

**Effects of Ultrasonication on the Antioxidant and Antidiabetic Properties of Hydrolyzed
Oat Proteins**

By:

Mallory Walters

Supervised by:

Dr. Apollinaire Tsopmo and Dr. William Willmore

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in
partial fulfillment of the requirements for the degree of

Master of Science in Chemistry

(Specialization in Food Science and Nutrition)

Carleton University

Ottawa, Ontario, Canada

© 2019 Mallory Walters

Abstract

The method of protein extraction from foods can affect yields, compositions, and functionalities of the isolated proteins. The first objective of this work was to determine the effects of ultrasonic treatments on protein yields and polypeptide compositions. Proteins were consequently extracted from oat brans under normal (CTL) conditions and after pre-treatments of brans with ultrasonic bath (UB), and high power (HP) sonication. There was no significant difference in extraction yields (2.06 – 3.79%), meanwhile gel electrophoresis and tandem mass spectrometry (LC-MS/MS) analysis showed differences in polypeptide compositions. Specifically, globulin and avenin proteins were present in all samples, however, ultrasonic treatments (UB and HP) released vromindoline, a starch-bound protein, while tryptophanin, a lipid-bound protein, was released after high power ultrasonic (HP) treatment. The second objective of this work was to determine the physical, chemical, and biological properties of the proteins after hydrolysis with proteases of various specificities. Proteins from CTL, UB, HP samples were hydrolyzed by three proteases (Flavourzyme, Papain, and Alcalase) and then characterized based on their sulfhydryl (SH) content, hydrophobicity and zeta potential. Data showed that HP ultrasonic treatments decreased SH concentrations in Papain and Alcalase hydrolysates but increased it in the Flavourzyme hydrolysate. The surface charge (i.e. zeta potential) was dependent on both the protein extraction and the protease. Papain had a much weaker surface charge (-0.78 to -1.32 mV) than the other proteases (-3.67 to -9.17 mV). UB treatments increased hydrophobicity for Flavourzyme and Papain hydrolysates, while HP treatments decreased the hydrophobicity. To further evaluate the hydrolyzed proteins (i.e. hydrolysates), their biological activities were determined. It was found that in general, ultrasonic treatments reduced the antioxidant activity of the protein hydrolysates. For example, peroxy radical scavenging activity was reduced by UB and HP sonication; however, HO• scavenging activity was reduced by HP and not UB sonication. In the antidiabetic tests, hydrolysates were evaluated for their ability to modulate the activity of dipeptidyl peptidase-4 (DPP-4) and alpha-amylase, as well as the secretion of GLP-1, a hormone that stimulates insulin response in NCI-H716 cells. Ultrasonication did not affect DPP-4 inhibition; however, concerning proteases, Papain hydrolysates had substantially greater (49.7 - 53.6%) DPP-4 inhibitory activity for all conditions. HP sonication slightly decreased the alpha-amylase inhibitory activity by 4.8 – 7.2% compared to UB and control. A two-fold increase in hydrolysate concentration resulted in a 13.7 – 76% increase in GLP-1 secretion, indicating a dose-dependent

response. Overall, the effect of ultrasonic treatments on the composition of proteins obtained from oats was the release of two proteins not found in the control. The hydrolysis of protein isolates with proteases produced hydrolysates with notable antidiabetic activity (DPP-4 and alpha-amylase inhibition) but this was not influenced by the difference in polypeptide compositions. The effect of ultrasonication of brans had a minimal effect on the physical properties of hydrolysates.

Acknowledgments

I would like to thank my supervisor, Dr. Apollinaire Tsopmo, for his guidance and supervision throughout this project. I would like to acknowledge Dr. William Willmore for his co-supervision over the cellular aspects of my work, and his team for their assistance. Additionally, Ramak Esfandi was always available to offer advice regarding my experiments and constantly supported me throughout my studies at Carleton. I would also like to acknowledge Dr. Farah Hosseinian, for the use of her high power sonicator, Dr. Alex Wong for his fluorometer, and Dr. Chibuike Udenigwe, for the use of his ZetaSizer. Lastly, I would like to thank my family and my friends, especially my husband, for supporting and encouraging me throughout my studies.

This work was supported by the Chemistry Department at Carleton University, Ottawa, ON and a grant from the National Sciences and Engineering Research Council of Canada (NSERC).

Table of Contents

Abstract.....	2
Acknowledgments.....	4
Table of Contents.....	5
List of Tables	8
List of Figures.....	9
List of Abbreviations	11
1. Literature Review.....	12
1.1 Overview	12
1.2 Nutritional value and composition of oats	12
1.2.1 Oat composition.....	13
1.2.2 Dietary fibers	14
1.2.3 Secondary metabolites/phytochemicals.....	14
1.2.4 Lipids	14
1.2.5 Proteins	16
1.3 Procedures for extraction and hydrolysis of proteins.....	18
1.3.1 Methods of extraction	18
1.3.2 Techniques to improve extraction yield and purity	19
1.4 Biological activities of protein hydrolysates.....	20
1.5 Type 2 diabetes mellitus.....	23
1.6 Contributors to T2DM.....	23
1.6.1 Oxidative stress.....	23
1.6.2 Hormone and enzyme imbalance.....	25
1.7 Treatment and management of T2DM.....	26
1.8 Methods to determine antidiabetic properties	28

1.9	Protein hydrolysates as active agents against T2DM.....	28
2.	Hypothesis and Objectives.....	32
3.	Materials and Methods.....	33
3.1	Materials.....	33
3.2	Protein extraction	33
3.3	Protein content.....	34
3.4	Free amino acid assay	34
3.5	Mass spectrometry.....	35
3.6	Gel electrophoresis.....	35
3.7	Enzymatic hydrolysis of protein extracts and dialysis of hydrolysates	36
3.8	Free thiol content.....	36
3.9	Zeta potential.....	37
3.10	Hydrophobicity assay	37
3.11	Fourier-transform infrared and ultraviolet spectroscopy.....	37
3.12	Peroxyl radical scavenging activity.....	37
3.13	Hydroxyl radical scavenging assay	38
3.14	Superoxide radical scavenging assay	38
3.15	Alpha-amylase inhibition assay.....	38
3.16	Dipeptidyl peptidase-4 inhibition assay	39
3.17	Cell culture conditions.....	39
3.18	Cell assays	39
3.18.1	Cell viability and cytotoxicity.....	40
3.18.2	GLP-1 secretion	40
3.19	Statistics.....	40
4.	Results and Discussion	41

4.1	Protein extraction and protein contents	41
4.2	Characterization of proteins	42
4.2.1	Protein content and molecular weight.....	42
4.3	Hydrolysis of proteins	45
4.4	Free thiol in hydrolyzed proteins	48
4.5	Zeta potential and hydrophobicity of hydrolyzed proteins	49
4.6	Spectroscopic characterization of hydrolyzed proteins.....	51
4.7	Antioxidant activities of hydrolyzed proteins	53
4.8	Inhibition of enzymes by hydrolyzed proteins.....	56
4.9	Effect of hydrolyzed proteins on cellular viability and the secretion of glucagon like peptide in colorectal cells.....	57
4.9.1	Viability of cells or cytotoxicity	57
4.9.2	Effects on the secretion of glucagon like peptide 1 (GLP-1).....	59
5.	Conclusion	61
6.	Future Studies	62
7.	References.....	63

List of Tables

Table 1: Fatty acid composition of oat lipids, as expressed as a percentage of total fatty acids. Hulled oats were used for the analysis, and each percentage is an average taken from five different cultivars ¹⁵	15
Table 2: Amino acid profile of oats and barley.....	18
Table 3: Antidiabetic properties of protein hydrolysates.....	31
Table 4: Composition of major proteins in each sample. (-) indicates an absence of protein, while (N/A) indicates an unknown coverage for the protein. Each sample was analyzed using MS/MS after digestion by trypsin. Myoglobin was used as a control, and the sequences were analyzed using Mascot TM Software.	44

List of Figures

Figure 1.2.1: Chemical structure of A) avenanthramides where R groups are typically hydrogen or hydroxyl groups, B) stearic acid and oleic acid, C) beta-glucan 16

Figure 1.6.1: Diagram of superoxide production in the electron transport chain. 24

Figure 4.2.1: SDS-PAGE gel electrophoresis results from samples (1mg protein/ml) dissolved in solution with and without a reducing agent. Samples (25 μ L) were run on a 12% resolving gel and 4% stacking gel for 1 hour at 120V and. Sample labels are as follows: CTL = control, UB = ultrasonic bath, HP = high power. 42

Figure 4.3.1: A) Percent protein of hydrolysates (dissolved at 120 μ g/ml) as determined by a Lowry assay, using BSA as a standard. B) Concentration of free amino acids within hydrolysates (1mg/ml), expressed as μ g FAA/g sample. Samples are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p-value < 0.05). 47

Figure 4.4.1: μ mol free sulfhydryl groups per gram of protein. Samples were prepared at 2.5 mg protein/ml and are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p-value < 0.05). 48

Figure 4.5.1: A) Zeta potential of hydrolysates and B) index of hydrophobicity for protein hydrolysates expressed as the initial slope of relative fluorescence versus concentration of protein. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p-value < 0.05). 49

Figure 4.6.1: A) UV absorbance of samples measured from 260 – 320 nm. B) FT-IR spectra of samples measured at 600 – 4000 cm^{-1} 51

Figure 4.7.1: A) Oxygen radical absorbance capacity, B) Hydroxyl radical scavenging activity, and C) Superoxide radical scavenging activity of samples prepared at 1.0 mg/mL. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p-value < 0.05). 53

Figure 4.8.1: A) alpha-amylase and B) DPP-4 inhibition by protein hydrolysates prepared at 1 mg/ml and 5 mg/ml, respectively. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with an LSD in a one-way ANOVA (p-value < 0.05). 56

Figure 4.9.1: A) Cell viability of NCI-H716 cells as determined by an MTT assay; B) GLP-1 secretion stimulated by hydrolysate treatment. Cells were plated at 5×10^4 cells in a 96 well microplate with Matrigel for 24 hours and were treated with hydrolysate fractions for 2 hours. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p-value < 0.05). 58

List of Abbreviations

(AT)-I	Angiotensin-I converting enzyme	GSH	Glutathione
(AT)-II	Angiotensin-II	HBP	Hexosamine biosynthetic pathway
ACE	Angiotensin-I converting enzyme	IL-1	Interleukin-I
AGE	Advanced glycation endproducts	IL-6	Interleukin-6
ATP	Adenosine triphosphate	LDL	Low-density lipoprotein
ATPase	Adenosine triphosphatase	MAPK	Mitogen-activated protein kinase
DPP-4	Dipeptidyl peptidase-4	MUFA	Monounsaturated fatty acid
DPPH	2,2-diphenyl-1-picrylhydrazyl	NF κ B	Nuclear factor κ B
EAA	Essential amino acids	PKC	Protein kinase C
ECM	Extracellular matrix	PPP	Pentose phosphate pathway
ETC	Electron transport chain	PUFA	Polyunsaturated fatty acid
G-3-P	Glyceraldehyde-3-phosphate	RAGE	Receptors for AGE
GFA	Glc-6-P amidotrasferase	ROS	Reactive oxygen species
GIP	Glucose-dependent insulintropic peptide	SFA	Saturated fatty acid
Gln	Glutamine	T1D	Type 1 Diabetes
GLP-1	Glucose-like peptide-1	T2DM	Type 2 Diabetes mellitus
		TNF α	Tumor necrosis factor α
		UFA	Unsaturated fatty acid
		WHO	World Health Organization

1. Literature Review

1.1 Overview

The primary role of foods is to provide nutrients that are required for the development, maintenance and repair of the human body. Through a delicate digestive system and a metabolic pathway, nutrients are released, absorbed and distributed throughout the body. A lack of required nutrients will result in poor health, therefore a varied and balanced diet is an adequate way to maintain health. All foods contain different levels of macronutrients, such as carbohydrates, fats, protein, and micronutrients (vitamins and minerals), together with secondary metabolites such as polyphenols. Among foods, cereals are mainly considered a source of carbohydrates and fibre, however, most cereals also contain proteins, lipids, and micronutrients. Over the years, research has shown that foods are not just a source of nutrients. In this regard, biological activities such as antioxidant, anti-inflammation, and anti-tumor activities have been reported for the secondary metabolites of food. Similarly, macronutrients possess functionalities and activities that are important to the quality of foods and the maintenance of human health. In addition to providing energy, polysaccharides in cereals (e.g. barley, oats), mainly fibre, are able to improve gastrointestinal health or reduce blood cholesterol. Food proteins not only serve as a source of amino acids, but they are also being used to produce peptides with biological functions. Some of the bioactivities from food-derived peptides include antioxidant, antihypertensive, anti-cancer, anti-inflammatory activities, as well as hormone regulation.

This study focuses on the antioxidant properties of hydrolyzed proteins, their ability to modulate the activity of enzymes, and the secretion of hormones involved in sugar metabolism. Hydrolyzed proteins and peptides that possess such functions can be useful in the management of diabetes. In fact, there are literature data, for example, on the antidiabetic properties of protein hydrolysates from quinoa ¹, rice bran ² and milk ³. Oats were used in this work because, relative to other cereals, they possess high proteins and better amino acid balance.

1.2 Nutritional value and composition of oats

Oats (*Avena*) are part of the *Poaceae* (grass) family and are classified into various species such as *Avena sativa*, *A. byzantine*, *A. fatua*, *A. diffusa*, and *A. orientalis*, to name a few ⁴. Oats were originally imported from Europe to Canada around the time of settlement but their origin can be traced back to Asia ⁴. Depending on the cultivar, oats can grow in a variety of climatic

conditions around the world. Meanwhile, they are mostly cultivated in Russia, North America, and mid – northern Europe ^{5,6}. In 2016, Canada produced 3.02 million tonnes of oats, second in the world to Russia, which produced 4.76 million tonnes. In Canada, oats are grown for straw, grain, feed, and fodder, however, they are primarily used as animal feed (74% of all oats grown in Canada) ^{6,7}. Human consumption has been increasing due to an increased awareness of the health benefits associated with the intake of oat products ^{4,8,9}. In 1998, the FDA recognized, based on available research data, that the consumption of 3 grams of soluble fibre from oats per day was sufficient to significantly decrease the risk of cardiovascular diseases ¹⁰. Since then, other studies have been conducted to determine the cholesterol-lowering properties of fibres from other cereals such as barley, however, those from oats are more efficient because of their high molecular weights. Today, more people are consuming oats and oat-related products containing brans or extracted fibres ⁸. Oat products include bakery products, oatmeal, ready to eat breakfast cereals, flour, bran, and fibres ¹¹. These components can also be added to other foods to obtain a desired texture or consistency ¹². Oat flour, for example, is often used in bakery products in combination with wheat flours to increase the nutritional value and improve the texture because oat flour has high protein content and high water retention capacity ^{11,13}. The high water holding capacity helps composite breads retain freshness for longer periods of time, as long as the ratio of oat-to-wheat flour is optimized ¹⁴.

1.2.1 Oat composition

Oats, like most cereals, are primarily composed of carbohydrates, starch, and soluble fibres. The oat kernel is a starchy endosperm that is covered by a nutrient-rich bran layer, enveloped in a hull, or husk layer. The hull comprises approximately 25 – 30% of the total oat grain ⁴ composed primarily of non-digestible fibres ¹⁵, and therefore greatly decreases the nutritional value of oat grain. For this reason, the hull is removed before processing. This is done as opposed to growing naked oats, a genetically modified version of *Avena sativa* that grows without husks. Naked oats are a desirable cultivar from a production perspective, however, the crops produce a lower yield, and are more prone to mold and mechanical damage ⁹. Removing the hull leaves bran covered oat kernel, rich in nutrients.

The bran layer covering the endosperm is typically removed in the milling step of processing but can be added to flour to obtain different bran levels (i.e. low bran flour or medium

bran flour). The bran can also be sold on its own to consumers as a food supplement, or to manufacturers as a food ingredient. As reviewed by Butt et al. (2008), oat bran contains 17.1% protein, 67.9% carbohydrates, 8.6% fat, 15-22% dietary fibre, 10.4% β -glucan and various vitamins and minerals⁴. High dietary fibre, protein, and mineral contents make oat bran a desirable product.

1.2.2 Dietary fibers

Beta-glucan (Fig. 1.2.1C), the most abundant soluble fibre in oats, is an unbranched polysaccharide with β -D-glucanopyranosyl monomers with β -1,4 and β -1,3 linkages¹⁶. The β -1,3 linkages cause a lack of uniformity and make β -glucan very soluble in water. Studies have shown that β -glucan can positively reduce obesity and weight gain. Beta – glucan increases satiety by absorbing water in the intestines, increasing intestinal bulk, and slows gastric emptying. Many studies have also shown a significant cholesterol lowering effect of β -glucan^{17,18}, lowering the risk of cardiovascular diseases. Beta – glucan achieves this by strengthening lipid micelles in the intestines, entrapping bile acids, and preventing them from returning to the liver via entero-hepatic circulation^{19,20}. The liver then uses blood cholesterol to restore bile acid levels, thus reducing plasma cholesterol concentration²¹.

1.2.3 Secondary metabolites/phytochemicals

Oat brans have a high concentration of antioxidant compounds that prevent oxidation of oat lipids⁹. These antioxidants include tocopherols, specifically vitamin E, phenolic acids (caffeic acid, *p*-coumaric acid, gallic acid, ferulic acid, vanillic acid, *p*-Hydroxybenzoic acid, etc.), and avenanthramides^{9,22,23}. Avenanthramides (Fig. 1.2.1A), a derivative of cinnamoyl-anthranilic acids, are unique to oats²⁴, and have up to 60% greater antioxidant activity than tocopherols⁹. There have been up to twenty-five different avenanthramides discovered in oats, and each has antioxidant and anti-inflammatory properties^{22,25}. Tocols are potent antioxidants due to their ability to scavenge free radicals, having an anti-proliferative effect on cancer cells²².

1.2.4 Lipids

Oats have the highest lipid content of all cereals²⁶, containing high levels of mono- and polyunsaturated fatty acids (MUFA, PUFA), approximately 75% of total lipids. Oat grains contain approximately 5.15 – 9.66%²⁷ lipids (Fig. 1.2.1B), primarily oleic acid (C18:1; 22-47% of total

lipid content), and linoleic acid (C18:2; 25-52%), and palmitic acid (C16:0; 13-26%)^{5,26}. Unsaturated fatty acids (UFA) are generally considered a ‘healthy fat’ while saturated fatty acids (SFA) are not considered nutritious. SFAs have been shown to increase blood cholesterol levels, specifically low-density lipoprotein (LDL), and increase risk of atherosclerosis (hardening of arteries) and other heart diseases, whereas MUFA and PUFA have plasma cholesterol lowering capabilities²⁸. Because of the high proportion of UFA, oats are considered highly nutritious. Although beneficial for human and animal nutrition, high lipid contents can be problematic, causing rancidity due to lipid peroxidation, and causing issues during processing, milling, and storage¹¹.

Table 1: Fatty acid composition of oat lipids, as expressed as a percentage of total fatty acids. Hulled oats were used for the analysis, and each percentage is an average taken from five different cultivars¹⁵.

Scientific Name	Common name	Nomenclature	%	SEM
Tetradecanoic acid	Myristic acid	C14:0	0.35	0.02
Hexadecanoic acid	Palmitic acid	C16:0	20.99	0.17
Octadecanoic acid	Stearic acid	C18:0	2.14	0.14
Eicosanoic acid	Arachidic acid	C20:0	0.17	0.01
Hexadec-9-enoic acid	Palmitoleic acid	C16:1	0.21	0.03
Octadec-9-enoic acid	Oleic acid	C18:1	43.06	0.42
Eicosenoic acid (cis-11)	Gondoic acid	C20:1	0.99	0.01
Docos-13-enoic acid	Erucic acid	C22:1	0.46	0.07
Octadecadienoic acid	Linoleic acid	C18:2	28.73	0.54
Octadecatrienoic acid	α -Linolenic acid (ALA); Linolenic acid	C18:3	0.71	0.09
SFA			23.66	0.06
UFA			74.16	0.05
MUFA			44.72	0.49
PUFA			29.44	0.47

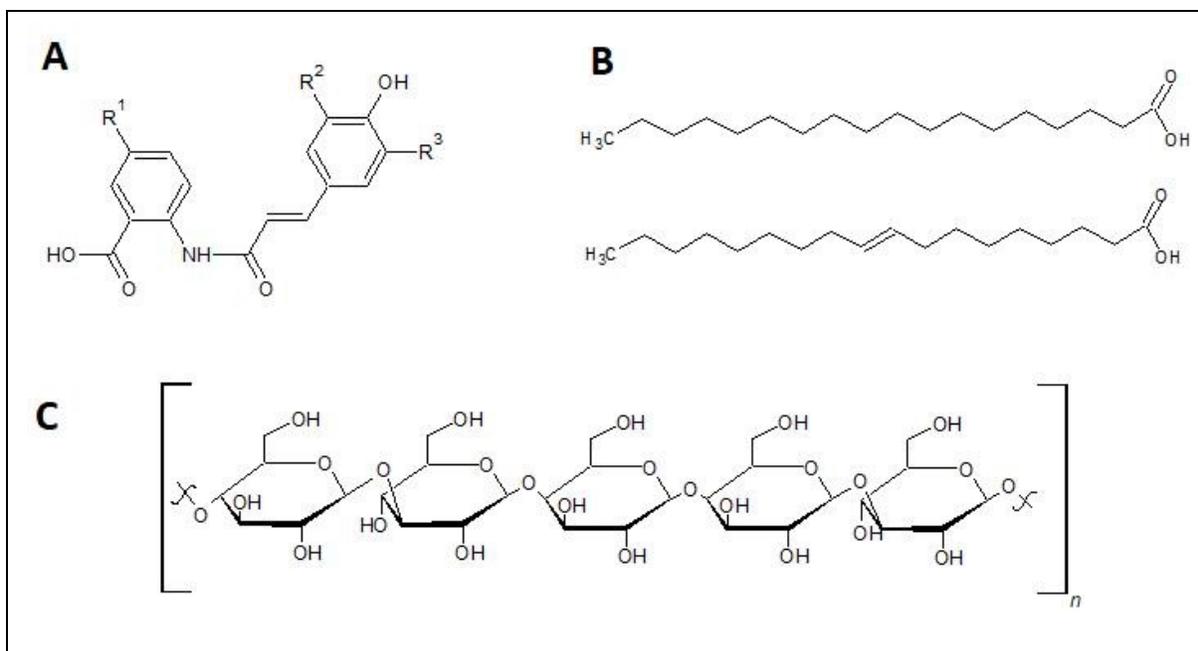


Figure 1.2.1: Chemical structure of A) avenanthramides where R groups are typically hydrogen or hydroxyl groups, B) stearic acid and oleic acid, C) beta-glucan

1.2.5 Proteins

Oat grains contain approximately 11-15% protein^{4,9,29} which is higher than most cereal grains³⁰. The two main classes of oat proteins are globulins and avenins (otherwise known as prolamins), which make up 50-80% and 10-20% of the total protein, respectively^{6,29,31}. Globulins are salt-soluble, whereas avenins are alcohol soluble proteins. The globulins are further classified based on their sedimentation values (2 to 12), the smaller number corresponding to a larger number to heavier proteins. The 12S globulin is the most abundant oat protein and is made up of two subunits A and B that are 32 and 22 kDa, respectively^{32,33}. The subunit A is acidic, and the subunit B is basic, and they are bound through disulfide bonds to form a 54 kDa dimer. Oats also contain 11S globulins, which are legumin-like proteins and are similar to the 12S globulins in their functionality³⁴. The 7S and 3S globulins are vicilin-like protein fractions; the 7S globulin contains 55 kDa polypeptides, while the 3S globulin contains two polypeptides of 15 and 20 kDa^{35,36}. Amino acids are classified into two categories: essential and non-essential amino acids. Essential amino acids (EAA) are considered indispensable because the body cannot produce them on its own, and therefore, these amino acids must be supplied from the diet. The EAA are leucine, isoleucine, valine, lysine, threonine, tryptophan, methionine, phenylalanine and histidine³⁷. The 12S and 11S globulins contain high levels of histidine, arginine, lysine, and aspartic and glutamic

acid, while the 3S and 7S globulin fractions are high in glycine and lower in glutamic acid ^{34,35}. Avenins are rich in glutamic acid and proline ^{34,38}. Overall, glutamic acid is the most abundant amino acid in oat protein, with leucine, arginine and asparagine in similar concentrations to each other. In table 2, the amino acid composition of oat protein is compared to barley protein. The percentages of amino acids are similar, however, oat proteins contain a higher concentration of arginine, phenylalanine, serine, and tyrosine. Although many of the EAA make up a smaller percentage of the overall protein content, oats still have a greater concentration of essential amino acids compared to other cereals ^{39,40}. In 2009, Liu et al. performed an analysis on the protein composition of oats and reported high levels of essential amino acids with respect to the ideal protein to meet nutritional needs outlined by the World Health Organization ⁴¹. According to their analysis, EAA in oats make up 33% of all amino acids, which includes seven out of the eight EAA. In comparison, barley and wheat grain proteins contain 21.65% and 22.48% EAA, respectively which are both slightly lower than the value for oat proteins ³⁹.

Table 2: Amino acid profile of oats and barley

	% amino acid in oat protein isolate ⁴¹	% amino acid in barley protein isolate ⁴²
Essential Amino Acids		
Arginine	6.93	5.50
Histidine	2.13	2.50
Isoleucine	3.48	3.94
Leucine	7.07	7.31
Lysine	3.46	4.18
Methionine	1.55	1.91
Phenylalanine	6.30	4.88
Threonine	2.65	3.78
Valine	4.77	5.71
Nonessential Amino Acids		
Alanine	4.08	4.57
Aspartic Acid	6.10	6.62
Proline	4.08	10.18
Cysteine	1.77	2.77
Glutamic Acid	21.45	23.46
Glycine	3.75	4.50
Proline	4.08	10.18
Serine	5.12	4.90
Tyrosine	4.60	3.29

1.3 Procedures for extraction and hydrolysis of proteins

1.3.1 Methods of extraction

In order to study food proteins or to use them in various applications, they must be extracted from the flour, for which different procedures exist in the literature. In 1972, Wu et al. ⁴³ published

an extraction method where sodium chloride, ethanol (70%), acetic acid, and sodium hydroxide solutions were used in succession to remove and separate proteins into fractions based on their preferred solubility. This method is effective because globulins are highly soluble in salt solutions, while avenins and prolamins are alcohol-soluble. Shortly after in 1973, Wu et al. developed an extraction technique based on pH. They found that protein extracts were 95% soluble at pH 11.4, 83% soluble at pH 2.1, and only 15% soluble at pH 5^{44,45}. These solubility patterns are caused by isoelectric points of polypeptides in oats which are between pH 4.5 and 7.0, depending on the protein fraction³³. Youngs et al. reported a different extraction technique in 1974, where the oat flour and bran slurries were centrifuged for 20 minutes at 12,000×g. The supernatant layer was removed and the pellet, containing protein, residue bran, and starch were separated with a spatula⁴⁶. In 1977, Wu et al. published an extraction method that is the most common protein extraction method used today. Hexane defatted oat flour is stirred in 0.02M NaOH solution for approximately thirty minutes. This solubilizes the protein into solution, and the starch and flour particles are removed via centrifugation. Next, the protein is precipitated out of solution by adjusting the pH to 5.0, close to its isoelectric point, and removed via centrifugation. This method has been adopted as the most widely practiced method due to its simplicity and ability to produce an adequate yield and purity.

1.3.2 Techniques to improve extraction yield and purity

There are various techniques that have been applied to facilitate and increase protein extraction and overall yield. Enzymatic treatment is commonly used to increase protein yield. Viscozyme-L, a carbohydrase enzyme mix, increases protein extraction by approximately 25 – 50% when applied to flour slurries prior to protein extraction⁴⁷. Viscozyme is a commercially available carbohydrase mix that contains a wide range of carbohydrate degrading enzymes; these enzymes work to break down polysaccharides within the food matrix to release protein during extraction. In a recent work, Jodayree et al. demonstrated that slurries hydrolyzed with amyloglucosidase, an amylase-degrading carbohydrase, yielded 82% protein, compared to the control which had 54% protein⁴⁸. Applying carbohydrases to flour slurries is an effective way at releasing proteins from the fibre and starch food matrix, facilitating extraction, and leading to a purer protein extract.

Ultrasonication is another technique that can be used to increase overall yield during extraction. In the ultrasonication, frequency sound waves are passed through the sample. Sonication can be categorized into high and low energy categories. Low energy sonication (low power, low frequency, >100 kHz, with intensities lower than $1 \text{ W}\cdot\text{cm}^{-2}$) is typically used for non-destructive quality assurance ⁴⁹. High energy sonication (high power, high frequency, 20-500 kHz, with intensities higher than $1 \text{ W}\cdot\text{cm}^{-2}$) is used to alter the physical state of a food product, for example: emulsification, defoaming, etc. It is suggested that the sonication treatments disrupt the covalent bonds and linkages between the protein and polysaccharide and fibre molecules, increasing extractability of proteins. Sonication has been shown to increase protein yield from sorghum flours ⁵⁰. Tang et al. observed protein extraction from rice bran after treatments of sonication, and found that sonication significantly increased the overall protein yield ⁵¹. Similar effects of sonication on soy protein extraction have also been observed ⁵², however, there is no data on the effects of sonication on oat protein extraction.

Much research has been done to observe the effects of sonication on the physical and biological activities of cowpea proteins ⁵³, vegetable proteins ⁵⁴, sorghum proteins ⁵⁵, and wheat flour proteins ⁵⁶. Sonication treatments do not typically affect the primary structure of protein extracts ^{53,54}; however, it can affect the tertiary and secondary structures of proteins, indicated by a change in functionality ^{54,57,58}. Prolonged high power sonication treatments can sometimes lead to an effect called ‘cavitation’, which is the formation and collapse of air bubbles, releasing energy and heat. Cavitation can change physical and chemical properties of protein ⁵⁹. Optimization of protein extraction is important as people look to food-derived nutraceuticals to manage diseases like diabetes.

1.4 Biological activities of protein hydrolysates

Protein hydrolysates are protein fragments of varying sizes that are produced in the body during digestion by protease enzymes. All protease enzymes cleave at different locations along a protein sequence, which results in a wide variety of peptides. The hydrolysis of protein creates bioactive hydrolysates that have important biological functionalities. These smaller peptide fragments are more easily absorbed into the bloodstream than their large, often multi-subunit, parent proteins.

Bioactive hydrolysates can be produced in the body naturally during digestion or can be made *in vitro* from many food sources. Some sources include cereals (oats ^{48,60–63}, wheat ^{64,65}, barley ^{62,63}, rice ^{2,66,67}, quinoa ^{1,68}), chickpeas ^{69,70}, peas ⁷¹, soybeans ^{72–74}, lentils ^{71,75}, dairy ^{3,76,77}, eggs ⁷⁸, and marine products ^{77,79–81}. The demand for bioactive peptides in the food and pharmaceutical fields is increasing, and therefore, economical sources of bioactive peptides are being studied.

Protein hydrolysates have many bioactivities, including antioxidant, antidiabetic, antihypertensive, hypocholesterolemic, anti-cancer and anti-inflammatory properties. Antioxidants are important for maintaining cellular homeostasis and preventing oxidation through the propagation of free radicals. Reactive oxygen species (ROS) are responsible for lipid oxidation and shorter shelf lives of food products. Cellular lipid oxidation is a common factor contributing to serious disorders, such as atherosclerosis, diabetes and cancer. Hydrolysates function as antioxidants through two mechanisms, including metal chelating activity and radical scavenging activity. The antioxidant activities of hydrolysates have been studied extensively in many compounds, including milk ^{3,82}, soy ⁸³, quinoa ^{1,68}, rice ⁶⁷, chickpea ⁸⁴, as well as oats ^{48,85–88}.

Transition metal ions are unstable and volatile and create many free radicals and other ROS; therefore, the chelation of these metal ions with protein hydrolysates reduces the formation of ROS in the body and the subsequent DNA damage and lipid oxidation. Commonly chelated metal ions are iron (Fe^{2+}), copper (Cu^{2+}), zinc (Zn^{2+}), and calcium (Ca^{2+}). Oat bran protein hydrolysates have iron and calcium binding capacities ⁸⁹. The iron chelating capacity of hydrolysates is most likely due to the presence of histidine amino acid residues, and its imidazole ring ^{69,84}. Acidic amino acids also contain negatively charged amino acids with extra electrons, facilitating iron chelation ⁸⁹. Although cysteine has been recognized as a proponent in iron and hydrolysate chelation, there is much discrepancy on the subject, and the literature on the subject is variable ^{69,89,90}. Likewise, calcium binding capacity has been observed due to the presence of histidine and in peptides with low molecular weights. Calcium binding occurs primarily at the carboxyl group of glutamic and aspartic acid ^{65,91}.

Protein hydrolysates are capable of scavenging many radicals, including superoxide anion radicals ($\text{O}_2^{\cdot-}$), hydroxyl radicals (HO^{\cdot}), oxygen radicals (ROO^{\cdot}), and DPPH radicals. For example, canola proteins hydrolyzed with Alcalase and Flavourzyme enzymes produced DPPH-radical

scavenging hydrolysates⁹². Likewise, chickpea protein hydrolysates showed DPPH, O₂^{•-}, and HO radical scavenging activities⁸⁴. Zou et al. reviewed the relationship between peptide structure and antioxidant activity. With respect to amino acid composition, they outlined that negatively charged amino acids act as strong antioxidants due to their excess electrons available for donation⁹³. Nongonierma et al reported that milk protein hydrolysates from whey, casein, and lactoferrin had stronger O₂^{•-} and DPPH scavenging activities, than dipeptides purified from the same sources³. This indicates a size and amino acid specificity for radical scavenging activity. Of interest for this study, oat bran protein hydrolysates also have radical scavenging activities. Tsopmo et al. reported DPPH, HO, O₂^{•-}, and peroxy radical scavenging activities of oat bran protein hydrolysates in various studies^{48,85,94}.

Bioactive hydrolysates act as antihypertensive agents through the inhibition of angiotensin I-converting enzyme (ACE). In normal conditions, the body maintains a healthy blood pressure within a homeostatic range of vasoconstriction and vasodilation. To maintain this, angiotensinogen is cleaved by renin to produce angiotensin (AT)-I. Afterward, ACE cleaves AT-I to produce AT-II, an important vasoconstrictor. In unhealthy conditions, where homeostasis is disrupted, AT-II levels are raised, causing high blood pressure. While there are synthetic ACE inhibitors available, consumers are seeking natural alternatives to help maintain normal blood pressure, food protein hydrolysates have shown ACE inhibitory activities. Most commonly studied sources for ACE inhibitory hydrolysates are milk and fish⁶¹. In a recent study by Rudolph et al., it was discovered that enzymatically hydrolyzed proteins from wheat, pea, soy, and rice produced hydrolysates with ACE-inhibitory properties⁹⁵. It was hypothesized that the presence of aromatic amino acids was the contributing factor to the inhibitory properties. Tyrosine was proposed as the main contributing factor of inhibitory properties since the IC₅₀ values increased as the tyrosine increased. Wheat showed the highest inhibitory activity, with an IC₅₀ of 39 mg/L, followed closely by soy, pea and rice protein hydrolysates⁹⁵. Legumes, such as chickpeas, lentils, lupins, and beans are another source of highly potent ACE-inhibitory protein hydrolysates, however, the extraction and hydrolysis methods may have an effect on the overall inhibitory activities⁹⁶. ACE inhibitors have also been derived from seaweed⁹⁷, fish⁹⁸, and marine products⁹⁹, and meat products.

Protein hydrolysates have marked antidiabetic properties through their antioxidant capacities, and their dipeptidyl peptidase-4 (DPP-4), α -amylase and α -glucosidase inhibition. Their specific antidiabetic properties are discussed further in section 1.9.

1.5 Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a worldwide burden and is currently one of the most common clinical disorders. The International Diabetes Foundation reported in 2011 that 366 million individuals were affected by diabetes and projected that number to reach 552 million by 2030¹⁰⁰. Canada had a national prevalence of 7.3%, and it was estimated that the cost to the healthcare system was 3.6 billion dollars in 2017 (Diabetes Canada, 2018)¹⁰². Type 1 diabetes (5 – 10% of cases) is a genetic disorder where pancreatic B-cells do not produce a sufficient amount of insulin, whereas T2DM is a complex disorder characterized by a lack of insulin sensitivity, resulting in high postprandial glucose levels. The cause of T2DM is still largely debated in literature, but some contributors are epigenetic factors (in embryonic, fetal, and early stages of life), environmental and lifestyle factors calorie-rich diets, sedentary lifestyles, smoking, alcohol, as well as other factors, such as ethnicity and socioeconomic status¹⁰³.

T2DM manifests itself through many physiological symptoms, and patients are at a high risk of morbidity. This is because of the effects on many organ systems, including the cardiovascular system, leading to heart failure, stroke, and various heart conditions caused by atherosclerosis. T2DM also affects the peripheral nervous system (neuropathy, ulcers), eyes (retinopathy), mouth, and kidneys, which often causes acute symptoms, such as polyuria and heightened thirst¹⁰⁴. T2DM patients are also otherwise more susceptible to other diseases, such as the liver and digestive complications, cancer, and infection.

1.6 Contributors to T2DM

The main physiological contributors to T2DM complications are oxidative stress and hormone imbalances. Oxidative stress is elevated due to an increase in glucose being metabolized through glycolysis and the electron transport chain (ETC). This hyperglycemia also causes a hormonal imbalance as the body tries to cope with the excess glucose.

1.6.1 Oxidative stress

Oxidative stress is caused by an increase in reactive oxygen species (ROS) or reactive nitrogen species (RNS). A ROS can either be a radical ($O_2^{\cdot-}$, HO^{\cdot} , RO_2^{\cdot} , RO^{\cdot} , HO_2^{\cdot}) or a non-radical species (H_2O_2 , $HOCl$, O_3 , 1O_2 , $ONOO^-$). Non-radical species are highly reactive oxygen-

containing molecules that are common precursors to ROS. ROS can cause damage to the body by disrupting the redox balance within the cells. The most common sources of ROS are in the mitochondria through the ETC, free transition metal ions, and exposure to ultraviolet light. ROS are normal byproducts of aerobic metabolism, and the body has mechanisms that it uses (antioxidants and antioxidant enzymes) to maintain homeostasis and prevent oxidative stress. Oxidative damage occurs when antioxidant mechanisms are unable to keep up with the overproduction of ROS.

Oxidative stress is an obvious consequence of T2DM because of a disproportionate flow through glycolysis and the ETC in the mitochondria. Glycolysis produces NADH and pyruvate, which is oxidized in the mitochondria through the tricarboxylic acid cycle (TCA). The byproducts of TCA are water, CO₂, NADH and FADH₂. NADH and FADH₂ are required for ATP production via the ETC. The ETC (Fig. 1.2) is composed of four enzyme complexes, cytochrome c and ubiquinone. As electrons are transferred from NADH/FADH₂ through the ETC, a proton gradient is formed which fuels ATP synthase, however, both the proton gradient and the complexes within the ETC are responsible for excess ROS production, specifically superoxide. The two main sites for superoxide production are at complex I (NADH dehydrogenase) and between ubiquinone and complex III (ubiquinol: cytochrome C oxidoreductase)^{105,106}. Complex I autoxidizes the reduced flavin mononucleotide of NADH dehydrogenase, while in complex III, superoxide is produced through the direct reduction of ubisemiquinone¹⁰⁷.

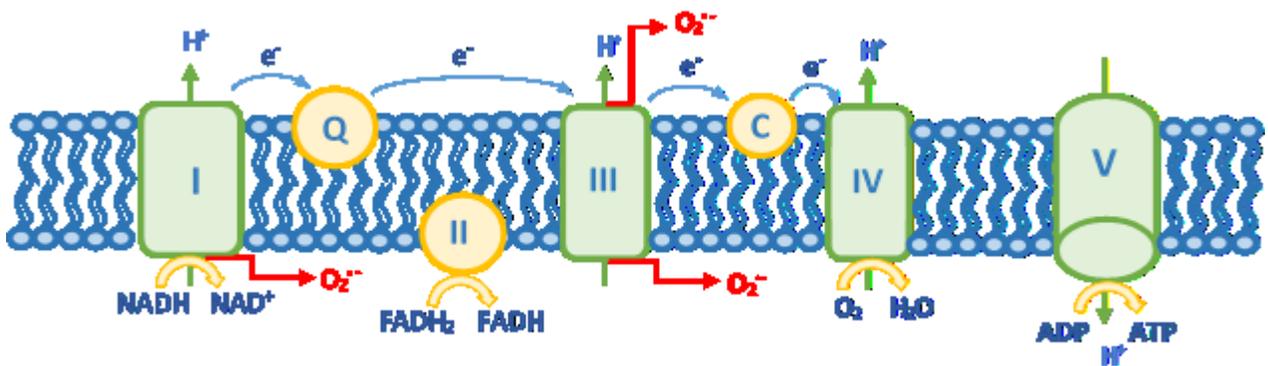


Figure 1.6.1: Diagram of superoxide production in the electron transport chain.

A study by Nishikawa et al. found that the excessive production of superoxide anion radical ($O_2^{\cdot-}$) in the mitochondrion increased glycolysis which is one of the driving factors for diabetic complications, while inhibition of the ETC down-regulated three of the four pathways leading to hyperglycemic damage (advanced glycation, polyol pathway flux, protein kinase C activation). One of the main enzymes in the glycolytic pathway, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is sensitive to changes in redox states. This is because excess ROS enzyme activates the poly(ADP-ribose) polymerase (PARP) enzyme, which in turn inhibits GAPDH¹⁰⁸. The inhibition of GAPDH forces glucose into metabolic and signalling pathways that favor the development of complications. Some of them result in the production of advanced glycation end (AGE) products, hexosamine biosynthetic pathway, and the activation of protein kinase C (PKC)¹⁰⁹⁻¹¹³. Some of the molecules involved in AGE formation are fibrinogen, albumin, collagen and immunoglobulin. The consequence is the disruption of the molecular structure of these proteins not only prevents them from functioning properly as carriers or enzymes, but also limits their capacity to resist degradation, and interact with receptors^{109,114}. AGE are also responsible for the production of pro-inflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α). The last mechanism for T2DM complications related to oxidative stress is through the hexosamine biosynthetic pathway, which can post-translationally modify proteins through glycosylation. Therefore, when considering the treatment of T2DM, the control of ROS production is critical.

1.6.2 Hormone and enzyme imbalance

The “incretin effect” is a term used to describe the increase in post-prandial insulin secretion and is caused mainly by several different (incretin) hormones. The two main incretin hormones are glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). GIP is secreted primarily from the K-cells in the small intestine, however, GIP can be secreted by other cells within the duodenum¹¹⁵. There are GIP receptors located on the pancreas, heart, within the adipose tissue and intestines, and in the brain. GIP is secreted in response to absorption of small carbohydrate and lipid molecules and can increase up to twenty times its normal level¹¹⁶. GLP-1 is another incretin hormone that stimulates insulin secretion. In the L-cells of the epithelial layer of the intestines, prohormone convertase – 1 (PC1) cleaves pro-glucagon to release GLP-1¹¹⁷. Depending on the cleavage and post-translational modifications, there are two forms of bioactive GLP-1: GLP-1 (7-37) and GLP-1 (7-36). They each start with an N-terminal histidine

residue, however GLP-1 (7-36) is missing a glycine residue at the C-terminal end. GLP-1 levels rise to notable levels within 15 minutes of eating, indicating that the release is stimulated by certain nutrients, specifically glucose, protein, and fat ¹¹⁸. Under normal conditions, GLP-1 persists in the bloodstream for up to three hours. The half-lives of GLP-1 and GIP, however, are significantly decreased in the presence of DPP-4 enzyme, shortening their half-lives to approximately two minutes ¹¹⁹. Inhibitors of DPP-4 are considered important in the treatment and management of T2DM. DPP enzymes preferentially cleave shorter peptides and peptides with proline or alanine in the second position from the N-terminal ¹²⁰. DPP-4 cleaves the bioactive GLP-1-(7-36) to its inactive form GLP-1-(9-36) ¹²¹. DPP-4 is primarily found in the intestines, on the endothelial cells where GLP-1 and GIP are secreted; it can also be found in the kidneys, lungs, pancreas, and liver, to name a few ¹²². Because of the proximity to DPP-4 enzymes, approximately half of all secreted GLP-1 is immediately inactivated ¹²¹.

Other enzymes included in metabolism of glucose are α -amylase and α -glucosidase. Alpha-amylase along with α -glucosidase are carbohydrase enzymes that are secreted from the salivary glands and the pancreas. Alpha-amylase hydrolyzes the α -D-1,4-glycosidic linkages in starch, cleaving it into smaller oligosaccharides ¹²³. Alpha-glucosidase enzymes hydrolyze these oligosaccharides into readily absorbable glucose monomers. These enzymes are important for healthy individuals, however, in hyperglycemic diabetic patients, they can be used to reduce glucose absorption.

1.7 Treatment and management of T2DM

There are different strategies to manage diabetes. Patients with type 1 diabetes receive insulin injections to supplement the limited natural insulin secretion. Optimal delivery, injection, and dosage are set based on extensive research data. Insulin injections are effective in the treatment of T1D since T1D patients are lacking the pancreatic beta-cells responsible for the production of insulin as a result of an autoimmune body response. The management of T2DM is more complicated; currently, there are strategies that focus primarily on lowering plasma glucose levels through a healthy lifestyle and diet choices, as well as the use of pharmaceutical drugs.

The current pharmaceutical treatments include metformin, sulfonylureas, meglitinides, DPP-4 inhibitors, thiazolidinediones, sodium-glucose cotransporter-2 (SGLT2) inhibitors, α -glucosidase and α -amylase inhibitors, dopamine agonists and bile sequestrants. Subcutaneous

treatments include GLP-1 receptor agonists, amylin analogues, and insulin. Metformin inhibits complex I of the ETC, which decreases ROS production and increases anaerobic metabolism. In the liver, metformin also prevents gluconeogenesis, which reduces glucose output. Sulfonylureas are also a glucose-lowering drug that is typically used to compliment metformin prescriptions. They stimulate insulin secretion by binding to ATP-sensitive potassium channels at the sulfonylurea receptor -1 (SUR1) ¹²⁴. This binding closes the potassium channel, which causes an influx of calcium; excess cytosolic calcium starts a series of reactions leading to glucose-independent insulin secretion ^{124,125}. Meglitinides act in a similar fashion as sulfonylureas, initiating insulin secretion, however, they bind to a different site on the SUR1 receptor ¹²⁶. Thiazolidinediones, another type of T2DM treatment, activate the nuclear peroxisome proliferator-activated receptor- γ (PPAR- γ). This receptor is primarily found in fat cells, and its activation produces adipocytes that are highly sensitive to insulin. This also reduces the fatty acid levels in the blood and helps regulate glucose metabolism ^{125,127}. SGLT2 is a transporter in the renal tube system that effectively removes 90% of glucose from the urine and transports it to the blood. Its inhibition allows glucose to be excreted in the urine, and is an effective method of lowering blood glucose ¹²⁸.

In 2007, DPP-4 inhibitors were introduced as a novel drug for the management of T2DM ¹²⁵. Inhibiting DPP-4 enzymes prolongs the half-lives of incretin hormones, accentuating the incretin effect, and increasing insulin secretion. Likewise, GLP-1 receptor agonists have also been used to supplement the baseline incretin effect. These receptor agonists have a heightened affinity to the GLP-1 receptors, and are unable to be degraded by DPP-4, and can, therefore, increase insulin secretion ^{125,129}.

Similarly, synthetic α -amylase and α -glucosidase inhibitors are currently prescribed for the treatment and management of T2DM. Inhibition of α -amylase and α -glucosidase slows down the absorption of glucose into the bloodstream, reducing post-prandial glucose levels ¹³⁰. Acarbose, originally introduced in the 1990s, is currently being administered as an α -glucosidase inhibitor. The success of the inhibitor drugs has prompted many researchers to focus their attention on the search of natural compounds of plant origin that can be used as an alternative in the regulation of blood sugars.

1.8 Methods to determine antidiabetic properties

There are many *in vitro* and *in vivo* techniques for determining the antidiabetic activity of different extracts and molecules. *In vitro* assays include antioxidant, enzyme, and glucose diffusion assays. Observing oxidative stress levels is an important method of determining antidiabetic effects since diabetes and oxidative stress are closely linked^{131–133}. Common antioxidant assays include peroxy radical, hydroxyl radical, superoxide radical, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and DPPH radical scavenging activities, several of which are utilized in this study. Other antidiabetic methods are based on the inhibition of enzymes that breakdown polysaccharides as well as the regulation of hormones involved in the regulation of glucose¹³⁴. As discussed above, key enzymes in diabetes include α -amylase, α -glucosidase, DPP-4, and angiotensin-converting enzyme (ACE). Inhibitors of these enzymes can be derived from many dietary sources^{135–141}. A recent study by Ayyash et al. observed that water-soluble extracts of fermented whole grains (lupin, quinoa, and wheat) caused α -amylase, α -glucosidase and ACE inhibition¹⁴¹.

In vivo assays including cellular and mice/rat models are also often used to determine antidiabetic properties. Ayyash et al. investigated the effects of the water-soluble extracts of fermented whole grains on the viability of two cancer cell lines (Caco-2 and MCF-7 cells). Cell types used in the literature include human intestinal cells (NCI-H716, Caco-2), mouse colon (GLUTag, STC-1)¹⁴², and pancreatic cells (α TC1-6)¹⁴³. Cell assays are typically used to determine the secretion of hormones such as GLP-1 and GIP, and the regulation of enzymes such as DPP-4 and antioxidant enzymes⁸⁶. In animal models, diabetes (i.e. hyperglycemia) is often induced using streptozotocin, a chemical that is toxic to the insulin-producing beta cells of the pancreas in mammals. The rats are fed a controlled diet with or without test agents, and plasma glucose is analyzed. This method has been used to test compounds including medicinal herbs^{123,144,145}, stevia¹³³, spices¹³⁴, fruit¹⁴⁶, and brown rice¹⁴⁶. One recent focus of research is the study of hydrolyzed proteins and peptides from foods.

1.9 Protein hydrolysates as active agents against T2DM

Proteins are well known for their nutritional values, however it is also now established that upon digestion with enzymes of the digestive tract system or other proteases, peptides with biological functions are released from food proteins. Data from the literature show that protein

hydrolysates contribute to human health through their capacity to act as antioxidant, anti-inflammatory, anticholesteremic, and antidiabetic agents. Protein hydrolysates control glucose levels through α -amylase and α -glucosidase inhibition, promoting incretin hormone and insulin secretion, DPP-4 inhibition, and promoting glucose uptake and decreasing the production of endogenous glucose.

Alpha-amylase inhibitors slow down the cleavage of complex starch molecules into oligosaccharides while α -glucosidase inhibitors inhibit hydrolysis of oligosaccharides to glucose monomers. These enzymes are critical in the maintenance of glucose homeostasis. Uraipong et al. reported α -amylase and α -glucosidase inhibition of rice bran protein hydrolyzed by Alcalase, Flavourzyme, Protamex, and Neutrase, with Alcalase producing the most effective inhibitors⁶⁷. In other works, hydrolysates from albumin¹³⁶, brewer's spent grain¹⁴⁷, pinto beans¹⁴⁸, and barley¹⁴⁹ all showed α -amylase and α -glucosidase inhibitory properties.

Incretin hormones play an important role in T2DM, stimulating approximately 50% of all insulin secreted. Since these hormones are quickly degraded, enhancing incretin hormone secretion and inhibition of incretin degrading enzymes is an effective mechanism against T2DM. A study by Cordier-Bussat et al. found that egg albumin hydrolysate and meat protein hydrolysate dose-dependently increased the secretion of GLP-1 in small intestine STC-1 cells¹⁵⁰. In a follow-up study, they found that the hydrolysates stimulated the transcription of proglucagon, leading to an increase in GLP-1 secretion¹⁵¹. Meanwhile, compared to STC-1, the stimulatory effect of hydrolysates on GLP-1 secretion on colon cell lines (GLUTag) was weak while no effect was found on pancreatic cells (RINm5F and INR1G9).

Protein hydrolysates are also natural sources of DPP-4 inhibitors. DPP-4 inhibition was reported for milk³, quinoa⁶⁸, and brewer's spent grain hydrolysates¹⁴⁷. Cereal hydrolyzed proteins from oats, barley, and buckwheat have also shown DPP-4 inhibitory properties⁶². In a study on fermented soybeans, Sato et al. isolated and characterized two peptides in an extract with DPP-4 inhibitory activity¹⁵⁰. The identified peptides Lys-Leu and Leu-Arg had dose-dependent inhibition values that are considered strong relative to other peptides from foods.

Lastly, hydrolysates impact glucose uptake and storage through their effects on glucose transporter GLUT-4. This glucose transporter is typically found on insulin-sensitive tissues, such as the heart, adipose tissue, and skeletal muscle. After glucose consumption in normal individuals,

insulin binds to insulin receptors which causes a series of phosphorylation reactions leading to GLUT-4 being redistributed to the cell surface to act as a channel for glucose uptake ¹⁵². In diabetic individuals, the insulin response and insulin sensitivity are affected, which then, in turn, affects GLUT-4 release, disrupting glucose uptake ¹³⁸. Morato demonstrated that whey protein hydrolysates increase the translocation of GLUT-4 to the plasma membranes in a rat model ¹⁵³. A similar effect was observed in a study on soy peptides ¹⁵⁴ and silk protein hydrolysates ¹⁵⁵. Protein hydrolysates, through the translocation of GLUT-4 to the cell surface, increase glucose uptake, metabolism and storage, thus ameliorating hyperglycemia and reducing the effects of T2DM.

Table 3: Antidiabetic properties of protein hydrolysates

Protein Source	Enzyme	Mechanism of Action	Method used	Reference
Rice Bran	Alcalase, Neutrase, Flavourzyme, Protamax	α -amylase inhibition; α -glucosidase inhibition	Enzymatic assays	[⁶⁷]
Albumin	Alcalase	α -amylase inhibition; α -glucosidase inhibition	Enzymatic assays	[¹³⁶]
Egg Albumin	Alcalase	α -amylase inhibition; α -glucosidase inhibition	Enzymatic assays	[¹⁵⁶]
Brewer's Spent Grain	Alcalase	α -glucosidase inhibition; DPP-4 inhibition; ACE inhibition	Enzymatic assays	[¹⁴⁷]
Pinto beans	Protamex	α -amylase inhibition	Enzyme assays	[¹⁴⁸]
Barley	Pepsin; trypsin	α -amylase inhibition	Enzyme assays	[¹⁴⁹]
	Trypsin/Pancreatin mixture	DPP-4 inhibition	Enzymatic assays	[⁶³]
Milk	Commercial gastro-intestinal preparations	DPP-4 inhibition	Enzyme assays, in silico models	[3], [74]
Quinoa	Papain; Papain-like preparation	DPP-4 inhibition	Enzymatic assays	[⁶⁸]
Oats	Trypsin/Pancreatin mixture	DPP-4 inhibition	Enzymatic assays	[⁶³]
Buckwheat	Trypsin/Pancreatin mixture	DPP-4 inhibition	Enzymatic assays	[⁶³]
Fermented Soy Beans	No enzyme	DPP-4 inhibition	Enzymatic assays	[¹⁵⁰]
Meat hydrolysates	-	GLP-1 secretion; proglucagon transcription	Cellular assays (STC-1, GLUTag, RINm5F, INR1G9)	[¹⁵¹]
Whey Protein Hydrolysates	-	GLUT-4 translocation	Rat model	[¹⁵³]
Silk Protein Hydrolysates	-	GLUT-4 translocation	Cell models (3T3-L1 fibroblast cells)	[¹⁵⁵]

2. Hypothesis and Objectives

The hypothesis of this study was that pre-treatment of oat flour with ultrasonication will affect protein extraction and the biological activities of their hydrolysates, namely antioxidant properties and antidiabetic properties.

The objectives of this study were to:

- i. Extract protein from oat flour after treatment with two degrees of ultrasonication, and further hydrolyze the proteins using three proteases: Flavourzyme, Papain, and Alcalase.
- ii. Characterize the proteins and hydrolysates (e.g. molecular weight, composition, free amino acid content, sulfhydryl content)
- iii. Determine the antioxidant properties of protein hydrolysates (oxygen radical absorbance capacity, hydroxyl radical scavenging activity, superoxide radical scavenging activity).
- iv. Evaluate antidiabetic properties of hydrolysates through enzyme inhibition and secretion of GLP-1 in colorectal cells.

3. Materials and Methods

3.1 Materials

The medium bran oat flour was donated by Richardson Milling (Portage La Prairie, Manitoba MB). The particle size distribution for medium bran flour is as follows: 2.00 mm (0.8%), 0.841 mm (61.5%), 0.595 mm (32.1%), 0.420 mm (5.0%), pan (0.6). The enzymes obtained from Sigma-Aldrich Ltd. (Oakville, ON, CA) were α -amylase from *Bacillus licheniformis*, Flavourzyme[®] from *Aspergillus oryzae*, Alcalase[®] from *Bacillus licheniformis*, and Papain, from papaya latex. Other chemicals and reagents also from Sigma-Aldrich were 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), 1,10-phenanthroline, iron(II) sulfate heptahydrate (FeSO₄·7H₂O), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 5,5'-Dithio-bis-(2-nitrobenzoic acid) (Ellman's Reagent), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 8-anilinoanthracene-1-sulfonic acid (ANS), ammonium bicarbonate, bovine serum albumin (BSA), calcium chloride, cupric sulfate pentahydrate, dibasic potassium phosphate, dimethylsulfoxide (DMSO), dithiothreitol, hydrogen peroxide, iodoacetamide, L-serine, 3,5-dinitrosalicylic acid, monobasic and dibasic sodium phosphate, monobasic potassium phosphate, phenylmethylsulfonyl fluoride (PMSF), potassium bromide, pyrogallol, reduced glutathione (GSH), sodium bicarbonate, sodium carbonate, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sodium potassium phosphate tartrate, sodium tartrate, starch, tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), trisaminomethane (Tris Base), and urea. Organic solvents, concentrated hydrochloric acid and Folin-Ciocalteu phenol reagent and fluorescein were purchased from Fischer Scientific Co. (Nepean, ON, CA). Potassium chloride was purchased from BDH, while magnesium sulfate and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) were purchased from BioShop. NCI-H716 cells (ATCC CCL[®]-251TM), RPMI 1640 medium (ATCC[®] 30-2001), and fetal bovine serum (ATCC[®] 30-2021) were purchased from American Tissue Culture Collection (Manassas, VA, USA).

3.2 Protein extraction

Medium oat bran flour was defatted using hexanes, stirring for one hour with a 1:4 ratio (w:v) and dried for 24 hours in a fume hood. The defatted flour was then mixed with 0.02M NaOH (1:10 ratio (w:v)), and adjusted to a pH of 9.5. For ultrasonic bath treatments, flour slurry was placed in an ultrasonic cleaner bath (Codyson PS-20 Ultrasonic Cleaner) for five minutes at 120

W with a frequency of 40 kHz. The slurries were stirred manually for the duration of the treatment, and the temperature was held steady at room temperature. For the high power sonication treatments, a UIP500hdT Transducer (Heilscher Ultrasound Technology, Germany) was used at 100 W and 20 kHz. The probe was submerged in the flour slurry 1.5 cm, and sonication was done for five minutes. The control samples received no sonication treatment. All slurries were then shaken (140 rpm) in an incubator (MaxQ 8000, ThermoFischer Scientific, MA, USA) for 2 hours at 25°C. Samples were centrifuged (2500 x g, 20 minutes, 4°C), the supernatant was collected, adjusted to pH 4.5 and centrifuged once more (10 000 x g, 40 minutes, 4°C). The pellet was collected, freeze-dried and stored at -20°C.

3.3 Protein content

Protein content was determined through a Lowry Assay, as described by Lowry et al. (1951) with some modifications¹⁵⁷. Reagents used were A (2.0% Na₂CO₃, 0.4% NaOH, 0.16% sodium tartrate, 0.5% SDS), B (4% CuSO₄), and C (1:100 ratio of B:A). Protein samples were dissolved in 0.5% SDS at 120 µg/ml. Bovine serum albumin was used as the standard and solutions were prepared in concentrations from 10 µg/ml to 200 µg/ml in 0.5% SDS. To 200 µl of each sample and standard, 600 µl reagent C was added and incubated for 25 minutes at room temperature. Next, 60 µl 1.0 M Folin-Ciocalteu phenol reagent was added, and reaction was incubated for 45 minutes at room temperature. The absorbance at 660 nm was measured using an Epoch Microplate reader (BioTek, VT, USA). Each sample was performed in triplicates. Protein content was expressed as the percentage of total protein within the isolate.

3.4 Free amino acid assay

The free amino acid content was determined using a modified method from Panasiuk et al. (1998)¹⁵⁸. A 75 mM potassium phosphate buffer (pH 7.4) was used to prepare samples and standards. Serine was used as standard, and was prepared in concentrations ranging from 0.00 – 140 µg/ml. To 150 µl sample, standard, or buffer (for blank), 150 µl 0.5% ninhydrin (in water) was added. Samples were heated in a 100°C water bath for 30 min. After samples had cooled, all solutions were diluted 1:5 in water. Triplicates of 200 µl were transferred to a 96 well microplate, and absorbances were read at 570 nm (Epoch Microplate Reader, BioTek, VT, USA). The

concentration of free amino acids was calculated, and results were expressed as percent free amino acids.

3.5 Mass spectrometry

Proteins were hydrolyzed prior to the analysis. Specifically, 1.0 mL of each protein isolate in 50 mM ammonium bicarbonate (1.5 mg protein/ml) were denatured in boiling water for 5 min. After cooling, 20 μ L 265 mM dithiothreitol was added to reach a final concentration of 5 mM. The samples were heated at 56°C for 1 h. After cooling a second time, 40 μ L of 265 mM iodoacetamide was added and samples were incubated at room temperature for 1 h. The digestion was then performed by addition of 30 μ L trypsin (1 mg/mL in ammonium bicarbonate; Sigma T-1426) to alkylated proteins (500 μ L of each). The hydrolysis was performed overnight at 37°C overnight and stopped with the addition of concentrated acetic acid.

The hydrolysates were filtered through a 0.22 μ m membrane followed by injection of 0.4 μ g into a 6550A iFunnel Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA). The elution was performed beginning with solvent A (H₂O with 0.1% formic acid) at 100%, and finishing with solvent B (acetonitrile with 0.1% formic acid) at 100% over 30 min. The mass range was 100-1600 m/z and the acquisition rate was 8098 transients/spectrum. Gas temperature was set to 125°C, drying gas at a rate of 11 L/min and nebulizer at 30 psig. The peak lists were analyzed using Mascot™ software. The NCBI nr database was used to identify sequences and the ion score cut off was set to 20.

3.6 Gel electrophoresis

The molecular weight of the protein samples was determined using sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) per the methods outlined in Walburg et al. (1983)³². The samples were prepared at a protein content of 1 mg/ml. One set of samples were dissolved in reducing buffer (0.125M Tris-HCl pH 6.8, 4% w/v SDS, 20% v/v glycerol, and 0.5% 2-mercaptoethanol), while the other set of samples was dissolved in buffer without the reducing agent (0.125M Tris-HCl pH 6.8, 4% w/v SDS, and 20% v/v glycerol)³². The samples (25 μ l), were loaded into a 4% stacking gel, and run on a 12% acrylamide gel (PROTEAN® Tetra Vertical Electrophoresis Cell) for 1 h at 120V. The gels were run in a 1X running buffer (0.303% w:v tris base, 1.44% w:v glycine, 0.1% w:v SDS) with the Precision Plus Protein™ Standards (BioRad,

CA, USA) used for the marker. The gels were immersed in coomassie blue staining solution for 60 min and de-stained overnight with a de-staining solution (20% MeOH, 10% glacial acetic acid, 70% ddH₂O).

3.7 Enzymatic hydrolysis of protein extracts and dialysis of hydrolysates

Protein extracts were hydrolyzed by three different proteases: Flavourzyme, Papain, and Alcalase. For all samples, 1.5 grams of protein was dissolved in 20 mL of ddH₂O and the solution was adjusted to the protease-specific pH (pH 7.0 for Flavourzyme and Papain; pH 8.0 for Alcalase). Protease was added to the protein solution at a 2% enzyme:substrate ratio. The samples were then incubated (at 50°C for Flavourzyme, 60°C for Papain and Alcalase) for three hours. After incubation, the proteases were inactivated by submerging samples in a hot water bath (85-90°C) for 5 min. After samples had cooled to room temperature, the solutions were centrifuged at 8000xg for 15 min at 4°C. The supernatant was then collected and dialyzed.

Hydrolyzed sample solutions were dialyzed immediately after hydrolysis using Spectra/Por[®] Dialysis Membrane Biotech Cellulose Ester Tubing (Spectrum[™], Rancho Dominguez, CA, USA) with a molecular weight cut off of 100-500 Da. Dialysis membranes containing hydrolyzed sample solutions were submerged in ddH₂O overnight with continuous stirring at room temperature. Solutions in dialysis membranes were collected, freeze-dried and stored at -20°C.

3.8 Free thiol content

Free sulfhydryl content of the hydrolysate samples was determined using a modified method from Puppo et al. (2005)¹⁵⁹. Samples (5 mg/ml), cysteine standards (0.0625-1.0 mM) and the Ellman's reagent (5,5'-Dithio-bis-(2-nitrobenzoic acid), 4mg/ml) were prepared in buffer (0.1M Tris, 1 mM EDTA, pH 8.0 with 8M urea and 1% SDS). To each sample (300 µL), 20 µL Ellman's reagent was added, and the sample was mixed and incubated at room temperature for 15 minutes. The absorbance was measured at 412 nm using an Epoch Microplate reader (BioTek, VT, USA), and free sulfhydryl groups were determined using the standard curve and reported as µM SH/g protein.

3.9 Zeta potential

Zeta potential was measured using a Zetasizer Nano Series Zen3600 (Malvern Instruments Ltd., UK) with a Millex[®] GP Filter and a 0.22 µM PES membrane. Protein samples were dissolved at 2.5 mg/ml in ddH₂O, pH 7.0. Samples were run in triplicates and reported in mV by the Zetasizer Software.

3.10 Hydrophobicity assay

Hydrophobicity was determined using the methods outlined by Hayakawa et al. (1985)¹⁶⁰. Samples were prepared at seven concentrations from 0.00125% - 0.03% protein in 0.01M phosphate buffer (pH 7). To 2 mL of sample, 10 µL 8.0 mM 8-anilino-1-naphthalene sulfonate (ANS) was added, and samples were immediately mixed. The fluorescence was measured at excitation and emission wavelengths of 390 and 470 nm, respectively (Synergy H1 Microplate Reader, BioTek, VT, USA). The fluorescence intensity was found using the following equation: $FI = F_1/F_0$, where FI is fluorescence intensity, F_1 is fluorescence of sample, and F_0 is fluorescence of the blank (phosphate plus ANS). The initial slope of the line from fluorescence intensity (FI) versus protein concentration was used as the index of hydrophobicity, H_0 .

3.11 Fourier-transform infrared and ultraviolet spectroscopy

FT-IR spectra were obtained by mixing hydrolysate samples (1.5mg) with 400 mg KBr, transforming them into pellets, and analyzing them at 600 to 4000 cm⁻¹ on a Bomem Michelson MB100 Spectrometer (with a deuterated triglycine sulfate detector; ABB Analytical, Thunder Bay, ON, Canada)¹⁶¹. Each spectrum is the compilation of 32 scans at 4 cm⁻¹ and analyzed using GRAMS 32Version 6.01 Software (Thermo Scientific, Mississauga, ON, Canada).

Ultraviolet absorbance spectra were obtained by measuring the absorbance of samples (1 mg/mL) in buffer (75 mM potassium phosphate buffer, pH 7.4) at 280 nm (Epoch Microplate Reader, BioTek, VT, USA). Samples were measured in triplicate.

3.12 Peroxyl radical scavenging activity

Oxygen radical absorbance capacity (ORAC) assay was used to determine peroxyl radical scavenging activity using the methods of Huang et al. (2002)¹⁶². Samples and GSH as control (0.1 mg/ml and 0.2 mg/ml) were prepared in buffer (75 mM potassium phosphate buffer, pH 7.4).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were serially diluted to prepare five dilutions ranging from 6.25-100 μM Trolox. To each sample, standard, blank and control (20 μl), 120 μl fluorescein ($8.21 \times 10^{-2} \mu\text{M}$) was added, and incubated in a Flx800 Fluorescence reader (BioTek, VT, USA) for 20 minutes at 37°C. Next, 60 μl 140 mM AAPH (2,2'-Azobis (2-amidinopropate) dihydrochloride) was added to all samples. Kinetic readings were taken for subsequent 50 minutes with excitation wavelength at 485 nm and emission wavelength of 528 nm.

3.13 Hydroxyl radical scavenging assay

The hydroxyl radical scavenging capacities of the samples were determined by dissolving samples in 75 mM potassium phosphate buffer (pH 7.4) at 1.0 mg/ml. GSH was used as standard. To each well, 50 μl sample, 3mM 1,10-phenanthroline, and 3 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added. Buffer was added to blanks and control instead of hydrolysates. To initiate the reaction, 50 μl 0.03% H_2O_2 was added (50 μl water to blank) and incubated at 37°C with shaking. After 1 hour, the absorbance was measured at 536 nm (Epoch Microplate Reader, BioTek, VT, USA), and the $\text{HO}\cdot$ scavenging activity was calculated using the following formula: $[(A_s - A_c)/(A_0 - A_c)] \times 100\%$, where A_s : absorbance of sample, A_c : absorbance of control, A_0 : absorbance of the blank.

3.14 Superoxide radical scavenging assay

Samples were prepared at 1mg/ml and 0.5 mg/ml in 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.3). Glutathione (GSH; 1 mg/ml) was used as a standard, and the buffer was used for blanks. In a 96 well plate, 80 μl sample was added (buffer for blanks). Next, 80 μl of buffer was added to each well, along with 70 μl 2.0 mM pyrogallol prepared in 10 mM HCl. Pyrogallol was substituted with 10 mM HCl for blanks. Absorbance was measured at 420 nm for 4 minutes at room temperature using an Epoch Microplate Reader (BioTek, VT, USA).

3.15 Alpha-amylase inhibition assay

Samples were prepared in concentrations ranging from 0 – 5.0 mg/ml in 0.02 M sodium phosphate buffer containing 6 mM NaCl (pH 6.8). The enzyme solution was prepared in the same buffer to achieve 1.0 U/ml. A 1.0 mg/ml acarbose solution was used for the positive control, and the buffer was used for blanks. Aliquots (100 μl) of sample, blank and control were mixed with 100 μl enzyme solution and incubated at 37°C for 5 minutes. Substrate (100 μl of 1% starch in

buffer) was added to each solution and were incubated 37°C for 10 minutes. Next, 200 µl DNS (1% 3,5-dinitrosalicylic acid, 12% sodium phosphate tartrate in 0.4 M NaOH) reagent was added to each solution. Samples were heated in boiling water for 5 minutes, after which they were placed in an ice bath to cool. Once at room temperature, samples were diluted 1:1 in buffer and 200 µl triplicates were transferred to a 96 well plate. Absorbance at 540 nm was measured using an Epoch Microplate Reader (BioTek, VT, USA).

3.16 Dipeptidyl peptidase-4 inhibition assay

DPP-4 inhibition assay was carried out using the DPP-4 Drug Discovery Kit (BML-AK499) from Enzo Life Sciences Inc. (Farmingdale, NY, USA). In a 96 well plate, 25 µl sample solutions and 10 µl inhibitor (0.1 mM) were added in triplicates. To all sample, inhibitor and control wells, 15 µl DPP-4 (17.34 µU/µl) enzyme solution was added. The plate was then incubated at 37°C for 10 minutes to allow complete interaction between all components. The reaction was initiated upon addition of 50 µl substrate (0.2 mM H-Gly-Pro-pNA) was added to all wells. Absorbance at 405 nm was read continuously for 60 minutes, and % DPP-4 activity was calculated as pmol/min using the following formula: [slope (OD/min) × conversion factor (µM/OD) × assay vol (µl)], where the conversion factor (µM/OD) = 50 / (average A₄₀₅ of control).

3.17 Cell culture conditions

NCI-H716 (ATCC CCL[®]-251[™]) is a human colorectal adenocarcinoma cell line that has been preserved from a 33 year old Caucasian male. The cells grow mainly as suspension cells with multi-cell aggregates. For growth and maintenance, cells were plated in 10 cm plates in Roswell Park Memorial Institute (RPMI) 1640 Medium (ATCC[®] 30-2001) supplemented with 10% fetal bovine serum (ATCC[®] 30-2021) and 2 mM L-glutamine. Cells are incubated in a Forma[™] Series II Water Jacketed CO₂ Incubator (ThermoFischer Scientific, MA, USA) at 37°C, 5% CO₂, and fed every two days, replacing 50% of the old media with fresh media.

3.18 Cell assays

To each well of a 96-well microplate coated with Matrigel (Extracellular matrix (ECM), Sigma E1270), 5 x 10⁴ cells in 100 µL were added and left to grow for 48 h. After the 48 h, media was carefully removed, and cells washed twice with 200 µL of Hank's Balanced Salt Solution (HBSS; 0.4 g/L potassium chloride, 0.06 g/L potassium phosphate monobasic, 0.35 g/L sodium

bicarbonate, 8.0 g/L sodium chloride, 0.048 g/L sodium phosphate dibasic, and 1.0 g/L D-glucose). Protein hydrolysates prepared at 0.4 and 0.8 mg/ml (final concentrations) in Krebs's Ringer Bicarbonate buffer (119 mM sodium chloride, 4.82 mM potassium chloride, 1.25 mM magnesium sulfate, 1.24 mM monosodium phosphate, 25 mM sodium bicarbonate, 2.0 mM HEPES, 1.0 mM calcium chloride) pH 7.4, and glucose (standard, prepared at 200 mM) were added and left to incubate at 37°C for 2 h.

3.18.1 Cell viability and cytotoxicity

To determine cell viability, after 2 h, 5 μ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in phosphate buffered saline (PBS)) was added to each well and further incubated for 1 h at 37°C. Protein hydrolysates were carefully removed from cells and 100 μ L dimethyl sulfoxide (DMSO) was added. Absorbance was measured at 570 nm with a background subtraction at 630 nm (Cytation 5 Imaging Reader, BioTek, VT, USA). Viability was calculated as a percentage of the control.

3.18.2 GLP-1 secretion

To determine GLP-1 secretion, after the 2 h incubation with samples, supernatants were collected with phenylmethylsulfonyl fluoride (PMSF) solution (50 μ g/ml final concentration) and frozen at -80°C until further analysis. GLP-1 secretion was determined using an ELISA kit (EGLP-35K, EMD Millipore Corporation, MA, USA). Samples were plated onto the antibody-coated plate for 24 h, a detection conjugate was added to bind to the bound sample, and lastly, the substrate was added. After 20 minutes of incubation with the substrate, fluorescence was measured at excitation/emission wavelengths of 355 nm and 460 nm, respectively (Cytation 5 Imaging Reader, BioTek, VT, USA). The concentration of GLP-1 secreted was determined using a standard curve.

3.19 Statistics

All experiments were performed in triplicate except for mass spectrometry, SDS-PAGE, and FT-IR. Statistical analyses were performed using SAS[®] Studio Online (© 2019 SAS Institute Inc., North Carolina, USA). The results are expressed as mean \pm standard deviation. The results were analyzed by one-way analysis of variance (ANOVA) and least significant difference (LSD) tests ($p < 0.05$).

4. Results and Discussion

4.1 Protein extraction and protein contents

Two different techniques were used to enhance the extraction of proteins from medium oat brans: ultrasonic cleaner bath and high power sonication. The extraction yield was 3.64% for the control bran, while the ultrasonic bath (UB) and high power (HP) treatments yielded 2.06% and 3.79%, respectively. The HP treatment of brans then had no effect on yields while UB treatment resulted in lower extraction amount. In the literature, sonication did increase yield of proteins from soy⁵⁰, rice⁵¹, and sorghum⁵⁰. Specifically, sonication increased the protein yield by 42.1% in rice bran in one study⁵¹ and by 10% in sorghum in another study⁵⁵. One reason why HP treatments used in this study did not increase protein yields is that the power level (100 W) was lower than those cited in the literature (750-1200W). Perhaps an increase in power intensity and a prolonged treatment time would result in an increase in protein yield.

The soluble protein content of the extracts were determined using the Lowry assay, with some modifications¹⁵⁷. The soluble protein contents were 90.1%, 89.4%, and 93.5% for control, ultrasonic bath, and high power sonicated protein samples, respectively. The high protein contents indicate a high level of purity within the protein extracts. These values are similar to protein contents in our other works, using similar extraction techniques⁸⁵.

4.2 Characterization of proteins

4.2.1 Protein content and molecular weight

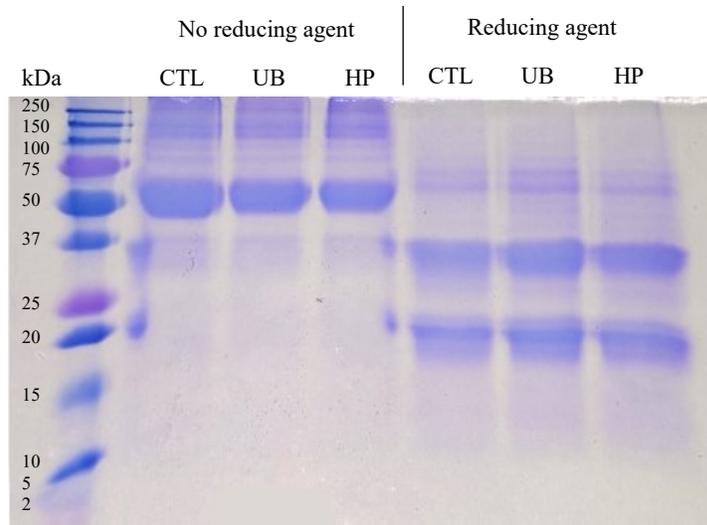


Figure 4.2.1: SDS-PAGE gel electrophoresis results from samples (1mg protein/ml) dissolved in solution with and without a reducing agent. Samples (25 μ L) were run on a 12% resolving gel and 4% stacking gel for 1 hour at 120V and. Sample labels are as follows: CTL = control, UB = ultrasonic bath, HP = high power.

Gel electrophoresis was used to determine the relative molecular weights of the protein extracts. This was done under non-reducing and reducing (2-mercaptoethanol) conditions. Overall, the three extracts had identical bands although, in certain cases there was a difference in intensities of some bands. In the absence of reducing agent, the major band was seen around 48-60 kDa while other bands were detected at 150 kDa, 125 kDa, 90 kDa, 75 kDa, and 37 kDa. In the presence of 2-mercaptoethanol, the major band (i.e. 48-60 kDa) almost disappeared; this was associated with the appearance of new intense bands at 35 kDa, 24 kDa, and 20 kDa, and 15 kDa. The results are consistent with those of previous works on oat proteins^{31,32,85,163-165}. 12S and 11S globulin proteins are the most dominant fractions in oat protein isolates and have a molecular weight of 50-58 kDa^{32,33}. This 12S globulin protein is represented by the major band at 48-60 kDa under non-reducing conditions. In their native forms, globulins are dimers made of two subunits, an acidic (A) and basic (B) subunit held together by a disulfide bond. This disulfide bond is cleaved by 2-mercaptoethanol to produce an acidic subunit with a molecular weight of 35-40 kDa, and a basic subunit (20-25 kDa)^{32,164}.

Oats also contain minor proteins, namely the 7S and 3S globulins^{35,164}. The 7S globulin fraction is 55 kDa, while the 3S globulin is made up of two subunits with molecular weights of 21 and 15 kDa. These proteins are visible in the reduced samples. It is probable that the 12S globulin band (58 kDa) in the non-reduced lanes overlap with the band for the 7S fraction (55 kDa). However, since there should be no 12S band in the reduced samples assuming the hydrolysis is completed, the 7S band can be seen. Likewise, under reducing conditions, the 3S globulin is cleaved into its two subunits that can be observed at 21 kDa, and 15 kDa however, the latter is very thin and not clearly visible, due to its poor resolution. The sonication of oat flour prior to protein extraction did not have a significant effect on molecular weights distribution based on the gel electrophoresis. The only difference is the higher intensity of band 48-60 in the control sample coupled to lower intensities its corresponding subunits in the reduced samples. This is likely because the sonication caused changes in secondary structures that increased the cleavage of disulfide bonds those samples relative to the control.

Table 4: Composition of major proteins in each sample. (-) indicates an absence of protein, while (N/A) indicates an unknown coverage for the protein. Each sample was analyzed using MS/MS after digestion by trypsin. Myoglobin was used as a control, and the sequences were analyzed using Mascot™ Software.

NCBI nr protein i.d.	Protein name	MW (Da)	Sample	Coverage (%)
gi 134918 P12615.1	12S Seed Storage Globulin 1	58964	CTL	33
			UB	34
			HP	31
gi 134919 P14812.1	12S Seed Storage Globulin 2	59038	CTL	34
			UB	36
			HP	35
gi 472867 CAA52764.1	11S Globulin	59768	CTL	33
			UB	30
			HP	30
gi 2764798 CAA54152	12S Globulin	53737	CTL	30
			UB	29
			HP	30
gi 2764800 CAA54153.1	12S Globulin	58531	CTL	25
			UB	25
			HP	25
gi 871982 CAA52763.1	11S Globulin		CTL	N/A
			UB	N/A
			HP	N/A
gi 114720 P27919.1	Avenin	24670	CTL	25
			UB	25
			HP	38
gi 327315253 CBL51494.1	Avenin protein	26116	CTL	24
			UB	24
			HP	24
gi 332368713 CBL51489.1	Avenin protein	26391	CTL	26
			UB	26
			HP	26
gi 166557 AAA32716.1	Avenin	25911	CTL	8
			UB	8
			HP	-
gi 386277748 AFJ04424.1	Vromindoline		CTL	-
			UB	N/A
			HP	N/A
gi 693794 AAB32025.1	Alcohol soluble avenin-3	23718	CTL	-
			UB	14
			HP	34
gi 75107166 Q09114.1	Avenin-E	21479	CTL	-
			UB	19
			HP	35
gi 155733233 ABU39831.1	Tryptophanin	16461	CTL	-
			UB	-
			HP	33

In addition to gel electrophoresis, the composition of protein extracts was determined using mass spectrometry. Peaks listed from MS/MS data of trypsin digests were analyzed using Mascot™ software. The determined sequences were matched with those present in the NCBI database. The software then compared the MS generated sequences to the sequences of known oat proteins and the percent coverage was reported. The percent coverage is a measurement of the amino acids present in the MS generated sequences and the amino acids in the sequences of previously-identified oat proteins and indicates the likelihood of the presence of that protein within the sample. The analysis (Table 3.0) identified four different 12S globulin proteins, two different 11S globulin proteins, and four avenin proteins as the most concentrated protein fractions. The mass spectrometry results confirm the presence of 12S globulins and their molecular weight within the range of 53-59 kDa. The high percent coverage indicates that 12S globulin is indeed the most dominant protein in each sample. There are several forms of the 12S globulin that were identified using mass spectrometry because there are many genes that encode for globulin proteins. This results in slight differences in amino acid sequences and molecular weights³². The 11S globulin and avenin proteins were also identified using mass spectrometry, which are both in accordance with literature^{34,85,164}.

The mass spectrometry results show that treatment with sonication did release two proteins (vromindoline and avenin-E) from UB and HP treated brans and one unique protein (tryptophanin) in the HP sample. These three proteins were absent in the control sample. Vromindolines are starch-bound proteins that can contribute to 50 % reduction of the oat grain hardness¹⁶⁶. Tryptophanin proteins also contribute to the oat grain softness because they are bound to lipids¹⁶⁷. It can be assumed that the sonication treatments disrupted the flour matrix to release these proteins, but this did not translate into an extraction of total proteins.

4.3 Hydrolysis of proteins

Hydrolysis of proteins is a common procedure to improve the functional and biological properties. Those functions are affected by both the composition of proteins (e.g. extraction procedure) and the nature of proteases used for enzymatic cleavage. This work tested the effect of three industrial food – grade proteases. Flavourzyme, a protease mixture, contains eight different proteases, namely leucine aminopeptidase A and 2, dipeptidyl peptidase 4 and 5, neutral protease 1 and 2, alkaline protease 1, and α -amylase A type 3¹⁶⁸. Papain, a cysteine protease, with catalytic

residues comprised of glutamine, cysteine, histidine, and asparagine cleaves many peptide bonds with low specificity ¹⁶⁹. The catalytic site does favor large hydrophobic side chains and leucine and glycine amino acids ¹⁶⁹. Alcalase, an alkaline protease (serine protease) that has catalytic sites containing aspartic acid, histidine, and serine, is able to cleave a wide variety of peptide bonds due to its low specificity ¹⁶⁹.

Protein samples (CTL, UB, HP) were hydrolyzed with each of the three proteases. The generated protein hydrolysates were assessed based on their soluble proteins (Figure 4.2A) and as well as based on their free amino acid contents (Figure 4.2B). Independently of the extraction procedure or the nature of the proteases, Flavourzyme hydrolysates had the lowest protein contents of (23.8 – 40.5%) while there was similarity in protein content of Papain (56.0 – 61.7%) and Alcalase (49.9 – 54.8%) hydrolysates.

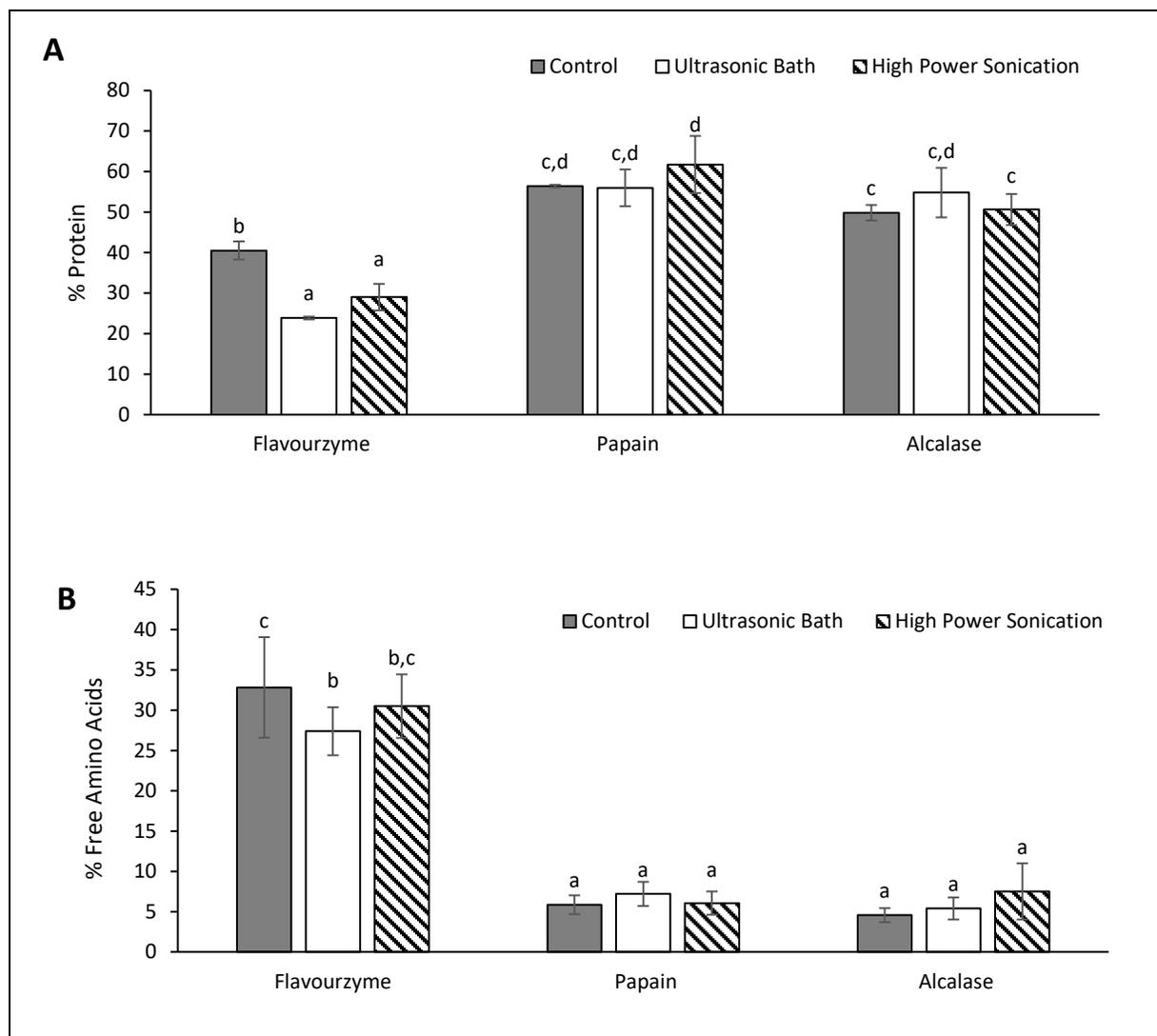


Figure 4.3.1: A) Percent protein of hydrolysates (dissolved at 120 $\mu\text{g/ml}$) as determined by a Lowry assay, using BSA as a standard. B) Concentration of free amino acids within hydrolysates (1mg/ml), expressed as μg FAA/g sample. Samples are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p -value < 0.05).

The free amino acid content data (Figure 4.2B) showed that Flavourzyme hydrolysates had higher concentrations of free amino acids (32.8% (CTL), 27.4% (UB), and 30.5% (HP)) than Papain (5.5 -7.2%) and Alcalase (4.6 -7.5%) hydrolysates. The high free amino acid concentrations of Flavourzyme extracts justify their low protein contents. Other works have reported greater free amino acid contents due the action of Flavourzyme compared to other proteases^{92,170}. This is because Flavourzyme contains endo- and exo-peptidase activities while Papain and Alcalase

contain endopeptidases only^{168,169}. The exopeptidase activity is associated with the release of free amino acid from the C- or N-terminal of proteins.

4.4 Free thiol in hydrolyzed proteins

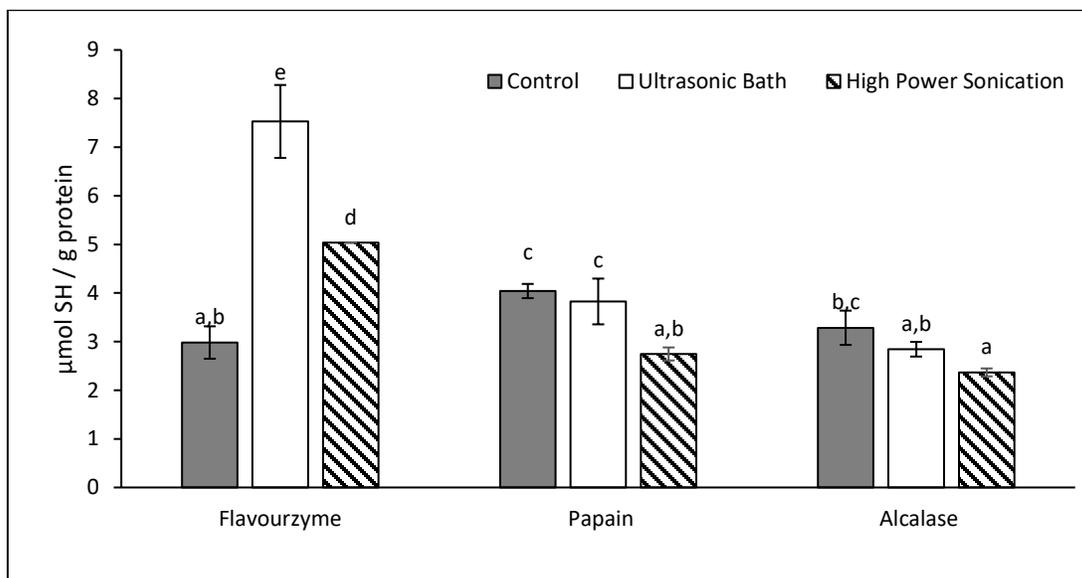


Figure 4.4.1: μmol free sulfhydryl groups per gram of protein. Samples were prepared at 2.5 mg protein/ml and are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p -value < 0.05).

The presence of disulfide bonds and sulfhydryl or thiol groups (SH) in proteins contribute to their overall structures. The disruption of these structures due to the cleavage of peptide bonds can make SH groups more available, which could, in turn affect the activity of the hydrolysates. Figure 4.3 showed that Flavourzyme samples that were pretreated with ultrasounds showed an increase in free sulfhydryls; the control sample had 2.98 μmol SH/g protein while ultrasonic bath and high power sonication samples had 7.53 μmol SH/g protein and 5.04 μmol SH/g protein, respectively. For proteins hydrolyzed by Papain and Alcalase, the use of ultrasonic bath had no effect on SH contents while the use of high power ultrasounds resulted in a decrease of SH amounts. Although typically sonication treatments can increase sulfhydryl groups by disrupting protein aggregates and changing protein conformation¹⁷¹, there is also evidence that ultrasonic treatment can decrease sulfhydryls⁵⁹. High power ultrasonic waves can generate reactive oxygen species which then oxidize SH groups^{59,172}.

4.5 Zeta potential and hydrophobicity of hydrolyzed proteins

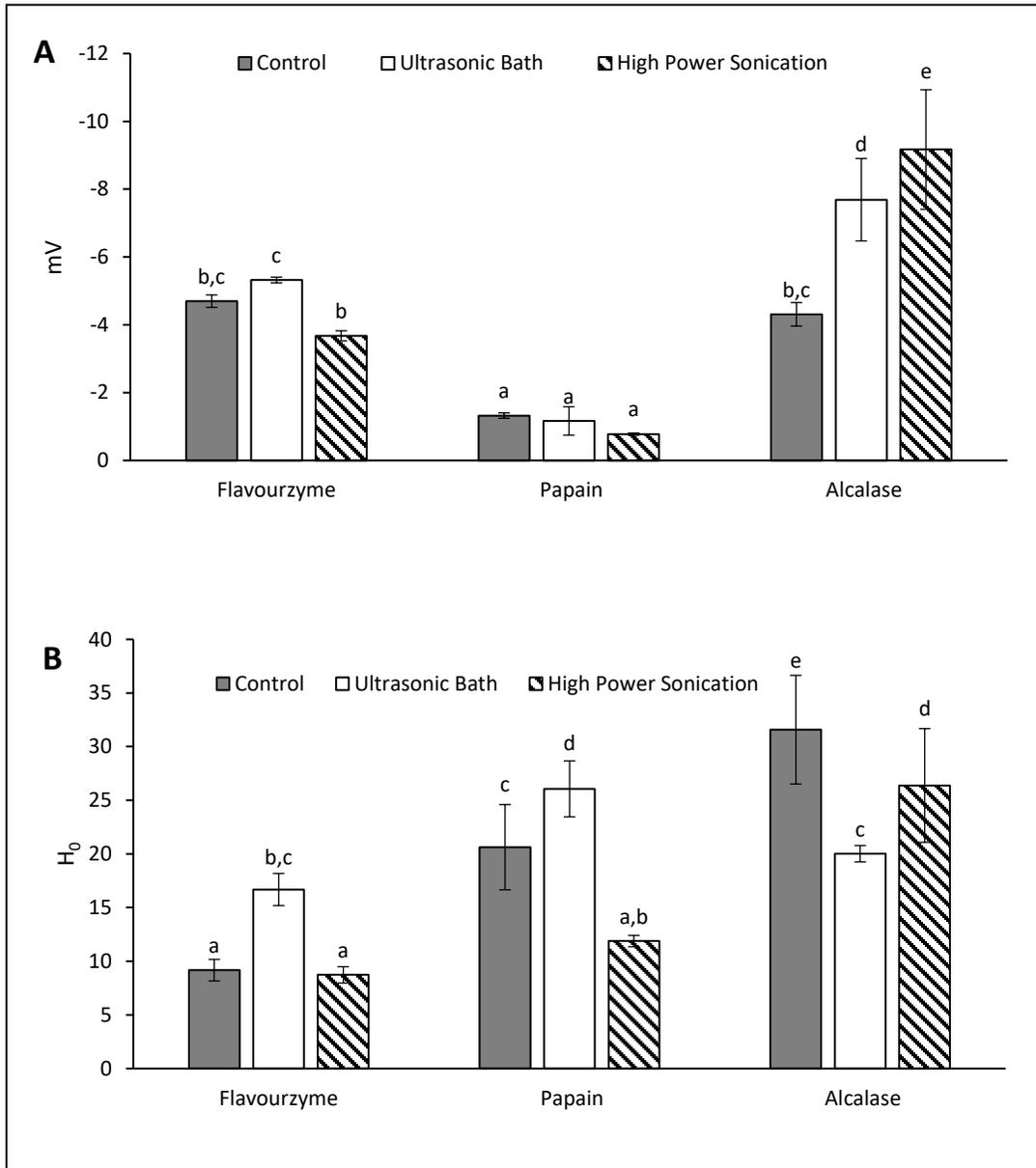


Figure 4.5.1: A) Zeta potential (mV) of hydrolysates and B) index of hydrophobicity (H_0) for protein hydrolysates expressed as the initial slope of relative fluorescence versus concentration of protein. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p -value < 0.05).

In addition to SH contents, the charge and the hydrophobicity can influence the functionality of the hydrolyzed proteins. The overall surface charge (i.e. zeta potential) of protein hydrolysates is displayed in Figure 4.4A. All hydrolysates had a negative zeta potential, which means that they contained more negative amino acids than positive ones. Alcalase hydrolysates

from sonicated oat brans had a greater zeta potential, -7.69 ± 1.21 mV for UB and -9.17 ± 1.76 mV for HP while all Papain hydrolysates had the smallest potential (-0.78 to -1.32 mV). One would expect zeta potential to change upon ultrasonic treatment because of the disruption of protein structure, but whether this increases or decreases the surface charge upon hydrolysis seems, based on our data, to depend on the nature of the proteases. This is illustrated by an increase in potential in the HP hydrolysates when Alcalase was used for hydrolysis, a decrease when it was Flavourzyme and no effect when Papain was used.

Charged amino acids are polar and their presence on the surface increases the solubility of proteins or polypeptides. An increase in zeta potential is then expected to be associated with a decrease in surface hydrophobicity. This is partially confirmed by the hydrophobicity data of the protein hydrolysates (Figure 4.4B) as two of the Papain hydrolysates (CTL, UB) with the lowest zeta potential had higher surface hydrophobicity than all Flavourzyme hydrolysates and the UB sample hydrolyzed with Alcalase. The surface hydrophobicity of the control proteins was the most affected by the nature of the protease. Specifically, the hydrophobicity index (H_0) was 9.17 ± 1.01 , 20.63 ± 3.97 , and 31.58 ± 5.06 after Flavourzyme, Papain and Alcalase treatment, respectively. There was little variation in H_0 ($16.68 - 26.06$) of the UB samples after proteolytic hydrolysis. Like the zeta potential, surface hydrophobicity can provide insight on structural changes of proteins or their hydrolysates in solution. There is no consistent pattern in hydrophobicity of samples; however, when hydrolyzed with Papain and Alcalase, the HP sonication treatment reduced the hydrophobicity index. The hydrophobicity index for Flavourzyme ranged from $8.73 - 16.68$, H_0 for Papain samples ranged from $11.88 - 26.06$, while H_0 for Alcalase samples ranged from $20.02 - 31.58$. The protease used to hydrolyze the samples was important to the hydrophobicity results. The low H_0 of Flavourzyme hydrolysates can be explained by their higher contents of free amino acids and lower protein contents (Figure 4.2). Since Flavourzyme contains endo- and exo-proteases, more protein was hydrolyzed, meaning there were fewer hydrophobic regions remaining. HP treated proteins hydrolyzed by Papain may have a lower H_0 because the Papain catalytic site favors large hydrophobic regions, which could have been exposed during sonication¹⁶⁹.

4.6 Spectroscopic characterization of hydrolyzed proteins

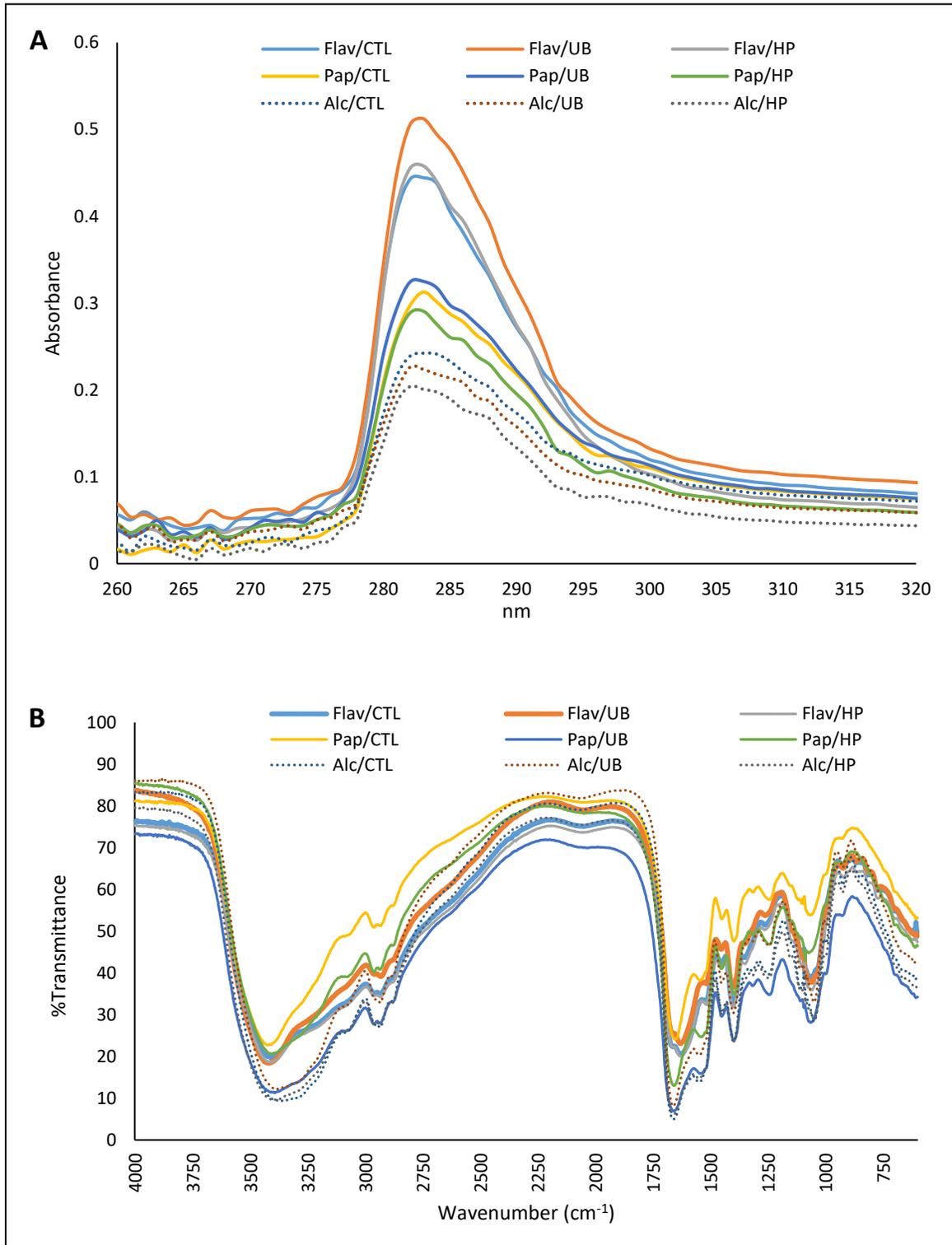


Figure 4.6.1: A) UV absorbance of samples measured from 260 – 320 nm. B) FT-IR spectra of samples measured at 600 – 4000 cm^{-1} .

The UV absorbance spectra and Fourier-Transform infrared spectra (Figure 4.5) were measured to characterize functional groups and side chains present in the hydrolysates. In figure 4.5A, the UV spectra shows the relative intensity of chromophores that had absorption between 280 – 285 nm. Amino acids that absorb UV light in this range are tyrosine, phenylalanine, and tryptophan, while others are undetectable. Therefore, these three aromatic amino acids are responsible for most of the UV absorbance of proteins, peptides and amino acids ¹⁷³. The UV spectra (Figure 4.5A) can be grouped into three based on the proteases. Alcalase hydrolysates had the lowest intensities followed by Papain hydrolysates. There appears to be little effect of the extraction procedure on the intensity of peaks. The UV data are somewhat consistent with some of the other data in this work (free amino acids, hydrophobicity). Flavourzyme hydrolysates had a higher degree of hydrolysis (i.e. higher free amino acids) and small peptides, and, therefore, more of the residual structure is exposed. In other words, the higher concentration of free amino acids meant that their aromatic side chains were available to absorb UV light, as opposed to the bound residues in larger peptides.

In figure 4.5B, the FT-IR spectra of hydrolysates are similar and displayed the characteristic proteins bands. There are four main peaks corresponding to functional groups on a protein FT-IR spectrum: amide I, II, A, and B bands ⁴¹. Amide I and amide II bands can be observed in the 1640-1650 cm^{-1} and 1530-1540 cm^{-1} ranges, respectively. The amide I band is characteristic of the C=O stretching in peptide bonds, and is related to the polypeptide backbone structure, specifically β -sheet conformations ¹⁷⁴. The amide II band results from the bending of N-H and C-N stretching. Amide A and B bands can be observed around 3445 cm^{-1} and 2970 cm^{-1} , respectively. These bands likely represent N-H vibrations, but could be due to C-OH stretching or the presence of water, both of which have similar ranges ⁴¹. These spectra and bands are similar to our previous findings, which looked at the structural characterization of proteins extracted from various milling fractions of oat flour ⁸⁵.

4.7 Antioxidant activities of hydrolyzed proteins

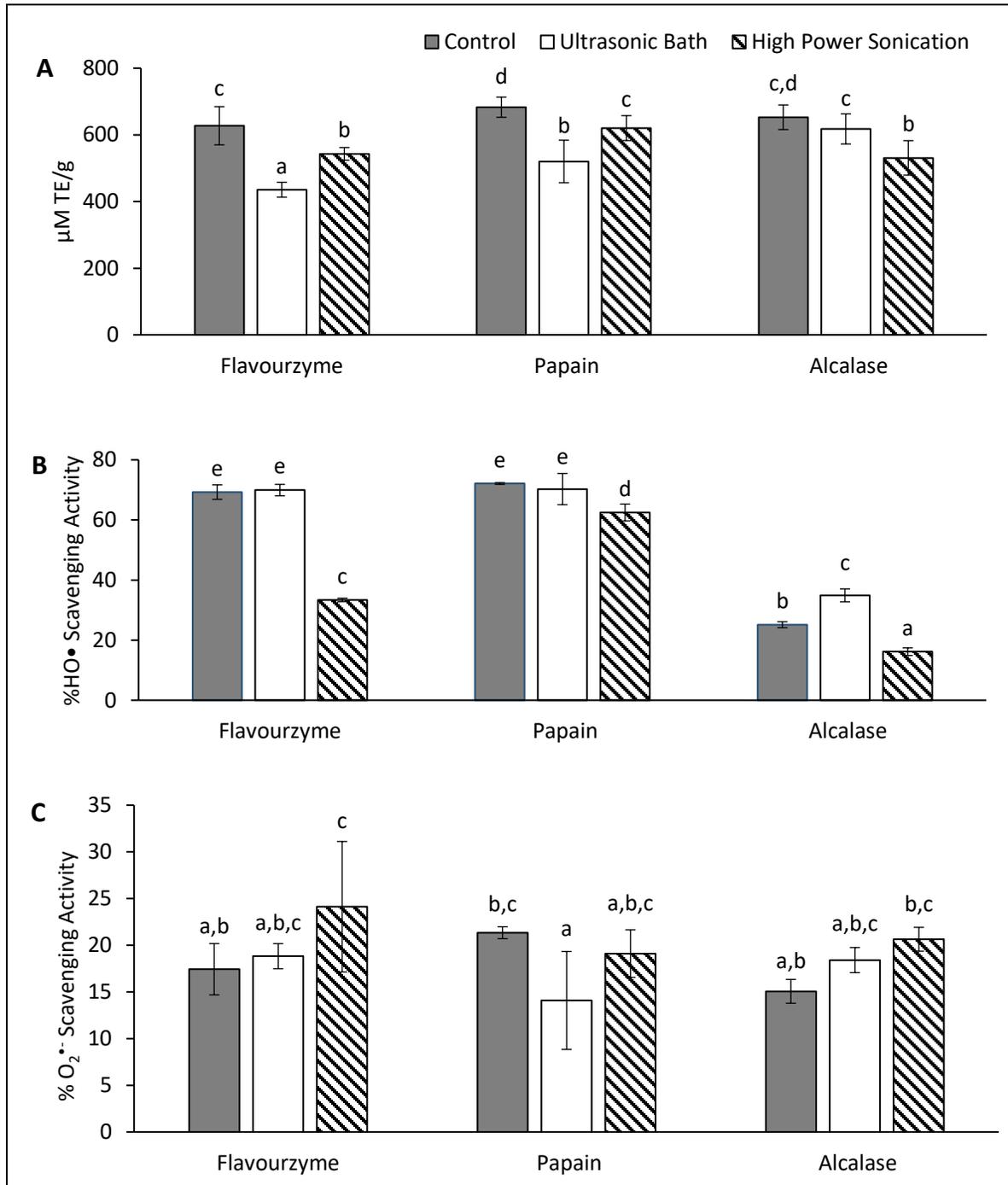


Figure 4.7.1: A) Oxygen radical absorbance capacity, B) Hydroxyl radical scavenging activity, and C) Superoxide radical scavenging activity of samples prepared at 1.0 mg/mL. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p -value < 0.05).

The effect of sonication on the peroxy (ROO^{\bullet}), hydroxyl (HO^{\bullet}), and superoxide ($\text{O}_2^{\bullet-}$) radical scavenging properties of protein hydrolysates was assessed (Figure 4.6). Sonication caused a statistically significant reduction in the peroxy radical scavenging activity for all protein hydrolysates (Figure 4.6A). The control samples scavenged ROO^{\bullet} with oxygen radical absorbance capacity (ORAC) values of $627.17 \pm 57.36 \mu\text{M TE/g}$, $682.90 \pm 30.21 \mu\text{M TE/g}$, and $652.67 \pm 36.94 \mu\text{M TE/g}$ for Flavourzyme, Papain, and Alcalase hydrolysate, respectively. Flavourzyme and Papain hydrolysates from brans treated with ultrasonic bath had statistically lower activities than those from brans treated with high power ultrasounds, while for Alcalase, the hydrolysate from HP – treated brans had lower activities relative to UB – treated brans.

The Flavourzyme and Papain hydrolysates used in this study were highly effective at scavenging HO^{\bullet} radicals, quenching approximately 70% of all hydroxyl radicals (Figure 4.6B). However, for Flavourzyme and Papain, HP sonication caused a significant reduction in scavenging activity. Flavourzyme hydrolysates from HP – treated brans scavenged only 33.38% of HO^{\bullet} radicals and Papain hydrolysates scavenged 62.50%. Alcalase hydrolysates were much less effective in scavenging HO^{\bullet} radicals, the lower value being 25.13% for hydrolysates from the control bran sample. From these results, it is clear that the ultrasonic bath treatment did not have an effect on the hydroxyl radical scavenging activity, while the high power sonication treatment reduced the activity.

Lastly, the superoxide radical scavenging activity was measured (Figure 4.6C). There is no obvious trend in the effect of ultrasonic treatments on superoxide radical scavenging activities. Superoxide radical scavenging activity of Flavourzyme hydrolysates (17.43 – 24.13%) from brans treated with high power sonication slightly increased compared to the control. A similar trend was observed in Alcalase samples (15.06 – 20.64%); however, the results were not statistically significant. The opposite effect was observed in Papain samples (14.09 – 21.34%), where ultrasonic treatment of oat brans decreased the superoxide radical scavenging activity of hydrolysates.

Overall, ultrasonic treatment of oat brans has a negative effect on the antioxidant activity of its protein hydrolysates. This trend is observed for all hydrolysates in the ORAC and hydroxyl radical assays and for Papain samples in the superoxide radical assay. The finding correlates with the decrease in sulfhydryl content and hydrophobicity. In a recent study by Wang et al. on the

effects of sonication of soy proteins, they found a positive correlation between antioxidant activities and SH groups as well as between antioxidant activity and hydrophobicity. Because sulfhydryl groups are one of the antioxidant mechanisms of proteins, the antioxidant activity decreased after ultrasounds caused a slight decrease in SH content ¹⁷⁵. Likewise, a decrease in hydrophobicity could mean that ultrasonication causes aromatic residues to relocate within the protein structure, making them unavailable to resonate free radicals. One would expect to see greater antioxidant activities in Flavourzyme hydrolysates compared to Alcalase hydrolysates since Flavourzyme samples had a higher degree of hydrolysis and aromatic amino acids; however this effect was not observed. This implies that the aromatic amino acids were not the primary source of antioxidant activity for these hydrolysates.

4.8 Inhibition of enzymes by hydrolyzed proteins

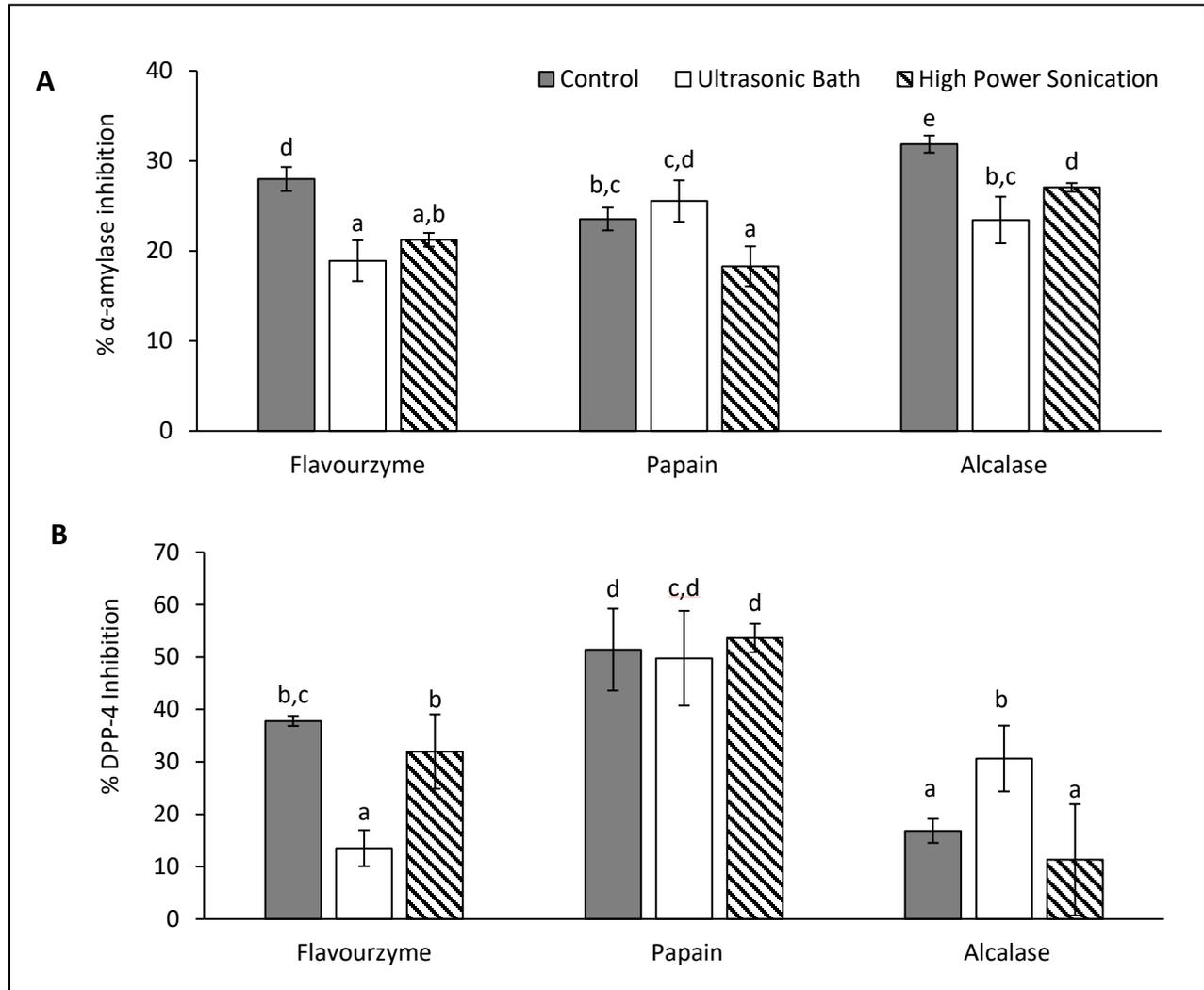


Figure 4.8.1: A) α -amylase and B) DPP-4 inhibition by protein hydrolysates prepared at 1 mg/ml and 5 mg/ml, respectively. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with an LSD in a one-way ANOVA (p -value < 0.05).

Bioactive peptides and hydrolysates have α -amylase and DPP-4 inhibitory activity (Table 2). These enzymes play important roles in glucose digestion and insulin secretion, and thus their inhibition is a measure of antidiabetic properties. Based on the data from the Flavourzyme and Alcalase hydrolysates (Figure 4.7A) from ultrasonic bath and high power treated brans reduced α -amylase inhibitory activity, while there was no effect of UB sonication on samples when hydrolyzed by Papain. Although the means are statistically different, the effect of sonication is quite small. All samples fall within the range of 18-32% inhibition, which is similar to levels

recorded in literature ¹⁷⁶. The mechanism of α -amylase inhibition has been widely researched. In a review by Payan ¹⁷⁷ on the structural interactions between plant protein inhibitors and α -amylase, it was concluded that inhibition occurs through competition for the active site between polysaccharides and (primarily) aromatic amino acids ¹⁷⁷.

DPP-4 inhibition is an important strategy for managing T2DM, and although synthetic DPP-4 inhibitors are typically administered to patients, there exist natural sources such as oat protein hydrolysates ^{3,63,68,76}. In this study, Papain hydrolysates inhibited up to 53.63% of all DPP-4 activity, while Flavourzyme and Alcalase samples had statistically lower activities (Figure 4.7B). There is no consistent pattern in the effects of ultrasonication on DPP-4 inhibition. Ultrasonic bath treatments decreased activity in Flavourzyme samples compared to the control; however, it increased the activity in Alcalase hydrolysates. Therefore, the choice of enzyme for hydrolysis is important with respect to DPP-4 inhibition. The intensity and power of sonication treatments also influence the ability of hydrolysates to inhibit enzymes. Wang, et al. ¹⁷⁸ assessed the effects of ultrasonic treatment (10-50 minutes at 250-1250 W) on the extraction yield and ACE inhibitory activities of oat proteins. Oat proteins reduced ACE activity after sonication at 750 W for 20 minutes ¹⁷⁸. In a study on amaranth protein hydrolysates, Velarde-Salcedo et al. found that hydrolysates with 13 or more amino acids inhibited DPP-4 enzymes by preventing the enzyme from dimerizing, disabling activation ¹⁷⁹. Since the hydrolysates in this work were primarily larger polypeptide sequences, their potent inhibitory activity is expected.

4.9 Effect of hydrolyzed proteins on cellular viability and the secretion of glucagon like peptide in colorectal cells

4.9.1 Viability of cells or cytotoxicity

The cellular model used for this study was NCI-H716 colorectal adenocarcinoma cells. The cytotoxicity of hydrolysates was determined based on the reduction of the MTT tetrazolium salt to formazan, by mitochondrial oxidoreductase enzymes. The concentration of formazan is proportional to the number of viable cells. The hydrolysates were tested at concentrations of 0.2 and 0.8 mg/mL, and data are presented in Figure 4.8A. There was no statistical difference in the cell viability of cells in cells treated with the hydrolysates relative the untreated cells. For some hydrolysates, the 0.8 mg/ml concentration of sample lowered viability of cells, but not to a significant level.

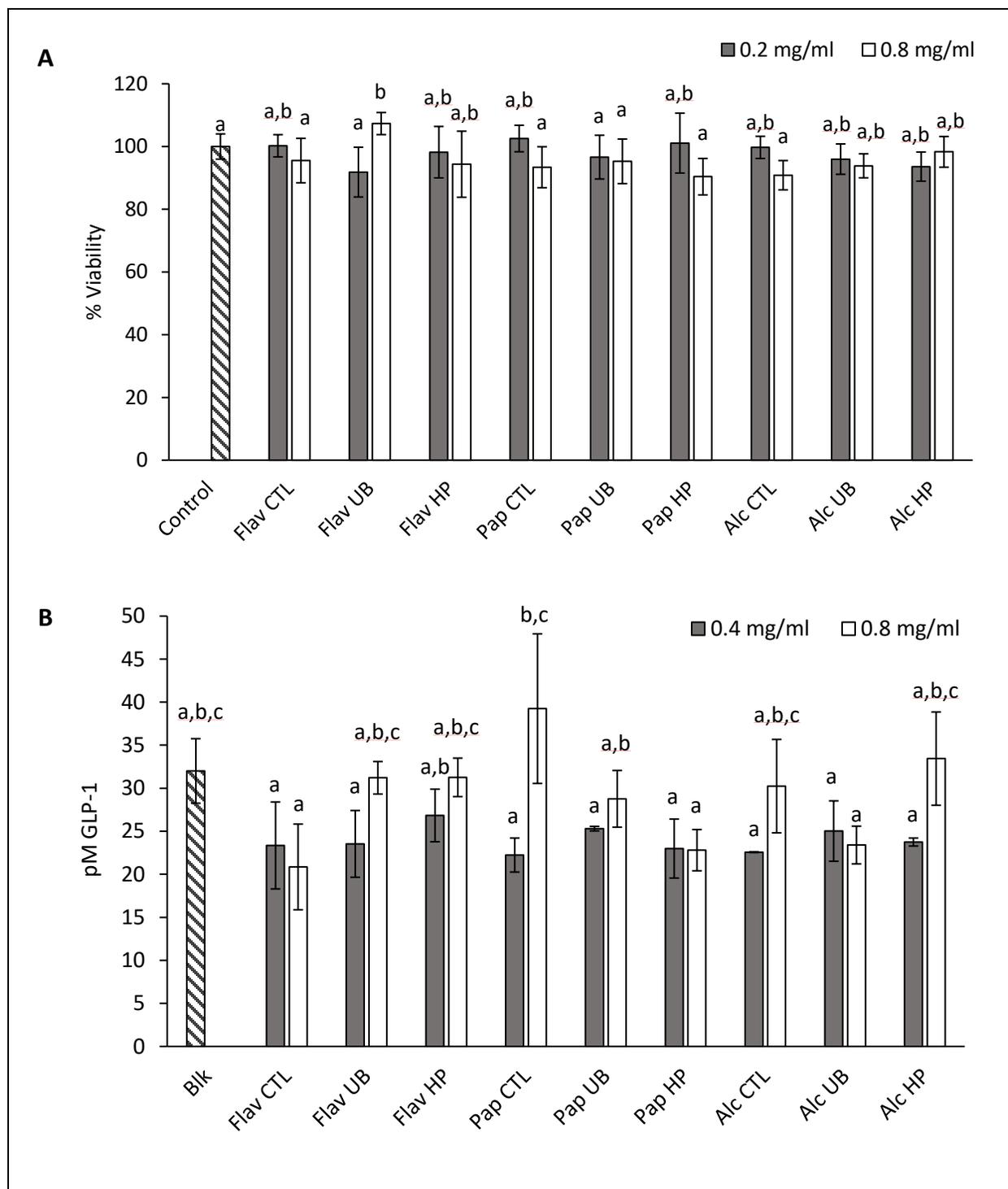


Figure 4.9.1: A) Cell viability of NCI-H716 cells as determined by an MTT assay; B) GLP-1 secretion stimulated by hydrolysate treatment. Cells were plated at 5×10^4 cells in a 96 well microplate with Matrigel for 24 hours and were treated with hydrolysate fractions for 2 hours. Data are expressed as a mean of triplicates \pm standard deviation. The letters above each bar represent significant differences as determined with a LSD in a one-way ANOVA (p -value < 0.05).

4.9.2 Effects on the secretion of glucagon like peptide 1 (GLP-1)

The NCI-H716 cell line is currently the only human model available for the *in vitro* study of GLP-1; however, there are two other GLP-1-producing cell lines from mice, namely GLUTag and STC-1. Another advantage of NCI-H716 cells is the higher secretion of GLP-1 hormone. In a comparison with native L-cells, GLUTag, and STC-1 cells, the NCI-H716 cell line produced statistically greater amounts of cellular GLP-1 (up to 20 times greater) and proglucagon, though the levels of secreted GLP-1 were similar¹⁴².

The GLP-1 secretion was measured in response to treatment with hydrolysates at 0.4 mg/ml and 0.8 mg/ml (Figure 4.8B). The range of GLP-1 secreted for all samples was 20.85 pM – 39.25 pM. The positive control treated with glucose had a GLP-1 concentration of 42.89 pM. Compared to the negative control (i.e. blank), no hydrolysate significantly increased the secretion of GLP-1. Additionally, dose dependency was not observed for all hydrolysates. Specifically, it was found that except for Papain HP, and Alcalase UB samples, an overall dose-dependent response can be observed, however, it is not large enough to be statistically significant. This effect is consistent with the literature^{180–182}; however, the exact mechanism by which peptides and hydrolysates control GLP-1 secretion is unknown.

The mechanisms described in literature include the upregulation of proglucagon transcription (a precursor for GLP-1) as shown by an increase in proglucagon mRNA¹⁵¹, and the activation of bile acid receptors¹⁸² observed by membrane depolarization¹⁸³. In a study on meat and egg albumin hydrolysates, treatment with hydrolysates significantly increased GLP-1 secretion in rat intestinal cells and STC-1 cells. The study reported that the protein hydrolysates stimulated pro-glucagon transcription in STC-1 cells, GLUTag cells, and rat intestines; however, the hydrolysates did not have an effect on non-intestinal cell lines. This suggests that intestinal cells are able to respond to hydrolysate stimulation, possibly through various peptide receptors and transcription activation mechanisms¹⁵¹.

It is also hypothesized that protein hydrolysates stimulate GLP-1 secretion through the activation of bile acid receptors (TGR5). According to literature, GLP-1 secretion is regulated, in part, through the activation of G-protein coupled receptors (GPCRs)¹⁸⁴ and bile acid receptors¹⁸². When activated, TGR5 leads to an increase in cytoplasmic Ca²⁺ levels. This is because binding to the GPCRs and TGR5 initiates a series of activations, leading to membrane depolarization caused

by an increase in intracellular Ca^{2+} release. In a study on the effect of ginseng extracts on the GLP-1 secretion of NCI-H716 cells, a corresponding increase in Ca^{2+} was observed with an increase in GLP-1 secretion, implying that the extracts activated TGR5. Kim et al. found that cells with inactive TGR5 did not secrete GLP-1 and that an increase in Ca^{2+} was not observed, demonstrating that activation of bile acid receptors is important in the regulation of GLP-1 secretion^{182,185}.

Further studies looking at DPP-4 inhibition, proglucagon transcription, and Ca^{2+} levels would be necessary in order to fully determine the mechanism by which oat protein hydrolysates affect the GLP-1 secretion in NCI-H716 cells.

5. Conclusion

This study determined the effects of ultrasonic bath and high power sonication treatments on protein extraction yields and the physical and structural characteristics of hydrolyzed proteins. The sonication treatments did not increase protein yield, possibly due to the use of a lower power intensity than has been reported in literature on sonication treatments. There is evidence that sonication treatments influenced the secondary structure of the hydrolyzed proteins. Gel electrophoresis demonstrated that sonication partially cleaved a disulphide bond in the main globulin fraction. Likewise, sonication disrupted the overall protein structure releasing novel proteins, namely vromindonline (in UB and HP) and tryptophanin (only in HP). Ultrasonication also reduced free thiol content and hydrophobicity for some samples, while it increased the overall surface charge for some hydrolysates. The surface charge was more dependent on the protease used for hydrolysis than the ultrasonication treatments, as Papain hydrolysates had much lower zeta potentials than Alcalase hydrolysates. With respect to antioxidant activities, UB and HP treatments reduced peroxy radical absorbance capacity, while only HP reduced the hydroxyl radical scavenging activity. There was no consistent pattern in the effect of sonication on the superoxide radical scavenging activities. Ultrasonication slightly reduced the hydrolysates' ability to inhibit α -amylase, limiting their antidiabetic activity. Interestingly, the protease used for hydrolysis had a greater effect on the DPP-4 inhibition than the sonication treatments. Lastly, after it was determined that the hydrolysates were not cytotoxic to NCI-H716 cells, the GLP-1 secretion was measured. A dose-dependent secretion was observed for most samples. Overall, sonication slightly altered the physical characteristics of the protein hydrolysates, which negatively affected the antioxidant activity but did not affect the antidiabetic activity of the hydrolysates.

6. Future Studies

This study considered the effects of sonication treatments on the bioactivities of oat proteins; however, only two types of sonication treatments were applied. The treatments applied did not increase protein extraction yield, which was contrary to what is typically seen in literature. Future studies should optimize sonication power and treatment length (for example, 100 – 1500W for 5 – 50 minutes) to obtain increased extraction yield and evaluate the effects of treatments on functionalities. To gain insight on the specific peptides responsible for the *in vitro* and *in vivo* effects, the hydrolysates should be fractionated by size using HPLC and identified using mass spectrometry. Furthermore, after fractionation, studying the susceptibility of ≥ 1 kDa peptides to degradation in cells would give insight on the bioactivity of peptides *in vivo*.

This study used NCI-H716 cells to observe GLP-1 secretion; however, STC-1 and Caco2 cell lines are also suitable cell models that should be studied. STC-1 also secretes high levels of GIP, another important insulinotropic hormone, which can be studied in the context of T2DM treatments¹⁴². Although a dose-dependent increase in GLP-1 secretion was observed, the exact mechanism by which oat protein hydrolysates increase GLP-1 secretion is unknown. To further understand the mechanisms, additional cellular processes should be assessed, including cytosolic calcium influx, levels of proglucagon mRNA, the prevalence of bile acid receptors, and changes in membrane polarization. Measurement of DPP-4 inhibition in a cellular model would also help further elucidate the mechanism of increased GLP-1 secretion.

7. References

1. Aluko RE, Monu E. Functional and Bioactive Properties of Quinoa Seed Protein Hydrolysates. *J Food Sci.* 2003;68(4):1254-1258. doi:10.1111/j.1365-2621.2003.tb09635.x
2. Hatanaka T, Inoue Y, Arima J, et al. Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran. *Food Chem.* 2012;134(2):797-802. doi:10.1016/j.foodchem.2012.02.183
3. Nongonierma AB, Fitzgerald RJ. Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides.* 2013;39(1):157-163. doi:10.1016/j.peptides.2012.11.016
4. Butt MS, Tahir-Nadeem M, Khan MKI, Shabir R, Butt MS. Oat: Unique among the cereals. *Eur J Nutr.* 2008;47(2):68-79. doi:10.1007/s00394-008-0698-7
5. Martinez MF, Arelovich HM, Wehrhahne LN. Grain yield, nutrient content and lipid profile of oat genotypes grown in a semiarid environment. *F Crop Res.* 2010;116(1-2):92-100. doi:10.1016/j.fcr.2009.11.018
6. Halima NB, Saad RB, Khemakhem B, Fendri I. Oat (*Avena sativa* L .): Oil and nutriment compounds valorization for potential use in industrial applications. *J Oleo Sci.* 2015;932(9):915-932. doi:10.5650/jos.ess15074
7. Strychar R. CHAPTER 1: World Oat Production, Trade, and Usage. In: Webster F, Wood PJ, eds. *OATS: Chemistry and Technology*. 3340 Pilot Knob Road, St. Paul, Minnesota 55121, U.S.A.: AACC International, Inc.; 2011:1-10. doi:10.1094/9781891127649.001
8. Martínez-Villaluenga C, Peñas E. Health benefits of oat: current evidence and molecular mechanisms. *Curr Opin Food Sci.* 2017;14:26-31. doi:10.1016/j.cofs.2017.01.004
9. Lásztity R. Oat grain—a wonderful reservoir of natural nutrients and biologically active substances. *Food Rev Int.* 1998;14(1):99-119. doi:10.1080/87559129809541150
10. Xu R. Oat fibre: Overview on their main biological properties. *Eur Food Res Technol.* 2012;234(4):563-569. doi:10.1007/s00217-012-1666-2
11. Girardet N, Webster F. Oat milling: specifications, storage, and processing. In: *Oats: Chemistry and Technology*. AACC International Inc.; 2011:301-309.
12. Yao N, White PJ, Alavi S. Impact of B-glucan and other oat flour components on physico-chemical and sensory properties of extruded oat cereals. *Int J Food Sci Technol.* 2011;46(3):651-660. doi:10.1111/j.1365-2621.2010.02535.x
13. Rozylo R, Dziki D, Laskowski J, et al. Texture and sensory evaluation of composite wheat-oat bread prepared with novel two-phase method using oat yeast-fermented leaven. *J Texture Stud.* 2014;45(3):235-245. doi:10.1111/jtxs.12069
14. Flander L, Salmenkallio-Marttila M, Suortti T, Autio K. Optimization of ingredients and baking process for improved wholemeal oat bread quality. *LWT - Food Sci Technol.* 2007;40(5):860-870. doi:10.1016/j.lwt.2006.05.004

15. Biel W, Jacyno E, Kawęcka M. Chemical composition of hulled, dehulled and naked oat grains. *S Afr J Anim Sci.* 2014;44(2):189-197. doi:10.4314/sajas.v44i2.12
16. Dais P, Perlin A. High field, ¹³C-NMR spectroscopy of B-D-glucan, amylopectin, and glycogen. *Carbohydr Res Chem.* 1982;71:301-307.
17. de Groot AP, Luyken R, Pikaar NA. Cholesterol-lowering effect of rolled oats. *Lancet.* 1963;282(7302):303-304. doi:10.1016/S0140-6736(63)90210-1
18. Oda T, Aoe S, Sanada H, Ayano Y. Effects of soluble and insoluble fiber preparations isolated from oat, barley, and wheat on liver cholesterol accumulation in cholesterol-fed rats. *J Nutr Sci Vitaminol (Tokyo).* 1993;39(1):73-79. doi:10.3177/jnsv.39.73
19. Ho HVT, Sievenpiper JL, Zurbau A, et al. The effect of oat β -glucan on LDL-cholesterol, non-HDL-cholesterol and apoB for CVD risk reduction: a systematic review and meta-analysis of randomised-controlled trials. *Br J Nutr.* 2016;116(08):1369-1382. doi:10.1017/S000711451600341X
20. Zhou X, Lin W, Tong L, et al. Hypolipidaemic effects of oat flakes and β -glucans derived from four Chinese naked oat (*Avena nuda*) cultivars in Wistar-Lewis rats. *J Sci Food Agric.* 2016;96(2):644-649. doi:10.1002/jsfa.7135
21. Ellegård L, Andersson H. Oat bran rapidly increases bile acid excretion and bile acid synthesis: An ileostomy study. *Eur J Clin Nutr.* 2007;61(8):938-945. doi:10.1038/sj.ejcn.1602607
22. Emmons CL, Peterson DM, Paul GL. Antioxidant capacity of oat (*Avena sativa* L.) extracts. 2. In vitro antioxidant activity and contents of phenolic and tocol antioxidants. *J Agric Food Chem.* 1999;47(12):4894-4898. doi:10.1021/jf990530i
23. Peterson DM. Oat antioxidants. *J Cereal Sci.* 2001;33(2):115-129. doi:10.1006/jcrs.2000.0349
24. Pellegrini GG, Morales CC, Wallace TC, Plotkin LI, Bellido T. Avenanthramides prevent osteoblast and osteocyte apoptosis and induce osteoclast apoptosis in vitro in an Nrf2-independent manner. *Nutrients.* 2016;8(7):2-15. doi:10.3390/nu8070423
25. Peterson DM, Hahn MJ, Emmons CL. Oat avenanthramides exhibit antioxidant activities in vitro. *Food Chem.* 2002;79(4):473-478. doi:10.1016/S0308-8146(02)00219-4
26. Zhou M, Robards K, Glennie-Holmes M, Helliwell S. Oat lipids. *J Am Oil Chem Soc.* 1999;76(2):159-169. doi:10.1007/s11746-999-0213-1
27. Sterna V, Zute S, Brunava L. Oat grain composition and its nutrition benefice. *Agric Agric Sci Procedia.* 2016;8:252-256. doi:10.1016/j.aaspro.2016.02.100
28. Fehily A, Pickering J, Yarnell W, Elwood P. Dietary indices of atherogenicity and thrombogenicity and ischaemic heart disease risk : the Caerphilly Prospective Study. *Br J Nutr.* 1994;(71):249-257. doi:10.1079/BJN19940131
29. van den Broeck H, Londono D, Timmer R, Smulders M, Gilissen L, van der Meer I. Profiling of Nutritional and Health-Related Compounds in Oat Varieties. *Foods.* 2015;5(1):2-11. doi:10.3390/foods5010002

30. Beloshapka A, Buff P, Fahey G, Swanson K. Compositional analysis of whole grains, processed grains, grain co-products, and other carbohydrate sources with applicability to pet animal nutrition. *Foods*. 2016;5(23):2-16. doi:10.3390/foods5020023
31. Robert LS, Nozzolillo C, Cudjoe A, Altosaar I. Total solubilization of groat proteins in high protein oat (*Avena sativa* L. cv. Hinoat): Evidence that glutelins are a minor component. *Can Inst Food Sci Technol J*. 1983;16(3):196-200. doi:10.1016/S0315-5463(83)72207-8
32. Walburg G, Larkins BA. Oat seed globulin: subunit characterization and demonstration of its synthesis as a precursor. *Plant Physiol*. 1983;72:161-165.
33. Ma C., Harwalkar VR. Chemical characterization and functionality assessment of oat protein fractions. *J Agric Food Chem*. 1984;32(1):144-149. doi:10.1021/jf00121a035
34. Klose C, Arendt EK. Proteins in oats; their synthesis and changes during germination: a review. *Crit Rev Food Sci Nutr*. 2012;52(7):629-639. doi:10.1080/10408398.2010.504902
35. Robert LS, Adeli K, Altosaar I. Homology among 3S and 7S globulins from cereals and pea. *Plant Physiol*. 1985;78(4):812-816.
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1064828&tool=pmcentrez&rendertype=abstract>.
36. Brinegar AC, Peterson DM. Synthesis of oat globulin precursors. *Plant Physiol*. 1982;70:1767-1769.
37. WHO, FAO UEC. *Protein and Amino Acid Requirements in Human Nutrition: Report of a Joint WHO/FAO/UNO Expert Consultation*. Vol 935. (World Health Organization, ed.). Geneva, Switzerland: World Health Organization; 2007.
38. Brinegar AC, Peterson DM. Separation and characterization of oat globulin polypeptides. *Arch Biochem Biophys*. 1982;219(1):71-79. doi:10.1016/0003-9861(82)90135-7
39. Folkes BF, Yemm EW. The amino acid content of the proteins of barley grains. *Biochem J*. 1956;62(1):4-11.
<http://www.ncbi.nlm.nih.gov/pubmed/13293145>
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1274504>.
40. Fontaine J, Schirmer B, Horr J. Near-infrared reflectance spectroscopy (NIRS) enables the fast and accurate prediction of essential amino acid contents. 2. Results for wheat, barley, corn, triticale, wheat bran/middlings, rice bran, and sorghum. *J Agric Food Chem*. 2002;50(14):3902-3911. doi:10.1021/jf011637k
41. Liu G, Li J, Shi K, et al. Composition, secondary structure, and self-assembly of oat protein isolate. *J Agric Food Chem*. 2009;57(11):4552-4558. doi:10.1021/jf900135e
42. Åssveen M. Amino acid composition of spring barley cultivars used in Norway. *Acta Agric Scand Sect B Soil Plant Sci*. 2009;59(5):395-401. doi:10.1080/09064710802263226
43. Wu Y, Sexson K, Cavins J, et al. Oats and their dry-milled fractions: protein isolation and properties of four varieties. *J Agric Food Chem*. 1972;20(4):757-761.
44. Wu YV, Cluskey JE, Wall JS, Inglett GE. Oat protein concentrates from a wet-milling

- process: composition and properties. *Cereal Chem.* 1973;50:481-488.
45. Wu YV, Sexson KR, Cluskey JE, Inglett GE. Protein isolate from high-protein oats: preparation, composition and properties. *J Food Sci.* 1977;42(5):1383-1386. doi:10.1111/j.1365-2621.1977.tb14504.x
 46. Youngs V. Extraction of a high protein layer from oat groat bran and flour. *J Food Sci.* 1974;39:1045-1046.
 47. Ansharullah, Hourigan JA, Chesterman CF. Application of carbohydrases in extracting protein from rice bran. *J Sci Food Agric.* 1997;74:141-146.
 48. Jodayree S, Smith JC, Tsopmo A. Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates. *Food Res Int.* 2012;46(1):69-75. doi:10.1016/j.foodres.2011.12.004
 49. Awad T, Moharram H, Shaltout O, Asker D, Youssef M. Applications of ultrasound in analysis, processing and quality control of food: A review. *Food Res Int.* 2012;48(2):410-427. doi:10.1016/j.foodres.2012.05.004
 50. de Mesa-Stonestreet NJ, Alavi S, Bean SR. Sorghum proteins: The concentration, isolation, modification, and food applications of kafirins. *J Food Sci.* 2010;75(5):90-104. doi:10.1111/j.1750-3841.2010.01623.x
 51. Tang S, Hettiarachchy NS, Shellhammer TH. Protein extraction from heat-stabilized defatted rice bran. 1. Physical processing and enzyme treatments. *J Agric Food Chem.* 2002;50(25):7444-7448. doi:10.1021/jf025771w
 52. Moulton KJ, Wang LC. A pilot-plant study of continuous ultrasonic extraction of soybean protein. *J Food Sci.* 1982;47:1127-1129.
 53. Quansah JK, Udenigwe CC, Saalia FK, Yada RY. The effect of thermal and ultrasonic treatment on amino acid composition, radical scavenging and reducing potential of hydrolysates obtained from simulated gastrointestinal digestion of cowpea proteins. *Plant Foods Hum Nutr.* 2013;68(1):31-38. doi:10.1007/s11130-013-0334-4
 54. O'Sullivan J, Murray B, Flynn C, Norton I. The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins. *Food Hydrocoll.* 2016;53:141-154. doi:10.1016/j.foodhyd.2015.02.009
 55. Bean SR, Ioerger BP, Park SH, Singh H. Interaction between sorghum protein extraction and precipitation conditions on yield, purity, and composition of purified protein fractions. *Cereal Chem.* 2006;83(1):99-107. doi:10.1094/CC-83-0099
 56. Singh N, Donovan G, Batey I, MacRitchie F. Use of sonication and size-exclusion high-performance liquid chromatography in the study of wheat flour proteins. I. Dissolution of total proteins in the absence of reducing agents. *Cereal Chem.* 1990;67(2):150-161.
 57. Krise KM. The effects of microviscosity, bound water, and protein mobility on the radiolysis and sonolysis of hen egg white. 2011. https://etda.libraries.psu.edu/files/final_submissions/3497.
 58. Arzeni C, Martínez K, Zema P, Arias A, Pérez OE, Pilosof AMR. Comparative study of

- high intensity ultrasound effects on food proteins functionality. *J Food Eng.* 2012;108(3):463-472. doi:10.1016/j.jfoodeng.2011.08.018
59. Zhang Z, Regenstein JM, Zhou P, Yang Y. Effects of high intensity ultrasound modification on physicochemical property and water in myofibrillar protein gel. *Ultrason Sonochem.* 2017;34:960-967. doi:10.1016/j.ultsonch.2016.08.008
 60. Ratnasari N, Walters M, Tsopmo A. Antioxidant and lipoxygenase activities of polyphenol extracts from oat brans treated with polysaccharide degrading enzymes. *Heliyon.* 2017;3(7):1-13. doi:10.1016/j.heliyon.2017.e00351
 61. Cheung IWY, Nakayama S, Hsu MNK, Samaranyaka AGP, Li-Chan ECY. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (*Avena sativa*) proteins by in silico and in vitro analyses. *J Agric Food Chem.* 2009;57(19):9234-9242. doi:10.1021/jf9018245
 62. Wang F, Yu G, Zhang Y, Zhang B, Fan J. Dipeptidyl peptidase IV inhibitory peptides derived from oat (*Avena sativa* L.), buckwheat (*Fagopyrum esculentum*), and highland Barley (*Hordeum vulgare trifurcatum* (L.) Trofim) proteins. *J Agric Food Chem.* 2015;63(43):9543-9549. doi:10.1021/acs.jafc.5b04016
 63. Yu G, Wang F, Zhang B, Fan J. In vitro inhibition of platelet aggregation by peptides derived from oat (*Avena sativa* L.), highland barley (*Hordeum vulgare* Linn. var. nudum Hook. f.), and buckwheat (*Fagopyrum esculentum* Moench) proteins. *Food Chem.* 2016;194:577-586. doi:10.1016/j.foodchem.2015.08.058
 64. Matsui T, Li CH, Osajima Y. Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. *J Pept Sci.* 1999;5(7):289-297. doi:10.1002/(SICI)1099-1387(199907)5:7<289::AID-PSC196>3.0.CO;2-6
 65. Wang L, Ding Y, Zhang X, et al. Isolation of a novel calcium-binding peptide from wheat germ protein hydrolysates and the prediction for its mechanism of combination. *Food Chem.* 2018;239:416-426. doi:10.1016/j.foodchem.2017.06.090
 66. Hatanaka T, Uraji M, Fujita A, Kawakami K. Anti-oxidation activities of rice-derived peptides and their inhibitory effects on dipeptidylpeptidase-IV. *Int J Pept Res Ther.* 2015;21(4):479-485. doi:10.1007/s10989-015-9478-4
 67. Uraipong C, Zhao J. Rice bran protein hydrolysates exhibit strong in vitro α -amylase, β -glucosidase and ACE-inhibition activities. *J Sci Food Agric.* 2016;96(4):1101-1110. doi:10.1002/jsfa.7182
 68. Nongonierma AB, Fitzgerald RJ. Quinoa (*Chenopodium quinoa* Willd.) protein hydrolysates with in vitro dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties. 2015;65:112-118. doi:10.1016/j.jcs.2015.07.004
 69. Torres-Fuentes C, Alaiz M, Vioque J. Iron-chelating activity of chickpea protein hydrolysate peptides. *Food Chem.* 2012;134(3):1585-1588. doi:10.1016/j.foodchem.2012.03.112
 70. Roy F, Boye JI, Simpson BK. Bioactive proteins and peptides in pulse crops: Pea,

- chickpea and lentil. *Food Res Int.* 2010;43(2):432-442. doi:10.1016/j.foodres.2009.09.002
71. Roy F, Boye JI, Simpson BK. Bioactive proteins and peptides in pulse crops: Pea, chickpea and lentil. *Food Res Int.* 2010;43(2):432-442. doi:10.1016/j.foodres.2009.09.002
 72. Singh BP, Vij S, Hati S. Functional significance of bioactive peptides derived from soybean. *Peptides.* 2014;54:171-179. doi:10.1016/j.peptides.2014.01.022
 73. Capriotti AL, Caruso G, Cavaliere C, et al. Identification of potential bioactive peptides generated by simulated gastrointestinal digestion of soybean seeds and soy milk proteins. *J Food Compos Anal.* 2015;44:205-213. doi:10.1016/j.jfca.2015.08.007
 74. González-Montoya M, Hernández-Ledesma B, Silván JM, Mora-Escobedo R, Martínez-Villaluenga C. Peptides derived from in vitro gastrointestinal digestion of germinated soybean proteins inhibit human colon cancer cells proliferation and inflammation. *Food Chem.* 2018;242(July 2017):75-82. doi:10.1016/j.foodchem.2017.09.035
 75. Boye JI, Roufik S, Pesta N, Barbana C. Angiotensin I-converting enzyme inhibitory properties and SDS-PAGE of red lentil protein hydrolysates. *LWT - Food Sci Technol.* 2010;43(6):987-991. doi:10.1016/j.lwt.2010.01.014
 76. Nongonierma AB, Fitzgerald RJ. An in silico model to predict the potential of dietary proteins as sources of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Chem.* 2014;165:489-498. doi:10.1016/j.foodchem.2014.05.090
 77. Yamamoto N. Chapter 12: Functional food products with antihypertensive effects. In: *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals.* Vol 169. ; 2002:169-177.
 78. Wu J, Majumder K, Gibbons K. Chapter 17: Bioactive proteins and peptides from egg proteins. In: *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals.* ; 2007:247-263.
 79. Ngo DH, Vo TS, Ngo DN, Wijesekara I, Kim SK. Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *Int J Biol Macromol.* 2012;51(4):378-383. doi:10.1016/j.ijbiomac.2012.06.001
 80. Ravallec-Plé R, Gilmartin L, Van Wormhoudt A, Gal Y Le. Influence of the hydrolysis process on the biological activities of protein hydrolysates from cod (*Gadus morhua*) muscle. *J Sci Food Agric.* 2000;80(15):2176-2180. doi:10.1002/1097-0010(200012)80:15<2176::AID-JSFA763>3.0.CO;2-G
 81. Charoenphun N, Cheirsilp B, Sirinupong N, Youravong W. Calcium-binding peptides derived from tilapia (*Oreochromis niloticus*) protein hydrolysate. *Eur Food Res Technol.* 2013;236(1):57-63. doi:10.1007/s00217-012-1860-2
 82. Bulatović M, Embiriekah S, Rakin M, Zarić D, Borić M. Antioxidant activity, functional properties and bioaccessibility of whey protein hydrolysates. *Int J Dairy Technol.* 2017;71(1):243-252. doi:10.1111/1471-0307.12428
 83. Reyes Jara AM, Liggieri CS, Bruno MA. Preparation of soy protein hydrolysates with antioxidant activity by using peptidases from latex of *Maclura pomifera* fruits. *Food*

- Chem.* 2018;264(October 2017):326-333. doi:10.1016/j.foodchem.2018.05.013
84. Zhang T, Li Y, Miao M, Jiang B. Purification and characterisation of a new antioxidant peptide from chickpea (*Cicer arietium* L.) protein hydrolysates. *Food Chem.* 2011;128(1):28-33. doi:10.1016/j.foodchem.2011.02.072
 85. Walters ME, Udenigwe CC, Tsopmo A. Structural characterization and functional properties of proteins from oat milling fractions. *J Am Oil Chem Soc.* 2018;95(8):991-1000. doi:10.1002/aocs.12101
 86. Du Y, Esfandi R, Willmore W, Tsopmo A. Antioxidant activity of oat proteins derived peptides in stressed hepatic HepG2 cells. *Antioxidants.* 2016;5(4):2-9. doi:10.3390/antiox5040039
 87. Vanvi A, Tsopmo A. Pepsin digested oat bran proteins: separation, antioxidant activity, and identification of new peptides. *J Chem.* 2016;2016:1-8. doi:10.1155/2016/8216378
 88. Esfandi R, Willmore WG, Tsopmo A. Peptidomic analysis of hydrolyzed oat bran proteins, and their in vitro antioxidant and metal chelating properties. *Food Chem.* 2019;279:49-57. doi:10.1016/j.foodchem.2018.11.110
 89. Baakdah MM, Tsopmo A. Identification of peptides, metal binding and lipid peroxidation activities of HPLC fractions of hydrolyzed oat bran proteins. *J Food Sci Technol.* 2016:1-9. doi:10.1007/s13197-016-2341-6
 90. Glahn RP, Van Campen DR. Iron uptake is enhanced in Caco-2 cell monolayers by cysteine and reduced cysteinyl glycine. *J Nutr.* 2018;127(4):642-647. doi:10.1093/jn/127.4.642
 91. Bao XL, Song M, Zhang J, Chen Y, Guo ST. Calcium-binding ability of soy protein hydrolysates. *Chinese Chem Lett.* 2007;18(9):1115-1118. doi:10.1016/j.ccllet.2007.07.032
 92. Cumby N, Zhong Y, Naczki M, Shahidi F. Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chem.* 2008;109(1):144-148. doi:10.1016/j.foodchem.2007.12.039
 93. Zou TP, He TP, Li HB, Tang HW, Xia EQ. The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules.* 2016;21(1):1-14. doi:10.3390/molecules21010072
 94. Tsopmo A, Cooper A, Jodayree S. Enzymatic hydrolysis of oat flour protein isolates to enhance antioxidative properties. *Adv J Food Sci Technol.* 2010;4(4):206-212.
 95. Rudolph S, Lunow D, Kaiser S, Henle T. Identification and quantification of ACE-inhibiting peptides in enzymatic hydrolysates of plant proteins. *Food Chem.* 2017;224:19-25. doi:10.1016/j.foodchem.2016.12.039
 96. Boschini G, Scigliuolo GM, Resta D, Arnoldi A. ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food Chem.* 2014;145:34-40. doi:10.1016/j.foodchem.2013.07.076
 97. Paiva L, Lima E, Neto AI, Baptista J. Angiotensin I-converting enzyme (ACE) inhibitory activity, antioxidant properties, phenolic content and amino acid profiles of fucus spiralis

- L. protein hydrolysate fractions. *Mar Drugs*. 2017;15(311):1-18. doi:10.3390/md15100311
98. Borawska J, Darewicz M, Vegarud GE, Iwaniak A, Minkiewicz P. Ex vivo digestion of carp muscle tissue - ACE inhibitory and antioxidant activities of the obtained hydrolysates. *Food Funct*. 2015;6(1):210-217. doi:10.1039/c4fo00621f
 99. Sadegh Vishkai M, Ebrahimpour A, Abdul-Hamid A, Ismail A, Saari N. Angiotensin-I Converting Enzyme (ACE) Inhibitory and Anti-Hypertensive Effect of Protein Hydrolysate from *Actinopyga lecanora* (Sea Cucumber) in Rats. *Mar Drugs*. 2016;14(10):176. doi:10.3390/md14100176
 100. Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Res Clin Pract*. 2014;103(2):137-149. doi:10.1016/j.diabres.2013.11.002
 101. DiabetesCanada. Diabetes in Canada. 2018;(March). <https://www.canada.ca/content/dam/phac-aspc/migration/phac-aspc/cd-%0Awww.fiahctes.ca/diabcles~ and—ynutknow-wmr-rightsr 'sunonri-lhc—diahetes-charter-tbr—camadaidiabetes-charter—for-canada>.
 102. Statistics Canada G of C. Health Fact Sheets: Diabetes, 2016. *Stat Canada*. 2017;82-625-X(82). doi:no.82-625-X
 103. Fradin D, Bougnères P. T2DM: Why epigenetics? *J Nutr Metab*. 2011;2011:1-17. doi:10.1155/2011/647514
 104. Paneni F, Cosentino F. *Diabetes and Cardiovascular Disease A Guide to Clinical Management.*; 2015.
 105. Edelstein D, Yamagishi S, Nishikawa T, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. 2002;404(6779):787-790. doi:10.1038/35008121
 106. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys*. 1985;237(2):408-414. doi:10.1016/0003-9861(85)90293-0
 107. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J*. 1980;191(2):421-427. doi:10.1042/bj1910421
 108. Du X, Matsumura T, Edelstein D, Rossetti L, Brownlee M. Inhibition of GAPDH activity. *J Clin Invest*. 2003;112(7):1049-1057. doi:10.1172/JCI200318127.Introduction
 109. Stitt AW, Jenkins AJ, Cooper ME. Advanced glycation end products and diabetic complications. *Expert Opin Investig Drugs*. 2005;11(9):1205-1223. doi:10.1517/13543784.11.9.1205
 110. Ahmed N. Advanced glycation endproducts - Role in pathology of diabetic complications. *Diabetes Res Clin Pract*. 2005;67(1):3-21. doi:10.1016/j.diabres.2004.09.004
 111. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res*. 2010;107(9):1058-1070. doi:10.1161/CIRCRESAHA.110.223545

112. Fantus IG, Goldberg HJ, Whiteside CI, Topic D. The hexosamine biosynthesis pathway. In: *The Diabetic Kidney*. Totowa, NJ: Humana Press; 2006:117-133. doi:10.1007/978-1-59745-153-6_7
113. Chung SSM. Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol*. 2003;14(90003):233S - 236. doi:10.1097/01.ASN.0000077408.15865.06
114. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414(6865):813-820. doi:10.1038/414813a
115. Mortensen K, Christensen LL, Holst JJ, Orskov C. GLP-1 and GIP are colocalized in a subset of endocrine cells in the small intestine. *Regul Pept*. 2003;114(2-3):189-196. doi:10.1016/S0167-0115(03)00125-3
116. Holst JJ, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *Am J Physiol Metab*. 2004;287(2):E199-E206. doi:10.1152/ajpendo.00545.2003
117. Kieffer TJ, Habener JF. The glucagon-like peptides. *Endocr Rev*. 1999;20(6):876-913. <http://www.ncbi.nlm.nih.gov/pubmed/10605628>.
118. Reimann F, Ward PS, Gribble FM. Signaling mechanisms underlying the release of glucagon-like peptide 1. *Diabetes*. 2006;55:S78-S85. doi:10.2337/db06-S010
119. Deacon CF, Nauck MA, Toft-Nielsen M, Pridal L, Willms B, Holst JJ. Both subcutaneously and intravenously administered glucagon-like peptide I are rapidly degraded from the NH₂-terminus in type II diabetic patients and in healthy subjects. *Diabetes*. 1995;44(9):1126-1131. doi:10.2337/diabetes.44.9.1126
120. Maes MB, Scharpé S, De Meester I. Dipeptidyl peptidase II (DPP_{II}), a review. *Clin Chim Acta*. 2007;380(1-2):31-49. doi:10.1016/j.cca.2007.01.024
121. Hansen L, Deacon CF, Ørskov C, Holst JJ. Glucagon-like peptide-1-(7-36) amide is transformed to glucagon-like peptide-1-(9-36) amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology*. 1999;140(11):5356-5363. doi:10.1210/endo.140.11.7143
122. Baggio LL, Drucker DJ. Biology of Incretins: GLP-1 and GIP. *Gastroenterology*. 2007;132(6):2131-2157. doi:10.1053/j.gastro.2007.03.054
123. Vinodhini S, Rajeswari VD. Exploring the antidiabetic and anti-obesity properties of *Samanea saman* through in vitro and in vivo approaches. *J Cell Biochem*. 2019;120(2):1539-1549. doi:10.1002/jcb.27385
124. Sturgess NC, Cook DL, Ashford MLJ, Hales CN. The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*. 1985;326(8453):474-475. doi:10.1016/S0140-6736(85)90403-9
125. Bailey CJ. The current drug treatment landscape for diabetes and perspectives for the future. *Clin Pharmacol Ther*. 2015;98(2):170-184. doi:10.1002/cpt.144
126. Dornhorst A. Insulinotropic meglitinide analogues. *Lancet*. 2001;358:1709-1716.

127. Lu M, Sarruf DA, Talukdar S, et al. Brain PPAR- γ promotes obesity and is required for the insuling-sensitizing effect of thiazolidinediones. *Nat Med.* 2011;17(5):618-622. doi:10.1038/nm.2332
128. Abdul-Ghani MA, Norton L, DeFronzo RA. Role of sodium-glucose cotransporter 2 (SGLT 2) inhibitors in the treatment of type 2 diabetes. *Endocr Rev.* 2011;32(4):515-531. doi:10.1210/er.2010-0029
129. Meier JJ. GLP-1 receptor agonists for individualized treatment of type 2 diabetes mellitus. *Nat Rev Endocrinol.* 2012;8(12):728-742. doi:10.1038/nrendo.2012.140
130. Puls W, Keup U, Krause HP, Thomas G, Hoffmeister F. Glucosidase inhibition. *Natuwissenschaften.* 1977;64:536-537.
131. Teugwa CM, Mejiato PC, Zofou D, Tchinda BT, Boyom FF. Antioxidant and antidiabetic profiles of two African medicinal plants: *Picralima nitida* (Apocynaceae) and *Sonchus oleraceus* (Asteraceae). *BMC Complement Altern Med.* 2013;13. doi:10.1186/1472-6882-13-175
132. Sabu MC, Kuttan R. Antidiabetic activity of *Aegle marmelos* and its relationship with its antioxidant properties. *Indian J Physiol Pharmacol.* 2004;48(1):81-88.
133. Shivanna N, Naika M, Khanum F, Kaul VK. Antioxidant, anti-diabetic and renal protective properties of *Stevia rebaudiana*. *J Diabetes Complications.* 2013;27(2):103-113. doi:10.1016/j.jdiacomp.2012.10.001
134. Perez Gutierrez RM. Antidiabetic and antioxidant properties, and α -amylase and α -glucosidase inhibition effects of triterpene saponins from *Piper auritum*. *Food Sci Biotechnol.* 2016;25(1):229-239. doi:10.1007/s10068-016-0034-6
135. Ngoh YY, Lim TS, Gan CY. Screening and identification of five peptides from pinto bean with inhibitory activities against α -amylase using phage display technique. *Enzyme Microb Technol.* 2016;89:76-84. doi:10.1016/j.enzmictec.2016.04.001
136. Yu Z, Yin Y, Zhao W, Liu J, Chen F. Anti-diabetic activity peptides from albumin against α -glucosidase and α -amylase. *Food Chem.* 2012;135(3):2078-2085. doi:10.1016/j.foodchem.2012.06.088
137. Siow HL, Tye GJ, Gan CY. Pre-clinical evidence for the efficacy and safety of α -amylase inhibitory peptides from cumin (*Cuminum cyminum*) seed. *J Funct Foods.* 2017;35:216-223. doi:10.1016/j.jff.2017.05.046
138. Oseguera-toledo ME, Mejía EG De, Reynoso-camacho R, Cardador-martínez A, Amaya-llano SL. Proteins and bioactive peptides: Mechanisms of action on diabetes management. 2014:147-157. doi:10.1007/s13749-014-0052-z
139. Li G-H, Le G-W, Shi Y-H, Shrestha S. Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutr Res.* 2004;24(7):469-486. doi:10.1016/j.nutres.2003.10.014
140. Khan A, Ghosh K. Phytic acid-induced inhibition of digestive protease and α -amylase in three Indian major carps: An in vitro study. *J World Aquac Soc.* 2013;44(6):853-859.

doi:10.1111/jwas.12084

141. Ayyash M, Johnson SK, Liu SQ, Al-Mheiri A, Abushelaibi A. Cytotoxicity, antihypertensive, antidiabetic and antioxidant activities of solid-state fermented lupin, quinoa and wheat by *Bifidobacterium* species: In-vitro investigations. *Lwt*. 2018;95(May):295-302. doi:10.1016/j.lwt.2018.04.099
142. Kuhre RE, Albrechtsen NJW, Deacon CF, et al. Peptide production and secretion in GLUTag, NCI-H716, and STC-1 cells: A comparison to native L-cells. *J Mol Endocrinol*. 2016;56(3):201-211. doi:10.1530/JME-15-0293
143. Whalley NM, Pritchard LE, Smith DM, White A. Processing of proglucagon to GLP-1 in pancreatic α -cells: is this a paracrine mechanism enabling GLP-1 to act on β -cells? *J Endocrinol*. 2011;211(1):99-106. doi:10.1530/joe-11-0094
144. Suba V, Murugesan T, Arunachalam G, Mandal SC, Saha BP. Anti-diabetic potential of *Barleria lupulina* extract in rats. *Phytomedicine*. 2004;11(2-3):202-205. doi:10.1078/0944-7113-00316
145. Sabu MC, Kuttan R. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *J Ethnopharmacol*. 2002;81(2):155-160. doi:10.1016/S0378-8741(02)00034-X
146. Wang Y, Xiang L, Wang C, Tang C, He X. Antidiabetic and antioxidant effects and phytochemicals of mulberry fruit (*Morus alba* L.) polyphenol enhanced extract. *PLoS One*. 2013;8(7). doi:10.1371/journal.pone.0071144
147. Connolly A, Piggott CO, FitzGerald RJ. In vitro α -glucosidase, angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory properties of brewers' spent grain protein hydrolysates. *Food Res Int*. 2014;56:100-107. doi:10.1016/j.foodres.2013.12.021
148. Ngoh YY, Gan CY. Enzyme-assisted extraction and identification of antioxidative and α -amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto). *Food Chem*. 2016;190:331-337. doi:10.1016/j.foodchem.2015.05.120
149. Alu'Datt MH, Ereifej K, Abu-Zaiton A, et al. Anti-oxidant, anti-diabetic, and anti-hypertensive effects of extracted phenolics and hydrolyzed peptides from barley protein fractions. *Int J Food Prop*. 2012;15(4):781-795. doi:10.1080/10942912.2010.503357
150. Sato K, Miyasaka S, Tsuji A, Tachi H. Isolation and characterization of peptides with dipeptidyl peptidase IV (DPPIV) inhibitory activity from natto using DPPIV from *Aspergillus oryzae*. *Food Chem*. 2018;261(April):51-56. doi:10.1016/j.foodchem.2018.04.029
151. Cordier-Bussat M, Bernard C, Levenez F, et al. Peptones stimulate both the secretion of the incretin hormone glucagon-like peptide 1 and the transcription of the proglucagon gene. *Diabetes*. 1998;47(7):1038-1045. <http://www.ncbi.nlm.nih.gov/pubmed/9648826>.
152. Olson AL. Regulation of GLUT4 and insulin-dependent glucose flux. *ISRN Mol Biol*. 2012;2012:1-12. doi:10.5402/2012/856987
153. Morato PN, Lollo PCB, Moura CS, et al. Whey protein hydrolysate increases translocation

- of GLUT-4 to the plasma membrane independent of insulin in Wistar rats. *PLoS One*. 2013;8(8). doi:10.1371/journal.pone.0071134
154. Lu J, Zeng Y, Hou W, et al. The soybean peptide aglycin regulates glucose homeostasis in type 2 diabetic mice via IR/IRS1 pathway. *J Nutr Biochem*. 2012;23(11):1449-1457. doi:10.1016/j.jnutbio.2011.09.007
 155. Lee HS, Lee HJ, Suh HJ. Silk protein hydrolysate increases glucose uptake through up-regulation of GLUT 4 and reduces the expression of leptin in 3T3-L1 fibroblast. *Nutr Res*. 2011;31(12):937-943. doi:10.1016/j.nutres.2011.09.009
 156. Yu Z, Yin Y, Zhao W, et al. Novel peptides derived from egg white protein inhibiting alpha-glucosidase. *Food Chem*. 2011;129(4):1376-1382. doi:10.1016/j.foodchem.2011.05.067
 157. Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin Phenol reagent. *J Biol Chem*. 1951;193:144-149.
 158. Panasiuk R, Amarowicz R, Kostyra H, Sijtsma L. Determination of α -amino nitrogen in pea protein hydrolysates: A comparison of three analytical methods. *Food Chem*. 1998;62(3):363-367. doi:10.1016/S0308-8146(97)00164-7
 159. Puppo MC, Calvelo A, Añón MC. Physicochemical and rheological characterization of wheat flour dough. *Cereal Chem*. 2005;82(2):173-181. doi:10.1094/CC-82-0173
 160. Hayakawa S, Nakai S. Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *J Food Sci*. 1985;50(2):486-491. doi:10.1111/j.1365-2621.1985.tb13433.x
 161. Li D, Mu C, Cai S, Lin W. Ultrasonic irradiation in the enzymatic extraction of collagen. *Ultrason Sonochem*. 2009. doi:10.1016/j.ultsonch.2009.02.004
 162. Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J Agric Food Chem*. 2002;50(16):4437-4444. doi:10.1021/jf0201529
 163. Anderson OD. The spectrum of major seed storage genes and proteins in oats (*Avena sativa*). *PLoS One*. 2014;9(7). doi:10.1371/journal.pone.0083569
 164. Nieto-nieto TV, Wang YX, Ozimek L, Chen L. Effects of partial hydrolysis on structure and gelling properties of oat globular proteins. *FRIN*. 2014;55:418-425. doi:10.1016/j.foodres.2013.11.038
 165. Peterson DM. Composition and nutritional characteristics of oat grain and products. In: Marshall H., Sorrells M., eds. *Oat Science and Technology*. Madison, WI: American Society of Agronomy; 1992:265-292.
 166. Gazza L, Taddei F, Conti S, et al. Biochemical and molecular characterization of *Avena indolines* and their role in kernel texture. *Mol Genet Genomics*. 2015;290(1):39-54. doi:10.1007/s00438-014-0894-5
 167. Kaukonen O, Sontag-Strohm T, Salovaara H, Lampi AM, Sibakov J, Loponen J. Foaming

- of differently processed oats: Role of nonpolar lipids and tryptophanin proteins. *Cereal Chem.* 2011;88(3):239-244. doi:10.1094/CCHEM-11-10-0154
168. Rabe S, Fischer L, Blank I, et al. Flavourzyme, an enzyme preparation with industrial relevance: automated nine-step purification and partial characterization of eight enzymes. *J Agric Food Chem.* 2015;63(23):5682-5693. doi:10.1021/acs.jafc.5b01665
 169. Barret AJ, Rawlings ND, Woessner JF, eds. *Handbook of Proteolytic Enzymes: Cysteine, Serine, and Threonine Peptidases*. Second Edi. Elsevier; 2004.
 170. Vioque J, Sánchez-Vioque R, Clemente A, Pedroche J, Bautista J, Millan F. Production and characterization of an extensive rapeseed protein hydrolysate. *JAOCS, J Am Oil Chem Soc.* 1999;76(7):819-823. doi:10.1007/s11746-999-0071-x
 171. Zhu Z, Zhu W, Yi J, et al. Effects of sonication on the physicochemical and functional properties of walnut protein isolate. *Food Res Int.* 2018;106(November 2017):853-861. doi:10.1016/j.foodres.2018.01.060
 172. Kang DC, Zou YH, Cheng YP, Xing LJ, Zhou GH, Zhang WG. Effects of power ultrasound on oxidation and structure of beef proteins during curing processing. *Ultrason Sonochem.* 2016;33:47-53. doi:10.1016/j.ultsonch.2016.04.024
 173. Goodwin TW, Morton RA. The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem J.* 1946;40(5-6):628-632. doi:10.1042/bj0400628
 174. Chirgadze YN, Nevskaya NA. Infrared spectra and resonance interaction of pleated sheet. *Biopolymers.* 1976;15:627-636.
 175. Yu HC, Tan FJ. Effect of ultrasonic pretreatment on the antioxidant properties of porcine liver protein hydrolysates. *Int J Food Sci Technol.* 2017;52(6):1392-1399. doi:10.1111/ijfs.13416
 176. Ngoh YY, Gan CY. Enzyme-assisted extraction and identification of antioxidative and α -amylase inhibitory peptides from Pinto beans (*Phaseolus vulgaris* cv. Pinto). *Food Chem.* 2016;190:331-337. doi:10.1016/j.foodchem.2015.05.120
 177. Payan F. Structural basis for the inhibition of mammalian and insect α -amylases by plant protein inhibitors. *Biochim Biophys Acta - Proteins Proteomics.* 2004;1696(2):171-180. doi:10.1016/j.bbapap.2003.10.012
 178. Wang B, Atungulu GG, Khir R, et al. Ultrasonic treatment effect on enzymolysis kinetics and activities of ACE-inhibitory peptides from oat-isolated protein. *Food Biophys.* 2015;10(3):244-252. doi:10.1007/s11483-014-9375-y
 179. Velarde-Salcedo AJ, Barrera-Pacheco A, Lara-González S, et al. In vitro inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of amaranth (*Amaranthus hypochondriacus* L.) proteins. *Food Chem.* 2013;136(2):758-764. doi:10.1016/j.foodchem.2012.08.032
 180. Chen Q, Ph D, Reimer RA, Ph D. Dairy protein and leucine alter GLP-1 release and mRNA of genes involved in intestinal lipid metabolism in vitro. *NUT.* 2009;25(3):340-349. doi:10.1016/j.nut.2008.08.012

181. Reimer RA, Darimont C, Gremlich S, Nicolas-Métral V, Rüegg UT, Macé K. A human cellular model for studying the regulation of glucagon-like peptide-1 secretion. *Endocrinology*. 2001;142(10):4522-4528. doi:10.1210/endo.142.10.8415
182. Kim K, Park M, Lee YM, Rhyu MR, Kim HY. Ginsenoside metabolite compound K stimulates glucagon-like peptide-1 secretion in NCI-H716 cells via bile acid receptor activation. *Arch Pharm Res*. 2014;37(9):1193-1200. doi:10.1007/s12272-014-0362-0
183. Choi EK, Kim KS, Yang HJ, et al. Hexane fraction of *Citrus aurantium* L. stimulates glucagon-like peptide-1 (GLP-1) secretion via membrane depolarization in NCI-H716 cells. *Biochip J*. 2012;6(1):41-47. doi:10.1007/s13206-012-6106-7
184. Behrens M, Meyerhof W. Gustatory and extragustatory functions of mammalian taste receptors. *Physiol Behav*. 2011;105(1):4-13. doi:10.1016/j.physbeh.2011.02.010
185. Thomas C, Gioiello A, Noriega L, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab*. 2009;10(3):167-177. doi:10.1016/j.cmet.2009.08.001