Identifying peptides from oat protein with potential hypocholesterolemic and satiety effects

by

Lijin K Litson

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Department of Agricultural, Food and Nutritional Science

University of Alberta

Abstract

The prevalence of cardiovascular diseases is a significant concern globally, and a move towards prevention through healthy diet and lifestyle modifications is recommended. Obesity and high cholesterol concentration in the blood are common risk factors for developing cardiovascular diseases. Excess weight is mainly due to poor food choices and lack of physical activity, increasing cholesterol concentration. Therefore, making healthy food choices is essential to reducing the risk of cardiovascular diseases. Currently, oat consumption is associated with cholesterol reduction, mainly due to the presence of dietary fiber, especially β-glucan. Oat protein has recently gained prominence for its nutritional value and functional properties. However, research on peptides generated from oat protein and their bioactivities is still limited. Therefore, this study aims to explore the feasibility of generating peptides from oat that can eventually impact cholesterol concentration in the blood and promote satiety effect in the gut.

Oat protein concentrate was hydrolyzed through simulated gut digestion using pepsin and trypsin as digestive enzymes. The hydrolysates were then fractionated as F1, F2, F3 and F4 based on their hydrophobicity using a reverse phase HPLC. Dipeptidyl-peptidase 4 (DPP4) enzyme inhibition assay was carried out using Caco-2 cells. DPP4 is an enzyme that cleaves and inactivates incretin hormones such as glucagon-like peptide 1 (GLP-1). Studies indicate DPP4 drug inhibitors allows the release of GLP-1, delays gastric emptying, and suppresses appetite. In this study, oat peptides fractions especially F4 showed 50% of DPP4 inhibition at the concentration of 50 ug/ml. Therefore, inhibiting the DPP4 enzyme would eventually lead in increase in GLP-1 levels which further increases the insulin level and enhances satiation Similarly, hypocholesteremic properties of oat peptides was carried out by evaluating HMG-CoA reductase (HMGCR) inhibition and micellar solubility of cholesterol. HMGCR is considered as the rate-limiting enzyme towards

cholesterol formation in the body. Fractions such as F3, F4 and HP (hydrolyzed peptide) inhibited HMGCR enzyme with 85%, 79% and 83% of inhibition at 200 ug/ml *in vitro* and F1 at 2 mg/ml showed 38% decreased solubilization of cholesterol micelles. Therefore, these results suggests that oat peptide could have an impact on cholesterol concentration in the blood.

These oat peptides were further identified using LC-MS/MS and sequences such as Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR) and Leu-Gly-Leu-Ser-Gln-Gln-Ala-Ala-Gln-Arg (LGLSQQAAQR) were found derived from 11S and 12S oat globulins. New *de novo* sequences rich in proline, arginine, phenylalanine, leucine, and valine were identified. The data suggests that the presence of hydrophobic amino acids could have favored the DPP4 inhibition. The presence of alanine and leucine in F3 and F4 with the constant presence of proline could provide structural flexibility to maximize the binding with the hydrophobic pockets on HMGCR. However, the presence of arginine, lysine and tyrosine which could possess an amphipathic nature could be the reason for higher binding capacity in F1 fraction with bile salts which are amphipathic in nature as well.

Currently, statin drugs, bile sequestrants are used as targets of cholesterol treatment. Therefore, the idea to generate peptides with hypocholesteremic and satiety improving properties from oat proteins provides a significant strategy to develop natural and healthy food products for hypercholesteremia and obesity prevention or management. In addition, this would further increase the acceptance of oats as part of a healthy diet and advance the revenue of oat production industries.

Keywords: oat protein; obesity; hypocholesterolemic peptides; satiation; DPP4 inhibition; HMGCR inhibition; micellar solubility.

Preface

The research project, of which this thesis is a part, is an original work by Lijin K Litson under the supervision of Dr. Lingyun Chen. The mass spectrometry and amino acid analysis was performed at the Alberta Proteomics and Mass Spectrometry Facility.

Dedication

This dissertation is dedicated to my family for their support, encouragement, strength, and love.

Above all, I thank God for enabling everyone to contribute to this thesis.

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1.1 **Cholesterol Metabolism**

1.1.1 Consumption of food and its role in energy production in the body

It is vital for the human body to produce energy to carry out essential functions and metabolic reactions. Macronutrients such as carbohydrates, lipids, and proteins, called metabolic fuels, mainly supply this energy within the cells. Their energy is transferred into a molecule termed adenosine 5' triphosphate (ATP) for use and storage within the cell (Dunn & Grider, 2023). ATP is primarily used in energy-dependent mechanisms such as muscle contractions, active-ion transport, synthesis of macromolecules and thermogenesis (Bonora et al., 2012). During digestion, the breaking down of proteins, lipids, and polysaccharides into smaller units, such as amino acids, fatty acids, glycerol, and glucose, leads to glycolysis reaction. Pyruvate and acetyl CoA is produced by glycolysis reaction, and as a result, ATP is produced. The smaller units from the macronutrients, such as glucose, and amino acids, through the chain of reactions, can produce pyruvate and fatty acids in the case of acetyl CoA. The oxidation and stepwise breakdown of fatty acids derived from lipids produce large amounts of acetyl CoA and, therefore, energy in the form of ATP (Alberts et al., 2002). In general, the human body is constantly in requirement of energy to carry out its essential functions and reactions.
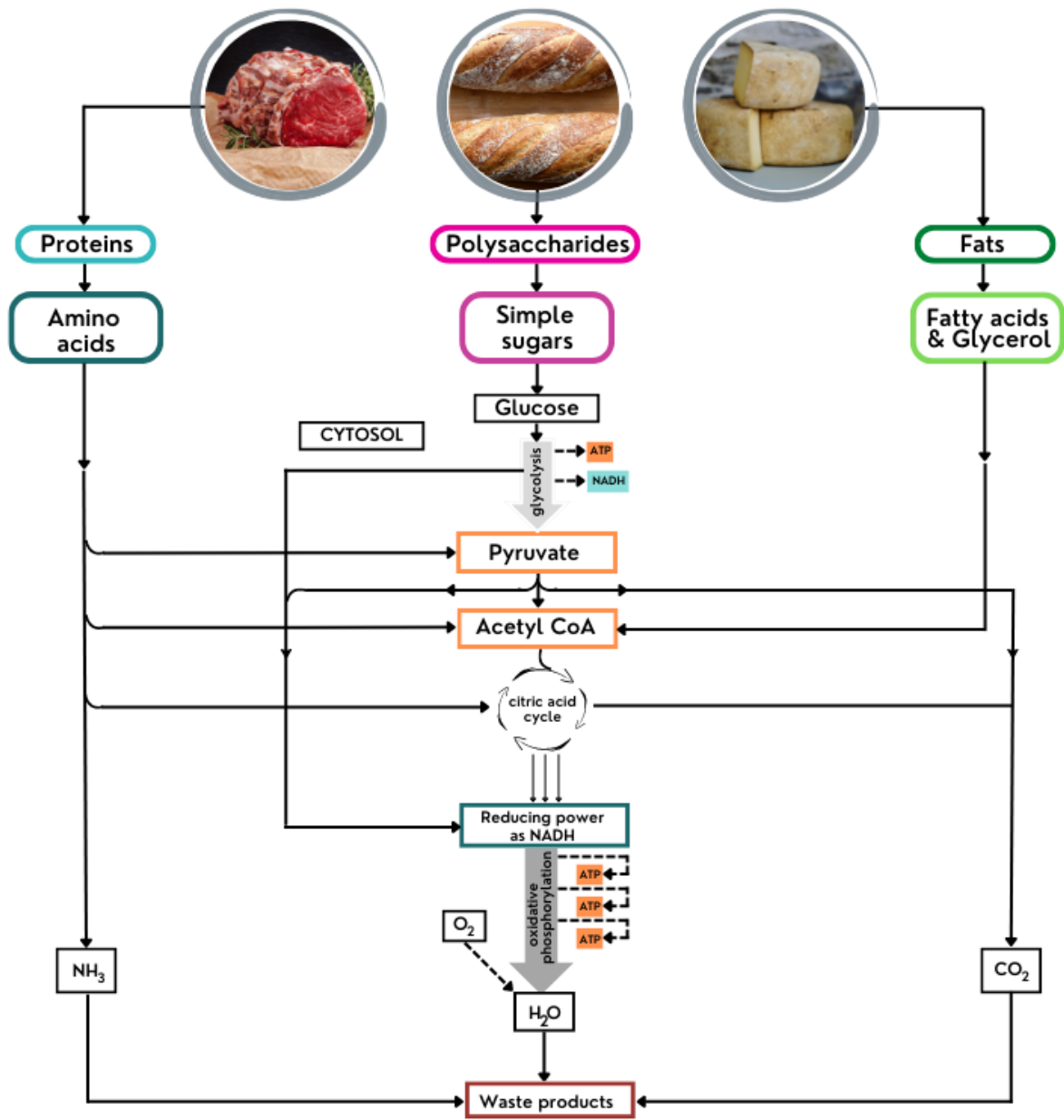
Figure 1.1 Food molecules broken down to produce ATP

Adapted from Alberts et al., 2002.

1.1.2 Lipolysis and intestinal lipid absorption

Fats present in food are called dietary fats, and the major types of dietary fats are saturated fats, trans fats, monounsaturated fats, and polyunsaturated fats (Hu et al., 2001). Some of these fats consist of a variety of polar and non-polar lipids, such as sterols (e.g., cholesterol), phospholipids, and other lipids like fat-soluble vitamins (Carey et al., 1983). Lipid digestion is initiated by digesting triglycerides present in the fat. This process happens in the oral cavity by lingual lipases secreted by glands in the tongue. The digestion is continued in the stomach with the help of gastric enzymes that break down fats into simpler units. After digestion, these by-products from lipid hydrolysis are absorbed by the epithelial cells present in the linings of the intestinal lumen called enterocytes, followed by transportation to the intracellular compartments (Iqbal & Hussain, 2009). Digestion of different fats happens differently, e.g., triacylglycerides are primarily digested in the jejunum by pancreatic lipase, phospholipids are digested by pancreatic phospholipase and cholesterol ester hydrolyzed by cholesterol esterase to yield free fatty acids and free cholesterol (Benzonana & Desnuelle, 1965; Bosch et al., 1965; Swell et al., 1958).

1.1.3 Cholesterol metabolism and its types

Cholesterol is a type of sterol mainly synthesized in animal cells and found in our diet, which is of animal source. The human body also produces cholesterol in the liver and then transports it to the bloodstream as spherical molecules called lipoproteins (van Heek et al., 2000). Cholesterol plays an important role in maintaining the fluidity and integrity of cell membranes and acts as a precursor in synthesizing bile acids, vitamin D, steroid hormones, and sex hormones (Di Ciaula et al., 2017). Since cholesterol is lipophilic in nature it is carried through blood plasma inside lipoproteins such as chylomicrons, high-density lipoproteins (HDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL). The presence of these

lipoproteins helps the detection and identification of the amount of cholesterol present in the blood (Huff et al., 2022).

The cholesterol concentration in the body is maintained by regulation of cholesterol synthesis (in the cell) or by absorption of cholesterol in the lumen (dietary intake). If the intake of cholesterol is high, then the rate of excretion will be high as the excess cholesterol will be excreted through feces (Huff et al., 2022). The cholesterol from the dietary intake present in the intestine is transported to the mucosal cell lining of the gut through processes dependent on emulsification and micellar solubilization by biliary lipids which possess detergent-like characteristics (Iqbal & Hussain, 2009). Bile acids are amphipathic in nature as the triglycerides present in the form of large aggregates hydrophobically interacts with the bile while the hydrophilic sides remain at the surface. Due to this interaction, the large-sized aggregates are broken down into smaller droplets as lipase enzyme can only act on triglyceride droplets. The lipase enzyme liberates monoglycerides and fatty acids which further acts with bile acids to form complexes with other lipids to generate a micelle. These micelles like structures are then taken in by the enterocytes present in the small intestine and further transported to the blood by particles called chylomicrons (Bowen, 2019). On the other hand, the biosynthesis of cholesterol that happens in the cell is followed by multiple complex processes. Some of the important interactions includes the formation of cholesterol from mevalonate. Initially, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA with the help of enzyme thiolase. The formed acetoacetyl-CoA interacts with another molecule of acetyl-CoA in the presence of HMG-CoA synthase which catalyzes the reaction to form HMG-CoA. HMG-CoA in the presence of HMG-CoA reductase (HMGCR) forms mevalonate. This process is the rate-limiting step towards the production of cholesterol. Finally, mevalonate through several sequential reactions forms cholesterol (Cerqueira et al., 2016).

The types of cholesterol present in the body can be determined based on the circulation of cholesterol with the help of lipoproteins. The major types of lipoproteins that transports the cholesterol are chylomicrons, VLDL, IDL, LDL and HDL. The consumption of saturated fatty acids significantly increases the level of cholesterol in the blood (Nettleton et al., 2017). It is very important to regulate the level of cholesterol in the blood. High cholesterol concentration in the blood can lead to fatty deposits in the blood vessels which would further increase the risk of cardiovascular diseases mainly heart attack and stroke (The National Health Science, 2022).

1.2   Prevalence of obesity

The increase in the prevalence of overweight and obesity in adults and children is a rapid and significant public health concern (Wang et al., 2011). According to The Global Burden of Disease, obesity is responsible for 4.7 million deaths as of 2017 (Dai et al., 2020). Obesity is mainly due to unhealthy lifestyles and food patterns comprising poor food choices, which can further lead to severe chronic diseases like cardiovascular conditions, high cholesterol concentration, and other health concerns (Dai et al., 2020). Obesity is commonly measured using the body mass index (BMI) scale. According to the World Health Organization (WHO), BMI is defined as a simple index of weight for height that helps to distinguish excess adiposity. Obesity is generally defined as a BMI value greater than 30 kg/m^2 (World Health Organization, 2022). In the case of North America, as of 2018, 71% of Americans are obese or overweight and research suggests that the consumption of fast and processed foods may be leading to more premature deaths than cigarette smoking (Fuhrman, 2018). The Canadian Health Measures Survey conducted in the year 2015 showed that more than 30% of adult Canadians suffer from obesity. It is also estimated that among the Canadians aged between 20 and 64, one in ten premature deaths are due to obesity (Obesity

Canada, 2022). Although multi-faceted, obesity can somewhat be tackled by maintaining a healthy lifestyle, and most importantly by consuming healthy food choices (Ritchie & Ross, 2017).

1.2.1   *Pathogenesis of dyslipidemia and potential risk factors*

Dyslipidemia can be defined as the elevation of serum cholesterol. It is characterized by increased levels of total cholesterol, LDL cholesterol, triglycerides, and reduced serum HDL cholesterol (Hedayatnia et al., 2020). Health behaviors like physical inactivity, excess tobacco use, high consumption of saturated fats and lack of proper nutrition i.e., low consumption of vegetables, fruits and nuts can increase the lipid levels in the body. These factors can further lead in developing cardiovascular diseases (CVD) with severe effects (Pappan & Rehman, 2022). Hypercholesteremia is a form of dyslipidemia that is attributed with increased risk of CVD. Hypercholesteremia is asymptomatic and can be detected only through a serum test. Although, there are no symptoms it is crucial to maintain the cholesterol concentration in the blood as high cholesterol concentration can develop fatty deposits in the blood vessels and can cause difficulty for the blood flow in the arteries. This can lead to a blood clot in the artery and may lead to a heart attack or stroke (MayoClinic, 2021). The process mainly begins when these fatty deposits form atherosclerotic plaques which further damages the endothelial cells and dysfunction them. This allows the LDL particles to permeate through the vascular wall and allows more lipid to accumulate within the wall. The atherosclerotic plaques further lead to decrease in blood flow (ischemia) or rupture the arterial vessel wall which can completely block the flow of blood causing myocardial infarctions (Huff et al., 2022). According to National Heart, Lung, and Blood institute, a cholesterol screening is recommended every five years for people aged between 9 to 11 and every one to two years for men aged between 45 to 65 and women aged between 55 to 65. More

frequent tests are recommended if their family history of hypercholesteremia or other factors such as diabetes and high blood pressure which constitutes to metabolic syndrome (NIH, 2022).

Hypercholesteremic values are dependent on the medical history of a particular individual. Cholesterol levels in the blood are measured in mg (milligrams) of cholesterol per dL (deciliter) of blood. For example, for a normal individual hypercholesteremia is of concern when the LDL cholesterol value goes beyond 190mg/dL. But for an individual who possess major risk factors like hypertension, diabetes, family history of premature ACD (atherosclerotic cardiovascular disease) and smoking, the cholesterol value would be lesser than the normal standard value i.e., < 190mg/dL. The value can also differ for individuals with low HDL cholesterol concentration i.e., < 40 mg/dL in male and < 55 mg/dL in a female and age i.e., male 45 years or older and female 55 years or older. Therefore, the cholesterol measuring value is dependent on an individual's medical history. A person is hypercholesteremic when LDL cholesterol value is greater than 160 mg/dL with one of the aforementioned risk factors and or if the value is greater than 130 mg/dL with any two of the mentioned risk factors (NIH, 2022).

1.2.2   Diagnosis and pharmacological treatments

Since hypercholesteremia is asymptomatic, lipid profiling or lipid panel is needed to determine cholesterol concentration present in the blood; this is ideally done after a 10 to 12 hour overnight fast. An average lipid panel test result normally includes the concentration of LDL cholesterol, HDL cholesterol, triglycerides, and total cholesterol (Huff et al., 2022). These values are further used to screen the abnormalities in different cholesterol concentration. According to U.S. Food and Drug Administration (FDA), the recommended total cholesterol level should be 200 mg/dl or less, the LDL cholesterol level less than 100 mg/dL, the HDL cholesterol level greater or equal to 40 mg/dL and the triglycerides level less than 150 mg/dL (U.S. Food and Drug Administration,

2018). The Friedewald formula as given below, is used to calculate the LDL cholesterol present in the blood and is accurate if the triglyceride (TG) concentration is not greater than 200 mg/dL. This formula is not recommended if the TG value is above 400 mg/dL (Ibrahim et al., 2022).

Friedewald formula:

LDL Cholesterol = Total Cholesterol – VLDL (TG/5) – HDL Cholesterol

High blood cholesterol concentration is often treated medically with medications and lifestyle modifications. The lifestyle modification mainly includes increase in physical activity, following a healthy diet and losing weight as obesity can lead to increased cholesterol concentration. Positive lifestyle changes can significantly improve body weight, hypertension and diabetes which plays an important role in regulating cholesterol concentration in the blood. Smoking cessation is also recommended for tackling hypercholesteremia (Huff et al., 2022; Ibrahim et al., 2022). For individuals with higher risk, a cholesterol lowering drug is commonly used with complementing with the lifestyle alterations. Statins are the most used drugs in the pharmacological market for the treatment of hypercholesteremia. Some of the most common statin drugs includes, Pravastatin, Atorvastatin, Rosuvastatin, Simvastatin, Fluvastatin, Lovastatin, Pitavastatin and Rosuvastatin (MayoClinic, 2021). For the past three decades, statin has been the most important treatment in tackling heart diseases. This is mainly done by the statins inhibiting the HMGCR enzyme which is a rate limiting step towards cholesterol formation in the cell and hence aids in the lowering of cholesterol concentration in the blood (Stancu & Sima, 2001). The decrease in cholesterol biosynthesis upregulates LDL cholesterol receptors and provides better movement for LDL cholesterol transport from the blood. Statin therapy is known to effectively reduce the level of LDL cholesterol, triglycerides and raise the levels of HDL cholesterol. Statins also help in stabilizing the atherosclerotic plaque which would further aid in decreased arterial

blood vessel rupture (Stone et al., 2014). Although, these drugs show significant effect, they also come with some major side effects. Statin drugs when taken display adverse reactions like myopathy, hepatotoxicity, rhabdomyolysis, and diabetes mellitus. These conditions can further cause renal and liver damage. It is recommended that patients prescribed with statin drugs must periodically conduct liver function tests because these drugs can raise the levels of transaminases (Brown & Watson, 2018; Sizar et al., 2022). Another series of drugs that aids in reducing hypercholesteremia are the bile acid sequestrants which are FDA approved. These drugs act by forming an insoluble complex with the bile acids and reduces the absorption of bile acids in the intestine. Since it cannot be digested in the intestines, it remains biochemically unchanged as it passes through the intestine and excreted in the feces. As there is depletion in the amount of bile acids present in the intestine, hepatic cholesterol is then converted to bile acids. This reduces the concentration of LDL cholesterol in the blood. The side effects caused by bile acid sequestrants are not as adverse as statin drugs as it is not absorbed in the gastrointestinal tract. Nevertheless, it can have a major effect on individuals with renal problems like renal impairment and other effects like stomach upset, constipation, vomiting, bloating, heartburn, and loss of appetite (Lent-Schochet & Jialal, 2022). Therefore, it is important to consider factors like drug intolerance, patient's medical history, psychological factors, and cost for treating hypercholesteremia.

1.3   Satiation and satiety

One of the major factors that contributes to excess body weight are unhealthy food choices. Consumption of fast food in excess amounts can increase the risk of overweight, obesity and cardiovascular diseases. Fast foods are high in fat and are commonly related to poor nutrition and higher energy intake (World Cancer Research Fund and American Institute for Cancer Research, 2007). The relationship behind obesity and body fat reflects positive energy homeostasis, where

the energy from food intake is greater than the energy expended by the means of physical activity, metabolism, and thermogenesis (Amin & Mercer, 2016). Hence, it is important to maintain energy balance. Reducing the intake of calories and consuming food which promotes satiety, satiation and energy compensation would be helpful in tackling overweight and obesity (Benton & Young, 2017). The sense of feeling hungry is the signal that initiates the meal consumption process, and this signaling occurs in the stomach due to the secretion of ghrelin hormone and other metabolic signals such as hypoglycemia. This action furthers as the vagus nerve sends electrical signals to the brain relating to the feeling of emptiness. Similarly, satiation is the feeling of fullness that leads to meal termination and possibly determines the meal size. Satiety is the feeling of fullness from the meal termination instance to the hunger initiation for the next meal (Amin & Mercer, 2016). The signaling after the meal also known as post ingestive feedback occurs in the stomach and the intestine providing the brain feedback based on the meal quantity. This allows gut peptide hormones like glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) act on meal processing and inhibits further intake of food (Van Kleef et al., 2012). CCK hormone is mainly released when protein and fat (mainly) are ingested as food (Wang et al., 2011). GLP-1 hormone is an incretin hormone which stimulates insulin secretion and inhibits glucagon secretion. GLP-1 drugs in the pharmaceutical market are agonists displaying anti-diabetes and anti-obese properties. These drugs are known to reduce appetite and feeling of hunger by delaying the release of food from stomach and elevate the sense of "feeling full" after eating (Ard et al., 2021).

GLP-1 hormone is produced by cleaving of pre-proglucagon gene. It is mainly expressed in the intestinal L-cells, α-cells of the pancreas and in the neural hypothalamus and in the solitary tract of the brainstem. The significant bioactive GLP-1 species in the humans are GLP-1 (7-36) amide and GLP-1 (7-37) (Baggio & Drucker, 2007; Jensterle et al., 2019). On ingestion of a meal,

GLP-1 is synthesized and released by the L-cells of the intestine. The primary response of GLP-1 secretion after meal ingestion occurs within minutes and remains for 30 minutes post ingestion followed by the second phase which occurs in 60 minutes or so later (Herrmann et al., 1995). GLP-1 hormone allows the satiation signals to be produced by activating the mechano-receptors and further relayed to solitary tract in the brainstem by the vagus nerve. This can be related to the medium-term satiety which occurs initially where the satiation signal is conveyed by the vagus nerve before nutrient absorption in the gut. This is partly because GLP-1 can delay gastric emptying and movement of food in the gut (Delgado-Aros et al., 2002). The second phase of the satiation signals occurs in the gut, which allows the intestine to release GLP-1 hormone because of the presence of food in the stomach (Rüttimann et al., 2009). Studies suggests that GLP-1 injections can lead to inhibition of food intake and this action can be related to the long-term satiety (Turton et al., 1996).

One of the most common effects of the GLP-1 hormone and other incretin hormones are the release of insulin which helps to maintain glucose concentrations in the blood and these hormones contribute to ~70% of insulin secretion (Nauck & Meier, 2018). Insulin plays a very important role in regulation of the glycemic index in the blood as an increase or a decrease in the glucose concentrations can cause adverse health risks. Hence, an increase in GLP-1 hormone can further stimulate insulin secretion and can inhibit glucagon secretion. But the half-life period of GLP-1 is of only few minutes, and this is because of the enzymatic degradation of GLP-1 happens rapidly. The enzyme responsible for this degradation is dipeptidyl peptidase IV (DPP4) and this increases blood glucose levels (Drucker & Nauck, 2006; Mentlein et al., 1993; Nauck & Meier, 2018). The incretin hormones are degraded by DPP4 linked with the hepatocytes during their passage across the hepatic bed (Deacon, 2004). Therefore, the introduction of DPP4 inhibitors in

the pharmaceutical market is a success in treatment of type 2 diabetes. These DPP4 inhibitors work by inhibiting the DPP4 enzyme, allowing more insulin secretion, and further regulating the glucose levels in the blood (Gallwitz, 2019). Some of the most common DPP4 inhibitors in the pharmacological market includes sitagliptin, linagliptin, alogliptin and saxagliptin (FDA, 2016).

Although, potential drugs like HMGCR inhibitors, bile sequestrants and DPP4 inhibitors are readily available in the pharmaceutical markets for the treatment and reducing the risk of metabolic syndrome, it is certain that there are adverse side effects connected to them. As a result, it is suggested that individuals can improve health outcome through healthy eating and other healthy lifestyle approaches (Tuso et al., 2013). According to U.S. Department of Agriculture (USDA), considering a shift to more plant-based diet would reduce the risk of cardiovascular diseases and obesity (U.S. Department of Agriculture, 2015). Research suggests that plant-based diets can promote healthy eating, are cost-effective and of low risk; it as an intervention that may help in lowering body mass index, cholesterol concentration, and blood glucose. They also reduce the number of medications taken associated with cardiovascular risks (Tuso et al., 2013). A balanced plant-based diet helps to reduce the intake of processed foods, oils, animal foods which are high in cholesterol and promotes the consumption of vegetables, beans, peas, lentils, fruits, and seeds which are generally low in fat (Blaney & Diehl, 2011). According to the food guide presented by Government of Canada, a plant-based diet is mainly considered as part of healthy eating pattern in order to maintain health (Government of Canada, 2022).
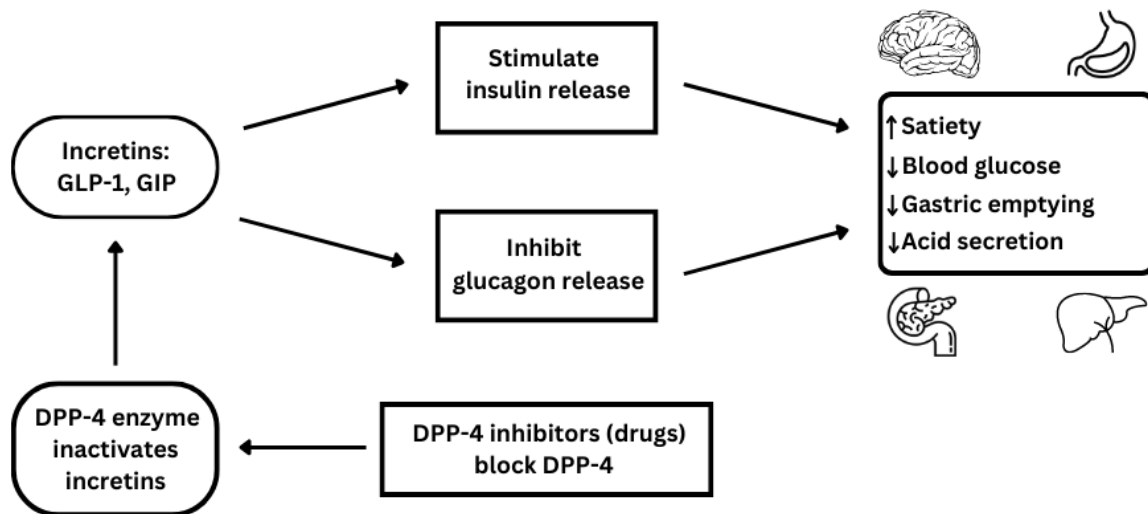
Figure 1.2 Incretin activity due to DPP4 inhibition.

1.4   Significance of protein consumption

The consumption of protein is critical and essential for development, growth, and health of humans. Protein deficiency can cause adverse health risks that affects the functioning of human body like growth, fatigue, swelling up of body and loss of muscles (Garg & Sangwan, 2019). The demand for dietary protein-based food has surged over the last few years. Animals, dairy and few plant proteins consists of high-quality proteins based on their essential amino acid profile. Despite animal proteins being considered 'complete proteins' as they contain all nine essential amino acids, there has been a significant increase in the popularity of plant-based proteins. Plant proteins can be combined with other protein sources in order to create a balanced and complete protein diet (Langyan et al., 2021). The trend towards plant-based diets is related to the association among continuous consumption of animal-based protein and increase in risk factors of metabolic syndrome (Chung et al., 2020), among other conditions. Research suggests that the consumption

of plant-based proteins can reduce cardiovascular risks, reduce LDL-cholesterol, obesity, and type 2 diabetes (Guasch-Ferre et al., 2019; Langyan et al., 2021). Plant protein also possess satiating effects which can further help in tackling obesity (Kristensen et al., 2016). Therefore, consumption of a diet that is majorly constitutes plant-based protein food could help in reducing the risk of metabolic syndrome. Foods like oats have been shown to be a good source of nutritional quality with high amount of protein. Oats possess hypocholesteremic and anti-obesity properties which may reduce the risk of cardiovascular diseases (Wouk et al., 2021).

1.5   Bioactive peptides and their properties

Bioactive peptides are molecules that can be found in the intact structure of food protein. These are specific components that exerts significant biological activities and can have positive effects on the human body (Daliri et al., 2017). Bioactive peptides can be released during the gastrointestinal digestion or food processing (Abdel-Hamid et al., 2017). The physiological role of the peptides based on their type, sequence, and specific amino acids have made them an efficient option for producing therapeutic compounds (Akbarian et al., 2022). Owing to their low side effects, bioactive peptides can be applied as a strategy to prevent/reduce the risk certain chronic diseases.

Studies suggests that peptides generated from protein sources possess different bioactivities such as anti-inflammatory, antimicrobial, antioxidant, wound healing, hypocholesteremic and anti-obese properties (Daliri et al., 2017). For instance, peptides from fermented soybean, black bean protein hydrolysates, salmon frame protein hydrolysates and silver carp showed potential anti-diabetic properties through in silico and *in vivo* studies (Kwon et al., 2011; Mojica et al., 2017; Roblet et al., 2016; Zhang et al., 2016). Peptide from cumin seeds also

showed anti-diabetic properties by inhibiting α-amylase enzyme, thereby inhibiting the breakdown of starch into sugars (Siow & Gan, 2016). Peptides from whey (bovine milk), casein, fermented β-casein and lactoferrin showed antihypertensive properties by inhibiting the angiotensin 1-converting enzyme (ACE). Peptides from tuna displayed anticancerous activity by inhibiting proliferation of cells in breast cancer cell line (Hung et al., 2014). Many bioactive peptides possess multifunctional bioactivties which makes them a potential factor to address different health risk at one time. For instance, peptides from lentil proteins, hemp seed and rice bran proteins showed both antihypertensive and antioxidant properties (García-Mora et al., 2017; Girgih et al., 2014; Wang et al., 2017). Peptides from cumin seeds displayed cholesterol lowering activity, antiamylase activity and antoxidant activity (Siow et al., 2016; Siow & Gan, 2016). Peptides isolated from egg yolk possessed antioxidant, antidiabetic and ACE inhibitory activities (Zambrowicz et al., 2015). This multifunctional properties of peptides could play an important role in addressing metabolic syndrome which is mainly due to the coexistence of different comorbodities. However, further research is required in this area as there are many factors that may influence the processing of bioactive peptides and the interactions between the bioactive peptide and the target molecule.

1.5.1 *Peptides with cholesterol-lowering effect*

There are several studies associated with the cholesterol-lowering effects of peptides from different sources of proteins. For instance, bovine milk peptides Ile-Ile-Ala-Glu-Lys (IIAEK) isolated from β-lactoglobulin inhibited the cholesterol absorption *in vitro* using Caco-2 cells and reduced serum cholesterol level using *in vivo* in animal experiments. The same peptide IIAEK also showed positive effect on cholesterol 7α-hydroxylase enzyme (CYP7A1) which is a rate-limiting enzyme in cholesterol catabolism and bile acid synthesis (Nagaoka et al., 2001). A novel hypocholesteremic peptide Val-Ala-Trp-Trp-Met-Tyr (VAWWMY) prepared from soybean

glycinin showed significant bile acid binding ability and micellar solubility of cholesterol. VAWWMY also acted as a potential inhibitor of cholesterol absorption *in vivo* (Nagaoka et al., 2010). Two peptides isolated from soybean β-conglycinin Tyr-Val-Val-Asn-Pro-Asp-Asn-Asp-Glu-Asn and Tyr-Val-Val-Asn-Pro-Asp-Asn-Asn-Glu-Asn (YVVNPDNDEN & YVVNPDNNEN) displayed statin-like properties by competitvely inhibiting the catalytic domain of HMGCR enzyme. Since, HMGCR is the rate limiting step towards cholesterol formation the inhibition of HMGCR could help in reducing the cholesterol in the blood. These peptides also showed potential in increasing the protein levels of LDL-receptor which would further help in smooth intake of LDL-cholesterol for cholesterol metabolism (Lammi et al., 2015). Peptides from soy Leu-Pro-Tyr-Pro-Arg and Trp-Gly-Ala-Pro-Ser-Leu (LPYPR and WGAPSL) proved to disrupt the cholesterol from mixed micelles and were compared to plant sterols and stanols which displaces cholesterol in a similar fashion and is a well-accepted mechanism (Zhang et al., 2013). Hempseed peptides displayed significant cholesterol lowering effects on human hepatoma cells (HepG2) by inhibiting the HMGCR enzyme and upregulating the proteins responsible for cholesterol reduction such as regulatory element binding proteins 2 (SREBP2) and LDL-receptor (Zanoni et al., 2017).

The presence of certain amino acids, the structure of their sequences and the positioning of amino acids in a sequence could play important roles in cholesterol-lowering activity of a specific peptide. For instance, peptides from bovine milk namely Ile-Ile-Ala-Glu-Lys (IIAEK), Ala-Glu-Lys (AEK), Glu-Lys (EK), Asp-Lys (DK) and Trp-Lys (WK) showed potential hypocholesteremic effect and the author concluded that the presence of C-terminal lysine could be important for peptides to have hypocholesteremic effect (Nagaoka, 2019). Peptides from sunflower hydrolysates which mainly contained hydrophobic amino acids especially the presence of Ala, Val, Leu, Lys or

Tyr showed higher rate of cholesterol inhibition by decreasing the micellar solubility of cholesterol (Megias et al., 2009). Similarly, Zhang et al. (2013), Kwon et al. (2011), and Prados et al. (2020), concluded that the presence of hydrophobic amino acids in the peptide could be the reason towards their cholesterol-lowering effects. In comparison, Sugano et al. (1988) suggested that the hypocholesteremic activity of the peptides was mainly due to the structure of the amino acid sequences and not based on the hydrophobicity of the peptide. The presence of both hydrophobic N-terminal side and hydrophilic C-terminal side in the peptide sequences showed potential hypocholesteremic activity (Nagaoka et al., 2001; Takenaka et al., 2003; Zanoni et al., 2017). This amphiphilic character of peptides can increase its potential as cholesterol/lipids lowering alternative. However, more research is required to understand the inhibitory interactions between the peptide and the substrates involved in cholesterol inhibitng mechanisms.

1.5.2 *Peptides with satiation/satiety effects*

Dietary proteins when taken in high amounts are generally considered to reduce food intake and induce satiety. The mechanism of action in inducing satiety can vary from stimulating GLP-1 or other incretin hormones production to inhibition of enzyme responsible in degradation of these hormones. As a result of this, the food intake decreases and promotes delayed gut motility and gastric emptying. Peptides isolated from fish protein hydrolysates induced GLP-1 production in STC-cell line and displayed a decreased food intake in male wistar rats. An evidence of decreased body weight gain was also shown in rats (Cudennec et al., 2012). Similarly, peptides derived from egg, whey, pea, wheat, casein and codfish stimulated GLP-1 release in the STC-1 cell line (Geraedts et al., 2011). Tyr-Pro-Phe-Pro-Gly-Pro (YPFPGP), Phe-Pro-Gly-Pro-Ile (FPGPI) and Tyr-Val-Pro-Phe-Pro-Pro-Phe (YVPFPPF) derived from casein protein hydrolysates and Ala-Leu-Met-Pro-His (ALMPH), Leu-Ile-Val-Thr-Gln-Thr-Met-Lys-Gly (LIVTQTMKG) and Leu-Ile (LI)

from whey protein hydrolysates showed potential satiety activity *in vitro* and *in vivo* (Aoki et al., 2017; Kondrashina et al., 2020; Osborne et al., 2014; Tulipano et al., 2017). Studies on satiating effect using cell line and mechanism connected to GLP-1 production especially using plant-based peptides are scarce, however research on peptides inhibiting DPP4 enzyme are of an appreciable amount. These studies mainly focus on antidiabetic peptides inhibiting DPP4 helping in insulin production which would further help in increased satiety effect.

DPP4 inhibitory activity was seen in peptides Ala-Lys-Ser-Pro-Leu-Phe (AKSPLF), Phe-Glu-Glu-Leu-Asn (FEELN), and Glu-Gly-Leu-Glu-Leu-Leu-Leu-Leu-Leu-Leu-Ala-Gly (EGLELLLLLLAG) retrieved from black bean protein hydrolysate in silico analysis against sitagliptin as drug (Mojica & De Mejía, 2016). Lys-Thr-Tyr-Gly-Leu (KTYGL), Lys-Lys-Ser-Ser-Gly (KKSSG), Gly-Gly-Gly-Leu-His-Lys (GGGLHK), Cys-Pro-Gly-Asn-Lys (CPGNK) from Brazilian carioca beans and Mexican black beans showed DPP4 inhibition (Mojica et al., 2017). Peptides such as Ile-Ala-Val-Pro-Gly-Glu-Val-Ala (IAVPGEVA), Ile-Ala-Val-Pro-Thr-Gly-Val-Ala (IAVPTGVA), and Leu-Pro-Tyr-Pro (LPYP) from soy glycinin increased glucose uptake and IAVPTGVA particularly showed good DPP4 inhibitory activity (Lammi et al., 2015). This interaction between IAVPTGVA, DPP4 and their regions were identified as Glu205 and GLU206 (amino terminal) and Arg358 (carboxyl terminal) using in silico molecular modeling study (Lammi et al., 2016). Similarly, studies on DPP4 inhibitory peptides derived from different sources like tuna cooking juice, egg, canary seeds, soybean, lupin, rice bran, amaranth and other sources are reported (Zani et al., 2018; Jao et al., 2015; Shaikh et al., 2021).

1.6   Enzymes in hydrolyzing peptides

The production of bioactive peptides have been drawing research and industry interest due to their biological activities. These bioactive peptides are only activated upon their release from the

parental protein and this action can be achieved by using different methods like digestive enzymes, chemical hydrolysis, commercial proteolytic enzymes, and other food processing techniques such as fermentation, curing or maturation (Chauhan & Kanwar, 2020; Daliri et al., 2017). The most favorable and used methods for the release of bioactive peptides are enzymatic hydrolysis and microbial fermentation. In microbial fermentation, microorganisms like bacteria, fungi or yeasts capable of releasing proteolytic enzymes are used as these enzymes further hydrolyzes proteins into shorter bioactive peptides (Cruz-Casas et al., 2021). However, microbial fermentation can produce other compounds such as bacterial living or dead cells, bacteriocins and exopolysaccharides and is susceptible to different problems like inconsistency of final products and poor yield between batches when compared to enzymatic hydrolysis (Martínez-Augustin et al., 2014; Rudnitskaya et al., 2016; Toldrá et al., 2018). Enzymatic hydrolysis involves breaking down of proteins into shorter chains of peptides at controlled pH and temperature for a certain period. These enzymes are then inactivated at high temperature and followed by separation of hydrolysates and characterisation (Ying et al., 2021). Enzymatic hydrolysis is cost effective, and the higher peptide yield, simple process and the high specificity of the enzyme makes it an advantageous method to release the bioactive peptides from the parent protein source (Martínez-Medina et al., 2019; Michalak et al., 2017). Some of the common enzymes used in the hydrolysis are alcalase, flavorzyme, pepsin, trypsin, chimotrypsin A etc (Cruz-Casas et al., 2021; Mora et al., 2018).
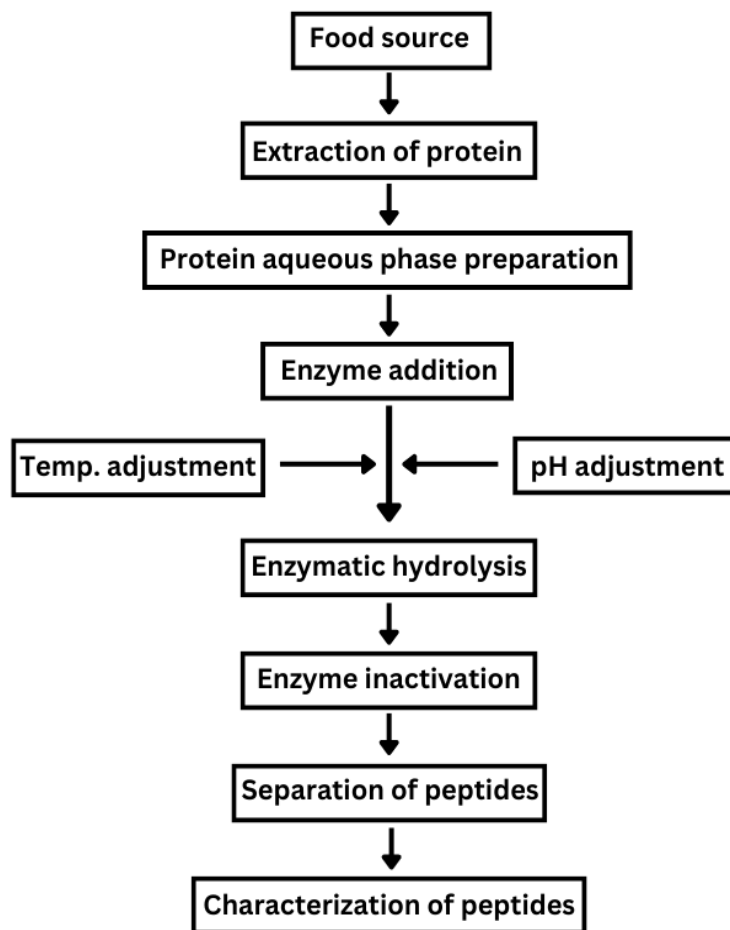
Figure 1.3 Bioactive peptides produced through enzymatic hydrolysis.

1.6.1   Protein hydrolysis in gut to generate peptides

Peptides are also generated by human gut digestion where the major digestion of proteins starts in the stomach and ends in the small intestine. Hydrochloric acid secreted by the parietal cells in the stomach and pepsin makes up the gastric fluid which further helps in digestion of protein into smaller peptides (Hsu et al., 2022). These peptides are then moved into the small intestine and further digested into smaller fragments by the intestinal fluid which includes trypsin or chymotrypsin (Dallas et al., 2017). Similarly, simulated gut *in vitro* digestion is done in glass containers and are preferred because of their practical, feasible and economical advantages. Dynamic conditions and mechanical forces can't be mimicked which could be considered as a

disadvantage (Sensoy, 2021). However, this does not compromise the process as bioactive peptides are still produced efficiently by the action of enzymes. The mixture of simulated gastric and intestinal fluid helps in the hydrolysis of the protein at optimal pH and temperature similar to the stomach and small intestine. This method of enzymatic hydrolysis can be useful as enzymes can be highly specific in cleaving certain amino acids. For instance, pepsin favors cleavage of leucine and phenylalanine and it rarely cleaves amino acids such as lysine and histidine unless they are placed adjacent to leucine and phenylalanine (Ahn et al., 2013; Hamuro et al., 2008). Trypsin prefers cleaving the peptide bonds at the C-terminal of arginine and lysine does not cleave if arginine and lysine is followed by proline. Similarly, the cleavage of trypsin can be slowed down if lysine and arginine are adjacent to amino acids that are acidic in nature (Manea et al., 2007; Simpson, 2006). However, factors such as exposure time to enzymes, pH of the digestion, concentration of the enzymes all play a role in the outcome of biopeptides produced (Ahn et al., 2013). Therefore, strict digestive conditions should be followed in order to produce an efficient hydrolysis of peptides.

1.7   Cell culture test

The bioavailability of a bioactive peptide is mainly dependant on the absorption of the peptide in the host and available for its cellular metabolism. The bioavailablity is normally tested *in vivo* (animal) and through clinical trials based on a specific dose (Santos et al., 2019). However, due to the cost, ethical issues and other issues related to animal and human experiments (Akhtar, 2015), the closest available option to *in vivo* experiments are the *in vitro* cell culture model which is a method considered to study the behavior of animal cells under a controlled environment. Over the years, cell culture method have been used to study basic cell biology and interactions between drugs, chemicals, proteins, vaccines etc with the cells (Arango et al., 2013). This helps in the

evaluation of absorption of food components which are broken down into simpler particles; in this case peptides and further determine their potential bioactivity. Caco-2 cell lines are the most widely used to determine bioactive peptide absorption and other ex vivo models like Ussing chamber, everted sac gut are used as tissue based models (Ozorio et al., 2020). Caco-2 cells are derived from colon carcinomas and can display morphological and functional characteristics of mature enterocytes. These cell lines have been widely used to simulate *in vitro* gastrointestinal model and can help in predicting the bioavailability of bioactive food nutrients and drugs in the cell line (Ozorio et al., 2020; Picariello et al., 2013; Santos et al., 2019). Several studies shows that Caco-2 cell line was used to study the biological effects such as DPP4 inhibitory activity, antioxidant activity, anti-inflammatory activity, antihypertensive activity and ACEI (angiotensin converting enzyme inhibition) activity of peptides from different protein sources (Álvarez-Olguín et al., 2022). Caco-2 cell models are also used to explore the cellular transport and mechanistic transport of specific bioactive peptides (Guha et al., 2021).

1.8   Oats

1.8.1   Oats as a healthy crop

Oat is a distinctive crop as it possesses multiple nutrients that is suitable for human food, animal feed, cosmetics and health care (Butt et al., 2008). Oat is one of the oldest crops known to humans and it has been cultivated for more than 2000 years in several parts of the world (Sang et al., 2017; Varma et al., 2016). Oat is considered as a cereal crop and it is a main source of dietary protein, carbohydrates, dietary soluble fiber, lipids, vitamins, minerals and other phenolic compounds (Joyce et al., 2019). There is an increase in attention towards oats by scientific researchers and food industries and this is mainly due to the growing awareness of the consumers towards healthy eating. For instance, the presence of soluble fiber – oat β-glucan is considered to possess anti-

diabetic and cholesterol lowering effects (Andrade et al., 2015; Whitehead et al., 2014). The phenolic compounds present in oats are associated with the role of defense mechanism against pathogens and help in preventing diseases such as cancer, heart diseases and stroke (Skoglund et al., 2008). The sterols and phytic acid present in oats have the potential to prevent the release of metal-mediated free radicals (Andrade et al., 2015). Phytochemicals such as steroidal saponin, avenacins and avenacosides display defense mechanisms against pathogens and have potential immunoregulatory, anticancer and cholesterol lowering activities (Sang et al., 2017). Similarly, the nutrient contents in oats are associated with their effect on cardiovascular diseases by reducing cholesterol and possess anti-diabetic, antioxidant, anticancerous, anti-inflammatory and antimicrobial effects. Therefore, food-based industries have shown an interest in developing novel food products using oats and incorporating it into snack bars, breakfast cereals, beverages and bread (Boukid et al., 2018).

1.8.2   Oats in Canada

Canada is the second largest producer of oats globally and is considered to be the world's largest exporter of high-quality oats (CerealsCanada, 2022). In 2020, oats worth $465 million was exported and over 3 million tonnes was produced (The Observatory Of Economic Complexity, 2020). Intially, oats were mainly used to feed the livestock, but over the years the consumption of oats by humans increased gradually due to the demand towards plant-based and gluten free products and other potential health benefits. Canada's top quality oats are popular in the domestic and international market for their health benefits (The Prairie Oat Growers Association, 2022). Oats are mainly cultivated in climates with cooler temperatures. Russia, Canada, Finland, Poland and Australia were ranked as top five countries in the world for producing oats (Zwer, 2004). There are mainly two kinds of oats – naked oats and husked oats. Husked oats contains hulls around the

kernel or groat after harvesting. Naked oats produce threshing character similar to wheat. Although, husked oats majorly represents the oat production, naked oats are also gaining its prominence in different markets (Zwer, 2004). However, the need in developing novel applications with regards to oats and its products is essential to enhance the oat production and reap all the possible benefits from this potential cereal crop.

1.8.3 Oats as a source of protein and its nutritional and functional properties

Avena sativa L. and *Avena nuda* L. are the most common varieties of oats and *Avena nuda* which is also known as naked oats are considered to be highly nutritious due to its high amino acid content (Arendt & Zannini, 2013; Zwer, 2004). Oat is generally consumed as a breakfast cereal flakes or using it as a flour to prepare different foods. However, the production of oat protein on an industrial scale in the form of beverages, snacks and other oat protein foods has gone up (Mäkinen et al., 2016). Oat mainly contains 12%-20% protein comprising globulin, prolamin, albumin and glutelin (Klose & Arendt, 2012). The nutritional value of oat protein is mainly based on protein concentration, digestibility of protein and the balance of essential amino acids present in the oats (Boczkowska et al., 2016). The digestibiltiy of oat protein was found to be 90% and this is similar to the digestibility of rice and corn (Welch, 2011). The presence of prolamin is less in oat proteins compared to that of rye, wheat and barley which can be a suitable cereal for individuals suffering from celiac diseases (Butt et al., 2008). Celiac disease or gluten intolerance is due to gluten which is a type of prolamin protein and found in grains and cant be digested completely by the gastrointestinal tract and can cause damage to the intestinal mucosa (Green & Cellier, 2007). The presence of protein content present in different grains is showed in **Table 1.1** and oat has the least amount of glutenins present in its protein content. Globulin is the major storage of protein in oats and is divided mainly into 12S, 7S and 3S globulins (Rafique et al., 2022; Zhang et al., 2015). The

distribution of amino acids in the oat protein can differ even among its own fractions, for instance; globulin proteins in oat carry the highest number of essential amino acids and also non-essential amino acids like arginine and glutamic (Jing et al., 2016). In a recent study, the amino acid profiling of naked oats showed higher number of hydrolyllic amino acid residues present in oats (Yue et al., 2021). Neverthless, the presence of essential amino acids in oat proteins constitutes to ~32.3% (Liu et al., 2009). In a study conducted by Zarkadas et al., (1995), three Canadian developed naked oat cultivars were assessed for their protein quality and amino acid composition. The presence of total essential amino acid content was found to be ~33.75% - **Table 1.3**. Although, the oat is considered to be a potential source of protein, it is still reviewed as an incomplete protein as it is low on lysine, which is an essential amino acid (Bonke et al., 2020). However, oat can be combined with different protein sources which are rich in lysine to provide a complete protein (Gorissen et al., 2018; Rasane et al., 2015).

Oat protein are also known for its potential functional properites. Oat proteins are generally soluble at alkaline pH and this suggests the potential to use alkaline extraction method for oat protein extraction (Ma & Harwalkar, 1984). The albumins present in oats possess high solubility at a wide pH range and globulins show highest solubility mainly at alkaline and acidic pH. At neutral pH oat globulins shows low solubility which could be a drawback in the developing aqueous food (Ma & Harwalkar, 1984; Mel & Malalgoda, 2022). However, it is possible to increase the solubility of oat protein by methods like succinylation and deamidation (Mirmoghtadaie et al., 2009). The foaming ability of oat protein and its stability is mainly due to the increased solubility of oat protein at higher pH. Oat protein isolate displayed foaming capacity similar to lupin protein and foaming stability that was twice as great as lupin protein (Mohamed et al., 2009). In a study conducted by Konak et al., (2014), oat protein showed the potential of being

used in beverages due to its foam stability and can act as a substitute for egg whites. Foaming

capacity in a protein is due to the protein network engulfing the air phase to increase the volume

and becomes stable after the processes like heating and mixing (Foegeding et al., 2006). Oat

protein is reported to demonstrate water holding capacity (WHC) (Mirmoghtadaie et al., 2009).

Albumins and glutelins present in oat proteins possess higher water holding capacity than globulins

(Ma & Harwalkar, 1984). This functional property of WHC can help proteins in moisture

absorption and can have an impact on the sensory attributes of food products (Walters et al., 2018).

Oat protein could make an impact in developing low-fat food products like snacks or salad dressing

due to its significant emulsion capacity and emulsion stability (Mohamed et al., 2009). In a study

conducted by Yang et al., (2017), cold-set oat protein gels displayed significant gelling properties

and showed potential to prevent early release of bioactive substances through by-passing the

stomach juices. These gels are then degraded in the gut and allows a gradual release of the bioactive

compounds. Similarly, different functionalities like fat binding capacity and gelling properties

studied on oat protein displayed the potential of oat protein from commercial food industry

perspective (Mel & Malalgoda, 2022).

Table 1.1 Distribution of protein content in oat and other grains.

Grains	Protein Content	Albumins	Globulins	Prolamins	Glutenins
	%	%	%	%	%
Oat	12-20	70-80	1-12	4-15	≤10
Rice	7-10	7-17	5-10	3-6	75-81
Wheat	11-15	20-25*	20-25*	30-40	45
Pea	23-31	2.47	7.01	1.52	87.47

Millet	7-11	11-17*	11-17*	6.8-9.3	39-54

*, Albumin and Globulin combined

Modified from Rafique et al. (2022); Žilić et al., (2011)

Table 1.2 Nutritional composition of oats

Nutrient (per 100g)	Oats	Oat Flour	Oat Bran
Energy (kcal)	389.0	404.0	246.0
Water (g)	8.2	8.6	6.6
Macronutrients			
Carbohydrates (g)	66.3	65.7	66.2
Protein (g)	16.9	14.7	17.3
Lipid (g)	6.9	9.1	7.0
Dietary Fiber (g)	10.6	6.5	15.4
Minerals (mg)			
Iron	4.7	4.0	5.4
Magnesium	177.0	144.0	235.0
Calcium	54.0	55.0	58.0
Phosphorous	523.0	452.0	734.0
Potassium	429.0	371.0	566.0
Sodium	2.0	19.0	4.0
Zinc	4.0	3.2	3.1
Vitamins (µg)			
Vitamin E	NR	700.0	1000.0

Vitamin K	NR	3.2	3.2
Vitamin B1	800.0	700.0	1200.0
Vitamin B2	100.0	100.0	200.0
Vitamin B3	100.0	1500.0	900.0
Vitamin B6	100.0	100.0	200.0
Folate	56.0	32.0	52.0

Modified from Menon et al. (2016); Rasane et al. (2015)

Table 1.3 Amino acid composition of Canadian developed naked oat cultivars (%).

Amino Acids (AA)	AC Hill*	AC Lotta*	AC Perry*
Essential AA			
Phenylalanine	5.37	5.30	5.28
Histidine	2.47	2.50	2.60
Methionine	1.49	1.38	1.45
Leucine	7.90	7.54	7.53
Lysine	3.70	3.84	3.96
Isoleucine	4.32	4.16	4.18
Threonine	2.88	3.23	2.87
Valine	5.62	5.31	5.57
Total	33.75	33.26	33.44
Non-essential AA			
Aspartic acid	7.25	7.95	7.38
Glutamic acid	23.20	22.31	22.87
Alanine	4.50	4.63	4.60

Cysteine	4.35	5.50	4.83
Arginine	6.19	6.80	6.77
Glycine	4.46	4.19	4.44
Proline	6.09	5.28	5.55
Serine	4.23	4.42	4.24
Tyrosine	4.28	4.05	4.22
Total	64.55	65.13	64.90

Modified from Zarkadas et al. (1995)

* Three cultivars of naked oats (Hill, Lotta and Perry) developed in Canada

1.8.4   Oat protein and its potential to reduce cholesterol and promote satiety.

The nutritional advantages of oats and its effect on cholesterol, cardioprotective, blood glucose and obesity are generally studied on oat components like β-glucans which is the dietary fiber found in oats. Consumption of diet with β-glucan rich oats is shown to decrease the levels of LDL cholesterol, total serum cholesterol and blood pressure (Beta & Camire, 2018; Braaten et al., 1991). The viscous nature of β-glucan allows the interaction with bile acids resulting in no adsorption of bile acids in the terminal ileum. This leads to elimination of bile acids through fecal excretion thereby increasing the requirement of bile acids from cholesterol available in the blood (McRorie Jr & McKeown, 2017). This leads to decrease in LDL-cholesterol. High viscosity of β-glucan is also attributed to delayed gastric emptying and slowing the digestion and absorption processes (El Khoury et al., 2012). Oat β-glucan has also been attributed in delaying the absorption of nutrients in the gut by reducing the blood glucose and increasing the insuling response which further reduce the blood pressure (Dreher, 2018; Keenan et al., 2002). However, the health benefits are mainly

attributed to oat components like dietary fiber and phenolic compounds, the study on effects of oat peptides and the biological response of these peptides on cholesterol and obesity is less known.

Current studies on oat peptides on different effects such as antioxidant activity, antidiabetic activity, antihypertensive activity, antiobese activity and cholesterol-lowering activity are studied *in vitro*, *in vivo* and in silico mainly (Rafique et al., 2022). *In vitro* and in silico studies on peptides obtained through enzymatic hydrolysis helps us to understand the possible sequences involved in hypocholesteremic activity (cholesterol micelle-binding and HMGCR inhibition) and satiety effects (DPP4 inhibition). To date, peptides from different sources of proteins have showed the potential for HMGCR inhibition, cholesterol micelle binding and DPP4 inhibition. As mentioned earlier, the presence of certain amino acids and the arrangement of a peptide sequence plays an important role in enzyme binding and other interactions. The amino acid profile in oat protein is more balanced and this makes oat protein a potential to reduce cholesterol and promote satiety (Zhang et al., 2021). For instance, in a study conducted by Walters et al., (2020), GLP-1 hormone secretion in NCI-H716 cell line was improved after treatments with oat bran protein hydrolysates. Oat protein fed to hamsters displayed significant decrease in LDL cholesterol and increased the excretion of bile acids (Tong et al., 2016). However, the study on cholesterol lowering activity of peptides from oats *in vitro* is limited. Sequencing of the peptides enhances the understanding of peptide chains and amino acids involved in the interactions. Therefore, a study involving peptides to counteract obesity, high cholesterol concentration and researching the possible mechanisms involved could reveal the potential of oat peptides in reducing the risk of cardiovascular diseases. Following a simulated gut digestion method using the enzymes pepsin and trypsin could help us to further relate to oat protein when consumed by humans in the form of beverages or food. The exploration of these characteristics of oat protein will help an individual towards healthy eating

and also pave the way towards the development of oat-protein based foods that would help in reducing the risk of cardiovascular diseases.

1.9   Research hypotheses and objectives

This research aimed at studying the effects of peptides derived from oats for hypocholesteremic acitivities and satiety effects, and characterize peptide fractions based on their structures to maximize their bioactivities.

Based on the literature review, the following hypothesis was proposed:

1. Peptides with potential hypocholesteremic activity and satiety effect could be generated from oats through simulated gut digestion.

The overall objective of this study was to generate bioactive peptides with potential cholesterol-lowering and anti-obese activities for weight management.

The specific objectives were:

1. To carry out oat protein hydrolysis and fractionation of peptides (e.g., RP-HPLC fractionation) to produce peptides with cholesterol lowering and satiety effect.

2. To investigate the effect of the derived oat peptides on cholesterol micelles, HMGCR enzyme and DPP4 enzyme through enzymatic assays and cell culture.

3. Identify the potential peptide sequences from oats that contribute to the cholesterol-lowering and satiety effects.

1.10   Significance of this work

This proposed study will provide insights on new peptide sequences derived from oat that can effectively inhibit the enzymes that promotes cholesterol formation and decreases satiety effect. The positive results will provide justification to investigate the health effects of oat peptides by using *in vivo* and clinical trial tests in collaboration with nutrition scientists. Exploring peptides

that can reduce the risk of obesity and cardiovascular diseases paves the way for creating a natural option towards healthy eating and weight management. Introduction of these peptides in food products and beverages like protein bars, snacks, smoothies, protein drinks or yogurts can help in managing cholesterol without taking therapeutics such as drugs which could have potential side effects. Overall, this research will provide new and improved opportunities for oat products in food development and potentially increase the consumption of oats, thus increasing the revenue of oat producers and oat industries in Canada.

Chapter 2 – Preparation of cholesterol lowering and satiety-promoting peptides from oat protein hydrolysates and identification of peptide structures

2.1 Introduction

The number of individuals having obesity and overweight is increasing rapidly in the world (Finucane et al., 2011; Gortmaker et al., 2011). According to the World Health Organization, more than 1 billion people across the world have obesity and these individuals are highly prone to cardiovascular diseases, diabetes, and certain cancers, and almost 3.4 million deaths per year are attributed to excess body weight (WHO, 2022). The development of obesity in an individual often leads to adipose and adipocyte dysfunction. Such dysfunction can lead to fat accumulation in the body and further lead to a condition called as hypercholesteremia (Aguilar & Fernandez, 2014; Bluher, 2009, 2013). Hypercholesteremia is the presence of high level of cholesterol in the blood and as of 2014, more than 10 million people had hypercholesteremia (Mundal et al., 2014). Statin drugs are most used in lowering the cholesterol concentration in the blood. However, statins have adverse effect on the human body such as in muscles and mitochondrion (Golomb & Evans, 2008). Weight loss causes neuroendocrine changes that happen along with changes in an individual's food intake, their appetite perception, and energy homeostasis; all of these makes maintaining and losing weight difficult (Rosenbaum & Leibel, 2010).

Suppressing an individual's appetite by following the food and dietary guidelines is intimidating, particularly in an environment that serves alluring food choices (Cohen, 2008). Appetite in humans is controlled by peripheral and central mechanisms that interact with the surrounding environment. Studies have suggested that specific food components can inhibit the

feeling of hunger after a meal, thus controlling appetite for a short duration. Therefore, a diet with these food components, combined with other changes in lifestyle such as physical activity, could lead to reductions in bodyweight (Halford & Harrold, 2012). Foods that increase satiety have actively been an area of investigation and have great promise in tackling obesity and reducing the health effects of excess body weight (Beck, Tapsell, Batterham, Tosh, & Huang, 2009; Lyly et al., 2009).

Among these food sources, proteins and peptides are of significant interest. The bioactive peptides released from the food proteins can exert many beneficial effects on bodily functions. Recent research interests include bioactive peptides that can contribute to weight management and cholesterol reduction. These includes peptides with alpha-glucosidase and dipeptidyl peptidase IV (DPP4) inhibitory effects and cholesterol lowering capacity. In addition, peptides possessing antioxidant, and anti-inflammatory properties have been used to prevent metabolic disorders such as obesity and type 2 diabetes (Zani et al., 2018). DPP4 is a type of transmembrane glycoprotein and exoprotease that cleaves and inactivates the incretin hormones – glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) (Deacon et al., 2008). GLP-1 hormone increases insulin secretion and delays gastric emptying, thus can exert satiety effect (Zander et al., 2002). Therefore, a DPP4 inhibiting peptide can help in inhibiting DPP4 enzyme in the body which further releases GLP-1 satiety hormone to help weight management and prevent obesity. Molecular docking studies revealed that the hydrogen bonding and hydrophobic interaction between the bioactive peptide sequence and DPP4 contributed to inhibition of DPP4 enzyme (Jin et al., 2020). Amino acid sequences directly determine their biological functions. Currently, dairy milk proteins are the one of the major food protein sources to prepare DPP4 inhibitory peptides. From bovine α-lactalbumin namely Glu-Leu-Lys-Asp-Leu-Lys-Gly-Tyr and Ile-Leu-Asp-Lys-Val-Gly-Ile-Asn-

Tyr displayed strong DPP4 inhibitory effect. The molecular docking analysis further showed that these amino acid sequences formed hydrogen bonds and salt bridges with DPP4 (Gao et al., 2020). Synthetic dipeptides and hydrolysates retrieved from milk protein i.e., Glu-Lys, Gly-Leu, Ala-Leu, Val-Ala, Trp-Val, Phe-Leu, His-Leu, Ser-Leu showed potential DPP4 activity. Another study from the same author concluded that DPP4 inhibition was observed with methionine, leucine, and tryptophan (Nongonierma & FitzGerald, 2013a; Nongonierma et al., 2013). These studies suggest the presence of both hydrophilic and hydrophobic amino acids could play a role in displaying strong DPP4 inhibition effect. However, the amino acids Leucine and Isoleucine are notably present in the peptide chains mentioned above and further studies on similar peptide chains could help us understand better the inhibitory effect.

High cholesterol concentration is also considered as a major risk factor of obesity related diseases. Cholesterol lowering peptides can aid in decreasing the solubilization of cholesterol into micelles and inhibiting the rate limiting enzyme -HMG-CoA reductase (HMGCR) involved in the cholesterol biosynthesis. In human liver, bile acids are synthesized from cholesterol and considered to be potent "digestive surfactants" that assists in lipid absorption (Lefebvre et al., 2009). In this process, the bile acid forms aggregate with digested food particles, cholesterol, monoglycerides, phospholipids and fatty acids (Wilson & Rudel, 1994). These aggregate forms a micelle and the micelle are then absorbed into the gut with the help of intestinal epithelial cells (Woollett et al., 2006). Statin drugs used to treat hypercholesteremia possess a strong binding affinity towards the catalytic region of HMGCR enzyme by hydrophobic interactions (Istvan & Deisenhofer, 2001). Previous studies suggests that the hydrophobicity of the bioactive peptides displays a similar hydrophobic interaction to bind with HMGCR enzyme (Pak et al., 2012). Peptide sequences namely Ile-Ala-Val-Pro-Gly-Glu-Val-Ala, Ile-Ala-Val-Pro-Thr-Gly-Val-Ala and Leu-

Pro-Tyr-Pro derived from hydrolyzed soy protein regulated cholesterol metabolism in HepG2 human hepatic cells (Lammi et al., 2015). Asn-Ala-Leu-Glu-Pro-Asp-Asn-Arg-Ile-Glu-Ser-Glu-Gly-Gly, Asn-Ala-Leu-Glu-Pro-Asp-Asn-Arg-Ile-Glu-Ser and Pro-Phe-Val-Lys-Ser-Glu-Pro-Ile-Pro-Glu-Thr-Asn-Asn-Glu derived from pigeon pea were considered to be strong peptide HMGCR inhibitors (Kumar et al., 2019). Also, smaller peptide sequence from cowpea protein namely Ile-Ala-Phe, Gln-Gly-Phe and Gln-Asp-Phe exhibited cholesterol lowering activity by binding with HMGCR (Silva et al., 2021). The commonality of these studies suggests that the presence of hydrophobic amino acids in at C or N terminus ends could be the reason for binding and inhibiting the HMGCR substrate. The presence of hydrophobic amino acids also increases the hydrophobicity which further favors in stability of the bonded structure (Dyson et al., 2006). However, further studies are required to establish the understanding of the relationship between the HMGCR substrates, and the different amino acids involved in the inhibition of the HMGCR. Therefore, discovering bioactive peptides with cholesterol lowering and satiety effects from food proteins provide opportunities to develop new food and natural health products to help weight management and prevent diseases related to hypercholesteremia and obesity.

Oat is known for its various health benefits as it contains a high dietary fiber content, especially oat β-glucan which is known to lower blood cholesterol and regulate sugar levels (Rasane et al., 2015). Due to the growing health concerns related to obesity, diabetes and other chronic conditions, the interest in oats has increased significantly (Izydorczyk, 2014). Oat is also a good source of plant protein with higher amount of the essential amino acid lysine compared to other cereals. In addition, oat is a good source of phytochemicals including phenolic acids, sterols, tocopherols and tocotrienols. The presence of protein, dietary fiber and phytochemicals makes the oat one of the healthiest grains. Oat proteins contain a significant amount of hydrophobic amino

acids, especially leucine, isoleucine, and phenylalanine, thus the peptides released from oat could play a potential role in DPP4 inhibition and HMGCR binding. However, studies on peptides from oats with regards to cholesterol lowering ability and satiety property is still very limited. Recently, preliminary work on oat peptides was done in our lab and the peptides showed good DPP4 inhibitory effect (Fuentes et al., 2021). These peptides were prepared from enzymatic hydrolysis using alcalase as the digestive enzyme. However, studies on oat peptides prepared from simulating gut enzymes – pepsin and trypsin are very limited. Therefore, a study on peptides prepared by simulating gut digestion and studying the effect of these peptides on cholesterol and DPP4 inhibition is needed.

Bioactive peptides can be prepared by enzyme degradation or gut digestion. This research aimed to study the peptide generation in the simulated human gut. Our findings could provide information on the peptide release after individual oral consumption of oat protein.

Oat protein was digested in a simulated human gut by pepsin and trypsin enzymes. Peptides were further fractionated based on their molecular weight (M_W) and hydrophobicity. The fractions were then evaluated for their cholesterol lowering and satiety effects by cholesterol solubilization, HMGCR and DPP4 inhibitory activities. The fractions that displayed greater inhibition effects were then characterized for their peptide sequences, thus, to provide critical information about the peptides exhibiting satiety and cholesterol lowering effects from oat protein. Research to identify and develop these biological functions from oat proteins will further support health benefits of oat-based food products for obesity and weight management.

2.2 Materials and Methods

2.2.1 Materials

Naked oat grains (*Avena Nuda L.*) were obtained from Wedge Farms Ltd., Manitoba, Canada. Extraction of oat protein was done using alkaline extraction at pH 10 followed by acidic precipitation at pH 5, according to our previous work (Nieto-Nieto, 2014). The protein content was 76% as analyzed using combustion nitrogen analyzer (Leco Corporation, St Joseph, MI, USA) using a conversion factor of 5.83. Pepsin from gastric mucosa, trypsin from porcine pancreas and β-glucan from baker's yeast was obtained from Sigma-Aldrich (USA). The Amplex® Red Cholesterol Assay Kit was purchased from Thermo Fischer Scientific (Canada). The HMG-CoA Reductase Assay Kit and the DPP4 Activity Assay Kit were purchased from Sigma-Aldrich (USA). Other chemicals used were of analytical grade.

2.2.2 Preparation of oat protein hydrolysates

The enzymatic hydrolysis of oat protein in the simulated gut was conducted according to the method reported by (Begoña, 2013) with slight modifications. Protein solution was prepared by dispersing 6g extracted oat protein concentrates in 250ml of distilled water. Acidic saline solution (150 Mm NaCl, pH 2.5) was prepared and readjusted to pH 2.5 using HCl as required. Pepsin (0.1%) was added to the acidic saline solution to prepare the simulated gastric fluid. The oat protein suspension was then added in the simulated gastric fluid (1:1, v/v). Continuous gastric digestion at pH 2.5 was performed at 37℃ for 2 h in a water bath with agitation set at 100 rpm/min. Right after pepsin digestion, pH was raised to 7.4 by adding 1M NaOH and finally trypsin (0.6%) was added for further digestion in the simulated intestinal fluid. The samples were digested for 6h continuously at 37℃ in a water bath with agitation set at 100 rpm/min. Aliquots of 30ml were taken at 30 mins and 1h after the digestion in the simulated gastric fluid, and then at 2h, 4h and 6h after digestion in the simulated intestinal fluid. The obtained samples were boiled at 90℃ for 10

mins to inactivate the enzymes, followed by centrifugation at 2504 g force for 20 mins and the supernatant was collected. After freeze drying the samples, the obtained hydrolysates were analyzed for their molecular weight (Mw) distribution using the size exclusion chromatography, phosphate buffer was used as the mobile phase, flow rate was set at 0.5 ml/min and detection set at 280 nm (Agilent 1200 series HPLC system equipped with a TSKgel G3000SWXL column (7.8 × 300 mm, Tosoh Corp., TO, Japan)).

2.2.3 Membrane filtration fractionation of oat protein hydrolysates

The protein hydrolysates were then fractionated by an ultrafiltration system (Centramate Cassettes filtration system, T-series Omega, Pall Life Sciences, Mississauga, ON, Canada) using membranes with molecular weight (Mw) cut off of 1 kDa and 5 kDa. Accordingly, 3 fractions with Mw > 5kDa, 1-5kDa and <1kDa were collected, followed by freeze drying to obtain powders of peptide fractions.

2.2.4 Reverse Phase- High Performance Liquid Chromatography (RP-HPLC) fractionation

After ultrafiltration, the selected fraction was further separated based on the peptide hydrophobicity using the Agilent 1200 series HPLC system equipped with a reversed-phase column (Zorbax SB-C18 column, 4.6 x 150 mm; 5 μm). The samples were injected into the HPLC system at a concentration of 1mg/ml. The linear gradient mixture composed of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in Acetonitrile) was used to elute the peptides. The gradient elution conditions were: 5% solvent B for 16 min and 30% solvent B for 6 min, 90 % B for 20 min, and finally 18 min at 5% B. Gradient elution was conducted at a flow rate of 0.5 ml/min, at 60℃. Peaks were monitored at a UV wavelength of 280 nm. Four fractions F1, F2, F3 and F4 were collected based on peaks found on chromatogram. After the collection of a suitable volume, samples were freeze-dried and used for further analysis.

2.2.5 Amino acid composition analysis

The amino acid composition was performed at the Alberta Proteomics and Mass Spectrometry Facility using the Waters AccQ-Tag system (Waters Corp., Milford, Mass.). The hydrolyzed samples were derivatized in borate buffer with Waters AccQ-Fluor reagent at 55 ℃ for 10 minutes. Chromatographic analysis of the derivatized amino acids was done on an Agilent 1200 series HPLC system (Agilent, Santa Clara, Calif.). Samples were separated by a Waters AccQ-Tag column (3.9 × 150mm) at 37 ℃ with a three-eluent gradient solvent system (AccQ-Tag eluent, ACN, and water) at a flow rate of 1.5 ml/min and detected at 254 nm using an Agilent G1365D multiple wavelength detector. Asparagine and glutamine were hydrolyzed to their corresponding acids and were quantitated as such.

2.2.6 Hypocholesteremic Properties

2.2.6.1 Inhibition of HMG-CoA reductase (HMGCR) by in vitro assay

The conditions recommended in the HMG-CoA reductase assay kit from Sigma-Aldrich (USA) by the manufacturer was followed in this experiment. Pravastatin was used as the reference statin drug and was considered as the positive control. The provided assay buffer was added with reference drug (0.005 μl/ml) and the fractionated peptides at different concentrations 50, 100 and 200 μg/ml into an UV compatible 96 well plate. 4 μl of NADPH and 12 μl of HMG-CoA substrate solution was added to all samples. The activity was initiated by adding 2 μl of HMGCR enzyme (concentration of the enzyme stock solution was 0.50–0.70 mg protein/mL). The samples were incubated at 37 ℃. The consumption of the NADPH molecules was monitored every 20 s for up to 600 s by reading the absorbance at 340 nm and 37 ℃ using a spectrophotometer. The results were expressed as μmol of NADPH oxidized/min/mg protein in the absence or presence of drug or peptides.

2.2.6.2 Cholesterol micelle preparation

The cholesterol micelles were prepared using the method found in (Zhang et al., 2012) with modifications. A lipid mixture was prepared by dissolving 0.0005mmol/ml cholesterol, 0.001 mmol/ml linoleic acid and 0.0024 mmol/ml phosphatidylcholine in methanol and dried. The dried lipid mixture was then combined with 15 mM sodium phosphate buffer containing 6.6 mM taurocholate salt and 132 mM NaCl at pH 7.4. The suspension was treated by sonication in an ice bath using a Transsonic Digital S (Elma) sonicator with a probe tip at 100% of maximum output (140 W) for 20 min. After sonication, 0.1 ml of the suspension was mixed with 0.2 ml of peptide solution of different fractions F1, F2, F3, F4 and HP (hydrolyzed peptide) at different concentration ranging from 0.02, 0.2 to 2 mg/ml. β-glucan was used as a reference for this experiment and considered as the positive control. The mixture was further treated by ultrasound for 30 min and incubated for 1 h at 37℃. The solution was then centrifuged at 1000xg for 10 min and filtered through a 0.20 μm Millex- GP filter (Millipore, Bedford, MA, USA) and the supernatant was collected. The cholesterol concentration in the samples was determined using the Amplex® Red Cholesterol Assay Kit (invitrogen, Paisley, UK) by a fluorescence spectroscopy (SpectraMax M3; Molecular Devices, San Jose, CA, USA). The measurement was conducted at the excitation wavelength of 555 nm and the emission wavelength of 590 nm. The cholesterol standard curve was acquired using the calibration standards and the micellar cholesterol uptake inhibition was calculated using the equation:

Inhibition Capacity in % = {(C$_0$ – C s)/ C$_0$ } x 100

2.2.7 DPP4 enzymatic assay

DPP4 assay was carried out using the method of (Lammi et al., 2018) and following the recommended conditions given by the DPP4 Activity Assay kit from the manufacturer Sigma

Aldrich (USA) with slight modifications. A total concentration of 5 x 10^4 Caco-2 cells was seeded in fluorescent 96-well plates. On the second day, the spent media was removed, and the cells were treated with 10 µl concentration of Sitagliptin or peptide fractions at the concentration of 50, 100 and 200 µg/ml in growth medium for 1 h at 37℃. After treatment, the cells were gently washed with 100 µl of PBS buffer without Ca^{2+} and Mg^{2+}. Finally, the cells were treated with 2 µl of DPP4 substrate mixed with 38 µl of assay buffer and incubated for 5 mins at 37℃. The fluorescence was measured for every 1 min up to 10 min, using a fluorescence spectrophotometer (SpectraMax M3; Molecular Devices, San Jose, CA, USA) where the excitation wavelength was set as 360 nm and the emission detection wavelength as 460 nm. DPP4 activity was reported as microunit/ml where 1 unit of DPP4 is the amount of enzyme required to hydrolyze the substrate to release 7-Amino-4-Methyl Coumarin (AMC) as a fluorescent product at 37℃.

2.2.8 MS/MS analysis

The mass spectrometry analysis was performed at the Alberta Proteomics and Mass Spectrometry Facility. For the digestions the samples - fractions (F3 and F4) were reduced (200mm DTT in 50mm bicarbonate) and alkylated (200mM iodoacetamide in 50mm bicarbonate) before trypsin (6ng/ul, Promega Sequencing grade) was added to a ratio of 1:20. The digestion was allowed to proceed overnight (~16 hrs.) at 37℃ and formic acid was then added to adjust the pH to 2-4. The samples were then desalted using C18 tips (Thermo Scientific).

Peptides were resolved and ionized by using nano flow HPLC (Easy-nLC 1000, Thermo Scientific) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Scientific) with an EASY-Spray capillary HPLC column (ES800A, 75um x 15cm, 100Å, 3µm, Thermo Scientific). The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey orbitrap spectra using external mass calibration, with a resolution of

35,000 and m/z range of 300–1700. The twelve most intense multiply charged ions were sequentially fragmented by using HCD dissociation, and spectra of their fragments were recorded in the orbitrap at a resolution of 17,500; after fragmentation all precursors selected for dissociation were dynamically excluded for 30 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the database was searched using SEQUEST (Thermo Scientific).   Search parameters included a strict false discovery rate (FDR) of .01, a relaxed FDR of .05, a precursor mass tolerance of 10ppm and a fragment mass tolerance of 0.01Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

De novo sequencing was done using Peaks X Pro (Bioinformatics Solutions Inc., Ontario, Canada). The de novo or the average local confidence score (ALC score) was calculated using the Peaks software algorithm based on the logistic regression model. The de novo peptide and the peptide from the database is compared and computed for confidence score for each amino acid present in the sequence.

2.2.9 Statistical analysis

General Linear Model (GLM) of the Statistical Analysis System (SAS Inst. Inc., Cary, NC Version 9.4) was used to analyze the data. Comparison of samples means were made through one-way analysis of variance (ANOVA) followed by Tukey pos-hoc test at a significance level of 0.05.

2.3 Results and Discussion

2.3.1 Enzymatic hydrolysis of oat protein

The size exclusion chromatography (SEC) traces in **Figure 2.1** show the molecular weight distribution of oat protein and its hydrolysates by pepsin and trypsin enzymes. The efficiency of pepsin in hydrolyzing the peptides was evident by observing the change in molecular weight (M_W)

distribution, as it reached the maximum potential of digestion after 30 mins. After oat protein digestion by pepsin, the molecular weight of the peptides ranged from 4 kDa to 10.6 kDa. Further, protein hydrolysis was observed by trypsin digestion. Although the samples were tested in the simulated intestinal tract for 6h, the digestion reached its maximum level after 2h. The SEC chromatograms show the M_w of oat protein hydrolysates were in the range of 1.2-5 kDa after pepsin and trypsin digestion. The chromatograms obtained after 6 hours of trypsin digestion showed no significant change in the major peaks and continued to remain in the M_W range less than 5 kDa.

To simulate the digestion in the human gut, pepsin and trypsin were applied in this research as they play an important role in digesting the protein molecules entering the human gut. The gastric phase in the human gut involves pepsin digestion at acidic pH and the small intestinal phase involves trypsin digestion at alkaline pH 7.4 (Ahmed et al., 2022). Both pepsin and trypsin are endopeptidases (i.e., initiates breaking of peptide bonds within the protein molecule) and highly efficient in breaking down and in cleaving the peptide bonds (Fu et al., 2021; Hustoft et al., 2011). Pepsin enzyme mainly cleaves the peptide bond containing phenylalanine, leucine, and glutamic acid residues (Luna-Vital et al., 2015). Trypsin cleaves at C-terminus of residues with long side chains and displays high specificity in cleaving arginine and lysine (Olsen et al., 2004).
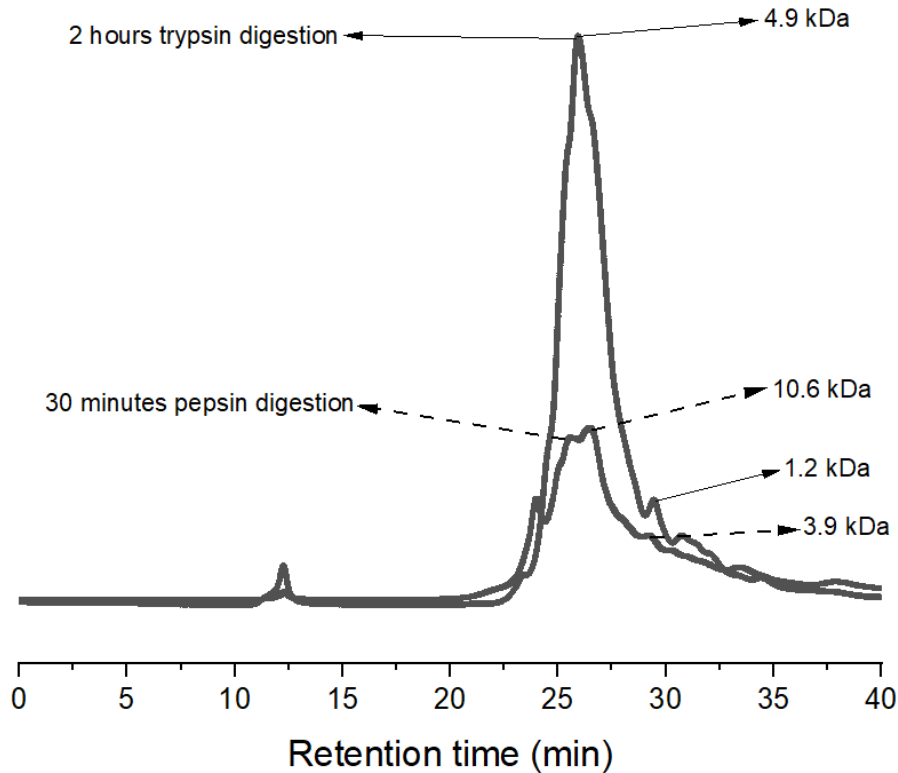
Figure 2.1 Size exclusion chromatogram of oat protein digested by pepsin and trypsin.

To better identify the desirable oat peptide fractions contributing to the cholesterol-lowing and DPP4 inhibition effects, the oat protein hydrolysates (HP) after 2h of digestion by pepsin and then another 2h by trypsin were then separated based on different hydrophobicity using RP-HPLC into four fractions including F1, F2, F3 and F4. Figure 2.2 shows the reverse phase chromatogram of the fractions obtained from hydrolyzed oat protein. The fraction F1 contained the most hydrophilic peptides, while the hydrophobic ones were accumulated in F4. All four fractions were further collected and tested for their effect on cholesterol micelle binding and inhibition of HMGCR and DPP4 enzymes.

Figure 2.2 Reverse-phase chromatogram of four fractions (F1-F4) obtained from oat protein hydrolysates.

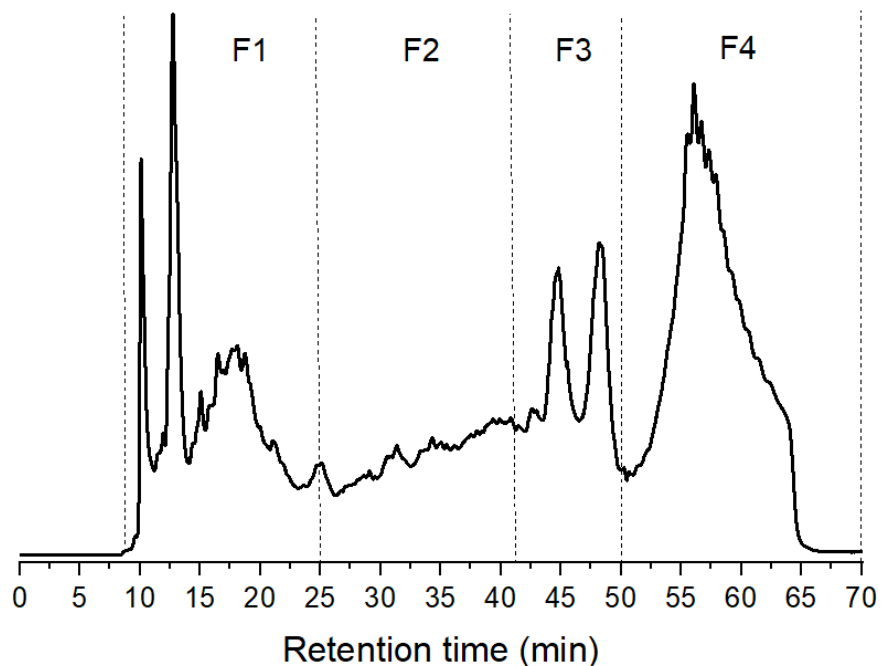### 2.3.2 Hypocholesteremic activity of oat protein hydrolysates

2.3.2.1 HMG-CoA Reductase activity (HMGCR activity)

In this research, pravastatin was used as a drug for comparison. The HMGCR inhibition activity treated by control, pravastatin as the drug and the peptide fractions was evaluated. Compared to F1 and F2, pravastatin, F3, F4 and HP were effective to inhibit HMGCR as reflected by significantly ($p<0.05$) reduced HMGCR activity. Pravastatin showed the highest inhibitory effect (88%). In general, as the concentration of HP, F3 and F4 fractions increased from 40 to 200 ug/ml, the inhibitory effect increased as reflected by the decreased HMGCR activity compared to the control.

The highest HMGCR inhibitory effects were observed for F3, F4 and HP at the concentration of 200ug/ml, reaching the values of 85%, 79% and 83% respectively. The highest inhibitory values were close to the inhibitory effect of the drug (pravastatin) (Fig. 2.3, 2.4 and 2.5). Even at lower

concentrations of 50 and 100 ug/ml, the HP, F3 and F4 samples reduced the HMGCR activity by 70-75% which is promising. Although, pharmacological intervention is effective at lower concentration, peptides have lesser side-effects and added value and thus can be added in the food formulations at higher concentrations to exert potential health benefits.

Figure 2.3 HMG CoA reductase activity relative to different concentrations of F3 (C50, C100 and C200 represent hydrolysate samples at the concentration of 50, 100 and 200 ug/ml). Drug represents pravastatin. Different letters (a, b, and c) above the bars indicate significant difference among samples.

At all concentrations, the HMGCR inhibitory activity of the F3 fraction was lower ($p<0.05$) than the control. There was no significant difference ($p>0.05$) between the drug and C200 in the F3 fraction which indicates that the F3 fraction at C200 is as effective as the drug activity on HMGCR.

Figure 2.4 HMG CoA reductase activity relative to different concentrations of F4 (C50, C100 and C200 represent hydrolysate samples at the concentration of 50, 100 and 200 ug/ml). Drug represents pravastatin. Different letters (a, b, and c) above the bars indicate significant difference among samples.

The HMGCR inhibitory activity of the F4 fraction was lower ($p < 0.05$) than the control at all concentrations. Although numerically, the inhibitory activity was slightly higher when compared to the activity in F3, there was no significant difference ($p > 0.05$) between the drug and C200 in the F4 fraction which again indicates that the F4 fraction at C200 is as effective as the drug activity on HMGCR.
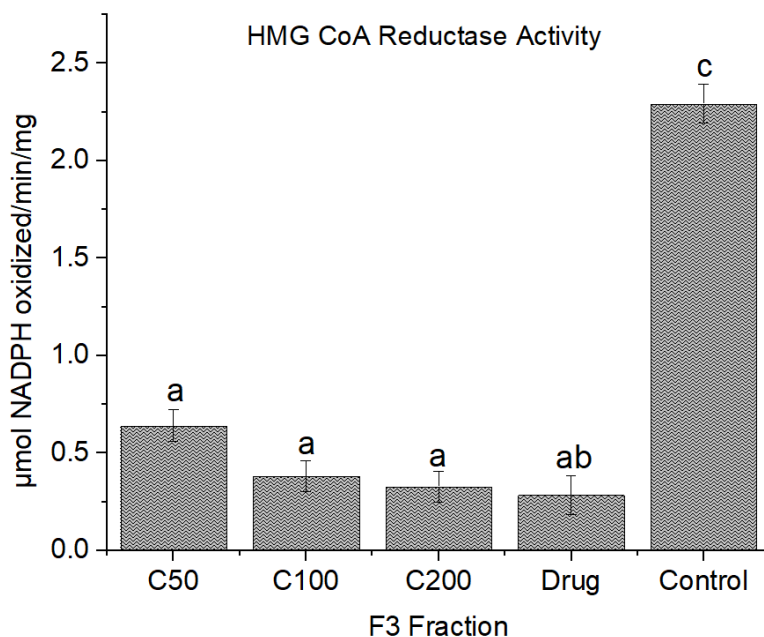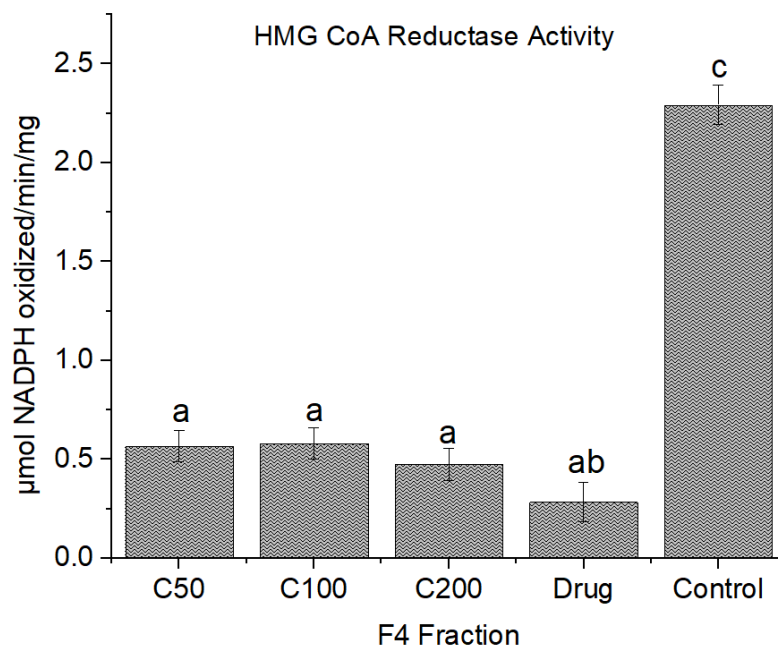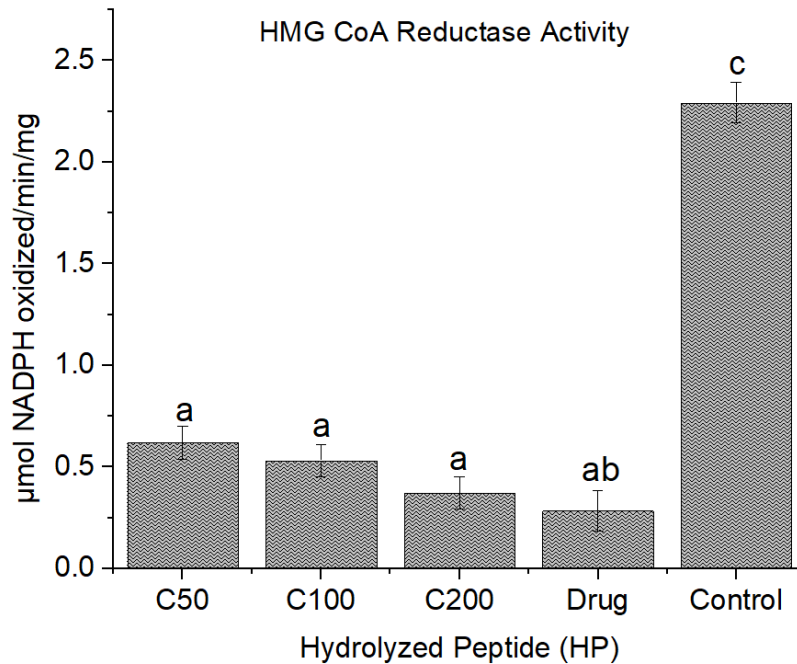
Figure 2.5 HMG CoA reductase activity relative to different concentrations of HP (C50, C100 and C200 represent hydrolysate samples at the concentration of 50, 100 and 200 ug/ml). Drug represents pravastatin. Different letters (a, b, and c) above the bars indicate significant difference among samples.

The HMGCR inhibitory activity of the HP fraction was lower ($p<0.05$) than the control at all concentrations. Although numerically, slightly higher when compared to the activity in F3, there was no significant difference ($p>0.05$) between the drug and C200 in the HP fraction which again indicates that the F4 fraction at C200 is as effective as the drug activity on HMGCR.

The cholesterol concentration in the human body is maintained through regulation of cholesterol synthesis followed by absorption. Cholesterol synthesis is initiated by acetyl-coenzyme A (acetyl-CoA) which acts as a precursor for cholesterol production. Two molecules of acetyl-CoA are condensed to form aceto-acetyl CoA with the help of enzyme thiolase. The formed aceto-acetyl CoA reacts with another molecule of acetyl-CoA, a process catalyzed by enzyme HMG-CoA synthase to form HMG-CoA. Furthermore, HMG-CoA oxidizes two molecules of

nicotinamide adenine dinucleotide phosphate (NADPH) which acts as a cofactor. Finally, the enzyme HMGCR in the presence of the oxidized molecules helps in achieving mevalonate which is a rate-limiting step towards cholesterol formation (Cerqueira et al., 2016). Therefore, inhibiting HMGCR enzyme essentially reduces the number of NADPH molecules oxidized and further affecting the overall cholesterol production. HMGCR inhibitors like pravastatin, simvastatin, lovastatin, and other stain drugs are mainly used to treat hypercholesteremia in the pharmaceutical market as they are effective in reducing blood cholesterol level (Ditschuneit et al., 1991). Generally, the statins act by binding to the catalytic domain of HMGCR which helps in blocking the conversion from HMG-CoA to mevalonate (Jiang, 2018).

In Fig. 2.3, 2.4 and 2.5, the drug showed the least amount of oxidation of NADPH molecules which symbolizes the inhibition of HMGCR enzyme. The fraction F3 at 200 ug/ml displayed a similar effect to that of the drug oxidizing the least amount of NADPH molecules among other fractions at different concentrations. The amino acid composition in **Table 2.2** shows that the fractions F3, F4 and HP were abundant in hydrophobic amino acids including leucine, proline, valine, alanine, and phenylalanine, with the percentage 62.5%, 66.8% and 56.6% respectively. Fractions F1 comprised 45% and F2 comprised 54.9% of hydrophobic amino acids. In the work by Kongo-Dia-Moukala et al., (2011) the cholesterol lowering peptides from defatted corn mainly consisted of proline, isoleucine, and alanine and both proline and isoleucine are mostly hydrophobic in nature. Similar conclusion can be seen where peptides containing leucine, isoleucine, valine, and proline showed strong HMGCR inhibition activity in the work done by Kwon and Soares (Kwon, 2002; Soares et al., 2015). In the case of statin interaction with HMGCR enzyme, a hydrophobic binding pocket is created near the active site of the enzyme. The hydrophobic groups present in the statin maximizes the contact with the hydrophobic pocket on the protein site which further helps with

the binding (Jiang, 2018; Istvan & Deisenhofer, 2001). A similar kind of binding by the bioactive peptides on HMGCR catalytic domain could be the reason for the inhibitory activity of F3, F4 and HP fractions. The structural and conformational analysis suggested that the bioactive peptides with inhibitory effect on HMGCR enzyme showed the structural flexibility (Pak et al., 2006). Dipeptides isolated from dry-cured ham showed HMGCR inhibitory activity ranging from 20% to 60% (Heres et al., 2021). Hydrolysates from raw cow pea protein hydrolysate showed 89% of HMGCR inhibition. The authors concluded that the constant presence of proline in the amino acid residues could be the reason for the structural flexibility present mainly in these peptides (Marques, 2015). Similarly, in a study conducted by Pak et. al (2010), HMGCR inhibition ranging from 37% to 75% was demonstrated by peptides isolated from 11S fraction of soybean protein. The presence of aromatic amino acids such as phenylalanine and tyrosine could more hydrophobic ring interactions similar to statin and further inhibit HMGCR enzyme. In addition, the presence of proline and other hydrophobic amino acids in the fractions F3 and F4 could favor inhibitory effect on HMGCR enzyme.

2.3.2.2 Micellar solubility of cholesterol

In this study, cholesterol micelle model was used to study the potential of oat hydrolysates and their fractions to lower the cholesterol level. β-glucan was used as a reference sample as it is known for its cholesterol binding and lowering capacity (Wang et al., 2017; Jesch & Carr, 2017). The results are shown in **Table 2.1** and Fig. 2.6. Fraction F1 showed the highest inhibition capacity of cholesterol solubilisation into micelles with the value of 16%, 30% and 38% at 0.02mg/ml, 0.2mg/ml and 2mg/ml respectively. The fractions F3 and HP also showed 10-20% inhibitory effect at the concentration ranging from 0.2-2mg/ml. The effect of F1 fraction at 2mg/ml was almost 2

times higher than F3 and β-glucan. Statistically it showed significance (p<0.02). It is worthy of mentioning that the peptides present in F1 fraction showed better inhibition effect than β-glucan.
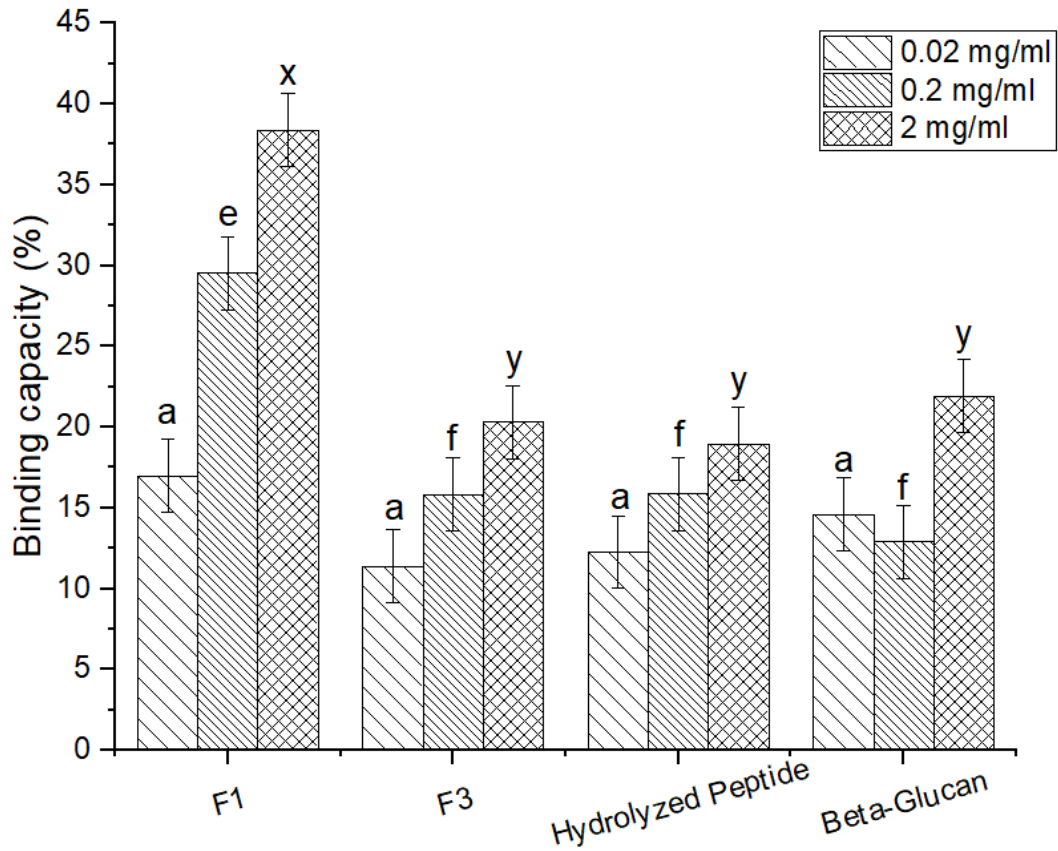
Figure 2.6 Binding capacity of β-glucan and peptide fractions on cholesterol micelles (*in vitro*). Different letters (a, e-f and x-y) above the bars indicate significant difference (p<0.05) among samples.

Table 2.1: Binding capacity of peptide fractions/β-Glucan at different concentrations.

Samples	Concentration (mg/ml)	Binding Capacity (%)
F1	0.02	16.97
	0.2	29.54
	2	38.38
F3	0.02	11.38
	0.2	15.82
	2	20.29
HP	0.02	12.26
	0.2	15.87
	2	18.95
β-Glucan	0.02	14.57
	0.2	12.89
	2	21.92

The consumption of certain food can reduce micellar lipids by binding the bile acids and the cholesterol removed from the micelles is excreted in the feces as they can't be absorbed in the intestine (Zhang et al., 2012). This action could happen by both disrupting the conformation of cholesterol micelles and bile acids competitively acting on the membrane proteins involved in cholesterol absorption (Marques, 2015). In the pharmaceutical market, bile sequestrants are used to reduce the blood cholesterol where the drug binds with the bile acid by disrupting the micelles and avoids the transportation of cholesterol and bile acid to the gut (Wang et al., 2015). This interaction causes the liver to use up cholesterol present in the blood and therefore leads to the

decrease in the blood cholesterol. Some of the well-known bile acid sequestrants are cholestyramine and colestipol (Wang et al., 2015).

In this study, β – glucan was used as a positive control as it is known to reduce cholesterol levels in the blood. β – glucan acts by binding with the bile salts and disrupting the micelles and further inhibits the reabsorption of bile in the intestinal lumen (Joyce et al., 2019). This forces the cholesterol to be transported through feces. Binding of fibre and the bile salts can occur both through hydrophobic and hydrophilic interactions (Florén & Nilsson, 1987). The effect of bile binding capacity was seen both with β – glucan and the peptide fractions. The amino acids present in the F1 fraction (**Table 2.2**) shows high amount of relatively hydrophilic amino acids such as arginine, lysine, serine, and tyrosine. In the work of Ito et al., (2020), peptides that contained arginine (R) and lysine (K) showed the highest bile acid micelle disruption activity. These amino acids were particularly found in peptides by gut digestion as trypsin can cause hydrolysis of peptide bonds at the C-terminal end of R and K, resulting in peptides containing at least one of these two amino acid residues. In another study, oligopeptides derived from hydrolysed sericin protein displayed good binding capacity with bile acids and reduced micellar cholesterol solubility *in vitro* (Lapphanichayakool et al., 2017). These oligopeptides mainly contained amino acids namely, lysine, serine, and tyrosine in high amounts. It was suggested that the relatively hydrophilic amino acids were likely to bind with the hydrophilic part of the bile salts. When the formation of micelles was disrupted through the binding of bile salt with the hydrophilic amino acids, the dietary cholesterol absorption reduces (Lapphanichayakool et al., 2017). The amphipathic nature of bile salt could promote both hydrophilic and hydrophobic interactions. In this study, taurocholate salt was used as the bile salt model and the presence of hydrophilic amino acid could be related to the high inhibition capacity of F1 fractions. On the other hand, bioactive peptides obtained from faba

beans, cowpea, sunflower showed reduction in cholesterol micellar solubility and the authors concluded that the presence of the hydrophobic amino acids was higher in the peptides (Ashraf, 2020; Marques, 2015). Boachie (2018) suggested that the hydrophobic peptides could bind with the hydrophobic part of the bile salt. This could be due to the higher binding capacity of the peptides to the bile acids which has higher surface hydrophobicity. For example, Casein plastein peptides also displayed hydrophobic binding on the bile acid – deoxycholate. The structure of deoxycholate suggests that it is more hydrophobic as it contains only one hydrophilic edge alongside the ring plane and likewise the presence of the sulfonate group in taurocholate makes it more hydrophilic in nature (Ghosh et al., 2015). Because of the amphipathic nature of cholesterol and bile acids, a deeper understanding of the interactions between specific amino acids and the hydrophobic and hydrophilic sectors in the cholesterol is required to better design peptides that can effectively bind to the cholesterol.

2.3.3 *Evaluation of DPP4 activity*

DPP4 activity of the fractions, drug, and the control (no inhibitor) samples is shown in Fig. 2.7. The drug showed the most significant effect by inhibiting 93% of the DPP4 enzyme present in the Caco2 cells at the concentration of 0.1 ml of drug in 1ml of culture media. Among the fractions, F4 possessed good inhibiting capacity as it reduced 50% of DPP4 activity at the concentration of 50 ug/ml. F1, F2 and F3 showed only 13% inhibition of DPP4 activity at the same concentration.
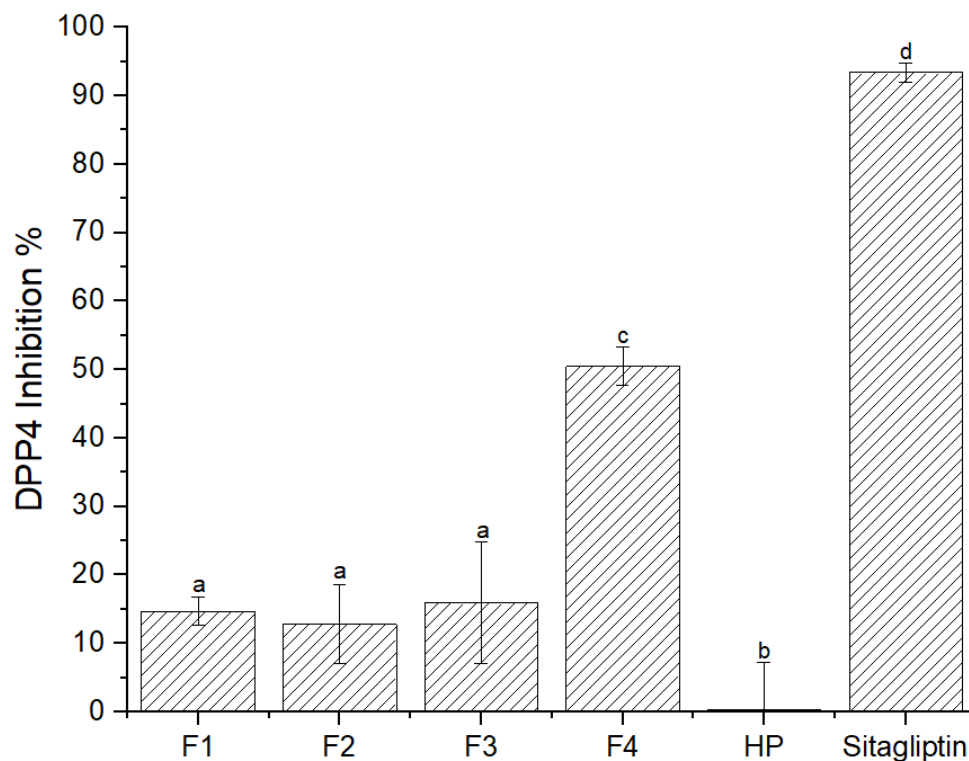
Figure 2.7 DPP4 inhibitory activity of sitagliptin and peptide fractions. Different letters indicated significance (p<0.05).

The DPP4 inhibitory activity for each of the fractions and drug is shown in Fig. 2.7. Sitagliptin used as a drug showed the highest inhibition effect compared to fractions. Among the fraction, F4 showed highest inhibition activity whereas F1, F2 and F3 displayed 42%,43% & 41% lesser activity than F4. Fraction F4 showed 86% lesser inhibitory activity than sitagliptin.

Dipeptidyl peptidase-4 (DPP4) is an enzyme that cleaves and degrades the incretin hormone glucagon-like peptide-1 (GLP-1) (Jose & Inzucchi, 2012). GLP-1 stimulates insulin secretion, delays gastric emptying, and increases satiation. The increase in satiation has been shown to benefit weight loss (Delgado-Aros et al., 2002; Zander et al., 2002). Therefore, inhibiting the DPP4 enzyme would eventually lead in increase in GLP-1 levels which further increases the insulin level and enhances satiation (Vella et al., 2008). Some of the well-known DPP4 inhibitors in the

therapeutic market are sitagliptin, vildagliptin, linagliptin and alogliptin (Jose & Inzucchi, 2012). DPP4 enzyme has high specificity and contains relatively large substrate binding sites (namely S1, S1', S2, S2 extensive) and the most common inhibitors bind stably by fitting the DPP4 binding site (Nabeno et al., 2013). Sitagliptin inhibitor was used as a drug for comparison in this study which is known for its effect on increase in GLP-1 hormone (Reimer, 2012). There are several studies on peptides and the effect on DPP4 enzymes using enzymatic assay, but in this work the study was furthered using a cell model (Caco-2) (Hatanaka et al., 2012; Huang et al., 2012; Li-Chan et al., 2012; Nongonierma & Fitzgerald, 2013b). Financial and ethical reasons of *in vivo* studies make cell model an improved *in vitro* DPP4 approach. Caco-2 cells display many functional and morphological features of enterocytes and have various membrane peptidases including DPP4 (Howell et al., 1992). Caco-2 cell line are considered to be a novel tool for DPP4 studies as these confluent living cells can be used as a DPP4 enzyme source and with the utilization of readily accessible DPP4 substrate and inhibitors, the DPP4 activity can be closely studied (Caron et al., 2017). As shown in the **Table 2.2**, F4 fractions contained peptides with high amount of hydrophobic amino acids such as leucine, phenylalanine, valine and proline. Araki et al., (2020), suggested that the N-terminus of the DPP4 inhibiting peptides consisted of aromatic/hydrophobic amino acids. The addition of hydrophobic amino acid at the C-terminus further increased the DPP4 inhibition activity by several folds. A study on the binding of DPP4 inhibitors (sitagliptin, linagliptin, alogliptin and teneligliptin) was conducted and compared. Inhibitors that interacted with the hydrophobic pocket of the DPP4 active site showed increased inhibitory activity (Arulmozhiraja et al., 2016). A QSAR-based in silico study conducted by Nongonierma et al., (2018) showed that DPP4 inhibitory activity by synthetic peptides was mainly due to the hydrophobic amino acid present at the N-terminal end. DPP4 cleaved all the peptides that had

bulky amino acid residues like phenylalanine at the C—terminal end. Interestingly, in our research the presence of phenylalanine was highest in the F4 fraction compared to other fractions. A previous work by Wang et al., (2015) showed high DPP4 inhibitory activity for the peptide Leu-Gln-Ala-Phe-Glu-Pro-Leu-Arg (LQAFEPLR) derived from oats and barley where sitagliptin was used a positive control. Interestingly, a similar peptide Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR) with a *de novo* score of 94. The presence of R (arginine) in the C-terminal can also be seen in the DPP4 inhibitory peptides derived from other food sources such as camel milk, manila clams, mare whey protein, bovine whey protein, buckwheat and barley (Nongonierma et al., 2018; Liu et al., 2017; Song et al., 2017; Lacroix & Li-Chan, 2014; Wang et al., 2015). Also, in the study conducted by Lacroix & Li-Chan (2012), alanine or proline were the preferred amino acids accepted by hydrophobic pocket of DPP4 substrate. This is because DPP4 selectively cleaves the N-terminal proline (penultimate) or alanine (Thoma et al., 2003). The hydrophobic nature of F4 fraction and the presence of specific amino acids like phenylalanine could be the reason why there was stronger inhibition of DPP4 activity compared to other fractions. However, there is requirement for deeper structural study of amino acids and its specificity towards the DPP4 substrate which would further enhance our understanding of the binding interactions between them.

2.3.4 Amino acid analysis and peptide sequencing of effective fractions

The amino acid compositions of oat protein and the fractions from the oat protein hydrolysates are shown in **Table 2.2**. The enzymes used during hydrolysis determine the exposure of certain amino acids. For example, the use of flavourzyme increases the exposure of aromatic amino acids and alcalase enzyme prefers cleaving at the hydrophobic amino acids (Nieto-Nieto, 2014; Walters et al., 2020). Similarly, pepsin enzyme prefers to cleave at the C-terminal side of aromatic amino acids and mainly shows a preference towards cleaving leucine and phenylalanine (Ahn et al.,

2013). In **Table 2.2**, the presence of leucine was most abundant in F3 and F4 fractions compared to other amino acids and phenylalanine was found to be high in F2, F3 and F4 fractions. The effect of pepsin cleavage could have increased the presence of aromatic amino acids such as phenylalanine. On the other hand, trypsin generally cleaves the peptide bonds between the carboxyl groups of arginine and lysine. When arginine and lysine is followed by proline, then the cleavage does not occur (Simpson, 2006). This explains the presence of arginine throughout the fractions and the hydrolyzed peptide. However, arginine content was highest in the F1 fraction compared to other peptide fractions, as F1 fraction was more hydrophilic in nature. Whereas fractions F3 and F4 also showed abundant presence of other hydrophobic amino acids such as alanine and valine. The total amount of hydrophobic amino acid present in F3, F4 and HP were found to be 62.5%, 66.8% and 56.6% respectively.

Table 2.3 shows the identified amino acid sequences from the most potent oat peptide fractions by LC-MS/MS analysis with average local confidence (ALC) > 90%. Due to their abundance in oat protein, the presence of proline and hydrophobic amino acids such as leucine, phenylalanine and valine is high in the identified sequences of this study. The presence of proline is distinct in all the mentioned sequences. The consistent presence of arginine in the above identified peptide sequences can be explained by the cleaving of trypsin at C-terminal to arginine or lysine (Olsen et al., 2004).

To achieve HMGCR inhibitory activity, it is considered important that the peptide chains contain hydrophobic amino acids such as proline, isoleucine, and leucine (Kongo-Dia-Moukala et al., 2011). For example, the sequences abundant in hydrophobic amino acids such as Leu-Pro-Tyr-Pro (LPYP), Leu-Pro-Tyr-Pro-Arg (LPYPR), Val-Gly-Val-Leu (VGVL), Gly-Gly-Val (GGV) and Ile-Val-Gly (IVG) derived from soy glycinin, and amaranth protein possessed potential HMGCR

inhibitory activity (Kwon, 2002; Soares et al., 2015). It is also suggested that the presence of valine and alanine residues could possess a steric effect due to the presence of aliphatic side chains at specific positions and this further promotes the HMGCR inhibitory activity (Heres et al., 2021). In addition, the presence of proline could promote peptide structural flexibility to favor their hydrophobic interactions on the hydrophobic pocket of HMGCR enzyme (Pak et al., 2006). In our study *de novo* sequencing of the samples helped in identifying sequences that are potential to have high HMGCR inhibitory effect **(Table 2.3)**. These sequences varied from 5-10 amino acid length and possessed some similarity in amino acid content and their positioning. For instance, Val-Pro-Phe-Leu-Arg (VPFLR), Phe-Glu-Pro-Leu-Arg (FEPLR), Met-Val-Pro-Phe-Leu-Arg (MVPFLR), Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR), all have Arg at the C-terminus and hydrophobic amino acids like Val, Ala and Phe at the N-terminus. Phe-Glu-Pro-Leu-Arg (FEPLR) and Tyr-Leu-Leu-Glu-Gly-Arg (YLLQGR) both contain aromatic amino acids such as phenylalanine and tyrosine at the N-terminal (Fig. 2.8). The ring structures present in the aromatic amino acids could possess a similar effect as that of statin drugs. Statin contains hydrophobic ring structures that mimic the HMG-CoA substrate, and these rings helps in binding of HMGCR enzyme for their inhibition (Murphy et al., 2020). This could possibly explain the inhibitory effect of F3 and F4 on HMGCR enzyme. These findings suggest the presence of aromatic amino acids at the N-terminal and the presence of hydrophobic amino acids and proline could promote the binding of oat peptides with HMGCR, although the exact mechanism of action would still need to be investigated.

Table 2.2: Amino acid composition of oat protein hydrolysates and fractions.

Amino Acid Residue (g/100g)	F1	F2	F3	F4	HP
Asx[a]	9.18	8.01	9.22	7.99	8.79
Serine	5.94	7.4	6.10	5.32	5.83
Glx[b]	19.31	26.37	21.59	21.17	25
Glycine	4.65	5.43	5.93	5.20	4.41
Histidine	1.9	2.82	2.09	2.10	2.52
Arginine	11.05	7.99	6.59	4.35	8.27
Threonine	3.46	4.09	6.27	3.29	3.42
Alanine	4.97	3.88	7.36	4.41	4.21
Proline	3.15	6.68	8.18	7.74	6.07
Cysteine	0.82	1.30	2.79	2.35	1.72
Tyrosine	3.40	5.29	4.76	4.35	4.42
Valine	4.44	5.47	7.12	6.02	5.39
Methionine	1.62	1.89	2.72	1.51	1.84
Lysine	5.87	3.59	4.57	2.19	3.65
Isoleucine	3.33	4.37	5.58	4.31	4.04
Leucine	4.9	7.50	10.09	9.85	7.41
Phenylalanine	2.8	6.88	6.86	7.33	6.02

[a] Asx (Asp + Asn); [b] Glx (Glu + Gln); HP represents hydrolyzed peptides.

For micellar solubilization of cholesterol, the presence of both hydrophilic and hydrophobic amino acids in the fractions could favor the binding interactions as bile salts are

generally amphipathic. For instance, four peptides namely, Thr-Asp-Val-Glu-Asn (TDVEN), Leu-Gln-Pro-Glu (LQPE), Val-Leu-Pro-Val-Pro-Gln (VLPVPQ), and Val-Ala-Pro-Phe-Pro-Glu (VAPFPE) derived from milk casein protein showed potential micellar cholesterol solubility inhibition activity. Out of these four peptides TDVEN and LQPE were hydrophilic, VLPVPQ and VAPFPE were hydrophobic (Jiang et al., 2020). In our study, F1 showed the most potential micellar binding activity followed by F3, and both showed relatively good activity compared to β-glucan. **Table 2.2** suggests the abundant presence of arginine and lysine in F1, which makes it more hydrophilic, and this could have possibly increased the binding capacity of the peptides with the micelles (Ito et al., 2020) due to the electrostatic interaction between the negatively charged functional groups of the bile acid and the positive charge on arginine or lysine (Ito et al., 2020). On the other hand, several studies suggest the presence of hydrophobic amino acids in a sequence favors the bile disruption which aids in the solubilization of cholesterol. This could explain the good activity observed for F3. Two steps of interaction could take place between the peptides and the micelles. The hydrophilic peptides could disrupt the micelles in the first step and then the hydrophobic peptides might hinder the cholesterol solubilization (Lapphanichayakool et al., 2017). Studies also suggest that the presence of aromatic rings could further lead to peptide stacking onto the carbon rings present in the cholesterol forming CH-π hydrogen bonds and this interaction is considered to be a trademark of protein-cholesterol interactions (Nishio et al., 2014). However, the binding interactions of peptides and bile salt would need further investigation to understand the mechanism involved.

Fraction F4 displayed the most potential DPP4 inhibition activity compared to other fractions. Diprotin A and B are two peptides known as strong inhibitors of DPP4 enzyme with the sequence of Ile-Pro-Ile and Val-Pro-Leu, respectively. Similarly, peptides isolated from soy Ile-

Ala-Val-Pro-Thr-Gly-Val-Ala (IAVPTGVA), lupin – Leu-Thr-Phe-Pro-Gly-Ser-Ala-Glu-Asp (LTFPGSAED) and dulse- Ile-Leu-Ala-Pro (ILAP) and Leu-Leu-Ala-Pro (LLAP) effectively inhibited DPP4 activity. These peptide sequences suggest the presence of hydrophobic amino acids such as isoleucine, leucine, and valine at the N-terminal position could play an important role in DPP4 inhibition. Interestingly, *de novo* peptide sequencing of F4 fraction (**Table 2.3**) shows the presence of sequences such as Val-Pro-Phe-Leu-Arg, Leu-Leu-Phe-Ala-Gly-Lys and Ala-Phe-Glu-Pro- Leu-Arg, which resemble the sequences of the peptides mentioned above. The amino acids at the N-terminal position and the penultimate N-terminal position play an important role in DPP4 inhibitory activity (Power et al., 2014). Sitagliptin, a competitive inhibitor, mainly bind at the active site of the DPP4 enzyme through hydrophobic interactions and hydrogen bonding (Berger et al., 2018). Therefore, it could be speculated that Val, Leu and Phe (Fig. 2.8) at the N-terminal position in the above sequences could have contributed to the stronger DPP4 inhibition activity in F4 fraction. Another known DPP4 inhibitor, gliptins possess multiple aromatic rings and tend to be rigid and hydrophobic (Berger et al., 2018). Hence, the presence of aromatic amino acids with ring structures such as phenylalanine and cyclic amino acids such as proline might also have contributed to the inhibition of DPP4 enzyme.

Table 2.3: Identification of amino acid sequences from the most potent oat peptide fractions by LC-MS/MS analysis with average local confidence (ALC) > 90%.

Fraction	Peptide	Tag Length	ALC[a] (%)	m/z	Mass
	VPFLR	5	98	316.20	630.38
	MVPFLR	6	96	381.72	761.42
F3	LGLSQQAAQR	10	96	536.30	1070.58
	FGVFTPK	7	93	398.22	794.43

	LLFAGK	6	92	324.70	647.39
	YLLQGR	6	91	375.22	748.42
	VPFLR	5	98	316.20	630.38
	FEPLR	5	97	331.18	660.35
F4	AFEPLR	6	94	366.70	731.39
	FGVFTPK	7	93	398.22	794.43
	LLFAGK	6	92	324.70	647.39
	YLLQGR	6	91	375.22	748.42

[a] – Average local confidence score

It is important to know the specific peptide sequences that contribute towards cholesterol lowering and satiety effect. The LC-MS/MS analysis suggested some sequences with high potential; however, their effects will still need to be validated. From the above discussions, peptide sequences such as Val-Pro-Phe-Leu-Arg (VPFLR), Phe-Glu-Pro-Leu-Arg (FEPLR) and Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR) could be promising for DPP4 inhibition. The sequences such as Tyr-Leu-Leu-Gln-Gly-Arg (YLLQGR) and Phe-Glu-Pro-Leu-Arg (FEPLR) could possess promising HMGCR inhibiting activity particularly when Y and F amino acids are present at the N-terminal end of the peptide sequence. Therefore, synthesizing these peptides could possibly be the next step and further testing the activity of the specific synthetic peptides on cholesterol lowering and satiety properties. It should be mentioned that the LC-MS/MS analysis of the F1 fraction was not conducted in this thesis. Considering its good cholesterol lowering effect, the peptide sequences in F1 could be investigated in the next step to better understand the peptide sequences that can contribute to the micellar solubilisation of cholesterol. To validate the sequence activities and further understand the action mechanisms, *in vitro* studies involving cell models can be used. For DPP4 inhibition, Caco2 cell lines are the most widely used model because they can be used to

simulate human intestinal enterocytes and can secrete DPP4 enzyme (Lammi et al., 2018). In addition, Caco2 cells also are used to test the amount of satiety hormones released such as GLP-1 (Song et al., 2015). For HMGCR inhibition, HepG2 cell lines can be used. HepG2 possess high proliferation rate, hepatic functions and is commonly used in drug metabolism studies (Donato et al., 2015). HepG2 cells are also used to test the effect of statin drugs such as simvastatin and atorvastatin for HMGCR activity (Cerda et al., 2021; Han et al., 2017). Positive results from cell lines will provide justification for further *in vivo* study using animal models followed by clinical study to validate the cholesterol lowering and satiety effect of the peptide sequences identified in this study and understand their effective dosage. Finally, molecular docking of peptides and can help us to understand the interactions between them, thus helping in selection of the most potential peptide sequences responsible for the DPP4 and HMGCR inhibitory activities.
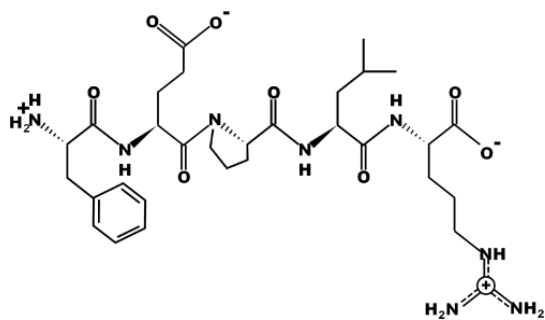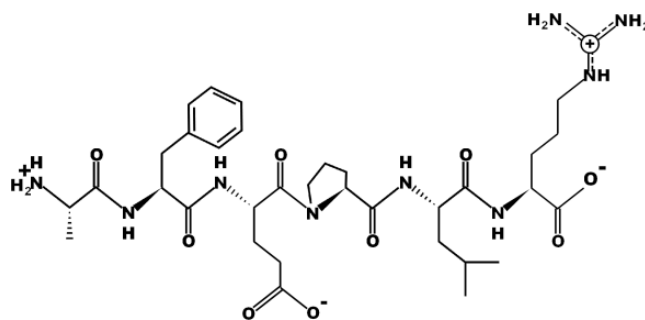
Peptide Structures
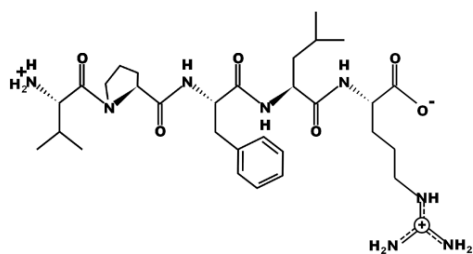
VPFLR

MVPFLR

FEPLR

AFEPLR

LLFAGK   YLLQGR

LGLSQQAAQR   FGVFTPK
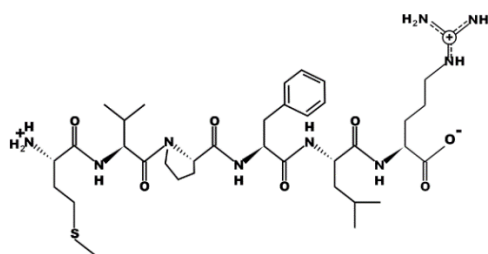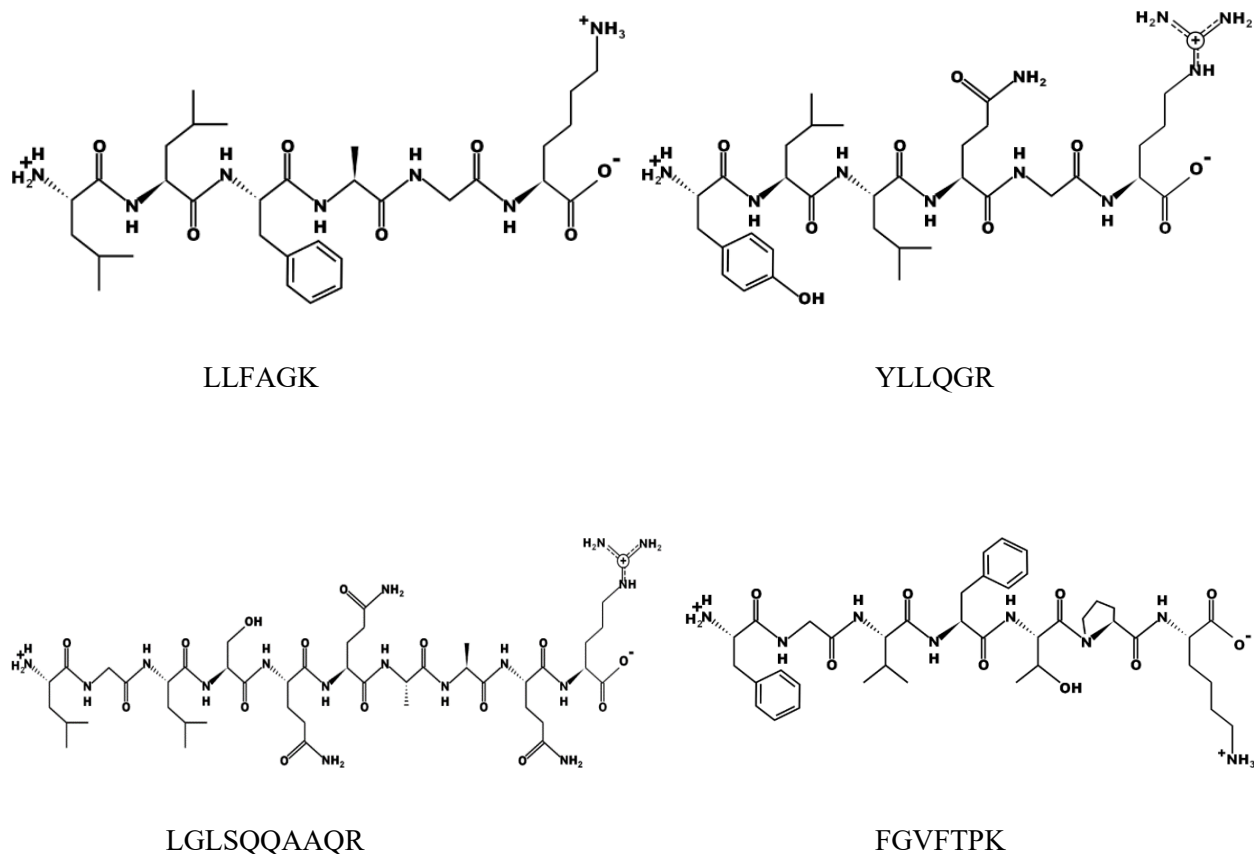
Figure 2.8 Primary structures of peptide sequences found in F3 and F4 RP-HPLC oat samples using PepDrawtool at https://pepdraw.com/

2.4 Conclusion

The findings of this research showed the cholesterol lowering properties of peptides obtained after simulated human gut digestion of oat protein by micellar solubilisation of cholesterol and inhibition of HMGCR enzyme. These peptides also demonstrated DPP4 inhibitory effect. The results suggested that the presence of aromatic amino acids and hydrophobic amino acids such as proline, valine, and leucine in the peptide sequences may play a significant role in contributing to the HMGCR activity and DPP4 inhibition. The presence of proline in most of the peptides present in the effective fractions (F3 and F4) could add flexibility to the peptides that facilitated their binding to the catalytic domain of the HMGCR enzyme. Similarly, the presence of proline and

alanine at positions 2 and 3 of the N-terminal end of the peptide could possibly increase the susceptibility of the substrate (peptide) to the DPP4 cleavage. Also, the presence of arginine at the C-terminal end could help with inhibition by binding to the active site of the DPP4 enzyme. The abundant presence of phenylalanine which has an aromatic ring could also aid in inhibiting the HMGCR enzyme and the DPP4 enzyme.

The specific peptides with high potential biological effects could be further synthesized and evaluated through *in vitro* and *in vivo* studies using cell and animal models to validate the peptide sequences contributing to the HMGCR and DPP4 inhibition effects and to understand their action mechanisms. Clinical trial is also important in the future research to understand the safety and effective dosage of the oat peptides. Such knowledge could allow the food and health industries to incorporate these peptides from oats to develop healthy food products (e.g., oat drinks) to help manage weight and cholesterol level of an individual.

Chapter 3 – Conclusion and Recommendations

3.1 Summary and Conclusions

Hypercholesteremia and overweight/obesity are two of the most common risk factors contributing to major cardiovascular diseases like atherosclerosis. The initiatives to reduce the impact of these risk factors have resulted in developing strategies to allow people to lead a healthy lifestyle. Pharmacological treatments like anti-cholesterol drugs and anti-obesity drugs have shown great effectiveness, but these medications are also influenced by factors such as side effects and costs. Regulating the cholesterol concentration and appetite can also be tackled through dietary choices, lifestyle, and physical activity with relatively low or no risk unlike the pharmacological treatments. Therefore, attempts to develop "heart-healthy" food products with bioactive peptides from food protein have gained significant attraction in regulating appetite with less calorie intake and lowering cholesterol in the last decade.

Oat is considered to possess high nutritional and beneficial properties; however, the consumption of oat by humans is still low as oat production is mainly related to produce animal feed. The nutritional properties of oats related to cholesterol reduction and appetite suppression are attributed mainly to the fiber component like β-glucan. The protein fraction of oat is an important source of essential amino acids. Studies on oat protein component for reducing cholesterol and improving satiety are still limited. Therefore, studying and exploring the capacity of oat protein to generate bioactive peptides that supports cholesterol regulation and satiety effects was investigated in this thesis.

The oat protein was hydrolyzed using gut enzymes like pepsin and trypsin following a simulated gut digestion. The hydrolyzed peptides were further investigated for its cholesterol

lowering activity by studying its effect on cholesterol micellar solubility and HMGCR enzyme inhibition. These peptides were also assessed to check its effect on satiety through DPP4 enzyme inhibition. The peptides were separated by using RP-HPLC fractionation into 4 fractions (F1, F2, F3 and F4) based on their hydrophobicity. Raw hydrolysate (HP) was considered as the 5th fraction. HMGCR inhibition was observed in F3, F4 and HP fraction with the inhibition effect of 85%, 79% and 83% respectively at 200 ug/ml concentration. DPP4 inhibitory effect was seen in F4 fraction as it reduced 50% of DPP4 activity at 50 ug/ml. Sitagliptin which was used as the positive control showed 93% of inhibition capacity at a lower concentration of 0.1ml of drug in 1ml of culture media. F1 fraction showed the highest binding capacity of 30% and 38% at 0.2mg/ml and 2 mg/ml respectively on cholesterol micelles, whereas F3 and HP showed ~20% of binding. β-glucan was used as the positive control, and it is noteworthy to mention that F1 peptide fraction showed better binding capacity than β-glucan. These results highlight the capacity of oat peptides to display potential cholesterol lowering and satiety effects at relatively low concentrations.

Peptide sequencing was done using LC-MS/MS analysis to understand the most abundant peptide sequences in the fractions. The *de novo* peptide sequences, and the peptides derived from 11S oat globulin and 12S oat globulin (Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR) and Leu-Gly-Leu-Ser-Gln-Gln-Ala-Ala-Gln-Arg (LGLSQQAAQR) showed that the presence of amino acids such as phenylalanine, leucine, proline, arginine, alanine, and valine may play an important role in inhibiting the DPP4 and HMGCR enzymes, therefore lowering cholesterol and promoting satiety effect. The inhibition of DPP4 enzyme allows the incretin hormones such as GLP-1 to be released and slows down the emptying of the stomach and this helps in promoting the feeling of fullness. This action, in turn, can help in reduced food intake and aid in weight loss. This research shows

that the oat protein has the potential to lower blood cholesterol by having an impact on cholesterol micelles and inhibiting HMGCR enzyme and promote satiety effect by inhibiting DPP4 enzyme.

3.2 Significance of this research

This research showed that oat can be used to generate potential peptides with hypocholesteremic and satiety promoting properties. This could help an individual with hypercholesteremia and/or excess body weight manage their cholesterol and appetite. This research also demonstrated that simulated gut digestion using gut enzymes such as pepsin and trypsin produced peptides with low molecular weight, inhibited cholesterol and enzymes that promotes anti-satiety effect. Simulated gut digestion also opens the possibility that the consumption of oat protein by an individual could potentially reduce cholesterol and promote satiety after digestion and post absorption state. This research also investigated the potential of the peptides by separating them by RP-HPLC fractionation based on their hydrophobicity. Initially the hypocholesteremic effects were studied by HMGCR enzyme inhibition and the peptide effects on cholesterol micelles. The satiety effect was studied based on DPP4 enzyme inhibition. The hydrophobicity of the peptides played a role in inhibiting the targeted enzymes. Due to the amphipathic nature of bile salts, both hydrophobic amino acids and hydrophilic amino acids could have an impact on cholesterol micelles. The LC-MS/MS analysis and *de novo* sequencing of the most effective fractions allowed to understand the desirable peptide sequences with the target. This presents an opportunity to further investigate individually the cholesterol lowering effects and satiety promoting properties of the derived bioactive peptides and the mechanisms involved in their interactions with the relevant enzymes.

Overall, these findings highlight the potential natural anti-cholesterol and satiety-promoting effects of food agents derived from plant proteins like oats. This research could also

help industries in formulating oat peptides in food products and developing products that help in weight management. Finally, the knowledge acquired from this research adds to the body of evidence of the opportunistic use of oat as part of a healthy diet and promote the revenue of oat production.

3.3 Recommendations

In this research, the hypocholesteremic evaluation of peptides was conducted using enzymatic models and DPP4 inhibition was studied using *in vitro* cell culture. However, it is necessary to synthesize the specific peptide sequences from the most potential oat protein hydrolysate fractions and perform the further tests.

1. Based on our study in this research, peptide sequences such as Val-Pro-Phe-Leu-Arg (VPFLR), Phe-Glu-Pro-Leu-Arg (FEPLR), Ala-Phe-Glu-Pro-Leu-Arg (AFEPLR), Tyr-Leu-Leu-Gln-Gly-Arg (YLLQGR) and other sequences with the presence of hydrophobic amino acids and aromatic amino acids at the N-terminal and arginine at the C-terminal ends could possibly favour the HMGCR and DPP4 inhibitions. Synthesizing these peptides and performing further tests would be recommended. Synthetic peptides can be obtained by using solid phase synthesis, which is a efficient method for peptide synthesis with high yield and purity. Liquid phase synthesis can also be considered and is faster than solid phase synthesis. It is mainly used to synthesize peptides which have shorter to medium-length peptides, however liquid phase synthesis could be more challenging in retrieving the final product due to the presence of side products compared to solid phase synthesis where the peptides would be attached to a solid support normally resins.

2. *In vitro* cell culture, *in vivo* and clinical trials are recommended in the next step to study the bioactivities of the above synthesized peptides. In this study, the effect of peptides was seen in dose dependant manner and the effect was observed even in the least dosage used i.e., 50 μg/ml. It is recommended to use a series of dosages starting from 50 μg/ml to perform the tests, in order to study the dose-response relationship of the peptides. Caco2 cells can be used as the cell model to study the effect of peptides on DPP4 inhibition. Whereas, in the case of HMGCR activity, it is recommended to use HepG2 cell lines which is a human hepatocellular carcinoma cell line commonly used as an *in vitro* model to study the cholesterol metabolism. HepG2 cells help in expressing HMGCR and aid in synthesizing cholesterol. Positive results from the cell models will give justification for the *in vivo* studies using animal models which can help us with more physiological relevance and help us understanding the complexity involved. It could also allow us to study the biological interactions and the responses in a realistic context. Finally, clinical trials would help us to evaluate the effective dosage and the safety of the peptides on humans. These trials could involve methods like venipuncture and testing of cholesterol concentration in the blood. The subjects can then be treated with food or a drink which contains our peptides of interest. After few hours, the blood is drawn out again to test the concentration of cholesterol in the blood. This would help us to know if the peptides derived from oat protein can have cholesterol lowering effect in humans. Similarly, for satiety, the subjects can be treated with food or drink that contains the oat peptides and survey can be taken from the subjects to test how satiated they feel after the consumption of the peptides. Overall, further tests using *in vitro*, *in vivo* and clinical trials would validate the activity of oat peptides on cholesterol lowering and satiety effects.

3. Additionally, the mechanisms involved in the interaction between bioactive peptides and enzymes should be elucidated. Methods such as molecular docking studies would enhance our understanding of the binding between peptides and enzymes. Molecular docking helps in simulating the interaction between peptides and enzymes and it identifies the most favorable binding sites. It also helps us in understanding the key interactions involved in substrate binding such as hydrogen bonding, hydrophobic interactions, and electrostatic interactions.

This research could also help in developing new oat protein-based food products to provide more health options in the market. Oat is mainly known for its fiber content and these days the consumer preferences towards plant protein has surged. Therefore, introducing oat protein foods such as snacks, breakfast cereals, oat protein milk products with cholesterol lowering and satiety properties could become a hit in the food market. The idea of incorporating the oat peptides in foods or drinks which are already available in the market could be another strategy towards food development.

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