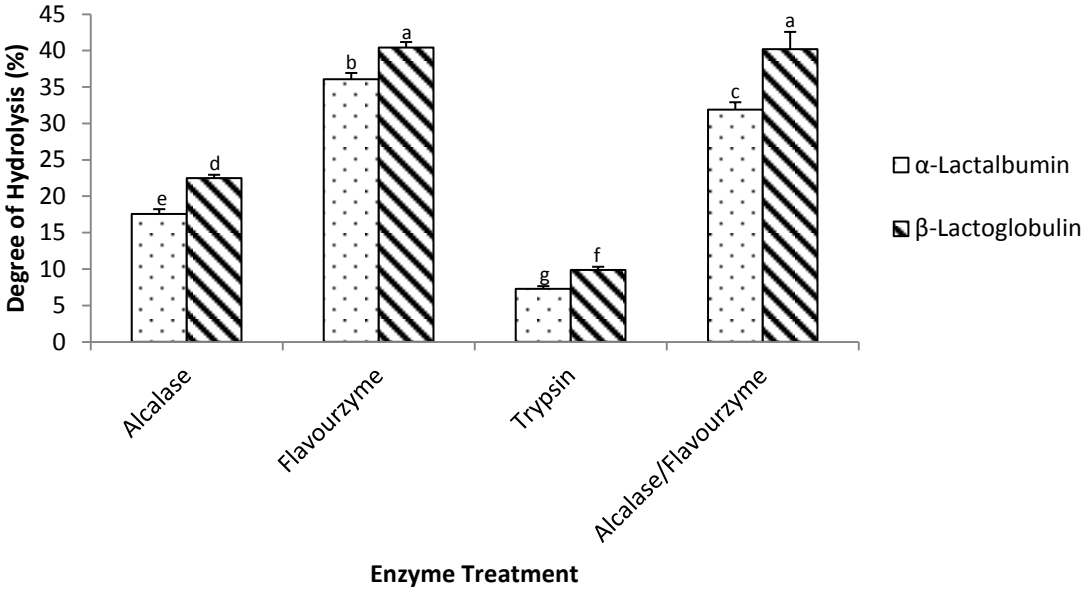


Figure 4. Degree of hydrolysis of α-La and β-Lactoglobulin hydrolysates. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

Degrees of hydrolysis have been reported previously by a number of different researchers. In 1993, Perea *et al* used the alcalase enzyme to hydrolyze both α-La and β-Lg with levels of hydrolysis of 16 % and 20 % respectively, results slightly higher than those found in this research. Other research has provided degrees of hydrolysis closer to those obtained in this study. With β-Lg approximately 9 % of the protein was hydrolyzed (Asselin *et al*, 1988; Amiot *et al*, 2004; Cheison *et al*, 2010) and for α-La the degree of hydrolysis was 8 % (Salami *et al*, 2009). Data for utilizing flavourzyme to hydrolyze dairy protein is minimal. Research conducted by

Dryakova *et al* in 2010 determined that whey proteins hydrolyzed for 180 min by flavourzyme had a degree of hydrolysis of approximately 35 %. This is in accordance with the results of the current study if we consider that the majority of whey is made of up β-Lg with a smaller percentage as α-La. The results obtained by the work completed by Dryakova *et al* (2010) falls between the degree of hydrolysis results obtained for α-La and β-Lg in this study. No research has been published to date utilizing both alcalase and flavourzyme to hydrolyze dairy proteins.

The fact that higher degrees of hydrolysis were found when hydrolyzing β-Lg compared to α-La shows us that β-Lg is much more susceptible to hydrolysis. This is most likely due to the structure of the protein. A large β-sheet (Creamer *et al*, 1983) gives the enzymes better access to cleavage sites as the amino acids are exposed on the surface of the sheet rather than being buried within an α-helix like that found in α-La (Permyakov, 2005). β-lactoglobulin contains 2 disulfide bonds and located away from the terminal ends of the protein (Thompson *et al*, 2009) leaving most of the protein exposed for hydrolysis. Flavourzyme used with or without alcalase provided similar degrees of hydrolysis when the substrate was β-Lg. This is possibly due to the exposure of the enzyme to the amino acid sequence. Alcalase is an endopeptidase and can cleave the peptide bonds within the structure of a protein. Flavourzyme, on the other hand has both endopeptidase and exopeptidase activity (Cumby *et al,* 2008). Flavourzyme would have more exposure to the ends of peptide chains formed due to the intra-molecular cleavage of the amino acid sequence by alcalase. This increase in the number of terminal ends accessible to flavourzyme increases the exopeptidase activity of flavourzyme. However, as flavourzyme possesses both endo and exopeptidase activity, the inclusion of alcalase does not increase the effect that flavourzyme has on the peptide bonds.The same is true for the α-La samples hydrolyzed with these two proteins. However research conducted by Graveland-Bikker *et al* in 2004 found that the presence of calcium can cause the structure of α-La to reassemble following

enzymatic hydrolysis with alcalase. This is an indication of incomplete hydrolysis of the proteins. Lower degrees of hydrolysis may be more favourable due to the decreased presence of free amino acids and an increased presence of peptides.

4.1.3 α-Lactalbumin and β-Lactoglobulin Hydrolysate Characterization

Extracts of α-La and β-Lg were hydrolyzed for a period of 4 h. Flavourzyme which is derived from *Aspergillus oryzae* has the capability of cleaving peptide bonds not only within the peptide chain but also from the N-terminus. The other enzymes trypsin and alcalase, a peptidase derived from *Bacillus lichenformis*, are endopeptidases but their access to the active sites can be limited by the presence of disulfide bridges. Trypsin cleaves the peptide bond on the carboxyl side of Arg and Lys (Cheison *et* al, 2011). Flavourzyme and alcalase both contain a number of enzymes as they are both crude extracts of enzymes obtained by *Aspergillus oryzae* or *Bacillus lichenformis*, repectively. The cleavage sites for flavourzyme are un-specific and possess both endo and exopeptidase activity (Raghavan & Kristinsson, 2009; Nchienzia *et al,* 2010). The cleavage sites of alcalase are numerous including the carboxyl side of aromatic amino acids such as phenylalanine (Phe), Tyr, and Trp. However cleavage has also been noted at glutamic acid (Glu), Met, Leu, Lys, and glutamine (Gln) (Doucet *et al*, 2003).

β-Lg contains 5 Cys residues, four of which are involved in intra-chain disulfide bridges for a total of 2 disulfide bonds (Thompson *et al*, 2009). There are two variations of the protein from bovine milk. The A variant contains aspartate at position 64 and Val at position 118 whereas the B variant contains glycine (Gly) and alanine (ALA) respectively at these two positions. The concentrations of each variant of β-Lg in the extract were not determined. The structure of β-Lg, however, contains a β-sheet (Creamer *et al*, 1983) and only 2 disulfide bonds

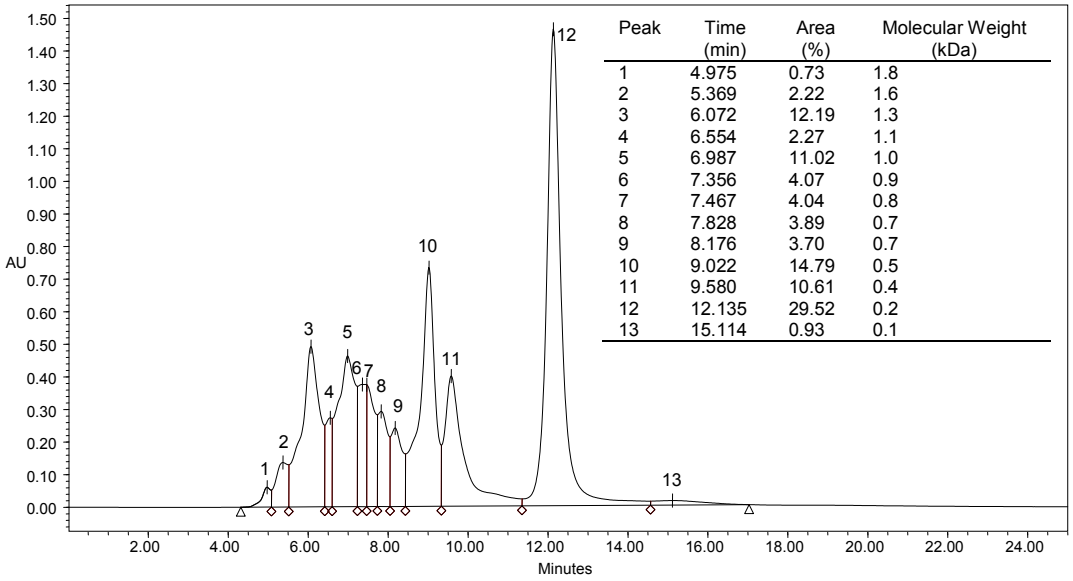
located in the center of the structure (Thompson *et* al, 2009) allowing proteases to hydrolyze the peptide bonds without breaking the disulfide bonds.

α-La contains 4 disulfide bridges, which form a large α-helix and a small β-sheet (Permyakov, 2005). This causes the protein to be resistant to proteolysis without first being denatured. This protein interacts with membranes, proteins, peptides, and other low molecular weight substrates. This is modulated by the binding of metal cations, especially calcium, making this protein a metalloprotein (Permyakov, 2005).

α-La hydrolyzed with various enzymes showed different molecular weight profiles. Trypsin and flavouryzme left approximately 24 % and 12 % of the α-La unhydrolyzed. There was no α-La peak left for hydrolysates formed with alcalase and the combination of alcalase and flavourzyme. The only enzyme which left some of the β-Lg intact (4 %) was flavourzyme. The profiles for the hydrolysates are shown in Figures 5 to 8 for α-La and Figures 9 to 12 for β-Lg. These hydrolysates were further fractionated using a stir cell with either a 10 kDa or 1 kDa membrane. Only the 1 to10 kDa and < 1 kDa fractions were further examined based on the theory that indicates that as the peptide chain length decreases to between 10 to 2 amino acids long the bioactivity increases. However the presence of free amino acids decreases bioactivity.

α-La hydrolyzed with flavourzyme contained 13 peaks as shown by gel permeation chromatography with approximately 55 % of these peaks having molecular weights between 5.5 and 0.5 kDa (Figure 5). Fractionation of this hydrolysate resulted in concentrating the small molecular weight peptides in the permeate of the 1 kDa fraction. The fraction contained peptides up to 2.4 kDa in size with 50 % of these peptides having a molecular weight range of 0.6 to 0.4 kDa (Figure 6). As the average molecular weight of an amino acid is 0.11 kDa, this means that theoretically based on the standard curve there are no free amino acids in the

fraction. That being said, 56 % of the peak area is contained at a molecular weight of 0.2 kDa. When free amino acids were injected onto the column the calculated molecular weight was approximately 0.2 kDa. This means that the amino acids are eluting outside of the linear range of the column and that the major peak in the < 1 kDa fraction may contain individual amino acids. The 10 kDa fraction contained 60 % of the peptides in the 11.7 to 9.4 kDa range (Figure 7). Some free amino acids may remain in the 10 kDa fraction but do not represent the dominant peak.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.975	0.73	1.8
2	5.369	2.22	1.6
3	6.072	12.19	1.3
4	6.554	2.27	1.1
5	6.987	11.02	1.0
6	7.356	4.07	0.9
7	7.467	4.04	0.8
8	7.828	3.89	0.7
9	8.176	3.70	0.7
10	9.022	14.79	0.5
11	9.580	10.61	0.4
12	12.135	29.52	0.2
13	15.114	0.93	0.1

Figure 5. Gel permeation chromatography chromatogram at a pH of 7.0 of an α-La fraction hydrolyzed with flavourzyme after 4 h at a concentration of 10 mg/mL.

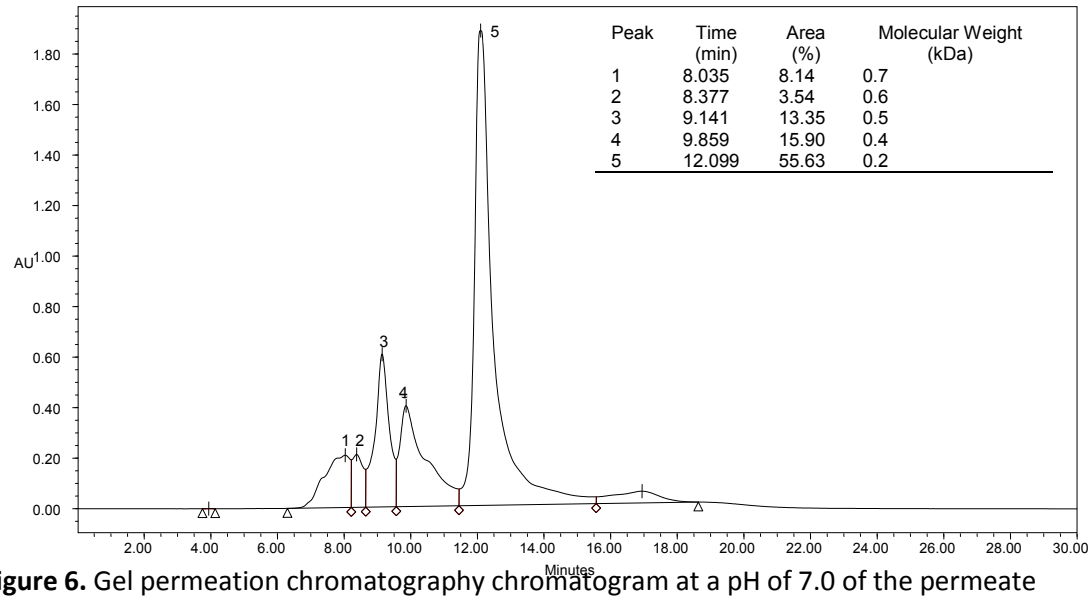


Figure 6. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 1 kDa membrane from an α-La fraction hydrolyzed with flavourzyme for 4 h at a concentration of 10 mg/mL.

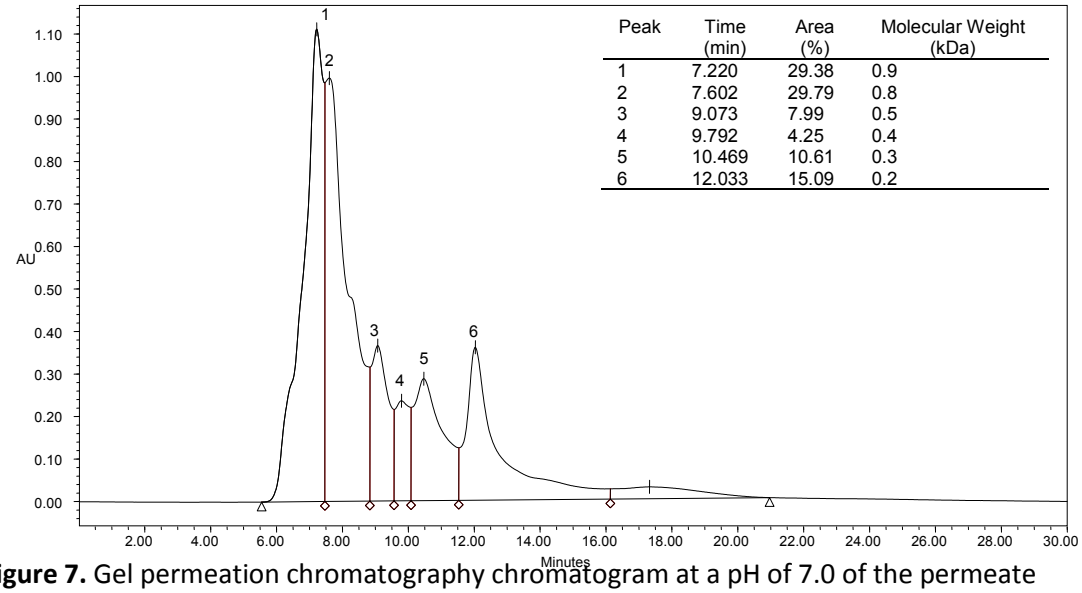
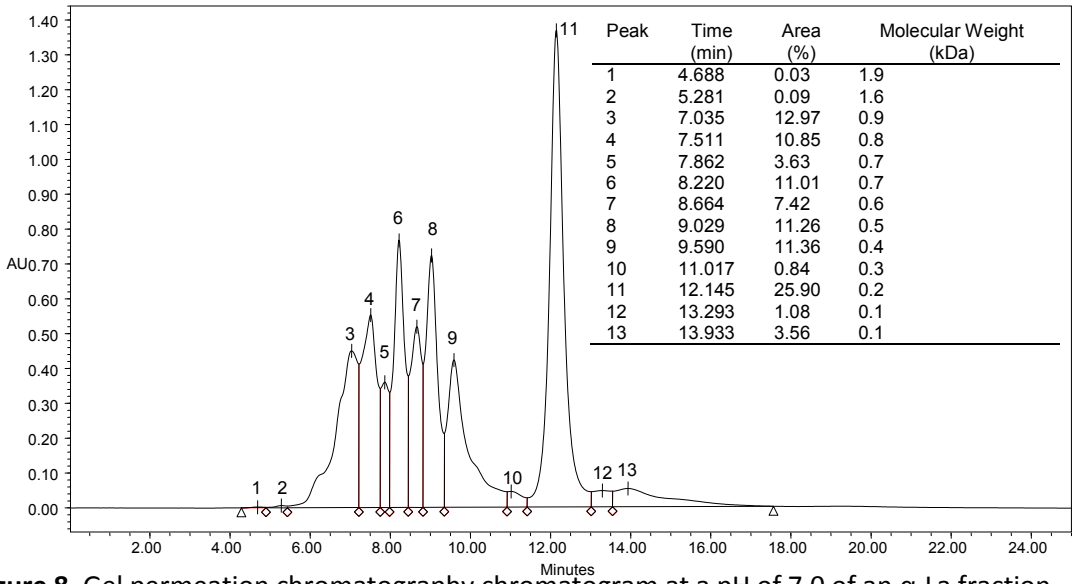


Figure 7. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 10 kDa membrane and the retentate of a 1 kDa membrane of an α-La fraction hydrolyzed with flavourzyme for 4 h at a concentration of 10 mg/mL.

The molecular weights of the peaks are difficult to determine even with calibration as the shapes of the protein or peptide are unknown. Peptides and proteins which have not been reduced down to their primary structure will move through the stationary phase differently than those which have been reduced. This is primarily due to the linear sequence having much more

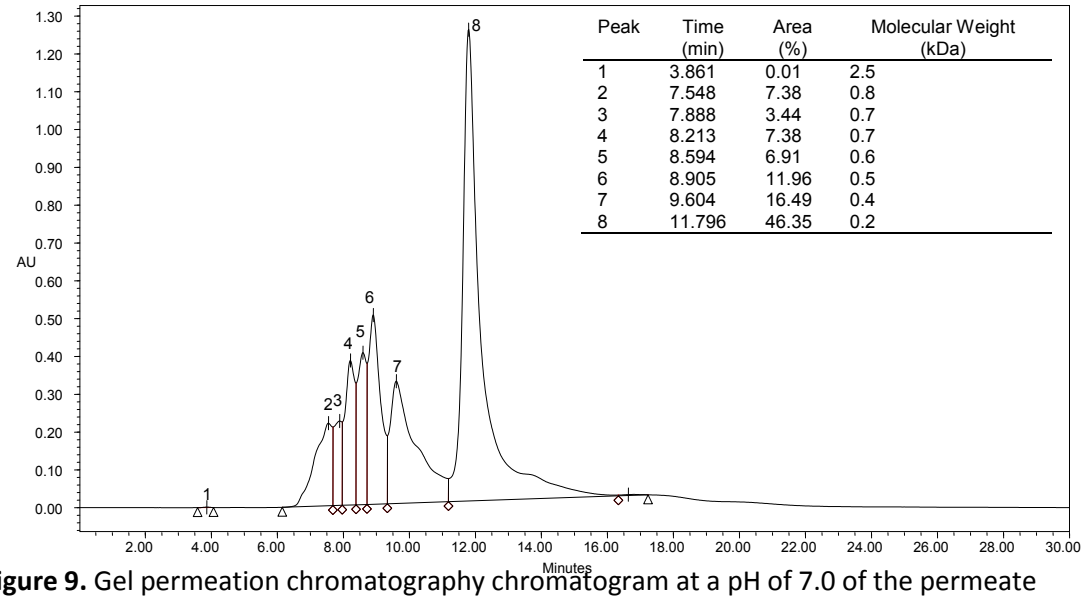
access to the pores within the stationary structure whereas those containing disulfide bonds will have less access therefore accessing fewer pores and eluting from the stationary phase more quickly than linear peptides.

α-La hydrolyzed with alcalase for 2 h followed by flavourzyme for 2 h shows similar molecular weight distribution when compared to flavourzyme alone (Figure 8). The hydrolysate contained 26 % of the peptides in the 0.2 kDa peak which most likely represents free amino acids. The other peaks are distributed between the molecular weights of 0.9 to 0.4 kDa. The 0.2 kDa peak was concentrated to represent 46 % of the peak area in the permeate of the 1 kDa membrane (Figure 9). This fraction consisted of peptides mainly (approximately 70 %) in the range of 0.5 to 0.2 kDa of which 24 % were in the peak identified as containing free amino acids. The 1 to 10 kDa fraction's molecular weight fractions contained 70 % of peptides in the 12.7 to 4.4 kDa range (Figure 10). Like the flavourzyme only fraction, the free amino acid peak was relatively minor.



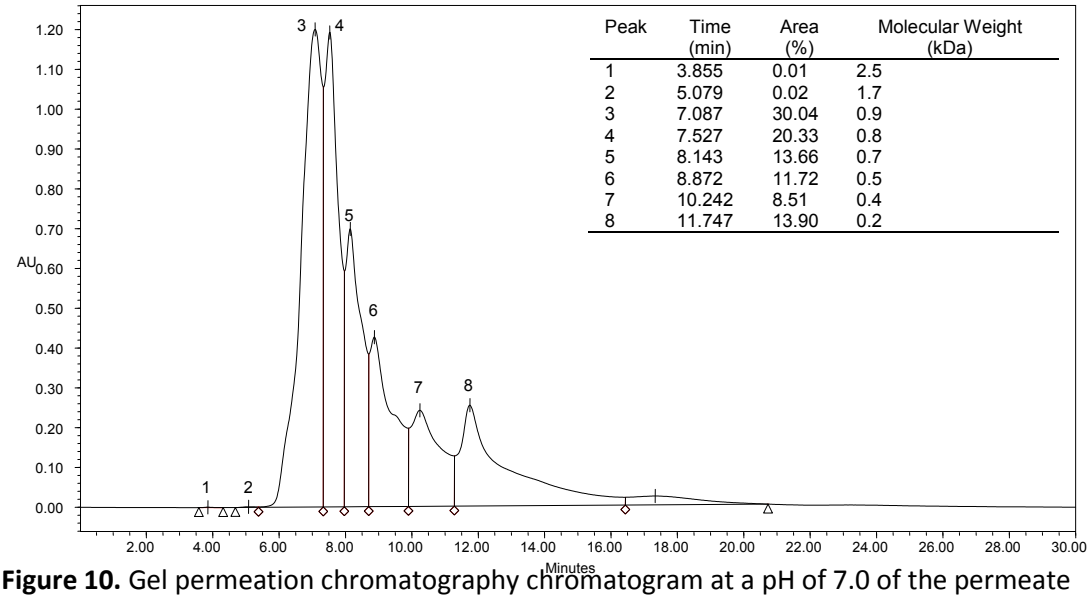
Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.688	0.03	1.9
2	5.281	0.09	1.6
3	7.035	12.97	0.9
4	7.511	10.85	0.8
5	7.862	3.63	0.7
6	8.220	11.01	0.7
7	8.664	7.42	0.6
8	9.029	11.26	0.5
9	9.590	11.36	0.4
10	11.017	0.84	0.3
11	12.145	25.90	0.2
12	13.293	1.08	0.1
13	13.933	3.56	0.1

Figure 8. Gel permeation chromatography chromatogram at a pH of 7.0 of an α-La fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	3.861	0.01	2.5
2	7.548	7.38	0.8
3	7.888	3.44	0.7
4	8.213	7.38	0.7
5	8.594	6.91	0.6
6	8.905	11.96	0.5
7	9.604	16.49	0.4
8	11.796	46.35	0.2

Figure 9. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 1 kDa membrane of an α-La fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL.

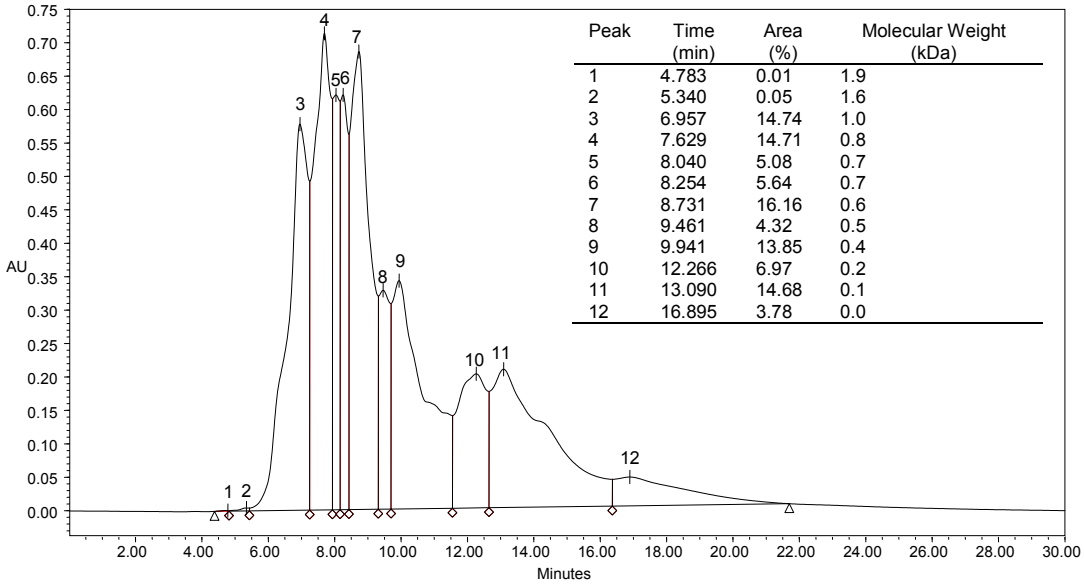


Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	3.855	0.01	2.5
2	5.079	0.02	1.7
3	7.087	30.04	0.9
4	7.527	20.33	0.8
5	8.143	13.66	0.7
6	8.872	11.72	0.5
7	10.242	8.51	0.4
8	11.747	13.90	0.2

Figure 10. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 10 kDa membrane and the retentate of a 1 kDa membrane of an α-La fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL

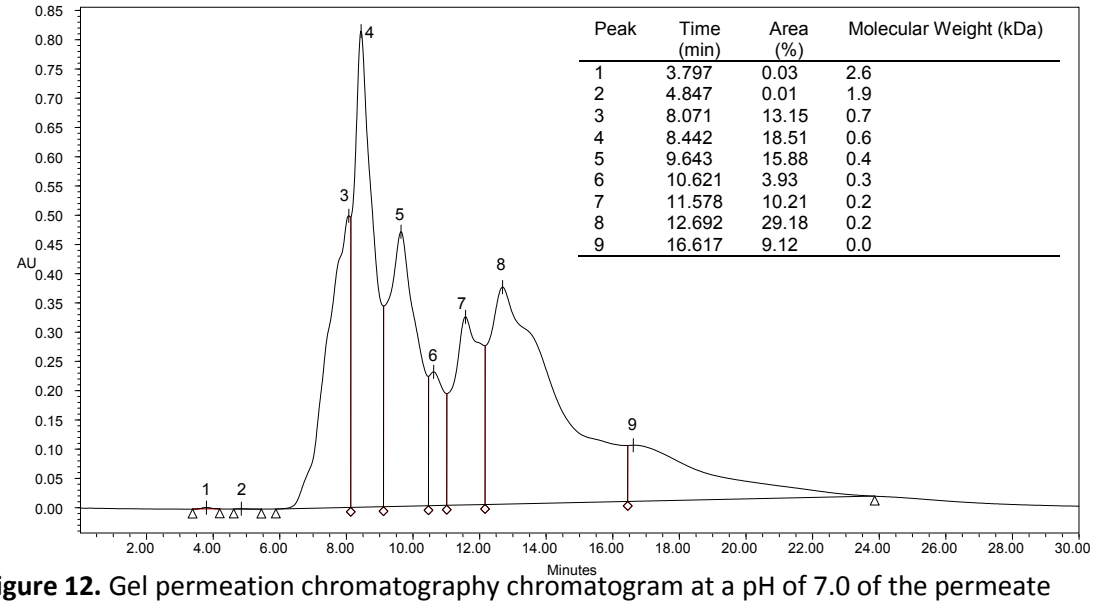
α-La hydrolyzed with alcalase showed a different molecular weight distribution than the alcalase flavourzyme combination hydrolysate. It contained one less peak and did not contain the large sharp peak of amino acids like the combination hydrolysate. The hydrolysate contained 5 peaks of varying molecular weight, each making up 15 % of the peak area as shown in Figure

11. The < 1 kDa fraction contains 40 % of peptides around the molecular weights of 0.2 kDa as shown in Figure 12. These may represent free amino acids due to their theoretical molecular weight. The 1 to 10 kDa fraction contained approximately 64 % of its peptides between the molecular weight of 14.9 and 6.2 kDa as shown in Figure 13. The possible free amino acid peak has been reduced in this fraction.



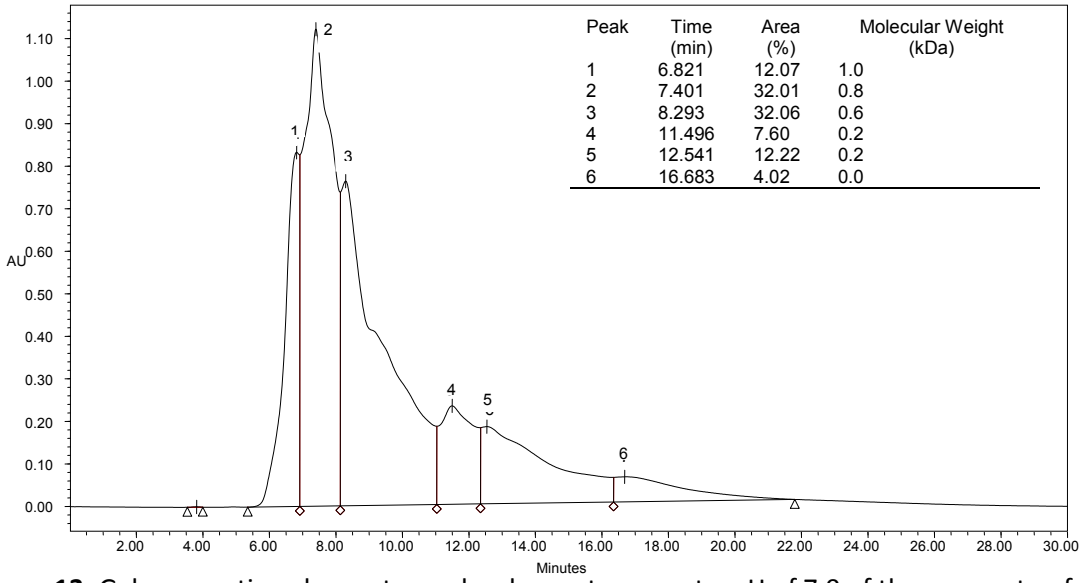
Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.783	0.01	1.9
2	5.340	0.05	1.6
3	6.957	14.74	1.0
4	7.629	14.71	0.8
5	8.040	5.08	0.7
6	8.254	5.64	0.7
7	8.731	16.16	0.6
8	9.461	4.32	0.5
9	9.941	13.85	0.4
10	12.266	6.97	0.2
11	13.090	14.68	0.1
12	16.895	3.78	0.0

Figure 11. Gel permeation chromatography chromatogram at a pH of 7.0 of an α-La fraction hydrolyzed with alcalase after 4 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	3.797	0.03	2.6
2	4.847	0.01	1.9
3	8.071	13.15	0.7
4	8.442	18.51	0.6
5	9.643	15.88	0.4
6	10.621	3.93	0.3
7	11.578	10.21	0.2
8	12.692	29.18	0.2
9	16.617	9.12	0.0

Figure 12. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 1 kDa membrane of an α-La fraction hydrolyzed with alcalase for 4 h at a concentration of 10 mg/mL.

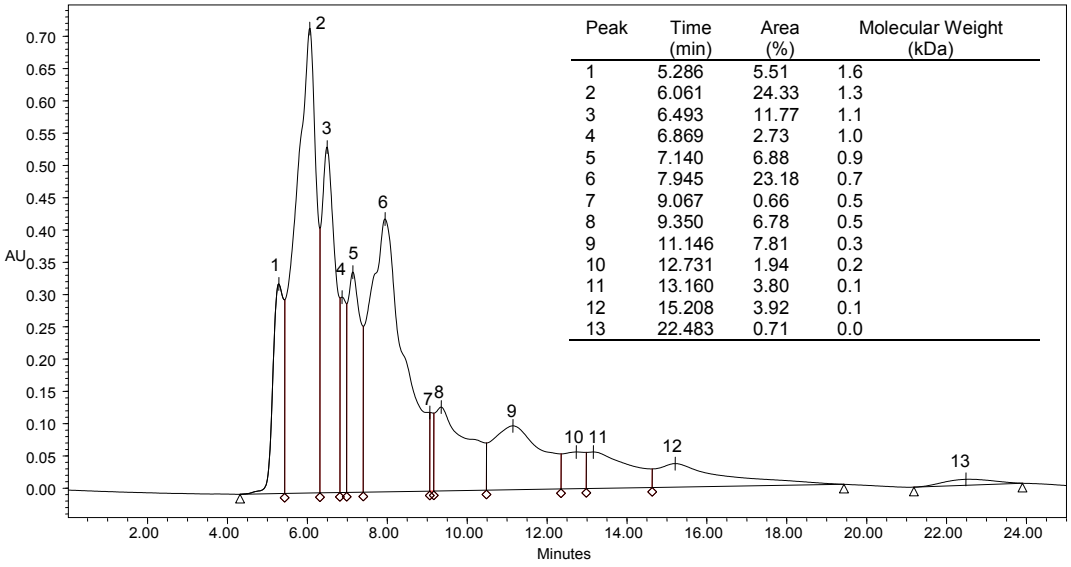


Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	6.821	12.07	1.0
2	7.401	32.01	0.8
3	8.293	32.06	0.6
4	11.496	7.60	0.2
5	12.541	12.22	0.2
6	16.683	4.02	0.0

Figure 13. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 10 kDa membrane and the retentate of a 1 kDa membrane of an α-La fraction hydrolyzed with alcalase for 4 h at a concentration of 10 mg/mL.

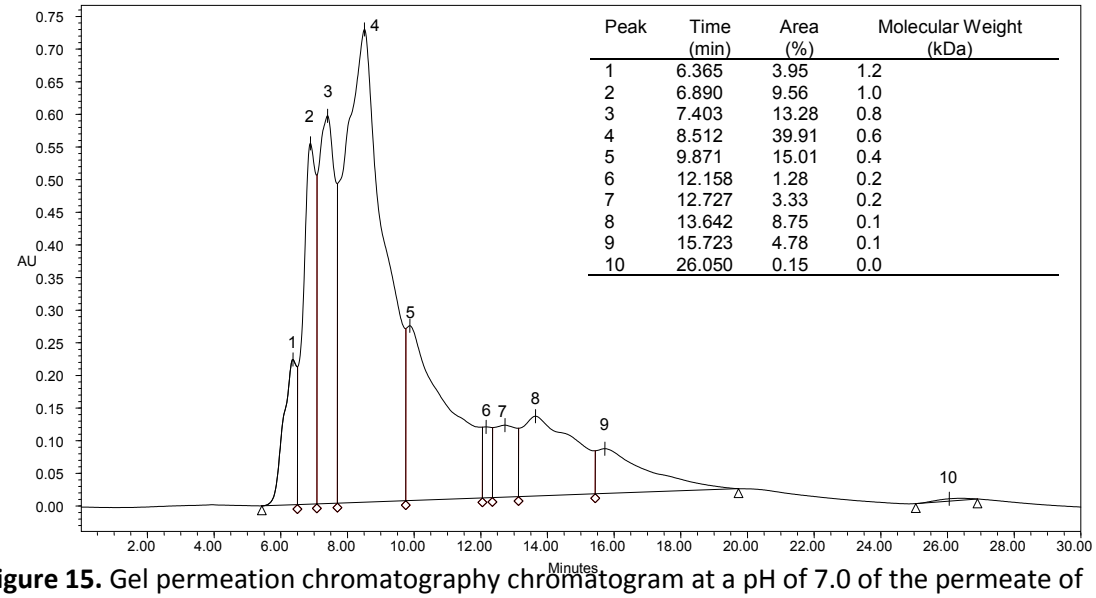
α-La hydrolyzed with trypsin contained a large number of smaller peaks with 2 peaks containing approximately 50 % of the peak area when combined as seen in Figure 14. This enzyme produced the least amount of free amino acids when compared to flavourzyme, alcalase, and the combination of alcalase and flavourzyme. The < 1 kDa fraction was composed

primarily of smaller peptides with about 50 % with a molecular weight range of 0.6 to 0.4 kDa (Figure 15). The hydrolysate also contained a number of low molecular weight broad peaks which probably make up the free amino acids from the hydrolysate. As expected, the 1 to 10 kDa fraction was composed primarily of larger molecular weight peptides with 70 % of the peptides in 3 peaks with a molecular weight range of 10.5 to 2.4 kDa as seem in Figure 16. These peaks have shoulder peaks on the sides of them which can probably be resolved by further fractionating the hydrolysate.



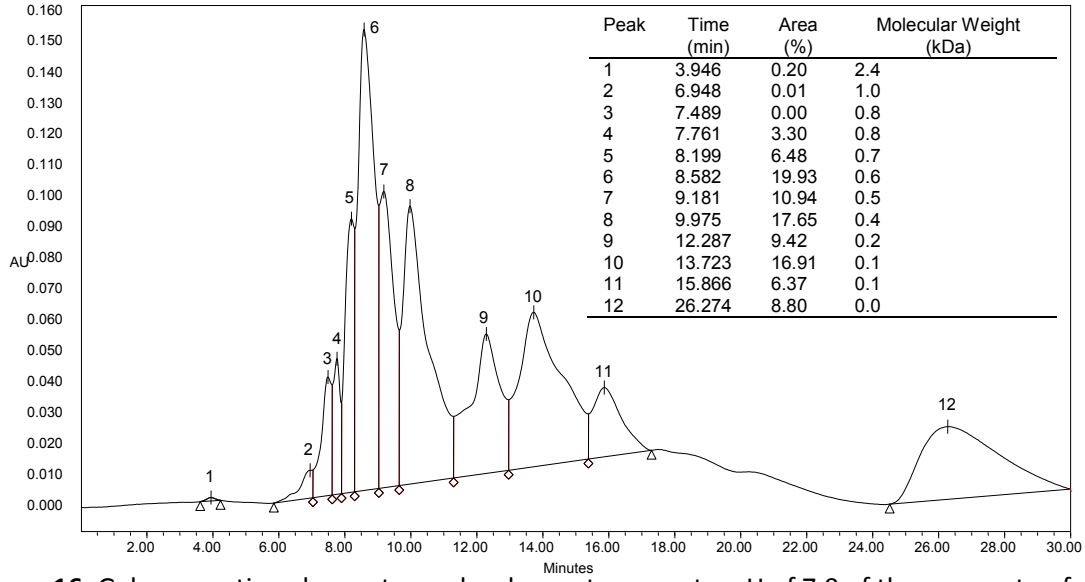
Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	5.286	5.51	1.6
2	6.061	24.33	1.3
3	6.493	11.77	1.1
4	6.869	2.73	1.0
5	7.140	6.88	0.9
6	7.945	23.18	0.7
7	9.067	0.66	0.5
8	9.350	6.78	0.5
9	11.146	7.81	0.3
10	12.731	1.94	0.2
11	13.160	3.80	0.1
12	15.208	3.92	0.1
13	22.483	0.71	0.0

Figure 14. Gel permeation chromatography chromatogram at a pH of 7.0 of an α-La fraction hydrolyzed with trypsin after 4 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	6.365	3.95	1.2
2	6.890	9.56	1.0
3	7.403	13.28	0.8
4	8.512	39.91	0.6
5	9.871	15.01	0.4
6	12.158	1.28	0.2
7	12.727	3.33	0.2
8	13.642	8.75	0.1
9	15.723	4.78	0.1
10	26.050	0.15	0.0

Figure 15. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 1 kDa membrane of an α-La fraction hydrolyzed with trypsin after 4 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	3.946	0.20	2.4
2	6.948	0.01	1.0
3	7.489	0.00	0.8
4	7.761	3.30	0.8
5	8.199	6.48	0.7
6	8.582	19.93	0.6
7	9.181	10.94	0.5
8	9.975	17.65	0.4
9	12.287	9.42	0.2
10	13.723	16.91	0.1
11	15.866	6.37	0.1
12	26.274	8.80	0.0

Figure 16. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 10 kDa membrane and the retentate of a 1 kDa membrane of an α-La fraction hydrolyzed with trypsin for 4 h at a concentration of 10 mg/mL.

All of the enzymes used in this experiment produced a similar number of peptide peaks as examined by gel permeation chromatography. Of these enzymes flavourzyme produced peaks with the smallest molecular weights, however the combination of flavourzyme and alcalase produced a similar peak. This indicates that hydrolysis with alcalase prior to hydrolysis with flavourzyme does not increase the activity of flavourzyme. Most researchers who have hydrolyzed whey proteins in the past have failed to fractionate the proteins prior to hydrolysis. The majority of the literature utilizing fractionated proteins has focused on the hydrolysis of major casein and whey proteins. As whole whey protein is primarily composed of β-Lg, results derived from whey as a whole cannot be accurately compared to the results obtained utilizing the α-La fraction of whey. All of the enzymes potentially produced bioactive peptides. However as hydrolysis increased, the concentration of free amino acids increased. Bioactive peptides generally contain 2 to 10 amino acids, although some research has indicated as many as 20 amino acids in a chain can be bioactive (Henle, 2005; Janin, 2003; Krause *et al*, 2006; Siemion *et al*, 2005). Therefore the presence of amino acids in the hydrolysates may decrease the bioactivity as a whole due to decreased concentration of bioactive peptides. This indicates that alcalase provided an appropriate level of hydrolysis without too high a concentration of free amino acids. Therefore this enzyme should provide the highest bioactivity even though flavourzyme hydrolysates had lower molecular weight peaks and a higher degree of hydrolysis. This may be due to the fact that flavourzyme has some exopeptidase activity which results in a larger number of free amino acids than treatment with alcalase.

The combination of flavourzyme and alcalase produced a number of peaks similar to the individual enzymes. This indicates that alcalase did not increase the number of cleavage sites available for flavourzyme to hydrolyze. However because of the combination of enzymes and

the difference between the cleavage sites, subtle differences in peptides produced exist when compared to either flavourzyme or alcalase alone.

While other combinations of enzymes were not utilized during these experiments, hydrolyzing α-La with either alcalase or flavourzyme followed by trypsin may have increased the number of cleavage sites for trypsin which could produce different peptides than those produced in this research without increasing the degree of hydrolysis too much like flavourzyme utilized alone does. It would be helpful to identify peptides by coupling gel permeation chromatography to MS to more accurately identify the molecular weights of the peptides. Further fractionation of peptides which contain less than 20 but more than 2 amino acids help identify the peptides which elicit the highest response.

The 1 to 10 kDa fractions were mainly composed of larger peptides and the < 1 kDa fraction was composed primarily of small peptides. It is expected that most of the bioactive peptides would be contained in the < 1 kDa fraction as the molecular weight range of peptides containing 2 to 10 amino acids is 0.22 kDa to 1.1 kDa. However this fraction would also contain salts produced during acid hydrolysis and any free amino acids. Both of these contaminants would decrease the bioactivity of the samples by decreasing the concentration of the bioactive peptides. It is also possible that some of the bioactive peptides will be larger in size and therefore found in the 1 to 10 kDa fraction. Some of the smaller peptides which contain the disulfide bonds may also be contained in the 1 to 10 kDa fraction due to the shape of the peptide. The linear peptides will pass through the 1 kDa membrane much easier than a globular protein. Non linear peptides may not pass through the membrane at all unless they are very small. An additional desalting step may have removed salt as a contaminant. This could potentially increase bioactivity, especially at a cellular level as too much salt can lead to cell

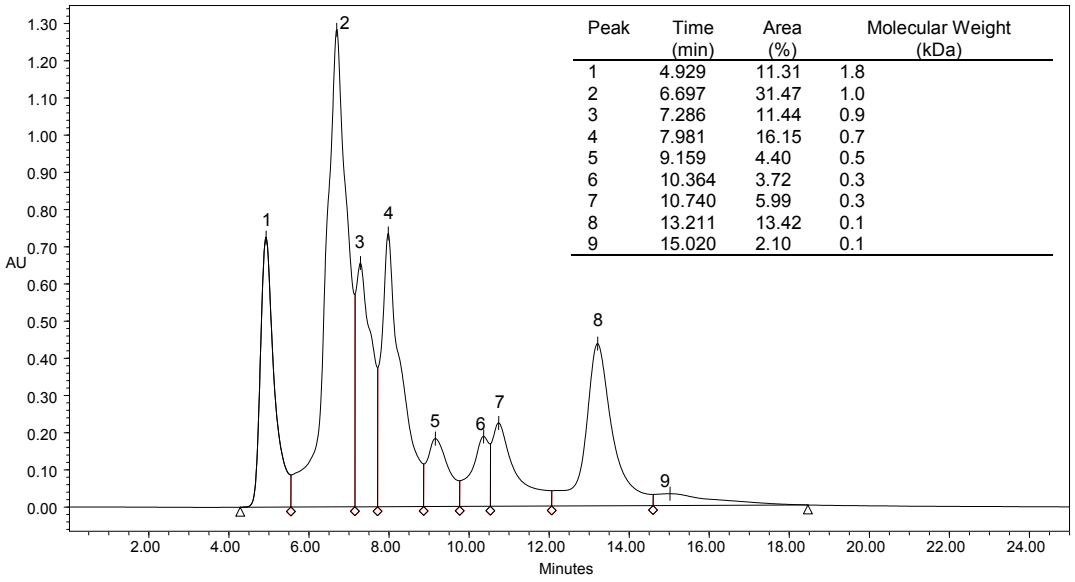
toxicity. Further fractionation of the 1 to 10 kDa fraction into a 1 to 3 kDa fraction and a 3 to 5 kDa fraction could lead to identification of larger molecular weight peptides with bioactivity.

β-Lg hydrolyzed with trypsin produced a large peak at 18.8 kDa which represented 31 % of the hydrolysate as shown in Figure 17. This peak potentially represents unhydrolyzed or minimally hydrolyzed β-Lg. As β-Lg acts as a dimer during gel permeation chromatography, this indicates that the hydrolysis with trypsin probably minimally cleaved the protein changing its ability to act as a dimer at a pH of 7.

The < 1kDa fraction of this hydrolysate contains a primary peak with a molecular weight of 0.2 kDa which represents 42 % of the peptides in the fraction as shown in Figure 18. As noted previously this peak potentially represents individual amino acids. A secondary peak was also identified in the hydrolysate making up about 21 % of the fraction with a molecular weight of 0.7 kDa. Most likely these peptides are made up of less than 10 amino acids but more than 2, and therefore should be capable of providing some bioactivity within a cell.

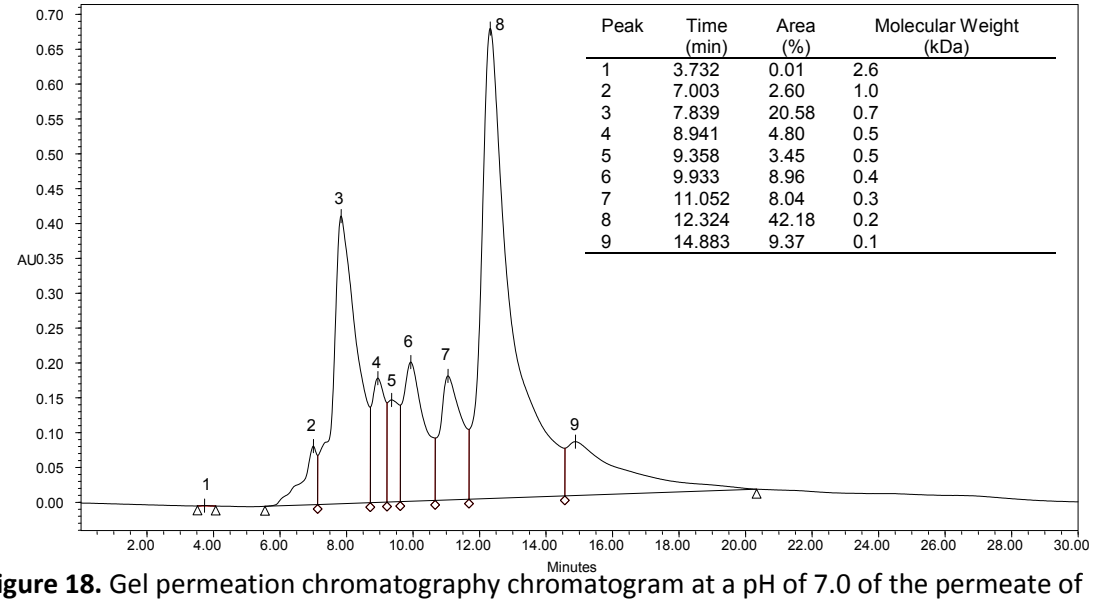
The 1 to 10 kDa fraction contains one primary peak which represents 53 % of the peak area of the fraction (Figure 19). This peak most likely represents unhydrolyzed β-Lg due to the fact that the molecular weight of the protein is lower than the molecular weight of the membrane. However this protein has been acting as a dimer during gel permeation chromatography and this peak may actually represent a slightly hydrolyzed version of the protein which decreased its ability to react as a dimer with other β-Lg proteins at a pH of 7. Unhydrolyzed protein may have eluted into this fraction as cellulose membranes can only retain proteins that are 2 times larger than the membrane's molecular weight cut-off. This means that some of the pores in the membrane will allow proteins up to 20 kDa to pass through. Work done by another research group determined that after 30 minutes a large amount of the β-Lg found in the sample had

been hydrolyzed forming 16 to 20 peaks (Madsen *et al*, 1997). Peaks increased in concentration up to 4 h but further hydrolysis only produced 3 more peaks after 24 h. Of the peaks examined, it was found that more than one peptide was contained within the peak area of the chromatogram. In the current work we found that 9 peaks were produced after 4 h of hydrolysis. It is probable that more than one peptide is contained in the peaks produced by gel permeation chromatography. The researchers in the Madsen (Madsen *et al*, 1997) study did not utilize peptide standards for calibration. Therefore their molecular weights may be inaccurate as peptides are linear and β-Lg is globular and therefore they would act differently when passing in and out of the pores of the stationary phase.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.929	11.31	1.8
2	6.697	31.47	1.0
3	7.286	11.44	0.9
4	7.981	16.15	0.7
5	9.159	4.40	0.5
6	10.364	3.72	0.3
7	10.740	5.99	0.3
8	13.211	13.42	0.1
9	15.020	2.10	0.1

Figure 17. Gel permeation chromatography chromatogram at a pH of 7.0 of a β-Lg fraction hydrolyzed with trypsin after 4 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	3.732	0.01	2.6
2	7.003	2.60	1.0
3	7.839	20.58	0.7
4	8.941	4.80	0.5
5	9.358	3.45	0.5
6	9.933	8.96	0.4
7	11.052	8.04	0.3
8	12.324	42.18	0.2
9	14.883	9.37	0.1

Figure 18. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 1 kDa membrane of a β-Lg fraction hydrolyzed with trypsin for 4 h at a concentration of 10 mg/mL.

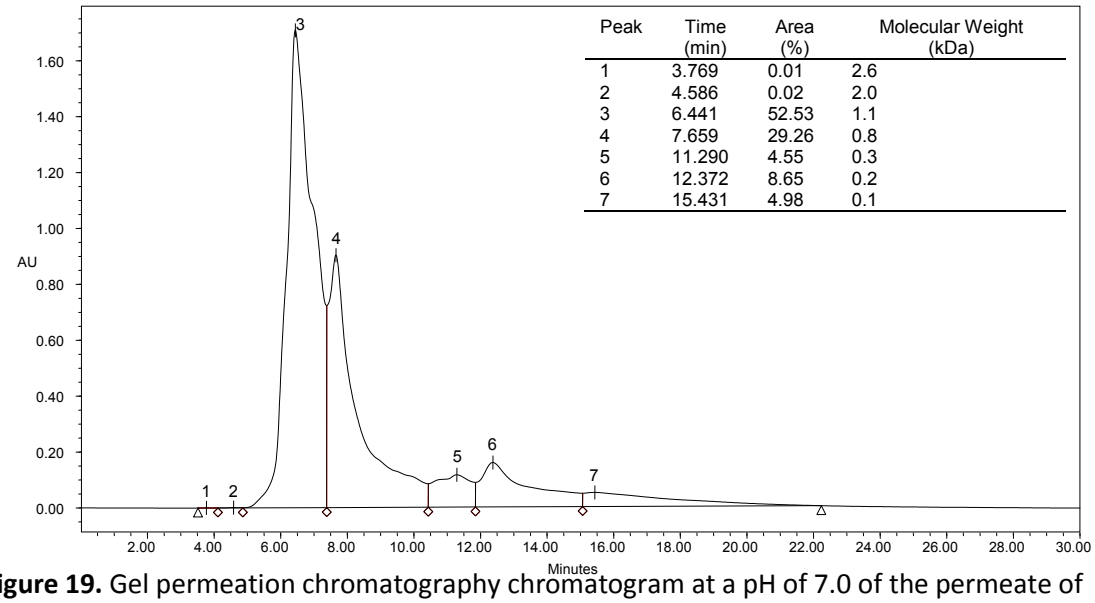
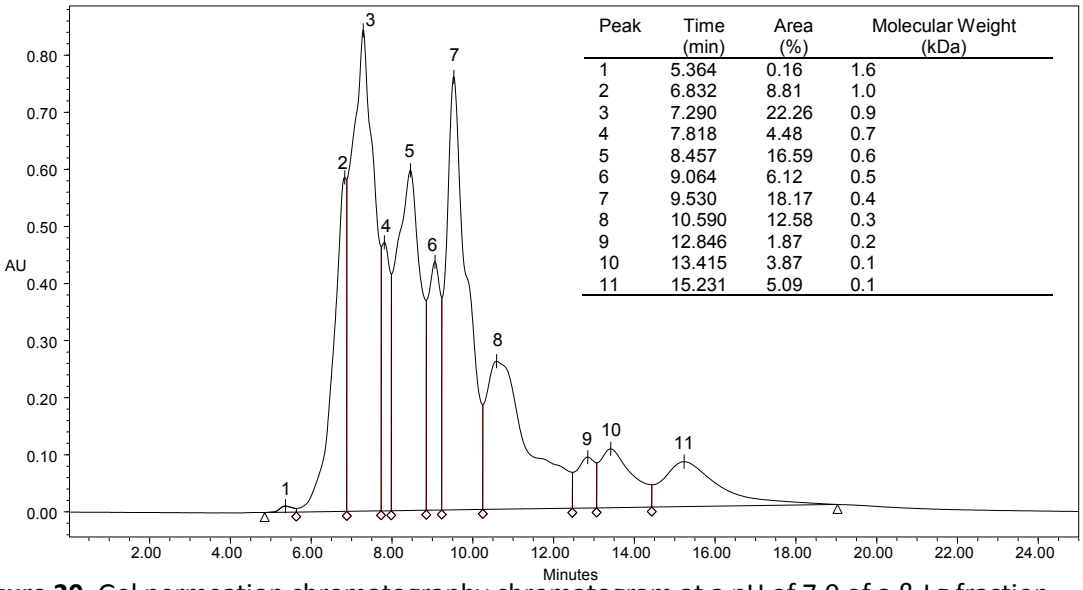


Figure 19. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 10 kDa membrane and the retentate of a 1 kDa membrane of a β-Lg fraction hydrolyzed with trypsin for 4 h at a concentration of 10 mg/mL.

β-Lg hydrolyzed with alcalase, the enzyme from *Bacillus lichenformis*, produced four main peaks with various molecular weights, each making up between 13 % and 22 % of the peak area of the gel permeation chromatography chromatogram as shown in Figure 20. In addition, small molecular weight peptides were produced which may be primarily free amino acids but their peak areas were quite low therefore not making up a major part of the hydrolysate. These free amino acids make up about 18 % of the < 1 kDa fraction as shown in Figure 21. The 0.5 kDa peak seen in the chromatogram for the hydrolysate was concentrated in the permeate of the 1 kDa fraction and represented approximately 30 % of the total peak area of this chromatogram. This peak probably represents peptides with very small molecular weights and chain lengths; however, the molecular weight is large enough that most likely no free amino acids are present in this peak. Approximately 70 % of the peptides in this fraction have a molecular weight between 0.3 and 0.7 kDa. All of the peptides in these peaks have the ability to potentially impart bioactive functions within cells.

The 1 to 10 kDa fraction contained 1 major peak with 30 % of the peptides in one peak with a molecular weight of 14.1 kDa as shown in Figure 22. Two other peaks were seen in the chromatogram. These represented approximately 13 % to 17 % of the total peaks in the fraction with molecular weights of 4.1 kDa and 8.5 kDa. These peaks contained partially hydrolyzed β-Lg. Most likely the enzyme can completely hydrolyze the β-sheet section of the protein and then have difficulty accessing the bonds within the α-helix of the portion of the protein due to the presence of disulfide bonds (Thompson *et al*, 2009). This would cause partially hydrolyzed proteins and peptides to be formed in the solution. Research conducted by Papiz *et al* in 1986 determined that the larger molecular weight proteins and peptides would most likely break down with increased hydrolysis time. The protein was found to be readily hydrolyzed with this proteinase due to the presence of Glu and Asp residues being mainly found in the β-sheet of β-Lg (Creamer *et al*, 1983). This would give the proteinase access due to the amino acid cleavage sites at the surface of the protein structure. Research completed by Madsen *et al* in 1997 determined that the majority of the peptides formed from the hydrolysis of β-Lg had a molecular weight of 2 kDa. The formation of free amino acids during hydrolysis can be attributed to the Glu and Asp affinity of the alcalase.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	5.364	0.16	1.6
2	6.832	8.81	1.0
3	7.290	22.26	0.9
4	7.818	4.48	0.7
5	8.457	16.59	0.6
6	9.064	6.12	0.5
7	9.530	18.17	0.4
8	10.590	12.58	0.3
9	12.846	1.87	0.2
10	13.415	3.87	0.1
11	15.231	5.09	0.1

Figure 20. Gel permeation chromatography chromatogram at a pH of 7.0 of a β-Lg fraction hydrolyzed with alcalase for 4 h at a concentration of 10 mg/mL.

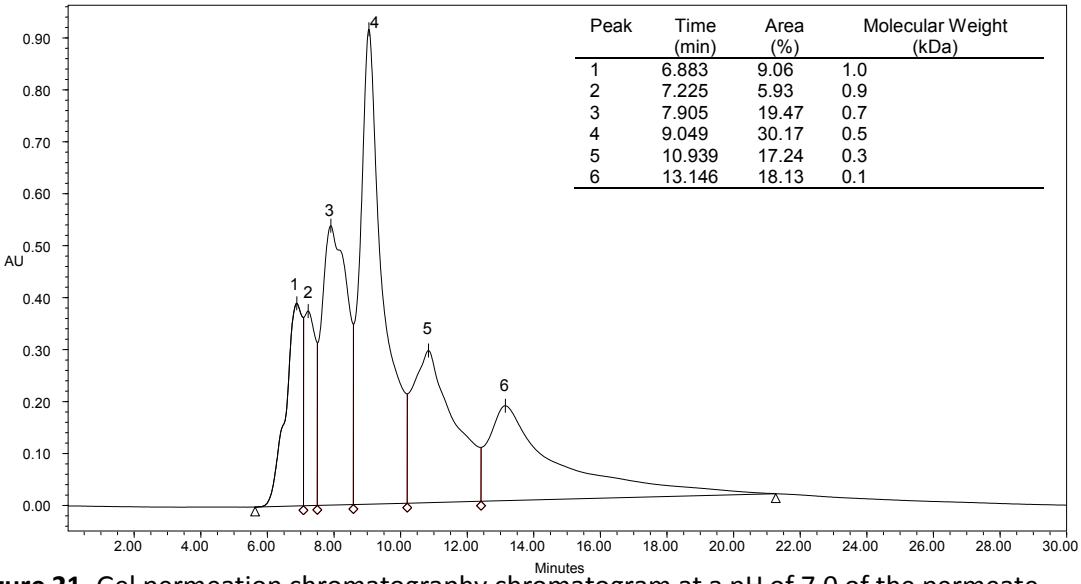


Figure 21. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 1 kDa membrane of a β-Lg fraction hydrolyzed with alcalase for 4 h.

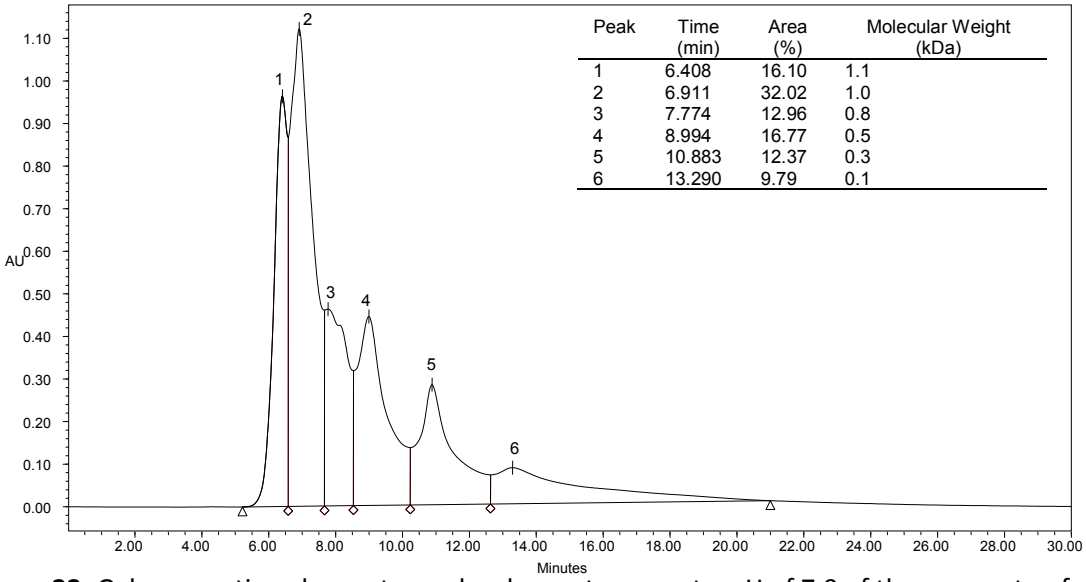


Figure 22. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate of a 10 kDa membrane and the retentate of a 1 kDa membrane of a β-Lg fraction hydrolyzed with alcalase for 4 h at a concentration of 10 mg/mL.

β-Lg was readily hydrolyzed with the flavourzyme, derived from *Aspergillus oryzae,* due to its combination of exopeptidase and endopeptidase activity. The hydrolysate shown in Figure 23 contained 27 % of the peptides in a single peak. This peak was broad with upwards of 2 shoulders to the right of the peak. This made the molecular weight of this peak difficult to

determine as peaks eluted with similar retention times in both the 1 kDa to 10 kDa and < 1 kDa fractions. The < 1 kDa fraction contained a lot of the peaks from the hydrolysate which were seen as shoulders in the original hydrolysate (Figure 24). There were 5 peaks in this fraction, with the four main peaks each representing 13 % to 32 % of the peak area and having molecular weights of 0.3 kDa to 0.9 kDa. The 1 kDa to 10 kDa fractions shown in Figure 25 contained larger molecular weight peptides with 85 % of these peptides having molecular weights ranging from 18.0 kDa to 13.8 kDa. These may actually be smaller than calculated using the globular molecular weight standards as small molecular weight proteins can be retained in the stirred cell ultrafiltration system due to concentration polarization. In this situation proteins interact causing a layer of protein to build up on the surface of the membrane; smaller peptides cannot pass through this protein and therefore cannot pass through the membrane. No research has been reported on the effects of this enzyme on the hydrolysis of β-Lg.

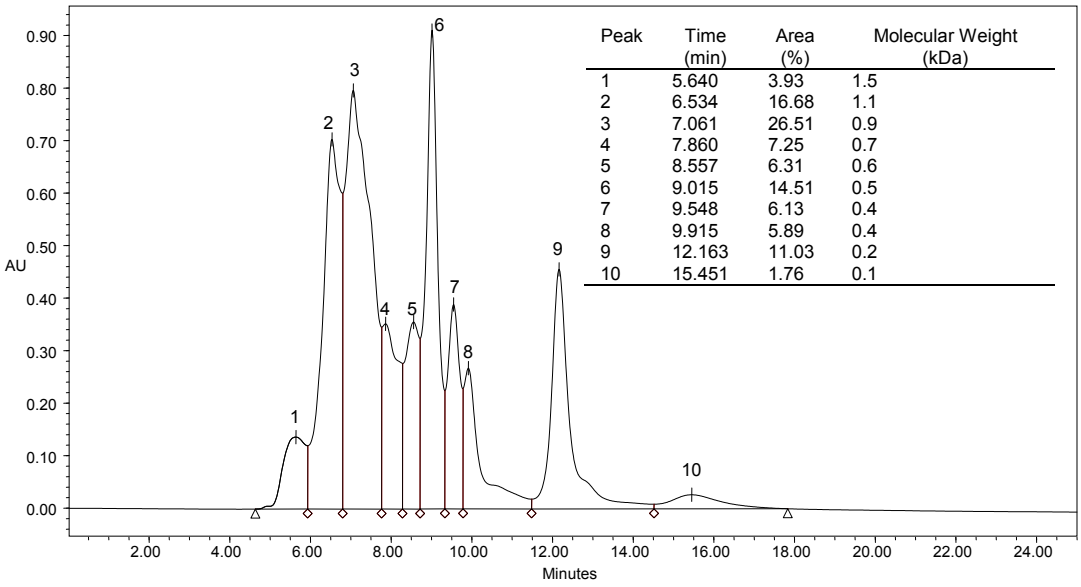
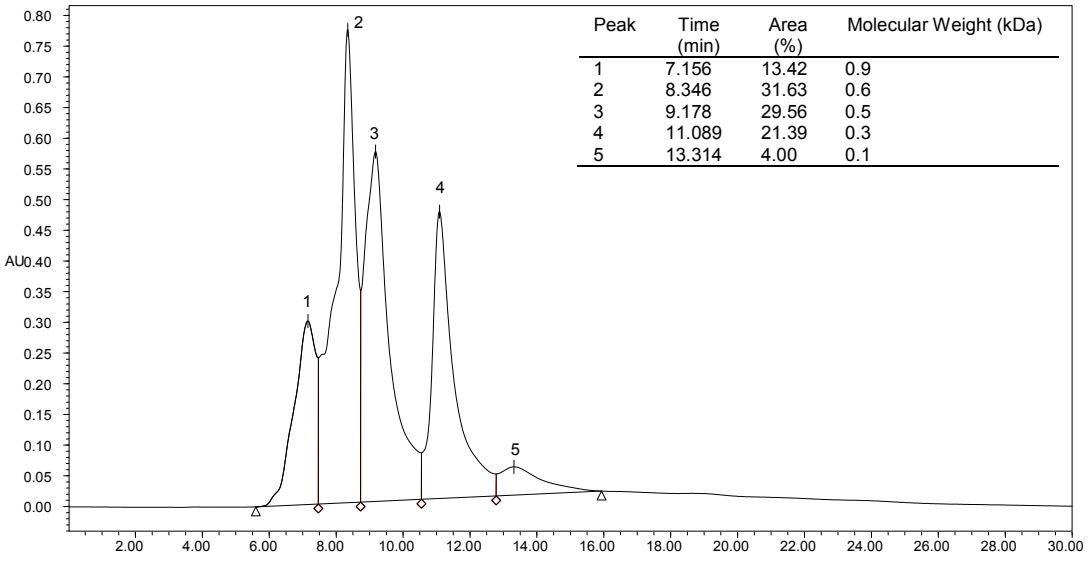
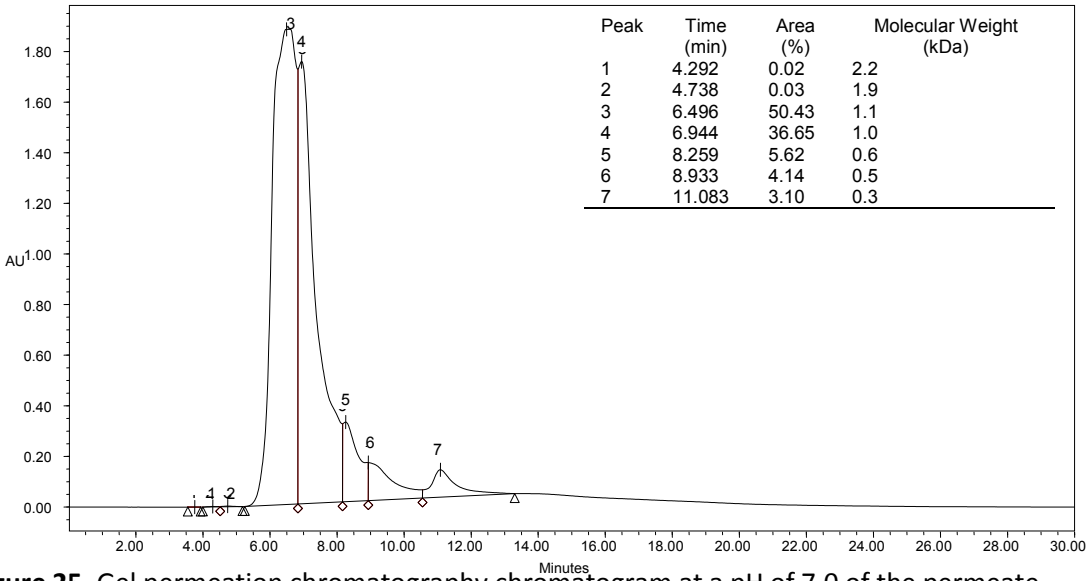


Figure 23. Gel permeation chromatography chromatogram at a pH of 7.0 of a β-Lg fraction hydrolyzed with flavourzyme for 4 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	7.156	13.42	0.9
2	8.346	31.63	0.6
3	9.178	29.56	0.5
4	11.089	21.39	0.3
5	13.314	4.00	0.1

Figure 24. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 1 kDa membrane of a β-Lg fraction hydrolyzed with flavourzyme for 4 h at a concentration of 10 mg/mL.

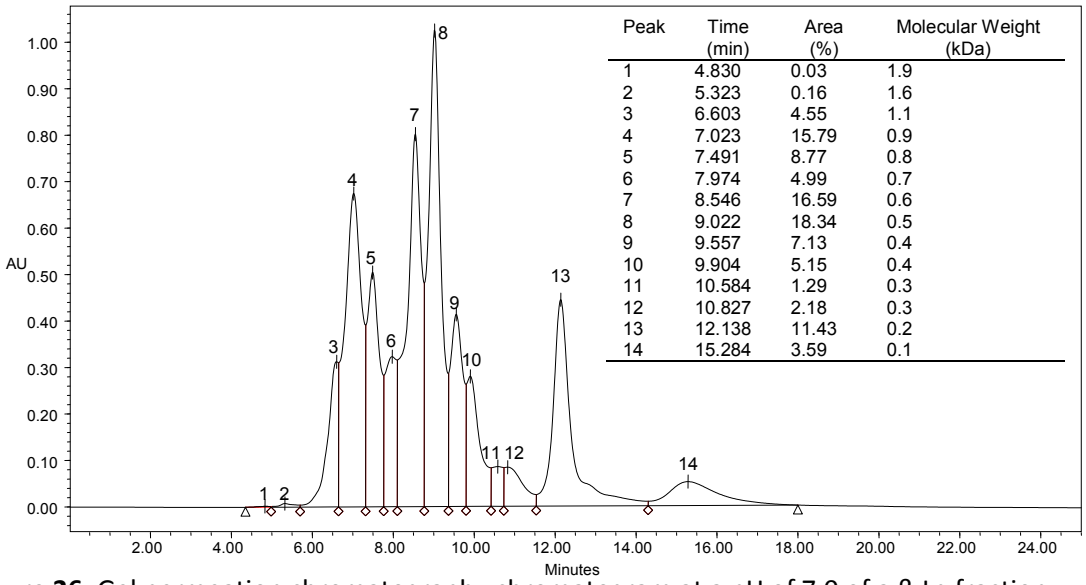


Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.292	0.02	2.2
2	4.738	0.03	1.9
3	6.496	50.43	1.1
4	6.944	36.65	1.0
5	8.259	5.62	0.6
6	8.933	4.14	0.5
7	11.083	3.10	0.3

Figure 25. Gel permeation chromatography chromatogram at a pH of 7.0 of the permeate from a 10 kDa membrane and the retentate of a 1 kDa membrane of a β-Lg fraction hydrolyzed with flavourzyme for 4 h at a concentration of 10 mg/mL.

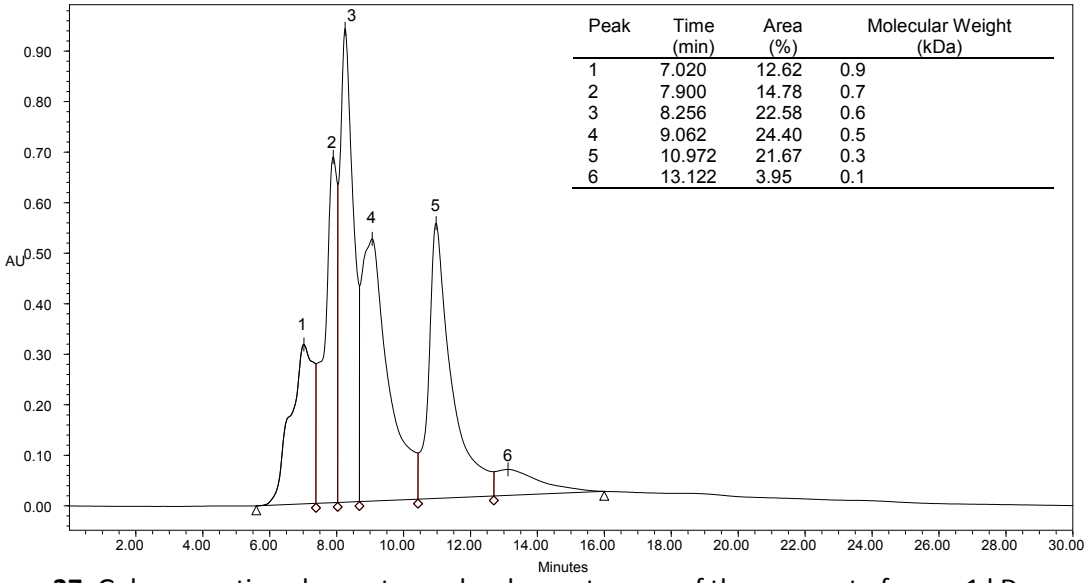
β-Lg hydrolyzed with alcalase for 2 h and flavourzyme for 2 h produced a peptide hydrolysate which contained 14 peaks as seen in Figure 26. The hydrolysate had 4 major peaks which were distributed throughout the hydrolysate. There are 2 very low molecular weight peaks which represented approximately 15 % of the hydrolysate and most likely consist of

individual amino acids. The < 1 kDa fraction contained 3 peaks and each peak contained 20 % or more of the peptides as shown in Figure 27. Theses peaks are small with molecular weights between 0.3 kDa and 0.6 kDa. The 1 kDa to 10 kDa fraction shown in Figure 28 consisted of 2 major peaks with about 37 % of the peak area each. The molecular weights of these peaks were 18.6 kDa and 14.2 kDa which are close to the molecular weights of β-Lg and α-La respectively. This fraction most likely corresponds with unhydrolyzed and minimally hydrolyzed starting material as a membrane with a molecular weight cut-off of 10 kDa can allow proteins with a molecular weight as large as 20 kDa to elute through the membrane. This means that the majority of the peptides are small due to the extensive hydrolysis resulting from this combination of enzymes. This also increases the likelihood that free amino acids exist in the hydrolysate due to over hydrolysis of the β-sheet (Creamer *et al*, 1983) due to the location and small number of the disulfide bonds (Thompson *et al*, 2009). Based on the increase in the number of peptide peaks when using the combination of flavourzyme and alcalase, it would appear that hydrolysis with alcalase followed by flavourzyme increases the number of accessible sites for flavourzyme. No research could be found which utilizes a combination of bacterial enzymes to hydrolyze dairy proteins.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	4.830	0.03	1.9
2	5.323	0.16	1.6
3	6.603	4.55	1.1
4	7.023	15.79	0.9
5	7.491	8.77	0.8
6	7.974	4.99	0.7
7	8.546	16.59	0.6
8	9.022	18.34	0.5
9	9.557	7.13	0.4
10	9.904	5.15	0.4
11	10.584	1.29	0.3
12	10.827	2.18	0.3
13	12.138	11.43	0.2
14	15.284	3.59	0.1

Figure 26. Gel permeation chromatography chromatogram at a pH of 7.0 of a β-Lg fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL.



Peak	Time (min)	Area (%)	Molecular Weight (kDa)
1	7.020	12.62	0.9
2	7.900	14.78	0.7
3	8.256	22.58	0.6
4	9.062	24.40	0.5
5	10.972	21.67	0.3
6	13.122	3.95	0.1

Figure 27. Gel permeation chromatography chromatogram of the permeate from a 1 kDa membrane of a β-Lg fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL.

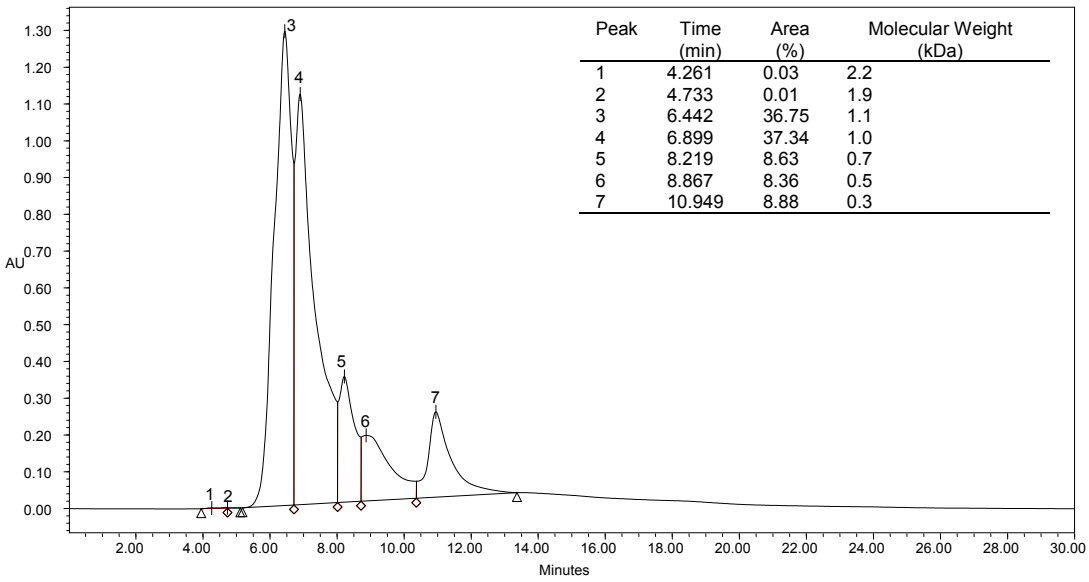


Figure 28. Gel permeation chromatography chromatogram of the permeate from a 10 kDa membrane and the retentate of a 1 kDa membrane of β-Lg fraction hydrolyzed with alcalase for 2 h followed by flavourzyme for an additional 2 h at a concentration of 10 mg/mL.

Overall, α-La was more resistant to hydrolysis than β-Lg, most likely due to the presence of a β-sheet (Creamer *et al*, 1983) and the location and number of the disulfide bonds (Thompson *et* al, 2009) in the structure of β-Lg and an increased number of disulfide bonds in the structure of α-La. Hydrolysis of both proteins potentially leads to the production of a large amount of free amino acids which have the potential to decrease the bioactivity of peptides by decreasing their concentration. Fractionation using chromatography to isolate potentially bioactive peptides would be beneficial in future research.

4.2 Angiotensin Converting Enzyme Inhibition

The ACE inhibition assay utilizes the substrate HHL, a tripeptide, and measures the formation of Hippuric acid in the presence of the catalyst ACE (Cushman & Cheung, 1971). This allows for the determination of how the activity of ACE is affected by the presence of a substance and ultimately provides an indication of the effect on blood pressure. This is due to ACE's role as a catalyst in the conversion of ACE-I to ACE-II and the inactivation of bradykinin

which are both known to be involved in the regulation of blood pressure in the kinin-kallikrein system (Shihabi & Scaro, 1981).

The protein extracts were tested to determine if they had an effect on the ACE inhibition activity. ACE activity was not significantly different from zero for both β-Lg and α-La. The hydrolysates and the permeates from the two membrane fractionation steps were also tested for their ACE inhibitory abilities (Figures 29 and 30). For α-La, the original hydrolysates and the < 1 kDa fraction from both alcalase and trypsin hydrolysis, as well as the 1 to 10 kDa fraction for trypsin inhibited approximately 90 % of ACE. The < 1 kDa fraction of all hydrolysates except those derived from flavourzyme showed the same if not increased ACE inhibition when compared to the 1 kDa to 10 kDa fraction. The 1 kDa to 10 kDa fraction for alcalase also showed a high level of inhibition at 85 %. While alcalase resulted in high ACE inhibition activity, flavourzyme hydrolysates displayed the lowest inhibitory activity. When using a combination of flavourzyme and alcalase, more of the activity was found in the permeate of the 1 kDa membrane with about 80 % inhibition whereas the 1 kDa to 10 kDa fraction inhibited about 65 % of ACE. This may be related to the partial exopeptidase activity of flavourzyme and the subsequent production of free amino acids.

Hydrolysates formed from the substrate β-Lg similarly showed that alcalase provided the highest ACE inhibition followed by the combination of alcalase and flavourzyme when comparing the initial hydrolysates. Fractionating the trypsin hydrolysate, which had an ACE inhibition of about 77 %, produced a higher level of activity in the < 1 kDa fraction with an ACE inhibition of about 85 % and lower activity in the 1 kDa to 10 kDa fraction (69 %). The trypsin fractions both produced higher activity when compared to flavourzyme. For all treatments, the 1 kDa to 10 kDa fraction had the lowest ACE inhibition. There were no difference seen between

the hydrolysates and the < 1 kDa fraction for both flavourzyme and the combination of alcalase and flavourzyme. Fractionation of the alcalase hydrolysate caused a decrease in ACE inhibition for both fractions with the < 1 kDa fraction having 95 % inhibition and the 1 kDa to 10 kDa fraction having 81 % inhibition.

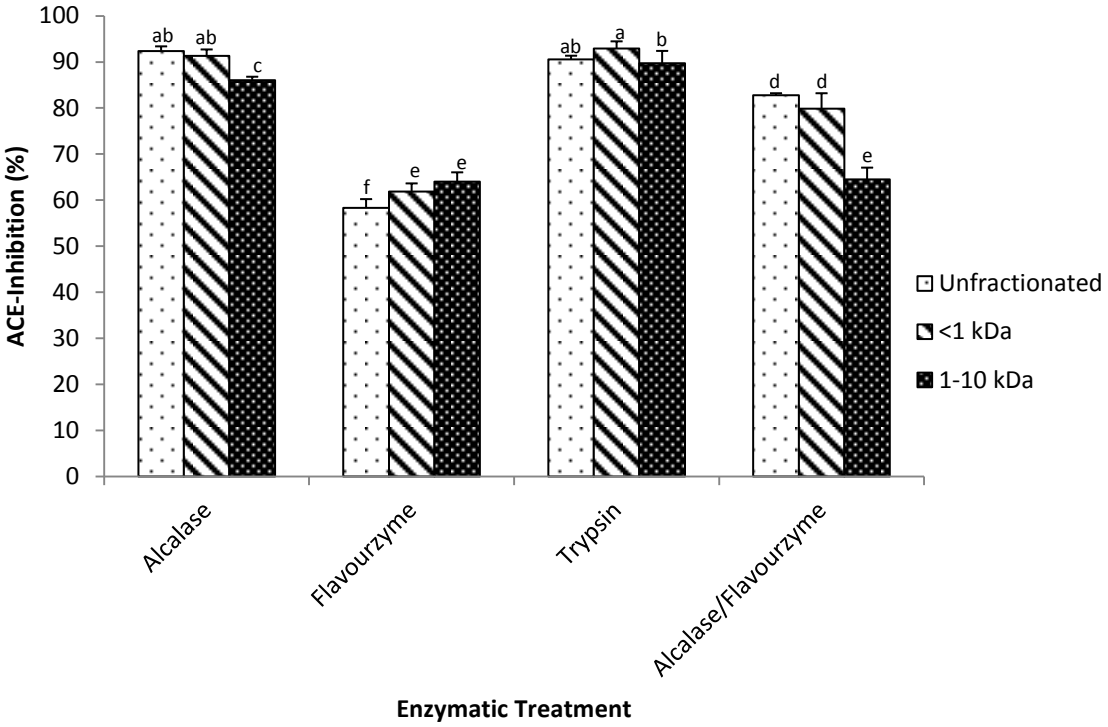


Figure 29. ACE-Inhibition of α-La extracts following hydrolysis and membrane fractionation. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

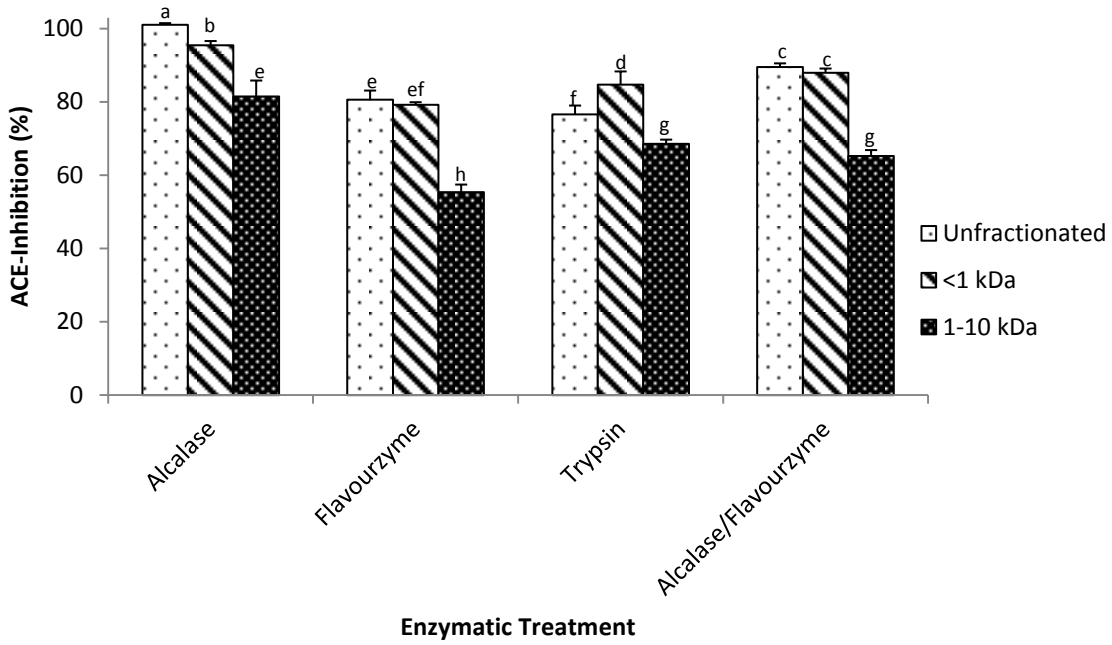


Figure 30. ACE-Inhibition of β-Lg extracts following hydrolysis and membrane fractionation. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

Other research has been conducted on these two proteins to determine their ACE inhibition activity following enzymatic hydrolysis. Research conducted by Otte *et al* in 2007 determined that α-La hydrolyzed with trypsin for 3 to 6 h produced ACE inhibitory activities of approximately 80 % and hydrolysates from alcalase hydrolysis produced ACE inhibition of 90 %. Otte *et al* (2007) also looked at both β-Lg A and B hydrolyzed with trypsin and alcalase. β-Lg A hydrolyzed with trypsin and alcalase produced ACE inhibitory activities of 80 % and 60 % respectively, whereas β-Lg B produced ACE inhibition of 75 % and 45 % respectively. The only results which are in accordance with the current research are those for the β-Lg hydrolyzed with trypsin. However the substrate utilized in Otte *et al's* (2007) research was N-[3-(2-Furyl)acryoyl]-Phe-Gly-Gly (FAPGG) as compared to the HHL used in the current study. This change in substrate may account for the differing results as FAPGG is more specific to ACE and may more closely replicate conditions *in vivo* (Gorski & Campbell, 1991). No information of the

ACE inhibition activity of flavourzyme or a combination of enzymes exists for whey but plenty of information exists for casein and components thereof. Researchers have not explored the potential of fractionating the peptides based on molecular weight to remove larger proteins and peptides as well as free amino acids in terms of the ACE inhibition of the bioactive peptides in dairy proteins.

ACE inhibition activities appear to correlate to the degree of hydrolysis for the α-La hydrolysates. As the degree of hydrolysis increased the ACE inhibition decreased. This is possibly due to the over hydrolysis of the protein with can lead to a large number of free amino acids in the solution. This is especially true for the < 1 kDa fraction and the initial hydrolysates. Due to the fact that the bioactive peptides are generally thought of as being 2 to 10 amino acids in length, a high degree of hydrolysis of small proteins, such as those found in whey, can be detrimental to the bioactivity. However isolation of specific peptides formed from hydrolysis may increase their activity due to the decreased presence of less bioactive substances. This however does not hold true for the peptides formed from β-Lg. With that being said, flavourzyme provided the highest degrees of hydrolysis but the lowest ACE inhibition overall. This is most likely attributed to the presence of amino acids due to extensive hydrolysis. In all cases peptides from alcalase showed the highest activity. This was followed by the combination of alcalase and flavourzyme. This is possibly due to the presence of a specific bioactive peptide with a strong affinity for ACE. Presumably it was present at a sufficient concentration to provide an increased level of activity even in the presence of flavourzyme. This may also indicate that this peptide or series of peptides which is formed from the hydrolysis with alcalase is not altered when followed by hydrolysis with flavourzyme. The decreased ACE inhibition due to the hydrolysis of trypsin may be due to the low hydrolysis of this protein. The peptides formed may be too large to produce sufficient inhibition of ACE. There is the possibility of unhydrolyzed

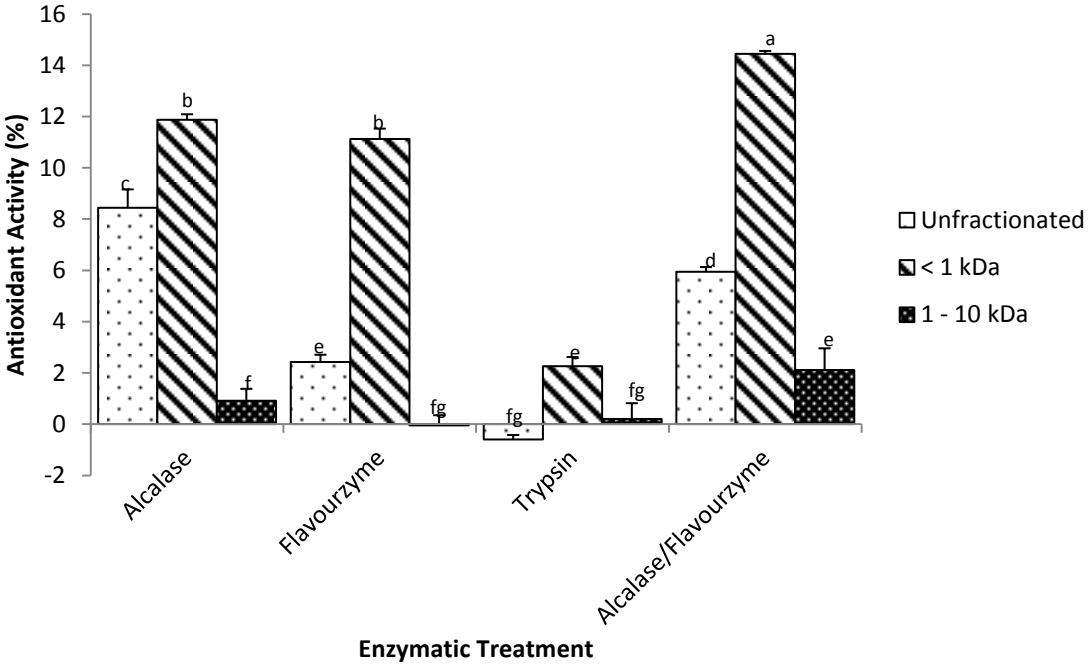
proteins present in the hydrolysate as well as the presence of free amino acids which will decrease the bioactivity of the relatively low hydrolyzed protein solution. Overall the fractionation of the β-Lg hydrolysates showed lower ACE inhibition in the 1 kDa to 10 kDa fraction when compared to the hydrolysate and the < 1 kDa fraction. The removal of the unhydrolyzed or minimally hydrolyzed proteins from the hydrolysates should concentrate the small peptides thereby increasing their bioactivity. This is something that should be considered in future research.

4.3 Antioxidant Activity

The antioxidant activity using DPPH measures the discoloration of a purple solution in the presence of radical scavengers. The change in color is measured and the antioxidant activity calculated. The antioxidant activity of all the hydrolysates was very low, as only some amino acids have free radical scavenging activity (Figures 4.31 and 4.32). α-La hydrolysates that showed the highest antioxidant activity were those treated with alcalase (12 %) and the combination of alcalase and flavourzyme (14 %), followed by flavourzyme (11%). Trypsin hydrolysates showed the lowest activity in all cases with the highest activity of approximately 2% in the < 1 kDa fraction. In the 10 kDa fraction where there was no significant difference between trypsin, flavourzyme, and alcalase treatments. The highest activity was noted to be in the < 1 kDa fraction followed by the unfractionated mixture. This is due to the peptides with antioxidant activity being small in nature. Additionally amino acids with antioxidant activity are present on the surface of the structure rather than being buried within a protein due to unravelling of the structure of the protein during hydrolysis.

The β-Lg hydrolysates that showed the highest antioxidant activity were those treated with a combination of alcalase and flavourzyme in all cases with upwards of 19 % inhibition. As

was the case with the α-La hydrolysates, trypsin hydrolysates of β-Lg displayed the lowest antioxidant activity in all cases with a high of 10 % inhibition. Overall flavourzyme (14 %) showed more activity than alcalase (13 %) except in the > 10 kDa fraction where the inhibitions were 7 % and 9 % respectively. Like the α-lactalbumin hydrolysates, the highest antioxidant activity was in the < 1 kDa fractions. However, the lowest activity was in the unfractionated hydrolysates. This is possibly due to the presence of larger molecular weight peptides with bioactivity in the > 10 kDa fraction. As noted earlier, β-Lg structure includes a large β-sheet (Creamer *et al*, 1983) and few disulfide bonds located near the centre of the protein (Thompson *et* al, 2009) which may expose amino acids with antioxidant activity due to their location at the surface. In general the higher the degree of hydrolysis the higher the antioxidant activity of the peptides produced from whey proteins during enzymatic hydrolysis.



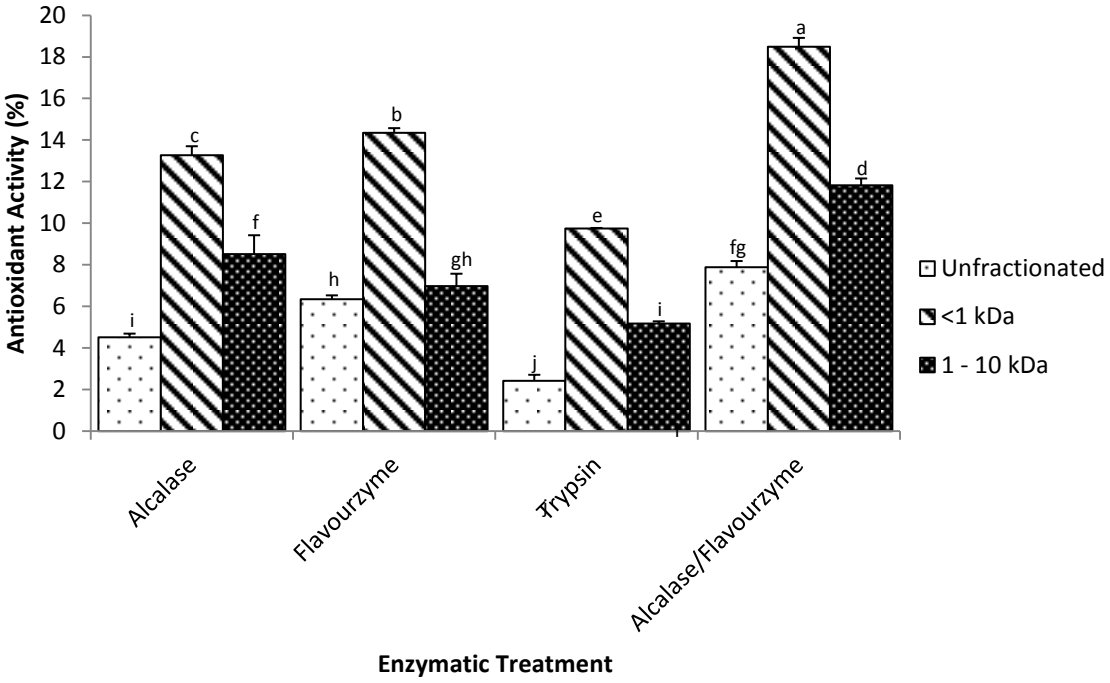


Figure 32. Antioxidant Activity of β-Lg extracts following hydrolysis and membrane fractionation. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

Other research has mostly utilized the oxygen radical absorbance capacity (ORAC) method of determining the antioxidant activity of dairy proteins and amino acids. Hernandez-Ledesma *et al* in 2005 determined that the antioxidant activity of α-La and β-Lg hydrolyzed by trypsin to be 1.031 and 0.979 µmol of Trolox equiv/mg protein. In order to compare this work accurately to work of others in the field, the same method of antioxidant activity must be used. However, research involving the DPPH antioxidant activity of milk and yogurt has been conducted. Aloglu & Oner in 2011 found that yogurts ranged in activity from 16 % to 31 % during aging from 3 to 4 weeks. Pritchard *et al* in 2010 found that fractions of peptides from cheese showed higher antioxidant activity in fractions larger than 5 kDa with upwards of 13 % inhibition. This result is similar to those in the research conducted here using whey proteins. Activity was slightly lower utilizing a single protein with the highest inhibition being 19 %; however, the presence of small

molecular weight proteins like lactoferrin may cause this to increase in yogurt and other whole milk product. Hogan *et al* in 2009 hydrolyzed milk with various bacterial enzymes, fractionated the hydrolysates, and determined the antioxidant activity. Milk hydrolyzed with a neutral protease from *Bacillus subtillis* showed highest activity in the 1 kDa to 10 kDa fraction with up to 30 % inhibition. Milk hydrolyzed with an alkaline protease from *Bacillus lichenformis* showed the highest activity in the same fraction with up to 27 % inhibition. Finally, milk hydrolyzed with a validase from *Aspergillus oryzae* showed the highest activity in the same fraction with up to 35 % inhibition. These results are in agreeance with my research as the highest antioxidant activity was in the 1 kDa to 10 kDa fraction for all of the hydrolysates.

In the research conducted by Elis *et al* in 2005, they found that the amino acids with the highest antioxidant activity to be Cys, Trp, and Met. Native α-La contains 8 Cys residues, all of which are involved in disulfide bonds within the α-helix of the protein structure. This protein also contains 4 Trp and 1 Met residue (Brew *et al*, 1970) which can contribute to the antioxidant activity of the peptides from this protein. The antioxidant amino acids found within the structure of β-Lg are 4 Cys, 2 Trp, and 3 Met residues. Like α-La, all of the Cys residues in β-Lg are involved in disulfide bonds (Sawyer & Kontopidis, 2000) within the protein structure of the β-Lg. It is unlikely that the disulfide bonds have been broken during hydrolysis leading to free Cys residues in the hydrolysate. However the residues possibly reside at the end of peptide chains in the hydrolysates which would give the free radicals access to the amino acids. It is also possible that the enzymes have a stronger affinity for breaking specific peptide bonds which leads to the formation of specific peptides with a higher concentration of antioxidant amino acids within a peptide chain. The high activity seen in the < 1 kDa fraction is most likely due to the presence of free antioxidant amino acids. Results from the antioxidant activity of proteins should be followed up with *in vitro* work using cell lines. This would give a better understanding

as to the antioxidant activity within a cell compared to giving a theoretical approach. This work indicates that the highest activity occurs in the hydrolysates which underwent the highest degree of hydrolysis possibly due to free amino acids and more antioxidant amino acids at the end of peptide chains.

4.4 Effect of α-Lactalbumin and β-Lactoglobulin Hydrolysates on Inflammation Markers in RAW 264.7 Murine Macrophage Cell Culture

4.4.1 Effect of hydrolysates on the cytotoxicity of murine macrophage cells

To determine the effect of hydrolysates of α-La and β-Lg on the inflammatory markers in murine macrophage cells, the effect they had on cell death needed to be determined. The cytotoxicity effect that samples had on unstimulated cells was determined by measuring the reduction of formazan crystals by utilizing the MTT assay. The cytotoxicity (%) of samples are shown in Figures 33 and 34. All of the α-La hydrolysates were significantly different than the control except for trypsin at a concentration of 500 µg/mL. Results varied from 113 % to 132 %. An increase in coloration indicates that more cells are reducing the MTT to a purple color than the control indicating more viable cells in these samples than the control. As a result, the α-La hydrolyates were not toxic to the cells.

Hydrolysates of β-Lg that were significantly different from the control were actually lower than the control as evidenced by the lack of reduction of MTT (Figure 34). Specifically all of the flavourzyme and trypsin hydrolysates were significantly lower than the control with 84 % to 91 % of the cells remaining viable. Also the combination of alcalase and flavourzyme at the concentrations of 50 µg/mL and 100 µg/mL showed a reduction in the conversion of MTT with values of 94 % and 88 % respectively. Unhydrolyzed β-Lg incubated with the cells at a

concentration of 200 µg/mL also showed reduced cell activity with 94 % of the viable cells. This would suggest that there was some cell toxicity with these samples. There were, however, some concerns with this experiment. If the results are plotted based on the time the wells' absorbances were determined, a stepwise increase in absorbance was observed. This may indicate that the wells were not all incubated for exactly the same amount of time. A decrease in the amount of time that the samples were incubated with MTT would lead to a decrease in the formation of the purple coloring due to the lack of time for this conversion to occur. This would be interpreted as cell toxicity. Research conducted by Lin *et al* in 2008 found α-La incubated with RAW 264.7 murine macrophage cells with concentrations of α-La above 50 µg/mL to be cytotoxic to cells after 48 h of incubation. This same research found that incubation with α-La for 24 h at a concentration of 100 µg/mL led to a decrease in the number of viable cells as well. It was thought that the protein inhibited cell proliferation by modifying the apoptosis and necrosis processes through the caspase-independent pathway. This ultimately lead to a decrease in B-cell lymphoma 2 (BCL-2) and cytochrome c which both regulate cell apoptosis and can lead to an increase in cell death. The results in the current research show the opposite to be true with α-La increasing the number of live cells. The decrease in cell viability by up to 15% with the β-Lg hydrolysates may be real or it may be a problem with the experiment. It is suggested that the incubation of hydrolysates with murine macrophage RAW 264.7 cells be repeated to ensure that the data is in fact representative of what occurred within the cells. As the cell culture used in this research was no longer available, this could not be completed in this research. Even if the toxicity values were reliable, there was sufficient media to continue with the experiment.

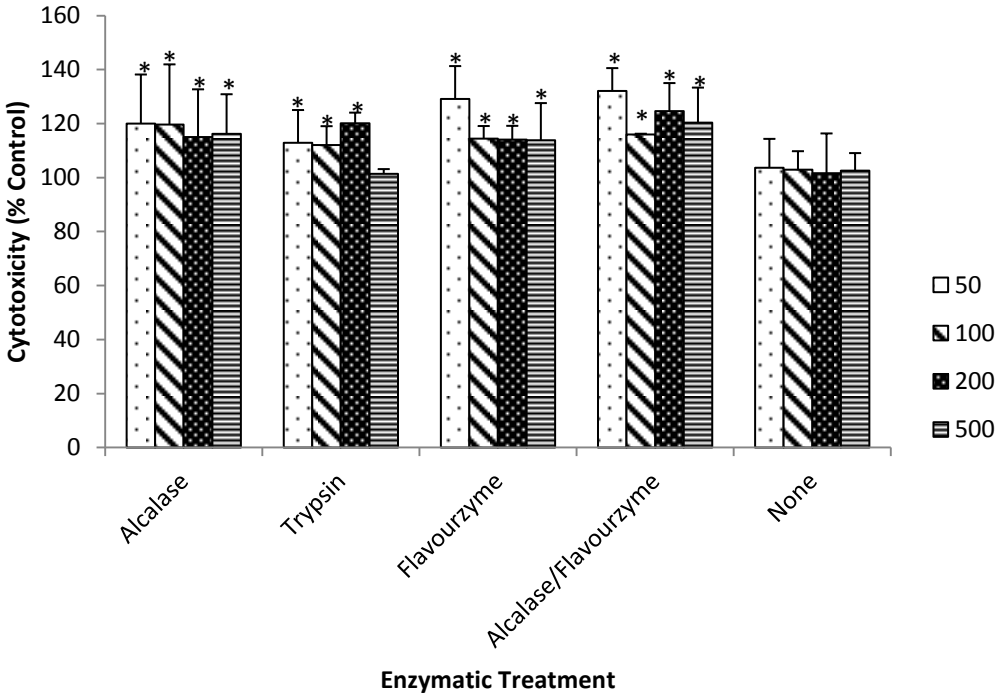


Figure 33. Cytotoxicity (%) of α-La hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of 100.00 ± 13.05 % was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and (*) are significantly different compared to the control (P>0.05).

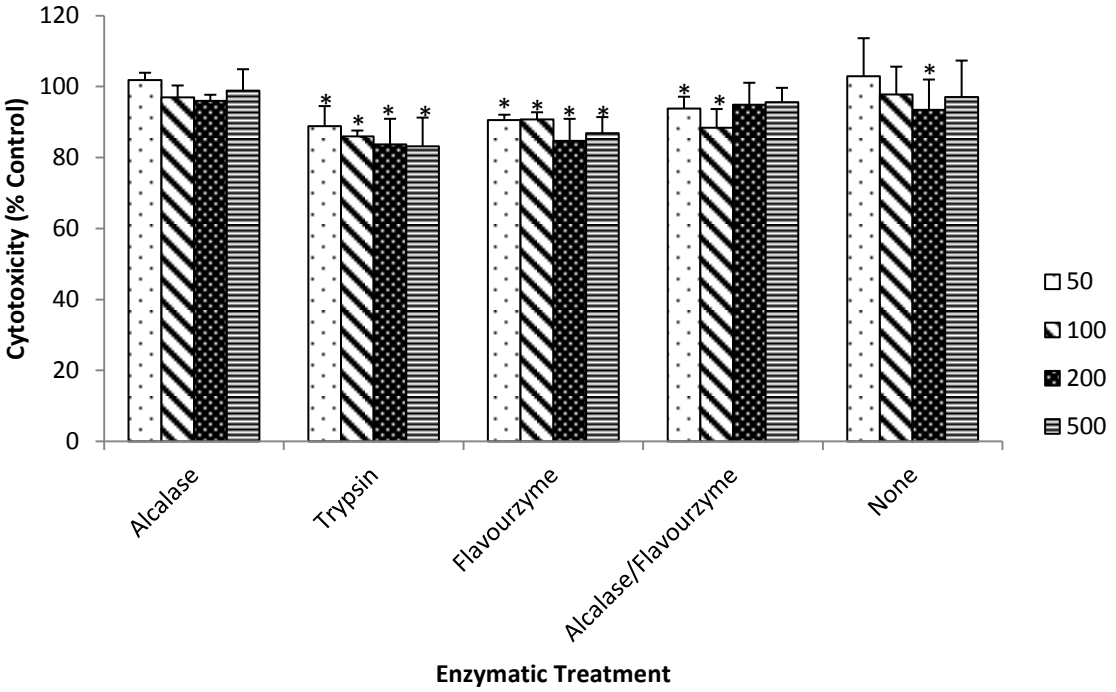


Figure 34. Cytotoxicity (%) of β-Lg hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of 100.00 ± 13.05 % was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and (*) are significantly different compared to the control (P>0.05).

4.1.2 NO in unstimulated murine macrophage cells

NO production within the cell is an important indicator of inflammation. NO is a free radical that increases in concentration within the cell during a period of immune response. Almost all of the samples were not significantly different than the control which indicates that the cells were not stressed or in a heightened state of inflammation. The three samples which were significantly different than the control were the β-Lg extract at concentrations of 100 and 200 µg/mL which produced 0.17 g/L and 0.39 g/L NO and the hydrolysate formed by alcalase treatment of β-Lg at a concentration of 50 µg/mL which produced 0.38 g/L NO. It should be noted that the majority of the samples and the control produced NO that was not stastically significant compared to zero. Again this may be due to experimental error and the experiment

should be repeated to confirm the results and utilize a positive control for comparison. Research conducted by Tellez *et al* in 2010 determined that the NO reduction of the supernatant obtained by milk hydrolyzed with *Lactobacillus helveticus* in an inflamed cell produced 28.98 μM NO when incubated with murine macrophage cells. Their result was found to be not significantly different than the lipopolysaccharide (LPS) control at a concentration of 1 μg/mL and demonstrated that the supernatant did not decrease NO inflammation within murine macrophage cells. However Tellez *et al* (2010) suggested that a peptide formed during hydrolysis may relieve cellular inflammation if applied following isolation. The results in the current research support the fact that the hydrolysates do not alter NO production within murine macrophage cells compared to the control.

4.1.3 Inflammation markers of whey protein hydrolysates incubated with unstimulated murine macrophage cells

TNFα is a well known marker of inflammation in murine macrophage cell culture work. This cytokine is secreted from activated macrophage cells and has been historically monitored as it represents changes in inflammation in the cells (Fitzgerald *et al*, 2001). Peptides were dissolved in distilled water at concentrations varying from 50 μg/mL to 500 μg/mL to determine the effect of increasing concentration on inflammation. For the unhydrolyzed proteins, an increase in protein concentration resulted in an increase in TNFα production within the cell as shown in Figures 35 and 36 for α-La and β-Lg, respectively. All concentrations of unhydrolyzed proteins incubated with the cells were significantly different than the control. This means that the protein itself caused an immuno-stimulatory effect. Hydrolysates formed from the hydrolysis of α-La showed this same increase in TNFα with an increase in the concentration of the peptides applied. However two of the hydrolysates were not significantly different than the media control. These hydrolysates were α-La hydrolyzed with alcalase at the concentrations of

50 µg/mL as well as the combination of alcalase and flavourzyme at a concentration or 50 µg/mL. The highest production of TNFα was seen with the α-La hydrolysates formed from the hydrolysis of trypsin at a concentration of 500 µg/mL.

Hydrolysates formed from the hydrolysis of β-Lg showed more samples with TNFα secretion levels in murine macrophage cells that were not significantly different than the control compared to the α-La hydrolysates. For all of the enzymes, hydrolysates from β-Lg at concentrations of 50 µg/mL and 100 µg/mL produced TNFα at levels that were not significantly different from the control (Figure 36). This was also true for the intact protein. It was also found that at a concentration of 200 µg/mL the hydrolysates formed by using alcalase and the combination of alcalase and flavourzyme did not produce statistically significant values compared to the control. This indicates that in order to produce an immuno-stimulatory effect on the murine macrophage cells with β-Lg hydrolysates, a concentration of peptides at or above 200 µg/mL is required. This may also indicate that the peptides may be candidates for reducing the level of inflammation in a stimulated murine macrophage. Like the α-La hydrolysates, trypsin produced the highest levels of TNFα to be secreted from the cells. However, the hydrolysates formed by hydrolysis with flavourzyme were not statistically significant from the trypsin peptides at all concentrations.

Research conducted by Tellez *et al* in 2010 examined the response of inflammation markers to incubation of *Lactobacillus helveticus* fermented milk supernatant in stimulated and unstimulated murine macrophage cells. They found that the supernatant incubated alone with the cells stimulated the cells more than LPS alone, where LPS was used as a positive control as it its known to stimulate cells. When incubating the supernatant with LPS, there was not a statistically significant difference compared to incubating with the supernatant alone. This

indicated that the supernatant upregulated the TNFα production within the cells. This result is true for some of the higher concentrations of peptides used in this research. However LPS was not used in the current research so the levels of inflammation cannot be compared to a positive control as was the case in the study of Tellez *et al* (2010).

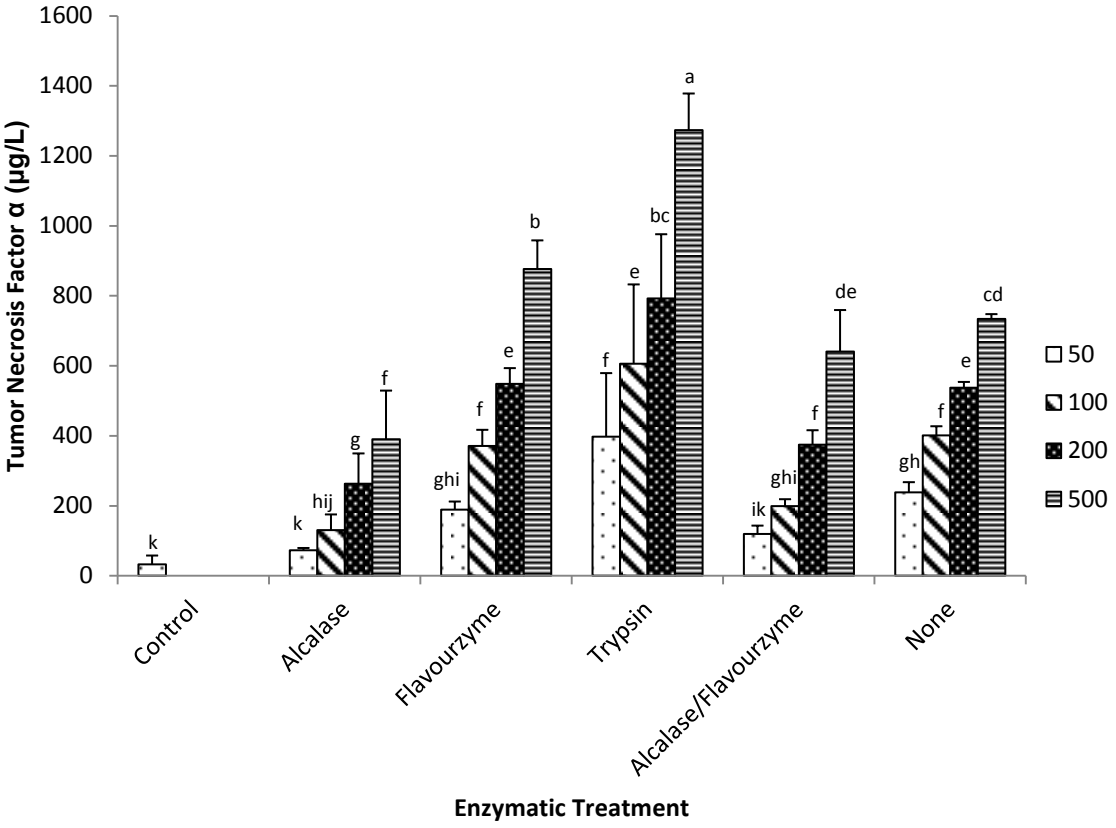


Figure 35. TNFα production of α-La hydrolysate incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of 32.06 ± 25.50 µg/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

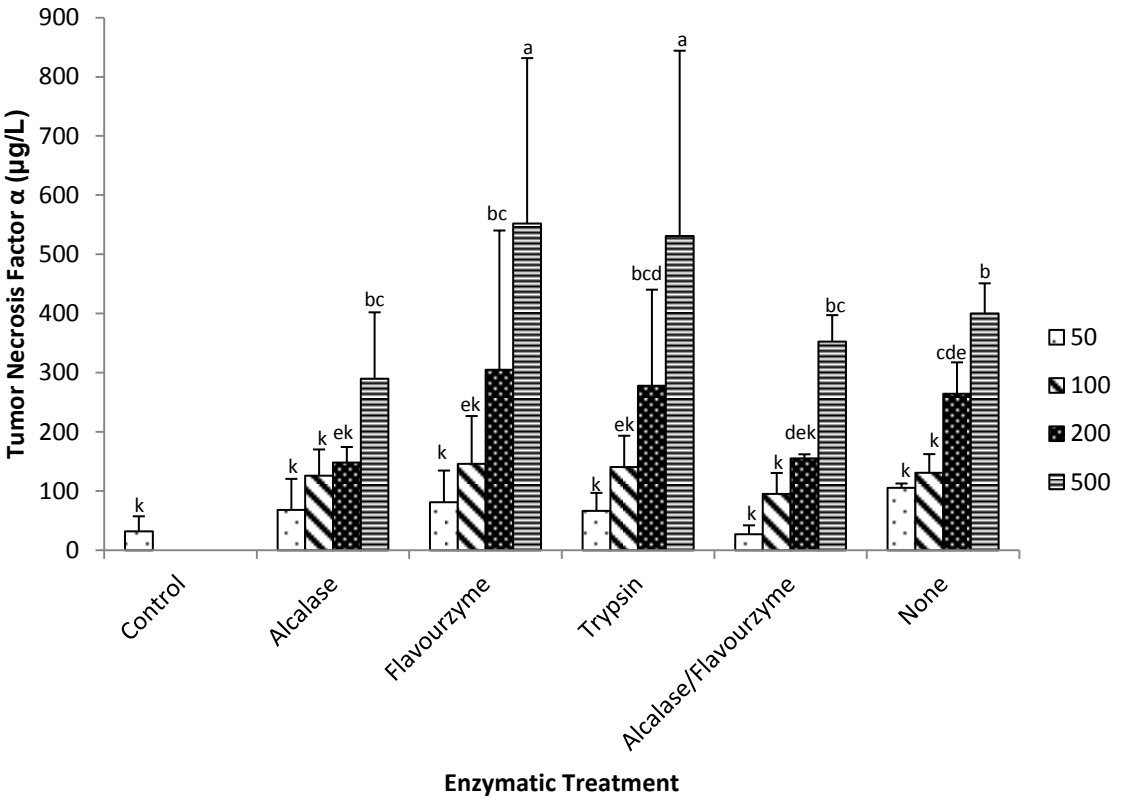


Figure 36. TNFα production of β-Lg hydrolysate incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of 32.06 ± 25.50 µg/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

IL6 is another marker of inflammation that was measured following the incubation of hydrolysates with murine macrophage RAW 264.7 cells. IL6 is a cytokine that is capable of stimulating the inflammatory response in cells. It is thought to activate signaling molecules in the cell membrane to upregulate the secretion of other cytokines, including TNFα (Fried *et al*, 1998; Mohamed-Ali *et al*, 1997). Protein extracts consisting of α-La and β-Lg did not cause a statistically significant effect on the production of IL6 production from murine macrophage cells. There may possibly be a trend of increasing IL6 production with an increase in the concentration of the sample. However, levels of IL6 are not significant compared to zero in this research so their accuracy is questionable. α-La hydrolyzed with either alcalase or trypsin both indicated

that this trend may exist, although most of the hydrolyzed samples were not significantly different than the control. The one α-La hydrolysate that was significantly different from the control was incubated with the cells at a concentration of 500 μg/mL following hydrolysis with trypsin. A similar result was obtained for the β-Lg hydrolysates except for the trypsin hydrolysate at a concentration of 100 μg/mL and the alcalase hydrolysate at 200 μg/mL. It was expected that as TNFα levels increased with concentration, the same would be true for IL6 due to the relationship that these two cytokines have to one another. However this was not the case. Research by Tellez *et al* in 2010 measured the production of IL6 in murine macrophage cells incubated with either LPS or the supernatant of milk fermented with *Lactobacillus helveticus*. The supernatant incubated with an un-inflamed cell produced significantly higher IL6 production compared to LPS, with levels of 15 ng/mL and 10 ng/mL respectively. When incubated with both the supernatant and LPS, levels of IL6 were statistically higher yet with a level of 80 ng/mL. This indicated that the supernatant causes an upregulation of IL6 production.

When you look at the results from Tellez *et al*'s research in 2010 as whole, it shows that as the levels of IL6 increase, so does TNFα, and NO. This trend is not seen in this research. In fact the only inflammation marker that increased was TNFα whereas the other markers were not much different from the control. This warrants further experimentation and a true duplication of the experiment to ensure that the results are accurate and reliable.

4.2 Effect of <1 kDa Fraction of α-Lactalbumin and β-Lactoglobulin Hydrolysates on Inflammation and Glucose Metabolism Markers in 3T3-L1 Adipocytes

4.2.1 Inflammation Markers

Adipocytes are the main producers of cytokines when the body is in a state of obesity. The overproduction of cytokines, specifically TNFα and IL6, is thought to lead to a reduction in insulin sensitivity in the affected individuals (Wellen & Hotamisligil, 2005). In this study, using 3T3-L1 adipocytes, only IL6 was examined as it is thought that IL6 regulates TNFα therefore the trend of IL6 secretion will likely cause the same trend to occur for TNFα secretions (Fried *et al*, 1998; Mohamed-Ali *et al*, 1997). The IL6 production from 3T3-L1 adipocytes incubated with the < 1 kDa fraction of the hydrolysates of α-La and β-Lg are shown in Figures 37, 38, and 39. As there were insufficient resources to determine the optimum concentration for incubation with the adipose cells, peptides were prepared to a concentration of 1 mg/mL based on the technician's previous experience working with 3T3-L1 adipocytes. The results shown in these figures could not be combined into a single figure as each experiment included a separate set of controls to which the treatments were compared. All of the α-La hydrolysates showed an increase in IL6 production in the cells when compared to the water control, which consisted of cells incubated with media and deionized water in place of the treatment. This indicates that the peptides did not cause a complete reversal of the IL6 production in the stimulated adipocytes. Comparing the α-La hydrolysates to the TNFα/IL1β control which consisted of cells incubated with IL1β and TNFα, the alcalase and the combination of alcalase and flavourzyme hydrolysates produced significantly lower IL6 secretion than the TNFα/IL1β control. This indicates that the peptides in the hydrolysates have an effect on lowering the inflammation caused by cytokines in adipose tissue. Comparison of the β-Lg hydrolysates with the TNFα/IL1β control showed that all the hydrolysates significantly lowered IL6 production. In addition, the trypsin and alcalase

hydrolysates produced IL6 values that were not stastistically significant from the unstimulated cells (water and media control). This indicates that all of the β-Lg hydrolysates are capable of decreasing the inflammation in adipose cells. However the alcalase and trypsin hydrolysates decreased the effect of IL1β and TNFα to the point where the response was similar to that of a normal unstimulated cell.

Research conducted by Zemmel and Sun in 2008 showed that IL6 production was significantly reduced by consuming a high calcium and soy diet or a high milk diet when compared to a soy only diet. This is in accordance for the results obtained for the hydrolysates originating from the whey protein β-Lg and the α-La hydrolysates in the current study. It should be noted that a substantial amount of the cytokines in adipocytes originate from other cells, such as macrophages (Bouloumie *et al*, 2005). This suggests that results may vary in a co-culture of adipocytes and macrophages when compared to individual culturing due to the effect of the secretions of one cell type on another. This would be a more realistic representation on what happens within the body due to better representation of the adipocyte-macrophage relationship and the inflammatory response. Other research is geared towards the effect of fats on adipocytes as adipose tissue is the main storage site of lipids within the body. In previous work, the levels of IL6 have been positively correlated with an increase in obesity and were reduced in periods of weight loss (Yudkin *et al*, 1999; Festa *et al*, 2001; Chiellini *et al*, 2004). TNFα and IL6 may cause the development of insulin resistance by stimulating the c-Jun amino-terminal kinase (JNK) and the nuclear factor-κB (NF-κB) pathways which upregulate mediators of inflammation and ultimately lead to insulin resistance (Wellen & Hotamisligil, 2005; Fain *et al*, 2004). This indicates that the most potent peptides, from β-Lg alcalase and trypsin hydrolysates, reduce the inflammation associated with obesity and may have an effect on the modulation of insulin resistance. Peptides from β-Lg hydrolyzed with flavourzyme and the combination of

flavourzyme and alcalase, as well as α-La hydrolyzed with alcalase, may also have an effect on the insulin resistance due to the decreased inflammation.

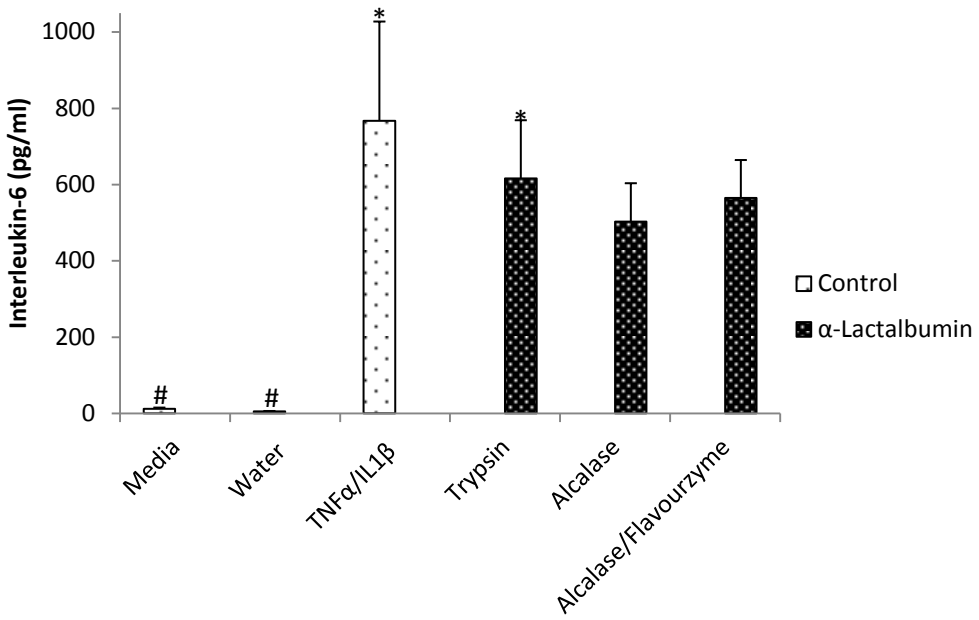


Figure 37. IL6 production (pg/mL) of α-La hydrolysates incubated with 3T3-L1 adipose cells on plate 1 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

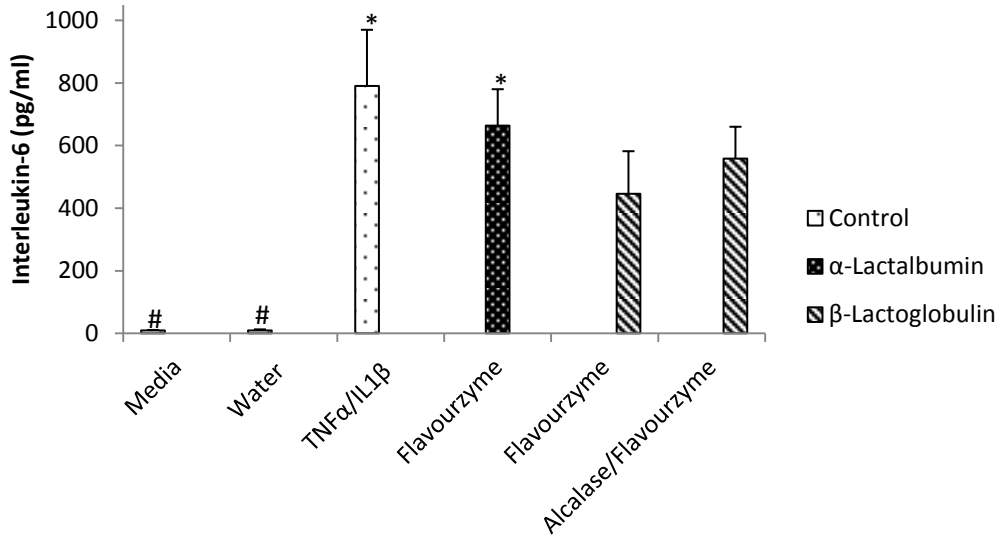


Figure 38. IL6 production (pg/mL) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipose cells on plate 2 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

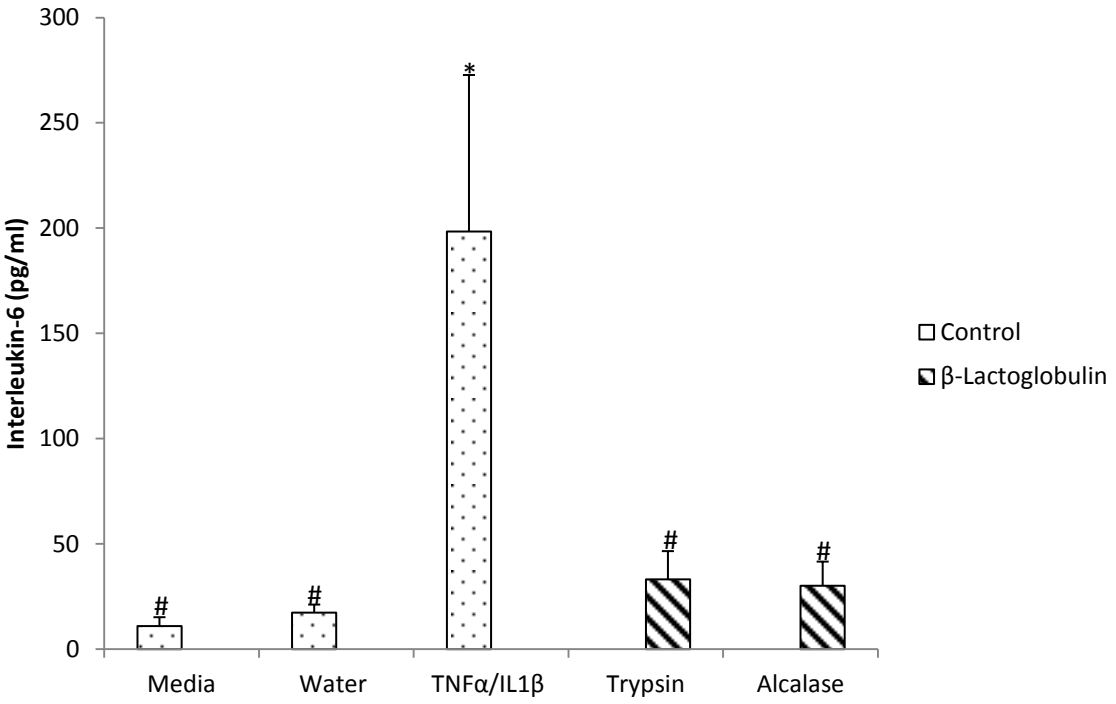


Figure 39. IL6 production (pg/mL) of β-Lg hydrolysates incubated with 3T3-L1 adipose cells on plate 3 for 24 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

4.2.2 Glucose Metabolism Markers

In a heightened state of inflammation due to obesity, it is not uncommon for insulin resistance to develop. Research has shown that GLUT, such as GLUT 4, are down regulated which can lead to an increase in circulating glucose due to a lack of translocation of the glucose to the cell surface (Oliver *et al*, 2010). The change in the gene expression of GLUT 4 was determined by RT-PCR. The results shown in these figures could not be combined into a single figure as each experiment included a separate set of controls to which the treatments were compared. All of the α-La < 1 kDa fractions were statistically different from the water/media control cells except for the hydrolysate obtained from flavourzyme, as shown in Figures 40 and 41. The flavourzyme hydrolysates from α-La and the combination of alcalase and flavourzyme from β-Lg (Figure 41) were not significantly different from either the unstimulated cells or the

stimulated cells. None of the hydrolysates (Figures 40, 41 and 42) were statistically different from the stimulated cells (TNFα/IL1-β control). These results would suggest that none of the hydrolysates were able to cause a statically significant elevation in the GLUT 4 levels in the stimulated cells. This may, in part, be due to large standard deviations for the measurements. As the aim was to see if the hydrolysates could reverse the effect of TNFα and IL1β on GLUT 4 production in adipocytes, it was interesting to see the effect of analyzing the data using preplanned comparisons. This had an impact on the second plate (Figure 41) where the variation of the water and media controls was particularly high. The flavourzyme hydrolysates from α-La and the combination of alcalase and flavourzyme from β-Lg were not significantly different when compared to the unstimulated cells (media control) as shown in Figure 45. However these hydrolysates were also not statistically significant compared to the TNFα/IL1β control. In this scenario, the peptides from the α-La flavourzyme hydrolysate and the β-Lg hydrolysate formed using a combination of alcalase and flavourzyme appeared to have the potential to increase GLUT 4 mRNA expression in adipocytes, however the large standard deviations in the media and water controls make the difference insignificant. To validate this observation, further work on the ability of some of these pepetides to cause an increase in GLUT 4 expression is warranted. Variations in hydrolysates levels used to treat the cells may confirm or disprove this potential.

Research conducted by Kim *et al* in 2009 and 2011 has shown that peptides derived from fibroin are capable of increasing insulin-stimulated glucose uptake in adipocytes. Research on the peptide Gly-Ala-Gly-Val-Gly-Tyr showed that glucose uptake was stimulated through the phosphoinositide 3-kinase (PI 3-K) pathway by phosphorylating phosphate kinase B (PKB) (Le Good *et al*, 1998; Pullen *et al*, 1998; Stephens *et al*, 1998). The phosphorylation of PKB can lead to an increase in the translocation of GLUT 4 at the plasma membrane which leads to a

biological response (Khan & Pessin, 2002) and ultimately an increase in glucose uptake by the cell. While the data in the current study was not conclusive with respect to an increase in the expression of GLUT 4 in adipocytes incubated with the peptides derived from whey proteins, based on previous literature and a non significant increase noted for two peptides when performing only preplanned comparisons in the current study, these hydrolysates may have the potential to lower blood glucose levels in those individuals that are insulin resistant.

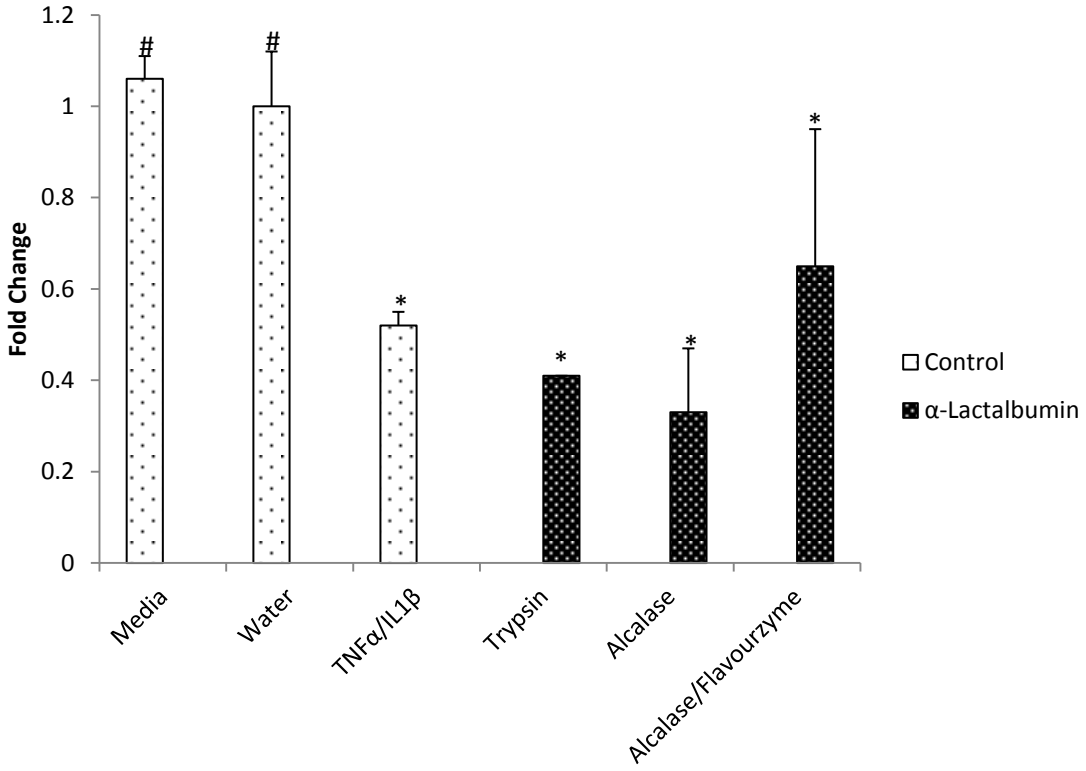


Figure 40. GLUT 4 mRNA expression (fold change) of α-La hydrolysates incubated with 3T3-L1 adipocytes on plate 1 for 24 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

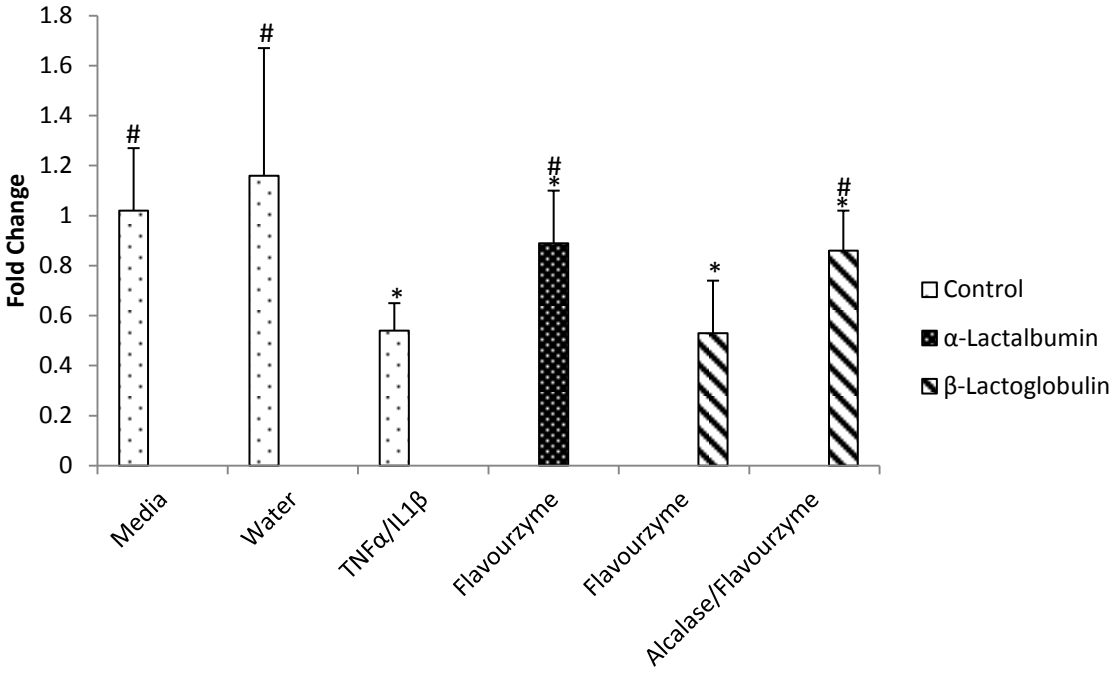


Figure 41. GLUT 4 mRNA expression (fold change) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipocytes on plate 2 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

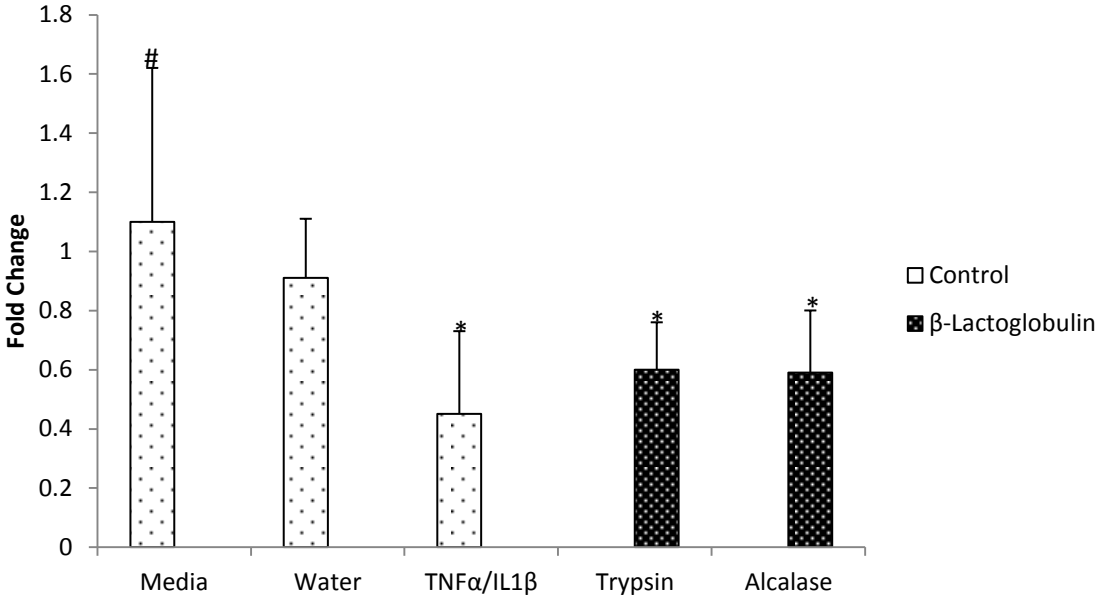


Figure 42. GLUT 4 mRNA expression (fold change) of β-Lg hydrolysates incubated with 3T3-L1 adipocytes on plate 3 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

The gene expression of adiponectin was also examined in this research. Adiponectin is a hormone that modifies the regulation of glucose (Oliver *et al*, 2010; Trayhurn & Wood, 2005). It is secreted mainly by white adipose tissue and has been shown *in vivo* to activate AMP-activated protein kinase (AMPK), increase fatty acid oxidation, and enhance glucose uptake leading to reducing circulating glucose levels (Zhang *et al*, 2009; Yamauchi *et al*, 2002). In individuals with insulin resistance, adiponectin secretion is impaired, and therefore an increase in secretion would indicate an improvement of a diabetic state. The results shown in Figures 43-45 could not be combined into a single figure as each experiment included a separate set of controls which the treatments were compared. None of the α-La hydrolysates showed statistically significant difference compared to the TNFα/IL1β control which consisted of stimulated cells as shown in Figures 43 and 44. This was also true for most β-Lg hydrolysates. The exception was the hydrolysate prepared with a combination of alcalase and flavourzyme which showed significantly higher expression of adiponectin than the stimulated cells (TNFα/IL1β control) and was not significantly different from the unstimulated cells (water/media control) as shown in Figure 44. Comparing the hydrolysates to the water and media controls showed a significant difference for the α-La hydrolysates prepared using flavourzyme and a combination of alcalase and flavourzyme. In addition, the β-Lg hydrolysate prepared using flavourzyme showed a significant decrease in adiponectin secretion when compared to the media and water controls. Results obtained on plate 3 shown in Figure 45 only show a significant difference between the water control and the TNFα/IL1β stimulated cells. This is due to the high variation in results obtained, particularly the controls.

Research conducted by Kim *et al* in 2009 and 2011 showed that the peptide from fibroin can increase adiponectin secretion in adipocytes in a state of inflammation. Their research suggested that adiponectin secreted from the adipocytes may have induced the activation of

AMPK in muscle cells. This indicates that the Gly-Ala-Gly-Val-Gly-Tyr peptide can enhance the insulin sensitivity of muscle and liver cells by increasing the secretion of adiponectin from adipocytes (Kim *et al*, 2009). This may explain what is happening when the stimulated cells are incubated with β-Lg hydrolyzed with a combination of flavourzyme and alcalase. This indicates that some peptides from β-Lg have the ability to decrease circulating blood glucose and increase fatty acid metabolism, both of which are factors in metabolic syndrome.

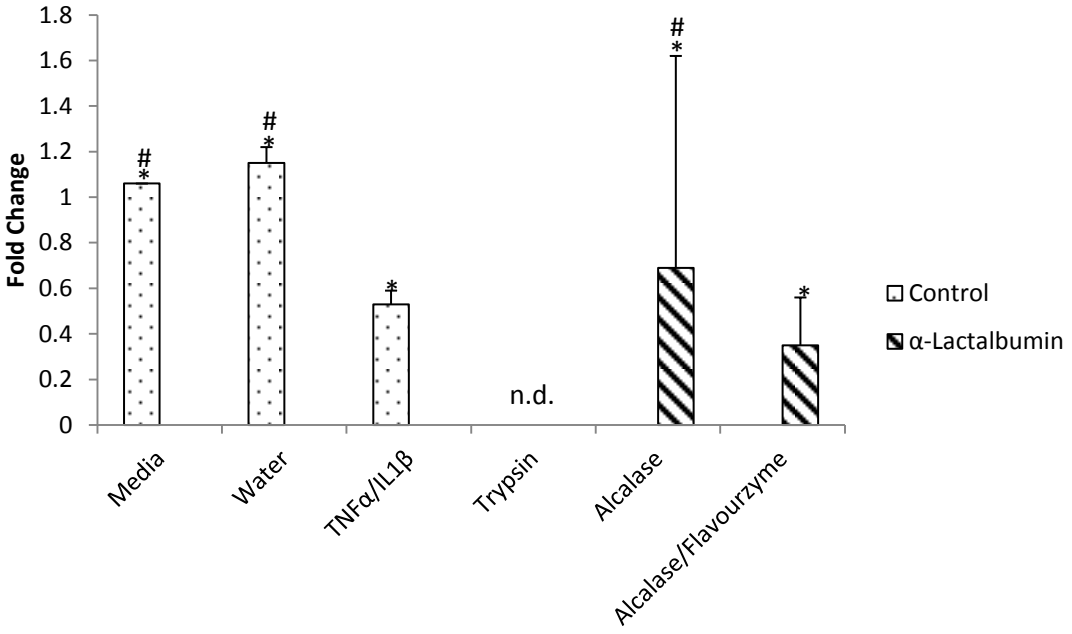


Figure 43. Adiponectin mRNA expression (fold change) of α-La hydrolysates incubated with 3T3-L1 adipocytes on plate 1 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

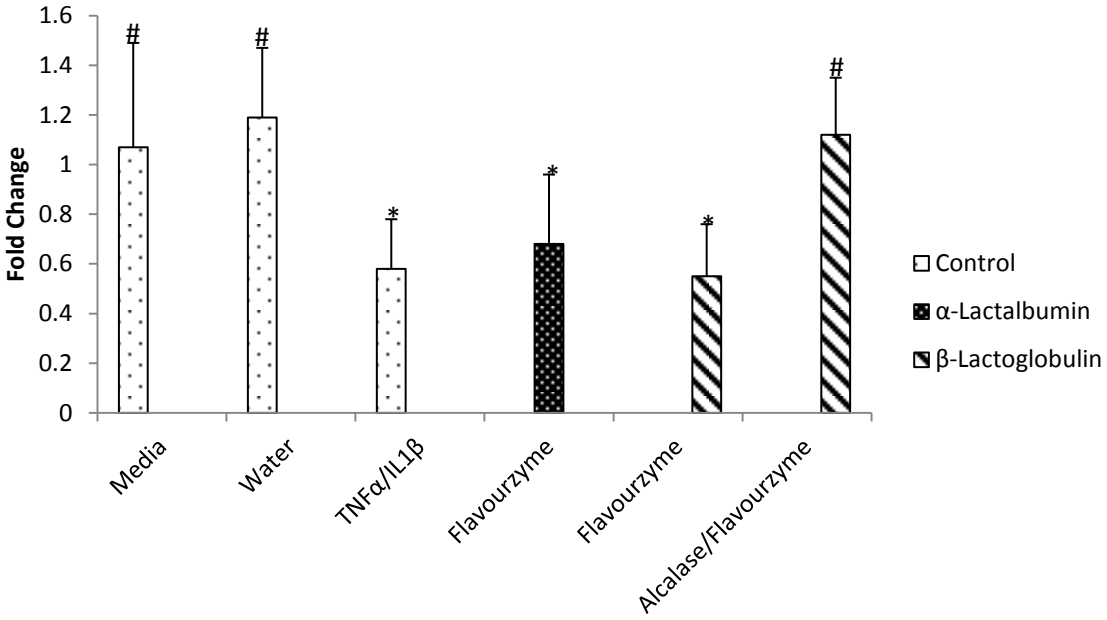


Figure 44. Adiponectin mRNA expression (fold change) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipocytes on plate 2 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

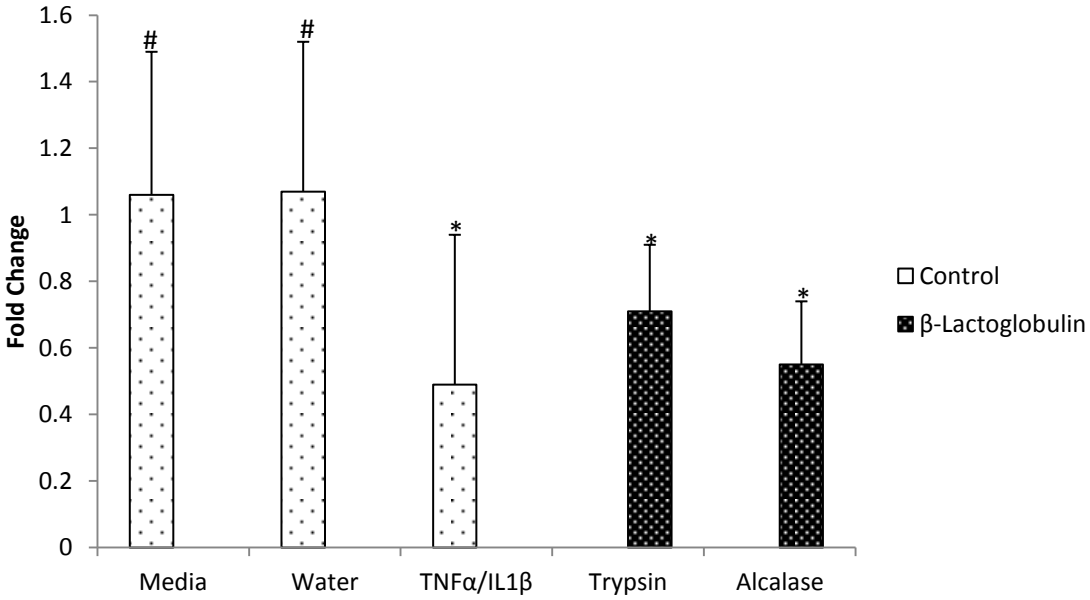


Figure 45. Adiponectin mRNA expression (fold change) of β-Lg hydrolysates incubated with 3T3-L1 adipocytes on plate 3 for 48 hours at a concentration of 1 mg/mL. Results are expressed as the mean ± standard deviation and (*) or (#) are not significantly different when compared to the media or TNFα/IL1β control (P>0.05), respectively.

In regards to glucose metabolism, the results obtained indicate that the < 1 kDa fraction obtained from hydrolysates formed using β-Lg and the combination of the enzymes alcalase and flavourzyme can increase the secretion of adiponectin in 3T3-L1 adipocytes. The peptides in this fraction examined by gel permeation chromatography earlier show a molecular weight range between 0.1 kDa and 0.9 kDa. If it is assumed that each amino acid adds 0.11 kDa, it can be estimated that the chain length varies anywhere between 1 amino acid to 8 amino acids in length. The peptides derived from β-Lg were hydrolyzed first with alcalase which is strictly an endopeptidase followed by flavourzyme which has both endo and exopeptidase activity. This creates very small peptides and potentially amino acids. Also the structure of β-Lg with a large β-sheet (Creamer *et al*, 1983) allows easy access for the exopeptidase activity of flavourzyme to extensively break down this portion of the peptide. The proteases are not restricted by the disulfide bonts in the structure as there are a limited number of disulfide bonds and they are located in the center of the structure (Thompson *et al*, 2009) allowing the terminal ends to be exposed to hydrolysis. This extensive break down will expose the hydrophobic amino acids buried within the structure of the easily digestible β-Lg. As adipocytes metabolize fats it would be assumed that hydrophobic amino acids would most likely influence the reversal of inflammation within the 3T3-L1 cells. Due to the resistance of α-La to hydrolysis and its large α-helix (Permyakov, 2005), few hydrophobic amino acids would be exposed during enzymatic breakdown. This would potentially reduce the ability of adipose tissues to be influenced by the peptides as they metabolize fats.

Due to the high variation in unstimulated (media and water controls) and stimulated cells (TNFα/IL1β controls) a number of hydrolysates did not show a stastically significant difference when compared to unstimulated or stimulated cells. For the GLUT 4 samples, these hydrolysates were α-La hydrolyzed with flavourzyme and β-Lg hydrolyzed with a combination of

alcalase and flavourzyme. For adiponectin samples, these hydrolysates were both the hydrolysates of α-La and β-Lg hydrolyzed and alcalase, as well as the trypsin hydrolysate obtained from β-Lg. These inconclusive results neither show that markers of diabetes were improved in stimulated cells (TNFα/IL1β control) nor that they were any different than unstimulated cells (water and media controls). This means that a conclusion as to the effect these hydrolysates had on the cells cannot be drawn. More work is required to determine if these hydrolysates with no significant differences are more similar to the unstimulated (water and media controls) and stimulated (TNFα/IL1β control) cells. Fractionation of peptides based on hydrophobicity would be essential in determining their effect on adipose tissue. Even further experiments utilizing a co-culture of macrophages and adipocytes would be more representative of what occurs within the body as macrophages secrete a large number of inflammatory markers which then influence the metabolism of fatty acids in adipose tissue.

5.0 Conclusions and Future Research Suggestions

5.1 Conclusions

Overall, β-Lg was broken down by enzymatic digestion more easily than α-La, especially with the bacterial enzyme flavourzyme which broke the most peptide bonds. β-Lg inhibited ACE to a greater degree than α-La for all peptides formed. In all cases the <1 kDa fractions outperformed the 1- 10 kDa fractions. The 1 kDa fraction of β-Lg provided higher ACE inhibition compared to α-La for all enzymes except for the combination of alcalase and flavourzyme. β-Lg hydrolyzed with alcalase and α-La hydrolyzed with trypsin or alcalase provided the most ACE inhibiton.

The antioxidant activity was higher in β-Lg hydrolysates compared to α-La hydrolysates except for the unfractionated hydrolysates formed with alcalase. In all cases the combination of alcalase and flavourzyme for either protein resulted in the highest activity.

β-Lg hydrolysates incubated with unstimulated murine macrophage cells stimulated TNFα secretion to a lower extent compared to α-La hydrolysates. Alcalase and a combination of alcalase and flavourzyme provided the lowest increase in secretion for both protein hydrolysates.

The < 1 kDa fraction of β-Lg hydrolysates increased IL6 production in stimulated 3T3-L1 adipoctes to a lower degree compared to α-La hydrolysates. β-Lg hydrolysates produced by hydrolysis with alcalase showed the lowest IL6 production in 3T3-L1 adipocytes with results that were not significantly different than water. Other hydrolysates, including α-La and β-Lg hydrolyzed with a combination of alcalase and flavourzyme, β-Lg hydrolyzed with flavourzyme,

and α-La hydrolyzed with alcalase significantly lowered IL6 but were significantly different when compared to water.

GLUT 4 mRNA expression in 3T3-L1 adipocytes was higher in β-Lg hydrolysates compared to α-La hydrolysates except for those hydrolysates derived from flavourzyme. α-La hydrolyzed with flavourzyme and β-Lg hydrolyzed with a combination of alcalase and flavourzyme had significantly higher expression compared to the stimulated cells but were significantly different compared to water. Adiponectin mRNA expression was higher in β-Lg hydrolysates compared to α-La hydrolysates except for those hydrolysates derived from a combination of alcalase and flavourzyme. α-La and β-Lg hydrolyzed with alcalase and β-Lg hydrolyzed with trypsin had significantly higher expression compared to the stimulated cells but were significantly different compared to water. β-Lg hydrolysates formed with flavourzyme were not significantly different than the water control. These results indicate that there is potential for some of these peptides to influence glucose levels and alleviate this component of metabolic syndrome.

The <1 kDa β-Lg hydrolysates, especially those formed with alcalase and a combination of alcalase and flavourzyme, showed the greatest potential to be applied to products creating functional foods and nutraceuticals which aid in the reversal of metabolic syndrome. However, more research is required to determine if the peptides remain unchanged during digestion and are effectively absorbed and utilized by the body.

5.2 Future Research Suggestions

The current research indicates that whey peptides, especially those derived from β-Lg can impart a bioactive response as determined by chemical and in cell culture analysis. It would

be useful to determine the bioactivity of smaller peptide fractions or individual peptides to determine which are capable of eliciting a greater response. It would also be beneficial to determine the effect of these peptides in a stimulated murine macrophage cell culture environment and a co-culture of murine macrophage cells and lipocytes as those with metabolic syndrome have a heightened state of inflammation and the macrophage cells influence lipocytes. Determination of the ability of the peptides to pass into the small intestine unaltered by digestion is essential to determine whether the peptides can be effectively utilized is essential. This additional research would allow the incorporation of peptides derived from whey proteins to be utilized as a functional food or a nutraceutical product.

6.0 References

Adler-Nissen, J. (1979). Determination of the Degree of Hydrolysis of Food Protein Hydrolysates by Trinitrobenesulfonic Acid. *Journal of Agriculture and Food Chemistry*. 27, 1256-1262.

Aloglu, H.S., & Oner, Z. (2011). Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. Journal of Dairy Science. 94: 5305-5314.

Amiot, J., Germain, L., Turgeon, S., Lemay, M., Ory-Salam, C., & Auger, F.A. (2004). Peptides from milk protein hydrolysates to improve the growth of human keratinocytes in culture. International Dairy Journal. 14: 619-626.

Apostolidis, E., Kwon, Y.I., & Shetty, K. (2006). Potential of Select Yogurts for Diabetes and Hypertension Management. *Journal of Food Biochemistry*. 30, 699-717.

Asselin, J., Amiot, J., Gauthier, S.F., Mourad, W., & Hebert, J. (1988). Immunogenicity and allergenicity of whey protein hydrolysates. Journal of Food Science. 53: 1208-1211.

Bouloumie, A., Curat, C.A., Sengenes, C., Lolmede, K., Miranville, A., & Busse, R. (2005). Rose of macrophage tissue infiltration in metabolic diseases. Current Opinion in Clinical Nutrition & Metabolic Care. 8:347-354.

Bramaud, C., Aimar, P., & Daufin, G. (1997). Optimization of a whey protein fractionation process based on the selective precipitation of α-Lactalbumin. Lait. 77:411-423.

Brand-Williams, W., Cuvelier, M.E., & Berset, C. (1995).Use of free radical method to evaluate antioxidant activity. LWT – Food Science and Technology. 28: 25-30.

Brew, K., Castellino, F.J., Vanaman, T.C., & Hill, R.L. (1970).The complete amino acid sequence of α-lactalbumin. Journal of Biological Chemistry. 245:4570-4582.

Cadee, J.A., Chang, C.Y., Chen, C.W., Huang, C.N., Chen, S.L., & Wang, C.K. (2007). Bovine casein hydrolysate (C12 peptide) reduces blood pressure in prehypertensive subjects. *American Journal of Hypertension*. 20, 1-5.

Caligiuri, M.A. & Lotze, M.T. (2007). *Cancer Drug Discovery and Development: Cytokines in the Genesis and Treatment of Cancer*. New Jersey: Humana Press.

Cheison, S.C., Schmitt, M., Leeb, E., Letzel, T., & Kulozik, U. (2010). Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC-ESI-TOF/MS. Food Chemistry. 121:457-467.

Cheison, S.C., Lai, M., Leeb, E., & Kulozik, U. (2011). Hydrolysis of β-lactoglobulin by trypsin under acidic pH and analysis of the hydrolysates with MALDI-TOF-MS/MS. Food Chemistry. 125: 1241-1248.

Chiellini, C., Santini, F., Marsili, A., Berti, P., Bertacca, A., Pelosini C., Scartabelli, G., Pardini, E., Lopez-Soriano, J., Centoni, R., Ciccarone, A.M., Benzi, L., Vitti, P., Del Prato, S., Pinchera, A., & Maffei, M. (2004). Serum haptoglobulin: A novel marker of adiposity in humans. Jounrnal of Clinical Endorcrinology and Metabolism. 89:2678-2683.

Chomczynski, P., & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry. 162: 156-159.

Cowherd, R.M., Lyle, R.E., & McGhee, R.E. (1999). Molecular regulation of adipocyte differentiation. Seminars in Cell and Developmental Biology. 10: 3-10.

Creamer, L., Parry, D., & Malcolm, G. (1983). Secondary Structure of β-Lactoglobulin B. Archives of Biochemistry and Biophysics. 227: 98-105.

Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water-holding capacity of canola protein hydrolysate. Archives of Food Chemistry. 109: 144-148.

Cushman, D.W., & Cheung, H.S. (1971). Spectrophotometric Assay and Properties of the Angiotensin-Converting Enzyme of Rabbit Lung. *Biochemical Pharmacology*. 20(7): 1637-1648.

Doucet, D., Otter, D.E., Gauthier, S.F., & Foegeding, E.A. (2003). Enzyme-Induced Gelation of Extensively Hydrolyzed Whey Proteins by Alcalase: Peptide Identification and Determination of Enzyme Specificity. Journal of Agriculture and Food Chemistry. 51: 6300-6308.

Driskell, J.A., ed. (2007). *Sports Nutrition: Fats and Proteins*. Florida: CRC Press.

Dryakova, A., Pihlanto, A., Marnila, P., Curda, L., & Korhonen, H.J.T. (2010). Antioxidant properties of whey protein hydrolysates as measured by three methods. European Food Research and Technology. 230: 865-874.

Elis, R.J., McClements, D.J., & Decker, E.A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase β-lactoglobulin in oil-in-water emulsions. Journal of Agriculture and Food Chemistry. 53: 10284-10253.

Fain, J.N., Madan, A.K., Hiler, M.L., Cheema, P. & Bahouth, S.W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous adominal adipose tissues of obese humans. Endocrinology. 145: 2273-2282.

Festa, A., D'Agostino, R., Williams, K., Karter, A.J., Mayer-Davis, E.J., Tracy, R.P., & Haffner, S.M. (2001). The relation of body fat mass and distribution to makers of chronic inflammation. International Journal of Obesity. 25: 1407-1415.

Fitzgerald, K.A., O'Neill, L.A.J., Gearing, A.J.H., & Callard, R.E. (2001). *The Cytokine Factsbook*. California: Academic Press.

Fitzgerald, R.J., & Meisel, H. (2000). Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. British Journal of Nutrition. 84: 33-37.

Frid, A.H., Nilsson, M., Holst, J.J., & Bjorck, J.M. (2005).Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. American Journal of Clinical Nutrition. 82: 69-75.

Fried, S.K., Bunkin, D.A., & Greenberg, A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. Journal of Clinical Endocrinology and Metabolism. 83: 847-850.

Graveland-Bikker, J.F., Ipsen, R., Otte, J., & de Kruif, C.G. (2004). Influence of calcium on the self-assembly of partially hydrolyzed alpha-lactalbumin. Langmuir. 20: 6841-6846.

Gassmann, M., Grenacher, B., Rohde, R., & Vogel, J. (2009). Quantifying western blots: Pitfalls of densitometry. Electrophoresis. 30: 1845-1855.

Gorski, T.P. & Campbell, D.J. (1991). Angiotensin-converting enzyme determination in plasma during therapy with coverting enzyme inhibitor: two methods compared. Clinical Chemistry. 37: 1390-1393.

Green, H., & Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell. 5: 19-27.

Gutierrez-Lopez, G.F., Barbosa-Canovas, G.V., Welti-Chanes, J., & Parada-Arias, E., ed. (2007). *Food Engineering: Integrated Approaches*. New York: Springer.

Hansen, B.C., & Bray, G.A., ed. (2008). *The Metabolic Syndrome Epidemiology, Clinical Treatment, and Underlying Mechanisms*. New Jersey: Humana Press.

Henle, T. (2005). Protein-bound advanced glycation endproducts (AGEs) as bioactive amino acid derivatives in foods. Amino Acids. 29: 313-322.

Hernandez-Ledesma, B., Davalos, A., Bartolome, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. Journal of Agriculture and Food Chemistry. 53: 588-593.

Hill, A.R. (Unknown). *University of Guelph: Dairy Science and Technology*. Guelph, Ontario. Retrieved January 6, 2009, from http://www.foodsci.uoguelph.ca/cheese/welcom.htm

Hogan, S., Zhang, L., Li, J., Wang, H., & Zhou, K. (2009). Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. Food Chemistry. 117: 438-443.

Humiski, L.M., & Aluko, R.E. (2007). Physiochemical and Bitterness Properties of Enzymatic Pea Protein Hydrolysates. *Journal of Food Science*. 72, S605-S611.

Janin, Y.L. (2003). Peptides with anticancer use or potential. Amino Acids. 25: 1-40.

Khan, A.H. & Pessin, J.E. (2002). Insulin regulation of glucose uptake: a complex of interplay of intracellular signaling pathways. Diabetologia. 45: 1474-1483.

Kim, E.D., Kim, E., Lee, J.H. & Hyun, C.K. (2011). Gly-Ala-Gly-Val-Gly-Tyr, a novel synthetic peptide, improves glucose transport and exerts beneficial lipid metabolic effects in 3T3-L1 adipocytes. European Journal of Pharmacology. 650: 479-485.

Kim, E.D., Bayaraa, T., Shin, E.J. & Hyun, C.K. (2009). Fibroin-derived peptides stimulate glucose transport in normal and insulin-resistance 3T3-L1 adipocytes. Biological and Pharmaceutical Bulletin. 32: 427-433.

Krause, C., Kirschbaum, J., & Bruckner, H. (2006). Peptaibiomics: an advanced, rapid and selective analysis of pepaibiotics/peptaibols by SPE/LC-ES-MS. Amino Acids. 30: 435-443.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P. & Parker, P.J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science. 281; 2042-2045.

Ley, M.D., ed. (2004). *Methods in Molecular Biology: Cytokine Protocols*. Volume 249. New Jersey: Humana Press.

Li, G.H., Le, G.W., Shi, Y.H., & Shrestha, S.(2004). Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins and their Physiological and Pharmacological Effects. *Nutrition Research*. 24, 469-486.

Lin, I., Su, S., & Kuo, C. (2008). Induction of cell death in RAW 264.7 cells by alpha-lactalbumin. Food and Chemical Toxicology. 46: 842-853.

Liu, H.C., Chen, W.L., & Mao, S.J.T. (2007). Antioxidant Nature of Bovine Milk β-Lactoglobulin. *Journal of Dairy Science.* 90, 547-555.

Madsen, J.S., Ahmt, T.O., Otte, J., Halkier, T., & Qvist, K.B. (1997). Hydrolysis of β-Lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. International Dairy Journal. 7: 399-409.

Maes, W., Camp, J.V., Vermeirssen, V. ,Hemeryck, M., Ketelslegers, J.M., Schrezenmeir, J., Ooostveldt, P.V., & Huyghebaert, A. (2004). Influence of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by endothelial cells. Regulatory Peptides. 118: 105-109.

Maubois, J.L., Pierre, A., Fauquant, J., & Piot, M. (1987). Industrial Fractionation of Main Whey Proteins. *Bulletin of the International Dairy Federation*. 212. Session VI: Chapter 24. Pages 154-159. International Whey Conference. Chicago, IL.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., & Coppack, S.W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. Journal of Clinical Endocrinology and Metabolism. 82: 4196-4200.

Mullally, M.M., Meisel, H., & Fitzgerald, R.J. (1997). Angiotensin-I-converting enzyme inhibitory activities of gastric and pancreatic proteinasedigests of whey proteins. *International Dairy Journal*. **7**, 299-303.

Nchienzia, H.A., Morawicki, R.O., & Gadang, V.P. (2010). Enzymatic hydrolysis of poultry meal with endo- and exopeptidases. Poultry Science. 89: 2273-2280.

Nilsson, M., Stenberg, M., Frid, A.H., Holst, J.J., & Bjork, I.M.E. (2004). Glycemia and insulinemia in healthy subjects after lactor-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. American Journal of Clinical Nutrition. 80: 1246-1253.

Novikoff, A.B., Novikoff, P.M., Rosen, O.M., & Rubin, C.S. (1980). Organelle relationships in cultured 3T3-L1 preadipocytes. Journal of Cell Biology. 87: 180-196.

Oliver, E., McGillicuddy, F., Phillips, C., Toomey, S., & Roche, H.M. (2010). The role of inflammation and macrophage accumulation in the development of obesity-induced type 2 diabetes mellitus and the possible therapeutic effects of long-chain n-4 PUFA. Proceedings of the Nutrition Society. 69: 232-243.

Oreopoulou, Y. & Russ, W., ed. (2007). *Utilization of By-Products and Treatment of Waste in the Food Industry*. New York: Springer.

Ostman, E.M., Elmstahl, E.G.M.L, & Bjorck, I.M.E. (2001). Inconsistency between glycemic and insulinemic responses to regular and fermented milk products. American Journal of Clinical Nutrition. 74: 96-100.

Otte, J., Shalaby, S.M., Zakora, M., Pripp, A.H., & El-Shabrawy, S.A. (2007).Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme, and time of hydrolysis. International Dairy Journal. 17-488-503.

Papiz, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C., Findlay, J.B., Sivaprasadarao, R., Jones, T.A., Newcomer, M.E., & Kraulis, P.J. (1986). The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. Nature. 324: 383-385.

Peng, X., Xiong, Y.L., & Kong, B. (2009). Antioxidant Activity of Peptide Fractions from Whey Protein Hydrolysates as Measured by Electron Spin Resonance. *Food Chemistry*. 113, 196-201.

Perea, A., Ugalde, U., Rodriguez, I., & Serra, J.L. (1993). Preparation and characterization of whey protein hydrolysates: Applications in industrial whey bioconversion processes. Enzyme Microbiology Technology. 15: 418-423.

Permyakov, E.A. (2005). α-Lactalbumin. Nova Publishers. 136 pages.

Pfeuffer, M., & Schrezenmeir, J. (2000). Bioactive Substances in Milk with Properties Decreasing the Risk of Cardiovascular Diseases. *British Journal of Nutrition*. 84, S155-S159.

Phillips, H.J., & Terryberry, J.E. (1957). Counting actively metabolizing tissue cultured cells. *Experimental Cell Research*. 13: 341-347.

Pihlanto-Leppala, A., Rokka, T., & Korhonen, H. (1998). Angiotensin I Converting Enzyme Inhibitory Peptides Derived from Bovine Milk Proteins. *International Dairy Journal*. 8, 325-331.

Pritchard, S.R., Phillips, M., & Kailasapathy, K. (2010). Identification of bioactive peptides in commercial Cheddar cheese.Food Research International. 43: 1545-1548.

Pullen, N., Dennis, P.B., Andjelkovic, M., Dufner, A., Kozma, S.C., Hemmings, B.A. & Thomas, G. (1998). Phosphorylation and activation of p70s6k by PDK1. Science. 279: 707-710.

Raghavan, S., & Kristinsson, H.G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. Food Chemistry. 117: 582-588.

Russel, T.R., & Ho, R. (1976). Conversion of 3T3 fibroblasts into adipose cells: triggering of differentiation by prostaglandin F2alpha and 1-methyl-3-isobutyl xanthine. Proceedings of the National Academy of Sciences of the United States of America. 73: 4516-4520.

Salami, M., Yousefi, R., Ehsani, M.R., Razavi, S.H., Chobert, J., Haertle, T., Saboury, A.A., Atri, M.S., Niasari-Naslaji, A., Ahmad, F., & Moosavi-Movahei, A.A. (2009). Enzymatic digestion and antioxidant activity of the native and molten globule states of camel α-lactalbumin: Possible significance for use in infant formula. International Dairy Journal. 19:518-523.

Saltiel, A.R, & Kahn, R. (2001). Insulin signaling and the regulation of glucose and lipid metabolism. Nature. 13: 799-806.

Sawyer, L. & Kontopidis, G. (2000). The core lipocalin, bovine beta-lactoglobulin. Biochimica et Biophysica Acta. 1482:136-148.

Schrezenmeir, J., Tato, F., Tato, S., Kustner, E., Krause, U., Hommel, G., Asp, N.G., Kasper, H., & Beyer, J. (1989). Comparison of glycemic response and insulin requirements after mixed meals of equal carbohydrate content in healthy, type-1 and type-2 diabetic man. Klin Wochenschr. 67:985-994.

Shihabi, Z.K. & Scaro, J. (1981). Liquid-chromatographic assay of angioteinsin-converting enzyme in serum. Clinical Chemistry. 27: 1669-1671.

Siemion, I.Z., Kluczyk, A., & Cebrat, M. (2005). The peptide molecular links between the central nervous and immune systems. Amino Acids. 29: 161-176.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P., Coadwell, J. & Hawkins, P.T. (1998). Protein kinase B kinases that mediate phophatidylinositol 3,4,5-triphosphate-depedent activation of protein kinase B. Science. 279: 710-714.

Tellez, A., Corredig, M., Brovko, L.Y., & Griffiths, M.W. (2010). Characterization of immune-active peptides obtained from milk fermented by Lactobacillus helveticus. Journal of Dairy Research. 77: 129-136.

Thompson A, Bowland M, & Singh H, ed. (2009). Milk *Proteins: from Expression to Food*. Amsterdam; Boston: Academic Press/Elsevier Inc.

Trayhurn, P., & Wood, I.S. (2005). Signalling role of adipose tissue: adipokines and inflammation in obesity. Biochemical Society Transactions. 33: 1078-1081.

Van, Q., Nayak, B.N., Reimer, M., Jones, P.J.H., Fulcher, R.G., & Rempel, C.B. (2009). Anti-inflammatory effect of *Inonotus obliquus, Polygala senega L.,* and *Viburnum trilobum* in a cell screening assay. Journal of Ethnopharmacology. 125: 487-493.

Wang, P., Mariman, E., Renes, J., & Keijer, J. (2005). The secretory function of adipocytes in the physiology of white adipose tissue. Journal of Cell Physiology. 216: 3-13.

Wang, W., & Zhang, J. (2008). Induction of renoprotective gene expression by hypoxia-inducible transcription factor-1alpha ameliorates renal damage. Medical Hypotheses. 70: 948-950.

Wellen, K.E. & Hotamisligil, G.S. (2005). Insulin resistance and β-cell drysfunction in aging: the importance of dietary carbohydrate. Journal of Clinical Investigation. 115: 1111-1119.

Yamauchi, T., Kamon, K., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B.B. & Kadowaki, T. (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nature Medicine. 8: 1288-1295.

Yudkin, J.S., Stehouwer, C.D., Emeis, J.J., & Coppack, S.W. (1999). C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue?. Arteriosclerosis, Thrombosis and Vascular Biology. 19: 972-978.

Yudkin, J.S. (2003). Adipose tissue, insulin action and vascular disease: inflammatory signals. International Journal of Obesity. 27: S25-S28.

Zemmel, M.B & Sun, X. (2008). Dietary calcium and dairy products modulate oxidative and inflammatory stress in mice and humans. Journal of Nutrition. 138: 1047-1052.

Zhang, B.B., Zhouo, G. & Li, C. (2009). AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metabolism. 9: 407-416.

7.0 Appendicies

Appendix 1. Enzyme reaction conditions and degree of hydrolysis of α-La and β- Lg hydrolysates. Rows with the same character are not significantly different (P>0.05).

	Reaction Conditions		Degree of Hydrolysis (%)
	pH	Temperature (°C)	
α-Lactalbumin			
Alcalase	8.5	50	17.6 ± 0.5^{e}
Flavourzyme	7.0	50	36.1 ± 0.7^{b}
Trypsin	8.0	37	7.3 ± 0.4^{g}
Alcalase/Flavourzyme	8.5 and 7.0	50	31.9 ± 2.4^{c}
β-Lactoglobulin			
Alcalase	8.5	50	22.5 ± 0.7^{d}
Flavourzyme	7.0	50	40.4 ± 0.9^{a}
Trypsin	8.0	37	9.9 ± 0.4^{f}
Alcalase/Flavourzyme	8.5 and 7.0	50	40.2 ± 1.0^{a}

Appendix 2. ACE Inhibition of α-La and β-Lg hydrolysates and their fractions. Cells with the same character are not significantly different (P>0.05).

	ACE-Inhibition (%)		
	Unfractionated	<1 kDa Fraction	1-10 kDa Fraction
α-Lactalbumin	0.0 ± 0.5		
Alcalase	92.4 ± 1.0^{ab}	91.3 ± 1.4^{ab}	86.0 ± 0.7^{c}
Flavourzyme	58.3 ± 1.9^{f}	61.8 ± 1.8^{e}	64.0 ± 6.1^{e}
Trypsin	90.6 ± 0.8^{ab}	92.9 ± 1.6^{a}	89.7 ± 2.0^{b}
Alcalase/Flavourzyme	82.7 ± 0.5^{d}	79.8 ± 3.4^{d}	64.5 ± 2.6^{e}
β-Lactoglobulin	0.0 ± 0.5		
Alcalase	101.0 ± 0.5^{a}	95.4 ± 1.2^{b}	81.5 ± 4.4^{e}
Flavourzyme	80.6 ± 2.5^{e}	79.2 ± 0.7^{ef}	55.3 ± 2.1^{h}
Trypsin	76.6 ± 2.4^{f}	84.7 ± 3.6^{d}	68.5 ± 1.2^{g}
Alcalase/Flavourzyme	89.5 ± 1.0^{c}	88.0 ± 1.1^{c}	65.2 ± 1.6^{g}

* Protein standards not significantly different compared to zero.

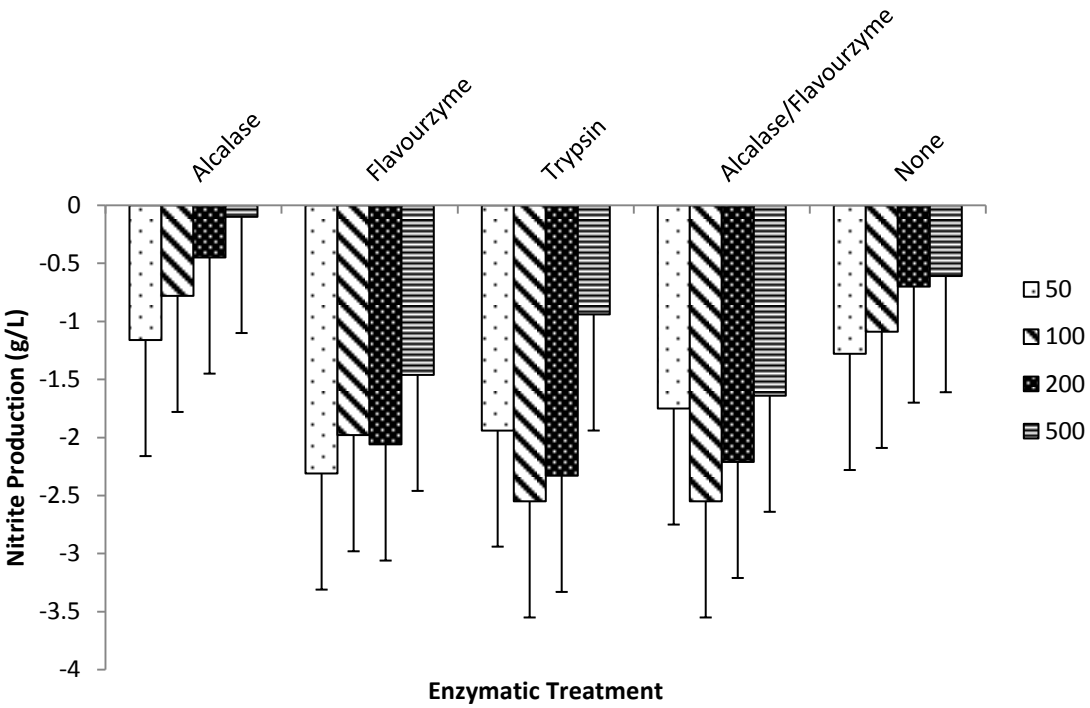
Appendix 3. Antioxidant activity of α-La and β-Lg hydrolysates and their fractions. Cells with the same character are not significantly different (P>0.05).

	Antioxidant Activity (%)		
	Unfractionated	<1 kDa	1-10 kDa
α-Lactalbumin	-0.06 ± 0.27^{fg}		
Alcalase	8.44 ± 0.72^{c}	11.87 ± 0.22^{b}	0.91 ± 0.47^{f}
Flavourzyme	2.42 ± 0.29^{e}	11.12 ± 0.41^{b}	-0.05 ± 0.40^{fg}
Trypsin	-0.59 ± 0.17^{fg}	2.26 ± 0.36^{e}	0.20 ± 0.62^{fg}
Alcalase/Flavourzyme	5.94 ± 0.19^{d}	14.44 ± 0.12^{a}	2.12 ± 0.84^{e}
β-Lactoglobulin	0.00 ± 0.00^{k}		
Alcalase	4.52 ± 0.17^{i}	13.27 ± 0.43^{c}	8.52 ± 0.90^{f}
Flavourzyme	6.34 ± 0.19^{h}	14.35 ± 0.22^{b}	6.98 ± 0.59^{gh}
Trypsin	2.42 ± 0.29^{j}	9.75 ± 0.02^{e}	5.18 ± 0.10^{i}
Alcalase/Flavourzyme	7.88 ± 0.30^{fg}	18.49 ± 0.42^{a}	11.82 ± 0.33^{d}

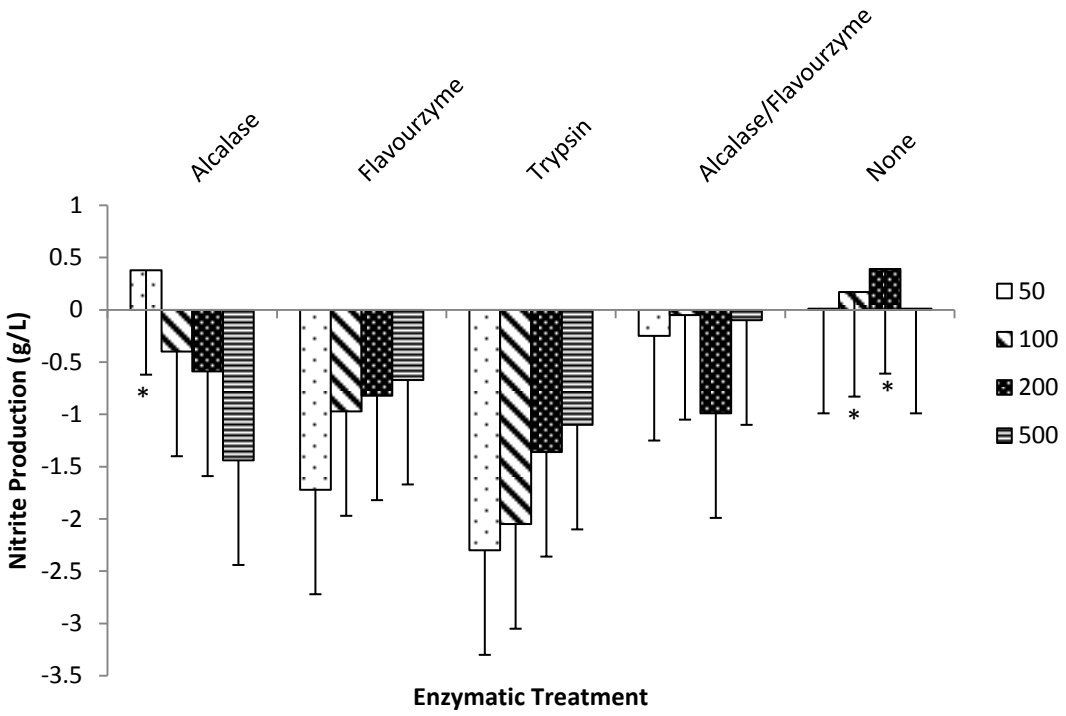
Appendix 4. Cytotoxicity (%) of α-La and β-Lg hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL.

	Concentration of Hydrolysates (µg/mL)			
	50	100	200	500
α-Lactalbumin	103.62 ± 1.18	103.72 ± 1.02	100.87 ± 2.33	102.34 ± 0.63
Alcalase	119.94 ± 18.24*	119.61 ± 22.33*	115.05 ± 17.64*	116.19 ± 14.68*
Flavourzyme	129.11 ± 12.19*	114.43 ± 4.67*	114.12 ± 5.05*	113.79 ± 13.80*
Trypsin	112.84 ± 16.57*	112.07 ± 6.97*	120.16 ± 3.90*	101.39 ± 1.76
Alcalase/Flavourzyme	132.07 ± 8.48*	115.94 ± 0.30*	124.65 ± 10.36*	120.37 ± 12.96*
β-Lactoglobulin	103.28 ± 1.11	97.89 ± 2.34	93.60 ± 0.99*	97.05 ± 2.14
Alcalase	101.87 ± 2.03	97.00 ± 3.31	96.01 ± 1.68	98.82 ± 6.07
Flavourzyme	90.56 ± 1.51*	90.71 ± 2.05*	84.68 ± 6.22*	86.90 ± 4.50*
Trypsin	88.83 ± 5.67*	85.98 ± 1.63*	83.67 ± 7.25*	83.19 ± 8.07*
Alcalase/Flavourzyme	93.78 ± 3.36*	88.38 ± 5.29*	94.89 ± 6.18	95.62 ± 4.02

*Significant compared to control 100.00 ± 13.05



Appendix 5. Nitrite production of α-La hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of -1.86 ± 0.65 g/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and (*) are significantly different compared to the control (P>0.05).



Appendix 6. Nitrite production of β-Lg hydrolysate incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of -1.86 ± 0.65 g/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and (*) are significantly different compared to the control (P>0.05).

Appendix 7. NO production (g/L) of α-La and β-Lg hydrolysates incubated with RAW 264.7 murine macrophage cells at concentrations between 50-500 µg/mL.

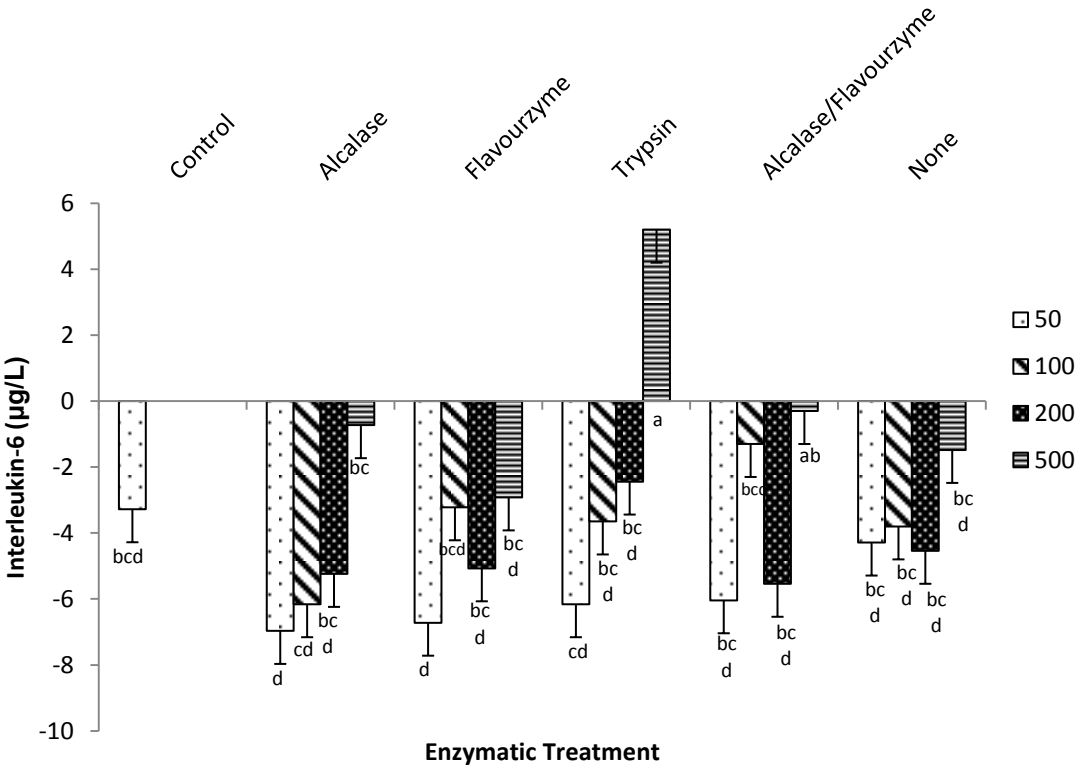
	Concentration of Hydrolysates (µg/mL)			
	50	100	200	500
α-Lactalbumin	-1.28 ± 0.55	-1.09 ±0.19	-0.70 ± 0.33	-0.61 ± 0.06
Alcalase	-1.16 ± 2.16	-0.78 ± 2.07	-0.45 ± 2.56	-0.10 ± 2.61
Flavourzyme	-2.31 ± 0.33	-1.98 ± 0.26	-2.06 ± 0.77	-1.46 ± 1.74
Trypsin	-1.94 ± 1.32	-2.55 ± 0.17	-2.33 ± 0.25	-0.94 ± 1.93
Alcalase/Flavourzyme	-1.75 ± 1.21	-2.55 ± 0.24	-2.21 ± 1.10	-1.64 ± 0.94
β-Lactoglobulin	0.01 ± 0.33	0.17 ± 0.18*	0.39 ± 0.30*	0.01 ± 0.30
Alcalase	0.38 ± 0.30*	-0.40 ± 0.72	-0.59 ± 0.42	-1.44 ±0.90
Flavourzyme	-1.72 ±0.79	-0.97 ± 0.65	-0.82 ± 0.86	-0.67 ± 1.29
Trypsin	-2.30 ± 0.62	-2.05 ± 1.15	-1.36 ± 1.16	-1.10 ± 3.08
Alcalase/Flavourzyme	-0.25 ±0.64	-0.05 ± 0.52	-0.99 ± 0.93	-0.10 ± 0.65

*Significantly different than control -1.86 ± 0.65

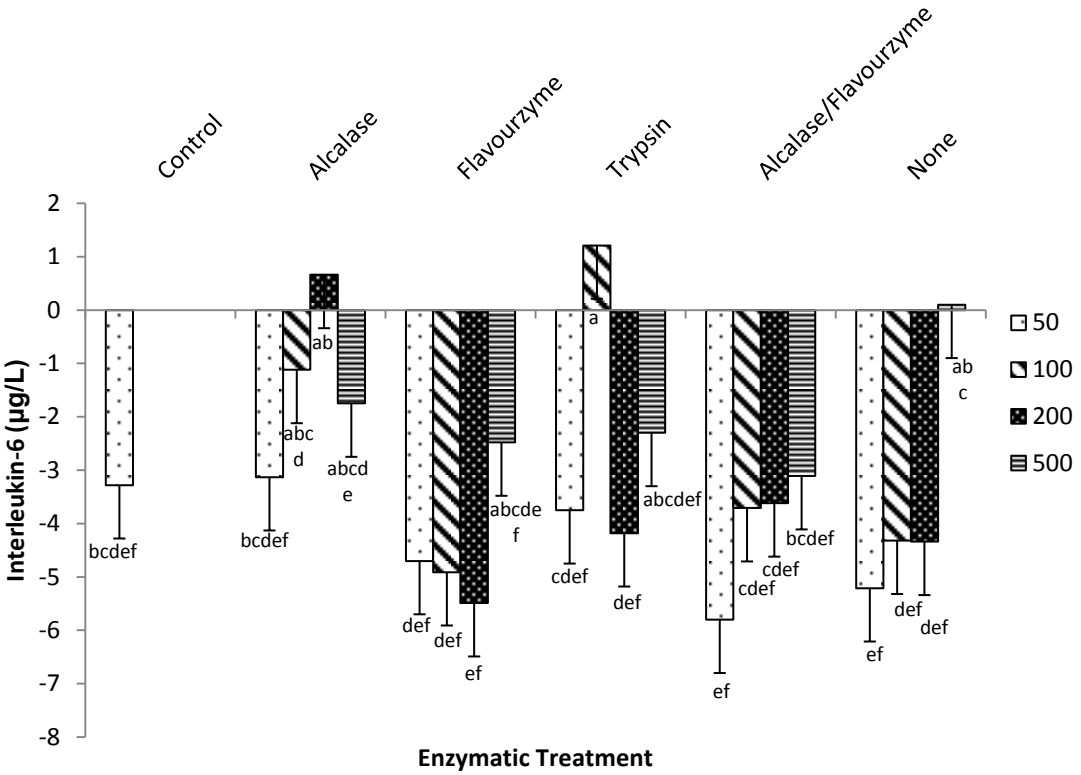
Appendix 8. TNFα production (μg/L) of α-La and β-Lg hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 μg/mL.

	Concentration of Hydrolysates (μg/mL)			
	50	100	200	500
α-Lactalbumin	237.71 ± 29.44^{gh}	400.98 ± 26.01^{f}	536.97 ± 16.62^{e}	733.24 ±14.15^{cd}
Alcalase	72.50 ± 6.62^{k}	130.34 ± 44.68^{hik}	262.30 ± 87.00^{g}	389.60 ± 139.21^{f}
Flavourzyme	188.99 ± 22.85^{ghi}	370.40 ± 46.37^{f}	547.63 ± 45.46^{e}	876.25 ± 81.82^{b}
Trypsin	397.54 ± 181.29^{f}	605.91 ± 226.33^{e}	792.55 ± 183.10^{bc}	1272.99 ± 104.81^{a}
Alcalase/Flavourzyme	119.27 ± 23.76^{ik}	199.00 ± 19.52^{ghi}	374.45 ± 41.20^{f}	640.71 ± 118.34^{de}
β-Lactoglobulin	105.64 ± 7.29^{k}	131.27 ± 31.27^{k}	264.74 ± 52.81^{cde}	399.97 ± 51.14^{b}
Alcalase	68.25 ± 52.48^{k}	125.91 ± 44.53^{k}	148.34 ± 26.22^{ek}	290.14 ±111.99^{bc}
Flavourzyme	81.09 ± 53.72^{k}	146.33 ± 80.53^{ek}	305.26 ± 235.08^{bc}	552.25 ±279.34^{a}
Trypsin	66.54 ± 30.56^{k}	140.58 ± 53.01^{ek}	278.07 ± 162.30^{bcd}	530.94 ± 313.12^{a}
Alcalase/Flavourzyme	27.49 ± 14.78^{k}	95.26 ± 35.44^{k}	155.10 ± 7.16^{dek}	352.60 ± 44.79^{bc}

*Control 32.06 ± 25.50^{k}



Appendix 9. IL6 production of α-La hydrolysate incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of -3.28 ± 1.44 µg/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).



Appendix 10. IL6 production of β-Lg hydrolysate incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL. A negative control value of -3.28 ± 1.44 µg/L was obtained by incubating murine macrophage cells with media overnight. Results are expressed as the mean ± standard deviation and like characters are not significantly different (P>0.05).

Appendix 11. IL6 production (µg/L) of α-La and β-Lg hydrolysates incubated with RAW 264.7 murine macrophage cells incubated overnight at concentrations between 50-500 µg/mL.

	Concentration of Hydrolysates (µg/mL)			
	50	100	200	500
α-Lactalbumin	-4.29 ± 0.27^{bcd}	-3.80 ± 0.41^{bcd}	-4.54 ± 0.30^{bcd}	-1.48 ± 0.94^{bcd}
Alcalase	-6.97 ± 3.64^{d}	-6.16 ± 0.40^{cd}	-5.24 ± 1.04^{bcd}	-0.73 ± 5.58^{bc}
Flavourzyme	-6.72 ± 2.56^{d}	-3.22 ± 5.43^{bcd}	-5.07 ± 3.05^{bcd}	-2.92 ±4.92^{bcd}
Trypsin	-6.16 ± 1.42^{cd}	-3.65 ± 4.39^{bcd}	-2.44 ± 4.63^{bcd}	5.20 ± 0.95^{a}
Alcalase/Flavourzyme	-6.04 ± 2.13^{bcd}	-1.30 ± 9.58^{bcd}	-5.54 ± 2.49^{bcd}	-0.30 ± 7.41^{ab}
β-Lactoglobulin	-5.21 ± 0.58^{ef}	-4.32 ± 0.60^{def}	-4.34 ± 0.28^{def}	0.10 ± 2.03^{abc}
Alcalase	-3.13 ± 0.85^{bcdef}	-1.12 ± 3.39^{abcd}	0.66 ± 5.33^{ab}	-1.75 ± 5.45^{abcde}
Flavourzyme	-4.70 ± 4.13^{def}	-4.91 ± 1.86^{def}	-5.49 ± 0.66^{ef}	-2.48 ± 1.87^{abcdef}
Trypsin	-3.75 ± 1.40^{cdef}	1.21 ± 7.88^{a}	-4.18 ± 0.34^{def}	-2.30 ± 3.23^{abcdef}
Alcalase/Flavourzyme	-5.80 ±0.78^{ef}	-3.71 ± 3.20^{cdef}	-3.62 ± 2.31^{cdef}	-3.11 ± 2.72^{bcdef}

*Control -3.28 ± 1.44^{bcdef}

Appendix 12. IL6 production (pg/mL) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipocytes for 24 hours at a concentration of 1 mg/mL.

Treatment	Plate	1	2	3
Control	Media	11.79 ± 3.23^{c}	9.09 ± 1.67^{d}	10.94 ± 4.26^{b}
	Water	4.82 ± 1.35^{c}	9.37 ± 3.30^{d}	17.33 ± 3.87^{b}
	TNFα/IL1β	767.11 ± 260.07^{a}	790.87 ± 179.37^{a}	198.36 ± 74.37^{a}
α-Lactalbumin	Trypsin	615.69 ± 152.74^{ab}		
	Alcalase	502.98 ± 100.29^{b}		
	Flavourzyme		663.99 ± 116.34^{ab}	
	Alcalase/Flavourzyme	564.35 ± 99.74^{ab}		
β-Lactoglobulin	Trypsin			33.20 ± 13.38^{b}
	Alcalase			30.18 ± 11.43^{b}
	Flavourzyme		446.43 ± 135.68^{c}	
	Alcalase/Flavourzyme		558.55 ± 101.77^{bc}	

Appendix 13. GLUT 4 mRNA expression (fold change) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipocytes for 48 hours at a concentration of 1 mg/mL.

Treatment	Plate	1	2	3
Control	Media	1.06 ± 0.05^{a}	1.02 ± 0.25^{a}	1.10 ± 0.52^{a}
	Water	1.00 ± 0.12^{a}	1.16 ± 0.51^{a}	0.91 ± 0.20^{ab}
	TNFα/IL1β	0.52 ± 0.03^{b}	0.54 ± 0.11^{b}	0.45 ± 0.28^{b}
α-Lactalbumin	Trypsin	0.41 ± 0.00^{b}		
	Alcalase	0.33 ± 0.14^{b}		
	Flavourzyme		0.89 ± 0.21^{ab}	
	Alcalase/Flavourzyme	0.65 ± 0.30^{b}		
β-Lactoglobulin	Trypsin			0.60 ± 0.16^{b}
	Alcalase			0.59 ± 0.21^{b}
	Flavourzyme		0.53 ± 0.21^{b}	
	Alcalase/Flavourzyme		0.86 ± 0.16^{b}	

Appendix 14. Adiponectin mRNA expression (fold change) of α-La and β-Lg hydrolysates incubated with 3T3-L1 adipocytes for 48 hours at a concentration of 1 mg/mL.

Treatment	Plate	1	2	3
Control	Media	1.06 ± 0.00^{ab}	1.07 ± 0.42^{ab}	1.06 ± 0.43^{a}
	Water	1.15 ± 0.07^{a}	1.19 ± 0.28^{a}	1.07 ± 0.45^{a}
	TNFα/IL1β	0.53 ± 0.06^{ab}	0.49 ± 0.45^{c}	0.49 ± 0.45^{b}
α-Lactalbumin	Trypsin	No Data		
	Alcalase	0.69 ± 0.93^{ab}		
	Flavourzyme		0.68 ± 0.28^{bc}	
	Alcalase/Flavourzyme	0.35 ± 0.21^{b}		
β-Lactoglobulin	Trypsin			0.71 ± 0.20^{ab}
	Alcalase			0.55 ± 0.19^{ab}
	Flavourzyme		0.55 ± 0.21^{c}	
	Alcalase/Flavourzyme		1.12 ± 0.23^{ab}	